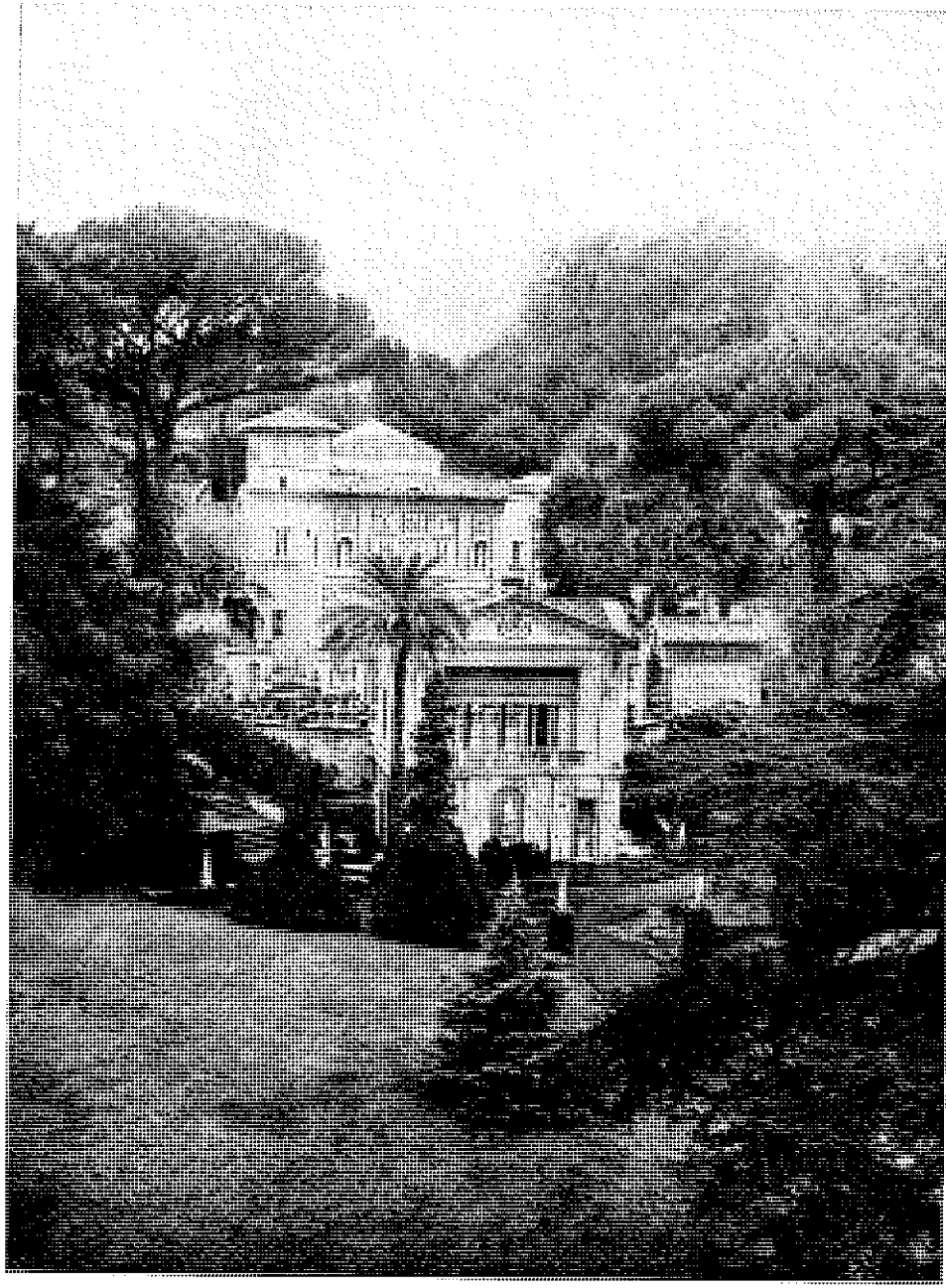

PONTIFICIAE ACADEMIAE SCIENTIARVM SCRIPTA VARIA

51

STUDY WEEK

ON:

MODERN BIOLOGICAL EXPERIMENTATION

October 18-23, 1982

EDITED BY

CARLOS CHAGAS

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SCIENTIFIC PAPERS

FOREWORD

Biology has in the last decades made extraordinary progress in all its domains. Some of the progress, as in human genetics or immunology, has a special interest for human life; others have had their impact in the field of agriculture and in industry. As a result of this development, humankind is looking forward to biotechnology as one of the ways in which many problems of welfare, the protection of natural resources, energy, etc., can be faced.

Along those lines of progress, we find as an important part the concepts and techniques which were brought up by the work done on the nucleic acids and on recombinant DNA. At the same time the development of techniques, such as cloning and tissue culture, has increased the perspectives of biomedical research. However, the hopes which modern biology yields are many times counterbalanced by apprehensions which come from the possibility of the misuse of the results obtained by it. This was the reason why the Pontifical Academy of Sciences chose as the theme of a Study Week "Modern Biological Experimentation".

It is clear that involved in biological experimentation there are some moral problems which are outside the realm of science proper. The Pontifical Academy of Sciences as a scientific body has treated the theme in scientific terms. However, at the innerness of each human being the misuse of modern biology is a constant preoccupation. The benefits of modern biology are dependent on the understanding that human life and dignity have to be protected.

The exciting presentations which were given at our meeting make this Study Week a very interesting one. It is a plesure for me to acknow-

ledge the zeal, the dedication with which the participants took part in the meeting. It always makes me feel a sense of being in the presence of grandeur when I see the good will of women and men who desert their laboratories and the competitive life that science has brought to its development, to come to the Casina Pio IV to discuss the multidisciplinary themes which are the object of our discussions. It is due to the collaboration of scientists from all over the world that this Academy can achieve one of its important functions, proposed on the bull of its creation by Pius XI, i.e., the promotion of science.

I want also to express my thanks to Father di Rovasenda, Chancellor of the Academy, and to Michelle Porcelli-Studer, Gilda Massa and Silvio Devoto for the help they have given. This help was extremely necessary in order that the meeting be held with no difficulty.

CARLOS CHAGAS
President of the
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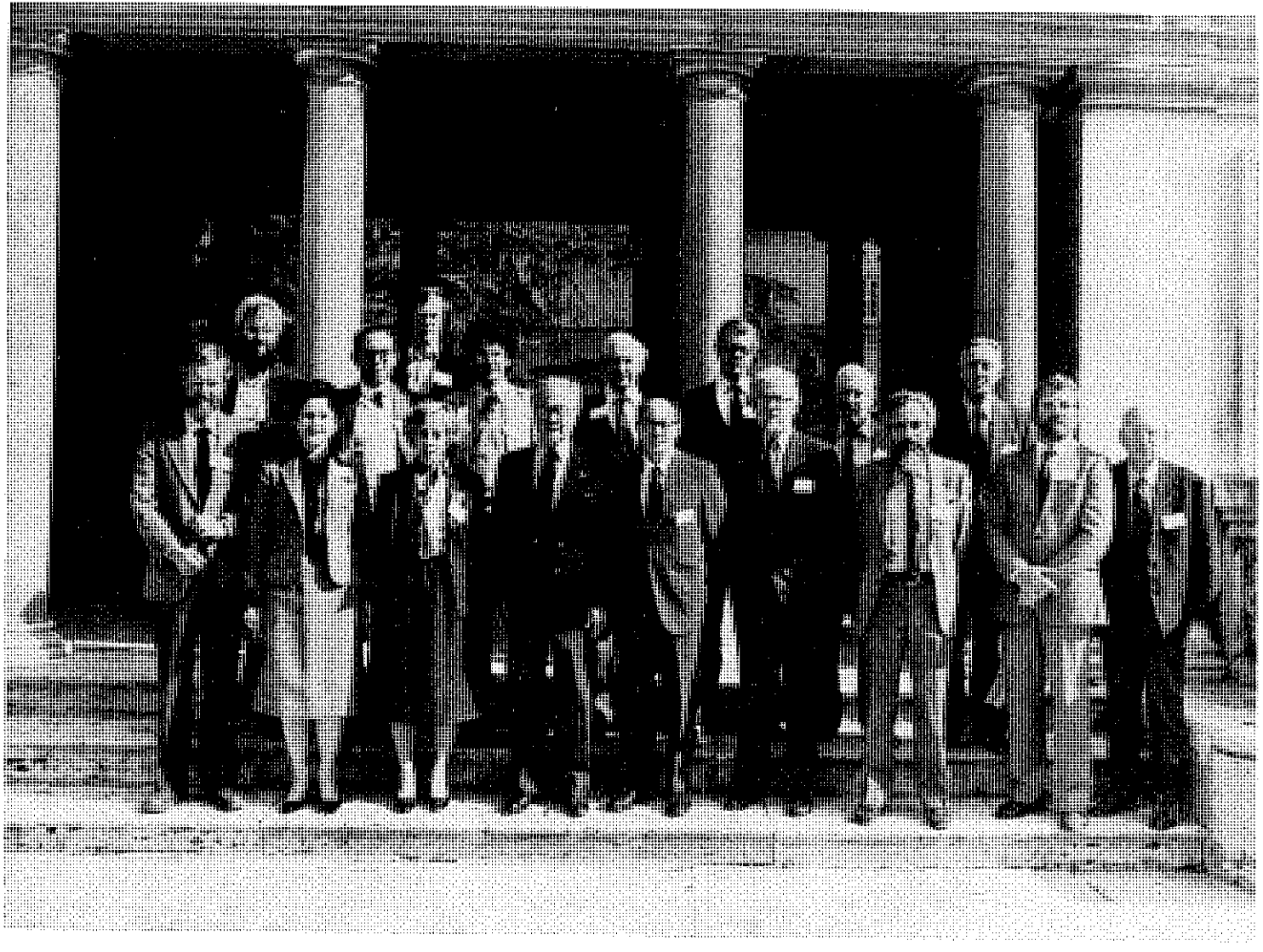

AUDIENCE OF THE HOLY FATHER

On October 23, 1982 His Holiness John Paul II granted an Audience in the Hall of the Throne of the Apostolic Palace to the Participants in the Study Week on "Modern Biological Experimentation", organized by the Pontifical Academy of Sciences.

The group, introduced in the Apostolic Palace by the President of the Academy, His Excellency Prof. Carlos Chagas, accompanied by the Director of the Chancellery, Rev. Father Enrico di Rovasenda, was paternally received by His Holiness, who at the end of the Audience wanted to greet personally all the Participants.

After having reached the throne, the Holy Father gave His consent to the President of the Academy Prof. Carlos Chagas, who delivered the following speech:

Holy Father,

Allow me first to thank You for the audience You have granted to the participants in the Study Week which has just been held at Your Academy. The participants have left their laboratories and their daily life to come to the Vatican and to present their knowledge and experience on a theme of very great societal interest. This is a gratifying gesture.

Modern experimental biology presents numerous "avant-garde" frontiers whose progress can undoubtedly bring to humankind great benefits. Biology during the second part of our century has made astonishing progress not only by the introduction of new concepts such as those of molecular biology, or the study of the specificity of biological interactions. Thus a new technology, either conceptual, as for instance the DNA excision analysis, or the introduction of a new instrumental methodology was deployed.

I would like to go further for a moment. Due to the contributions of modern biological experimentation, certain domains, like those of genetics, pharmacology, neurobiology and pathogeny, have acquired new dimensions and have opened new ways for the recognition of vital phenomena, either in animals and men, or in plants.

It is interesting to point out that at the basis of this progress comes, as a breakthrough, the use, as an experimental model, of a minute bacteria, the E. Coli. Among many examples of how technology has made biology advance I would like to point out how tissue culture, which was quite an isolated venture when I began my scientific career, has turned out to be one of the most current techniques in a great proportion of biological laboratories. This could also be said of the use of selected genetic strains of animals used for experimentation.

In our Study Week a vast spectrum has been covered because one of the objectives was to see the perspectives and ethical implications of the experiments which are on the borderline of scientific progress and which have made biology the science of today and of tomorrow because its final objective is the understanding of man in his immanency.

In a broad perspective a panorama of scientific achievement, from the gene structure up to the development of an embryo, has been presented. Thus we can say that we went from the molecular basis of life to the most complex phenomenon of differentiation.

The papers and the discussions presented opened new perspectives as they show, for instance, how you may insert in a genome a new gene or its active part, thus creating a source of new genetic information. This can be done either by DNA recombination, by the transfer of genes, or other technologies. As an interesting visualization of what can be done, it has been shown that in animal eggs we can create specimens with a double heredity, or, as it was also shown, that by new techniques we can expect to provide new methods for vaccination or to understand better the mechanism of the human defense against aggression. Also, some of the work presented is of great interest in cancer research.

The work on embryos of animals will also make it possible to understand the mechanics of cell differentiation and ontogenic evolution. Experiments on extra-corporeal fecundation have also been debated.

The Study Week has ended with a full day of discussion on the perspectives and ethical problems involved. I hope to send to You in the near future the conclusions which were reached by the scientists.

Some of the subjects which were discussed have in the past been susceptible to a publicity which is unjustifiable. It is a disservice rendered to man to misinform him or to put in a false light scientific discoveries.

The true scientist is humble and in his humility he faces the mysteries of our day-to-day life and tries to understand them, knowing perfectly well that those mysteries will take their distance as you get

near to them. The true scientist does not like a vain publicity but in his preoccupation he attaches himself solely to the presentation of facts. He elaborates hypotheses and he creates theories. Some of those will be valid forever; others for a long or shorter time; some, as a French poet says, will last, as does the rose, for a morning. The true scientist is humble because he knows he will never attain the primary causes.

The participants in this Study Week are all true scientists, and I am proud to have had them as my companions for a week. I am sure that they are as anxious as I am to hear the expression of Your wisdom on the so relevant subjects we have discussed.

Holiness, in renewing our thanks, I wish to express again our admiration for the faith, serenity and strength with which You face the problems of humankind today, and say that Your statements, which reach the most remote boundaries of our globe, will give us hope and assure us that a great leader is fighting for peace and for human dignity.

I would like also to express in the name of all of us our congratulations to the Church and to the world on the 4th Anniversary of Your pontificate, which has been full of endeavours and realizations and has extended throughout the world the message of peace and brotherhood which we find in the Gospels.

Very humbly, Holy Father, I ask for Your Apostolic Benediction.

The Holy Father answered with the following discourse:

Mr. President, Ladies and Gentlemen,

1. *I desire to express to you my deep gratitude for your visit and to present my best wishes for your activities, of which Professor Chagas has spoken. Permit me, first of all, to offer my felicitations to the President of the Pontifical Academy of Sciences for the intense work performed in various areas of science and for the initiatives undertaken for the well-being of all humanity, such as the recent appeal against nuclear war, endorsed by approximately forty Presidents of Academies throughout the world and by other scientists who gathered on 23-24 September last in the Casina Pio IV, the headquarters of our own Academy.*

2. *The work which you have accomplished during these days, besides having a* high scientific value, *is also of* great interest for religion. *My predecessor Paul VI, in his discourse to the United Nations Organization on 4 October 1965, spoke from the viewpoint of being an "expert in humanity". This expertise is indeed linked with the Church's own wisdom, but it likewise comes from culture, of which the natural sciences are an ever more important expression.*

In my talk to UNESCO on 2 June 1980, I mentioned, and now I wish to repeat it to you scientists, that there exists "an organic and constitutive link between culture and religion". I must also confirm before this illustrious assembly what I said in my address of 3 October 1981 to the Pontifical Academy of Sciences, on the occasion of the annual Study Week: "I

have firm confidence in the world scientific community, and in a very particular way in the Pontifical Academy of Sciences, being certain that, thanks to them, biological progress and research, as also all other scientific research and its technological application will be accomplished in full respect for the norms of morality, safeguarding the dignity of people and their freedom and equality". And I added: "It is necessary that science should always be accompanied and guided by the wisdom that belongs to the permanent spiritual heritage of humanity, and which is inspired by the design of God inscribed in creation before being subsequently proclaimed by His Word".

3. *Science and wisdom, which in their truest and most varied expressions constitute a most precious heritage of humanity,* are at the service of man. *The Church is called, in her essential vocation, to foster the progress of man, since, as I wrote in my first Encyclical: "...man is the primary route that the Church must travel in fulfilling her mission:* he is the primary and fundamental way for the Church, *the way traced out by Christ Himself"* (Redemptor Hominis, 14). *Man is also for you the ultimate term of scientific research, the whole man, spirit and body, even if the immediate object of the sciences that you profess is the body with all its organs and tissues. The human body is not independent of the spirit, just as the spirit is not independent of the body, because of the deep unity and mutual connection that exist between one and the other.*

The substantial unity between spirit and body, and indirectly with the cosmos, is so essential that every human activity, even the most spiritual one, is in some way permeated and coloured by the bodily condition; at the same time the body must in turn be directed and guided to its final end by

the spirit. There is no doubt that the spiritual activities of the human person proceed from the personal centre of the individual, who is predisposed by the body to which the spirit is substantially united. Hence the great importance, for the life of the spirit, of the sciences that promote the knowledge of corporeal reality and activity.

4. *Consequently, I have no reason to be apprehensive for those* experiments in biology *that are performed by scientists who, like you, have a profound respect for the human person, since I am sure that they will contribute to the* integral well-being of man. *On the other hand, I condemn, in the most explicit and formal way, experimental manipulations of the human embryo, since the human being, from conception to death, cannot be exploited for any purpose whatsoever. Indeed, as the Second Vatican Council teaches, man is "the only creature on earth which God willed for itself"* (Gaudium et Spes, 24). *Worthy of esteem is the initiative of those scientists who have expressed their disapproval of experiments that violate human freedom, and I praise those who have endeavoured to establish, with full respect for man's dignity and freedom, guidelines and limits for experiments concerning man.*

The experimentation that you have been discussing is directed to a greater knowledge of the most intimate mechanism of life, by means of artificial models, such as the cultivation of tissues, and experimentation on some species of animals genetically selected. Moreover, you have indicated some experiments to be accomplished on animal embryos, which will permit you to know better how cellular differences are determined.

It must be emphasized that new techniques, such as the cultivation of cells and tissues, have had a notable development which permits very important progress in biological

sciences, and they are also complementary to experimentation done on animals. It is certain that animals are at the service of man and can hence be the object of experimentation. Nevertheless, they must be treated as creatures of God which are destined to serve man's good, but not to be abused by him. Hence the diminution of experimentation on animals, which has progressively been made ever less necessary, corresponds to the plan and well-being of all creation.

5. *I have learned with satisfaction that among the themes discussed during your Study Week you have focused attention on* in vitro *experiments which have yielded* results for the cure of diseases related to chromosome defects.

It is also to be hoped, with reference to your activities, that the new techniques of modification of the genetic code, in particular cases of genetic or chromosomic diseases, will be a motive of hope for the great number of people affected by those maladies.

It can also be thought that, through the transfer of genes, certain specific diseases can be cured, such as sickle-cell anaemia, which in many countries affects individuals of the same ethnic origin. It should likewise be recalled that some hereditary diseases can be avoided through progress in biological experimentation.

The research of modern biology gives hope that the transfer and mutations of genes can ameliorate the condition of those who are affected by chromosomic diseases; in this way the smallest and weakest of human beings can be cured during their intrauterine life or in the period immediately after birth.

6. *Finally, I wish to recall, along with the few cases which I have cited that benefit from biological experimentation, the important advantages that come from* the increase of food

products *and from the formation of new vegetal species for the benefit of all, especially people most in need.*

In terminating these reflections of mine, which show how much I approve and support your worthy researches, I reaffirm that they must all be subject to moral principles and values, which respect and realize in its fullness the dignity of man. I express the hope that the scientists of those countries which have developed the most advanced modern techniques will take into sufficient account the problems of developing nations and that, outside of every economic or political opportunism which reproduces the schemes of an old colonialism in a new scientific and technical edition, there can be had a fruitful and disinterested exchange. This exchange must be that of culture in general and of science in particular, among scientists of nations of different degrees of development, and may there thus be formed, in every country, a nucleus of scholars of high scientific value.

I ask God, who is the merciful Father of all, but especially of the most abandoned and of those who have neither the means nor the power to defend themselves, to direct the application of scientific research to the production of new food supplies, since one of the greatest challenges that humanity must face, together with the danger of nuclear holocaust, is the hunger of the poor of this world.

For this intention and for the overall genuine progress of man, created in the image and likeness of God, I invoke on you and on your scientific activities abundant divine blessings.

SCIENTIFIC PAPERS

CENTROMERES AND MINICHROMOSOMES

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The role of the centromere in cell division

During normal cell division in eukaryotic cells, the chromosomes are replicated and a single copy of each is passed on to the daughter cells. The centromere region on each chromosome is responsible for this segregation process. In the absence of a centromere, most genetic units are unstable; even though the DNA replicates, the duplicate copy is not always transported into the daughter cell.

In addition to its role in maintaining proper segregation of the chromosomes during mitotic and meiotic cell division, the centromere might well be involved in various other processes, such as chromosomal copy number control, meiotic and mitotic recombination, and replication (for reviews, see Lewin, 1980; Pickett-Heaps *et al.*, 1982). The molecular mechanisms involved in these processes are still largely obscure, although progress is now being made with the aid of the powerful tools offered by modern recombinant DNA technology.

The centromere is seen in higher eukaryotic chromosomes as a constricted region that contains a structure known as the kinetochore, to which the spindle fibers attach during mitosis. In the cells from most organisms, several spindle fibers (microtubules) attach to the kinetochore on each chromosome; however in the yeast, *Saccharomyces cerevisiae*, only a single microtubule is bound to each centromere (Peterson and Ris, 1976). Again, the mechanism by which the spindle fibers attach to the kinetochore and the protein composition of the kinetochore itself are unknown (Pickett-Heaps *et al.*, 1982).

This paper presents a review of our published results on the develop-

ment of yeast as a system for the study of chromosome and centromere structure, and the mechanism of chromosome segregation in eukaryotic cells.

Yeast as an experimental organism

Common bakers' or brewers' yeast, *Saccharomyces cerevisiae*, has been developed during the past 6-7 years as an extremely powerful experimental system for the study of the mechanism of cell division and chromosome segregation in eukaryotes. Yeast has been used for genetic studies since the 1930's, and a wide range of mutant strains are available, including strains harboring mutations that block cell division at various stages of the cell cycle (reviewed by Pringle and Hartwell, 1982). Extensive genetic maps of each of the sixteen chromosomes are available and the position of the centromere on each chromosome is known with respect to the location of nearby markers (Mortimer and Schild, 1980). Yeast grows in either the haploid or diploid state, so it is possible to isolate recessive mutations with relative ease.

In 1976, it was shown that many yeast genes could be isolated (cloned) in the bacterium, *Escherichia coli*, by selecting for complementation of auxotrophic mutations in the bacterial host cell by segments of yeast DNA covalently sealed into plasmid or bacteriophage vectors. For example, the yeast *LEU2* and *HIS3* genes were isolated by complementation of the corresponding *leuB* and *hisB* mutations in *E. coli* (Carbon *et al.*, 1977; Struhl *et al.*, 1976; Ratzkin and Carbon, 1977). Shortly after the isolation of the yeast *LEU2* gene in our laboratory, Hinnen, Hicks and Fink at Cornell University succeeded in transforming a yeast *leu2* mutant to *LEU2*$^+$ using plasmid DNA containing the cloned *LEU2* gene (Hinnen *et al.*, 1978). This discovery opened the way for an extremely rapid development of yeast as a system for studying eukaryotic molecular genetics.

Yeast transformations with DNA segments containing the *LEU2* or *HIS3* genes occur with low frequency and require integration of the transforming DNA into the host cell genome. It was discovered, however, that when certain specific regions of DNA, either from yeast or other eukaryotic cells, are present within the transforming plasmid DNA, the plasmid would replicate autonomously within the host cell without integrating into the genome (Struhl *et al.*, 1979; Hsiao and Carbon, 1979). These replicator segments were called *ARS* (autonomously replicating segment) (Stinchcomb

et al., 1979). It is thought that these DNA sequences serve as origins of DNA replication at scattered points along the chromosome arms, although this remains unproven to date.

Various "shuttle" plasmid vectors have been constructed that can replicate in either *E. coli* or yeast and that contain various genes used for selection of cells containing the plasmid. These vectors usually contain *ARS1* (Struhl *et al.*, 1979; Kingsman *et al.*, 1979), *ARS2* (Hsiao and Carbon, 1979, 1981b), or the replicator region from the yeast 2-micron plasmid (Beggs, 1978; Struhl *et al.*, 1979). Yeast genes can now be isolated by selecting for complementation of mutations in yeast with genomic DNA segments sealed into these shuttle vectors, a procedure that was used to advantage in our experimental strategy for the isolation of a yeast centromere (Clarke and Carbon, 1980a, 1980h).

Assays for centromere function

Our attempt to isolate a functional centromeric DNA segment from yeast required suitable assays for proper centromere function of DNA segments cloned into the appropriate shuttle vector. The *ARS* vectors mentioned above provide convenient assays for both the mitotic and meiotic functions of the centromere.

Mitotic function is assayed by taking advantage of the fact that *ARS* vectors lacking a centromere DNA sequence are quite unstable mitotically; the lack of a suitable attachment site results in a high level of plasmid non-disjunction during cell division. The rate of segregation loss of an *ARS1* plasmid (pLC544) is about 18% per generation in haploid yeast (Kingsman *et al.*, 1979). With the addition of a functional centromere DNA sequence, however, an *ARS* plasmid becomes mitotically quite stable and is lost at the rate of only 1-3% per generation (Clarke and Carbon, 1980b; Fitzgerald-Hayes *et al.*, 1982). This stabilizing effect of the centromere on *ARS* plasmids not only is used as a convenient assay for centromere function, but also is the basis of one experimental strategy for the isolation of functional yeast centromere DNA sequences. In this procedure, centromeres are obtained by selecting directly for cloned DNA sequences that effectively stabilize the *ARS1* vector, YRp7 (Hsiao and Carbon, 1981a).

Meiotic function of an isolated centromere DNA segment is tested by scoring the distribution of a genetic marker on tbe centromere plasmid among the four haploid meiotic progeny of suitable a/α diploid yeast cells. A genetic cross of the type shown in Table 1 is carried out; both parents

TABLE 1 — *Meiotic segregation of various minichromosomes.*

Minichromosome in cross	Size of DNA insert, kb	Distribution in tetrads of genetic marker on minichromosome (%) 4+:0−	3+:1−	2+:2−	1+:3−	0+:4−	Test for centromere linkage of marker on minichromosome PD	NPD	T	Reference centromere marker (chromosome)
1. pYe (*CDC10*) 1	8.0	1 (6 %)	0	10 (63%)	0	5 (31%)	2	8	0	*met14* (XI)
2. pYe (*CDC10*) 1	8.0	4 (21%)	0	11 (58%)	0	4 (21%)	ND	ND	ND	—
3. pYe (*CDC10*) 1	8.0	1 (7 %)	0	11 (79%)	0	2 (14%)	4	7	0	*met14* (XI)
4. pYe (*CEN3*) 41	1.6	3 (14%)	3 (14%)	13 (63%)	1 (5%)	1 (5 %)	7	6	0	*trp1* (IV)
5. pYe (*CEN3*) 30	0.63	4 (20%)	2 (15%)	8 (40%)	1 (5%)	5 (25%)	4	3	1	*leu1* (VII)
6. pYe (*CEN3*) 30	0.63	15 (44%)	8 (24%)	5 (15%)	1 (3%)	5 (15%)	2	3	0	*met14* (XI)
7. pYe (*MET14*) 2	5.2	0	1 (2 %)	38 (72%)	2 (3%)	12 (23%)	9	14	1	*leu1* (VII)
8. pYe (*MET14*) 2	5.2	3 (16%)	1 (5 %)	10 (52%)	1 (5%)	4 (21%)	4	6	0	*leu1* (VII)
9. pYe (*MET14*) 2	5.2	5 (19%)	0	13 (48%)	1 (3%)	8 (30%)	7	4	2	*ade1* (I)
10. pYe (*CEN11*) 12	1.6	6 (27%)	3 (13%)	10 (46%)	0	3 (13%)	3	6	1	*ade1* (I)
11. pYe (*CEN11*) 12	1.6	8 (17%)	3 (6 %)	18 (38%)	3 (6%)	15 (32%)	8	7	3	*met14* (XI)
12. pYe (*CEN11*) 10	0.86	5 (13%)	3 (8 %)	17 (46%)	2 (5%)	10 (27%)	6	8	3	*leu1* (VII)
13. pYe (*CEN11*) 10	0.86	6 (24%)	1 (4 %)	8 (32%)	2 (8%)	7 (28%)	2	5	0	*met14* (XI)
14. pYe (*CEN11*) 5	0.86	12 (38%)	4 (13%)	10 (31%)	1 (3%)	5 (16%)	3	6	0	*met14* (XI)

In all the above crosses, both parents contained inactivating mutations in the gene carried as a wild-type marker on the minichromosome. The minichromosome was present in only *one* of the parents (the first strain mentioned in each cross). The crosses were: 1. XSB52-23C with X2928-3D-1A; 2. SB17B with X2928-3D-1A; 3. SB1C with XSB52-23C; 4. XSB52-23C with X3144-1D; 5, 6, 7, and 8. J17 with Z136-1-13C; 9 and 10. J17 with RH218; 11, 12, 13, and 14. J17 with Z136-1-13C. The genotypes of the strains are given in Fitzgerald-Hayes, Clarke and Carbon (1982). Details of the procedure used for the tetrad analyses are given in Clarke and Carbon (1980b). PD, parental ditype; NPD, nonparental ditype; T, tetratype; ND, not determined. (Taken from Fitzgerald-Hayes, Clarke and Carbon, 1982).

in the cross are mutant in the gene carried on the plasmid, and the plasmid is present in only one of the two parents. If the centromere plasmid is behaving as a typical aneuploid chromosome in the cross and is controlled to a single copy, the plasmid will appear in only two of the four haploid progeny, in sister spores that are the result of the second meiotic division. The sister spores are identified by scoring the tetrads for the distribution of a genetic marker known to be tightly linked to tbe centromere on any of the host chromosomes; such markers nearly always undergo first division segregation.

When centromere plasmids are analyzed by the above procedure, the genetic marker on the plasmid distributes 2+:2− as predicted in about 60-90% of the tetrads, always in two sister spores (Table 1). Abnormal 1+:3− or 3+:1− tetrads are rarely seen. A relatively large number of tetrads completely lacking the plasmid (0+:4−) are always observed, presumably due to plasmid non-disjunction occurring during growth of the diploid prior to sporulation. In addition, a variable number of 4+:0− tetrads are seen, depending on the number of cells in the cross that contain more than one copy of the plasmid.

Isolation of functional centromere DNA from yeast

Functional centromere DNA segments have been isolated from total yeast DNA by two methods: (a) by isolating DNA segments containing known centromere-linked genes and using chromosome "walking" or overlap hybridization procedures to obtain the flanking regions (Clarke and Carbon, 1980; Fitzgerald-Hayes, Buhler, Cooper and Carbon, 1982; Stinchcomb *et al.*, 1982); and (b) by selecting directly for DNA segments capable of stabilizing *ARS* vectors (Hsiao and Carbon, 1981a).

The strategy used for the isolation of the centromere (*CEN3*) from chromosome III of yeast is shown in Figure 1. The yeast *LEU2*, *PGK*, and *CDC10* genes, known to be located very near the centromere on III, were first isolated on separate segments of DNA using various types of DNA cloning procedures (Ratzkin and Carbon, 1977; Hitzeman *et al.*, 1980; Clarke and Carbon, 1980a). Plasmids containing regions of the DNA flanking these genes were obtained by screening yeast genomic libraries for plasmids containing overlapping fragments of DNA (Chinault and Carbon, 1979). The DNA segments thus obtained were screened for centromere activity in *ARS* plasmids by the mitotic and meiotic procedures

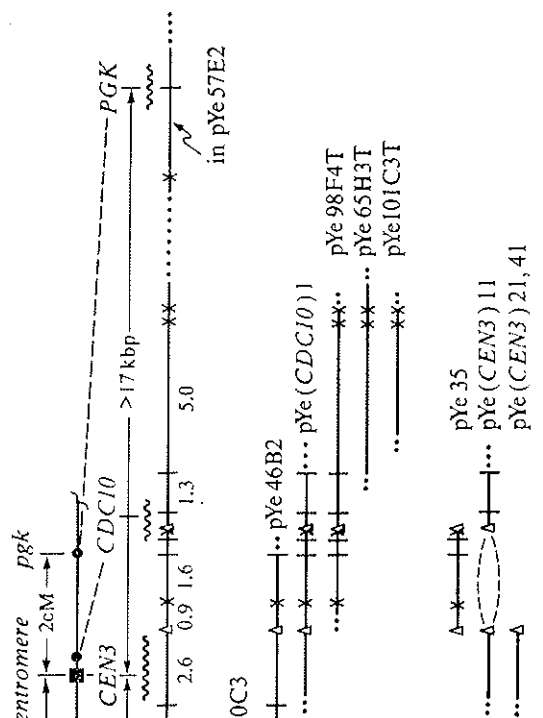

FIG. 1 Genetic and physical maps of the centromere region of yeast chromosome III. The numbers refer to kilobase pairs (kb). The yeast *LEU2* and *CDC10* genes are located on plasmids pYe (*LEU2*) 10 and pYe (*CDC10*) 1, respectively. The functional centromere (*CEN3*) is located on plasmids pYe (*CDC10*) 1, pYe 46B2, pYe (*CEN3*) 11, and has been subcloned into plasmids pYe (*CEN3*) 21 and pYe (*CEN3*) 41. The relationship between physical and genetic distances across the centromere region is about 3 kb/centiMorgan, about the same as the average value along the chromosome arms. (Taken from Clarke and Carbon, 1980b)

described above. *CEN3* was localized to the plasmid, pYe (*CDC10*) 1 (and by inference to plasmid pYe 46B2). Subcloning experiments showed that a 1.6 kb DNA segment from pYe (*CDC10*) 1 contained all of the centromere activity (pYe (*CEN3*) 41, see Figure 1).

Similarly, tbe centromere (*CEN11*) from yeast chromosome XI has been isolated by examining flanking regions occurring near the centromere-linked *MET14* gene (Fitzgerald-Hayes, Buhler, Cooper and Carbon, 1982). In addition, Stinchcomb, Mann and Davis (1982) have isolated the centromere (*CEN4*) from chromosome IV of yeast by making use of the centromere-linked *TRP1* gene, and *CEN6* has been isolated by analogous methods (L. Panzeri and P. Phillippsen, personal communication).

The direct selection procedure (Hsiao and Carbon, 1981a) has been used to isolate several as yet unidentified centromeres in our laboratory, and *CEN3* (Stinchcomb *et al.*, 1982) and *CEN5* (B. Tye, personal communication). This method has also been used to isolate from organisms other than yeast segments of genomic DNA that possess the property of stabilizing *ARS* vectors replicating in yeast. Mitotic stabilizing sequences (*MSS*) of this type have been isolated from the nematode, *Caenorhabditis elegans* (D. Stinchcomb, personal communication), and from the unicellular algae, *Chlamydomonas reinhardtii* (W. Cipriano and J. Carbon, unpublished results). Any relationship these *MSS* elements might bear to the centromeres in the parent organisms has not been established, however.

Localization of centromere activity on cloned DNA segments

Relatively short segments of double-stranded DNA that contain the *CEN3* and *CEN11* activities have been obtained by subcloning various restriction fragments from plasmids with larger DNA inserts, and testing for the mitotic stabilizing activity in an *ARS* vector (Fitzgerald-Hayes, Buhler, Cooper and Carbon, 1982; Fitzgerald-Hayes, Clarke and Carbon, 1982). In this manner, *CEN3* activity was localized to a 627 bp *Bam*HI-*Sau*3A restriction fragment (cloned into the vector YRp7′ to give pYe (*CEN3*) 30). Similarly, *CEN11* activity was subcloned on an 858 bp *Bam*HI-*Sau*3A restriction fragment (also in YRp7′ to give pYe (*CEN11*) 5).

The mitotic stabilizing activity of these smaller DNA fragments is quite similar to that of longer fragments derived from the centromere regions of chromosomes III and XI (Figure 2). When plasmid segregation is measured over a relatively short period of time (5 generations of growth

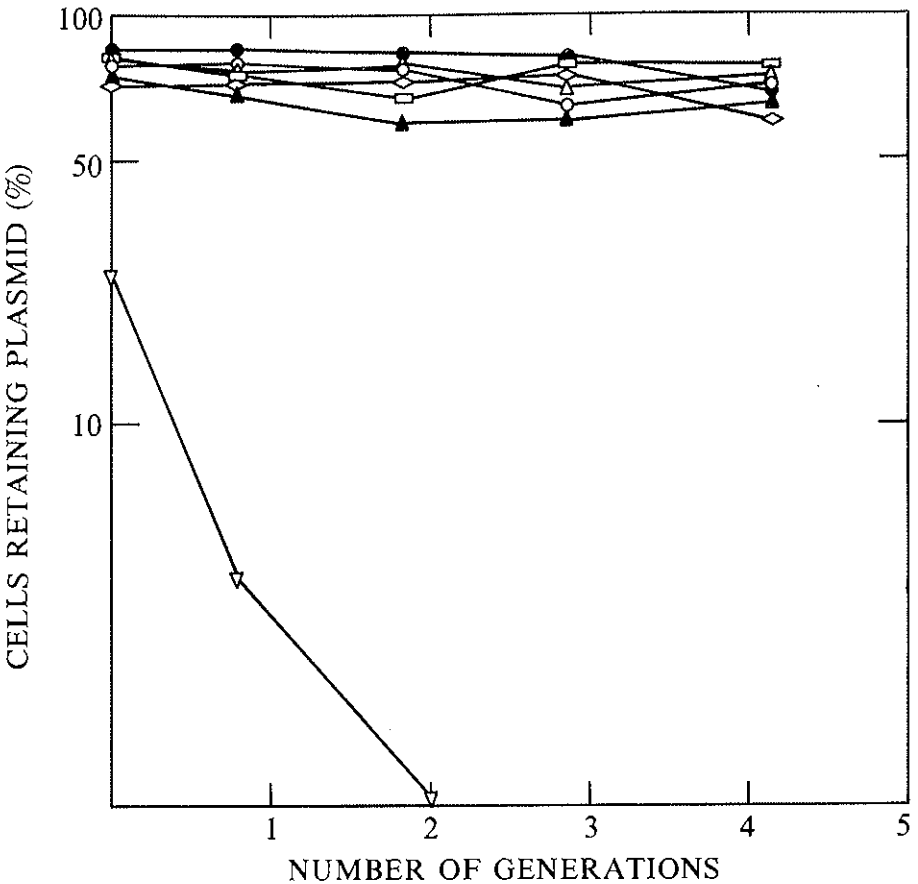

FIG. 2. Mitotic stabilities of various centromere-containing plasmids in yeast. The mitotic stabilities of plasmids pYe (*CDC10*) 1 (●); pYe (*CEN3*) 30 (◇); pYe (*MET14*) 2 (○); pYe (*MET14*) 27 (△); pYe (*CEN11*) 12 (▲); and pYe (*CEN11*) 10 (□) were compared to plasmid YRp7′ (▽) in yeast strain J17. See Fitzgerald-Hayes, Clarke and Carbon (1982) for details of the experimental procedure. See Table 1 for the sizes in kb of the *CEN* DNA fragments cloned in these plasmids. (Taken from Fitzgerald-Hayes, Clarke and Carbon, 1982).

in a non-selective medium), no significant differences can be seen in the mitotic stabilities of plasmids containing various sizes of DNA inserts (e.g., pYe (*CDC10*) 1, 8 kb insert; pYe (*CEN3*) 30, 627 bp insert; see Figure 2 for further details). However, if the yeast strains bearing *CEN* plasmids are grown for longer periods of time non-selectively, significant small differences in plasmid stability can be observed. For example, plasmid pCH4

contains a 7 kb insert (in YRp7) that contains roughly equal lengths of flanking region DNA on both sides of the 627 bp *Bam*HI-*Sau*3A *CEN3* fragment (Hsiao and Carbon, 1981b). Plasmid pCH4 is lost from budding yeast cultures at a rate of 1.6% per generation, whereas pYe (*CEN3*) 30 is lost at 3.5% per generation and YRp7 is lost at 12.9% per generation (W. Cipriano, unpublished data). The exact significance of these results is unclear, however, since other factors such as the overall size of the plasmid could have small effects on plasmid stability.

The relationship between the size of the *CEN3* DNA insert and the meiotic segregation behaviour of the resulting minichromosomes is shown in Table 1. Regardless of insert size down to the small 627 bp insert present in pYe (*CEN3*) 30, when the plasmid segregates 2+:2−, it appears in sister spores, indicating that the minichromosome genetic marker is segregating as expected for a centromere-linked gene. However, the relative proportion of 4+:0− and 3+:1− tetrads increases in crosses involving minichromosomes with the smaller *CEN* inserts (Table 1). This could be due to nondisjunctive events occurring during growth of the diploid prior to sporulation, which would give rise to the presence of parent cells containing multiple copies of the minichromosomes, or to some defect in copy number control.

Structural properties of yeast centromeres: Nucleotide sequences

The nucleotide sequences of the 627 bp *CEN3* and 858 bp *CEN11* DNA fragments have been determined (Fitzgerald-Hayes, Clarke and Carbon, 1982). These sequences are shown in Figures 3 and 4.

When the two sequences are compared, no long regions of perfect sequence homology are found. A prominent feature of both sequences, however, is an extremely (A+T)-rich region approximately 90 bp in length (93% A+T in *CEN3* and 94% A+T in *CEN11*), designated sequence element II in Figures 3 and 4. This high A+T region is bounded on both ends by short sequences that are completely homologous in the two centromeres (termed sequence elements I and III in Figure 3 and 4). A third region of short homology (element IV) occurs about 250 bp to the right of element III in both sequences. The element I-IV region is compared in Figure 5. An overall sequence homology of 71% is seen in the element I through III regions of these two centromeres.

Highly significant homologies were found among the element I area

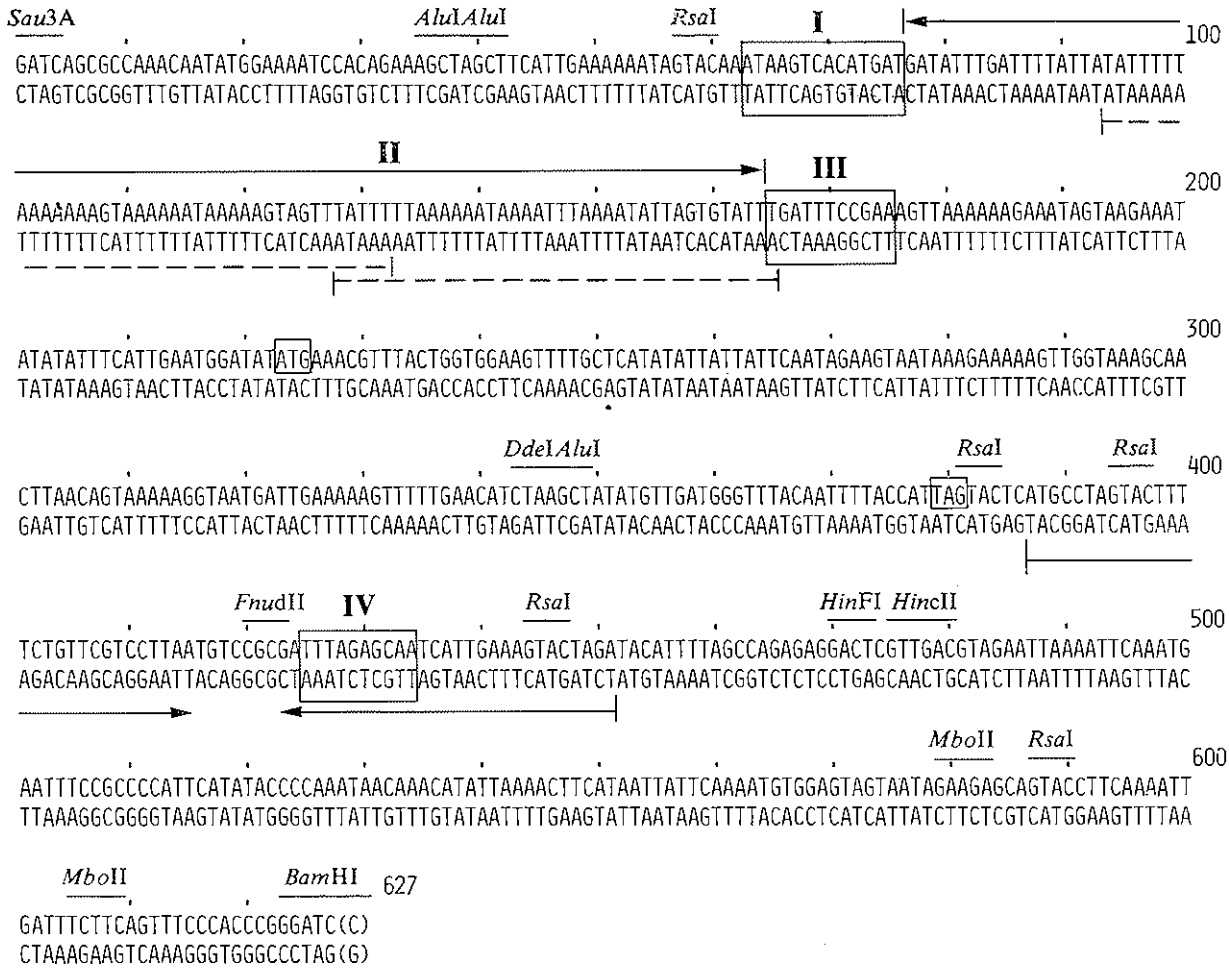

FIG. 3. Nucleotide sequence of the centromere region from yeast chromosome III. The sequence shown is that of the 627 bp fragment cloned in pYe (*CEN3*) 30. The sequence is numbered along the upper strand, from 5′ to 3′ (left to right). Short lines above the sequence indicate restriction enzyme cleavage sites. The boxes enclose regions of perfect homology between *CEN3* and *CEN11*, referred to as sequence elements I, III and IV. Element II is the extremely high AT region flanked by elements I and III. The dotted lines indicate the most significant internal homology within the sequence; the arrows indicate a region of dyad symmetry. The largest open reading frame extends from positions 223 to 379. (Taken from Fitzgerald-Hayes, Clarke and Carbon, 1982)

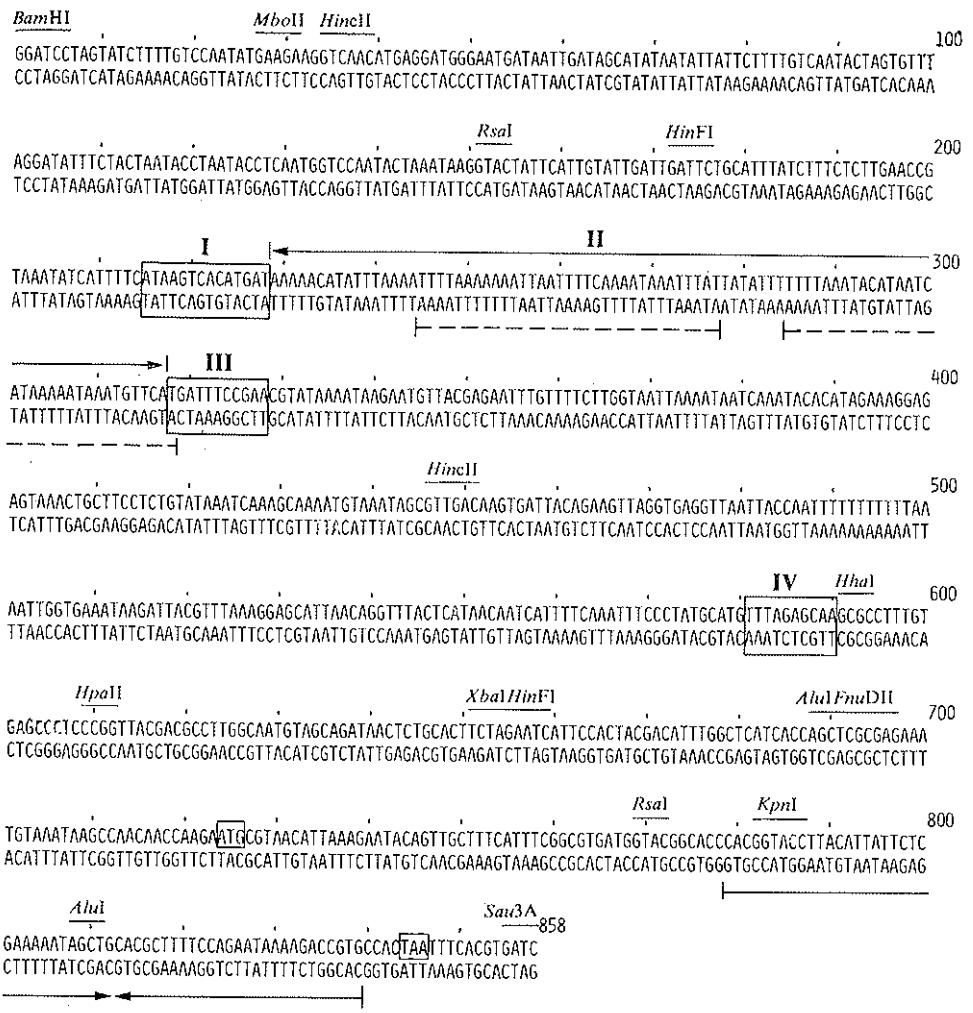

FIG. 4. Nucleotide sequence of the *CEN11* region. The sequence of the 858 bp DNA fragment cloned in pYe (*CEN11*) 5 and pYe (*CEN11*) 10 is shown. For explanation of the various symbols, see Figure 3. (Taken from Fitzgerald-Hayes, Clarke and Carbon, 1982)

of *CEN3* and various satellite (highly repetitive) DNAs from higher eukaryotic organisms (Fitzgerald-Hayes, Clarke and Carbon, 1982). The biological significance of these coincidences of sequence remains unclear. Although satellite DNA sequences are often located in the regions flanking the centromeres of higher eukaryotic chromosomes, yeast DNA contains

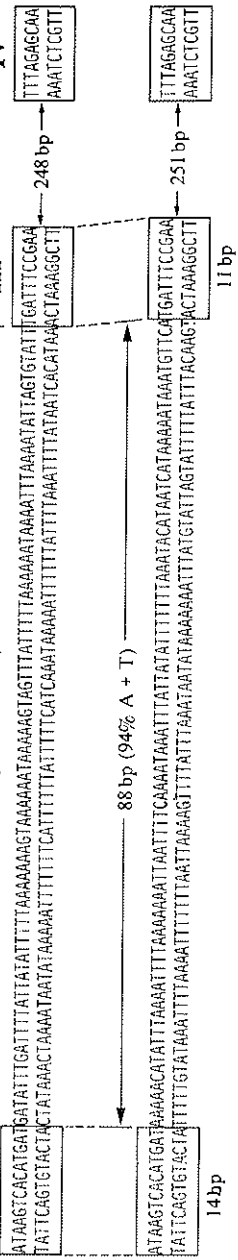

FIG. 5. Spatial relationship of sequence elements common to *CEN3* and *CEN11*. An overall homology of 71% is seen in the element I through III regions of *CEN3* and *CEN11*. (Taken from Fitzgerald-Hayes, Clarke and Carbon, 1982)

no repetitive sequences of this type. It is possible that an evolutionary relationship exists between certain satellite sequences in higher eukaryotes and sequences from the centromere region of yeast.

Structural properties of yeast centromeres: Chromatin structure

Recent studies in our laboratory have shown that a region of approximately 250 bp of DNA containing the element I through III region is in a unique conformation in the yeast chromatin, as distinguished from the usual 160 bp nucleosomal spacing (Bloom and Carbon, 1982). When yeast nuclei are digested with micrococcal nuclease or pancreatic DNAase I and the purified DNA fragments are separated electrophoretically and blotted onto nitrocellulose filters, the centromeric nucleosomal subunits are resolved into significantly more distinct ladders than are those from the bulk of the chromatin. In these experiments, the centromeric nucleosomal subunits are visualized by hybridization with a radioactively labeled DNA fragment containing the centromere. The nucleosomes in the centromere region appear to be uniformly spaced for a distance of about 2.0-2.5 kb surrounding the element I-III region (see Bloom and Carbon, 1982 and Figure 7 for details).

The nucleosome mapping method of Wu (1980), an indirect end labeling technique, was used to map the exact location of nuclease-sensitive sites in the centromere area (Bloom and Carbon, 1982). In this method, the chromatin is digested under partial conditions with either micrococcal nuclease or DNAase I, and, after deproteinization, the DNA is digested with a restriction enzyme that cleaves at a site adjacent to the area being mapped. After separation of the fragments by gel electrophoresis and blotting onto nitrocellulose, fragments from the desired region are visualized by hybridization to a labeled DNA probe homologous to the region immediately proximal to the chosen restriction site.

The results of a typical mapping experiment of the type described above are shown in Figure 6. In this experiment, the spacing of nuclease-sensitive sites in the chromatin is measured with respect to the *Hind*III site located about 1300 bp from the element I-III area of *CEN3* (see the restriction site map shown in Figure 6). The experiment indicates that a 250 bp region of DNA located between 1250 and 1500 bp in a centromere-proximal direction from the *Hind*III site is completely resistant to digestion by the nuclease, and this region is flanked on both sides by hypersensitive

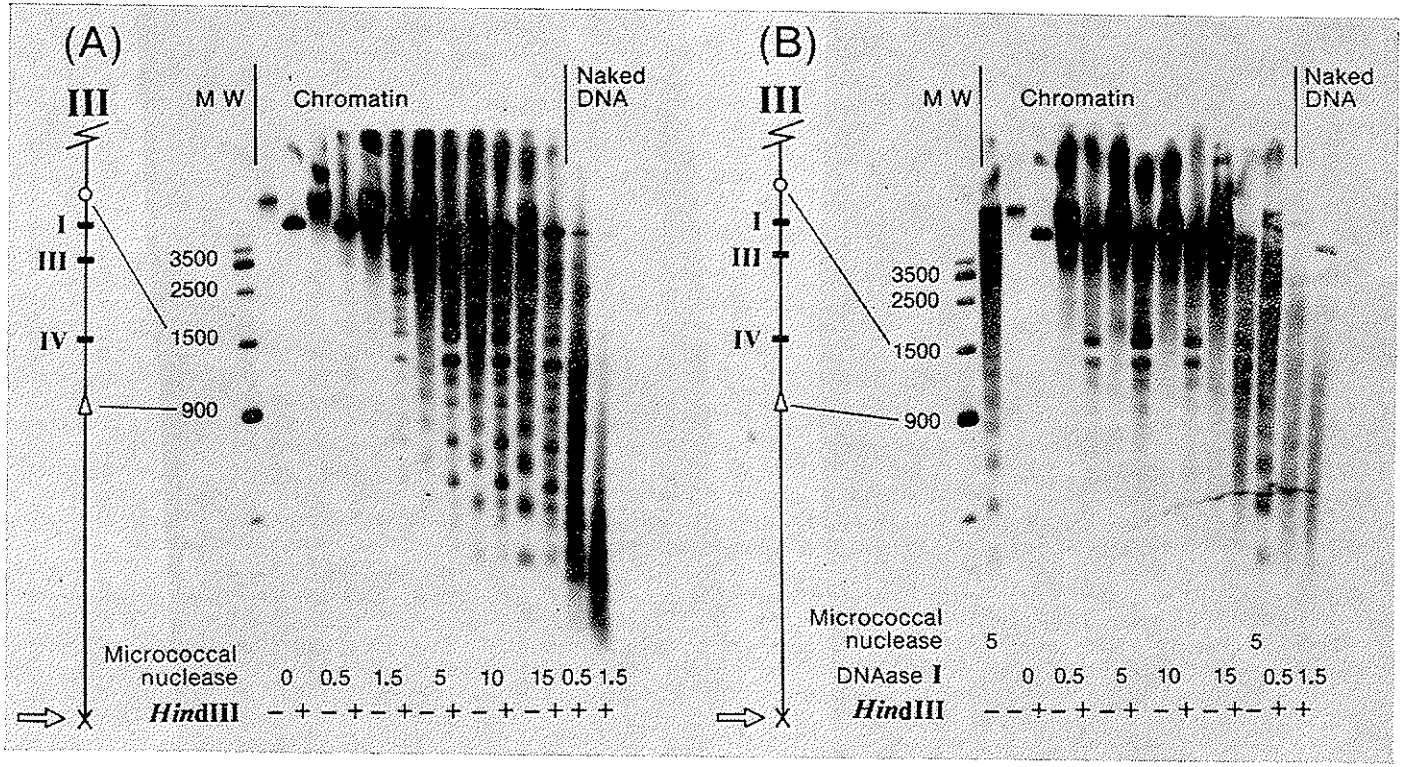

FIG. 6. Mapping nuclease-sensitive sites on the centromeric chromatin from yeast chromosome III. Intact yeast nuclei were digested with micrococcal nuclease (A) or DNAase I (B) for the times (minutes) as indicated. After deproteinization with phenol, the DNA samples were incubated in the presence (+) or absence (−) of restriction endonuclease *Hind*III, and fractionated by electrophoresis through 1.4% agarose gels. The DNAs were transferred to nitrocellulose filters and visualized by hybridization with a ^{32}P-labeled 900 bp DNA fragment that includes the region between the *Hind*III and *Bam*HI sites as shown. See Bloom and Carbon (1982) for details of the experimental procedures. To the left is shown a restriction map of the centromere region of yeast chromosome III; *Bam*HI (△), *Sau*3A (○), *Hind*III (×). The large arrows indicate the *Hind*III site on chromosome III immediately proximal to the labeled 900 bp probe. (Taken from Bloom and Carbon, 1982)

cleavage sites. This specific pattern of cleavage sites is not seen when naked deproteinized DNA is subjected to the same treatment. Significantly, the sequence element I-III region is included within the 250 bp protected region. Regardless of the restriction site used as reference point for the nucleosome mapping experiments, the unique 250 bp protected region is seen to occur at the same position surrounding the element I-III region (Bloom and Carbon, 1982).

Nucleosomal mapping experiments of the type described above have shown that the centromere areas of both chromosome III and XI maintain this unique conformation whether present in the genome or on autonomously replicating plasmids in yeast cells. In addition, an examination of plasmids in which foreign DNA sequences have been substituted for the normal centromere flanking sequences has shown that the highly phased nucleosomal organization seen on both sides of the 250 bp protected core is a property of the normal flanking DNA and is not propagated through the foreign DNA (Bloom and Carbon, 1982).

A schematic representation of the centromere region of yeast chromosome XI is presented in Figure 7. The highly phased 160 bp nucleosomal subunits extend about 1.5 kb on one arm of the chromosome, but only for a distance of 0.5 kb on the other arm, where the *MET14* gene occurs closely adjacent to the centromere. Since deletion analysis has shown that all or a portion of the sequence element I-III area is essential for activity of the centromere in terms of maintaining stable segregation of the minichromosomes (see below), we have suggested that the unusual 250 bp protected region is a type of kinetochore to which the spindle fiber attaches during mitotic cell division (Bloom, Fitzgerald-Hayes and Carbon, 1982).

The structural and functional boundaries of the yeast centromere

Preliminary deletion analysis has been carried out in an attempt to delineate the structural and functional boundaries of the yeast centromere region (Bloom, Fitzgerald-Hayes and Carbon, 1982). For example, the plasmid pYe (*CEN11*) 5, containing an 858 bp *Sau*3A fragment that includes *CEN11*, was digested with nuclease *Bam*HI, thus converting the DNA to a full-length linear molecule. This cleavage point is immediately adjacent to the sequence element I-III area and to the "left" of element I (see Figure 8). The linear DNA was digested with the exonuclease, *Bal31*, which trims away double-stranded DNA from both ends of the linear

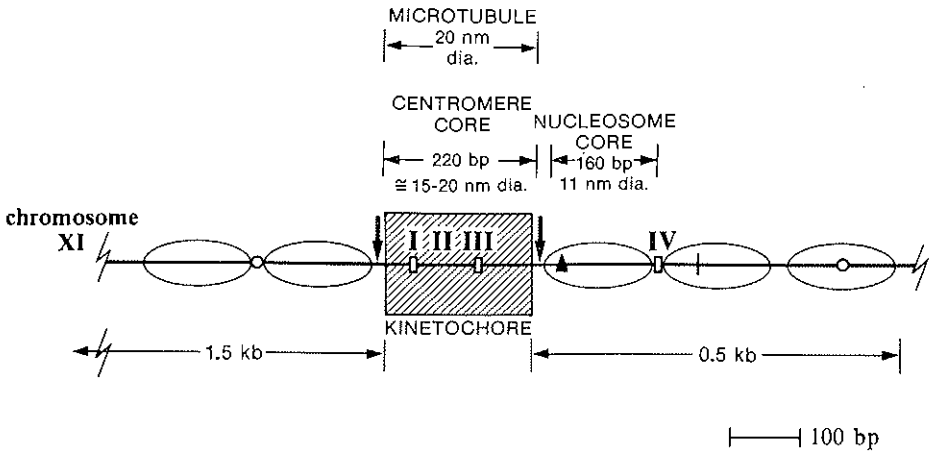

FIG. 7. Schematic representation of the centromere region of yeast chromosome XI. The diagram shows the relative positions of the sequence elements I, III, and IV (see Figure 5). The high AT region (II) lies between elements I and III. Restriction enzyme sites are *Sau*3A (○), *Hinc*II (▲), and *Hinf*I (+). Micrococcal nuclease and DNAase I hypersensitive cleavage sites that bound the nuclease-resistant centromere core (shaded box) are indicated by the arrows. The DNA fiber is presented in linear form to visualize the position of the 220-250 bp nuclease-resistant core and the nucleosome cores relative to the restriction map of the DNA. In chromatin the nucleosomal DNA is wrapped around the histone proteins to form a roughly cylindrical particle about 11 nm in diameter (McGhee and Felsenfeld, 1980). By extrapolation, we estimate the centromere core particle to be at least 15-20 nm in diameter, however the conformation of this structure is unknown. The arrows below the restriction map indicate the distance that the ordered nucleosomal arrays extend from the *CEN* core. (Taken from Bloom, Fitzgerald-Hayes and Carbon, 1982)

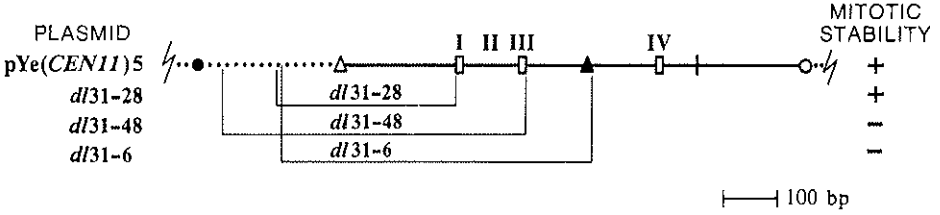

FIG. 8. Diagrammatic representation of deletion mutants constructed in pYe (*CEN11*) 5. The relevant portion of plasmid pYe (*CEN11*) 5 containing the 858 bp *CEN11* DNA fragment from chromosome XI is indicated. Sections of DNA removed by the deletions are designated by the brackets. Solid lines indicate yeast DNA, dotted lines indicate vector pBR322 sequences. Restriction sites are *Bam*HI (△); *Sau*3A (○); *Sal*I (●); *Hinf*I (+); and *Hinc*II (▲). The deletion in *dl*31-28 extends to the leftward boundary of element I (see Figure 5); the deletion in *dl*31-48 extends to the rightward boundary of element III. (Taken from Bloom, Fitzgerald-Hayes and Carbon, 1982)

molecule. After religation to form circular double-stranded DNA molecules, the plasmids containing deletions of various lengths surrounding the *Bam*HI site were recovered as individual clones by transformation of *E. coli*. Nucleotide sequence analysis revealed that deletion 31-28 extends immediately up to the leftward boundary of element I, but the element I-III area is intact. Deletion 31-48 extends completely through the I-III area, terminating at the rightward boundary of element III; 31-6 extends past element III with a boundary in the region between III and IV (see Figure 8).

The mitotic segregation rates of plasmid pYe (*CEN11*) 5, the three deletion plasmids, and the control plasmid, YRp7, were determined as shown in Figure 9. The results were very clear; only the plasmids with an intact element I-III region (pYe (*CEN11*) 5 and *dl*31-28) retained the mitotic stability characteristic of centromere-bearing plasmids. The plasmids in which the element I-III region had been deleted were as mitotically unstable as is plasmid YRp7, which completely lacks a centromere. This experiment does not establish that the entire I-III region is necessary for the mitotic stability function, however, since we still lack deletions that terminate between I and III. Since the exonuclease *Bal*31 proceeds very rapidly through high A+T DNA, other methods might have to be used to examine this question further.

Deletion plasmid 31-28 segregates meiotically in a manner very similar to the parent plasmid pYe (*CEN11*) 5. However, as shown below, centromere plasmids with small *CEN* inserts tend to maintain a copy number between 1 and 2 in yeast cells, leading to a relatively high percentage of 4+:0− distributions in tetrads resulting from crosses where only one of the parents carries the plasmid. When the plasmid does give a 2+:2− distribution, however, the plasmid genetic marker shows the expected centromere linkage and appears only in sister spores.

Similar deletion studies with plasmids containing *CEN3* have completely confirmed the above results; the sequence element I-III region (or a portion thereof) is absolutely essential for centromere activity. Deletion plasmids showing partial function have not been observed, although, as stated above, the plasmids with smaller inserts can be seen to be somewhat more unstable mitotically when segregation rates are measured over many generations.

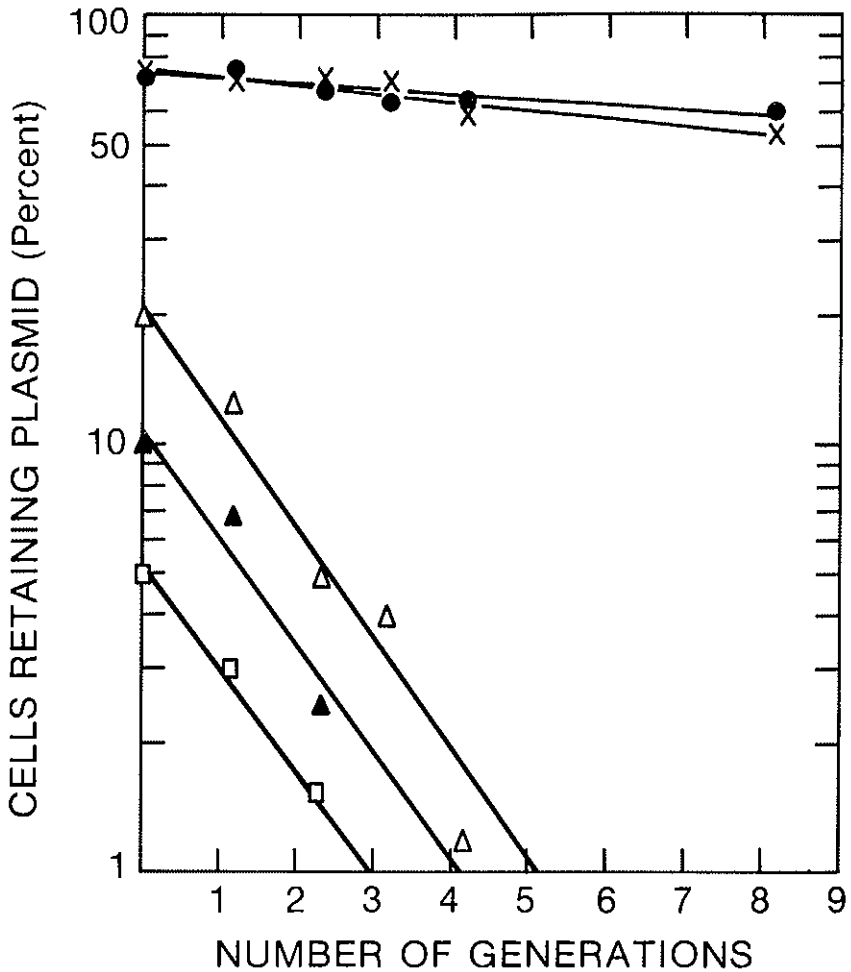

FIG. 9. Mitotic stabilities of pYe (*CEN11*) 5 and various deletion mutant plasmids in yeast. The mitotic stabilities of plasmids pYe (*CEN11*) 5 (●), *dl*31-28 (×), *dl*31-48 (△), and *dl*31-6 (□) were compared to the parent plasmid YRp7′ (▲) in yeast strain J17. See Bloom, Fitzgerald-Hayes and Carbon, 1982, for details of the experimental procedure. Deletions that remove the element I-III region inactivate the centromere with regard to its ability to stabilize the YRp7′ vector. (Taken from Bloom, Fitzgerald-Hayes and Carbon, 1982)

Copy number control by the centromere

An extremely interesting, but as yet unexplained, property of the centromere is its apparent ability to control the copy number of autonomously replicating plasmids to a low value, usually one or two copies per cell. This is true even if the plasmid contains a replicator system that normally produces a high copy number, as in the yeast 2-micron plasmid (G. Tschumper and J. Carbon, unpublished results).

Recently, we have measured the copy numbers of various plasmids replicating in yeast cells (Bloom, Fitzgerald-Hayes and Carbon, 1982). These measurements were carried out by the genomic Southern blot technique (Southern, 1975), using as hybridization probe a DNA segment that would hybridize both to the plasmid and to a region in the genome that is known to be single copy. Measurement of the relative intensities of the two hybridization signals gives the plasmid copy number (see Bloom *et al.* (1982) for details). Under the conditions used, plasmid YRp7 is present at an average copy number of about 25-50 per cell, but is present in only a small percentage of the total cell population. The centromere-bearing plasmid, pYe (*MET14*) 27, with a 3.4 kb *CEN11* insert, is present at one copy per cell. Plasmids pYe (*CEN11*) 5 and *dl*31-28 are present in approximately one to two copies per cell, whereas the inactive deletion plasmids, *dl*31-48 and *dl*31-6 (see Figure 8), are maintained at 50 and 25 copies per cell, respectively, but are present in only about 10% of the cell population.

Similar studies using plasmids containing all or a portion of the 2-micron replicator plus *CEN3* have shown that the centromere is dominant with regard to copy number control (G. Tschumper and J. Carbon, manuscript in preparation). Whereas the 2-micron plasmids are normally maintained at copy numbers ranging from 20 to 50 per cell, the hybrid *CEN* 2-micron vectors are stably maintained at about one to two copies per cell. Thus, the centromere in some way counteracts the function of the 2-micron genes, *REP1* and *REP2*, which are thought to be responsible for the high copy number of the yeast plasmid (Jayaram, Li and Broach, 1983). The molecular mechanism of this unique effect of the centromere is currently under investigation in our laboratory.

Summary and conclusions

The yeast centromere is a relatively small structure containing an essential core of at most only a few hundred hase pairs of DNA. Since it is known that yeast chromosomes attach to only a single spindle fiber at the centromere (Peterson and Ris, 1976), we postulate that the 250 bp region of DNA containing sequence eleuents I-III and existing with a unique conformation in the chromatin is the yeast kinetochore, a hinding site for the spindle fiber. This region is ahsolutely essential for proper centromere function *in vivo*: deletion of elements I-III results in inactivation of the centromere.

Centromere-bearing plasmids (minichromosomes) are maintained in controlled low copy numher, by a mechanism as yet unexplained. This copy number control is in some way dominant over the *REP1* and *REP2* functions of the yeast 2-micron plasmid. Since, in higher eukaryotes, the sister chromatids of replicated chromosomes are held together at the centromere until anaphase occurs late in mitosis, it is possible that the centromere maintains a block on DNA replication that is not released until the sister chromatids separate and cell division (or budding) occurs. Two DNA replication blocks would be necessary, one on each side of the centromere core area, which would be replicated early to offer duplicate attachment points for the opposing spindle fibers. This replication block system, in conjunction with a mechanism to prevent multiple initiation events at the origins of DNA replication, would help to insure stable maintenance of chromosomes in controlled low copy number.

Although the circular minichromosomes containing small *CEN* DNA fragments possess many of the properties usually associated with normal chromosomes, a few important differences should be noted. The most apparent of these involves the mitotic stability of the minichromosomes; they are unusually stable when compared with *ARS* plasmids, but actually quite unstable when compared to intact aneuploid chromosomes in yeast. The minichromosomes undergo nondisjunction at the rate of about 1-3% per generation, whereas an extra copy of a yeast chromosome (an n+1 or 2n+1 aneuploid) is lost at rates at least one to two orders of magnitude lower (Campbell *et al.*, 1981). Several possible reasons for this discrepancy could be proposed: the minichromosomes are extremely small, they are circular rather than linear, they lack telomeric attachment sites, or the *CEN* DNA fragments that we have worked with represent only incomplete portions of the true centromere. We feel that the latter possihility is

unlikely; many of the DNA fragments we have used contain defined genes on both sides of the active *CEN* area, although the possibility of stabilizing sequences interspersed between structural genes along the chromosome arms cannot be excluded.

Another important distinction between normal chromosomes and the synthetic minichromosomes lies in the area of meiotic pairing. Minichromosomes containing *CEN3*, for example, do not pair with either of the normal chromosomes III in meiosis, as evidenced by the complete absence of pairs of dead sister spores in the tetrads (Clarke and Carbon, 1980b). This could be due to the circular structure of the minichromosomes or to lack of sufficient DNA sequence homology, although, again, the existence of hypothetical pairing sequences distributed along the chromosome arms is a possibility. Experiments in progress in several laboratories should eventually clarify these important issues.

The principal value of the yeast minichromosomes lies in their use as a model experimental system for the study of the mechanism of chromosome segregation and cell division in higher eukaryotes. They are currently being used as probes for the isolation and characterization of proteins that bind selectively to the centromere DNA (for example, see Bloom, Fitzgerald-Hayes and Carbon, 1982). Eventually, it should be possible to define all of the molecular components necessary for proper spindle fiber attachment to the centromere and for chromosome segregation. Similar experimental strategies will also be used to isolate and study centromeres from the chromosomes of higher eukaryotic cells.

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GENETIC ENGINEERING IN LABORATORY MAMMALS

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INTRODUCTION

Our perception of the mammalian embryo has changed drastically in the last couple of decades: Starting with the view that the developmental plan is laid out in a fixed and irrevocable fashion shortly after cleavage begins, we have progressed to the realization that the opposite is true, that there is in fact great flexibility for a considerable period of time. Thus, we can easily separate the early blastomeres in a mouse embryo and obtain from each cell a complete and normal individual. Or all the cells from two (or more) entire cleavage-stage embryos may be aggregated *in vitro* to produce a giant embryo that will become, after implantation *in vivo*, a single normal-size individual in which two unrelated genetic subpopulations of cells are harmoniously integrated (Mintz, 1965, 1978b). We even know that the ectoderm cells of the embryo are still capable, for a few days after implantation, of generating the major tissue types in tumors formed after transplantation to an ectopic site (Diwan and Stevens, 1976). Even more remarkable, we know that cells from some malignant teratocarcinomas can be made to participate in normal embryogenesis (Mintz and Illmensee, 1975). And, from recent experiments, we have learned that it is surprisingly easy to introduce foreign pure recombinant DNA directly into the egg without perturbing development, and subsequently to derive new strains of mice with those foreign genes (e.g., Stewart *et al.*, 1982). The genetic material itself is now known — from evidence that will not be reviewed here — to have substantial lability.

With this degree of flexibility of early mammalian development and of the genome, genetic manipulations made possible by modern DNA technology now enable us to change the genes or the expression of genes in laboratory mice, and presumably in other mammals as well. Such experimental changes in laboratory animals may supply us with the tools needed to analyze *in vivo* the mechanisms underlying tissue-specific orderly expression of genes during differentiation. They may also enable us to produce animal models of human genetic diseases in order to dissect the etiology of those diseases and to test possible cures.

Introduction of recombinant DNA sequences into mice

The first successful attempt to introduce foreign DNA into a mammal was carried out by microinjection of purified simian virus 40 (SV40) DNA into the fluid-filled cavity of early mouse embryos at the blastocyst stage (Jaenisch and Mintz, 1974). The aims were to investigate the mechanisms of incorporation of tumor viral genetic material, its vertical transmission, and, ultimately, control of its expression. The DNA from some tissues in approximately 40% of the mice produced from the injected embryos contained SV40-specific DNA sequences, as judged from reannealing kinetics in molecular hybridization tests with a radiolabeled SV40 DNA probe.

With the more recent recombinant DNA technology, it is now possible to introduce any cloned gene in intact or specifically modified form. Injection into the uncleaved fertilized egg, rather than the blastocyst, would be expected to increase the number of tissues in which the donor genetic material might be found. The pronucleate egg provides a convenient stage for injection of DNA before cleavage.

A bacterial plasmid obtained from T. Maniatis and used in our first series of injections (E. Wagner *et al.*, 1981) contained the human β-globin gene (Fritsch *et al.*, 1980), which would be expected to have tissue-specific expression in erythroid cells; and the herpes simplex viral (HSV) thymidine kinase (*tk*) gene, which should be constitutively expressed. Both genes were ligated in the pBR322 plasmid designated PtkHβ1. The vector included the 7.6-kb *Hind*III fragment of the human adult genomic β-globin gene (including the entire gene plus approximately 6 kb of flanking sequences) and the 3.6-kb *Bam*HI fragment of the HSV *tk* gene.

Approximately 2,500-3,000 copies of the DNA were injected into the male (larger) pronucleus and the eggs were surgically transferred to the

oviducts of pseudopregnant females. In the first series, 33 animals were examined in late fetal stages. The high-molecular-weight DNA of five individuals (15%), and of their corresponding placentas, was positive for the foreign globin and *tk* genes (and also for pBR322 sequences). The five positive fetal-placental pairs contained 3-50 copies per cell of the human β-globin gene, of which the 4.4-kb *Pst* I diagnostic fragment spans the entire coding region; and 3-20 copies per cell of the 3.6-kb *Bam*HI fragment of the HSV *tk* gene (E. Wagner *et al.*, 1981).

Both the globin and *tk* genes were present as intact copies and, in some individuals, also in higher-molecular-weight DNA fragments. These larger fragments probably arise as a result of changes such as deletions and duplications. Such rearrangements could involve breakage and loss of at least one specific restriction site and utilization of a corresponding cleavage site in adjacent host DNA. The evidence is therefore consistent with occurrence of integration of the foreign sequences into mouse chromosomal DNA.

A later series of postnatal mice from eggs injected with DNA from the same plasmid resulted in animals in which the donor gene sequences had persisted into adult life in stable form in all somatic tissues tested (Figure 1). In one animal, the intact human and viral genes were found in its tissue DNA; in another, at least some of the 3′ sequences flanking the coding region of the human β-globin gene were present. Transmission of these sequences occurred through the germ line of the latter individual, to approximately half of its progeny. This frequency is indicative of a Mendelian pattern of inheritance for a single gene at a heterozygous locus and is strong evidence for integration in host chromosomal DNA (Stewart *et al.*, 1982).

Among the five prenatal animals that were positive for the HSV *tk* gene, one clearly positive case of function of that gene was identified (E. Wagner *et al.*, 1981). An initial test was based on differences in substrate specificity of HSV and mouse *tk* enzymes; the result was confirmed in an independent test involving enzyme neutralization with antiserum specifically directed against the HSV-type of enzyme. Thus, the donor gene was mediating production of its specific functional protein *in vivo*, even in the absence of any selective pressure or experimental induction.

Successful introduction and apparent integration and germ-line transmission of recombinant DNA from other species into mice, after microinjection into eggs, has also recently been reported for the rabbit β-globin gene (Costantini and Lacy, 1981), the HSV *tk* gene fused to the promoter

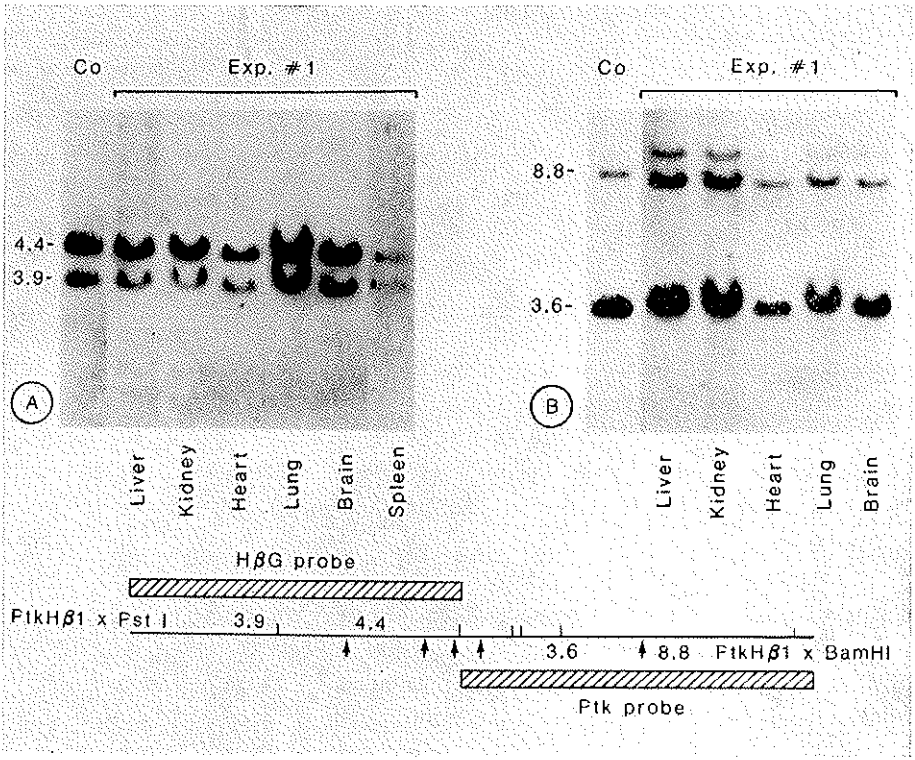

FIG. 1. The human β-globin (HβG) and herpes simplex viral (HSV) thymidine kinase (tk) genes in various tissues of an adult mouse (Exp. #1). The animal was derived from a fertilized egg injected with DNA of a recombinant plasmid (PtkHβ1) containing both genes. (A) Hybridization of tissue DNA (after *Pst* I digestion) with a ^{32}P-labeled HβG probe demonstrates the two diagnostic fragments (4.4 kb and 3.9 kb) spanning the entire coding region of the HβG gene. (B) Hybridization (after *Bam* HI digestion) with a labeled probe representing the remainder of the plasmid reveals the intact HSV tk gene sequences (as 8.8. kb and 3.6 kb bands) and also a higher molecular weight band absent from the plasmid DNA (Co lane) and indicative of integration into mouse chromosomal DNA. (From Stewart *et al.*, 1982).

of the mouse metallothionein gene (Brinster *et al.*, 1981), and some SV40, HSV *tk*, and human interferon sequences (Gordon and Ruddle, 1981). The fusion gene comprising the foreign *tk* also gave evidence of function (Brinster *et al.*, 1981). In another report on injection of the rabbit β-globin gene (T. Wagner *et al.*, 1981), preliminary data were presented for expression of the rabbit gene. However, a Southern blot analysis of the DNA was carried out for only one experimental mouse and the hybridization

revealed only internal sequences of the probe; there were no changes in the flanking regions, of the sort that might indicate the presence of integrated (rather than free) DNA of the donor type.

Experiments with introduction of recombinant DNA into mouse eggs have thus had a very promising beginning. They have led to retention and transmission of foreign genes, as well as some gene expression. It remains to be seen whether integration into a favorable chromosomal site will play a major role in the usefulness of this approach for analyzing control of tissue-specific expression of genes.

Teratocarcinoma stem cells as vehicles for genetic engineering of mice

Mouse teratocarcinoma stem cells are apparently the malignant counterparts of normal early somatic embryo cells (Mintz, 1978a; Mintz *et al.*, 1978) whose proliferation has been sustained while their differentiation has become restricted and chaotic. Various tissues are found in the solid tumors formed when the cells are grown subcutaneously in a syngeneic host; however, these tissues are usually immature and there are many types that are never present (Stevens, 1967). The fact that the differentiated cells are derived from the primitive stem cells was demonstrated by the range of types obtained after single cells were transplanted to adult recipients (Kleinsmith and Pierce, 1964). The stem cells are capable of being propagated as transplant lines *in vivo* (either in the solid or modified-ascites form) and also as cell culture lines.

If the stem cells were in fact the neoplastic derivatives of developmentally totipotent embryo cells, it seemed possible that their differentiation might become more normal if they were in the company of the corresponding normal stem cells, i.e., if they were placed in early embryos. Such an experiment would be comparable to the production of allophenic mice — animals with cells of different genotypes, produced by bringing together blastomeres from two different embryos (Mintz, 1965, 1974). In this case, however, the teratocarcinoma stem cells would be used instead of one of the normal embryonic contributors.

The experiment was carried out by injecting tumor stem cells from a karyotypically normal *in vivo transplant* line into blastocysts of another strain identifiable by numerous genetic markers. The tumor cells were indeed stably "normalized" by the accompanying embryo cells (Figure 2)

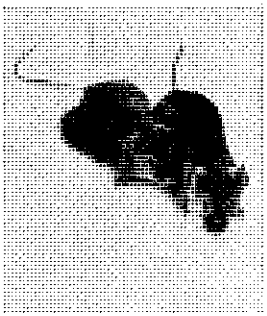

FIG. 2. Orderly differentiation *vs.* malignant proliferation of mouse teratocarcinoma cells as a function of the tissue environment *in vivo*. The normal animal on the *right* was derived from a blastocyst (C57BL/6 black strain) injected with teratocarcinoma stem cells (129 agouti strain). In all of its tissues, including the largely agouti-colored coat, tumor-lineage cells predominated and developed normally. However, when tumor stem cells were injected subcutaneously into a syngeneic adult, the cells formed a large tumor (*left*). (From Mintz, 1978a.)

after almost a decade of serial transplantation in the malignant state (Mintz and Illmensee, 1975; Cronmiller and Mintz, 1978). The neoplastic defect was evidently one of aberrant control of gene expression. Tumor-lineage cells were able to contribute, in the best cases, not only to all somatic tissues (along with embryo-lineage cells) but also to functional germ cells and, therefore, to progeny in which the tumor-cell genome was perpetuated. Successful but more limited differentiation was obtained in related experiments in which other tumor stem cells contributed to formation of one (Brinster, 1974) or of several (Papaioannou *et al.*, 1975) somatic tissues, but not of all somatic tissues and never to germ cells.

The capacity for normal and complete somatic and germinal differentiation in our experiments formed the basis for a novel experimental scheme: Inasmuch as the stem cells could be grown in culture, they might there serve as "surrogate eggs" in which selected genetic changes could be made at will; after injection of the cells into blastocysts, the change could ultimately be represented in the germ line and lead to new strains of mice of predetermined genotype (Mintz, 1978a,b; 1979). Large numbers of stem cells could be subjected to specific selection or screening procedures *in vitro* and only cells with the gene change of interest would be injected into blastocysts. In the case of DNA transformation, the teratocarcinoma route would have certain advantages over the egg-injection route: Many tumor cells could be treated, and selected or screened; moreover, selected cell clones could be pre-characterized, e.g., to ascertain the chromosomal

site of DNA integration, by means of *in situ* hybridization. However, direct injection of DNA into the egg might still offer the advantage of immediate transition into all cells of an individual.

Among the range of options that the teratocarcinoma experimental system offers is production of animals with murine mutations which would yield mouse models of a corresponding human genetic disease. Production of animals with mutations in mitochondrial genes would also be possible (Mintz, 1979; Watanabe *et al.*, 1978).

An example of a relevant human genetic disease is Lesch-Nyhan disease, due to a severe deficiency of the enzyme hypoxanthine phosphoribosyltransferase (HPRT). The human disorder results from an X-linked recessive defect that is usually fatal in affected males (Lesch and Nyhan, 1964; Seegmiller *et al.*, 1967). By first selecting mutagenized mouse teratocarcinoma stem cells of an *in vitro* culture line for resistance to the purine base analogue 6-thioguanine, an HPRT-deficient cell clone was isolated. When these cells were injected into blastocysts of another strain, mosaic animals were obtained (Dewey *et al.*, 1977). The tumor-strain cellular population (distinguishable by an independent strain-specific isozyme marker) had retained the HPRT defect throughout differentiation. Strong selective pressure against the defective cells in blood of the mosaic animals provided an interesting parallel to selection against cells of the defective phenotype in the blood of human heterozygous female carriers.

The cell line used in this HPRT-mutant experiment, and in all other teratocarcinoma mutagenesis experiments until recently, was not karyotypically normal. The abnormality in the HPRT-deficient case was a trisomy that would be incompatible with production of viable germ-line progeny (Gropp, 1975), although it obviously permitted formation of all somatic tissues (Dewey *et al.*, 1977). We shall return to this important question of the requirement for a euploid culture line of teratocarcinoma stem cells in such experiments.

We have also isolated mutant teratocarcinoma cells deficient in other enzymes, such as adenine phosphoribosyltransferase (Reuser and Mintz, 1979) or thymidine kinase (Pellicer *et al.*, 1980); and have laid some of the groundwork for seeking teratocarcinoma mutants with deficiencies in specific receptor systems, such as the low-density lipoprotein receptor (Goldstein *et al.*, 1979) and the transferrin receptor (Karin and Mintz, 1981).

Transfer of recombinant DNA into teratocarcinoma cells has been accomplished for selectable as well as unselectable genes. In our earlier

experiments, mutant teratocarcinoma cells that were stably deficient in thymidine kinase activity (tk^-) were first isolated. They were then used as recipients, after addition of DNA in a calcium phosphate precipitate, and selection in HAT (hypoxanthine/aminopterin/thymidine) medium, to obtain cell clones that had taken up the foreign HSV *tk* gene (Pellicer *et al.*, 1980), as in previous experiments (Wigler *et al.*, 1977; Wigler *et al.*, 1978) carried out with fibroblast cell lines. Cotransfer of the unselectable human β-globin gene, by inclusion in the precipitate along with tbe HSV *tk* gene, was successful in some tk^+ transformants (Pellicer *et al.*, 1980).

More recently, we have treated tk^- teratocarcinoma stem cells with DNA from the same plasmid vector, PtkHβ1, as was used for DNA injection into the pronucleus of fertilized eggs. In this case, the unselectable human β-globin gene was linked, in the same plasmid, to the selectable HSV *tk* gene. A high transformation efficiency was obtained after selection in HAT (Wagner and Mintz, 1982). Hybridization tests disclosed the presence of intact copies (3-6 copies per cell) of the human gene in the majority of transformants. That these donor sequences were associated with cellular DNA was inferred from the presence of some new high-molecular-weight fragments, seen in Southern blots, and from stability of the donor sequences in tumors resulting from subcutaneous injection of the cells.

In order to test for production of mRNA transcripts of the human gene, total polyadenylate-containing RNA was examined in four of the transformed cultured cell clones. Two of these showed hybridization to the human-gene probe. The RNA species from one of them resembled mature transcripts of the human β-globin gene; the others were larger in size (Wagner and Mintz, 1982). Erythroid development was seen in some of the tumors, although it is not yet known whether any of the hemoglobin in them was attributable to the foreign gene.

Dominant-selection vectors enable recombinant genetic material to be transferred to wild-type cells, thereby circumventing the initial mutagenesis and selection steps (e.g., to obtain tk^- mutant recipient cells). This could help to avoid occurrence of extraneous and undesirable genetic changes during the initial phases of gene transfer into teratocarcinoma cells in culture. The plasmid DNA vectors (pSV-*gpt*, from P. Berg), carrying the *E. coli* bacterial gene for xanthine-guanine phosphoribosyltransferase and some regulatory sequences from SV40, in addition to pBR322 (Mulligan and Berg, 1980, 1981), have provided such an opportunity. We isolated numerous transformants after addition of pSV2-*gpt* DNA in calcium phos-

phate to the cell culture medium and growth in mycophenolic acid, xanthine, hypoxanthine, aminopterin, and thymidine (Wagner and Mintz, 1982). The donor DNA sequences were apparently stably integrated into the stem cells and their differentiated tumor derivatives. We may conclude that any cloned gene can be introduced into teratocarcinoma stem cells, even without mutagenesis of the cells, by cotransfer with, or linked transfer in, such vectors. New hybridization methods allowing *in situ* chromosomal visualization of genes (e.g., Robins *et al.*, 1981) should make it possible to characterize the transformants and to choose those of interest for experiments involving further differentiation in the soma and in the germ line *in vivo*.

A limitation in the teratocarcinoma "portal of entry" scheme has heretofore been the lack of a cell culture line characterized by karyotypic normalcy and also by developmental totipotency. Until recently, the only teratocarcinoma stem-cell sources capable of yielding all somatic tissues as well as germ cells, after injection into blastocysts, were two *in vivo* transplant lines — one chromosomally male (X/Y) and one female (X/X); these generated germ-line progeny in some mosaic phenotypic males and females, respectively (Mintz and Illmensee, 1975; Cronmiller and Mintz, 1978). We have now been able to surmount this difficulty by establishing a cell culture line with the desired properties (Mintz and Cronmiller, 1981). The line originated from a tumor produced by the method (Stevens, 1970) of transplanting an embryo to an ectopic site under the testis capsule, where embryogenesis becomes disorganized and early stem cells can persist. The grafted embryo from which the cell line arose was fortuitously X/X. The cell line has been designated METT-1 (the first Mouse Euploid Totipotent Teratocarcinoma cell line) (Mintz and Cronmiller, 1981).

The developmental potential of these stem cells (of the 129 strain) was assayed by microinjecting them into blastocysts of the C57BL/6 strain (Stewart and Mintz, 1981). Biochemical markers documented the capacity of tumor-lineage cells to contribute to formation of all somatic tissues and to remain normal. Of ten mosaic-coat females that were test-mated to males of the blastocyst (recessive-color) strain, two have produced some offspring of the diagnostic tumor-strain agouti color; F_1 heterozygotes in turn transmitted their tumor-strain genes to F_2 homozygous segregants (Figure 3) (Stewart and Mintz, 1981, 1982). The METT-1 line of teratocarcinoma stem cells therefore bridges the gap between soma and germ line, and between propagation and genetic manipulation of cells *in vitro* and embryogenesis *in vivo*.

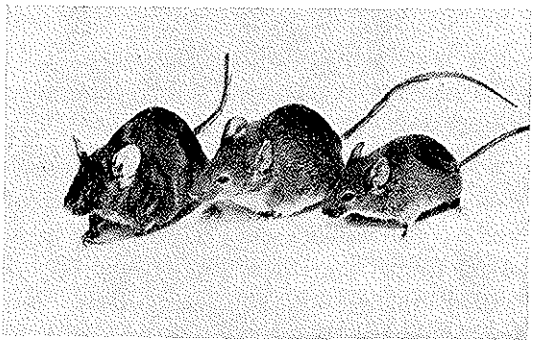

FIG. 3. Three generations of mice derived from the karyotypically normal METT-1 teratocarcinoma stem cell line. The animal at the left is a female that was produced from a blastocyst (C57BL/6 strain) injected with teratocarcinoma cells of this line; those cells developed normally, along with embryo-lineage cells, and contributed the agouti hair clones to its black-and-agouti coat. Some of the mosaic animal's germ cells were also of tumor-strain lineage, as seen in the all-agouti color of an offspring (center) from a mating to the recessive-color C57BL/6 strain. The agouti animal at the right, from the following generation, also displays the tumor-strain color, transmitted from the F_1. (From Stewart and Mintz, 1981.)

EARLY PRENATAL REPLACEMENT OF TOTIPOTENT HEMATOPOIETIC STEM CELLS

The hematopoietic lineage is of special interest with respect to its differentiation, pathology, and normal and defective genes. It is not yet possible to introduce recombinant genes into fetal liver hematopoietic stem cells, from which the definitive bone marrow and spleen cells are derived. However, we have been able to replace the cells themselves *in utero* with genetically marked donor cells that permit many questions concerning gene control of blood development and disease to be analyzed *in vivo*.

Microinjection into efferent blood vessels of the fetal placenta results in rapid entry of the injected material into the circulation of the early fetus. Allogeneic normal fetal liver cells have been successfully introduced by this route (Fleischman and Mintz, 1979). In order to provide the relatively small number of donor cells with a selective advantage over the endogenous proliferating hematopoietic cells, a genetically disadvantaged recipient fetus was used, i.e., one whose own hematopoietic stem cells were defective. The *W*-series anemic mutants have such a stem-cell defect (McCulloch *et al.*, 1964). When recipients at 11 days' gestation were injected with normal fetal liver cells from slightly older donors, strain-specific

hemoglobin markers demonstrated that the erythroid lineage had been successfully replaced — in some cases completely so, even before birth (Fleischman and Mintz, 1979). The genetically defective recipients were then no longer anemic and were able to live a full lifespan. The rate of replacement varied directly with the severity of the genetic defect in the *W*-allelic series (Figure 4). Segregants of non-anemic genotypes did not undergo detectable replacement (Fleischman *et al.*, 1982). Further analyses with concurrent markers in granulocytic cells (with *beige*-genotype giant lysosomes) and lymphoid cells (by means of serum allotypes) have shown that cell replacement in fact occurred at the *totipotent hematopoietic stem-cell* level (Fleischman *et al.*, 1982). These stem cells were capable of sustained long-term self-renewal as well as differentiation. Thus, this experimental system uniquely provides a means of placing early-stage, genetically marked, totipotent hematopoietic stem cells in the normal developmental sequence of tissues and microenvironments, progressing from fetal liver to spleen, thymus, and bone marrow. There is no graft-*vs*.-host reaction or immuno-

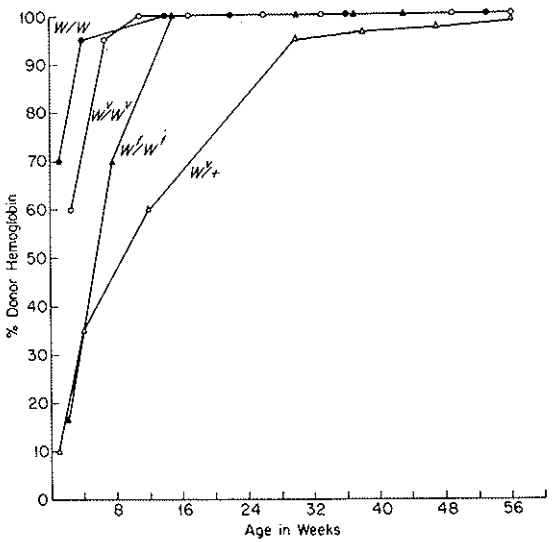

FIG. 4. Postnatal kinetics showing complete substitution of donor- for host-strain red blood cells (judged from hemoglobin variants') after injection of fetal liver cells into early fetuses via the placenta. Donor cells are genetically normal; hosts are of the *W*-mutant series characterized by a hematopoietic stem cell defect. Rate of replacement is directly correlated with decreasing severity of the defect in recipients of the W/W, W^v/W^v, W^f/W^f, and $W^v/+$ genotypes. (From Fleischman *et al.*, 1982.)

logical rejecton of these young cells and no irradiation of the host is needed. Cell and tissue interactions critical for normal differentiation and gene expression can therefore he examined in the various hematopoietic derivatives, and the etiology of many blood and immune diseases or malignancies can be analyzed *in vivo*.

One of the questions thus far investigated is whether hematopoietic cells from adult hone marrow can resume fetal differentiation if they mature in a fetal environment. An antigen, Ft, present on fetal erythrocytes and ahsent on adult erythrocytes, was used as the discriminant. Adult marrow cells were introduced into fetuses via the placenta and, after birth, erythrocytes were fractionated by fluorescence-activated cell sorting based on antibody binding to Ft. Lysates of Ft-positive, i.e., fetal, cells did not detectably contain donor-strain hemoglobin. The adult cells therefore did not resume fetal-specific Ft expression (Blanchet *et al.*, 1982). The longer-term fate of allogeneic bone marrow cells in fetal recipients is currently under investigation.

Summary

We have demonstrated that pure genes can be introduced into mouse eggs, can remain into adult life, and can be transmitted to progeny. Moreover, at least one foreign gene (HSV-*tk*) can be fully functional in the animals that develop. A second route for predetermined genetic change is defined by mouse teratocarcinoma stem cells, of which a new cell culture line (METT-1) is developmentally totipotent. Changes to mutant forms of murine genes, or introduction of recombinant cloned genes, can be effected while these cells are in culture; after transfer of the cells to early embryos, it should be possible to produce, through the germ-line, strains of mice whose genotype has been established in advance. An entry route specific for early totipotent hematopoietic stem cells has also been shown to be feasible, by microinjection of these cells into the early fetus via the placental circulation *in utero*. Allogeneic combinations are accepted with impunity.

Thus, even in an organism as complex as a mammal, there are now promising new options for analyzing *in vivo* the developmental regulation of gene expression and for realizing the experimental replacement of defective genes.

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HIGHLY REPEATED SEQUENCES IN THE AFRICAN GREEN MONKEY GENOME

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More than a decade ago, Britten and Kohne (1968) concluded that eukaryotic genomes typically include a very large number of families of DNA segments that are repeated multiple times. From studies on the rates of renaturation of a variety of DNAs, they deduced that the repetition frequencies of the different families vary from small numbers like 100 to hundreds of thousands. Furthermore, the families fall into two broad classes, those whose members are interspersed with single-copy DNA sequences or with other repeated sequences and those whose members are arranged in long randem arrays. The latter class was also described by others who noted that variable portions of eukaryotic genomes separate from the bulk of the DNA upon isopycnic centrifugation; the separation depends on the characteristic density imposed by long tandem arrays of repeated units having a particular base composition. This class became known as satellite DNA and is localized primarily to the centromeres (for review see Singer, 1982). The interspersed repeated sequence families are found between genes, within intervening sequences, interrupting satellites arrays, and clustered together with members of other families of interspersed repeats.

An extensive body of work on the molecular level has now confirmed the earlier conclusions. These experiments have also revealed an extraordinarily complex situation that raises fundamental questions about how eukaryotic genomes have evolved. However because the function of the very abundant repeated families remains an enigma, we are unable to inform structural investigations by reference to function.

The situation typical of many different species from many phyla can be illustrated by data on primate genomes, although each species has its own peculiarities. I will focus on work with the genome of the African green monkey (*Cercopithecus aethiops*) since this has been the central interest of our laboratory. The monkey genome includes the two major categories of highly repeated sequences, those that are arranged in long tandem arrays, the satellites, and those that are spread throughout the genome among other sequences.

Monkey satellite DNAs

Several satellite fractions were separated from the monkey genome by isopycnic centrifugation (Maio, 1971; Kurnit and Maio, 1974) but to date only one of these, the α-satellite, has been characterized at the molecular level (Table 1). The α-satellite comprises between 13 and 20 percent of the monkey genome. The length of the α-satellite repeat unit is 172 base pairs (Rosenberg *et al.*, 1978) and there are as many as 5×10^6 copies of the segment (Singer, 1979). The α-satellite is localized mainly, if not entirely, in or near centromeres; *in situ* hybridization showed that each of the 60 monkey chromosomes contains α-satellite sequences (Kurnit and Maio, 1974; Segal *et al.*, 1976). The many copies comprise a family of sequences and individual family members vary from one another in one or more base pairs. Typically, randomly selected individual repeat units vary about 3 percent (Thayer *et al.*, 1981) from the consensus

Table 1 - *Properties of two monkey satellites.*

	α-satellite [1]	*deca-satellite* [2]
Percent of genome	13-20	< 1
Location	centromeric	centromeric
Number of chromosomes	60/60	27/60
Repeat unit	172 bp	5'-AAACCGGNTC
Copy number	3-5 × 10^6	n.d.

[1] Data for α-satellite are taken from Rosenberg *et al.*, 1978; Singer, 1979; Kurnit and Maio, 1974; Fittler, 1977; Segal *et al.*, 1976.

[2] Data for deca-satellite are taken from Maresca and Singer, 1982; T.N.H. Lee, unpublished.

sequence determined on a bulk preparation of 172 base pair long monomer units. The variations are of two types: 1) randomly dispersed single base pair changes and 2) recurring specific base pair changes that appear in many family members. For example, while the typical repeat unit has no sequence 5′-GAATTC (an EcoRI restriction endonuclease site), about ten percent of the units do have such a site and it is always at the same place. Repeat units with an EcoRI site are not randomly distributed, but tend to be clustered together in long arrays (Fittler, 1977). Arrays of the satellite which include such recurring deviations from the typical sequence are referred to as domains and several examples of such unusual domains are shown in Figure 1. In one case, it is known that particular domains (the ones shown in Figure 1) are associated with a specific chromosome (Lee and Singer, 1982).

These data suggest that each chromosome is at least partly isolated from other non-homologous chromosomes with respect to satellite evolution. On the other hand it is also clear that in the course of evolution, the α-satellite of different chromosome pairs was not completely isolated since the bulk of the monkey α-satellite is distinctly different from the related α-satellites of closely related organisms. For example, the baboon *Papio papio* also contains a very large amount of a specific version of α-satellite (Donehower *et al.*, 1980); the major repeat unit is not 172 base pairs, but 343 and it is composed of two different variants of the typical monkey repeat. Human DNA has another species-specific version of α-satellite that is distinct from but related to that of both monkeys and baboons (Wu and Manuelidis, 1980). This type of situation is typical of

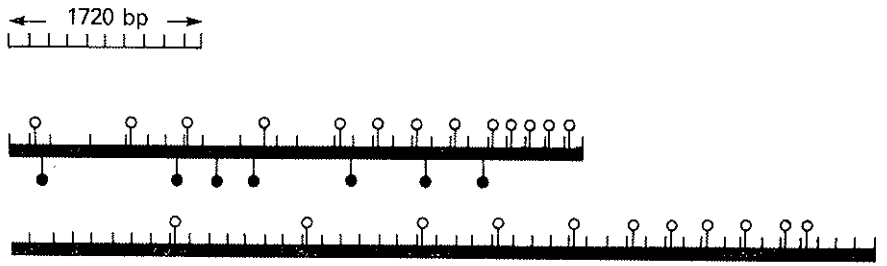

FIG. 1 — Two domains within α-satellite on a single monkey chromosome (from T.N.H. Lee and M.F. Singer, 1982). The open circles indicate EcoRI sites; the closed circles indicate HaeIII sites; the short lines indicate HindIII sites which occur within almost all the 172 base pairs monomer of α-satellite. The diagrams are schematic and do not represent precise restriction endonuclease maps.

satellites; closely related species even in the same genus or order have species-specific forms of a satellite. In fact, satellites are the most species-specific DNA sequences known, suggesting that they evolved rapidly. How rapidly? Our studies on a second monkey satellite provide some preliminary information about the time scale.

A second monkey satellite, the deca-satellite (Table 1), is unrelated to α-satellite, is estimated to comprise less than one percent of the genome, has a ten base pair repeat length and was discovered by molecular cloning (Maresca and Singer, 1982). It is not known whether deca-satellite corresponds to any of the isopycnic fractions identified by Maio (1971). Baboon DNA hybridized with deca-satellite but no hybridization was detected with human DNA. Deca-satellite too is localized to centromeric regions, as shown by *in situ* hybridization (T.N.H. Lee, unpublished), but only about half the monkey chromosomes contain detectable deca-satellite by this technique.

The centromeric location of both α- and deca-satellite suggests that the two might be joined together in some or all of the chromosomes in which they both appear. There is direct molecular evidence for their joining. When a recombinant library of monkey DNA in λ-bacteriophage vector is constructed by fragmenting the monkey DNA with EcoRI, long stretches of α-satellite are eliminated from the library (McCutchan *et al.*, 1982) because some domains are devoid of EcoRI sites. Those phage in such a library that do hybridize with α-satellite are enriched for genomic segments in which α-satellite is joined to other kinds of DNA sequences. Using this approach, we found that three phage out of 17 chosen at random because they hybridized with α-satellite also contained deca-satellite. In one case, a direct joining of α- and deca-satellite was demonstrated by sequence analysis (Maresca and Singer, 1982). These results suggest that α- and deca-satellite junctions are not uncommon. This finding was recently confirmed by experiments with another library that is more representative of the entire genome than is the EcoRI library. Of the phage in the library, 2.8 percent hybridized to deca-satellite and fully 12 percent of these also hybridized to α-satellite consistent with there being frequent junctions between the two sequences (A. Maresca, unpublished). We do not know if one satellite interrupts stretches of the other at any of these intersections.

The most interesting fact regarding deca-satellite is its extraordinary polymorphism (Maresca and Singer, 1982). This was revealed by digesting genomic DNA with restriction endonucleases that have only occasional sites in the satellite. The resulting fragments were separated by electrophoresis

and those with deca-satellite were detected by hybridization. As expected, many bands were seen (Figure 2), and each of these represents multiple fragments of that size within the genome; in addition, a large percent of the deca-satellite remained undigested. The striking thing in Figure 2 is that none of the DNA samples shown, each of which comes from a different monkey, has the same pattern. When other enzymes beside EcoRI were used, again the patterns were different for different individuals. The pat-

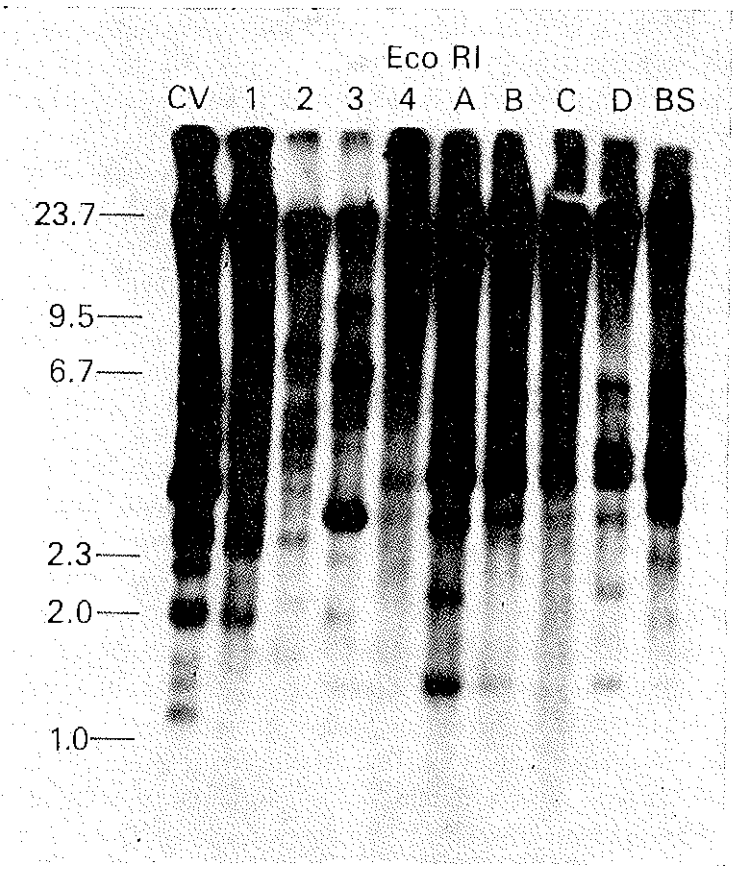

FIG. 2 — The pattern of bands hybridizing to deca-satellite after digestion of monkey genomic DNA with EcoRI (from Maresca and Singer, 1982). After digestion with EcoRI, the DNA was electrophoresed on agarose, transferred to nitrocellulose and hybridized with a ^{32}P-labeled segment of deca-satellite cloned in pBR322. The mobility of known marker fragments is shown on the left. Each lane represents DNA from a different monkey (1-4 and A-D) or from different tissue culture cell lines (CV and BS).

terns were, however, constant when DNA from different tissues of a single individual were analyzed.

These results indicate that no one single organization of deca-satellite has become fixed during the evolution of the monkey. Homologous chromosomes must contain divergent forms of deca-satellite. To what extent is the contemporary bank of deca-satellite changing and on what time scale? If it can change from one generation to another, then the pattern in an offspring might contain bands not found in either parent. Therefore we have begun to look at the deca-satellite patterns within families. In one preliminary experiment, all the distinguishable bands in the patterns from two male offspring were present either in the mother or father, suggesting that no major organizational features were lost or gained within the single generation. The patterns of the two offspring themselves were different, not so much in which bands occurred but in the relative quantities of the bands. This is consistent with homologous chromosome pairs in the parents containing different amounts or arrangements of deca-satellite. We are continuing to survey additional families as they become available.

Interspersed and highly repeated monkey DNA sequences

Among the 17 α-satellite-containing phage isolated from the monkey genomic library as described above, 12 contained junctions between α-satellite and non-satellite sequences. In every case examined so far, these non-satellite sequences have proven to be members of interspersed and highly repeated sequence families. Family members occur in many additional genomic sites beside the satellite regions. In some of the library segments, the interspersed repeats have a single junction with α-satellite but in others, the interspersed repeats interrupt the satellite as though they had been inserted into a satellite array. Two conclusions follow from these observations. First, satellite arrays are not necessarily "pure"; they can be interrupted by other kinds of DNA sequences. Second, a sequence that interrupts satellite is likely to be a movable element since satellite arrays are quite species-specific and therefore recent in an evolutionary sense while the interspersed repeats are probably older since they are generally conserved among primates. These conclusions are illustrated by several specific examples.

The Alu family of interspersed repeated sequences.

One of the sequences found to interrupt α-satellite represents an abundant family of 300 base pair long sequences called Alu that are interspersed throughout old world primate genomes (Schmid & Jelinek, 1982). It is estimated that there are about 3×10^5 Alu sequences in primate genomes. They have been found between genes and in introns as well as within satellite. Figure 3A shows (schematically) a monkey Alu segment and its immediate flanking sequences. Typically, the identifiable Alu sequence is surrounded by short direct repeats. Another monkey Alu sequence that illustrates this principle is shown in Figure 3B. For each of the two Alus the direct repeat is different both in length and in sequence. In more extensive data with humans this principle is maintained (Schmid and Jelinek, 1982). Such short direct flanking repeats are typical of the transposable DNA elements that have been characterized in prokaryotes, Drosophila and yeast (Calos and Miller, 1980). In the latter case it was proven that the flanking repeats represent a duplication of a sequence that was present only once before the insertion of the transposable element. It is difficult to isolate the same site with and without a movable element from mammals; in yeast, Drosophila and prokaryotes, genetic manipulation permits the isolation of such "empty" and "filled" sites and thus the molecular characterization of the flanking target site duplication. The fortuitous finding of an Alu sequence interrupting α-satellite in one of our cloned monkey segments (Figure 3B), however, allowed analysis of such a pair of sites, since the consensus sequence of the α-satellite sequence is known (Rosenberg *et al.*, 1978). Comparison of the satellite sequences flanking the Alu in Figure 3B with the α-satellite consensus sequence showed that a target site duplication had indeed occurred (Grimaldi and

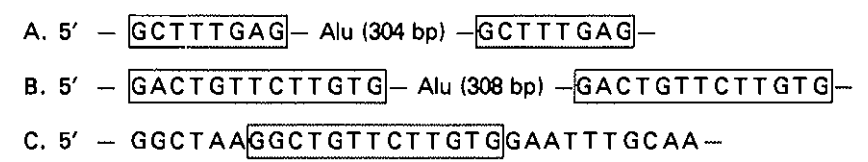

FIG. 3 — Short direct repeats flank Alu sequences. A) A monkey Alu segment and its flanking sequences (boxed) (Grimaldi *et al.*, 1981). B) A monkey Alu segment that interrupts α-satellite (Grimaldi and Singer, 1982). The flanking direct repeats (see text) are boxed. C) shows the consensus sequence of uninterrupted α-satellite in this region; the 13 base pairs repeated on either side of the Alu are boxed; note that the interrupted satellite diverges from the consensus sequence at one position.

Singer, 1982). This finding supports the notion that Alu sequences are movable.

Nevertheless, Alu segments are quite different from typical transposable elements. Alu sequences are much shorter and contain neither the characteristic short inverted terminal repeats nor long direct repeats at the two ends of the element itself. Also, the target site duplications associated with transposable elements are always a fixed size characteristic of each individual element. Alu sequences are, however, reminiscent of the structure of some pseudogenes (Van Arsdell *et al.*, 1981; Hollis *et al.*, 1982), which appear to have been inserted into distant genomic segments. Pseudogenes also contain a long terminal poly A segment and are flanked by short direct repeats. Similarly, there is a known movable element in Drosophila which shares the poly A terminus and variable size target site duplication with Alu (Dawid *et al.*, 1981). Thus, in addition to the classical transposable elements, a new class of movable sequences is emerging. The mechanism by which such units move about in genomes is unknown and may be quite different from the mechanism used by classical transposable elements (Jagadeeswaran *et al.*, 1981; Denison & Weiner, 1982).

The RET sequence.

Another one of the cloned α-satellite segments is interrupted by a sequence we call RET (R.E. Thayer & M.F. Singer, in preparation). RET is 829 base pairs long and like Alu it ends in a long 3′-terminal stretch of A residues and is flanked by short direct repeats of the α-satellite sequence it interrupts (Figure 4); otherwise it is unrelated to Alu. Of the phage in a monkey library, 16 percent hybridize with RET, suggesting a minimum of about 40,000 copies of the sequence within the genome. Together, the data suggest that RET is a member of a highly repeated family of interspersed sequences that includes moveable members. However the analysis of

A. 5′ — TC[AAAACTGGAAAGAA]— RET (824 bp) —[AAAACTGGAAAGAA]GCTT—

B. 5′ — TC[AAAACTGGAAAGAA]GCTT—

FIG. 4 — A. Schematic diagram of the RET segment (see text) that interrupts α-satellite. The flanking direct repeats of the satellite are boxed. The 829 base pairs of RET are indicated and include an A-rich stretch at the right side, as written (from R.E. Thayer and M.F. Singer, in preparation). B. The consensus sequence of uninterrupted α-satellite in the relevant region; the 14 base pairs repeated on either side of RET are boxed.

another cloned monkey DNA segment that hybridizes with RET showed that the situation is more complex.

The LS-1 sequence.

The 1300 base pairs long LS-1 segment was subcloned from a 17 kilobase pair long monkey DNA segment present in a phage selected from a genomic library for other reasons (Queen *et al.*, 1981). A highly repeated DNA segment, LS-1 was shown to hybridize with RET and with 20 percent of the phage in a monkey library (M. Lerman, unpublished) but not with Alu. Complete sequence analysis of LS-1 and comparison with RET showed that it is, in part, a scrambled version of RET (Figure 5); its ends are each homologous to the two ends of RET, but these are inverted relative to one another in LS-1 compared to their orientation in RET. Furthermore, the center region of LS-1 is unrelated to RET. We

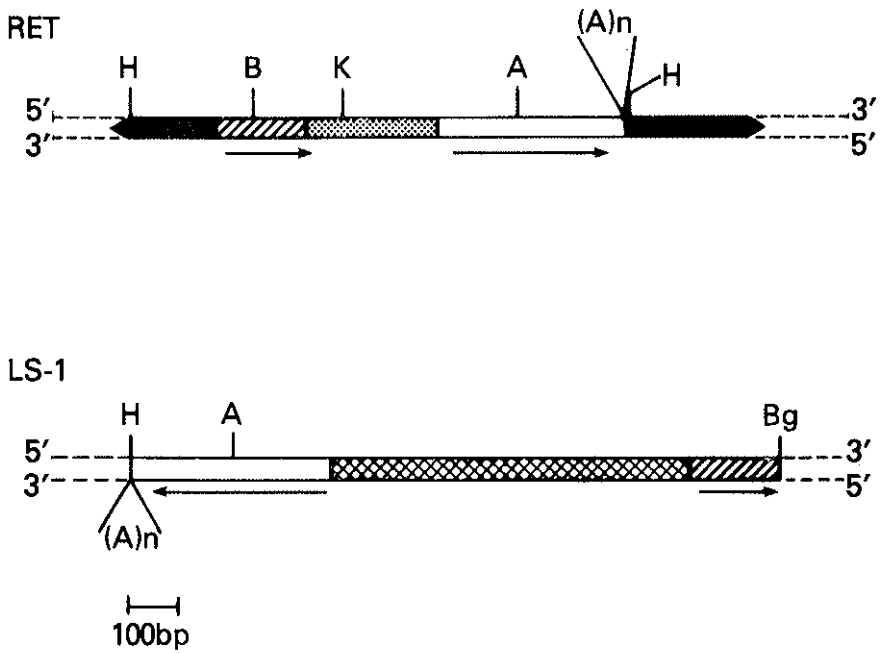

FIG. 5 — Schematic diagram showing the relation between LS-1 and RET (M. Lerman and R.E. Thayer, unpublished). The open and slashed bars represent homologous sequences; the filled bars represent the α-satellite sequences surrounding RET. Arrows indicate the direction of the nucleotide sequence. $(A)_n$ indicates a stretch of A residues.

are unable at this time to estimate the repeat frequency in the genome of the individual portions. We also do not know whether either the RET or the LS-1 element is, in its entirety, repeated elsewhere in the genome.

The KpnI family.

A third cloned segment of α-satellite contained a much longer interruption, one about 6 kilobase pairs in length (Figure 6; G. Grimaldi and M.F. Singer, submitted). Subclones representing the left (L), center (C), and right (R) hand segments of this interruption were used as probes to investigate the organization of the sequences in the genome. These analyses involved hybridization to restriction endonuclease digests of total monkey DNA, to representative phage in a monkey genomic library and to two isolated phage that contain homologous sequences. The data can be summarized as follows. Sequences homologous to left, center and right occur on the order of 10^4 times in the monkey genome. Those on the left are almost always associated with those in the center but those in the center also occur independently of those on the left. Also, the central sequences frequently occur independently of those on the right and furthermore almost half of the genomic sequences homologous to the right hand segment are not associated with the center. Thus the segment starting at the leftmost end of the segment shown in Figure 6 and extending to the distal

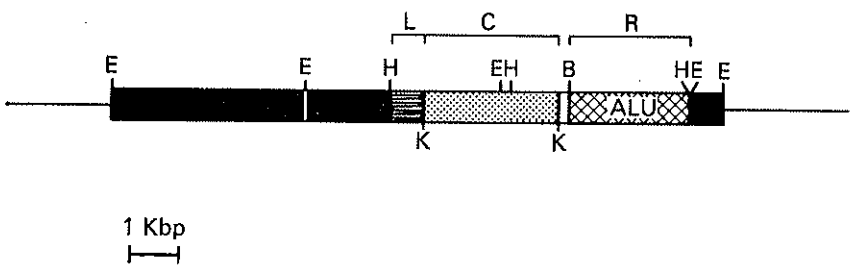

FIG. 6 — Schematic diagram of a KpnI family member that interrupts α-satellite (from G. Grimaldi and M.F. Singer, submitted). The filled bars represent flanking α-satellite sequence. Three portions of the family member are distinguished: left (L), horizontal lines; center (C), dots; right (R), cross-hatched. Each of the three has been subcloned. The right end segment includes an Alu family member (which was not included in the subclone). The position of various restriction endonuclease sites is shown: E, EcoRI; H, HindIII; B, BamH1; K, KpnI.

KpnI site is an abundant repeated element as such. This left-hand stretch (about 3.5 kilobase pairs) is homologous to a complex repeated sequence family in human and other primate genomes previously described by others (Schmeckpeper *et al.*, 1981; Manuelidis, 1982; Manuelidis and Biro, 1982; Shafit-Zagardo *et al.*, 1982). The best characterized human family member occurs about 3 kilobase pairs downstream from the human β-globin gene (Adams *et al.*, 1980). In both human and monkey DNA there are restriction site polymorphisms that distinguish subfamilies of the major family. The subfamilies in the two primates are related in structure but vary markedly in relative abundance (G. Grimaldi and M.F. Singer, submitted). Therefore there is a definite and easily discernible species specificity.

General comments about interspersed repeats.

The analysis of interspersed repeat units that are embedded in satellite has several advantages. First, the beginning and end of a unit is readily defined by the junctions with known satellite sequence. Secondly, it is likely that the units represent insertions into the satellite and therefore they provide direct evidence for the transposition of the unit at some time in the past. Moreover when direct repeats of a known satellite segment flank a unit, its characterization as a movable element is supported.

The data with Alu are relatively straightforward. A large body of work defines the Alu unit as a discrete element. Of those that have been studied, most Alu units are known to be flanked by short direct repeats and in the case of the Alu embedded in monkey α-satellite it is clear that these repeats are duplications of the target site. However the data with RET, LS-1 and the KpnI family are complex. The structure of RET is consistent with it too being a transposed segment. But in what sense is RET a unit? We do not know how many such units occur in the genome. We do however know that segments within RET can also appear in a shuffled configuration. Thus, while RET has the structure XZ, LS-1 has the structure X′WZ (where X′ indicates an inverted sense). Similarly with the KpnI family member we have described. In some places in the genome the left, center and right portions occur together; in others the center and right occur without the left and perhaps individually. Thus a variety of sequence elements occur in assorted clusters. Since it is likely that additional repeated elements remain to be discovered, the number of possible genomic combinations is probably very large. To what extent are individual clusters fixed as to the assortment and alignment of units? To what extent

are clusters fixed at specific genomic positions? And what, if any, is the role of the clusters in modulating genomic functions? These unanswered questions remain for future research.

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CHROMOSOMAL TRANSCRIPTION: FUNCTIONAL IMPLICATION OF CHROMATIN STRUCTURE

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INTRODUCTION

Due to the typical band interband pattern of polytene chromosomes particularly suited for cytological studies, they have been frequently chosen for analytical studies of chromosomal transcription and replication. Moreover these chromosomes have received wide interest because the application of *in situ* hybridization directed to the DNA or chromosomal RNA has revealed the topological location of specific genes and allowed to measure their relative syntbetic activity [1, 2, 3, 4]. In addition, the studies on the relationship between specific cell functions, changes in pattern of protein synthesis and puffing have indicated that there is a direct correlation between the appearance of specific proteins, decondensations of the chromatin and transcriptional activity [5, 6, 7]. This fact, of course, implies a large scale synthesis of RNA which might be correlated with conformational changes occurring at the level of the chromatin and which may underlie the observed cytological decondensations. In fact several reports have shown that active genes are in a different conformation than genes in an inactive state and that active conformations are probably maintained by specific proteins among which, in eukaryotes, the HMG may be the principal component [8, 9, 10]. Most of our knowledge, however, about conformational changes associated with active transcription comes from studies of tissue-specific chromatin. Very little is known about

conformational changes occurring in whole chromosomes as a result of increased transcription.

Based on thermal denaturation data it was previously suggested that a large fraction of histone bound DNA in chromosomes destabilizes during puffing [11]. In the present paper we extend this type of analysis to a region of the chromosomes apparently inactive in RNA synthesis. This study was carried out, moreover, because the use of a high resolution method able to detect transcriptional activity *in situ* has revealed that the control region which was taken as being in resting stage and to which the puffing associated conformational changes were compared, transcribes RNA at a significant high rate. Since, in addition, it is known that the DNA concentration and packing ratios of bands and interbands widely differ and we have data showing that the melting point of bands and interbands is not the same, we have tried to correlate these facts with the distribution of nascent RNA molecules on the chromosomal structure in order to position the place of their genetic activity. These studies prolong previous autoradiographic analysis of polytene chromosomes which showed that labeled RNA precursors incorporate not only in puffs but in many other chromosomal sites. We hope that they will provide new insights into the transcriptional regulation system used in higher organisms.

Materials and methods

A stock of *Drosophila hydei* was used. In order to induce puffing at region 2-48B_4C_5 middle third instar larvae were treated at 37°C for 1/2 hr. Puff at region 4-78AB was induced *in vivo* by injection of whole larvae with β-ecdysterone at a concentration of 10^{-3} μg/larva. After 30 min the salivary glands were fixed in 10 mM $MgCl_2$-50% ETOH for 2 min followed by 3 min in PBS-3% formaldehyde (PBS: 0.01 M Na-phosphate, 0,15 M NaCl-pH 7.2). Afterwards they were squashed in 45% acetic acid on quartz slides and postfixed in 3% formaldehyde in PBS. For RNA estimations some of the slides, after UV measurements, were treated with a mixture of pancreatic RNase A (1 mg/ml) and 100 units/ml of RNase T_1 in 2 × SSC (SSC: 0.15 M NaCl-0.015 M Na citrate) for 2 hrs at 37°C. The UV absorption was measured at 260, 280 and 310 nm. The quantity of nucleic acids and proteins was calculated from absorption values [11]. RNA quantity was determined by DNA subtraction from the total nucleic acids content of the region. A Zeiss UV 03 scanning microspectrophoto-

meter connected to a PDP 12 computer and an ultrafluar 100x immersion objective under glycerol were used to measure absorption values. The scanning raster was adjusted to correspond to the puffed and non-puffed areas. The scan lines were 0.5 μ apart. Each point was measured 20 times and the average value was taken. Four different regions from the distal part of the gland were measured. Background contamination was allowed for by measuring at different wavelengths the absorption of areas close to the chromosomes. The level of polytenization of the chromosomes measured was similar. This level was estimated from the amount of DNA from bands 4-73D.

Thermal denaturation

The formaldehyde fixed and RNase treated chromosomes were denaturated stepwise hy immersing the slides for 10 sec. in 0.25 mM Na EDTA-0.3% formaldehyde (pH 8 with solid Tris) at a temperature ranging from 25 to 100°C. The increase in hyperchromicity was recorded at 1°C intervals. After heating at each temperature the slides were dehydrated in ice-cold 90% ETOH for 5 min. Corrections for light scattering were made by measurements at 310 nm. Each point was measured 20 times. The average value was taken. The scanning raster was added to the chromosomal areas. Data were analyzed by means of a desk computer HP 85 to obtain the derivative melting curves.

From every set of five consecutive values of H (t), the hest-fitting line was adjusted by linear regression. The resulting slope was taken as $\Delta Ht/\Delta t$ at the middle temperature.

Indirect immunofluorescence

The induction of chromosomal DNA:RNA hybrids was achieved by denaturation of the chromosomes in 100 ml solution of 0.1 × SSC at 95°C for 20 sec and further incubation of the chromosomes in 100 ml of hybridization huffer (50% formamide −3SSC) for 10 min. This method has been coined as an endogenous RNA hybridization technique (EHT, reference 12). Following the incubation period in the reannealing buffer the chromosomes were washed in PBS and incubated for 1 hr with 20 μl of goat-antihybrid IgG at a dilution of 1:100 [13, 14]. Afterwards the chromosomes were extensively washed in PBS and incubated for 1 hr with 20 μl of fluorescein isothiacynate labeled rabbit-antigoat IgG at a dilution of 1:40. After a

subsequent wash in PBS the slides were mounted in glycerine: 1 M Tris (9:1) pH 7.0. The resolution power of this method of analysis of chromosomal transcription lies in the fact that it undoubtedly detects regions engaged in transcription because the fluorescence observed reveals DNA-RNA hybrids induced *in situ* which have been formed between nascent RNA molecules and their DNA templates. Only nascent RNA molecules are competent to hybridize with their templates with the observed kinetics of less than 2 sec. This kinetics is similar to the unimolar hybridization of inverted DNA repeats. The chromosomes were photographed in a Zeiss epiilluminated fluorescent microscope using a 35 mm Kodak Tri-X pan film. The precise location of the chromosomal hybrids was determined by densitometric scanning of photographic negatives from chromosomes at a magnification of 8.500x. The chromosomes which had been stained with fluorescein for DNA:RNA hybrids were afterwards stained with lactoacetic acid for DNA. They were rephotographed again. The scanning raster was adjusted to cover 0.16 mm along the chromosomal longitudinal axis. The scan lines were 100 μ apart. Using stained cytoplasmic markers the photographic films could be perfectly matched. The absorption values of the fluorescence over chromosomal regions were also determined on photographic films. In these films the scanning rasters were adjusted to correspond to the puffed and non-puffed areas. An Optronics P 1700 microdensitometer on line to a PDP 11/45 computer was used.

Results

I. DNA, RNA and protein content of regions 2-48B_4C_5 and 4-78AB in puffed and non-puffed form and of region 4-73D.

From a cytological point of view, region 2-48B_4C_5 can be subdivided in 3 distinct zones. One is formed by 5 "dotted" bands ($B_4B_5B_6C_1$ and C_2), a second one which has 2 continuous bands (C_3 and C_4) and a third one which contains the telemore. It is known that puffing at this region may be induced by several treatments [15, 16] and that incubation of whole larvae or salivary glands at 37°C for 1/2 hr induces only decondensation of the first zone. Bands $B_4B_5B_6C_1$ and C_2 are no longer observed when the puff has attained its maximum diameter [16]. Region 4-78AB is formed by 1 dense and 6 thin bands. Late during the third instar this region develops into a puff and it may also be triggered on *in vitro* by ecdysone. Even in a non-puffing

configuration both regions accumulate radioactive precursors after 10 min of a ^3H uridine pulse, but the heat-shock inducible subregion 2-48B_4C_5 incorporates more RNA precursors than the hormone inducible 4-78AB. A similar observation has been reported by Zhimulev for the heat-shock inducible region 63BC in the polytene chromosomes of Drosophila melanogaster in which significant incorporation of ^3H-uridine takes place without the typical swelling [17]. Figure 1 shows the pattern of fluorescence (DNA/RNA chromosomal hybrids) of regions 2-48B_4C_5 and 4-78AB in puffed and non-puffed state. As an inlet the total absorption values of the fluorescence of each region are given. It appears, in agreement with autoradiographic data, that unpuffed division 2-48B_4C_2 has a large amount of nascent RNA molecules able to form hybrids with their templates. In both puffed and unpuffed forms, the positioning of the fluorescence seems to be restricted to bands $B_4B_5B_6$ This observation suggests that only the DNA of these bands is actually transcribed in the puff even though the decondensation reaches other bands as well. It was previously reported that at about 10-20 min after the onset of puff formation, typical RNP characteristics for this puff can be found in the zone occupied by bands B_4 and B_5 and that after longer periods of activity the RNP distributes over the entire puff zone to band C_2, as an indication that bands B_{4-5-6} are the origin of the puff [16]. A similar observation concerns region 4-78AB in which fluorescence is restricted to subregion B. Even within that subregion the fluorescence does not cover the entire decondensed zone which extends over part of zone A. Probably this phenomenon has to be correlated with functional domains within tbe puffs since in a large puff (region 5-95D) the fluorescence covers its entire decondensed area [18]. In zone B there are two fluorescent bands indicated by bars in Figure 1C. Probably the puff originates from the thin one named P. Tbe cytology and transcriptional activity of these two regions contrast with those of region 4-73D. This region is formed by 4 dense bands and 3 narrow interbands and it does not incorporate radioactive labeled precursors after 10 min of a ^3H uridine pulse. It only shows very weak fluorescence in endogenous RNA hybridization experiments. This region moreover was never observed to form a puff.

These cytological observations led us to analyze the chemical composition and DNA conformation of experimentally induced chromosomal regions (puffs) relative to the same regions in non-induced form. Table 1 shows the DNA, RNA and protein content of regions 2-48B_4C_5, 4-78AB and 4-73D. There is not any significant difference between the amount of DNA of region 2-48B_4C_5 or 4-78AB either in puffed or unpuffed form

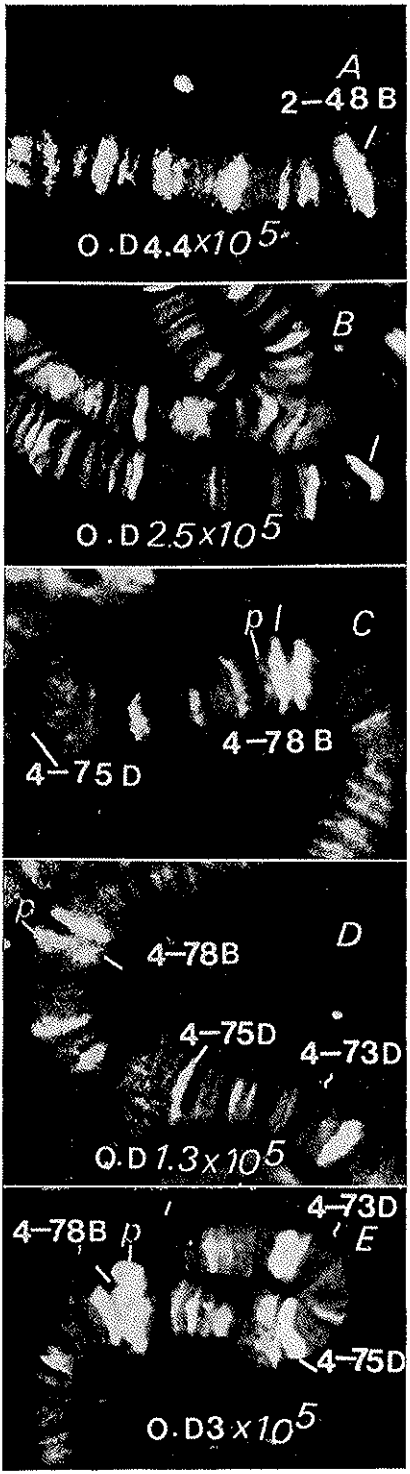

FIG. 1. Indirect immunofluorescence of transcribing regions 2-48B_4C_5, 4-78AB and 4-73D. (A) 2-48B_4C_5 in puffed form. (B) 2-48B_4C_5 in unpuffed form. (C) 4-78AB in unpuffed form. (D) 4-78AB at the onset of puff formation. (E) 4-78AB in puffed form. In C and D region 4-73D is also shown. Note that in region 4-75D a bright fluorescent band develops at the developmental stage corresponding to the onset of puff 4-78B. The O.D. is expressed in arbitrary units.

TABLE 1 - *DNA, RNA and protein content of regions 2-48BC and 478AB in puffed and non-puffed form and of region 4-73.*

Region	DNA	RNA	Protein	DNA/Protein
2-48BC (no puff)	0.47	0.29	3.52	0.13
2-48BC (puff)	0.46	0.84	7.36	0.06
4-78AB (no puff)	0.70	0.45	5.92	0.11
4-78AB (puff)	0.73	1.50	8.76	0.08
4-73D (no puff)	2.21	0.32	6.63	0.33
4.73D (heat-shocked)	2.10	0.29	6.54	0.32

The numbers represent mean values of 4 measurements. The measurements were carried out in chromosomes from the distal part of the glands. The numbers are given in picograms. The S.D. was within 10% of each value.

while the amount of RNA clearly differs with the morphological state of the region. The amount of RNA in region 2-48B_4C_5 in non-puff state was 0.29 pg while it was 0.84 pg in puffed configuration. Therefore the relative quantity of RNA differs by about a factor of 3. This relation is similar to that observed in region 4-78AB where a factor of 3.3 was observed (1.5/0.45). Regions 2-48B_4C_5 and 4-78AB contain an RNA/DNA ratio of about 0.6.

This ratio is significantly higher than that observed in whole chromosomes, where the RNA/DNA is about 0.25. On the other hand the RNA/DNA ratio in region 4-73D is even lower than the average value of whole chromosomes and it did not experience any change whether the chromosomes were taken from larvae raised at 25°C or heat-shocked.

Table 1 shows moreover that the protein mass also changes with puffing. The amount of protein in region 2-48B_4C_5 in unpuffed configuration was 3.5 pg but it was 7.36 pg when the puff had its maximum diameter. A similar increase was observed in region 4-78AB. In region 4-73D, however, the ratio DNA/Protein was significantly higher than in unpuffed regions 2-48B_4C_5 or 4-78AB and remained constant after the heat-shock treatment.

These estimates of RNA and protein content of experimentally activated regions indicate that during puffing extensive changes in the macromolecular composition of local chromatin do take place, which are unlikely to be due to the inducing treatment since in region 4-73D a heat-shock incubation of whole larvae failed to produce any similar effect. They

also indicate that the amount of proteins, relative to the DNA content, is lower in regions of highly condensed bands than in regions rich in interbands or less condensed bands.

By a different approach Izquierdo *et al.* [4] in our laboratory have measured the relative transcriptional activity of a chromosomal region in puffed and unpuffed form in the polytene chromosomes of Drosophila melanogaster. They hybridized *in situ* a cloned labeled DNA probe, mapping at the early ecdysone inducible region 63F, to the chromosomal nascent RNA in order to determine whether the homologous chromosomal sequence was transcribed at a given time. The hybridization of the pDm 63F ^{3}HDNA showed that the chromosomal sequence transcribes RNA whether the region was in puff stage or not. The experiment moreover indicated that the number of RNA molecules accumulated in the puffed region able to hybridize to the labeled DNA was two to three times higher than the number of RNA molecules in the unpuffed region.

Thermal denaturation of region 2-48B$_4$C$_5$ and 4-73D.

Since the amount of DNA and histones does not appreciably vary during gene activation [19] it is likely that the observed increase in protein mass in a region of puffing is primarily due to an accumulation of non-histones including the RNA polymerase. Accumulation of non-histones in regions of puffing was suggested by Alfert and Geschwind [20], Swift [21] and Gorowsky and Woodard [22] and demonstrated by Holt [23]. Accumulation of RNA polymerase has been shown by Jamrich *et al.* [24] and Lamb and Daneholt [34]. The significance of the data reported in table 1 would stand, therefore, in the fact that they not only reveal that during gene activation non-histones accumulate in regions of puffing but that the amount accumulated is considerably large. Thus, it is probable that they may entail conformational changes on the DNA as a result of their binding. In fact it has been reported that the presence of non histone proteins may loose the interaction of histones with the DNA allowing the unfolding of the nucleosomes [25]. At present, however, we cannot determine which proportion of the newly accumulated protein/s is DNA or RNA binding. Since electron microscopy of transcriptionally "active" chromatin has revealed an absence or marked reduction in the number of nucleosomes relative to the "inactive" form, we have tried to visualize potential conformational changes which may occur during puffing. For these studies the DNP in the chromosomes had to be fixed in formaldehyde

in order to prevent protein extraction by squashing in 45% acetic acid. Under these experimental conditions the thermal denaturation profiles shown in Figure 2 will not represent the native situation of the chromosomal DNP, but for comparative purposes they will be able to reveal relative conformational differences underlying puffing cytological transformations. Figure 2 shows that most of the DNA fraction of region 4-73D is highly stable because its melting occurs between 70° and 87°C with a peak at 82°C. There are in addition 3 very small transitions at 40°C, 50°C and 65°C but the DNA of these fractions accounts at most for 10% of the total. The melting profile of region 2-48B_4C_5 shows large similarities to the melting profile of region 4-73D since most of its DNA melts at high temperature with a peak at 80°C. This fraction, however, starts to melt at about 55°C instead of at 70°C. Moreover the second and third small transitions at 50°C and 65°C are not observed. On the other hand the melting fraction with a peak at 40°C increased significantly in area relative to the same fraction of the DNA from region 4-73D. More extensive differences are observed

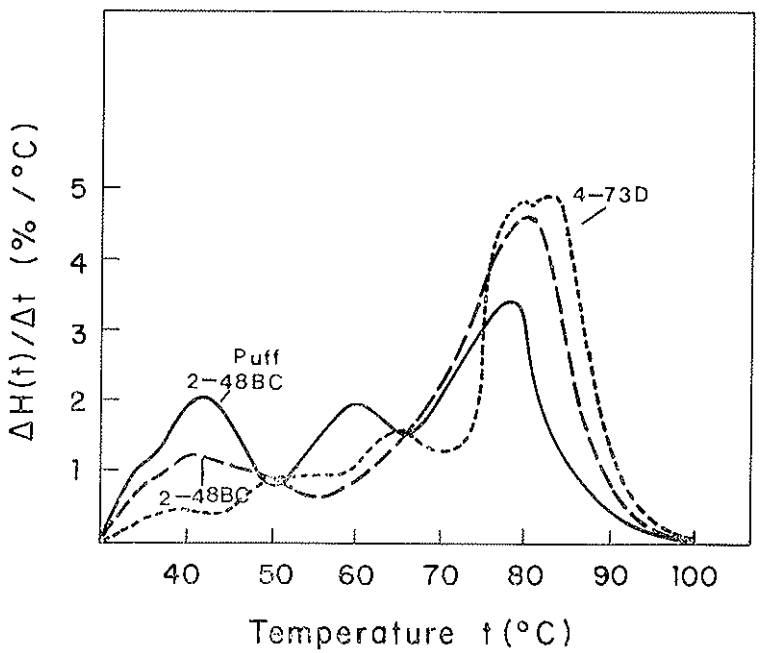

FIG. 2. Derivative melting profiles of regions 2-48B_4C_5 and 4-73D. ———— 2-48B_4C_5 in puffed form. — — — 2-48B_4C_5 in unpuffed form. - - - - - 4-73D.

when the melting profiles of the DNA from regions 4-73D and 2-48B_4C_5 in unpuffed form are compared with the melting profiles of the DNA from region 2-48B_4C_5 in puffed form. In this activated region the fraction of DNA melting at high temperature, with a peak at 77°C, significantly decreased in size and instead two relatively large melting fractions are observed at 40°C and 61°C. These fractions may represent 50% of the total DNA. We are trying to draw cytological maps of the DNA denaturation of puffing regions in order to determine possible melting domains within them.

Localization of RNA synthesizing regions

The precise localization of transcriptional domains in chromosomes is a crucial element for understanding their structural and functional organization. Most of the studies carried out using ^3H RNA precursors or antibodies against RNA polimerase have suggested that interbands and the fraction of the chromatin with a low degree of DNP fiber packing represent active genes [26, 27). Semeshin *et al.* have indicated that about 40% of uridine incorporation occurs over interbands [28]. However, the assumption that interbands in polytene chromosomes only contain a minor fraction of the chromosomal DNA (about 5%) [6], has been the hasis for the construction of theoretical models which impose necessary restrictions to the coding information possibilities of interbands [29, 30]. The question even arises as to whether all the silver grains or RNP observed over specific regions of the chromosomes represent RNA molecules synthesized *in situ* or they also manifest redistribution of chromosomal products synthesized some place else. We have approached this problem by studying the distribution of nascent RNA molecules *in situ.* Figure 3 shows the DNA/nascent RNA hybrids distribution on the chromosomes of Drosophila hydei. In *a* the DNA/RNA hybrids were stained with fluoresceine and in *b* the same chromosomes were afterwards stained with lacto-acetic orcein. An overall view of the distribution of the fluorescence indicates that probably all or at least most of the chromosomal subdivisions are active in RNA synthesis. Since similar patterns of fluorescence are observed at different developmental stages, we have suggested that a large fraction of the functional genome of even a specialized, terminally differentiated tissue as the salivary gland, is active in RNA synthesis. Figure 4 shows a close analysis of the cytological distribution of transcriptionally active domains detected by densitometer tracing of the fluorescence and DNA of a portion of chromosome 2. In *a* the profile

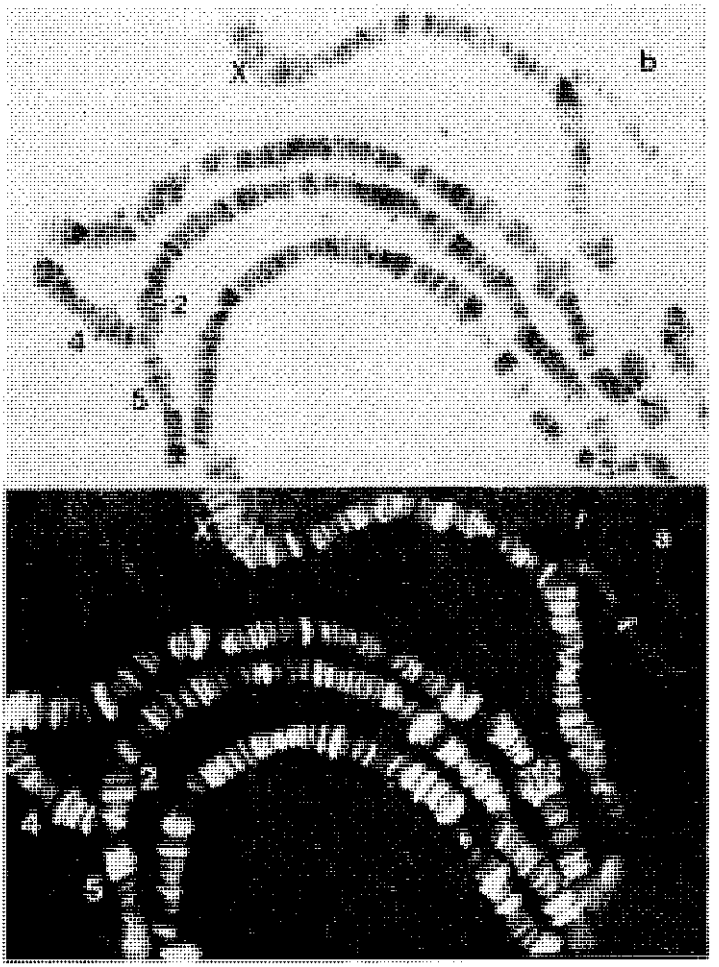

FIG. 3. Indirect immunofluorescence of transcribing chromosomes. In *a* the chromosomes were stained with fluoresceine (for DNA: RNA hybrids). In *b* the same chromosomes were stained with lactoacetic orcein (for DNA).

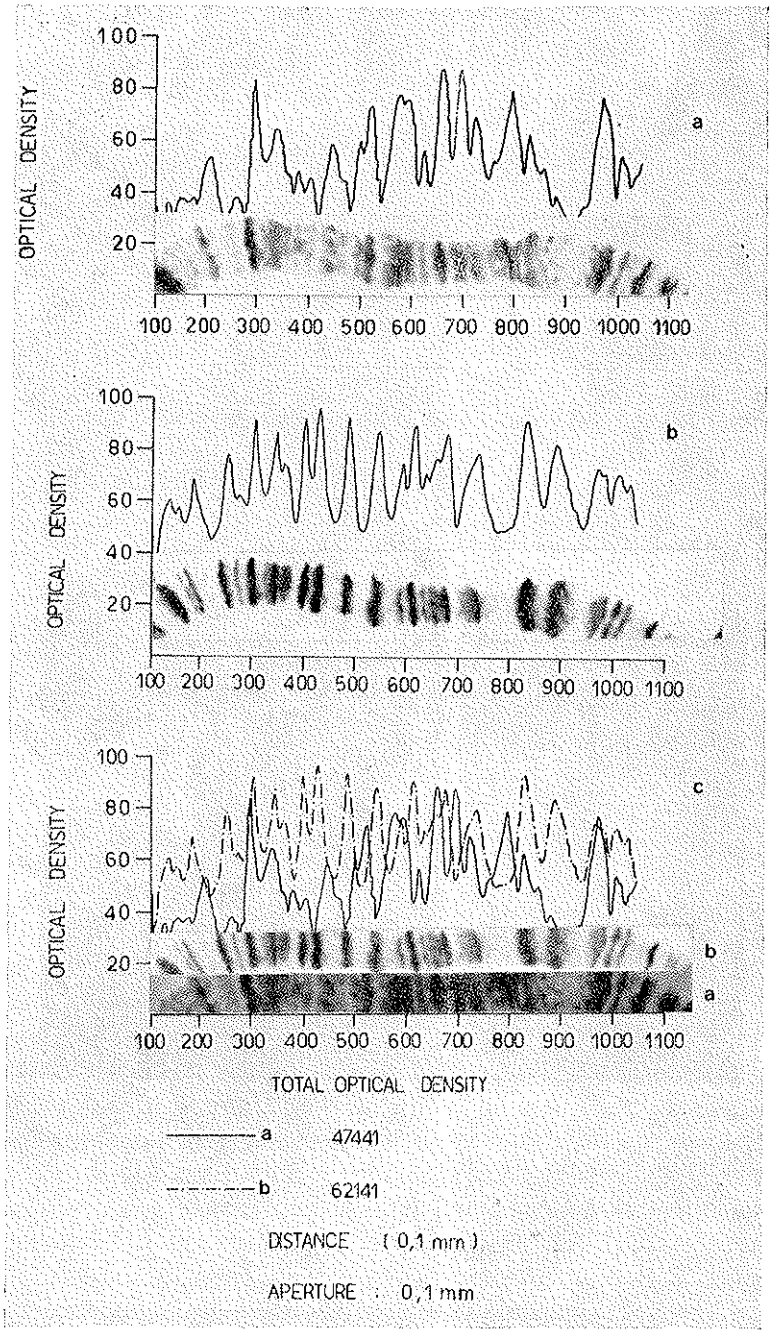

FIG. 4. Densitometric profiles of a fraction of chromosome 2. In *a* the chromosome was stained for DNA. In *b* the same chromosome was stained for DNA:RNA hybrids. In *c* both profiles are shown together.

indicates the distribution of the DNA and in *b* it reveals the presence of transcribing regions (DNA/nascent RNA hybrids). In *c* both profiles are shown together.

It may be observed that the profile of the transcribing domains is in general indirectly related to the distribution of the DNA in that the peaks of transcriptional activity (high density of fluorescence) correspond to regions of low chromatin condensation in between densely packed DNA. Some bands however seem to retain large amounts of fluorescence as an indication that their DNA is also being transcribed. Several analyses of these situations have shown that only a fraction of their DNA is involved in transcription since the fluorescence does not cover the entire band but it is slightly shifted towards one side of it. Simultaneous double labeling experiments in which the DNA is made to fluoresce red by ethidium bromide and in which the DNA/RNA hybrids exhibit yellow-green fluorescence by fluoresceine have shown, moreover, that the fluoresceine-labeled bands are in close apposition to the ethidium bromide labeled DNA but not on top of it. These experiments, moreover, also suggest, in agreement with the DNA profile of Figure 4 a, c, that a substantial part of the chromosomal DNA may remain transcriptionally inactive. We can say at least that it does not serve as template for RNA molecules able to form hybrid complexes in amounts large enough to be observed by the method used. Preliminary data allows us to make a rough estimate of the amount of inactive DNA which may account for about 60% of the total chromosomal DNA. From a cytological point of view it is therefore conclusive evidence that the chromatin from actively transcribed regions exhibits a structure different from the one which is not transcribed.

Discussion

Since regulation at the transcriptional level is one of the mechanisms involved in the control of gene expression, the problem concerning whether chromatin chemical or conformational changes are a prerequisite for transcription or are only consequences of the transcriptional event has been the object of extensive studies. Based on biochemical data it was proposed by Alfert and Geschwind [20], Swift [21] and Gorowsky and Woodard [22] that associated with puff formation specific changes in the protein content of particular regions of the chromosomes do take place but that histones do not undergo obvious changes in a quantitative sense. The non-histone character of the puff protein was ascertained by Holt [23].

In agreement with these observations our measurements show that, in addition to a local accumulation of RNA, the quantity of protein over the heat-shocked or hormone-activated gene/s increases by about a factor of 2 (see also Holt: reference 23) and that this increase is large relative to the DNA since the ratio DNA/protein changes from about 0.13 in unpuffed form to 0.06 in puffing configuration (2-48BC).

These cytological observations have been enlightened by the recent progress in nuclease digestion analysis of chromatin which suggests that in addition to histone modification, non-histone proteins may modulate active chromatin conformation or transcription process. It has been recently reported that in Drosophila melanogaster a non-histone protein doublet (44.000 and 48.000 dalton) is associated with the less compact region of the chromatin which at the same time is highly sensitive to DNase I digestion [31]. Particularly relevant is the demonstration of the chromosomal localization of antibodies directed against a 63.000 dalton protein fraction solubilized after limited DNase I digestion of Drosophila melanogaster embryo nuclei. These experiments showed that all salivary gland polytene chromosome puffs showed intense fluorescence as did many other non-puffed loci known to puff at other times during third instar or prepupal stage. Heat-shocked loci, however, which stained brigbtly with the antiserum were not stained prior to induction [32].

Altbough the hypothesis that template active chromatin contains a specific population of NHP is highly suggestive, it has not been convincingly demonstrated that individual NHPs are components of chromatin subunits associated with specific transcribing genes and that they are involved in specifying their altered chromatin conformation. Thus the DNA conformational changes deduced from the thermal denaturation curves shown in Figure 2 may either be interpreted as directly induced by the puff newly accumulated proteins or totally independent from them. It is true that these proteins may not necessarily be involved in the generation or maintenance of the "open" or transcribing conformation because a large fraction of the puff protein may be RNA binding involved in the formation of the RNP, known to accumulate at puff loci [16], but it is reasonable to assume that the presence of large amounts of NHP in the puff introduces a new electrostatic environment in the local nucleoprotein capable of substantially altering the DNA/Histone binding energies. These alterations will affect the DNA melting profile. All in all, whether the puff proteins induce the puffing conformational changes or not, we may presume that their presence in large amounts in the puffs will have

a specific effect on the DNA spatial conformation. In this case they may influence the propagation of the molecular mechanism of chromatin decondensation, which in turn modulates the transcriptional process. In this case the puff proteins would act in a cooperative manner with the initial triggering of the decondensation event. The old dilemma of whether chromatin decondensation is prior to or a consequence of increased transcription remains unanswered. However, puffed chromatin can not be implied to be *per se* necessarily transcribed since in the puffed regions studied nascent RNA molecules do not cover the entire decondensed DNA zone. In these puffs the incorporation of ^{3}H uridine over the whole decondensed zone could be accounted for by the accumulation of puff RNPs [16]. There are however data from large puffs in which there is not any redistribution of labelled RNA over the decondensed zone [18]. Probably there is no contradiction between these observations because they may be due to the different transcriptional and morphological complexities of various puffs. In this context the terms of "place of origination" and of "activity zone" [33] may have a specific meaning reflecting the functional situation of the transcriptional domain/s.

At present based on autoradiographic and biochemical data, there is conclusive evidence that puffs are not only morphological signs of genetic activity but that in them a given population of mRNA molecules are actively transcribed [3]. The molecular mechanism controlling the triggering of the increase in transcriptional activity of specific loci, however, remains unanswered in spite of extensive studies. Lamb and Daneholt have inferred, by comparing the properties of the active 75S RNA gene in *Chironomus tentans* with those of active genes in other systems, that the loss of nucleosome beads and the extension of the fiber in active units is probably directly related to the level of their transcriptional activity [34]. Moreover, since the DNA of "active" decondensed chromosome zones is more extended than that of "inactive ones" (DNA packing ratios of $\pm$ 1.6 versus $\pm$ 1.9) it has been proposed that the degree of chromatin extension in "active" non-ribosomal units might be directly proportional to the density of RNA polymerase molecules actually present in the transcribed locus [35].

Since RNA polymerase causes a local strand separation of the DNA the thermodynamic properties of the transcribed DNA would be different from that non-transcribed. Moreover these specific thermodynamic pro-

perties may be important in the efficiency of transcription. The absence of RNA polymerase from bands and its presence in interbands [24] support these biochemical evidences. In addition, the location of the transcriptional process, mainly in decondensed zones, strengthens the idea of a direct functional correlation between DNP decondensation and transcription. However, the degree of decondensation and transcription does not have to be correlated in a quantitative sense [17]. If the denaturation curves are related to decondensation of the DNP it is not surprising to observe that the denaturation profiles shown in figure 2 are also related to the transcriptional process with a lowering of the DNA melting point in puffs. It is likely that even in these highly activated regions (puffs) the DNA denaturation profiles would be time-dependent and related to their degree of activity.

The accumulation in the cytoplasm of large amounts of the protein products of the puffs would not be explained by a 3-fold increase of RNA synthesis in the puffs relative to the transcription of unpuffed forms (Table 1). A differential rate of chain elongation and termination in the puffs would have a multiplied net effect in the cytoplasmic concentration of their specific RNAs. It seems that this should be the case, at least, for region 2-48B_4C_5 since in puffed form it only contains 3 times more RNA than in unpuffed form. A close analysis of the fluorescence from region 4-78AB indicates, in contrast, that the puff RNA originates also from a very thin fluorescent band in the first subdivision within the B zone. Several analyses of the fluorescence observed during progression of the puff indicate that the thickest fluorescent band does not increase during puffing. On the contrary the thinnest band (Figure 1C) develops into a large-sized fluorescent area. Since the thinnest fluorescent band accounts for only about 1/20 - 1/25 of the total fluorescence of the zone, the newly synthesized puff RNA accumulated in the region would represent an increase of about 45 fold. A similar situation was never observed in region 2-48B. The significant conclusion from these cytological data is that in the inducible systems studied, transcription is not regulated up from a completely "off" state. Whether this situation is a general phenomenon in all inducible chromosomal regions remains to be observed.

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RECOMBINATION PROMOTED BY PAPOVAVIRUSES: MODEL SYSTEMS FOR A NONHOMOLOGOUS RECOMBINATION PATHWAY

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It is now well recognized that gene mobility — resulting from nonhomologous recombination — is an important parameter in the evolution of prokaryotic and some eukaryotic organisms (Calos and Miller, 1980). We have chosen the papovaviruses, simian virus 40 (SV40) and polyoma virus, to construct model systems intended to provide a better understanding of the nonhomologous recombination pathways in animal cells. The rationale for the use of these tumor viruses to probe the cellular recombination process derives from the wide range of recombination-like activities that can occur in infected monkey and rodent cells. This range includes the integration of viral genes at multiple sites in the cell's chromosomal DNA (Topp *et al.*, 1980; Krieg *et al.*, 1981) the incorporation of host DNA into the viral genome (Lavi and Winocour, 1972; Kelly and Nathans, 1977; Wakamiya *et al.*, 1979) and extrachromosomal recombination with unrelated DNA such as that of bacterial plasmids or the bacterial virus ØX174 (Winocour and Keshet, 1980). Indeed, in its capacity to mediate or promote nonhomologous DNA exchange in animal cells, SV40 and other tumor viruses exhibit some properties reminiscent of bacterial transposable elements.

We describe below results from two of our current SV40-based systems. The first system analyses recombination between SV40 DNA and prokaryotic DNAs transfected into monkey cells; the second monitors the ability of an SV40 DNA sequence to promote plasmid fusion and/or release from the plasmid incompatibility stricture in *E. coli* bacterial cells. We suggest that both the monkey and the bacterial cells recognise putative

recombination control elements that are specified by the SV40 genome. A fuller account of these studies is being published elsewhere (Winocour *et al.*, 1982; Dorsett *et al.*, 1982; Vernade *et al.*, 1982).

A. *Recombination between papovavirus DNA and prokaryotic transfected into monkey and mouse cells*

The animal cell model is based upon the finding that cotransfection of monkey cells with SV40 DNA and unrelated DNAs (bacterial plasmid or bacterial virus ØX174 DNAs) generates recombinant genomes encased within SV40 capsids which replicate in the presence of wild type SV40 virions (Winocour and Keshet, 1980). The recombinant virus is detected by *in situ* plaque hybridization procedures (Villarreal and Berg, 1977). An essentially analogous system exploits polyoma virus and mouse cells rather than SV40 and monkey cells.

The experimental protocol is illustrated diagrammatically in Fig. 1. Suspensions of African green monkey kidney cells (the established BSC-1

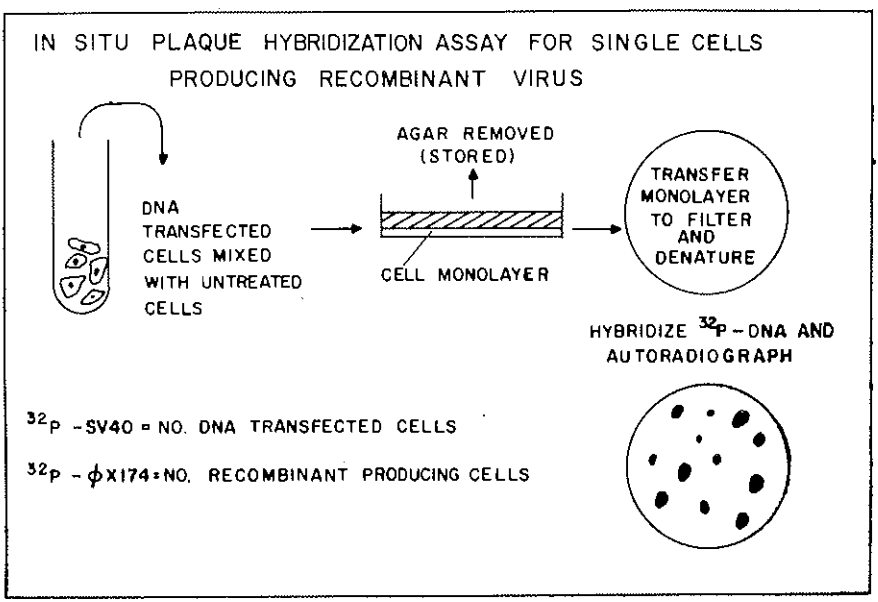

FIG. 1 — An infectious-center assay for titrating recombinant producing cells (see Text).

line) are exposed to a mixture of SV40 and ØX174 RF1 DNAs (or other DNAs, as designated) using DEAE-dextran as the facilitator. Known numbers of the transfected cell population are then mixed with a large excess of untreated BSC-1 cells, plated for monolayer formation and overlaid with agar-nutrient medium. Following an incubation period of 6 days, the agar is removed and the underlying cell layer is transferred, physically intact, to a nitrocellulose membrane filter (Villarreal and Berg, 1977) which is processed for recombinant plaque identification by *in situ* plaque hybridization (Winocour and Keshet, 1980). In each experiment, one set of filters is hybridized with ^{32}P-labelled SV40 DNA to determine the number of successfully transfected cells; another set of filters is hybridized with a ^{32}P-labelled ØX174 DNA probe (or other probe homologous to the DNA cotransfected with SV40) to determine the number of successfully transfected cells which give rise to recombinant foci. We define the efficiency of recombination as the ratio between those two values: that is, the fraction of transfected cells which give rise to amplifiable recombinant genomes. Examples of the *in situ* plaque-hybridization autoradiographic signals are shown in Fig. 2. The recombinant plaques (Fig. 2, panels B and C) are heterogeneous in size because of the varying rates with which different recombinant structures replicate. Nevertheless, the foci are discrete and replicate counts show standard deviations no greater than ± 25%.

The key feature of the protocol illustrated in Figs. 1 and 2 is that it is an infectious-center assay which measures the number of recombinant producing cells rather than the yield of recombinant virus. This is a useful feature which can be exploited to identify cells in which recombination activities are high. The limitation of tbe assay is that the recombinant structures must be amplifiable with the aid of the wild type helper virus. However, since the only SV40 function required for helper-mediated amplification is the replication origin, tbis limitation is of minimal significance.

That the autoradiographic signals shown in Fig. 2, panels B and C, do in fact arise from the replication of SV40/ØX recombinant genomes has been confirmed by isolating the bybrid virus (from the portion of the agar overlay corresponding to the autoradiographic signal; Villarreal and Berg, 1977) cloning the DNA in prokaryotic vectors and mapping the recombinant structures by restriction endonuclease and heteroduplex electron microscopy procedures (Winocour *et al.*, 1982). We have mapped 4 SV40/ØX recombinant genomes, each of which arose from a different recombinant

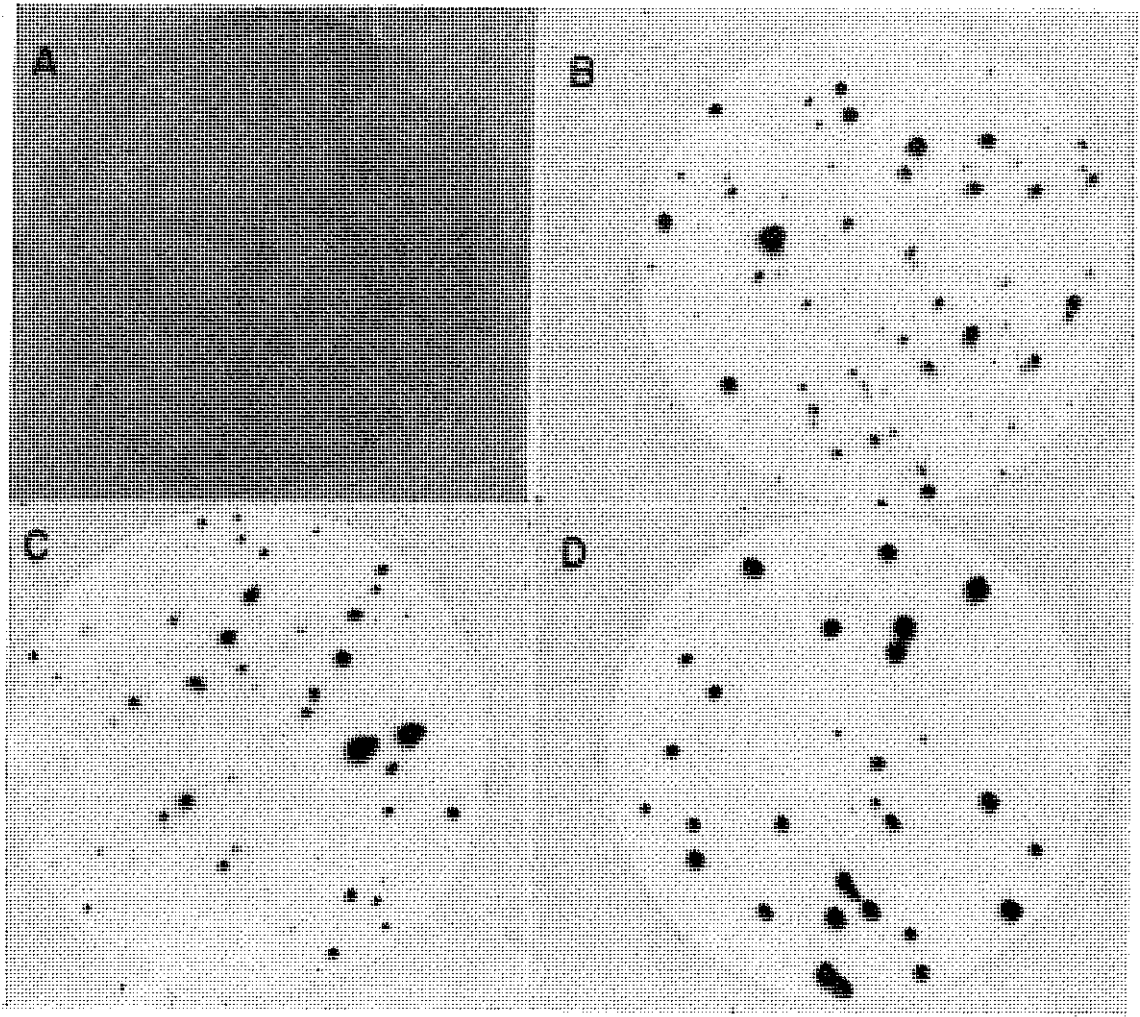

FIG. 2 — Autoradiograms showing recombinant plaques detected by infectious-center *in situ* plaque hybridization. Monkey BSC-1 cells were transfected with 1 μg/ml each of SV40 DNA and ØX174 RF1 DNA (panels B, C, and D) or with ØX174 RF1 DNA alone (panel A) and processed through the infectious-center assay described in the text and in Fig. 1. B and C show SV40/ØX174 recombinant plaques derived from plating 100,000 cotransfected cells together with 3×10^6 uninfected cells and visualized by hybridization with ^{32}P-ØX174 DNA. Panel D shows plaques visualized by hybridization with ^{32}P-SV40 DNA after plating 1,000 cotransfected cells together with 3×10^6 uninfected cells. Panel A shows the lack of hybridization reaction with ^{32}P-ØX174 DNA when SV40 DNA was omitted from the transfection mixture (other conditions as in B and C). Hybridization details are described in Winocour and Keshet, 1980. Data from Dorsett *et al.*, 1982. Autoradiographic exposure times were: Panel A, 7 days; B and C, 5 days; D, 1 day.

producing cell. The ØX174 DNA sequences (800-1200 base pairs) are present as single inserts, located in either the early or late regions of the SV40 genome. Figure 3 shows the restriction map of one such recombinant genome in which the ØX insert is located in the late region of the SV40 genome. The entire SV40 early region (and the replication origin) has been retained in an unscrambled form, as shown by the mapping procedures and by the ability of the hybrid DNA to replicate itself when transfected into monkey cells *in the absence* of the wild type helper virus. Hence, the

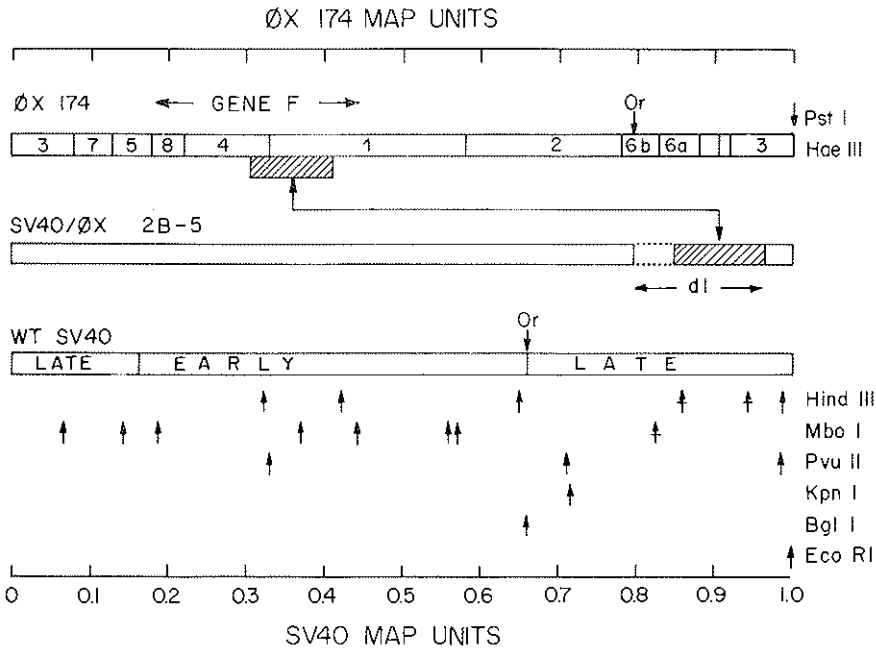

FIG. 3 — Map of the cloned SV40/ØX recombinant genome 2B-5. SV40/ØX recombinant plaques were generated as shown in Figs. 1 and 2 and as described in the text. Hybrid virus was isolated from a portion of the agar overlay corresponding to a single well-separated autoradiographic plaque (Villarreal and Berg, 1977). Supercoiled DNA progeny was prepared by published methods (Winocour and Keshet, 1980). The recombinant DNA molecules were separated from the wild type helper virus DNA by molecular cloning in lambda charon phages followed by subcloning in pBR322 vectors, using standard procedures. The above map was constructed on the basis of data from restriction analysis and heteroduplex electron microscopy (Winocour *et al.*, 1982). The cross-hatched areas show the position of the ØX174 DNA insert in the SV40/ØX 2B-5 genome and the derivation of the insert with respect to the ØX174 genome. As indicated, the deletion in SV40/ØX 2B-5 is larger than the insert. SV40 restriction sites retained in 2B-5 are denoted as arrows (↑); those that are missing are denoted as crossed-arrows (-↑-). The restriction maps before and after molecular cloning were identical.

recombinant structures generated by the DNA cotransfection procedure and isolated from single cells as illustrated in Fig. 1, are relatively simple and do not involve sequence rearrangements outside the area of the insert. In each case examined so far, the restriction maps before and after molecular cloning are identical, indicating that each recombinant-producing cell generates only 1 predominant species of recombinant structures.

We have exploited the technology described in Figs. 1 and 2 to address the following 3 questions about the nonhomologous recombination process in animal cells: 1) Is the efficiency of recombination influenced by the presence of homologous DNA sequences?; 2) Does recombination occur prior to SV40 DNA replication?; and 3) Does the SV40 genome contain sequences which promote or regulate its own recombination?

1) *DNA homology and the efficiency of recombination.*

We have carried out a series of dosage-response experiments in which we measured the frequencies of recombinant producing monkey cells after exposure to a fixed concentration of SV40 DNA and increasing concentrations of ØX174 RF1 DNA, bacterial plasmid DNAs, or the partially homologous polyoma virus DNA. The characteristics of the dosage-response curves were essentially identical in all cases. At the saturation level, 1-2% of the transfected cell population (10% of the total number of cells) gave rise to recombinant genomes. Recombination between SV40 and the totally unrelated prokaryotic DNAs occurred just as frequently as recombination with the partially homologous polyoma DNA. Similar conclusions have been reached in analogous studies in mouse cells. Neither the limited sequence homology between SV40 and polyoma DNAs (Soeda *et al.*, 1980) nor the source of the cotransfected DNA — be it animal virus, bacterial virus, or bacterial plasmid — influences the SV40 or polyoma recombination efficiency. It should be emphasized, however, that the experimental evidence, upon which this conclusion is based, relates to *gross* nucleotide sequence homology (> 30 base pairs) and does not therefore exclude the possible effect of short homologous stretches that might serve as recombination control elements.

2) *Does recombination occur prior to DNA replication?*

Two lines of evidence indicate that the initial recombination event occurs prior to SV40 DNA replication. If monkey cells are exposed to a

fixed concentration of ØX174 DNA and variable concentrations of SV40 DNA, the proportion of SV40/ØX recombinant producing cells is strongly dependent upon the input dosage of SV40 DNA at low DNA concentrations. Since monkey cells are fully permissive for SV40 DNA replication (each transfected cell yields approximately 10^6 progeny DNA molecules) the dependence upon the *initial* SV40 DNA concentration can only be explained on the basis that recombination occurs prior to SV40 DNA replication. The second line of evidence derives from studies using SV40 deletion mutants which lack the sequences comprising the origin of replication (Gluzman *et al.*, 1980). The addition of SV40 *ori*$^-$ DNA to cells transfected with wild type SV40 and ØX174 DNA reduces the number of recombinant producing cells. Analysis of the dosage response data indicates that the SV40 *ori*$^-$ DNA competes with wild type DNA for recombination with ØX174 DNA. Hence a functional replication origin is not required for the initial recombination event; the SV40 replication origin is only required for the amplification of the recombination products.

3) *Does the SV40 genome encode elements which regulate its own recombination?*

In the system described above, SV40 serves both as one partner in the recombination process and as the vector which amplifies the recombination products. The total lack of discrimination with regard to the second partner in the recombination process (ØX174 or bacterial plasmid DNA) together with the strong dependence upon the SV40 gene dosage input, suggested that this recombination pathway may be controlled by elements in the SV40 genome itself. To examine this possibility, we have added defined restriction-endonuclease fragments of the SV40 genome to "standard" recombination reactions consisting of intact SV40 DNA and intact ØX174 DNA; the objective being to determine if, at appropriate dosage-response conditions, the addition of any particular SV40 DNA sequence enhances the recombination frequency. The sectors of the SV40 genome studied so far are shown in Fig. 4. We find that the addition, to the "standard" recombination reaction, of the A or B segments consistently increases the recombination frequency in contrast to other SV40 segments of equivalent size. The stimulation of the recombination frequency is also observed when the B-segment is derived from origin-deletion mutants of SV40 (Gluzman *et al.*, 1980; see map at the bottom of Fig. 5 for the location of the deletions) indicating that the origin

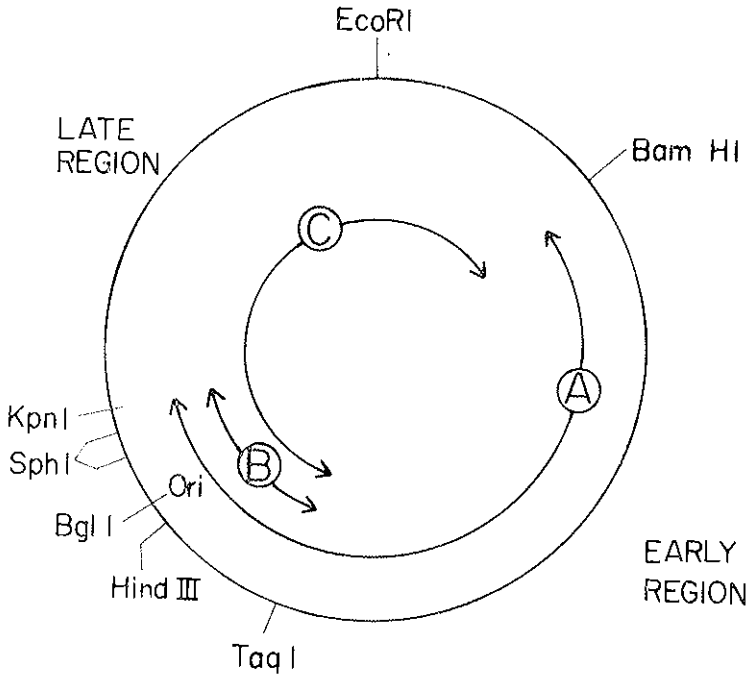

FIG. 4 — Map showing the SV40 segments tested for their ability to enhance recombination between intact SV40 DNA and ØX174 (RF1) DNA (see text).

sequences are not directly involved. Further experiments are required to identify the location of the recombination enhancer sequence(s) flanking the replication origin.

Although the C-segment shown in Fig. 4 contains all the sequences present in the B-segment, we find that it is a poor enhancer of recombination. The C-segment, in contrast to the B-segment, contains DNA derived from the late region of the SV40 genome. Conceivably, the late SV40 region may encode an element which suppresses the recombination enhancing properties of the B-segment.

B. *An effect of SV40-encoded DNA sequences on plasmid fusion and/or plasmid incompatibility in Escherichia coli cells.*

Bacterial plasmid incompatibility has been defined as the inability of 2 related plasmids to be stably coinherited in a single clone of dividing

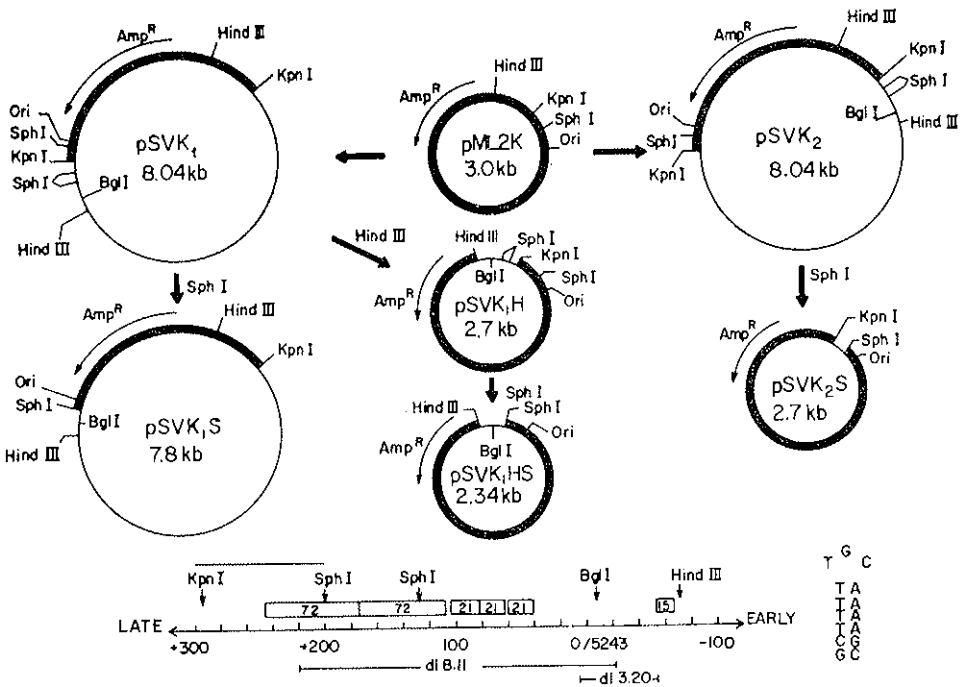

FIG. 5 — Construction of plasmids bearing different inserts of SV40 DNA. pML2K Amp^r is the pML2 plasmid derived from pBR322 by Lusky and Botchan, 1981, and further modified by the introduction of a single Kpn1 site (we are grateful to Dr. Ch. Cahana for the gift of this plasmid). $pSVK_1$ is derived from pML2K by the insertion at the Kpn1 site of unit-length SV40 (linearized at the single Kpn1 site on the circular viral genome). $pSVK_2$ is the same as $pSVK_1$ but with the unit-length SV40 insert in opposite orientation. The remaining plasmid constructs are derived by the restriction cleavages indicated followed by religation and selection on ampicillin-containing media. Light lines denote SV40 DNA and heavy lines denote plasmid DNA (not drawn to scale). The plasmid replication origin is marked as "*Ori*" and the SV40 replication origin is designated by the Bg11 restriction site. All of the Amp^r SV40/plasmid constructs shown, *except* $pSVK_2S$, give rise to Tet^r/Amp^r colonies when cotransfected with pMB9 Tet^r (Vernade *et al.*, 1982).

The map at the bottom shows the SV40 replication origin and flanking regions. Nucleotide numbers are marked as + on late side of the origin (0/5243) and as — on the early side of the origin. Thus the HindIII site at nucleotide 5171 [SV system; Buchman *et al.*, 1980] on the early side of the origin is drawn above at position — 72. The map also shows the positions of the deletions in the SV40 *ori*⁻ mutants dl 8·11 and dl 3·20 (Gluzman *et al.*, 1980). The deletion in dl 8·11 extends from nucleotide positions + 220 to — 21 (220-5221 in SV system); that of dl 3·20 extends from positions — 5 to — 54 (5-5189 in SV system). The sequence at the right of the map (drawn as a stem and loop structure) is the 15 base pair inverted repeat located at nucleotide positions — 55 to — 72 (nucleotide numbers 5188 to 5173 in SV system).

bacteria (Hashimoto-Gotoh and Timmis, 1981). Incompatibility can be recognized if the 2 related plasmids carry different antibiotic resistance markers. Thus, if *E. coli* cells are cotransfected with the two related plasmids, pMB9$^{Tet^r}$ (which imparts resistance to the antibiotic tetracycline) and a pBR322 derivative which imparts resistance to ampicillin but not to tetracycline, some transfected bacteria will grow into colonies in the presence of tetracycline; others will plate in the presence of ampicillin; but very few or none (depending upon the strength of the incompatibility stricture) will plate in the presence of both antibiotics. However, if the 2 incompatible plasmids recombine or fuse together such that both antibiotic resistance markers are carried on the same DNA molecule (linked to a common replicon) then a substantially higher number of bacterial colonies resistant to both antibiotics should arise. It occurred to us that this quick and convenient bacteriological test might be exploited to screen for putative SV40 DNA sequences which promote plasmid recombination or plasmid fusion. We chose to work with the plasmids pMB9$^{Tet^r}$ and pML2K$^{Amp^r}$ (a pBR322 derivative) because initial tests showed that they display a particularly strong degree of incompatibility when cotransfected into either *E. coli* RecA$^-$ strains (HB101 or DH1) or RecA$^+$ strains (MM294). A series of pML2K$^{Amp^r}$ constructs containing intact SV40 DNA, or defined fragments thereof, were made as shown in Fig. 5. When these Amp^r plasmid-constructs were transfected together with pMB9$^{Tet^r}$ DNA into *E. coli* cells (either RecA$^+$ or RecA$^-$ strains) the following results were obtained.

1. Intact SV40 DNA linearized at the KpnI site and inserted (either orientation) into the KpnI site of pML2K$^{Amp^r}$ (designated pSVK$_1$ and pSVK$_2$ in Fig. 5) gave rise to a substantial proportion of colonies resistant to both antibiotics when cotransfected with pMB9$^{Tet^r}$. The number of colonies resistant to both antibiotics was 1-10% of the number resistant to either antibiotic alone. In contrast, bacterial cells cotransfected with similar amounts of pMB9$^{Tet^r}$ and pML2K$^{Amp^r}$ (no SV40 insert) gave rise to no Tet^r/Amp^r colonies.

2. To identify the SV40 DNA region responsible for the above effect, we reduced the size of the SV40 DNA insert in pSVK$_1$ by the series of restriction cleavages and religations shown in Fig. 5. The SV40 DNA insert in pSVK$_1$H comprises 366 base pairs extending from the KpnI site at

nucleotide 294, through the SV40 replication origin, to the first HindIII site on the early side of the origin at nucleotide 5171 (see map at bottom of Fig. 5). The SV40 DNA in pSVK$_1$HS is further reduced to 201 base pairs extending from the Sph1 site at nucleotide 129 to nucleotide 5171. Both pSVK$_1$H$^{Amp^r}$ and pSVK$_1$HS$^{Amp^r}$ gave rise to Tet^r/Amp^r colonies when cotransfected with pMB9$^{Tet^r}$.

3. To evaluate the role of the SV40 replication origin sequences, we made a similar series of pML2K constructs using, instead of wild type SV40 DNA, the DNA of the SV40 origin-deletion mutants dl 8·11 and dl 3·20 (Gluzman *et al.*, 1980). All such Amp^r constructs gave rise to Tet^r/Amp^r colonies when cotransfected together with pMB9$^{Tet^r}$. Hence, SV40 origin sequences are not required.

4. The locations of the deletions in SV40 dl 8·11 and dl 3·20 are shown on the map at the bottom of Fig. 5. pSVK$_1$H constructed with SV40 dl 8·11 DNA contains 74 base pairs of SV40 DNA derived from the late side of the origin (nucleotides 220-294) and 50 base pairs from the early side of the origin (nucleotides 5171-5221). The responsible SV40 sequence must reside in either of these two regions. To assess the role of the DNA sequence between nucleotides 220-294, we constructed pSVK$_2$S which is derived from pSVK$_2$ (intact wild type SV40 in pML2K in the opposite orientation to pSVK$_1$) by Sph1 digestion followed by religation. The SV40 complement of pSVK$_2$S extends from nucleotide 201 to nucleotide 294. pSVK$_2$S *did not* promote the generation of Tet^r/Amp^r colonies when cotransfected together with pMB9$^{Tet^r}$ and hence SV40 sequences from nucleotides 201-294 cannot be involved. We conclude, therefore, that the SV40 sequence responsible for the effect in bacteria must reside within the 50 nucleotide stretch, nucleotide positions 5171-5221. It may be noted that the pSVK$_2$S construct also serves as a negative control since it contains the same plasmid-derived information as pSVK$_1$S which promotes, together with pMB9$^{Tet^r}$, the generation of Tet^r/Amp^r colonies.

5. The SV40 mutant dl 3·20 is currently being used to further pinpoint the responsible SV40 sequences. This 58 base pair deletion extends from nucleotide 5 to nucleotide 5189, that is to within 18 nucleotides of the HindIII site at position 5171 (Fig. 5, map at bottom). pSVK$_1$H

constructed with dl 3·20 DNA still gives rise to Tet^r/Amp^r colonies, suggesting that the responsible sequence lies between nucleotides 5171 and 5189. Inspection of the SV40 sequence (Fiers *et al.*, 1978) in this region reveals the 15 base pair inverted repeat capable of forming the stem and loop structure shown in Fig. 5, bottom right. This inverted repeat is now being synthesized to facilitate a direct test of its ability to promote plasmid fusion and/or relaxation of the incompatibility stricture.

6. The expression of the SV40 sequence described above appears to be controlled *via* a spatial relationship with another SV40-encoded signal located in the late region. We have observed that if circular SV40 DNA is linearized at the EcoRI site, instead of the Kpn1 site, and inserted into the EcoRI site of pML2K, the resulting plasmid construct does not give rise to Tet^r/Amp^r colonies when cotransfected with $pMB9^{Tet^r}$. It appears unlikely that the position of the SV40 insert within the plasmid is crucial since other experiments show that the insert can operate in either orientation and at several plasmid sites. Furthermore, if the SV40 encoded sequence signal located between the origin and the HindIII site at nucleotide 5171 is attached to DNA from the late SV40 region (DNA between the Kpn1 and EcoRI sites) its expression is neutralized and no Tet^r/Amp^r colonies arise. We propose therefore that the late SV40 region contains a sequence encoded element (as yet unidentified) which negatively controls the expression of the positively acting signal(s) located between the origin and nucleotide 5171.

7. The molecular basis for the emergence of Tet^r/Amp^r colonies is currently being investigated. The plasmid DNA isolated from one Tet^r/Amp^r colony is clearly a pMB9/pML2K fusion product, as judged by restriction analysis and the nonsegregation of the antibiotic resistance markers upon retransfection of *E. coli* cells. On the other hand, other Tet^r/Amp^r colonies contain multiple species of plasmid DNA which have yet to be identified and in retransfection of *E. coli* with these DNA preparations, the antibiotic resistance markers segregate out. It is possible that the initial pMB9/pML2K fusion products were unstable in these latter colonies.

Concluding remarks

The dissection of the nonhomologous recombination pathways in animal cells is crucial to the analysis of viral carcinogenesis and the presumed role of gene mobility in development and differentiation. A major obstacle to our understanding of gene exchange in higher organisms is the lack of experimental systems amenable to molecular analysis. Some of the better characterized tumor viruses provide an encouraging approach to this problem. The entire nucleotide sequence of the SV40 and polyoma genomes is known (Fiers *et al.*, 1978; Reddy *et al.*, 1978; Soeda *et al.*, 1980) and ingenious methodologies for the introduction of site-specific mutations have been discovered (see article by D. Nathans, this volume). The objective of the studies discussed in this article is to understand how cellular recombination activities are mobilized during the SV40 and polyoma infection.

At the nucleotide sequence level, SV40 and polyoma exhibit little or no discrimination in the recombination acts which can occur in monkey or rodent cells. Recombination with unrelated prokaryotic DNAs occurs just as frequently as that with related viral DNA. This mobilization of a nonhomologous recombination pathway is not an antifact of the DNA cotransfection technique since equally indiscriminate recombination events have been observed after infection with different unrelated virus particles (work in progress). Two types of observations suggest that recombination in SV40 infected monkey cells is controlled at least partly by the SV40 genome itself: First, the dependence of the frequency upon the initial SV40 gene dosage; Second, the enhancement of the frequency when specific sectors of the SV40 genome are used to increase the gene dosage. The recombination enhancing region of the viral genome flanks the replication origin but does not involve the origin sequences themselves. Whether the "enhancer" is a DNA sequence-specified signal or a gene product remains to be determined.

We have also observed that the insertion of an SV40 DNA fragment into a bacterial plasmid promotes plasmid fusion and/or a reduction in the plasmid incompatibility stricture in *E. coli* cells. The identification of the very small amount of SV40 DNA responsible for this phenomenon (within the 50 nucleotide stretch between positions 5171 to 5221 on the early side of the replication origin) suggests that a sequence specified signal rather than gene product is involved. The expression of this signal is influenced by its spatial relationship with an as-yet-un-

identified element in the late region of SV40. The fact that the viral DNA elements which influence the recombination frequency in monkey cells are located in similar sectors encourages us to assume, as a working hypothesis, that the SV40 DNA-encoded putative recombination signals are universal in the sense that they are recognized by both prokaryotic and eukaryotic cells. The validity of this assumption depends upon the outcome of our current efforts to define the molecular basis for the SV40-promoted plasmid fusion and reduction in plasmid incompatibility in the bacterial cell. If our assumptions are estahlished, then the bacterial assay described in this article should provide a rapid screening procedure for the identification of similar recomhination-enhancing signals encoded in the chromosomal DNA of higher organisms.

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PRODUCTION OF HUMAN INTERFERONS BY DNA RECOMBINANT METHODS AND REGULATION OF THE INTERFERON-β1 GENE

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INTRODUCTION

Recombinant DNA technology has had two major impacts in biology. First, it has opened new possibilities for obtaining gene products in larger quantities than by classical tissue or cell cultures. Isolated genes can be propagated, amplified and expressed in microorganisms or in eukaryotic cells, using a variety of DNA vectors equipped with defined replication, transcription and translation signals. By optimalizing gene expression, it is possible to create situations in which a normally minor protein product can become a sizeable fraction of the total proteins made by the cell.

The second major application of recombinant DNA technology is in the study of gene expression. The control mechanisms which regulate transcription, translation and processing of genes in cells, can be examined by modifying the structure of the gene and its environment. This gives information that could not be readily obtained by classical genetic methods. The function of the different segments of a gene can be analyzed and these functions can be tested by reassembling the segments in various synthetic arrangements. Ultimately, these techniques should allow gene replacements, therapy of genetic diseases and improvement of existing genomes.

Using the human Interferon IFN-β_1 gene, several examples of these applications of genetic engineering will be illustrated here.

The IFN-β_1 gene as a model of inducible gene

Expression of the human fibroblast interferon gene IFN-β_1 is switched on, i.e. IFN-β_1 mRNA accumulates in cells when fibroblasts or other cells from solid tissues are infected by viruses or exposed to double-stranded (ds) RNA [1, 2]. Expression of several other cellular genes is also induced by ds RNA [3] and, in particular, a minor second species of interferon (IFN-β_2) is made in addition to the major IFN-β_1 species [4-6). Leucocyte IFN-α genes can be expressed at low levels in virus-infected fibroblasts [7]. The IFN-β genes in fibroblasts are subject to additional regulation, such as superinduction by RNA and protein synthesis inhibitors [8, 11] and priming by IFN treatment [11, 12]. It is likely that these controls do not all operate at the transcriptional level; some could affect RNA processing, other RNA stability [10, 11] and RNA transport from the nucleus to the cytoplasm.

The biochemical mechanisms which control gene expression in the cell nucleus probably involve complex interactions between proteins or other structural elements, and the nucleotide sequence of the gene. It has become possible to analyze the role that these nucleotide sequences play, since genes or gene fragments can be isolated by cloning in a bacterial host, and transferred into recipient cells to examine their expression [13, 14]. Although this approach cannot reveal all the reactions involved in switching-on or -off the genes, it can indicate what are the important nucleotide sequences which regulate the gene's activity, and verify the role of recognizable signal sequences in the DNA.

Gene transfer experiments into eukaryotic cells, can be done in several ways, resulting in either 1) gene integration in the recipient cell genome, as in the co-transfection system of Wigler *et al.* [13], or 2) propagation of the gene as an extrachromosomal element in the nucleus of the recipient cell. In the latter case, two possibilities exist: either 1) the gene is permanently propagated in the cell, as by the papillomavirus vector [15], or 2) the gene is transiently amplified on a replicating virus vector, which eventually kills the cell, as in the case of SV40 in permissive monkey cells [16, 17]. Each of these methods has its own advantages for specific applications. For the study of the IFN-β_1 gene induction, we chose [18] the latter method with a transiently replicating SV40 vector, because 1) each

experiment can be completed within 3 days without having to wait for the establishment of transformed clones, and 2) the extrachromosomal nature of the vector allows to better define the structure of the gene introduced into the recipient cell. In this way, modification of the genes can be studied without the danger that unknown rearrangements occur during the experiment. Several studies on induction of the IFN genes using cotransformation [19, 23] or the papillomavirus system [24, 25] have also been reported.

Regulated expression of the transferred IFN-β_1 gene

The experimental scheme adopted to study the expression of the IFN-β_1 gene in SV40 vectors is outlined in Fig. 1. Among the various monkey cell lines permissive for SV40, CV-1 cells exhibited most clearly the induction of human IFN-β_1 gene expression after treatment with the inducing mixture of poly (rI) (rC), DEAE-dextran and cycloheximide [18]. The number of SV40-IFN β_1 recombinant genomes in the CV-1 cells at the time of induction (i.e. 56 hours post transfection) was estimated to be about 10,000 copies per cell, which is less than in other monkey cell lines.

In induced human cells, the majority of the IFN-β_1 transcripts are 900 bp long and their 5′ ends ("cap site") map about 75 bp upstream from the initiator ATG codon, and 30 bp downstream from a typical Hogness TATAA box [26, 27], as shown in Fig. 2 and 3. A small percentage of longer IFN-β_1 transcripts are present in these cells and discrete bands at 1.4, 3.8 and 5.1 kb were detected (Nir, Maroteaux and Revel, unpublished). These transcripts appear to have the same 5′ ends as the major 0.9 kb RNA, but extend further down on the 3′ side of the gene. Induction of the IFN-β_1 gene can, therefore, be defined by the presence of transcripts which start at the cap site. These transcripts can be measured in total cell RNA, by single-stranded nuclease mapping [28].

The human IFN-β_1 gene was isolated on a 1.84 kb *Eco* R1 genomic DNA fragment [29], which contains 285 bp of 5′ flanking sequences upstream from the cap site, and 700 bp of 3′ flanking sequences downstream from the polyadenylation site (Fig. 2B). This fragment retained its normal expression and inducibility when introduced into the monkey CV-1 cells as part of one of the SV40 plasmid vectors of Fig. 4A. Correctly initiated transcripts identical to those found in human cells, accumulated when the CV-1 cells were induced for IFN production (Fig. 4B lane 2). The *Hinf* 1-labeled DNA probe used here was such that correctly initiated

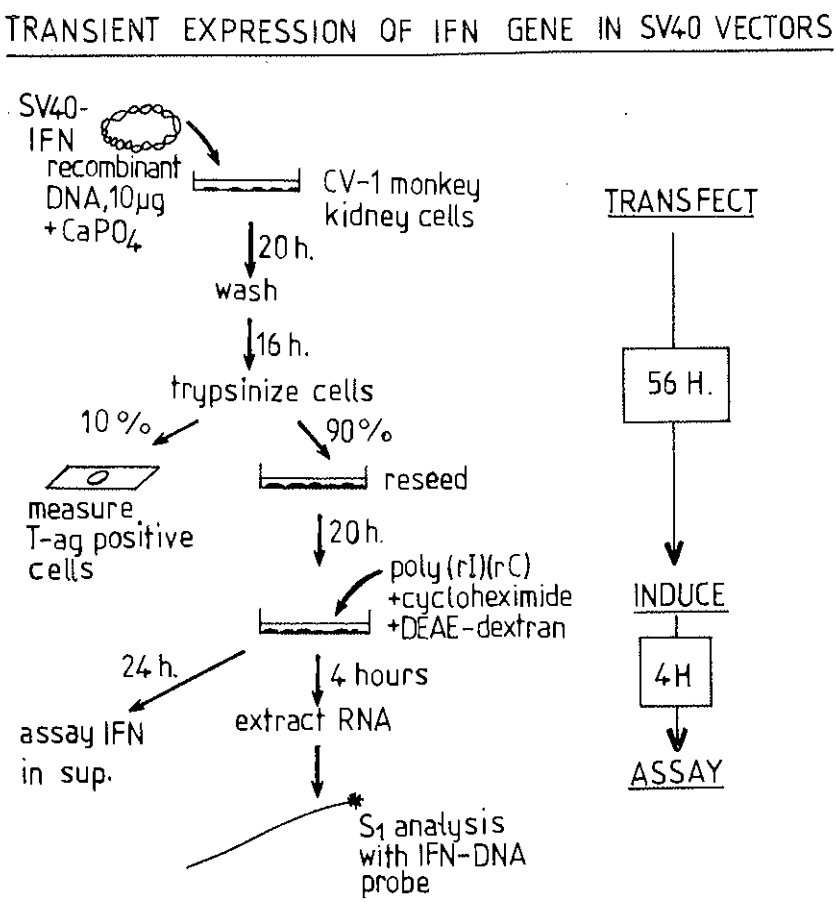

FIG. 1. *Outline of the procedures used for human gene transfer and measure of its expression* (for details see text).

IFN-β_1 RNA protects 160 bp against S1 nuclease (Fig. 2B). In uninduced cells, almost no protection of the 160 bp fragment probe was seen (Fig. 4B lane 1). This inducibility was independent of the position in which the IFN-β_1 gene was inserted in the vector (first 4 lines of Fig. 4A). When cytosine arabinoside was added to the cells 24 hours before induction, to block replication of the SV40 DNA vector, the amount of IFN-β_1 RNA produced was reduced to a very low level, indicating that induction did occur on the replicating SV40-IFN β_1 recombinant DNA.

Induction in CV-1 cells induces also monkey IFN-β RNA, which dif-

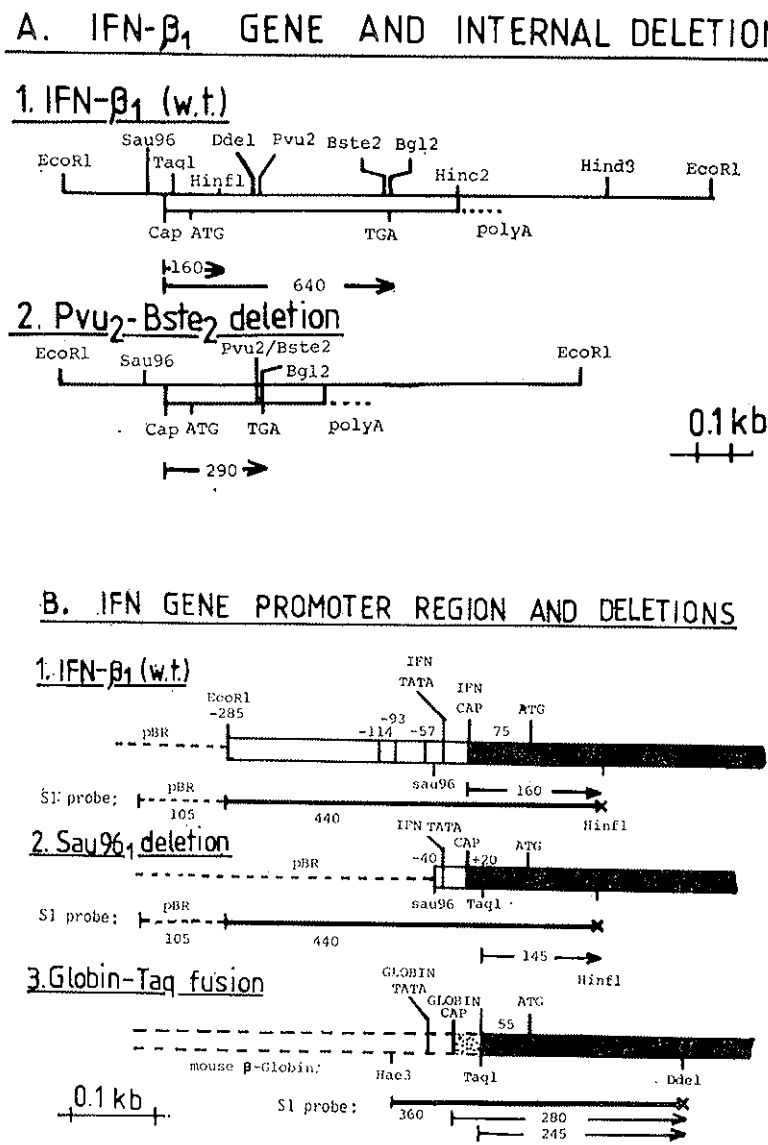

FIG. 2. *Structure of the human IFN-β_1 gene and the various deletions studied.*

A1. Wild type IFN-β_1 gene is isolated on 1.84 kb *Eco* RI human genomic fragment [29]. The relevant restriction sites are indicated relative to the boundaries of the gene. Arrows show the distance from the 5′ end of the mRNA (cap) to the *Hinf*$_1$ and *Bgl*$_2$ sites.

A2. IFN-β_1 gene with deletion in the coding region. Arrow shows cap-*Bgl*$_2$ distance in the deleted gene.

B1. Enlarged drawing of the wild type IFN-β_1 promoter region. Arrow shows the S_1 probe used. RNA initiated outside the gene protects 440 bp; RNA starting at cap site protects 160 bp. of the S_1 probe.

B2. Promoter deleted up to the *Sau* 96$_1$ site.

B3. Promoter fused at *Taq*$_1$ site to a mouse ß-globin promoter. Lower arrows show the S_1 probe used. RNA initiated at globin cap site protects 280 bp; RNA not initiated at glohin cap would protect only 245 bp. Upper arrow shows that with the *Hinf*$_1$-labeled S_1 probe, the globin-*Taq* fused gene would protect 145 bp instead of the normal 160 bp.

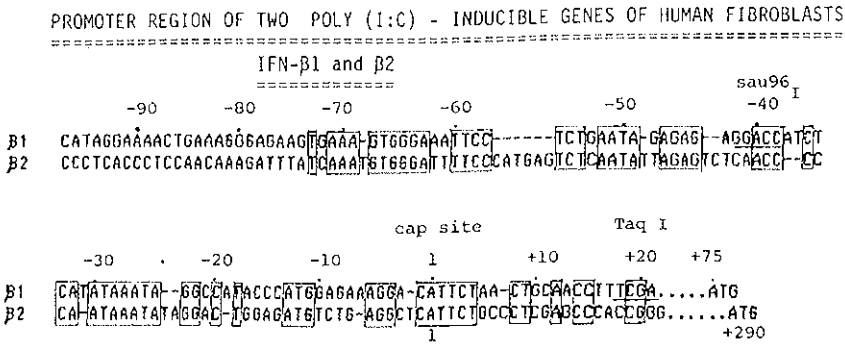

FIG. 3. *Nucleotide sequence of the promoter region of the IFN-β_1 gene.* The sequence is given in comparison with that of the IFN-β_2 gene expressed in human fibroblasts [4]. The common features of the two promoters are boxed. Restriction sites used for constructing deletion of the IFN-β_1 promoter are indicated.

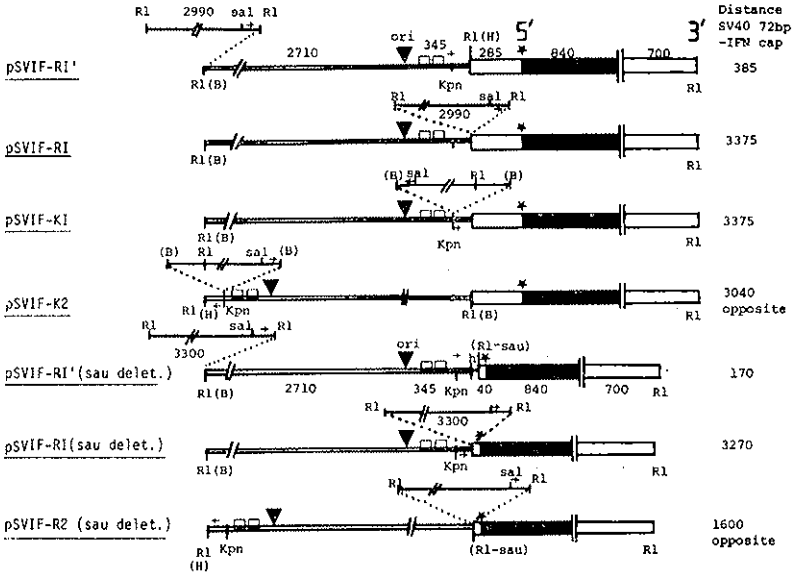

FIG. 4A. *Transfer and expression of the human IFN-β_1 gene by SV40 vector in monkey cells.* Structure of IFN-β_1-SV40-pML2 plasmid recombinant genomes. The 4 upper lines represent constructions with the wild type IFN-β_1 gene. The 3 lower lines are constructions with *Sau* 96$_1$-deleted IFN-β_1 promoter. The small arrows show the SV40 late promoter and an RNA start site present in the plasmid pML2 sequences. The squares represent the 72 bp repeat enhancer of SV40; the black triangle is the origin of SV40 replication. The asterisk marks the cap site of the IFN-β_1 gene.

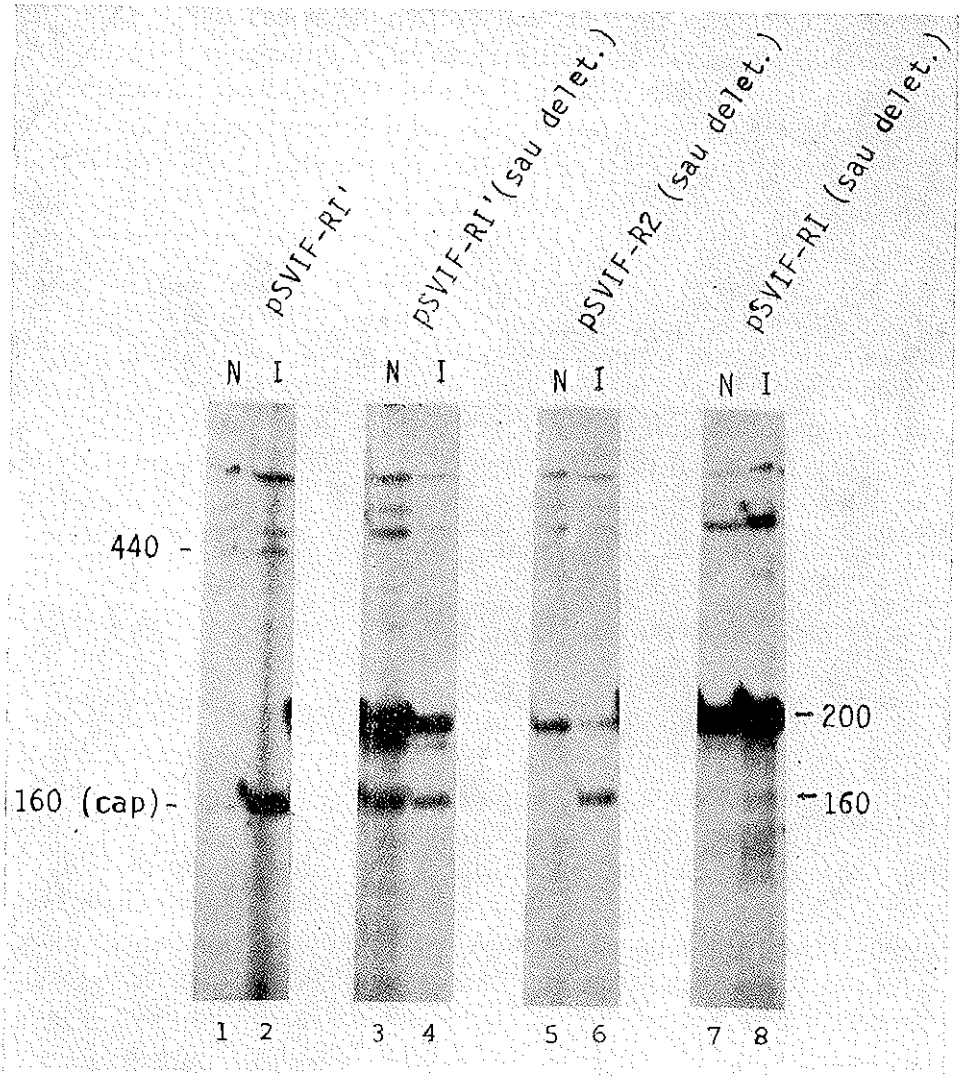

Fig. 4B. Single-stranded nuclease analysis of IFN-β_1 RNA expressed in monkey CV-1 cells transfected by IFN-SV40 recombinants. Cells were transfected and RNA analysed as outlined in Fig. 1. Cells were induced (I) with 50 μg/ml each of poly (rI):(rC), DEAE-dextran and cycloheximide or left uninduced (N). For the structure of recombinant DNAs see 4A. Protection of the 160 bp fragment in the S_1 probe indicates the RNA starting at the IFN cap site (see Fig. 2). The 440 bp and 200 bp fragment indicate RNA initiated outside the gene in the case of the wild type (lanes 1, 2) and *Sau* 96_1 deleted genes respectively (lanes 3-8).

fers probably only by a few nucleotides from the human IFN-β_1 RNA. In most experiments, the S1 nuclease analysis discriminates between the human and monkey IFN RNA, and monkey RNA did not protect the S1 DNA probe [18]. It is, however, essential to demonstrate that the IFN-β_1 RNA detected in transfected cells indeed originates from the human IFN-β_1 gene introduced by the SV40 vector. We therefore made a deletion in the IFN-β_1 gene which removed 350 bp between the *Pvu* 2 and *Bste* 2 sites of the coding sequence (Fig. 2A). An S1 nuclease probe derived from the deleted gene and labeled in the *Bgl* 2 site, should be protected over 290 bp if the mRNA derives from the deleted gene. Fig. 5 (lanes 3 and 4) demonstrates that the induced transcripts are produced by the deleted transfecting human gene. This experiment also shows that a large part of the coding region can be deleted without prejudice to the inducibility of the IFN-β_1 gene. The same result was obtained when the entire 3′ region of the gene downstream from the termination codon was removed.

Role of the 5′ flanking region in the expression and inducibility of the IFN-β_1 gene

To examine if the 5′ flanking region of the IFN-β_1 gene was important for the induction phenomenon, we replaced it by the 5′ flanking region of the mouse β-globin gene. The 5′ flank of the globin gene was cut by *Hinc* 2 at nucleotide 24 downstream from the globin cap site [30], and fused through *Eco* R1 linkers to the 20th nucleotide downstream of the IFN cap site in the IFN-β_1 gene (cut with *Taq* 1, see Fig. 2B). The hybrid globin-IFN gene was cloned in the SV40-plasmid vector and used to transfect CV-1 cells. Analysis by ss-nuclease of the RNA produced, showed that the IFN-β_1 gene was now transcribed constitutively, and the same amount of specific RNA (145 bp band, Fig. 5 lanes 7, 8) was found before and after induction with poly (rI) (rC). The RNA produced was indeed initiated at the globin cap site, as shown (Fig. 5 lanes 9, 10) by the protection of 280 bp from the *Dde* 1-labeled S1 probe used (see Fig. 2B, line 3). These experiments clearly demonstrate, therefore, that the absence of expression of the IFN-β_1 gene in uninduced cells, and its inducibility, are a property of the IFN-β_1 gene 5′-flanking region. When this region is replaced by the globin promoter, inducibility is lost and the gene is expressed continuously.

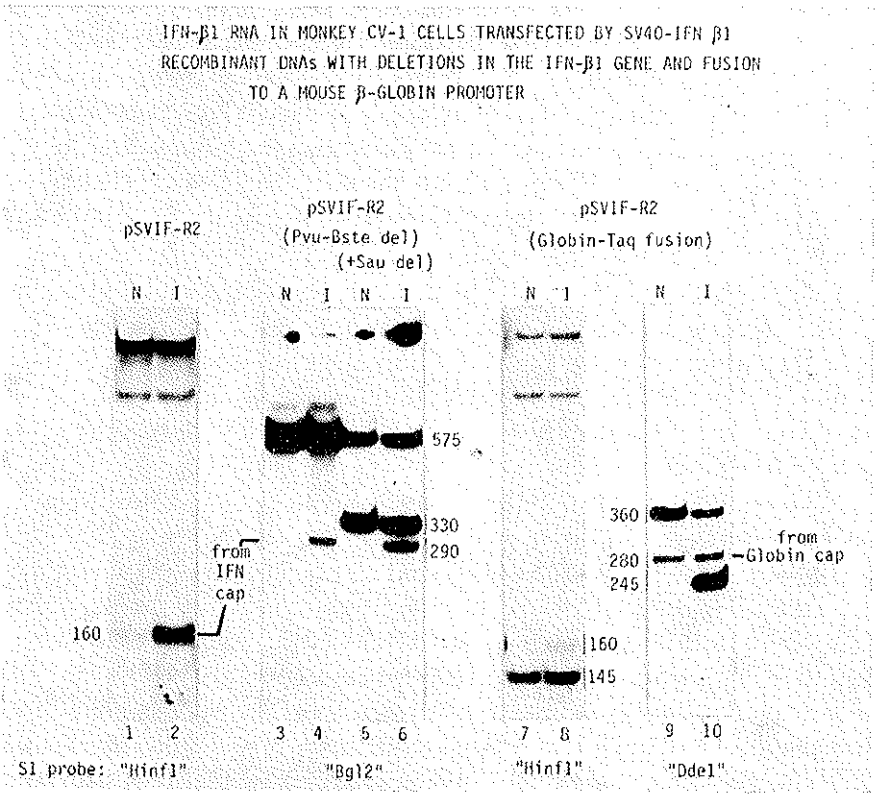

FIG. 5. *Expression of modified IFN-β_1 gene in transfected monkey cells.* CV-1 cells were transfected and RNA analyzed as in Fig. 1. Structure of pSVIF-R2 is the same as that shown in the last line of Fig. 4A, but with the intact 285 bp 5′ flanking region of the IFN-β_1 gene. The structure of the other deletions, and the fusion to globin promoter is described in Fig. 2. The S_1 probe used in each experiment is indicated, and explained in Fig. 2. In lanes 1, 2, IFN cap-initiated RNA protects 160 bp while in lanes 3-6, it protects 290 bp. The 330 bp represents RNA initiated outside the gene, 575 bp is the reannealed probe. In lanes 7, 8, IFN RNA initiated at the globin promoter protects 145 bp (the 160 bp originating from monkey RNA). In lanes 9, 10, globin cap initiates RNA protects 280 bp (the 245 bp originating from monkey IFN RNA and the 360 bp being the reannealed probe).

Effect of deletions in the 5′-flanking region of the IFN-β_1 gene

The 285 bp region preceding the IFN-β_1 cap site, contains several recognizable transcription signals: in addition to the TATAA box at −30, a possible CAAT box is seen at −57, and additional sequences at −93 and −114 could be present [27]. We made use of the presence of a *Sau*96$_1$ restriction site at −40 to construct an SV40-IFN β_1 recombinant genome with a deletion of the entire 5′-flanking region, leaving only 10 nucleotides upstream from the TATAA box (Fig. 3). The 5′-deleted gene was introduced in 3 different orientations with respect to the SV40 vector, as shown in Fig. 4A (3 lower lines).

The *Sau*96-deleted IFN-β_1 gene was still clearly inducible by the poly (rI) (rC) mixture as shown in Fig. 4B (lanes 5 and 6) in the case of the pSVIF-R2 construction of Fig. 4A (lower line). This finding was also confirmed using the *Pvu*2-*Bste*2 deletion in the coding region (Fig. 5B lanes 5, 6). Thus, the 1,84 kb IFN-β_1 gene can be reduced to about 300 bp and retain its inducibility.

Although the 5′-deleted IFN-β_1 gene retains its regulation, there are differences between the deleted and the original wild-type gene. While the wild-type gene is inducibly expressed in all the constructions studied, the regulated expression of the 5′-deleted gene depends on its position in the SV40 vector. In plasmid pSVIF--R1′ (sau deleted) (Fig. 4A), in which the RNA start site of the deleted gene is very close to the SV40 late promoter region (170 bp), transcription from the IFN cap site can be seen even in uninduced CV-1 cells (Fig. 4B lanes 3, 4). In contrast, in the pSVIF-R1 (sau del.) construction (Fig. 4A), where the IFN cap site is 3,300 bp away from the SV40 promoter region, expression and induction of the IFN-β_1 gene is reduced to very low levels (Fig. 4B lanes 7, 8).

The induced expression is optimally observed in the pSVIF-R2 (sau del.) construction of Fig. 4A, where the distance of the 2 promoters is 1,600 bp.

These results suggest that in the deleted 5′-flanking sequences of the IFN-β_1 gene, there are signal sequences which enhance transcription from the IFN cap site. The function performed by these signals can be replaced by some element in the SV40 promoter region, most likely the 72 bp repeat enhancer sequence [31] which can act at a certain distance, and is believed to create a nucleosome-free gap in the episomal genome [32, 33]. The poly (rI) (rC)-dependent induction phenomenon seems, however, to be a property of the sequences remaining in the *Sau*96$_1$-deleted IFN-β_1 gene.

The simplest interpretation of all the results, would be that induction exerts a positive effect on transcription (or stabilisation) of RNA starting at the cap site. In non-induced cells, this site is normally poorly active, but can be activated by the close proximity of a strong transcription enhancer. The 5′-flanking region of the IFN-β_1 gene would prohahly contain weak transcription signals, which require further activation by induction. It cannot, however, be excluded that there is also a repression effect by the 5′-flanking sequences, since the SV40 enhancer does not activate the IFN cap site in the intact IFN-β_1 gene (as in pSVIF-R1′) unless the cells are induced. Fig. 6 presents a summary of these findings.

In conclusion, the regulated expression and induction of the human

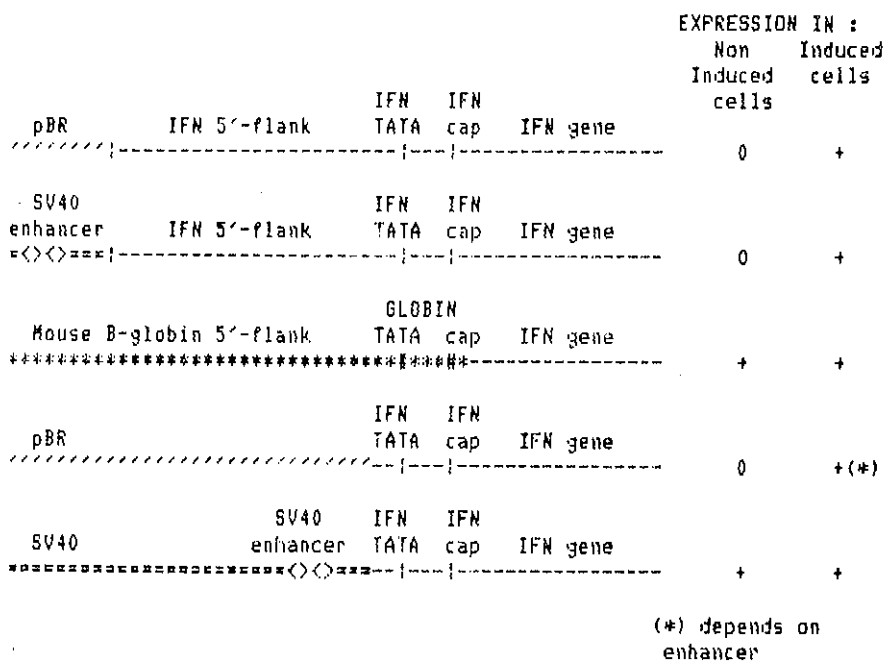

FIG. 6. *Summary of the experiments on transfer and expression of the IFN-β_1 gene.* The schematic structure of the various constructions is shown, together with their expression, measured by the presence of cap initiated RNA in induced or non-induced monkey CV-1 cells. Asterisk shows that this promoter-deleted gene is expressed only if the SV40 72 bp enhancer is not too distant. For details see text.

IFN-β_1 gene seems to be due to nucleotide sequences close to the TATA box and RNA start site of the gene. A comparison of these sequences in the IFN-β_1 gene and in another poly (rI) (rC)-inducible gene (IFN-β_2 in Fig. 3) shows a high degree of conservation, although the overall structures of these two genes are quite different [4, 29]. It would now be interesting to test the function of the sequences around the TATA box and α cap site of the IFN-β_1 gene, and to create hybrid promoters with other genes. Such experiments have not yet been done in the eukaryotic system, but are possible in prokaryotes as described in the next section.

Expression of the human IFN-β_1 gene in E. coli *with a hybrid tryp-lac promoter*

E. coli promoters often contain two conserved sequences at -10 (TATAA box) and at -35 (TTGACA), which were shown to lie on the same side of the DNA helix and to interact with the RNA polymerase [34, 35]. Not all the promoters, however, contain these consensus sequences. The Lac UV5 promoter of *E. coli* has the -10 consensus TATAA (the wild-type Lac promoter does not), but differs from the consensus at -35. The tryptophan operon promoter has the consensus sequence at -35 but not at -10. A hybrid tryp-lac promoter can be constructed which has both consensus sequences (Fig. 7). It becomes, therefore, possible to test the functions of these two signals.

The sequence coding for the mature human IFN-β_1 was linked either to the Lac UV5 promoter or to the hybrid tryp-lac promoter [36, 37]. The ribosomal binding site was, in both cases, that of the Lac mRNA followed by the ATG codon of the IFN gene. Table 1 shows that the efficiency of IFN synthesis was at least 10 times higher with the hybrid tryp-lac promoter than with the Lac promoter. The same was found for the expression of another human interferon gene, IFN-α_C (Table 1). Another advantage of the tryp-lac promoter is its insensitivity to catabolite repression by glucose in the medium, resulting from the removal of the DNA segment which in the Lac promoter binds the catabolite activated CAP protein [38]. The tryp-lac promoter is still repressed by the Lac repressor, but because of the promoter's strength, this occurs only in *E. coli* strains that overproduce the repressor (Lac i^q mutation) (Fig. 8). Induction by isopropylthiogalactoside, at the end of the log phase of growth, produces optimal amounts of IFN, and this system can be used for industrial production of human interferon in recombinant bacteria. Other bacterial promoters

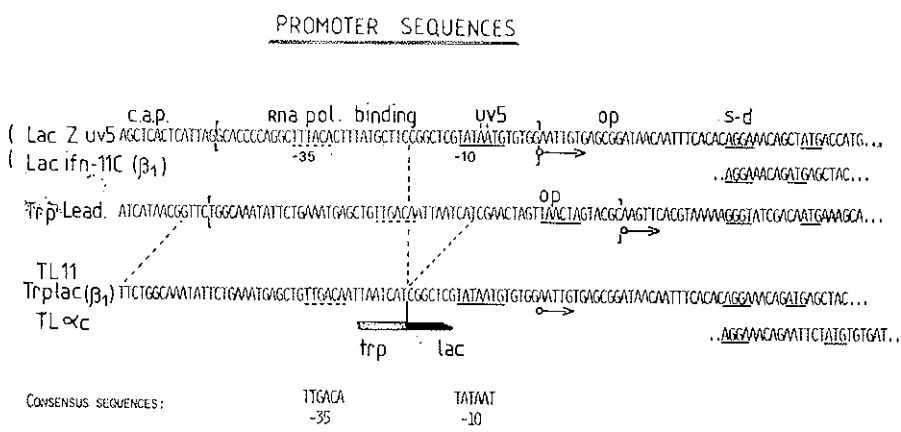

FIG. 7. *Nucleotide sequence of the promoter and ribosomal binding sites of the lac- and tryp-lac-IFN recombinant DNA used for expression in* E. coli.

Line 1: *E. coli* lac UV5 promoter sequence [38]. c.a.p. is the binding site of the catabolite activated protein; the RNA polymerase binding site is shown in brackets. The −35 and −10 signals are underlined. The 2 nucleotides changed in the UV5 mutation are shown. Op, lac operator. S-D, nucleotides presumed to base-pair with 16S ribosomal RNA. The ATG codon of the ß-galactosidase cistron is underlined. Arrow shows start of mRNA.

Line 2: Junction of the lac ribosomal binding site to the ATG, corresponding to the first amino acid of the mature IFN-β_1 sequence.

Line 3: *E. coli* tryp operon promoter sequence [39].

Line 4: Hybrid tryp-lac (TL) promoter and ribosomal binding site in the case of IFN-β_1 (TL-11).

Line 5: Same as line 4 for the IFN-α_C gene. The first amino acid of the mature sequence is the cysteine (TGT) which follows the ATG codon.

TABLE 1 - *Expression of human interferon α and β genes by tryp-lac hybrid promoter.*

STRAIN CHARACTERISTICS	IFN ACTIVITY (UNITS/LITER/O.D. BACTERIAL CULTURE)	
	IFN-β_1	IFN-α_C
3 LAC UV5 PROMOTERS	LAC-11C: 0.5×10^6	LAC-α_C: 0.4×10^6
1 TRYPLAC HYBRID PROMOTER (TL)	TL-11 5×10^6	TL-α_C-647: 11×10^6
2 TL-IFN GENES PER PLASMID	TL-218: 10^7	
TL PROMOTER + TRP TERMINATOR	TL-44: 2.4×10^7	

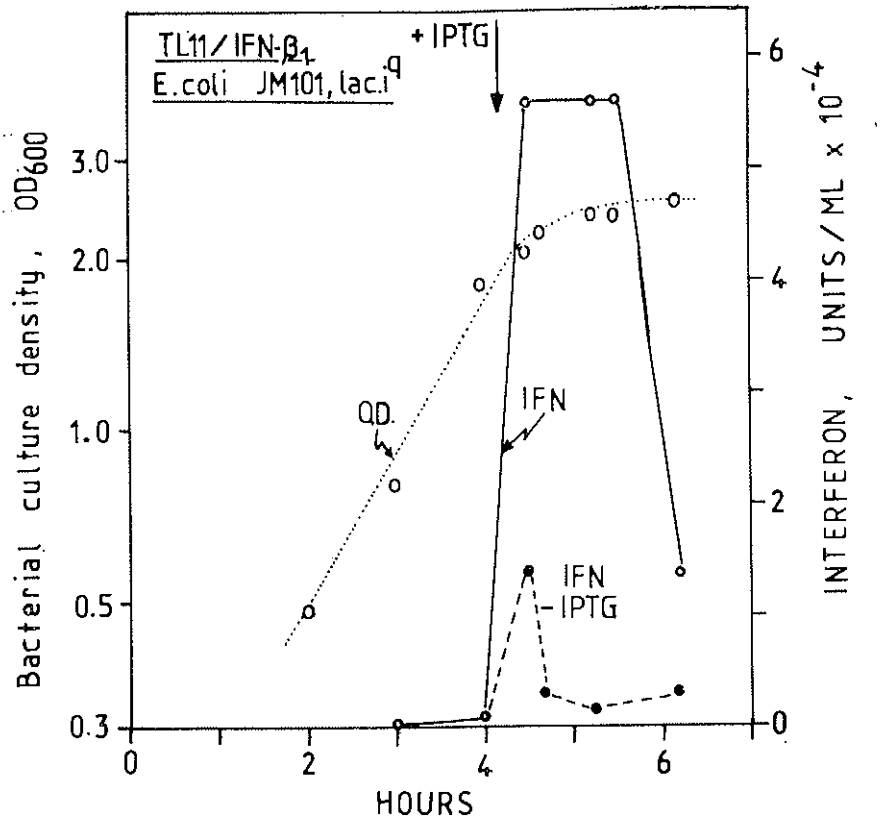

FIG. 8. *Production of human IFN-β1 in* E. coli *by tryp lac plasmid TL-11.* The growth curves of the *E. coli* strain JM101/TL-11 in a 0.5 1 fermentor run is shown by O.D. at 600 nm. Lac inducer IPTG was added where indicated (full line) or omitted (dotted line). IFN activity measured by inhibition of Vesicular Stomatitis virus cytopathic effect on human fibroblasts and calculated per ml of bacterial culture.

have been used by various groups to produce high levels of human IFN in bacteria [40, 43].

The examples described in this review, show the potential benefits of a better understanding of regulatory signals in genes, to optimalize and modulate their expression by recombinant DNA manipulations.

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GENE TRANSFERS INTO PLANTS AS A NATURAL AND EXPERIMENTAL PHENOMENON

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ABSTRACT

The main limitations of conventional plant breeding are the length of time required to produce new, useful varieties and the relatively small gene pool available for sexual crossings. In plants, the length of time can be reduced by employing haploid tissue cultures and plants: elimination of undesirable phenotypes and selection of genetic variants is simplified, and the diploid state can be restored, providing the breeder with suitable material for further crossings. Somatic hybridization of plant cells is a first step to enlarge the available gene pool: Protoplasts from sexually incompatible plants are fused to form a new genetic unit which contains the genomes of both parent plants.

The direct introduction of genes as DNA molecules with the help of gene vectors represents a system of minimal interference with existing, stable genomes. With dicotyledonous plants, the Ti-plasmid system is available for direct transfer of genes, and improvement of crop plants can be expected in the near future. Gene vectors for monocotyledonous plants and preferably also for chloroplasts and mitochondria need to be developed. The chief limitation for progress in genetic engineering is insufficient knowledge on structure, function, and regulation of genes.

Introduction

Conventional plant and animal breeding has been of great importance in the last decades for the production of better and more useful plants and animals, and there is little doubt that it will continue this role in the future.

However, it also has limitations: It takes a long time to produce new and better varieties (with plants for example between eight and 30 years), and the gene pool available for new combinations is limited by the fact that under normal conditions only closely related species can be crossed sexually. The vast pool of potentially valuable genes in other organisms cannot be tapped by conventional breeding techniques.

Therefore much of the current interest is focussed on the development of techniques which either shorten the time required for breeding or which enlarge the gene pool available for production of new and useful gene combinations. The last few years have seen important developments in both of these areas, and although much is still in the experimental stage and many fundamental questions still have to be resolved, it is nevertheless worthwhile and timely to assess the possible applications.

Plant Tissue Cultures

Haploid Cells and Plants

In contrast to higher animals, many plants can be regenerated from tissue cultures or even from single cells. With the advancement of these techniques it also became possible to establish tissue cultures from haploid male or female gametes and to regenerate haploid plants. Since these cells or plants contain only one set of chromosomes instead of two, they offer significant advantages over diploid plants in several steps necessary in plant breeding. Recessive traits which are otherwise not detected because of complementation by the second chromosome are immediately visible in the phenotype of haploid plants, and thus can be detected much faster than by crossing of diploid plants.

If undesirable they can be recognized and eliminated directly in the haploid stage, and this leads to a considerable reduction in the time required to select for optimal gene combinations. Another advantage of haploid cells and plants is that the screening for genetic variants, for example

resistance to pathogens, is simplified. Since haploid cells often return spontaneously to diploidy in culture or can be induced to do so by suitable treatments, the original diploid chromosome number can be restored. In these cells the two chromosome sets are identical, and this material provides the plant breeder with good material for further improvement by conventional plant breeding techniques.

Successful application of haploid cells requires that it is possible to produce protoplasts which are capable of growth and division and which can be regenerated to plants. The breeding of plant species amenable to these procedures can be expected to be accelerated considerably by the use of haploid cell techniques.

Hybridization of Somatic Cells

After removal of plant cell walls, the naked cells (protoplasts) often fuse spontaneously or they can be induced to do so, even with protoplasts from other plants. Since it has been shown that with a certain frequency not only the cytoplasms but also the nuclei merge to form a new unit which combines both genomes and which is capable of growth and division, this technique can be used to produce genetic combinations from distantly related plants which cannot be crossed sexually. However, in many cases it has been found that the new combination was not stable. Progressive loss of chromosomes has been observed in culture, sometimes to the extent that only one or a few chromosomes of one parent plant were left in the hybrid, and the properties of the finally stabilized hybrids cannot be predicted. Accordingly it is also not possible to predict the genotype and phenotype if plants can be regenerated from such hybrids. To obtain plants with specific, desirable properties it is necessary to apply large and time-consuming screening or selection programs, and it has to be shown that the phenotype is stable.

Hybridization of somatic cells can be performed only with plants from which viable protoplasts capable of plant regeneration can be obtained. It seems unclear whether this approach to fuse complete genomes will be useful in the near future for crop plants. It is possible, however, that the full potential of these techniques can be realized when they are used in combination with genetic engineering methods.

Gene Transfer in Plants

The development and practical use of so-called gene transfer methods or "gene vectors" represents a major breakthrough. These systems aim for a minimal disturbance of existing stable genomes by introduction of a single gene or a set of genes for desirable properties. The most developed of a number of potential gene-vector systems has been derived from a natural system for genetic manipulation of plants.

Agrobacterium tumefaciens is a gram-negative soil bacterium which infects wounds in dicotyledonous plants and in gymnosperms, and it induces tumorous outgrowths (crown galls). Cells from such tumors grow and divide — in contrast to most normal plant cells — in simple media without added growth hormones like auxins and cytokinins; they are hormone-autotrophic. Since growth and differentiation in plants are largely controlled by these hormones, this property of the cells appears to be one of the major factors in tumorous growth.

The cells also produce a number of low molecular weight compounds (opines), often unusual aminoacid derivatives, which are not detected in normal cells, and these substances specifically support the growth of the inducing Agrobacteria. Thus the biological system essentially represents a parasitic relationship in which the Agrobacteria force the plants to divert some of their anabolic capacity towards the production of opines. The capacity of the bacteria to induce tumorous growth and opine synthesis resides in large plasmids (Ti-plasmids, Fig. 1) which are found in all pathogenic Agrobacteria. A large body of evidence shows that the bacteria use the Ti-plasmids as a tool for genetic engineering of plants: All tumor cells contain a specific part of the plasmid (the T-DNA) which had been transferred from the bacteria into the plant cells and which is stably incorporated, maintained and transmitted in the chromosomal DNA of the nuclei. The T-DNA contains defined genes which are expressed in plant cells, and the actions of the gene products are responsible for tumorous growth and opine synthase. Thus, the interaction between Agrobacteria and plants can be described as a "genetic parasitism" or "genetic colonization" of plants which benefits the Agrobacteria (Fig. 2).

The key to this interaction is the Ti-plasmid; it is a natural gene vector. Intensive research has been undertaken in the last years to investigate whether Ti-plasmids can also be used to introduce other, well-defined genes into plants. The results clearly show that Ti-plasmids containing additional genes in the T-DNA will transfer these genes unchanged into

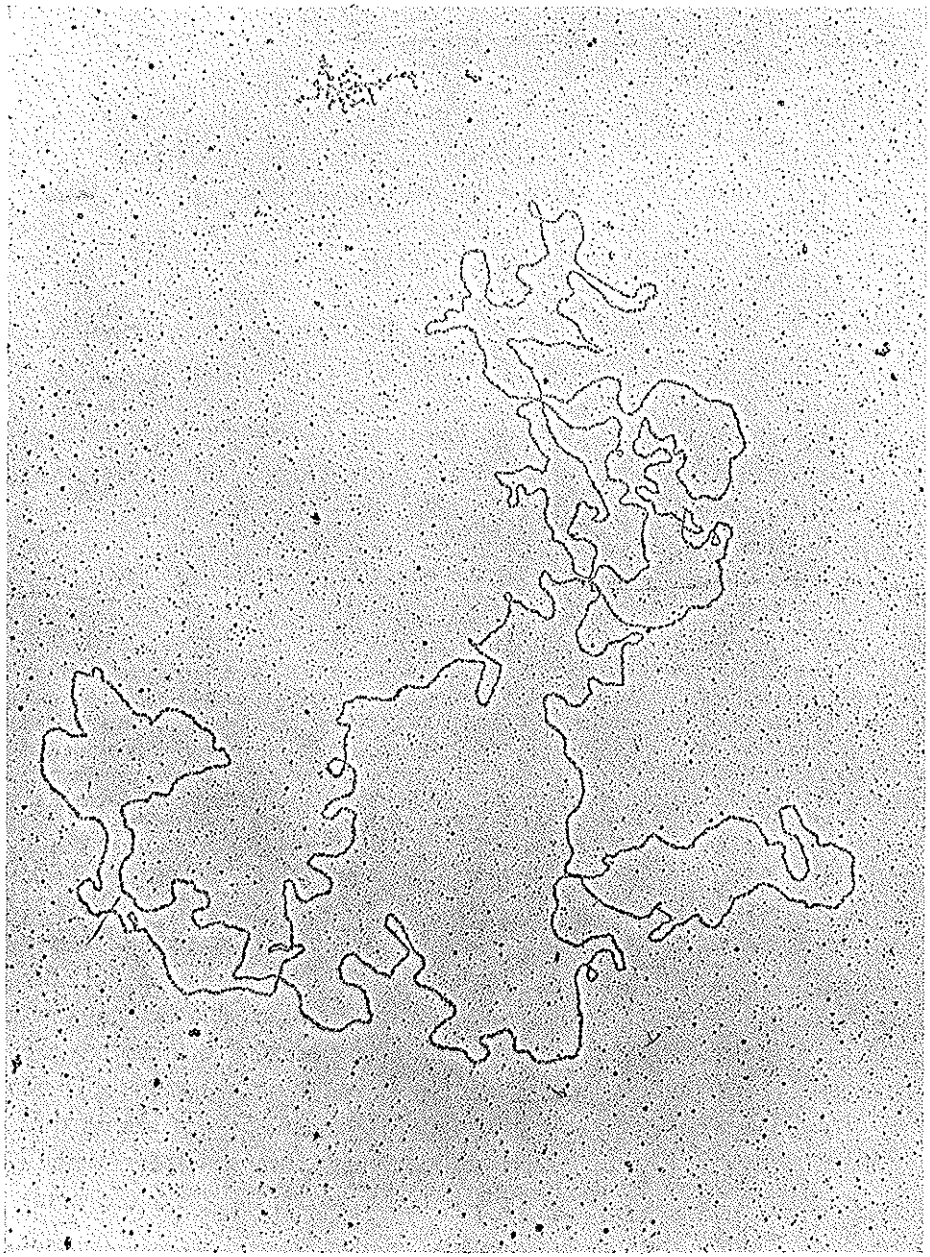

FIG. 1. Electronmicrographic picture of a tumor-inducing plasmid (Ti-plasmid) isolated from Agrobacteria (15 000 x magnification).

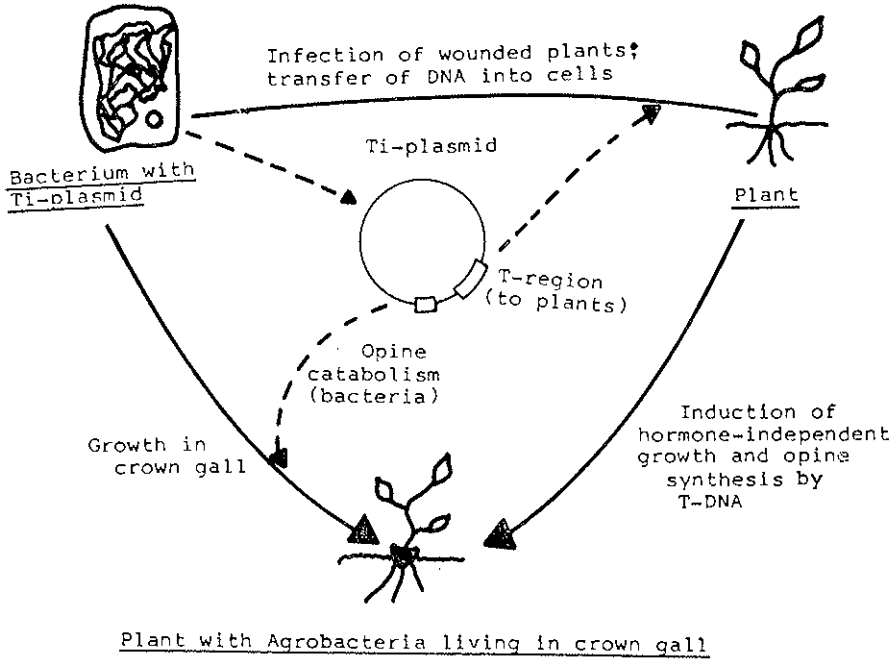

FIG. 2. Ti-plasmids as natural gene vectors: Genetic colonization of plants by Agrobacterium tumefaciens. After infection of a wound by the bacteria, a specific part of the Ti-plasmid is transferred into the nuclei of plant cells where it is stably incorporated and maintained in the genome. The transferred DNA (T-DNA) contains genes which are responsible for tumorous growth of the infected plant cells and synthesis of various compounds (opines) which specifically support the growth of the Agrobacteria. The capacity of the bacteria to catabolize the opines produced in the tumor cells is also encoded on the Ti-plasmids.

plant cells where they become integrated into the chromosomes and where they are stably maintained and transmitted to progeny of the cells. If the genes responsible for tumorous growth are still present and active, however, such plant cells usually cannot regenerate to normal plants since the tumor genes affect hormonal control of differentiation processes. These genes were identified and mapped in the T-DNA, and it was shown that they have no function in the actual process of gene transfer from bacteria to plants nor in the integration of the transferred DNA into the plant genome. It was therefore possible to eliminate these genes without affecting the genetic engineering capacity of the Ti-plasmid. Plant cells containing such modified T-DNA were shown to regenerate into normal plants which

were fertile and trasmitted the transferred gene to progeny in a Mendelian fashion.

In the last few years, more than 15 model genes have been transferred via Ti-plasmids into plant cells, and therefore a generalization seems to be justified that Ti-plasmids will transfer any DNA sequence into plants.

It can be expected that its application will yield in the short term (5-10 years?) plants with improved biological pest resistances, for example against fungi and viruses. The chief limitation to fast results appears to be that disease resistant genes — as defined hy the plant breeder — are very often not defined by their biochemical mechanism, and without this basic knowledge it is very difficult to isolate the genes as DNA molecules.

Since understanding of the biochemistry of disease resistances is crucial, active support of basic research in this field appears to be important. One can also envision significantly new strategies for a more efficient use of chemical pest control methods. For example, soil organisms contain genes for degradation of herbicides or to produce peptides that are highly and specifically toxic for insects and these genes can be isolated, suitably engineered for expression in eucaryotic cells, and then introduced into important crop plants. This might supply the plants with resistance against specific herbicides or with resistance against insects often used in agriculture.

Crucial for this approach was the demonstration that bacterial genes can be introduced, stably maintained and also efficiently expressed in plant cells.

In a number of experiments we have shown that normally bacterial genes, introduced in the plant cell nucleus via the Ti-plasmid, are not expressed (silent). It was therefore decided to construct "chimeric genes" consisting of so-called promotor sequences derived from isolated genes that are known to be expressed in plants and of the polypeptide coding sequence of bacterial genes. Thus chimeric genes which produce enzymes which can detoxify drugs like neomycin, kanamycin, chloramphenicol or which make cells insensitive to very toxic compounds such as methotrexate, have recently been constructed in one laboratory. When introduced in plants via the Ti gene-vector these chimeric genes were shown to be expressed with the result that the plants containing these chimeric genes became resistant to the toxic drugs. These important results open the way for the expression of a large number of useful "foreign" genes in plants.

A wide open field for genetic engineering techniques is the use of plant cell cultures for the production of valuable proteins, enzymes and

high value drugs. Plant cells can now be grown like microorganisms in large suspension cultures using fairly standard fermentation procedures, and many attempts have been made to produce with them rare chemicals or proteins. However, these substances are usually formed by complex biosynthetic pathways which are not necessary for rapid growth of the cells, and expression actually represents a metabolic load which slows growth down. Such cells are fairly quickly lost since the standard culture conditions select for rapidly growing cell types. The problem is therefore to change the regulation of the genes for valuable proteins or drugs in such a way that they can be activated under controlled conditions when high cell density has been reached by rapid growth. Genetic engineering techniques are undoubtedly critical and powerful tools to achieve these goals.

The natural host range of Agrobacteria is very broad and encompasses probably all dicotyledonous plants and most gymnosperms. Accordingly, Ti-plasmids are useful gene vectors for all these plants. However, monocotyledonous plants are not attacked by Agrobacteria, and so far it seems not possible to transfer genes via Ti-plasmids into these plants. Since this group contains the most important crop plants it remains of high priority either to adapt the Ti-plasmid system to these plants or to develop entirely new gene vectors.

New perspectives for the development of gene vectors for cereals have been opened by our finding that chimeric genes can be constructed to function in plants. Since cereals are sensitive to drugs like kanamycin and methotrexate we hope that the chimeric genes producing resistance against these toxic products can be used as "selectable vector genes" i.e. that one could covalently attach other genes to the selectable marker genes and introduce these gene sets in cereals via infection or by fusion between plant and bacterial protoplasts. The cereal cells that would have taken up both the selectable marker gene and the gene one wants to transfer, could be identified because of their resistance to the toxic drugs. It would seem desirable to develop gene vectors for chloroplasts and mitochondria, since Ti-plasmids transfer genes exclusively into the nucleus. One of the problems with direct transfer of genes appears to be that the transferred genes are often not active in the host cells. Further basic research on the structure, function, and regulation of plant genes is essential for the practical application of gene transfer techniques.

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MUTATIONAL ANALYSIS OF A VIRAL REGULATORY PROTEIN, THE T ANTIGEN OF SIMIAN VIRUS 40

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The small DNA containing papovaviruses, among them Simian Virus 40 (SV40), continue to attract the attention of molecular biologists interested in genetic regulatory mechanisms of mammalian cells. These viruses develop in cell nuclei and both the replication and expression of their genomes, which consist of circular duplex DNA of about 5000 base pairs, depend on cellular enzymes. In nuclei of infected cells, viral genomes may undergo recombination, both with themselves and with chromosomal DNA, and viral gene products induce marked changes in cellular regulation, including changes that lead to tumorigenicity. We have been studying some of these regulatory processes in SV40-infected cells by constructing and characterizing viral mutants with regulatory defects. In this paper we summarize our recent findings. For details the reader should consult the original publications cited in the bibliography.

The genome of SV40 has 5243 base pairs that code for six identified proteins and regulatory signals concerned with DNA replication and transcription and RNA processing (Figure 1). (For recent reviews, see references 1 and 2). The structural genes are divided into two sets, early and late, based on the time of predominant synthesis of gene products in virus infected cells. Between the start of early and late gene segments is a control region of about 400 base pairs containing the origin of DNA replication and promotors for the early and late genes. We focus here on one of the early gene products, the large T antigen (hereafter referred to as T antigen), a phosphorylated protein of about 90,000 daltons (708 amino acids) that has several known regulatory activities related to the SV40 growth cycle

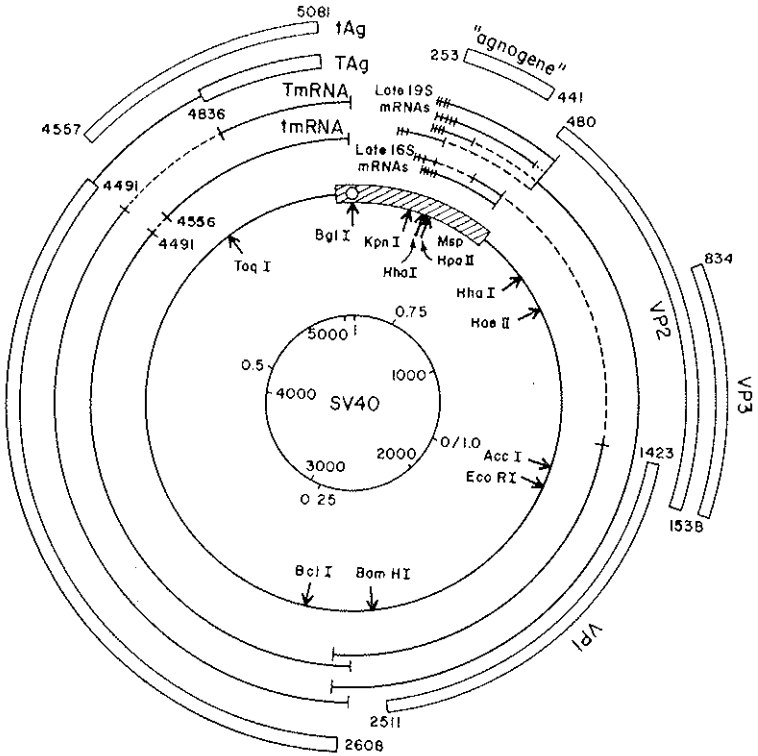

FIG. 1 — Organization of the SV40 genome. At the top is the control region, containing the origin of DNA replication, promoters, and T antigen binding sites. The six structural genes are the overlapping large and small T antigen genes, transcribed counterclockwise, and the agnogene and the three overlapping virion protein genes, transcribed clockwise. (From reference 2)

or to cellular effects of the virus. During the growth cycle in permissive monkey cells, T antigen is involved in the initiation of viral DNA replication, and in the decrease in early gene transcription that is accompanied by a shift in the start site of transcription. These activities are thought to be mediated by binding of T antigen to the control region of viral DNA, an activity demonstrated in vitro. In non-permissive rodent cells T antigen stimulates cell DNA and RNA synthesis and cell proliferation, and after integration of the T antigen gene into chromosomal DNA, causes cell transformation. Additional biochemical properties of T antigen, whose significance is not yet understood, are its ATPase activity and its ability to bind to a cell protein (p53) present at high concentration in transformed cells.

The initial objective of our studies was to determine whether the various activities of T antigen can be ascribed to different domains of the protein. For this purpose we constructed a series of T antigen deletion mutants in bacteria starting with a pBR322 plasmid/SV40 recombinant, defined the mutational changes, and determined residual activities of the altered T antigens [3]. Each of the mutated DNAs was analysed by restriction and subsequent nucleotide sequencing to determine the predicted change in T antigen structure. As seen in Figure 2, four of the fifteen mutant DNAs contained in-phase deletions, ranging from 30 to 438 nucleotides, and the remainder contained out-of-phase deletions that would result in premature termination of translation. The overall result was a collection of mutants coding for T antigens missing internal amino acids or different amounts of the protein from its carboxyl end (see Figure 2). In several instances it was shown that the predicted polypeptide was present in infected cells.

The following activities of the mutants were assessed: the presence of T antigen in cell nuclei; the ability to complement a temperature-sensitive T antigen mutant or a late deletion mutant of SV40; mutant DNA replication in permissive cells; stimulation of cellular DNA synthesis and activation of silent ribosomal RNA genes (in collaboration with Galanti, Soprano, Jonak, McKercher, and Baserga - reference 4); ATPase activity and ability to bind to the control region of SV40 DNA (in collaboration with Clark and Tjian - reference 5). The results of these various studies are summarized in Figure 2.

The most significant results are those in which one or more activities are retained by a mutant. As seen in Figure 2, as the C-terminus of T antigen was progressively deleted, first viral DNA replication, transformation, and ATPase activities were lost; next, activation of ribosomal RNA genes; and finally, stimulation of cell DNA synthesis and binding to the viral control region. On the assumption that the differential effects on activity are due to qualitative changes in T antigen structure, we conclude that T antigen has separable functional domains.

In regard to viral DNA replication, cell transformation, and ATPase activities, it is apparent that only the very C-terminal part of T antigen was dispensable, and that these activities were lost in parallel when more of the C-terminus was lost or when internal amino acids were deleted at the positions defined by three of the in-phase deletion mutations. Although one can conclude that ATPase, viral DNA replication, and cell trans-

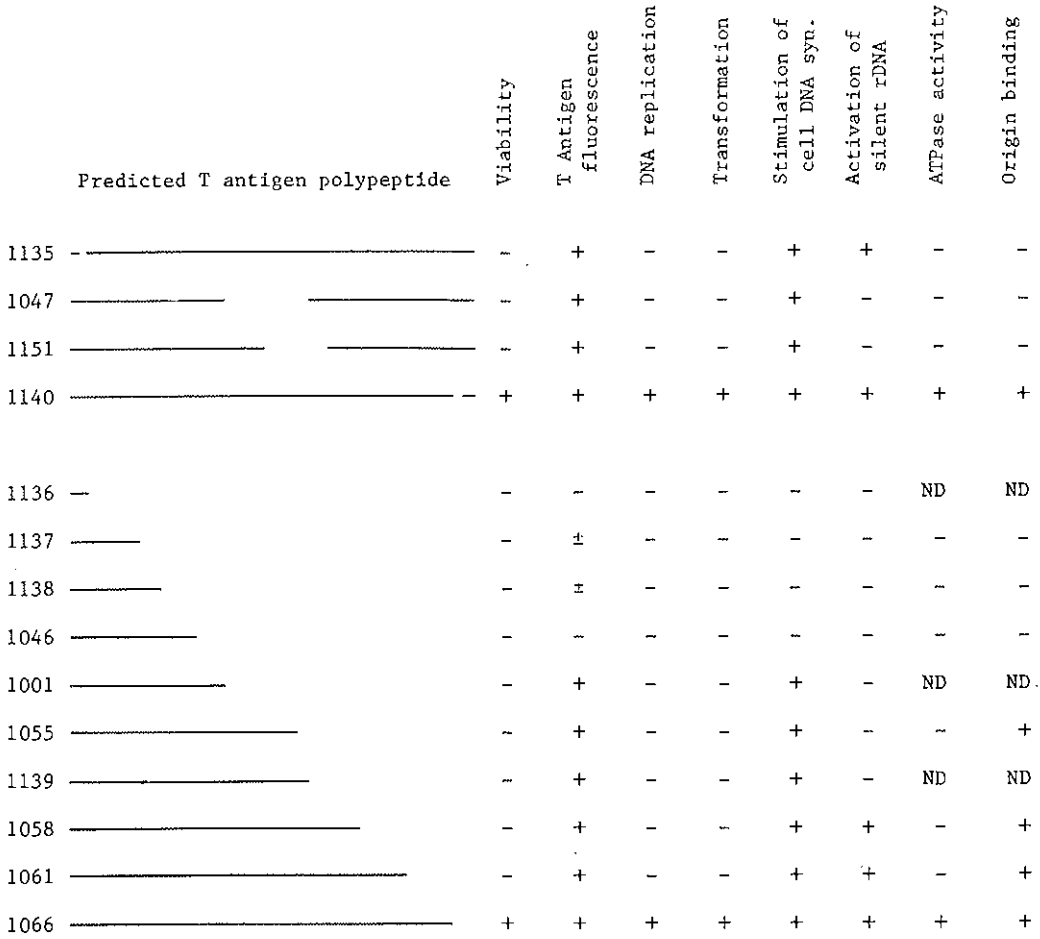

FIG. 2 — Activities of mutant T antigens. On the left are diagrams of the mutant proteins inferred from the nucleotide sequence analyses; the four mutants at the top have in-phase deletions depicted as gaps in the protein sequence, and the others have out-of-phase deletions that would lead to formation of the truncated T antigens depicted. +, activity present; —, activity undetectable; ±, barely above background; ND, not done.

formation functions require different or additional T antigen activities than do stimulation of cellular DNA and rRNA synthesis or binding of T antigen to the control region, one cannot infer that the activities most sensitive to deletion of terminal amino acid sequences are directly related to each other. Indeed, we and others have recently isolated point mutants of SV40 that map in a segment of the T antigen gene (somewhat beyond

the distal splice site) previously shown to be related to T antigen interaction at the viral origin of replication. Such mutants are defective in viral DNA replication but retain the transformation function. Therefore, these two activities are distinct.

In the T antigen map shown in Figure 3 our results are combined with those of others that better define the amino-terminal limits of T antigen involved in its various functions. As can be seen, for some T antigen activities, the amino-terminal segment of the protein may also be dispensable. Thus it is clear that T antigen can be divided into functional domains by mutational analysis. To define these domains in more precise biochemical terms is a challenge for the future.

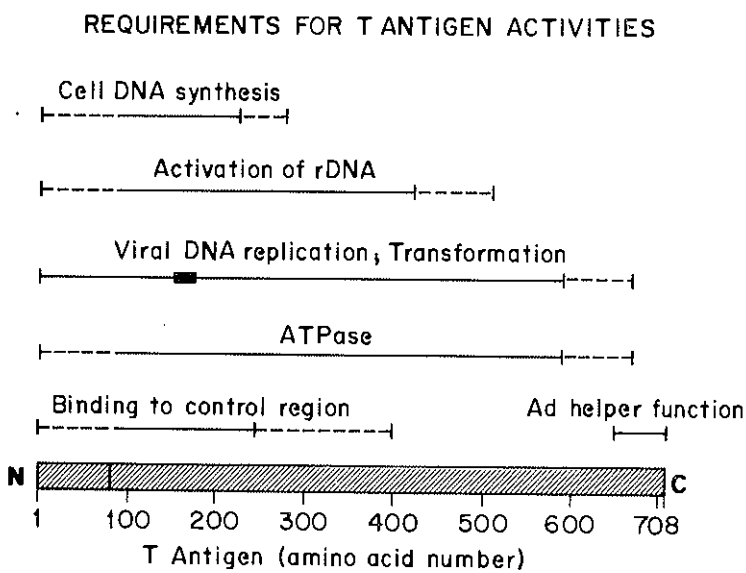

FIG. 3 — Map of the T antigen, showing the parts of the protein that are sufficient for the given activity (with limits indicated by the dashed line), based on the results presented in the text and data reported in references 6 and 7. On the bar representing T antigen, the vertical line is the junction between amino acids coded by the two exons of the T antigen gene. The thickened segment of the line representing requirements for viral DNA replication indicates the location of point mutations that result in loss of viral DNA replication with retention of transforming activity, as noted in the text.

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NEW METHODS FOR MOLECULAR DNA CLONING AND CHROMOSOME MANIPULATION

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ABBREVIATIONS:

SDS, sodium dodecyl sulfate
Xgal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside
IPTG, isopropyl-thio-β-D-galactoside
bp, base pairs
kbp, kilobase pairs

INTRODUCTION

The isolation of protein-coding genes from large recombinant DNA libraries can be achieved, in principle, by using antibodies to detect antigen produced by specific recombinants. Several expression vectors have been used with various antigen screening techniques to identify recombinant DNA containing clones [1-6]. However, these techniques lack the sensitivity and efficiency necessary to isolate specific recombinant molecules from cDNA libraries containing 10^5-10^7 clones.

A useful recombinant expression vector should have properties that permit the construction and maintenance of large cDNA libraries. The recombinant should be propagated in its host cell as a single-copy genomic insert to enhance its stability and to facilitate repression of foreign genetic information. The expression vector should also respond to induction with a rapid increase in copy number and high level transcription of the foreign

DNA. Since the ability to detect antigen will depend on its stability, the expression vector and its host should include features which minimize the degradation of the foreign eucaryotic protein.

These properties have been included in the phage expression vector λgt11. The general ability of λ lysogens to produce large quantities of phage products upon induction has been exploited in λgt11 to enhance the sensitivity and efficiency of antigen screening. The construction of a library of recombinant DNA-containing lysogens with λgt11 permits the growth, induction and lysis of antigen producing cells directly on nitrocellulose filters.

Since bacteria rapidly degrade most unusual polypeptides [7-11], eucaryotic proteins, and especially portions thereof, are potentially unstable in procaryotic cells. Therefore, fusion of the eucaryotic moiety with a stable procaryotic protein, β-galactosidase, encoded by λgt11, should minimize hybrid protein instability. The use of host cells defective in protein degradation pathways (*lon* mutants) should also increase the lifetime of novel proteins produced from the induced λgt11 lysogens.

In summary, a method is presented for the isolation of gene sequences using antibody probes. A λ phage expression vector, λgt11, has been constructed whose properties permit insertion of foreign DNA to produce a recombinant DNA library, high frequency lysogeny in particular *E. coli* strains, induced synthesis of β-galactosidase fused to protein specified by the foreign DNA and reproducible detection of antigen in populations of up to 10^6 lysogens per 82 mm nitrocellulose filter.

Thus far, most cloning techniques in vector construction have involved the manipulation of small DNA sequences, usually on autonomously replicating plasmids. A new technique has been developed which allows manipulation of large sections of chromosomal DNA. This technique relies on the ability to select rare homologous recombinants between two sequences which reconstruct a functional gene. The use of point mutations for such a selection is inadequate since the point lesions can revert to functionality or gene conversion can occur between the two sequences and not result in reciprocal exchange. We have developed a 3′- and a 5′ deletion of the *HIS3* gene such that gene conversion is blocked and only reciprocal recombination is selected.

Materials and Methods

Enzymes

T4 DNA ligase and *E. coli* DNA polymerase I were generous gifts of S. Scherer (California Institute of Technology). DNA restriction endonucleases were from New England Biolabs, Inc.

Bacterial Strains

Strains are listed in Table 1. BNN98 results from the spontaneous loss of the F′ episome in BNN94. BNN100, BNN101, BNN102 and BNN103 were constructed by P1 transduction of the relevant alleles (*hfl*A150 from BNN92, *sup*F from BNN99) into BNN93, BNN96 or

Table 1. Bacterial strains. (chr: :Tn10) designates a chromosomal insertion of Tn10 which exhibits 65% cotransduction with *hfl*A150.

Strain	*Alias*	*Genotype*	*Source*
BNN45	LE392	*hsd*R⁻*hsd*M⁺ *sup*E44 *sup*F *thi met lac*Y	This laboratory
BNN91	MA150	Δ(*pro-lac*) *hfl*A150 *gal*K *str*A	A. Hoyt (Berkeley)
BNN92	MA156	MA150 [chr: :Tn10]	»
BNN93	C600	*hsd*R⁻*hsd*M⁺ *sup*E *thr leu thi lac*Y1 *ton*A21	This laboratory
BNN94	CSH41	F′ *lac*I *lac*P *pro*A⁺ *pro*B⁺/Δ(*pro-lac*) *gal*E *thi*	M. Howe (Wisconsin)
BNN95	AB1899	*thr*-1 *leu*B6 *thi*-1 *arg*E3 *his*-4 *pro*A2 *lon*-1 *lac*Y1 *gal*K2 *mtl*-1 *xyl*-5 *ara*-14 *str*A31 *tsx*-33 *sup*E44	B. Bachman (Yale)
BNN96	SG1041	Δ(*lac*IPOZYA) U169 *pro*A⁺ Δ*lon ara*D139 *str*A *thi*	S. Gottesman, D. Court (N.I.H.)
BNN97	Y1004	BNN93 (λgt11)	This work
BNN98	Y1048	F⁻ Δ(*pro-lac*) *gal*E *thi*-1 *sup*F58	»
BNN99	Y1059	F⁺ *sup*E57 *sup*F58 *mel*-1 [*trp*C22: :Tn10] (λ)	»
BNN100	Y1068	F⁻ Δ(*pro-lac*) *gal*E *thi*-1 *sup*F58	»
BNN101	Y1070	F⁻ Δ(*pro-lac*) *gal*E *thi*-1 *sup*F58 *hfl*A150 [chr: :Tn10]	»
BNN102	Y1073	BNN93 *hfl*A150 [chr: :Tn10]	»
BNN103	Y1083	BNN96 *hfl*A150 [chr: :Tn10]	»

BNN98. The medium used was Luria Broth, pH 7.5, unless otherwise specified. All media and strain construction were as described by Miller [12]. λ phage were from our collection and are described by Williams and Blattner [13].

Construction of λgt11 (lac5 nin5 cI857 S100)

λgt11 is essentially a derivative of λgt7-*lac5* (*b*522 *nin5*) and λgt4 (*c*I857 *S*100 *nin5*). To construct this phage, λgt7-*lac5* and λ540 (Δ*B imm*21 *nin5*) were cleaved with HindIII endonuclease and the restriction fragments were pooled and then ligated with T4 DNA ligase. The desired phage recombinant produced turbid (*imm*21), blue (*lac5*) plaques when the DNA was transfected into *E. coli* BNN93 and cells were plated on medium containing the chromogenic indicator Xgal. The λgt7-*lac5*/λ540 hybrid was then crossed with λgt4 (*c*I857 *S*100 *nin5*) and recombinants, grown at 42°, were scored for the formation of clear (*c*I857), blue plaques on Xgal plates. The presence of the amber mutation *S*100 was confirmed by examining relative plating efficiency on hosts which contained or lacked the amber suppressor *sup*F (BNN45 or BNN93, respectively). Finally, the *lac5* *c*I857 *S*100 phage were mapped for EcoRI restriction endonuclease cleavage sites. λgt11 contained a single EcoRI cleavage site and was mapped in detail with other enzymes.

Preparation of Antibodies

Rabbit antisera made against the pancreatic α-amylase of C57 BL/6J mice was a gift of M. Miesler (U. of Michigan). Rabbit antisera made against chick ovalbumin was provided by M. Wickens (M.R.C., Cambridge). IgG was purified from the sera as described by Broome and Gilbert [4] and stored at a concentration of 5-10 mg/ml.

Examining Lysogens for Antigen Production using Antibody Probes

BNN91 or BNN103 is grown to stationary phase in Luria Broth pH 7.5 plus 0.1% maltose. Up to 5×10^6 cells per plate are infected at an m.o.i. of 1.0 for 30′ at 32° in 0.1 ml of 10 mM tris, pH 7.5, 10 mM $MgSO_4$. The infected cells are diluted with 0.5 ml of Luria Broth and poured carefully on a Schleicher and Schuell BA85 82 mm nitrocellulose

filter which had been placed previously on an LB plate. The liquid culture is spread carefully over the filter and permitted to soak through the filter into the plate (relatively dry plates are useful here). The plate is incubated at 32° for 8 hours, and a replica is made in the following manner. The master filter is removed from its plate and excess liquid is blotted from its underside. Another filter is wetted on an LB plate, blotted, placed over the master, pressed evenly against it and marked with a needle. Both master and replica filters are then replated and incuhated at 32°. After one hour, one of the two plates is removed to 42° for 2 hours, the other plate is refrigerated. The induced cells are then lysed on the filter by inverting the plate over a small vessel of chloroform for 15 minutes to create a chloroform-saturated atmosphere. The nitrocellulose filter is removed from the plate and submerged in 3 ml Buffer A (0.17M NaCl, 0.01M tris HCl pH 7.5) plus 0.01% SDS for 1 hour (this and all subsequent steps are performed at 24°). The solution often becomes viscous at this stage and DNA, if not removed, appears to reduce antigen availability. Therefore, the filter is rinsed in 3 ml buffer A, incubated in 3 ml buffer A plus 2 μg/ml DNaseI for 10 minutes and then rinsed once again in buffer A. To reduce nonspecific protein binding to the nitrocellulose, filters are incubated in 3 ml buffer A plus 3% BSA for 1 hour. IgG is generally diluted to 50 μg/ml in buffer B (buffer B = buffer A plus 0.1% SDS, 0.1% triton X100 and 1 mM EDTA) and the filter is gently agitated in this solution for 3 hours. After washing the filter twice in Buffer B (10 minutes each wash), the bound antibody is allowed to react with approximately 5×10^6 cpm of ^{125}I-laheled (1-2 $\times$ 10^6 cpm/μg) Protein A (*Staphilococcus aureus*) in 3 ml buffer B for 1 hour. Finally, the filter is washed 5 times in 5 ml buffer B, 15 minutes per wash. Good autoradiographic signals are usually obtained overnight in a screen of 10^6 colonies/filter.

Preparation of Lysates from Induced Recombinant Lysogens

Lysogens were grown at 30° to a cell density of 2×10^8, induced at 42° for 15 minutes, then well aerated by shaking at 38° for 2 hours. (Induced lysogens containing some recombinants will lyse after 2 hours at 38° [unpublished data], presumably because of the detrimental effects of the high levels of some hybrid proteins). Cells were pelleted and quickly resuspended in gel sample buffer (50 mM tris, pH 6.8, 1.5% SDS, 50 mM

DTT and 4M urea) at 3% the original volume and mixed well by passaging several times through a 21 gauge needle. The solution was then heated to 70° for 2 minutes and insoluble material was removed by centrifugation for 3 minutes in a microfuge.

Results

The expression vector λgt11 (*lac5 nin5 c*I857 *S*100) was constructed as described in Materials and Methods (Figure 1). The site used for insertion of foreign DNA is a unique EcoRI endonuclease cleavage site located within *lac*Z, 53 base pairs upstream of the β-galactosidase termination codon [14, 15]. Phage containing inserts generate an inactive β-galactosidase fusion protein; these phage can be distinguished from nonrecombinant phage by their inability to produce blue plaques on a *lac*Z⁻ host on Xgal plates. The vector can accommodate up to 8.3 kb of insert DNA, assuming a maximum packageable phage DNA length of 52 kb. λgt11 cDNA libraries containing 10^5-10^7 recombinant phage (in which recombinants account for 4-30% of total phage) have been constructed using polyadenylated RNA isolated from *S. cerevisiae* strain X2180 (unpublished data), *C. elegans* strain CB1490, rat preputial gland and human placenta (B. Meyer, T. Chappell and D. Pauza, personal communication).

The ability to form lysogens from the λ *c*I857 *S*100 expression vector can be exploited to maximize the yield of protein synthesized from transcripts of the foreign DNA. The phage vector produces a temperature sensitive repressor and contains an amber mutation which renders it lysis defective [16, 17]; consequently, lysogens can be induced by temperature shift to accumulate large quantities of phage products in the absence of lysis. To obtain efficient lysogeny, strains containing the mutation *hfl*A150 [18, 19] have been used. Essentially every *hfl*A mutant cell is lysogenized when infected with λ, yet *c*I857 prophage induction remains unhindered. Moreover, *hfl*A affects neither the relative plating efficiency nor the plaque size of λgt11 or λgt11 phage containing recombinant DNA in the otherwise isogenic pair BNN100 and BNN101 (unpublished data). Thus, *hfl*A strains can be lysogenized efficiently with λgt11 recombinant DNA libraries and these lysogens can be induced to produce normal phage yields.

Construction of model recombinants

To test the ability of λgt11 to express foreign DNA as a fusion product and as a detectable antigen, model recombinants were constructed by inserting mouse α-amylase and chick ovalbumin cDNAs into the EcoRI site of the vector. Since the DNA sequence surrounding the *lac*Z EcoRI site and the amino acid sequence of β-galactosidase are known [14, 15] the foreign DNA insertion could be engineered to obtain a continuous (or noncontinuous) coding frame from β-galactosidase into α-amylase or ovalbumin. The origin of the DNA used in these recombinants and the portion of eucaryotic protein they encode are given in Table 2.

Detection of eucaryotic antigens

The relative level of antigen detection was investigated with induced lysogens containing λgt11 recombinant phage whose inserts vary in orientation and fusion frame. Purified IgG was used to detect antigen produced by 2×10^6 lysogenized cells in 4 mm dots on nitrocellulose filters as described in Materials and Methods. Good signals were obtained with both α-amylase and ovalbumin IgG. All lysogens containing inserts in the proper transcriptional orientation (αP3, T81, T104 and P82) produce

TABLE 2. Model recombinants were constructed according to methods described in [20]. α-amylase (amyl) cDNA was isolated from pMPa21 [21, 22] and ovalbumin (oval) cDNA was isolated from p0v230 [23, 24]. The table indicates the transcriptional orientation of the cDNA and its coding frame relative to that of *lac*Z. The predicted size of the hybrid protein produced by recombinants whose coding frames coincide is also given. The β-galactosidase portion of the hybrid accounts for 114,000 daltons [15].

Name	*cDNA*	*Fragment*	*Orientation*	*Frame*	*Hybrid length*
αP2	amyl	1.5kb EcoRI	reversed	—	—
αP3	amyl	1.5kb EcoRI	proper	in	142,000
T81	oval	2.0kb TaqI	proper	—	153,000
T83	oval	2.0kb TaqI	reversed	—	—
T104	oval	2.0kb TaqI	proper	out	—
P82	oval	3.2kb PvuII	proper	in	140,000

detectable antigen; in contrast, αP2 and T83, whose DNA inserts are in the opposite orientation, yield signals comparable to those in control spots. T104, an out-of-frame ovalbumin cDNA insert, produced approximately 1/8 the signal obtained with the lysogen containing the fused polypeptide, T81 (see discussion). This experiment indicates that antigenic detection is dependent upon proper orientation of the insert DNA with respect to the β-galactosidase transcription unit and that the relative signal strength is greatest when the reading frames of *lac*Z and insert DNA coincide.

Screening λgt11 recombinant DNA libraries with antibody probes

The following reconstruction experiment illustrates the screening procedure. Approximately 25 MA150 cells lysogenized with a recombinant DNA phage (either α-amylase or ovalbumin) were added to 10^5, 10^6 or 5×10^6 λgt11 lysogens of MA150. Each culture was plated immediately on 82 mm nitrocellulose filters, grown for 8 hours at 32°, replica-plated, induced and probed with antisera as described in Materials and Methods. Antigen produced by cells containing recombinant phage was detected even at the highest cell density tested (5×10^6). However, replica filters retained fewer signals with greater cell density: approximately 5%, 10% and 60% of the signals obtained from the master filter were lost at respective cell densities of 10^5, 10^6 and 5×10^6 per plate. Thus, satisfactory signal reproducibility, at least for the antigen-antibody interaction examined here, is obtained at a plating density of 10^6 cells/filter.

Production of fusion polypeptides

The amount of hybrid protein which accumulates in BNN91 cells containing the λgt11 recombinants αP3, T81 and P82 were determined by subjecting lysates of these lysogens, prepared as described in Materials and Methods, to SDS polyacrylamide gel electrophoresis. Bands exhibiting the mobility predicted for the hybrid proteins were observed when stained with silver [25], but not with the less sensitive Comassie Brilliant Blue, suggesting that very small amounts of the novel proteins accumulate. To improve the yield of these proteins the recombinant phage were lysogenized in the *lon*Δ mutant strain, BNN103. Since BNN103 increases the stability of β-galactosidase peptide fragments [26], its lysogens might be expected to accumulate larger quantities of the unstable β-galactosidase hybrids. Indeed, the gel in figure 4 shows that BNN103 lysogen lysates contain proteins of the predicted size for both α-amylase (αP3) and ovalbumin

(P82) hybrids. The T81 fusion protein, with a predicted molecular weight of 153,000, could not be resolved from the RNA polymerase subunit β. Similar results were obtained with another *lon* mutant, BNN95.

To determine whether the enhanced yield of the full-length fusion polypeptide in *lon* mutants increases the level of detectable antigen, 4 mm dots of BNN91 and BNN103 lysogens of αP3 (4×10^5 cells/dot) were probed as before. Figure 5 shows that the BNN103 *lon*Δ lysogen generated about 3-fold greater signal than did the BNN91 lysogen. Thus, BNN103 should be useful for enhancing signal-to-noise ratios in high cell density screening of lysogen libraries.

Discussion

High density lysogen screening

λgt11 is a general recombinant DNA expression vector capable of producing polypeptides specified by inserted DNA. Antibodies can be used to detect reproducibly the antigen produced in single colonies of induced λgt11 lysogens when up to 10^6 lysogen colonies are examined on an 82 mm nitrocellulose filter.

Proper expression of foreign DNA in λgt11 recombinant lysogens will depend upon the insert DNAs orientation and reading frame with respect to those of *lac*Z. Thus, 1/6 of the λgt11 recombinants containing a specific cDNA will produce β-galactosidase fused to the protein of interest. cDNA quality will further affect the ability to detect antigen produced by the recombinant; nearly full-length cDNAs specify more potential antigenic determinants than shorter cDNAs made against the same mRNA.

Antibody quality plays an important role in a successful screen. The α-amylase and ovalbumin IgG used here produced good signal-to-noise ratios without additional purification. However, antibodies directed against coliform proteins are found in many preparations and must be removed from the specific probe. In fact, the specific signal obtained with ovalbumin IgG could be improved by preincubation with a λgt11 lysogen (BNN97) lysate bound to nitrocellulose and others have successfully used column bound lysates with similar results [6]. The best specific reaction with antigens produced by λgt11 recombinants should be obtained with affinity purified antibodies. Affinity purified anti-k light chain immunoglobulin antibody has been used successfully to detect k-producing λgt11 plaques

at densities of up to 10^5 plaques/90 mm plate (T. St. John, manuscript in preparation).

The strength of the signal observed when probing the α-amylase recombinant BNN91 lysogen at high cell density was greater than might be expected given the amount of hybrid protein accumulation judged by gels. Kemp and Cowman [6] were unable to observe *trp*D fusion polypeptides in polyacrylamide gels after staining with Comassie Brilliant Blue, yet detected full length hybrid protein as well as peptide fragments in protein blots examined with antibody probes. Moreover, the ability to detect antigen produced by recombinants whose coding sequences are inserted out of frame suggests that some antigen is produced by anomalous translation initiation. These data indicate that complete hybrid protein and other forms of the antigen contribute to antibody binding.

Isolation of Unknown Native Proteins

λgt11 can facilitate the identification and isolation of proteins which are specified by previously cloned DNA. The hybrid protein produced by cells containing the recombinant DNA can be purified and used to obtain antibodies. Antibodies produced against these proteins should include activity against the eucaryotic portion of the protein fusion; this antibody could be used as a tool to isolate the native protein from the organism of interest.

The hybrid proteins examined here accumulate in *lon* mutant strains to amounts amenable to purification. For example, a BNN103 lysogen of αP3 contains approximately 10^{-15} g of α-amylase hybrid protein (about 0.5% of total protein), as estimated by comparison with known amounts of β-galactosidase on SDS polyacrylamide gels. Thus, 1 mg of this protein could be obtained from 10^{12} cells, or about 2 liters of induced culture. Quite possibly the hybrid protein could be purified by standard techniques, taking advantage of its size (using, for example, ammonium sulfate fractionation and gel filtration) and the charge properties of its β-galactosidase moiety (binds DEAE tightly) [12, 15]. In addition, our preliminary experiments suggest that hybrids can be purified on sepharose columns bound with anti-β-galactosidase antibody. Finally, the more stable and abundant fusion proteins can he purified directly by preparative polyacrylamide gel electrophoresis.

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MONOCLONAL ANTIBODIES AS TOOLS FOR THE STUDY OF HUMAN TUMORS

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Although tumors develop from normal organs of an organism, the existence of tumor-associated antigens has been suspected for many decades. There were no tools, however, to prove the existence of such antigens since cancer victims did not seem to produce circulating tumor-specific antibodies, and sera of animals immunized with tumor tissue cross-reacted with normal tissue.

This situation changed with the development of a procedure that made possible the definition of tumor antigens through interaction with monoclonal antibodies (Mab). These antibodies, in contrast to those produced by immunization of experimental animals, originate from one cell of the immune system, a B cell, which is fused with another cell to produce a hybrid cell. This hybrid cell replicates in vitro (in tissue culture) and the progeny, referred to as a hybridoma, gives rise to a permanent culture which produces antibodies ad infinitum outside the body of an animal. To date, mice are the preferential hosts for immunization with human tumor tissues. The B lymphocytes of these animals are then fused with cells of a variety of variants of mouse myeloma to form Mab-secreting hybridomas useful in the characterization of human tumor antigens.

A variety of human tumors, either in the form of living cells or cell extracts, have been used to produce Mabs. The list includes melanomas, adeno-carcinomas of the gastrointestinal tract, lung cancer, breast cancer, bladder cancer, gliomas and a variety of leukemias. The discussion here will be limited to Mabs reactive with melanoma and cancers of the gastrointestinal tract.

The specificity and/or cross-reactivity of Mabs is usually determined in radioimmunoassay using either living or fixed human tumor cells or cell

extracts as targets. Out of hundreds of hybridomas analyzed, only a fraction reacts more or less specifically with a given tumor antigen. For instance, in the case of Mabs against human melanoma, seventeen hybridomas out of hundreds produced were found to bind to cells of all melanomas either surgically removed or maintained in tissue culture. One group of these Mabs also showed cross-reactivity with tumors of nervous tissue such as glioblastomas. Since both melanomas and tumors of nervous tissue originate from cells which during fetal life derive from cells of the neural tube, it is quite possible that the antigen(s) defined by these Mabs is an oncofetal antigen which is expressed during fetal life by cells of the neural tube, is suppressed after birth and is re-expressed in tumor tissue.

Another group of Mabs against human melanomas cross-reacts with fetal but not with adult melanocytes. Although it is not difficult to immunoprecipitate proteins extracted from melanoma cells using Mabs and to determine molecular weights of the antigens precipitated, the amount of antigenic material obtained in a research laboratory is too small to allow its detailed characterization by polypeptide analysis, amino acid sequencing, etc. Hopefully, in the near future large-scale production methods of hybridomas will circumvent this difficulty.

Apart from defining a tumor-associated antigen, melanomas present a unique situation as far as human carcinogenesis is concerned in permitting the study of precancerous lesions. These are dysplastic nevi which in a certain proportion of cases show malignant transformation into melanomas. Cells derived from such nevi can be grown in tissue culture and express antigens recognized by Mabs against melanomas. However, they do not show the patterns of indefinite growth in tissue culture that are characteristic of melanomas; instead, they have a finite lifetime similar to normal human fibroblasts. Thus, there are obviously factors that influence the malignant transformation of nevus cells in vivo but that are inactive in vitro. One such factor might be a growth-promoting factor produced by human cells such as T cells which are known to produce these substances. Starting from this point, cells from dysplastic nevi grown in tissue culture in the presence of human T cells acquire a capability for indefinite growth, express some antigens of melanomas but, similar to the cultures of normal skin cells, have a diploid number of chromosomes.

Whereas antigens of melanomas defined by Mabs are proteins, antigens expressed by cells of carcinomas of the gastrointestinal tract such as stomach, pancreas, colon, and rectum, are glycolipids, one of which contains a carbohydrate identified as sialyl-N-lacto-fuco-pentaosyl II. This monosialo-

ganglioside can be identified in gastrointestinal tract tumors and their metastases but in no normal tissues except for meconium, the first stool of a newborn child which contains among other muck, intestinal cells shed from the 7th week of gestation on. So in this case again we are dealing with an oncofetal glycolipid. The carbohydrate part of this antigen is nothing but a Lewis A oligosaccharide sialylated by a hitherto unknown sialyl-transferase. The sialic acid is the actual antigenic determinant recognized by the Mab since treatment with neuraminidase abolishes the reaction. Lewis (a^-b^-) individuals cannot express the antigen, and the susceptibility of this population to gastrointestinal cancers merits investigation.

Sera of nearly 50% of colorectal cancer patients and nearly 100% of patients with either pancreatic or gastric cancer show the presence of the sialyl-lacto-N-fuco-pentaosyl II determinant. The same antigen can be detected on the surface of the tumor cell in the immunoperoxidase reaction. Both assays are of diagnostic value. In addition, Mabs labeled with either ^{131}I(odine) or ^{111}In(dium) localize specifically to the corresponding tumors in patients or in nude mice implanted with tumors.

Mabs of the IgG2a isotype, but not of any other class, have distinct tumor-killing properties as shown in experiments in nude athymic mice implanted with human tumors. Tumors are not destroyed by Mab alone but rather by effector cells "armed" with IgG2a Mab. These effector cells have been identified as macrophages which, following activation through contact of their Fc receptors with the IgG2a Mab, destroy the tumor cells that express the antigens recognized specifically by the Mab.

Human macrophages derived from blood monocytes and kept in culture also develop Fc receptors for mouse IgG2a Mabs and also become specifically tumoricidal for cancer tissue reactive to the Mab.

Patients with metastases after removal of their primary colorectal cancer have been treated with IgG2a Mab admixed, in most cases, with the patient's own buffy coat. The outcome of this procedure relates very strongly to the size and number of metastases, i.e., treatment is ineffective in patients with a large number of metastases, and somewhat beneficial in patients with a small number of metastases.

In conclusion one can freely say that monoclonal antibodies against human tissues have opened the way to more sophisticated approaches in the study of human tumors and the possibility of immunotherapy.

MONOCLONAL ANTIBODIES FOR THE ANALYSIS OF COMPLEX ANTIGENIC SYSTEMS. ILLUSTRATIONS FROM THE WORK ON MAJOR HISTOCOMPATIBILITY ANTIGENS

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INTRODUCTION

The immortalization of antibody-producing cells, introduced by Köhler and Milstein in 1975 [1], has made it possible to dissect a heterogeneous antibody response into its individual components and to obtain continuously growing hybridoma cell lines which secrete large amounts of pure monoclonal antibody (mAb). The ample supply and high specificity of mAb has firmly established this new biotechnology in biological and medical sciences. Today mAb are used for many different purposes, such as diagnosis and possibly therapy of infectious diseases, isolation of rare natural products such as interferon, detection and quantitation of hormones, diagnosis of cancer, etc. [for overviews see 2, 3, 4].

In this article the use of mAb for the dissection of the major histocompatibility complex (MHC) system is described. The MHC system has been of central interest to immunologists and geneticists for a long time, not only because MHC antigens lead to vigorous transplant rejection, but also because of its enormous degree of polymorphism. There are probably more than 50 or 100 alleles for each locus. Very recent analysis of gene sequences suggests that besides point mutation gene conversion could be one of the mechanisms involved in the generation of this unique polymorphysm [5].

A major breakthrough in the study of MHC antigens was the detection

that cytotoxic T lymphocytes (CTL) recognize foreign antigens, such as tumor or viral antigens, only in conjunction with their own MHC products [6]. This observation has raised many questions, for example if and how the foreign antigen combines with MHC structures; if T lymphocytes have two receptors, one for foreign and another one for their own MHC or if they have only one receptor which can recognize neodeterminants created by association of foreign antigen and self-MHC.

Since it is a frequent phenomenon in nature that specialized functions are confined to particular regions on protein molecules (e.g. enzymes, antibodies, etc.) the expectation is that MHC molecules mediate their function also via specific regions. Therefore, we searched for mAb which could define different epitope regions on histocompatibility (H-2) molecules. For this purpose a large panel of mAb against H-2 antigens was generated [7].

Results

Allodeterminants are clustered in spatially distinct regions on H-2 molecules.

Antibody blocking studies were performed for investigation of the spatial relationship of allodeterminants on H-2 molecules. A set of mAb [7, 8] was labeled with ^{125}Iodine, and the ability of cold mAb to block the binding of labeled mAb was determined. The results show that the mAb fall into two or three distinct groups, depending on the set of mAb and the respective H-2 allele analyzed. Thus, only mAb of one group block each other but they do not interfere with the binding of mAb of the other groups. From these studies it was concluded that allodeterminants are clustered on H-2 molecules in several spatially separate epitope regions. Investigation of several different H-2 antigens gave analogous results [8, 9].

In order to analyze which aminoacids of the H-2 protein sequence would structurally influence the three epitope regions of the H-2K^b molecule several well defined H-2K^b mutant strains (bm strains of mice) with known aminoacid sequence were utilized. Some representative results are schematically summarized in Fig. 1 [9]. In this figure, the boxes below the schematic diagram of the H-2K^b polypeptide chain contain the members of different mAb which crossblock each other and therefore define an epitope region. It can be seen that on the wild type H-2K^b antigen with mAb against H-2K^b three epitope regions could be identified, designated arbitrarily A, B and C. When the same set of mAb was tested against the bm3 mutant which has two aminoacid substitutions at Pos. 77 and 89, only

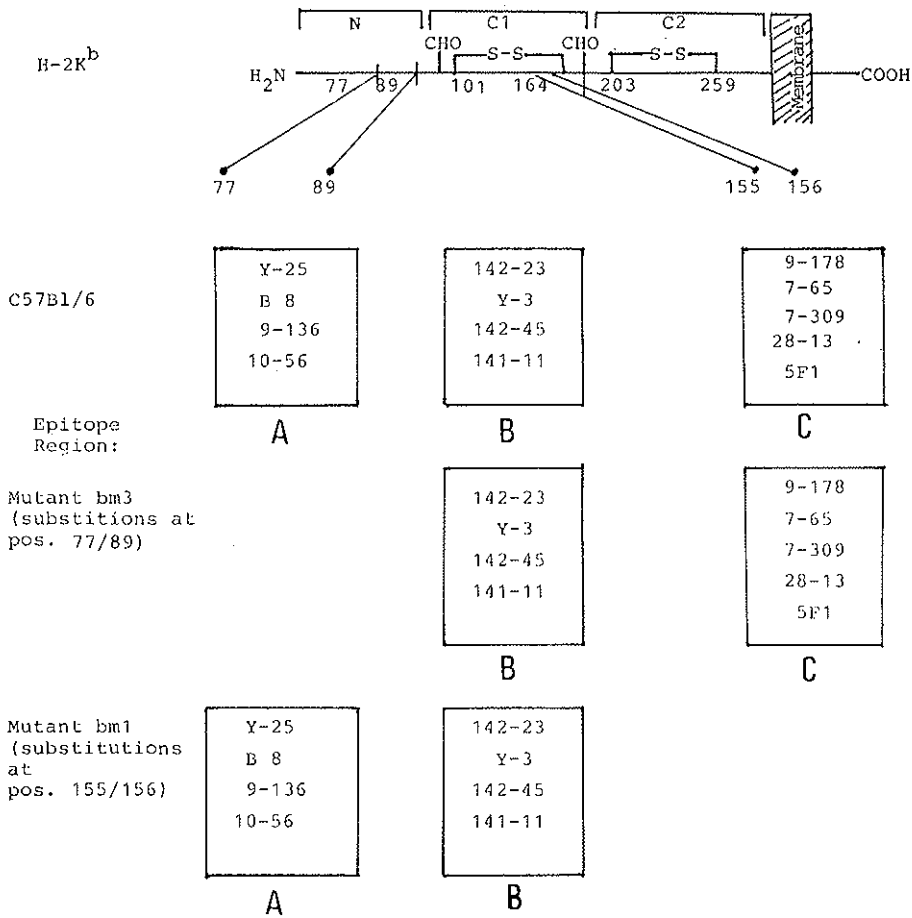

FIG. 1. *Epitope regions on the H-2K^b molecule and influence of aminoacid composition.* The boxes contain the code numbers of anti-K^b mAb which crossblock each other and thus define an epitope region. The wild type K^b molecule expresses all three epitope regions, A, B and C, whereas the bm3 mutant expresses only regions B and C, and the bm1 mutant only regions A and B. For details see text.

members of groups B and C were still reactive. These data demonstrate that the substitutions 77/89 modify only epitope region A but not the other two regions B and C. In contrast substitutions 155 and 156 (mutant bm1) destroy only the target determinants for mAb of group C, but not those of groups A and B (see Fig. 1, lower part). On the other hand

mutations at Pos. 116 and 121 (mutant bm6) do not affect the binding of mAb of any group (not shown).

Altogether such data indicate that some aminoacid substitutions change only the structure of one antigenic region but not the other ones.

Different CTL subpopulations recognize distinct H-2 epitopes.

It is clear from the preceding section that mAb define distinct epitope regions on H-2 molecules. On the molecular level these epitope regions appear to be predominantly located on the first two domains (N and C1) of the H-2 polypeptide chain (see Fig. 1). In contrast very little is known as to which parts of the H-2 molecule are recognized by alloreactive and H-2 restricted cytoxic T lymphocytes (CTL). The experimental approach was to generate a large number of CTL clones and to examine their fine specificity for H-2 epitopes by attempting to block their reactivity with the target H-2 antigen with different anti-H-2 mAb [10, 11]. It might be added as a technical comment that it is very difficult to establish long-term CTL clones but it is relatively easy to obtain short-term clones in a limiting dilution system. Such short-term clones, which have to live only for the duration of the experiment, were generated in the following limiting dilution system.

Increasing numbers of DBA/2 responder cells were stimulated with 1×10^6 A/J stimulator cells in the presence of TCGF for seven days. Lytic activity of the resulting H-2K^k specific CTL was then measured against appropriate target cells (L929, H-2^k). A certain percentage of these microwells can contain more than a single CTL clone, but the resulting degree of clonal distribution is good enough for the subsequent fine specificity analysis. In a large number of limiting dilution experiments we found frequently triphasic Poisson distribution curves. A typical Poisson plot is presented in Fig. 2 which depicts three sets of experimental values, each fitting to a straight line. The lines cross the ordinate at approximately 1.0 suggesting that in each case only one cell type is limiting. Statistical evaluation of the Poisson distribution curves reveals that the three lines represent three CTL precursor populations which differ by their precursor frequency. At a certain number of responder cells the lines turn back suggesting the existence of an as yet unknown suppressive mechanism. Because of this suppression of a high frequent precursor population now another CTL population can become visible with a lower precursor frequency.

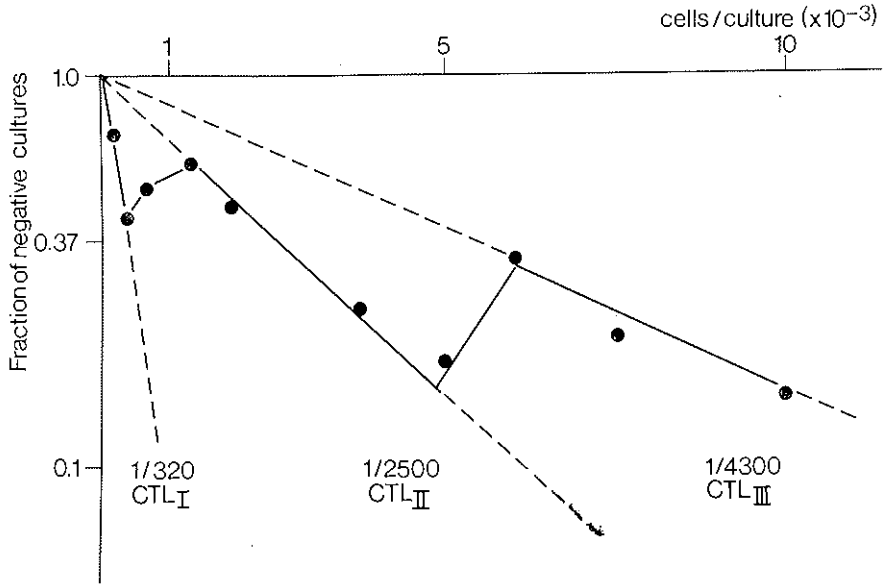

FIG. 2. *Three alloreactive H-2K^k specific CTL populations revealed by limiting dilution analysis.* The Fig. shows a Poisson plot of a limiting dilution experiment in which graded numbers of DBA responders were stimulated with a constant number of A/J stimulator cells for 7 days in the presence of T cell growth factor. Cytolytic activity was measured against ^{51}Chromium labeled L99 target cells. (Adapted from ref. 10).

Next, target inhibition by anti-H-2 mAb against distinct epitope regions was used for determination of H-2 epitopes with specificity of CTL clones in the three CTL subpopulations. Each limiting dilution well was split into two aliquots and cytolytic activity was measured in the presence and in the absence of excess mAb. From the resulting data the percentage of CTL can be calculated which are directed against that area of the H-2 molecule which is covered or modified by binding of the respective mAb. The results are summarized in Table 1.

It can be seen that each mAb inhibits only a certain percentage of microwells. These findings demonstrate the existence of distinct CTL clonotypes with specificity for different H-2 determinants. The most surprising observation is the differential inhibition obtained with mAb against the two clusters (epitope regions) of H-2K^k determinants. Thus, only mAb against cluster A were able to block CTL clones from the high frequent CTL population. In contrast, the third low frequent CTL population was much more efficiently blocked by cluster B antibodies.

TABLE 1 — *Target inhibition with anti-H-2K^k mAb of H-2K^k specific CTL clones.*

Inhibitor (anti-H-2 mAb)	Cluster	% microwells inhibited of CTL subpopulations		
		CTL_I	CTL_{II}	CTL_{III}
H100-5/28 (m3)	A	74	53	29
H100-27/55 (m4)	A	52	41	24
H116-22/7 (m1)	A	48	10	8
H100-30/23 (m5)	B	0	55	65
H141-23/3 (m9)	B		52	68
H142-45/2 (m10)	B	0	59	72
Mixed mAb	A+B	85	90	95
Balb/c ä-A/J serum	A+B	76	85	80
Ratio of A:B		1:0	1:1.6	1:3.6

CTL clones were generated in a limiting dilution system (DBA/2 anti-A/J). Wells were split into two equal aliquots and cytolytic activity was measured in the absence or in the presence of anti-H-2 mAb (ascites fluid 1:25). CTL subpopulations correspond to those shown in Fig. 2.

Similar studies with corresponding results were performed with H-2 restricted CTL generated in limiting dilution systems and also with some longterm H-2 restricted CTL. The conclusion from these CTL experiments is that indeed a single H-2 molecule carries not only one but several distinct epitope regions which can be recognized by CTL. However, it is not yet known if the epitopes recognized by alloreactive CTL, H-2 restricted CTL and by antibodies are the same ones. Target inhibition by CTL is a difficult approach for answering this question because it is not clear in which cases blocking of CTL and reaction with a target is due to induction of conformational changes on the H-2 molecule or to direct blockade of the determinant.

Therefore a new approach has to be considered which comprises modification of H-2 genes and gene transfer studies. Some preliminary results are described.

Transfer and expression of H-2 genes in allogeneic cells.

The recent successful isolation of H-2 genes bas demonstrated their exon-intron structure. Of these the exons 2, 3 and 4 correspond to the

three H-2 domains on the outside of the cell membrane which are defined by aminoacid sequences and which are probably the most important parts for recognition of H-2 by the immune system.

A genomic clone with specificity for the H-2K^d gene [12] has been used for transfection of thymidine kinase (tk) deficient mouse L cells (H-2^k) together with the herpes simplex tk gene. Most cells which have taken up the tk gene have also incorporated the H-2 gene and actively synthesize the new H-2 antigen. This could be demonstrated by the reactivity of transfected cells with CTL and mAb specific for the H-2K^d antigen (see Table 2). Reactivity with a panel of mAb and biochemical analysis of the transferred H-2K^d antigen suggest that it is identical to its counterpart on normal cells.

The availability of genomic H-2 clones and the transfection technique make it now possible to modify defined parts of the H-2 gene, for example by site-specific mutation or exchange of exons between different H-2 genes. Such hybrid genes could then be used to answer the question: which parts of the H-2 molecule are of functional significance for association with foreign antigen and for T cell recognition? Such studies are presently in progress.

TABLE 2 — *Ltk$^-$ cells (H-2^k) transformed with a cloned H-2K^d gene express H-2K^d which can be recognized with CTL and monoclonal antibodies against H-2K^d.*

	Cell line	mAb K^d % lysis	CTL K^d % lysis
Parental	Ltk$^-$	0	0
Transf.	L 7.6	100% (strong)	33%
	L 7.4	100% (strong)	24%
	L 8.4	100% (weak)	12%
	L 9.3	100% (weak)	6%
	L 6.9	0	0

Parental Ltk$^-$ cells were transfected with a cloned H-2K^d gene [12] together with a herpes simplex thymidine kinase (tk) gene. Transformants were selected in HAT medium which permits only growth of cells carrying the tk gene. The transformants were cloned and analysed with monoclonal anti-K^d antibodies by complement-dependant cytotoxicity or with killer cells in chromium release assays. The antibody assay is usually much more sensitive than the killer assay which explains the discrepancy in the % lysis. Unrelated antibodies or killer cells did not lyse the transformants. (Data from H. Burkhart, S. Kvist, B. Dobberstein, and E. Meyer, Heidelberg, manuscript in preparation).

Concluding remarks

In conclusion the studies here have demonstrated the existence of distinct functional epitope regions on histocompatibility complex antigens which are important for recognition by antibodies or by cytotoxic T lymphocytes. Genetic modification of H-2 genes will make it possible to correlate the aminoacid sequence with the various functional properties of histocompatibility molecules, and thus will provide a better understanding of this class of molecules which are so essential for cellular interaction in the immune system.

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CHAGAS' DISEASE: HUMAN ANTIBODY RESPONSE TO A SURFACE GLYCOPROTEIN ISOLATED FROM *TRYPANOSOMA CRUZI*

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More than seventy years after Carlos Chagas' original description [1] of American Trypanosomiasis, the disease which now bears his name has become a major public health problem in Latin America. According to recent estimates [2], more than 24 million people are infected by *Trypanosoma cruzi*, the parasitic protozoan which is the causative agent of Chagas' disease. Primitive housing in rural areas of the continent has long been identified as an important factor in vectorial transmission of *T. cruzi* by hematophagic triatomine insects. Many of its species are well adapted to artificial ecotopes such as poor human dwellings and animal dens, thereby establishing conditions for a domiciliary cycle of parasite transmission. Human and other mammals are contaminated by metacyclic trypomastigotes released in the insect's intestinal droppings, following a blood meal. The life cycle of the parasite starts with the invasion of muscle and/or nerve fibers, and of tissue macrophages of various organs. Thereafter, the flagellates reorganize to amastigote forms before the onset of reproduction by binary fission; by the time of host cell death, the amastigotes are transformed into infectious trypomastigotes which disseminate the infection after gaining access to the bloodstream [reviewed in 3]. High blood parasitemia, extensive tissue parasitism and inflammation are characteristic features of acute Chagas' disease, but the course of the disease may be asymptomatic in many individuals. Years later, 20 to 50% of infected individuals of various geographical areas develop chronic inflammatory

lesions in the heart; this invariably causes myocardial damage, and often leads to cardiac failure. Chronic inflammatory lesions are sometimes found in the autonomic nervous system, and are associated with marked enlargement of esophagus or colon (mega syndrome). The progressive course of chronic lesions, the lymphocytic nature of inflammatory infiltrates, in addition to scarcity of tissue or bloodstage forms of the parasite suggest the participation of immunopathogenic processes in the chronic form of the disease [4]. Auto-antibodies to heart and skeletal muscle, to blood vessels and to peripheral nerves are commonly detected [5, 6, 7]; in fact, the recent identification of anti-laminin antibodies in chagasic serum [8] has provided a molecular basis for at least some of the auto-reactivities described. Several cell-mediated reactions against host tissues have been reported in humans and experimental infections [9, 10], but the absence of a more satisfying animal model for chronic disease has limited the investigation of the cellular basis of the postulated auto-immune reactivities; it is conceivable that the failure to reproduce chronic human pathology in experimental animals stems from as yet undefined influences of host genotypes [11] and from the large heterogeneity of natural parasite populations. These factors probably underlie the clinical pleomorphism of human Chagas' disease.

Recent studies have placed strong emphasis on *T. cruzi* molecular biology [12, 13], trying to relate it to the biological differences of strains in terms of their virulence, tissue tropism, and morphology [3]. The application of cloning techniques to *T. cruzi* has extended these observations to intra-strain differences, and to fresh parasite isolates from man, animals, and infected triatomines [14, 15, 17]. For example, Postan *et al.* [15] have recently shown that the pluripotential nature of a strain may be due to genetic heterogeneity of the parasite populations which constitute the strain: clones isolated from insects used to xenodiagnose an acute case of human disease showed very disparate behavior when injected in experimental animals. Moreover, the pattern of infection with the original strain was compatible with that of the superimposed cloned sub-populations. Antigenic differences, which were long appreciated within *T. cruzi* strains [16], have been recently extended to intra-strain level [17].

In the light of these considerations, the balance between immune responses to "common" (shared) or "restricted" *T. cruzi* antigens would influence the host-parasite relationship in benefit/or prejudice of the former. Interestingly, chagasic serum has been reported to either enhance [18] or inhibit [19] *T. cruzi* infectivity of non-phagocytic cells *in vitro*; whether

this relates to host inadequacy to attain sterile immunity is not known, but the results emphasize again the need to characterize specific components of the antibody response. Similar considerations should be raised to account for the apparent contradiction in the finding of complement-dependent lytic antibodies to bloodstream forms of *T. cruzi* in the serum of chronic patients [20, 21] which invariably show low grade parasitemia. This could be explained by the exercise of antibody escape manoeuvres by *T. cruzi* [22], as suggested by the experimental studies of Krettli and Nussenzweig with strains Y and CL [23]. Notwithstanding this hypothesis, it is conceivable that antibody effectiveness also depends on the nature of the target antigen, the isotype (and sub-class) of the corresponding antibodies, and their effective concentrations at any given moment of infection.

The isolation of *T. cruzi* surface antigens such as the 90 K glycoprotein described by Snary and Hudson [24], or the 25 K glycopeptide recently investigated in our own laboratories [25, 26] provide the first opportunity to undertake studies of antigen specific immune responsiveness in human chagasic patients. Our efforts have been directed towards the (i) identification of human antibody responses to well-defined surface antigens *shared* by vast *T. cruzi* populations and, (ii) investigation of their functional role in host defence to the parasite.

A direct approach to this project recently became realistic with the development of reproducible procedures for the isolation of well characterized glyco-conjugates of *T. cruzi* [25] by L. Mendonça-Previato, J.O. Previato and co-workers at the Instituto de Microbiologia da Universidade Federal do Rio de Janeiro (Mendonça-Previato *et al.*, manuscript in preparation). Our studies are presently confined to a highly purified glycoprotein of 25 K (Gp25) whose main immunochemical [26] (J. Scharfstein; J.O. Previato; M.M. Rodrigues; C.A. Alves; W. de Souza, and L. Mendonça-Previato, manuscript in preparation) and biological properties are as follows: Gp25 is highly immunogenic to infected humans, since antibodies to Gp25 are found in the serum of 325/332 (97.8%) of chronic chagasic patients from widely different endemic areas. Human antibodies to Gp25 were specifically purified on Gp25-affinity columns and used to demonstrate the corresponding antigenic sites at the surface of *T. cruzi*, and at various stages of parasite development. We also observed that anti Gp25 reactivity is selective for *T. cruzi*, not being observed so far in other well characterized trypanosomatid species. The absence of anti-Gp25 reactivity in the serum of several patients with visceral or tegumental leishmaniasis is in contrast to the cross-reactivity usually detected with other antigen-antibody systems.

These findings suggest that Gp25/anti-Gp25 may be a valuable system to be applied in serodiagnosis of Chagas' disease, particularly in endemic areas where leishmaniasis is also prevalent.

The remarkable high frequency of antibodies to Gp25 in the chagasic population leads to an important inference, if one takes into account the large inter-strain heterogeneity (now extended to intra-strain differences) of *T. cruzi*, and the widely different geographic regions from where our patients originate: Gp25 is necessarily part of the antigenic repertoire of a large proportion of *T. cruzi* populations which commonly infect the human host. Taken together, our data support the prediction that the determinants defined by human anti-Gp25 may contain epitopes that are highly conserved in the species. Obviously, the biological role of a surface molecule with such characteristic can only be a matter of speculation, but the present availability of human oligoclonal antibodies, with the forthcoming isolation of monoclonal species, should give us the opportunity to investigate this problem. Our recent findings that human (and rabbit) antibodies to Gp25 protect primary cultures of human smooth muscle cells from *T. cruzi* infection *in vitro* [27], and that immunization of rabbits with purified Gp25 confers partial protection to acute challenge with *T. cruzi* (L. Ramirez, Z. Brener, L. Mendonça-Previato, R. Borojevic and J. Scharfstein, manuscript in preparation), support the notion that the highly prevalent anti-Gp25 response may play a role in immune resistance to *T. cruzi* infection. This hypothesis will be pursued in future studies.

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IMPLEMENTATION OF A DEVELOPMENTAL PROGRAM

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Introduction

Experiments involving both nuclear and cellular transfer to an early embryo have repeatedly shown the extreme flexibility of the cellular genomes. Genetic material from advanced embryos and from some neoplastic cells becomes, when introduced into an early embryo, adjusted to its development and will, subsequently, contribute to various host tissues without marked deviations from the normal (e.g. Mintz, Illmensee, this volume). But such observations also indicate the existence of a strict *regulatory system* within the embryo providing the transplanted material with "epigenetic" clues for its spatially and temporally synchronized expression within the host organism. This general building program and the mechanism of its implementation are only fragmentarily known, but one central principle explored since the beginning of this century is *embryonic induction*, i.e. a morphogenetically significant communication between cells. According to the definition by Grobstein (1956), "inductive tissue interaction takes place whenever in development two or more tissues of different history and properties become intimately associated and alterations of the developmental course of the interactants result".

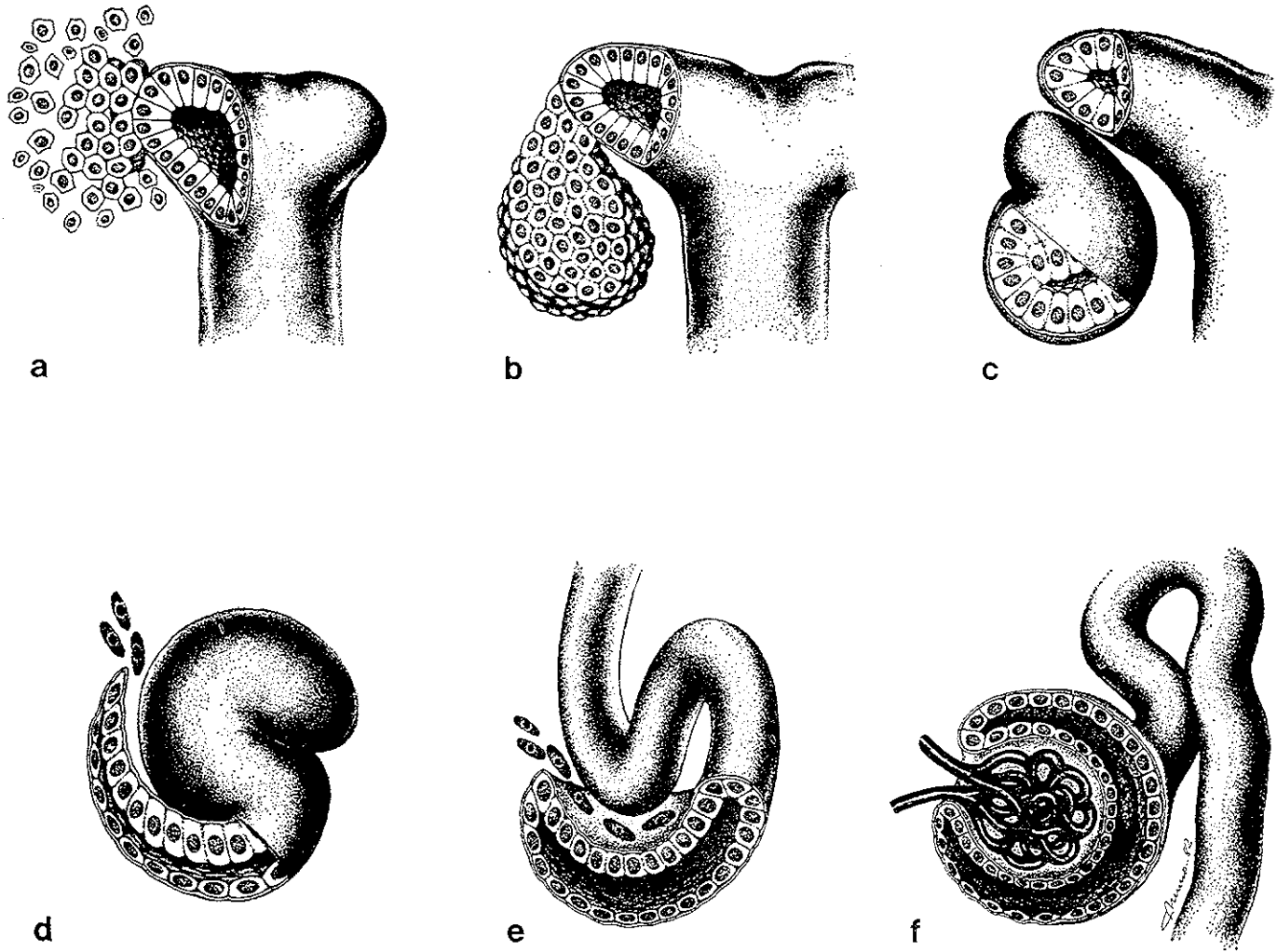
In fact, both the flexibility of embryonic cells and the existence of a regulatory system based on cell interactions were experimentally demonstrated already in 1918 by Hans Spemann: when a piece of the presumptive neuroectoderm is dissected from a young Amphibian gastrula and transplanted to the belly side of a host of the same developmental stage, the transplant becomes "regulated" and develops according to its new site. The result is a completely normal embryo. If, however, the very same

experiment is performed on gastrulae of more advanced stages, a secondary neural plate develops from the transplant, and the host carries a secondary anlage of the CNS at the site of the transplant. The conclusion is apparent: the cells of the transplant had become irreversibly determined (committed) during development between the two stages. As shown later, this commitment is a response to an inductive stimulus released by the invaginating axial mesoderm establishing contact with the still multipotent ectoderm (Spemann and Mangold, 1924).

This demonstration of a morphogenetically meaningful dialogue between two cell lineages has been followed by very many similar, experimentally documented examples of such interactions, and they seem to be the rule rather than an exception in the regulation of embryonic development. Such interactions are known to govern a variety of cell functions during embryogenesis including gene activation (determination), permissive enhancement of metabolic activities, proliferation, polarization and aggregation of cells as well as migration of cells and cell clusters. There is no reason, however, to believe that these different regulatory actions are implemented through similar molecular mechanisms, and until common denominators are shown, each interactive situation should be explored separately. In this overview, I will summarize observations on one model-system for organogenesis governed by sequential inductive interactions.

The Model-System

The permanent mammalian kidney develops from three cell lineages: the epithelium of the Wolffian duct, the mesenchymal blastema and the endothelial cells of the vasculature. The differentiation and organization of the functional unit of the kidney, the nephron, is illustrated in the figure: while the epithelial bud invades the mesenchymal blastema, cells become aggregated around the tips of the branches. The mesenchymal cells soon become polarized and are transformed to epithelial cells aligning a basement membrane. A central lumen opens and the anlage converts gradually to what is known as the S-shaped body. At this stage the first endothelial cells are detected in the lower crevice of the S-shaped body, and ultimately they contribute to the formation of the filtering apparatus, the glomerulus. This seemingly simple example of organogenesis requires a strict coordination of development of the three cell lineages and strongly implies interactions between them. In fact, analysis of this development



A schematic illustration of the early morphogenesis of the functional unit, the nephron, of the metanephric kidney (from Saxén *et al.*, 1983 b).

and its experimental fractionation into single steps has unravelled several consecutive interactions not only between the various cell types but also between cells of the same developmental fate. While these findings are briefly summarized, the reader is referred to the following reviews on the topic: Grobstein, 1956, 1967; Saxén *et al.*, 1968, 1981; Wartiovaara, 1966; Lehtonen, 1976; and Ekblom, 1981).

Determination of the Mesenchymal Cells

The interdependence of the epithelial and the mesenchymal cells prior to overt morphogenesis was demonstrated by Grobstein (1953) by surgical separation of the two cell populations. Subcultivation of these separated tissues showed no further differentiation of the nephric components, but their recombination initiated branching of the ureter and epithelial transition of tbe mesenchyme. The signal substances transmitting this dialogue have remained unknown. While many embryonic tissues (e.g. spinal cord) possess this capacity to trigger epithelial differentiation and tubule formation in the mesenchyme, efforts to mimic their effects with devitalized tissues or cell-free fractions have failed. There is good evidence that the interaction requires close apposition of the cells, and that it is blocked by contact-preventing filters introduced between the interactants (Wartiovaara *et al.*, 1974; Lehtonen, 1976; Saxén *et al.*, 1976). Such observations seem to exclude signal substances transmitted by free diffusion and should focus attention to either surface-associated molecules or direct channels between the cells.

The use of filters not preventing contacts between the epithelial and the mesenchymal cells has shed some light on the *kinetics* of this inductive interaction. After an initial transfilter recombination of the mesenchyme and a heterotypic inductor (spinal cord), the contact can be broken after various time intervals and the response evaluated from isolated, subcultivated mesenchymes. Such experiments have shown tbat the first cells become irreversibly committed after a 12-hour contact with the inductor. Gradually new cells enter the program, and at 24 h the induction is completed and the response does not differ from the controls (cultured in uninterrupted transfilter contact) (Saxén and Lehtonen, 1978). Knowledge of this time course of determination has proved useful when developmental cbanges at various levels are temporally (and causally?) correlated.

Stimulation of Proliferation

Early aggregation of the determined cells is preceded by a stimulation of their DNA synthesis as shown by incorporation experiments using radioactive thymidine. After an initial decline, the incorporation rate increases from 12 h on and reaches its maximum at 24 h, i.e., when also the induction is completed. The stimulation can be attributed to a contact-mediated *mitogenic interaction* between the inductor and the target cells, since isolated mesenchymal cells show no increased incorporation nor do cells separated from the inductor by filters preventing cell contacts.

The close association between the determination of the target cells and their mitotic stimulation has not allowed an experimental separation of the two events. Thus, alternative explanations remain to be explored: either the two are separate events, or the mitogenic stimulus recruits a predetermined cell population which after one or several cell cycles expresses a new phenotype. According to our calculations, the "induction time" in our experimental conditions is sufficient to allow the completion of one cell cycle (Saxén *et al.*, 1983a).

Recognition and Aggregation

One of the new characteristics expressed by the induced cells is their acquired capacity to *recognize similarly determined cells and to adhere to them.* After the contact-mediated induction, cells proliferate rapidly, and they have been shown to move into the still uninduced portion of the mesenchyme, where they become aggregated to form pretubular condensates (Saxén and Karkinen-Jääskeläinen, 1975). Again the molecular mechanism of this "homotypic interaction" (Grobstein, 1962) has remained unknown, but we have recently demonstrated by immunomicroscopy a series of changes in the extracellular matrix (ECM) which parallel the aggregation. Induction is followed by the disappearance of a set of interstitial type proteins from the induced areas: these are collagen type I and type III and fibronectin. In the in vitro system, this change is completed at 24 h and, hence, shows a good temporal correlation with the completion of induction. Simultaneously with this change, a set of "new" proteins is expressed, now belonging to the basement-membrane-type compounds: collagen type IV, laminin and a heparan-sulfate-rich proteoglycan (Ekblom *et al.*, 1981). Immunofluorescence depicts these molecules first as scattered dots in the induced areas, but following ag-

gregation they become confined around the condensates and contribute, subsequently, to the basement membrane of the tubular anlagen.

The causal correlation of these profound changes in the ECM with the parallel movement and aggregation of the induced cells can only be speculated upon. The disappearance of the interstitial proteins might facilitate the motility of the cells within the induced portion of the mesenchyme, and the new set of proteins might carry adhesive properties resulting in recognition and aggregation of similarly induced cells synthetizing these molecules (Ekblom *et al.*, 1980; Saxén *et al.*, 1983b).

Migration Conducted by Cell Interactions

As illustrated in the figure, after aggregation the epithelialized cells form the typical S-shaped body, whereafter endothelial cells are seen in the lower crevice of this nephric anlage. These cells will ultimately form the vascular component, but their origin has remained controversial: they might be mesenchymal cells trapped into the crevice, or cells of the mesenchymal blastema migrating into the presumptive glomerular space, or, finally, cells of a different cell lineage. To distinguish between these alternatives, we grafted young, avascular kidney anlagen from mouse embryos onto the chorionallatoic membrane (CAM) of the quail. Such kidney grafts become regularly richly vascularized, and, in contrast to the in vitro cultures, the glomeruli now show an endothelial, vascular component. The nuclei of these endothelial cells invariably carry the "quail marker" (LeDouarin, 1973), indicating that hybrid glomeruli are formed in which the epithelial podocytes are of mouse origin, but the endothelium is derived from a host source (Sariola *et al.*, 1983).

The vascularization of the kidney by invasion of outside cells might be a two-step process: first the graft stimulates the proliferation of the endothelial cells, and, subsequently, they are conducted to be homed at a precise location in the glomeruli at a strictly determined stage of development. The path conducting this migration has not yet been found. It might be created by the regularly branching ureter, because an experimentally induced mesenchyme with random distribution of tubules becomes, indeed, vascularized, but the glomeruli remain avascular. It remains to be clarified, whether this failure is due to a disorganized basic architecture of the nephric blastema or to a confused timing of the development of the two cell lineages.

Comment

Analysis of the above model-system for organogenesis, experimentally singled out from the complex process of embryogenesis, demonstrated how multipotent cells are regulated by a precise developmental program of the embryo implemented by consecutive intercellular dialogues. The poor understanding of the molecular mechanism of these vital events is an enigma. While, as shown by the excellent reports in this volume, our present methods allow sophisticated analyses and manipulations at the level of DNA and of the intracellular compartment, knowledge of the communicative events in the intercellular compartment is clearly lagging behind. Perhaps the *biological framework* of these processes has remained too superficial for a meaningful molecular approach. Until such efforts become feasible, we should at least know where to search for the hypothetic signal substances and their target sites (receptors), when these are created and when and where they act. The extreme complexity of embryogenesis and its dynamic nature hamper experimental analyses of these questions and has left many of them still open.

It is to be hoped that the wide gap in our knowledge between events at the genetic and the epigenetic levels, between the cellular and the multicellular levels, will gradually narrow down. A more profound understanding of the regulative mechanisms during embryogenesis is also a prerequisite for a better understanding of abnormal development, be it genetically determined, caused by exogenous insults, or resulting from an "idiopathic" lability of the delicate developmental program of an embryo.

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EXPERIMENTAL GENETICS OF THE MAMMALIAN EMBRYO *

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In recent years, several approaches suitable for the analysis of gene activity during mammalian embryogenesis have been realized in the mouse and I should like to briefly summarize these new results and discuss their relevance and applicability to experimental embryology. Firstly, enucleation by removing either both pronuclei from the fertilized egg or the female pronucleus from the unfertilized egg provides an experimental means to analyze the protein pattern derived from maternal mRNA stored during oogenesis. Secondly, microsurgical removal of one of the two pronuclei from the fertilized mouse egg makes it possible to independently study the maternal or paternal genome for its protein synthesis capacity during development. Thirdly, in nuclear transplantations the egg genome is replaced by a somatic cell nucleus in order to reveal the developmental potential of nuclei originating from different cell types and lineages at various stages of differentiation. Fourthly, in gene transplantations cloned recombinant DNA molecules are introduced into the developing organism via injection into the pronucleus of the fertilized egg to examine their presence, expression and regulation during mouse development.

1. *Protein synthesis from maternal message*

Although it has been known for some time that mouse eggs and early embryos synthesize proteins (reviewed hy van Blerkom, 1981), only recent-

* This article is dedicated to the memory of Robert Briggs.

ly there have been attempts to determine whether the mRNA coding for these proteins is derived from transcription during oogenesis or from embryonic gene activity (Braude *et al.*, 1979; reviewed by Schultz *et al.*, 1981). However, it is not yet entirely clear, to which extent these proteins coded by maternal mRNA contribute to early embryogenesis and interact with proteins derived from newly synthesized mRNA of the embryonic genome (reviewed by Sherman, 1979).

In this way, we tried to further investigate the presence of a maternally derived mRNA pool by analyzing the translational capacity of the cytoplasm of unfertilized and fertilized mouse eggs in the absence of the nucleus. For this purpose the eggs were microsurgically enucleated and the surviving cytoplasts analyzed quantitatively and qualitatively for protein synthesis at different times during culture in vitro (Petzoldt *et al.*, 1980). Some remarkable differences were observed between the polypeptide pattern synthesized by fertilized and unfertilized eggs following enucleation. Furthermore, cytoplasts of fertilized eggs showed a more rapid decrease of incorporation than those of unfertilized eggs. Assuming that the pool of total mRNA is originally rather similar between the two egg types, our data may imply that a higher depletion rate of active RNA takes place after fertilization and/or less active RNA is available for translation before fertilization. Both fertilized and unfertilized enucleated eggs reached approximately the same low level of protein synthesis after three days in culture. Newly synthesized proteins could only be detected in enucleated fertilized eggs when cultured for 2 days and appeared similarly to those polypeptides expressed in normal 2-cell embryos (Fig. 1). The differences in protein synthesis between fertilized and unfertilized eggs might result either from newly synthesized mRNA (Piko and Clegg, 1981) or from "masked" maternal mRNA being activated by specific developmental events, e.g. fertilization. Maternally derived mRNA is shown to he present in fertilized eggs, gradually decreases during subsequent cleavage divisions and eventually disappears during the blastocyst stage (Bachvarova and De Leon, 1980).

For some of the polypeptides newly appearing in fertilized eggs, activation of maternally derived mRNA seems a plausible mechanism. Braude *et al.* (1979) extracted mRNA from unfertilized mouse eggs and translated it in vitro. Besides the pattern normally expressed by unfertilized eggs, they also obtained some polypeptides typical for late fertilized eggs and early 2-cell embryos. The mRNA coding for these proteins might remain inactive in our enucleated unfertilized eggs due to the lack of

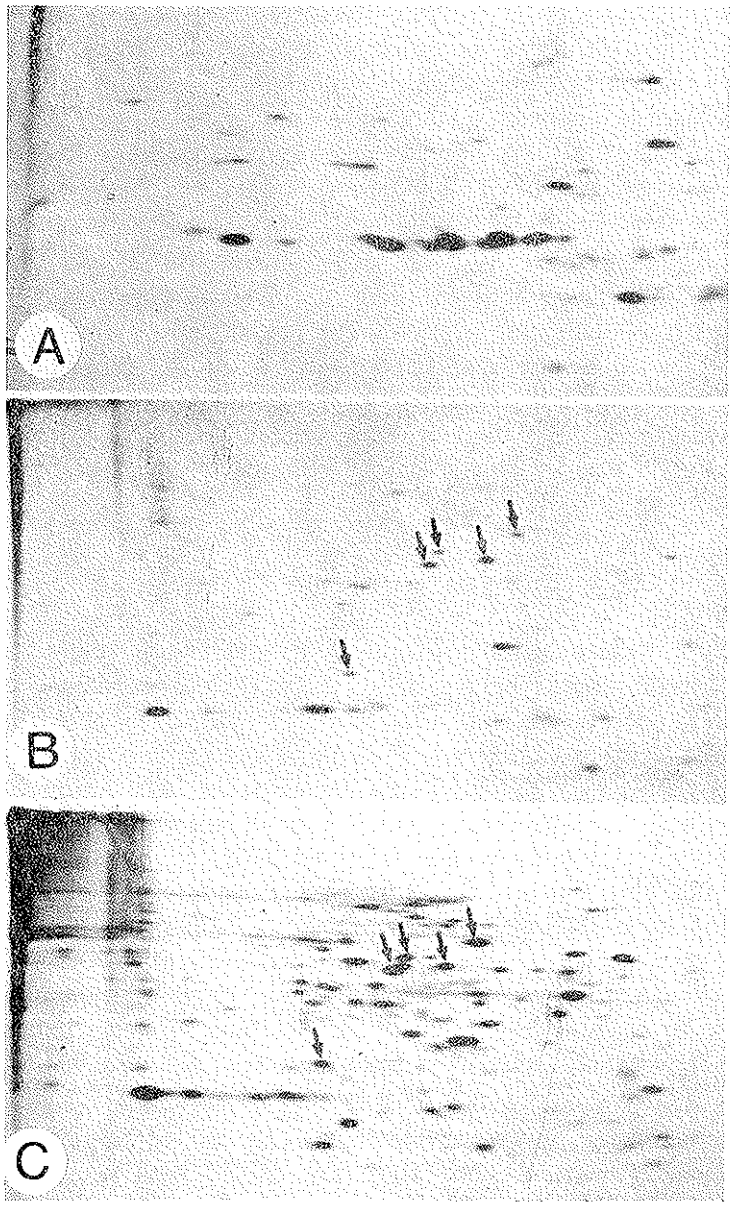

FIG. 1. Protein synthesis in enucleated unfertilized eggs (A), enucleated fertilized eggs (B), and nonoperated 2-cell embryos (C), using 2-D polyacrylamide gel electrophoresis. Both kinds of enucleated eggs continue to synthesize proteins, though qualitative and quantitative differences are observed after two days of culture *in vitro*. Additionally, in enucleated fertilized eggs only, proteins appear that correspond to those of normal 2-cell embryos (after Petzoldt *et al.*, 1980).

appropriate "activation signals". Recently, Schultz *et al.* (1978) demonstrated that artificial maturation of cytoplasmic oocyte fragments could be initiated with special media. Under these conditions the protein synthesis pattern changed qualitatively, and some meiosis-specific polypeptides appeared. It therefore seems reasonable to conclude that activation of maternal mRNA is feasible in oocytes and eggs if the proper stimulus is used.

In future experiments, we shall attempt to artificially activate enucleated unfertilized eggs in order to investigate whether equivalent changes of the polypeptide pattern occur in these cytoplasts. It will also be important to isolate and characterize the different populations of maternal mRNA stored in the egg and, eventually, the genes coding for these messages in order to approach at the molecular level the problem of gene activity and its control during mammalian oogenesis.

2. *Maternal and paternal gene activity*

Early preimplantation development of the mouse embryo seems to be supported by both the translation of mRNA made during oogenesis and the activation of the embryonic genome (reviewed by Sherman, 1979). During this transient period when oogenetic and embryonic gene products seem to function in a temporally coordinated way, it is rather difficult to separate these two processes under normal developmental conditions. Although there is accumulating evidence for early activation and expression of the embryonic genome, the paternal and maternal gene products can usually not be distinguished from each other. Only in a few instances, paternal gene contributions have been documented in preimplantation mouse embryos (reviewed by Chapman *et al.*, 1977). It would therefore be desirable to study the paternal and maternal genome independently as far as their genetic activity is concerned.

The two parental genomes which reside independently as two pronuclei within the egg soon after fertilization can microsurgically be separated from each other, thereby producing androgenetic and gynogenetic mouse embryos (Markert and Petters, 1977) which after transfer into foster mothers have developed into normal isodiploid mice (Hoppe and Illmensee, 1977). Following this experimental scheme (Fig. 2), androgenetic and gynogenetic emhryos were subsequently analyzed for their protein synthesis using high resolution 2-dimensional gel electrophoresis.

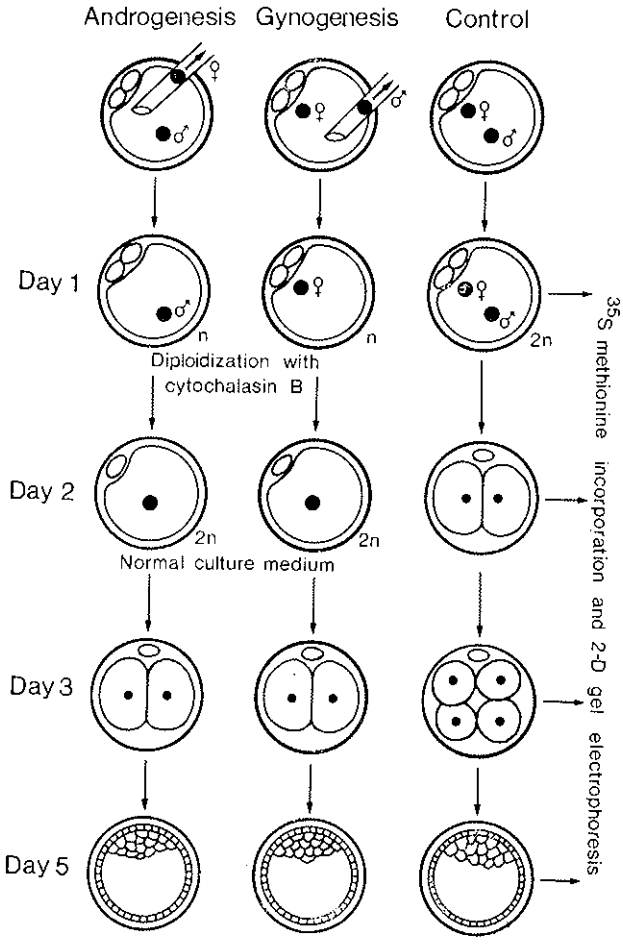

FIG. 2. Experimental scheme for the production of andro- and gynogenetic mouse embryos. Fertilized C57BL/6 eggs were placed in culture medium containing cytochalasin B (CB). Subsequently either the maternal or the paternal pronucleus was removed by sucking it into a small glass pipette. The resulting haploid eggs were diploidized with CB and then cultured *in vitro* in normal medium. As in nonoperated controls they developed to blastocysts at day 5. Androgenetic, gynogenetic and control embryos of day 1, 2, 3 and 5 were used for incorporation with ^{35}S-methionine and further processed for protein analysis using 2-D polyacrylamide gel electrophoresis (after Petzoldt *et al.*, 1981).

We have found that both types of embryos irrespective of the parental genetic origin synthesized very similar patterns of polypeptides throughout different stages of preimplantation development (Petzoldt *et al.*, 1981). In this respect, the paternal as well as the maternal nuclear genome seems to be equally competent in controlling translation during cleavage and blastocyst formation. While certainly some proteins are derived from maternal mRNA (as previously discussed in this article), a significant proportion of newly synthesized proteins has to come from embryonic gene activity and it is therefore important to emphasize that the paternal genome by itself is fully capable of promoting normal early embryogenesis. In this context, further studies on X-chromosomally linked gene expression during subsequent post-implantation stages should provide new insights into the process of maternal versus paternal sex-chromosomal determination (reviewed by Takagi, 1978). Recently, we have demonstrated a temporal difference in the onset of X-linked phosphoglycerate kinase (PGK-1) synthesis depending on whether this particular gene product is of maternal or paternal origin (Krietsch *et al.*, 1982). The maternal PGK-1 gene is already activated during late pre-implantation development, whereas the paternally inherited gene locus remains silent at the pre-implantation stage but is expressed soon after implantation of the embryo into the uterine wall.

Contrary to the similarities in protein synthesis between androgenetic and gynogenetic embryos, a remarkable discrepancy in stage-specific protein patterns occurred in these uniparental embryos when compared with control embryos. The diploidized eggs expressed a polypeptide pattern which very much resembled that of normal 2-cell embryos, and when the experimental eggs cleaved to the 2-cell stage they synthesized proteins typical of the normal 4-cell embryos. These differences between stage-specific protein synthesis and the actual cell stages are most likely caused by the temporal discordance between nuclear and cellular division, since cytochalasin B treatment of the haploid eggs prevented the first cleavage but did allow chromosomal replication to proceed normally to an isodiploid genome (Hoppe and Illmensee, 1977). After having removed these eggs from cytochalasin B, their asynchrony to normal embryos with respect to cytokinesis was maintained during the second and subsequent cleavage divisions. Are these changes in protein synthesis causally related to nuclear replication rather than cellular division? Do they possibly result, at least to some extent, from a pool of maternal mRNA like-

wise involved in early embryonic protein synthesis and/or originate from newly transcribed mRNA after fertilization?

To clarify these questions, we first omitted from the protein pattern of normal 2-cell embryos all polypeptides presumably derived from maternal mRNA (see Petzoldt *et al.*, 1980) or being ubiquitously present during pre-implantation. In doing this, we were indeed left with a few characteristic proteins which appeared stage-specifically and were also correlated with nuclear replication (Fig. 3). Following the next cleavage division, another set of particular proteins was regarded as characteristic for normal 4-cell embryos as well as uniparental 2-cell embryos.

From our data we conclude that the appearance or increased synthesis of some stage-specific polypeptides is correlated with the replication of the genome independent of whether cytokinesis is occurring. To more intensively investigate the effect of chromosomal and DNA replication on stage-specific protein synthesis, it will be important to further characterize

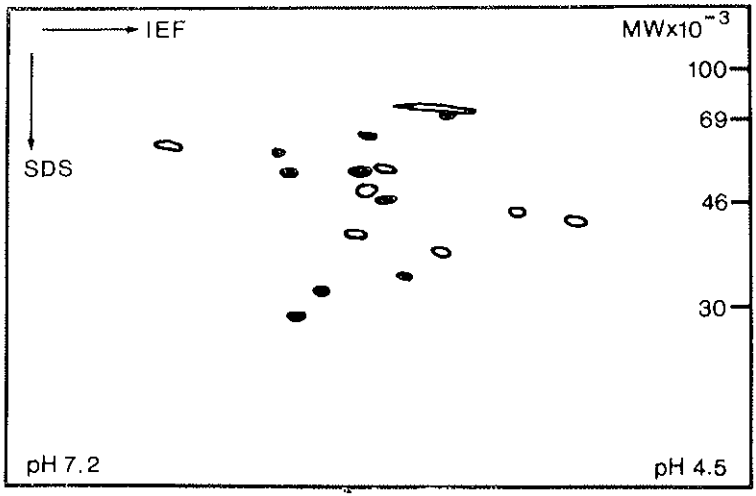

FIG. 3. Schematic presentation of proteins whose synthesis is correlated with nuclear replication at the first and second division but independent of cytokinesis. After having eliminated all proteins presumably translated from maternal mRNA (see Petzoldt *et al.*, 1980) or being present ubiquitously during pre-implantation, only those proteins showing developmental stage specificity have been retained in the diagram. Following the first nuclear replication, 9 proteins (filled spots) appear in andro- and gynogenetically diploidized eggs that are also specific for normal 2-cell embryos. After the second nuclear replication, 8 proteins (open spots) including groups of polypeptides are found in androgenetic and gynogenetic 2-cell embryos which are typical for normal 4-cell embryos (Petzoldt *et al.*, 1981).

these proteins and eventually reveal their function during early mouse embryogenesis.

3. *Developmental potential of transplanted nuclei*

The transfer of a somatic cell nucleus into a fertilized egg provides a unique opportunity of experimentally approaching a long-standing problem in biology that has so far remained unsolved: Are changes in gene expression and possible genomic rearrangements during differentiation gradually restricting the original potential of the egg nucleus, and if so how and when do they occur in the developing organism? Does the cellular and ontogenetic program, whatever its molecular nature may be, also irreversibly alter the genomic potential?

In our attempts to transplant somatic cell nuclei into fertilized but enucleated mouse eggs, we probed this bioassay on cells of the day-4 embryo, the blastocyst, and could demonstrate that only nuclei derived from the inner cell mass were able to promote development to the adult stage (Illmensee and Hoppe, 1981a). Live-born mice were genetically derived from the transplanted nuclei as judged by coat color, karyotype and enzyme analysis. In breeding tests, the nuclear-transplant mice proved to be fertile and functionally transmitted the genome of the donor embryo to their progeny. These results demonstrate that nuclei from particular cells of the late pre-implantation embryo still retain a fully functional genome which is able to provide all the gene products required for normal adult differentiation.

More recently, we extended our ontogenetic analysis into the post-implantation period with an attempt to determine the developmental potential of nuclei derived from different cell types of the day-7 embryo (Illmensee and Hoppe, 1981b). During this particular post-implantation stage, the mouse embryo comprises an outer monocellular layer of distal endoderm, an inner layer of proximal endoderm and, more internally, a multicellular layer of embryonic and extraembryonic ectoderm as well as the ectoplacental cone (reviewed by Snell and Stevens, 1968). These regions of the embryo may conveniently be separated from one another by mechanical means and digestive enzyme treatment and be further dissociated into single cells so that each cell type can then be tested in nuclear transplantation experiments. Do these different cells, some of which already show differentiated epithelial properties (Jackson *et al.*, 1981),

also exhibit different nuclear potencies or, alternatively, are their similarities in developmental capacities between the various nuclei irrespective of their cellular origin and fate?

Although this extensive series of nuclear transplantation has not yet been completed and has only been published in part recently (Illmensee and Hoppe, 1981b), the results so far obtained may be already presented and discussed concerning the nuclear potential of the different cell types. Nuclei from distal andoderm cells did not promote development of the enucleated eggs beyond early cleavage divisions. Similarly, nuclei taken from cells of the extra-embryonic ectoderm and ectoplacental cone were not able to bring about normal development of recipient eggs which usually cleave but then invariably arrest after a few divisions. Contrary to these restrictions in nuclear potential, when nuclei from cells of the embryonic ectoderm were transplanted into enucleated eggs, they gave rise to fertile mice bearing exclusively the genetic markers of the transplanted nuclei (Fig. 4). Our data suggest that nuclei from cells of the embryonic ectoderm, which will eventually contribute to the fetus proper, are still able to express in an orderly sequence all genes necessary for normal adult development. In contrast, nuclei derived from the other cell types of the day-7 embryo show a very restricted potential, presumably due to their cellular specialisation. Future studies are aimed at substantiating these biological findings at the molecular level.

We also have applied nuclear transplantation to the study of parthenogenesis. Diploid parthenogenetically activated oocytes can regularly be obtained after gonadotropin-induced ovulation of virgin females of the LT/Sv inbred mouse strain. These oocytes cleave spontaneously and develop into blastocysts which implant in the uterus but then die within a few days (Stevens, 1975). On the other hand, when the activated LT/Sv oocytes do not ovulate but remain in the ovary, they can obviously not implant but continue to grow and develop into ovarian teratomas composed of many differentiated cell types (Stevens and Varnum, 1974). The reasons for the developmental arrest of parthenogenetic embryos soon after their implantation into the uterus are not known. It has been suggested that, among other possibilities, the presence of recessive lethal genes or deleterious gene combinations becoming homozygously uncovered in the diploid parthenotes may be responsible for their premature death (reviewed by Graham, 1974).

We therefore examined the developmental potential of nuclei from parthenotes after their transplantation into fertilized but enucleated eggs.

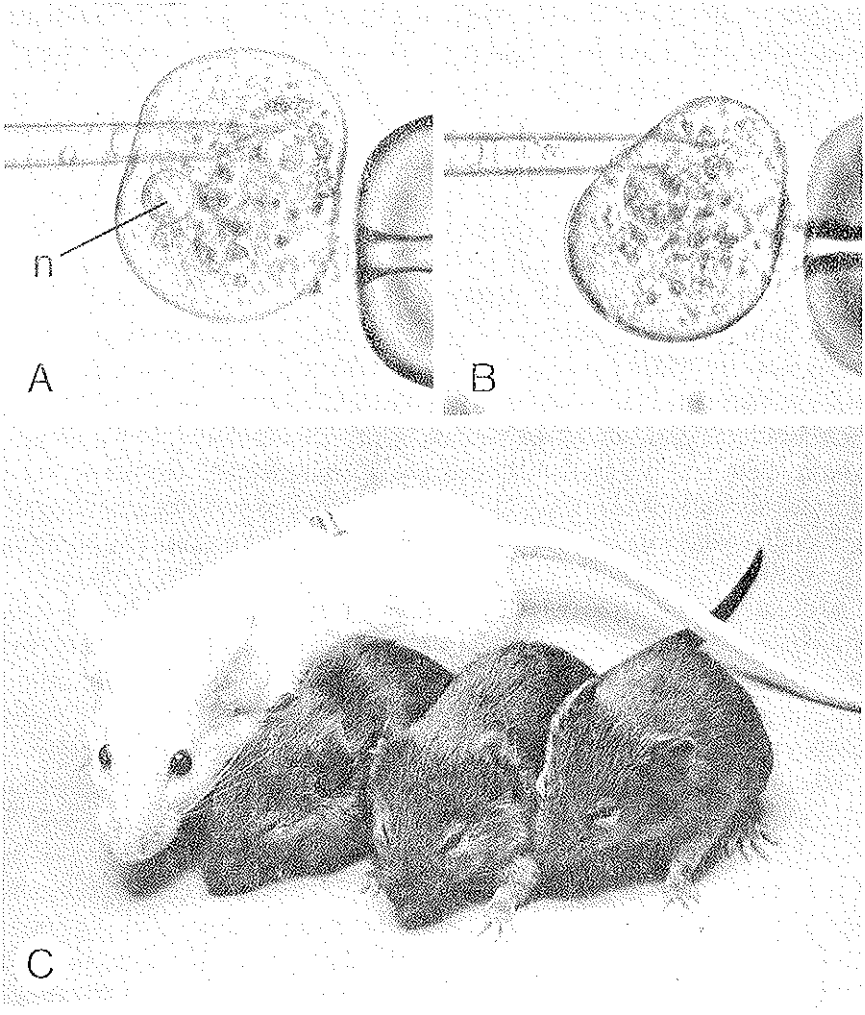

FIG. 4. Nuclear transplantation into a fertilized mouse egg. The egg (0.06 mm in diameter) is attached to a blunt holding pipette (right) in the proper position, and the nucleus (n) of an embryonic cell is transferred into the egg using a thin injection pipette (A). Subsequently, the male and female pronucleus are removed from the egg by sucking them into the same pipette (B). The operated eggs are then cultured *in vitro* and transferred into the uterus of a surrogate mother for further development (C). The three cloned nuclear-transplant mice, shown here together with their surrogate mother, are derived genetically from cell nuclei of the embryonic ectoderm of a *single* day-7 mouse embryo. Their agouti coat, diploid karyotype with the chromosomal T6 translocation and several enzyme markers, all characteristic of the nuclear donor strain, document their genetic origin from the transplanted nuclei (Illmensee and Hoppe, 1981b).

We have recently reported that nuclei originating from cells of parthenogenetic embryos were able to promote normal development beyond the lethal stage of the nuclear donors and gave rise to normal adult mice (Hoppe and Illmensee, 1982). From these nuclear transplantations, four female offspring were born and all of them showed a diploid karyotype and expressed enzyme activities of only the LT/Sv genotype. One female proved to be fertile and transmitted the parthenogenetic genome to the next generation. Therefore the early post-implantation death of parthenotes does not seem to be primarily related to an aberrant genome but rather to some yet undefined mechanisms associated with fertilization and normal morphogenetic processes.

4. *Gene transplantation*

In mammals, pre-implantation embryos of the mouse seem to be suitable recipients for gene transplantation because of their well investigated genetic background and the relative ease with which they can be collected from pregnant females, manipulated and cultured in vitro, and then retransferred into surrogate mothers for further development. In this way, the integration and possible expression of the foreign genes can be most effectively studied at various stages of embryonic, fetal and adult life and, eventually, transmitted to the next generation for genetic mapping of their location in the host genome. Recombinant DNA plasmids composed of segments of Herpes simplex virus, simian virus 40 and the bacterial vector pBR322 were injected directly into the pronuclei of fertilized mouse eggs in order to increase the probability of integration of donor DNA into cells of the developing embryos (Gordon *et al.*, 1980; Gordon and Ruddle, 1981). Similarly, cloned plasmids containing the human β-globin gene and thymidine kinase gene of Herpes simplex virus were transferred into mouse eggs (Wagner, Stewart and Mintz, 1981). Recombinant DNA plasmids carrying the rabbit β-globin gene were found to be integrated in the host's genome, transmitted to the next generation (Costantini and Lacy, 1981) and presumably expressed during *in vivo* development (Wagner *et al.*, 1981). In another study, the mouse metallothionein gene has successfully been transferred into mouse eggs and found to be expressed in adult mice (Brinster *et al.*, 1982; Palmiter *et al.*, 1982).

We recently attempted to introduce recombinant DNA molecules containing a defined eukaryotic gene, the human insulin gene, into develop-

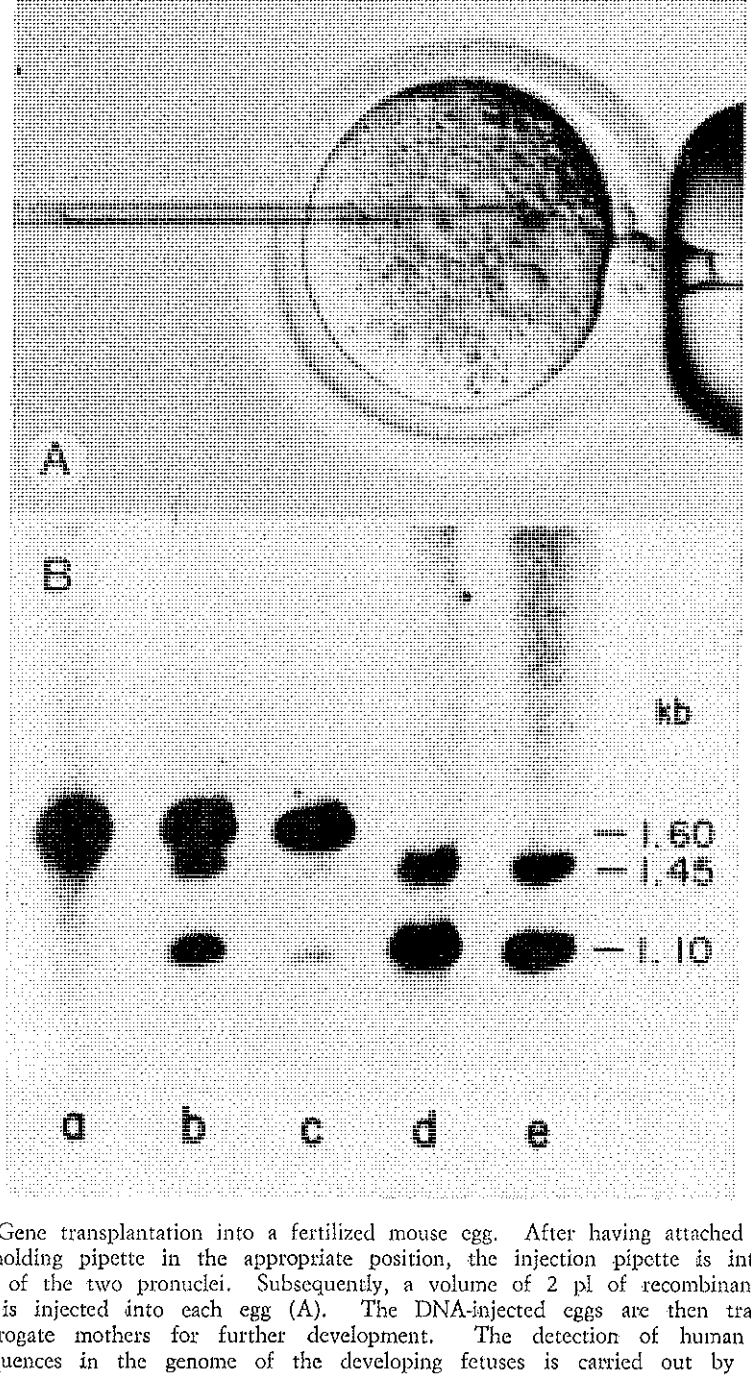

FIG. 5. Gene transplantation into a fertilized mouse egg. After having attached the egg to the holding pipette in the appropriate position, the injection pipette is introduced into one of the two pronuclei. Subsequently, a volume of 2 pl of recombinant DNA solution is injected into each egg (A). The DNA-injected eggs are then transferred into surrogate mothers for further development. The detection of human insulin gene sequences in the genome of the developing fetuses is carried out by Pvu II restriction enzyme digestion and Southern hybridization using a 310 bp fragment of the human preproinsulin gene as probe (B). One fetus (b) and its placenta (c) both show the 1.6 kb human DNA hybridizing fragment (see human DNA control in a), whereas another fetus (d) and its placenta (e) remain negative. In addition, they all show two bands of 1.45 and 1.1 kb in size corresponding to the two endogenous mouse insulin genes which are detected by cross-hybridization (after Illmensee *et al.*, 1981).

ing mouse eggs via microinjection (Illmensee *et al.*, 1981). The cloned plasmid we employed is comprised of a 12.5 kb region of the human genome including the entire insulin gene (Ullrich *et al.*, 1980) and the 4.3 kb bacterial vector pBR322. Following DNA injection, the developing embryos were then transferred into the oviduct or uterus of pseudopregnant foster females in order to allow normal implantation to proceed. Fetuses and their corresponding placentas were isolated from the uteri at late pregnancy and their DNA was extracted and screened for the presence of the injected DNA sequences by using a radioactively labeled 310 bp probe that contains most of the human preproinsulin. With the aid of restriction endonucleases in conjunction with Southern blot hybridization we found that in two normally developed fetuses at day 18 of pregnancy, both fetal and placental tissues contained the human insulin gene including the flanking regions and bacterial plasmid sequences (Bürki and Ullrich, 1982). Digestions with Pvu II and Bgl II endonucleases revealed a hybridizing fragment that corresponded in size to the human insulin gene, indicating that the original size of the injected gene was maintained during in situ development (Fig. 5).

Although we have demonstrated the presence of the human insulin gene in mouse fetuses, and more recently in the adult mouse genome as well (unpublished data), it remains to be shown whether this particular gene is functionally expressed during cellular differentiation, organ-specifically controlled at the adult stage, and eventually transmitted through the germline to the progeny.

Acknowledgments

I should like to thank Drs. P.C. Hoppe and A. Ullrich for their invaluable collaboration and all my associates at the Laboratory of Cell Differentiation for their help and assistance. Inbred mice were kindly donated by the Füllinsdorf Institute of Biomedical Research, Switzerland. This research was supported by the Swiss Science Foundation (FN 3.442.0.79), the March of Dimes Birth Defects Foundation (1-727), and the National Institutes of Health (CA 27713-03).

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ON SOMITE INDUCTION IN VERTEBRATE EMBRYOS

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Ribonucleoproteins and Cytodifferentiation

In 1961, I presented at the Study Week of this Academy some data on ribonucleoprotein action in cellular differentiation [18].

My group extracted ribonucleoproteins from liver, heart, muscles, kidney and the glandular stomach of adult chicken. The ribonucleoproteins were extracted following the Niu method. An allantois explanted from a 4-day-old chick embryo was filled with these ribonucleoproteins and grafted on the chorioallantois membrane of an 8-day chick embryo. Some cells of the chorioallantois were transformed in 30% of the treated embryos. Glandular cells synthesizing glycogen are induced with liver ribonucleoproteins in the chorioallantois. Multinucleate elements with fibrillar cytoplasm are induced by heart ribonucleoproteins. The skeletal-muscle ribonucleoprotein induces the transformation of mesenchime cells into muscular structures, on which cross-striated elements may also appear. Hollow vesicles covered with epithelia, that remind us of the epithelia of the nephric ducts, are induced by kidney ribonucleoprotein. Secreting epithelial cells are induced by glandular stomach ribonucleoprotein [20].

Somite induction in chick embryo

More recently, a lot of work has been done to identify the messenger ribonucleic acids (mRNAs) that direct the synthesis of the proteins typical of differentiated tissues (Brawerman [22]) and the role of these messengers

during cell differentiation has been clarified. Particular attention has been paid to the synthesis of myosin, which accounts for 7-15% of the total proteins synthesized in the fully differentiated myotubes. Heywood and Rourke [10] and Merlie *et al.* [12] made the first steps in defining the 26S mRNA that codifies for the myosin heavy chain and its role during the differentiation of the striated muscle. During myogenesis, myosin is, in fact, rapidly accumulated.

The purpose of our study (1) was to examine the ability of messenger RNA from homospecific or heterospecific sources to modify the shape of mesodermic cells and to organize them to form somite masses.

Fertilized chicken eggs (Ross Italia) were incubated at 38.5°C for 24 hours to the primitive streak stage (stage 4 of Hamburger and Hamilton [9]). Older embryos were discarded. The embryos at the primitive streak stage were explanted and cultured "in vitro" by New's method [14]. The anterior part of the embryo used for the experiment was cut away 0.6 mm behind the node and the remaining part (postnodal piece = PNP) was cultured in Pannett-Compton solution and albumen (Fig. 1). In table I and in the figures these explants are called controls if no other additions were made to the media.

Treated postnodal pieces were cultivated adding to the culture medium (2 ml per embryo) mRNA at the final concentration 4/10 μg/ml. 26S mRNA and the pool of mRNA from muscle induced somites (Fig. 1). The action is organospecific because the mRNA pools extracted from liver or kidney did not induce somites. The induction is not related to a transcription process because actinomycin D did not prevent somite formation, although the somite was small. Somite formation depends on a translation process, since puromycin added to mRNA inhibited the induction (Tables I, II). The induced somites were larger than the somites in the control embryos. The data presented here are for the sixth somite of the control embryo. The mean volume (± standard error) of the induced somite was 302,333 ± 17,800 $m\mu^3$ that of sixth somite of chick embryo at 48 hours is 240,000 ± 10,900 $m\mu^3$.

These results support the hypothesis that induction of somites must be caused by the translation of 26S mRNA into myosin and subsequently by the action of myosin itself on the cell shape. PNPs treated with chicken soluble myosin induced a good percentage of somites, even when the

(1) Research of M. Cigada Leonardi, F. De Bernardi, A.M. Bolzern, U. Fascio, R. Maci, C. Sotgia and myself [1], supported by C.N.R. grants.

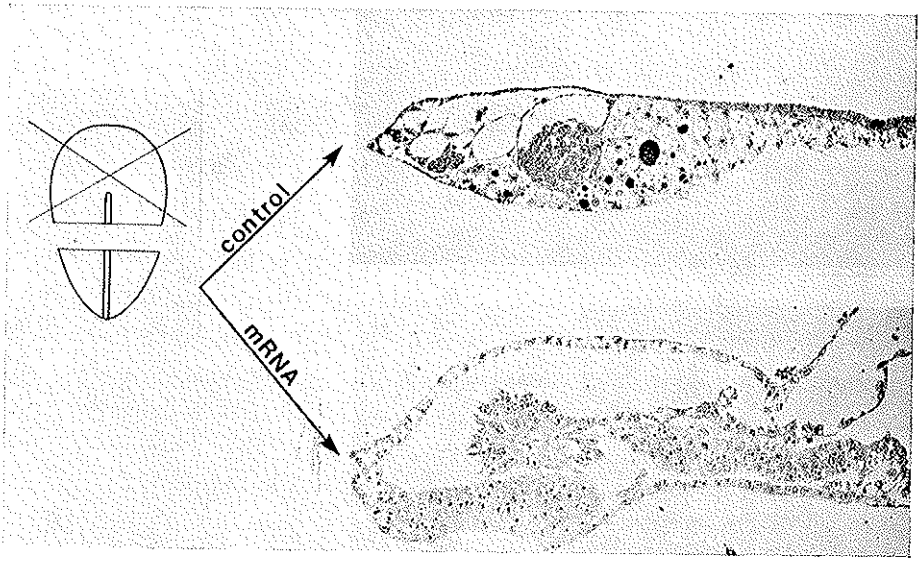

FIG. 1. Experiment on action of 26S mRNA or of myosin on the development of postnodal explants of chick embryo.

myosin was combined with actinomycin D or puromycin (Tables I, III). The heavy chain of myosin (codified for by 26S mRNA) was also able to induce somites.

We induced somite structures also by treating the PNPs with pool of mRNA and with 26S myosin mRNA extracted from duck, rabbit, rat and even from trout.

The somites were well organized when induced by 26S mRNA or pool mRNA from chicken muscles or by myosin (Fig. 1, 2). Sometimes these substances induced very large fused somite masses (Fig. 2). Small somites were induced by chicken 26S mRNA or myosin plus actinomycin D. Similar small somites could sometimes be induced with heterospecific Vertebrate RNA (Fig. 2). 26S mRNA from duck, rabbit, rat or trout induced somites often built up of cells separated by large spaces and joined around a large myocoele (Fig. 2). Even the 26S mRNA of crayfish muscles induced elongated cells joined in the basal region and connected around a cavity (Fig. 2). The same elongated cells were obtained by treating the PNPs with the myosin light chain from chicken muscle.

These results raise the problem of the degree of induction by chick 26S mRNA as related to concentration. Consequently we tested 26S mRNA

TABLE I

	explanted embryos	surviving explants	somite induction	% induction
Controls	192	133	0	0
Myosin mRNA of chick	289	206	76	36.9
mRNA pool of chick muscle	204	144	76	52.8
mRNA pool of chick liver	40	30	0	0
mRNA pool of chick kidney	42	22	0	0
Myosin mRNA of duck	25	20	5	25.0
Myosin mRNA of rabbit	67	48	7	14.5
Myosin mRNA of rat	30	19	2	10.5
Myosin mRNA of trout	30	27	10	37.1
Myosin mRNA of crayfish	31	23	2*	(8.7)
Myosin mRNA + actinomycin D	64	44	9	20.5
Myosin mRNA + puromycin	30	9	0	0
Myosin mRNA heated at 60°C	10	10	1	10.0
Myosin mRNA ribonuclease-treated	18	12	0	0
Myosin mRNA trypsin-treated	23	15	2	13.1
Soluble myosin of chick	64	51	17	33.3
Myosin + actinomycin D	6	3	2	66.6
Myosin + puromycin	9	6	3	50.0
Myosin of rabbit	15	12	0	0
Heavy chain of chick myosin	41	24	8	33.3
Light chain of chick myosin	8	7	3*	(42.9)
Myosin denatured by NaSCN	12	7	0	0
F-actin	5	4	0	0
G-actin	18	14	0	0

* elongated mesodermic cells joined in the basal region.
When not otherwise indicated the substances were extracted from chicken.
The results of the experiments here presented are summarized in Tables II and III.

at final concentration of 8,4 and 2 μg/ml. Somites were induced by 8-4 μg/ml 26S mRNA but not by 2 μg/ml. The somites induced by these concentrations looked alike and were well developed, with the same frequency as the somites induced by 10 μg/ml of 26S mRNA. We conclude that the different characteristics of the somites induced by nonspecific mRNA result only from the nonspecificity of the messenger.

TABLE II - *Action of RNA on somite induction.*

The somites are induced by:
Myosin 26S mRNA of chick
mRNA pool of chick muscle
26S mRNA with actinomycin D
26S mRNA treated with tripsin
26S mRNA heterospecific (of duck, rabbit, rat, trout) induces some somite structures. Also 26S mRNA of crayfish induces elongated mesodermic cells joined in the basal region.
No induction has been observed with:
mRNA pool of chick liver
mRNA pool of chick kidney
26S RNA of chick with puromycin
26S RNA of chick treated with ribonuclease

TABLE III - *Action of myosin and actin on somite induction.*

The somites are induced by:
Chick myosin
Chick myosin with actinomycin D
Chick myosin with puromycin
Heavy chain of chick myosin
No induction has been observed with:
Denatured chick myosin
Rabbit myosin
F actin
G actin

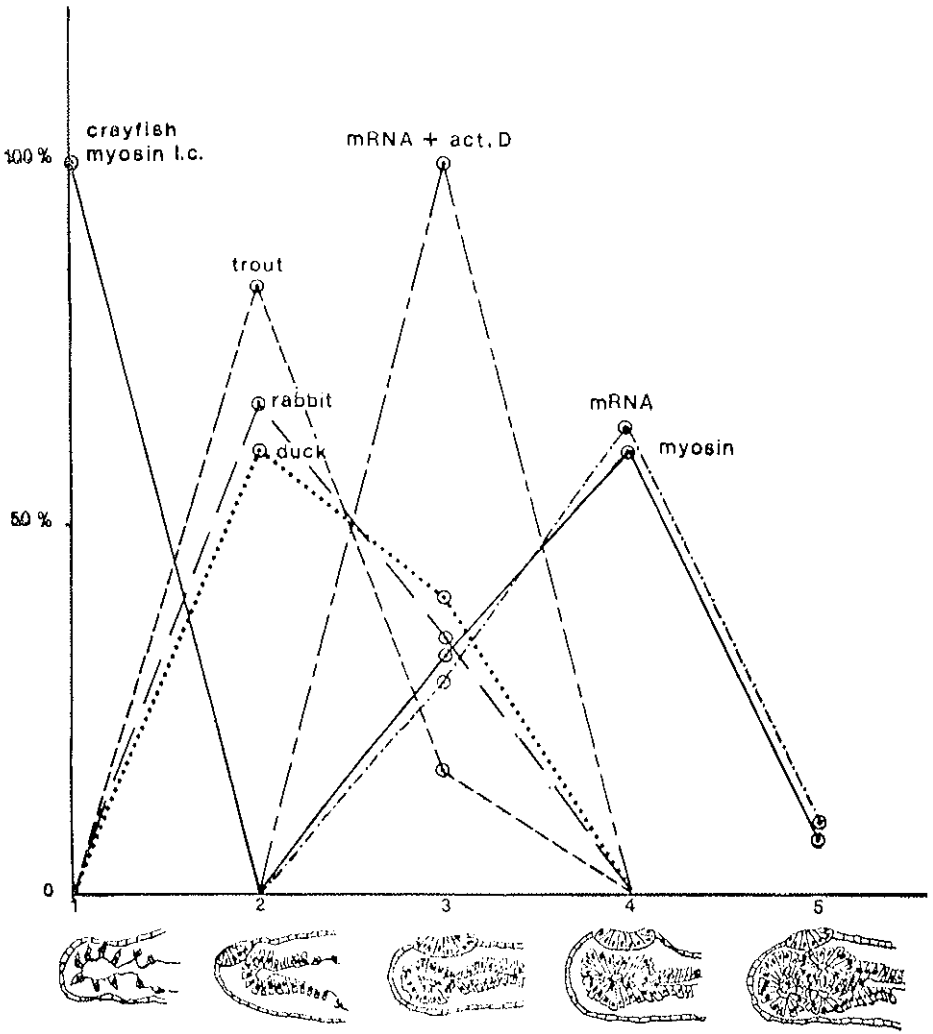

FIG. 2. Percentages of different types of somites induced by 26S mRNA extracted from various animals and by chick myosin.

Cbick myosin denatured by NaSCN did not induce somites. The results were also negative after culture with myosin extracted from a different species such as rabbit, or with chick actin, whether in globular (G-actin) or polymerized form (F-actin). It is probable that rabbit myosin does not have any action because it cannot penetrate the cells of a different zoological species.

Notochord and the ventral part of the neural tube are necessary for the further development of the somites [17]. The factor that comes from these rudiments was not present in our explants. Consequently, within 24 hours the somites became small vesicles built of dedifferentiated cells.

Ebert [7] discovered that heart myosin is present in chick embryos at the primitive streak stage. We have been able to demonstrate myosin in the chick embryo at tbe primitive stage (stage four of Hamburger and Hamilton [9]) both by Ouchterlony immunodiffusion and by gel electrophoresis. These methods have also enabled us to demonstrate the presence of myosin heavy chain.

When the somites were induced by treatment with 26S mRNA or with myosin, the ectoderm overlaying the induced somites was in many cases thickened, with columnar cells and the cell arrangement of a typical early neural plate (Fig. 1, 2). This did not appear in control embryos and it was never present in treated embryos without somites. In some cases, a neural plate did not appear even in the presence of somite induction. No neural plate was observed when the ectoderm was at a critical distance from the induced somite. We conclude that the neural plate is induced by the somite, as Holtfreter also observed in Urodela embryos [11]. This conclusion is corroborated by the observation that when the somites were not well developed, as happened with nonspecific inductors, the neural plate was also less developed.

From the experiments described here, we can conclude that mRNA induces the formation of somites in PNP transected 0.6 mm posterior to Hensen's node. The use of PNP as experimental material was introduced by Niu and Deshpande and they obtained contracting heart [16]. Both Chauhan and Rao [3] and Deshpande, Niu and Niu [6] reported the formation of some neuroid tissue and/or notochord, in low frequency (3-6%), in the controls. We never observed any differentiation in our controls.

From our experiments with myosin mRNA plus transcription or translation inhibitors, we conclude that myosin mRNA is translated to produce myosin and the myosin itself promotes the differentiation of the mesodermic cells, which have the characteristic form of myoblasts when examined by electron microscopy. These myoblasts aggregate to form somite masses. The ability of the cells to take up and to correctly translate exogenously-supplied mRNA had already been demonstrated in a critical review by Mroczkowski *et al.* of myoblast culture data [13]. Somite induction in the presence of puromycin, in our experiments, seems to

indicate that translation of mRNA also occurs in our explants. On the other hand we never obtained induction of somites by ribonuclease-digested mRNA and in only one explant by heat-denaturated mRNA.

The inductor is mRNA: even 26S mRNA treated with trypsin (10 μg/ml at 37 °C, reaction stopped after 90 minutes with 10 μg/ml soybean trypsin inhibitor) induces somites (Table I). Tbe mRNAs from heterospecific sources also have an inductive effect, as we have shown for duck, rabbit, rat, trout and, up to a certain point, for crayfish. The translational possibilities of a heterospecific messenger in *Xenopus* oocytes are well known (Gurdon *et al.* [8] for globin mRNA of rabbit; Woodland, for bistone mRNA from sea urchins [21]; see also Ranzi [18]). Now we have observed that 26S mRNAs from different species (rabbit, rat, trout, duck) induce some transformation of chick mesenchyme cells, up to a certain degree of somite formation, and that the mesenchyme cell shape can be changed by 26S mRNA of crayfish (Fig. 2). The induction obtained with dilute 26S messenger suggests that induction with a homospecific messenger is an all-or-nothing process and that the results obtained with heterospecific messenger are different because the messenger is different.

A second step in our research was the culture of PNPs directly with the translation product, myosin. When the myosin was homospecific, the results were identical with those obtained after myosin mRNA treatment.

These results confirmed that the translation of mRNA into myosin is an important step in the induction process and that myosin itself is able to arrange mesodermal cells into somite shape. No difference was observed between somites induced by entire myosin or by myosin heavy chain alone. Induction in this case takes place even in the presence of actinomycin D or puromycin and this indicates that myosin itself is the inductor for somites.

Heterospecific myosin (rat and rabbit) failed to induce somites: one explanation could be that the antigenic properties of the plasma membranes do not allow rat or rabbit myosin to penetrate the cells. When mRNA is translated inside the cells, the myosin produced is inside the membrane and could act to modify the surface components and promote the aggregation of the cells (Fig. 1).

The first step observed in induction is a change in the shape of the cells. This appears to be obtained with 26S mRNA of all the origins tested. The second, more species-specific step, is the aggregation of the cells into a somite.

The presence in chick blastoderm at the primitive streak stage of myosin, with its heavy chain, suggests that the pathway of induction in normal development is: DNA transcription → 26S mRNA → myosin heavy chain → somite.

26S mRNA in Amphibian embryo

Another research series carried out in this field by De Bernardi [4] led to the recognition of the presence of 26S myosin mRNA in the early stages of development of *Xenopus laevis* embryos. This was by means of hybridization of the polyadenylated segment with ^{3}H-poly (U). Myosin mRNA can be shown only in the "dorsal" (ectomesodermal) region of the embryo from the gastrula stage onwards. Treatment with actinomycin D indicated that this mRNA is stable and had been transcripted long before the translation moment (De Bernardi and Bolzern [5]).

These data may be linked with the results obtained by Ranzi and Citterio [19], who showed the presence of myosin in *Rana* embryos at late gastrula stage by using an immunological approach. Thus it may be

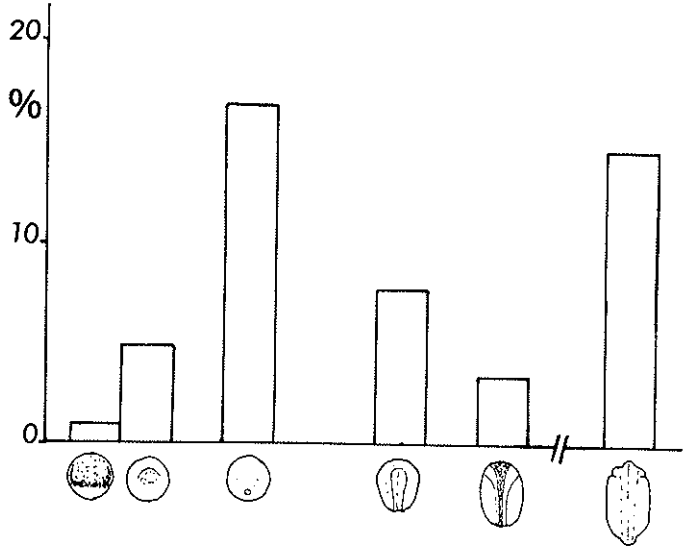

FIG. 3. Percentage of ^{3}H-poly(U) hybridized in 26S mRNA compared with the ^{3}H-poly(U) hybridized in total polyadenylated mRNA in each stage.

concluded that in amphibian embryo, too, at a stage comparable to the primitive streak of chick embryo, i.e. when the somites have to be induced, the myosin is present, and myosin mRNA is then translated in the normal embryo. If we compare the quantity of labelled poly (U) hybridized in 26S RNA to the total poly (U) hybridized in total RNA in each stage, we can observe two peaks of concentration of 26S mRNA (Fig. 3). The second of these arises at stage 22 of Nieuwkoop and Faber [15], shortly before the beginning of differentiation of muscular cells from myotomes, when a great quantity of myosin mRNA is translated into muscular myosin in the myotubes.

The first maximum is already observed at stage 12.5, and we wonder what use this active transcription of 26S mRNA is and, consequently, what is the use of myosin at this stage. If we remember both the experiments of the induction of somites in chick embryos and the fact that the somite mesoderm begins to thicken at stage 13.5 (1.5 - 2 hours later at 18-20 °C), we can suggest the hypothesis that the myosin, or better an increased quantity of the myosin inside the cells, can transform the mesodermic cells into somitic cells in Amphibian embryo too, as experimentally shown in chick embryo.

Further experiments were carried out to investigate the action of 16S mRNA coding for tubulin. This mRNA was extracted from chick embryo brain. Adding this 16S mRNA to the albumen medium of the culture, the cell shape of PNP changes. The mesenchime cells are elongated and packed like a cylindrical epithelium. No coelomatic or myocelic cavities are present. The ectoderm epithelium is also thickened and the blood island elements are packed in compact masses.

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THE ETHICAL, SCIENTIFIC AND MEDICAL IMPLICATIONS OF HUMAN CONCEPTION IN VITRO

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It is a great pleasure to respond to the invitation to speak on human conception *in vitro* to the Vatican Academy of Sciences, and so contribute to its long history of ethical debates on issues influencing society.

During the course of our work, we have to come to terms with fundamental concepts such as the rigbt of patients to the results of research, the duties of scientists and doctors, church and state in framing society's viewpoint of rapidly-expanding research and the rights of the embryo and fetus at various stages of gestation. The introduction of method of fertilizing human oocytes *in vitro*, and to grow embryos in culture have thrust many of these issues into the forefront of ethical debate. I welcome the chance to give a brief overview on the ethical, clinical, and scientific aspects of work on human conception *in vitro* to such a distinguished audience.

1. THE SCIENTIFIC, MEDICAL AND ETHICAL HISTORY OF IN VITRO FERTILIZATION

The early years

The possibility of human conception *in vitro* first became tangible in 1965, wben the stages of meiosis in human oocytes maturing *in vitro* were classified. This observation formulated the chromosomal basis of matura-

tion and fertilization in human eggs, established a supply of oocytes ready for fertilization, and indicated the precise time to collect ripening eggs directly from the ovary (Edwards, 1965a,b) (Table 1). Before 1965, sporadic studies into oocyte maturation, ovulation and fertilization had been made, but were somewhat misleading. The conclusions of Pincus and Enzmann, in 1935, on the maturation of rabbit oocytes was correct, but their identification of a similar period of 12 hours for the maturation of human oocytes was not. Reports that human eggs had been fertilized and cleaved *in vitro*, e.g. by Menkin and Rock (1948) and Shettles (1955) were almost certainly erroneous, since there was no evidence of proper oocyte maturation, the entry of spermatozoa into oocytes, pronucleus formation or syngamy, and it is well known that occasional oocytes will cleave *in vitro* as a result of parthenogenesis.

After 1965, a succession of steps into human embryology were virtually predictable. Almost all of these studies were carried out in our own laboratory: fertilization and the growth of embryos through cleavage into blastocysts (Fig. 1) (Edwards *et al.*, 1969, 1970; Steptoe *et al.*, 1971), the first human pregnancy following the replacement of an embryo, which was an ectopic (Steptoe and Edwards, 1976), and finally the birth of Louise Brown and two other babies (Edwards and Steptoe, 1979; Edwards *et al.*, 1980; Steptoe and Edwards, 1979; Steptoe *et al.*, 1980). During this period, sporadic contributions came from other laboratories, e.g. on the ultrastructure of human fertilization (Soupart and Strong, 1974, 1975), the reported growth of a human blastocyst (Shettles, 1971), and a single report was published of an embryo replacement into a woman (De Kretzer *et al.*, 1973). But there were no detailed back-up studies to support these isolated contributions, and no convincing evidence of an ongoing research and clinical programme to sustain them.

TAB. 1 - *Duration of meiosis in human oocytes* (Edwards, 1965a).

	Hours after release of oocytes from follicles
Germinal Vesicle	0 - 24
Diakinesis	25 - 28
Metaphase - I	36 - 43
Metaphase - II / Polar Body	36 - 43

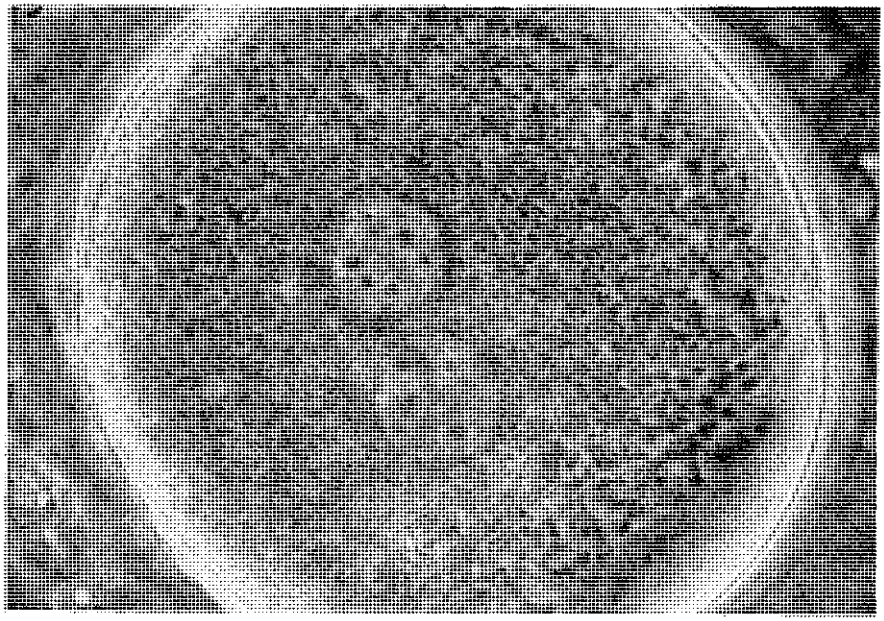

FIG. 1a. Human embryos *in vitro*. Two pronuclei in a fertilised egg.

Ethical debates on human conception *in vitro* have followed the scientific and clinical work, step by step. Initially, comments on the emerging ethical issues were first included amongst our scientific papers, in both specialist and more widely read non-professional journals (Edwards, 1966; Edwards and Fowler, 1970). As the work advanced rapidly, in 1969 and 1970, more contributions were made to the ethical issues, with papers devoted entirely to them (Edwards, 1969; Edwards and Sharpe, 1971). The potential importance of *in vitro* fertilization in alleviating human infertility and averting inherited disorders, the normality of embryonic growth *in vitro*, and the scientific and ethical consequences were discussed in detail. Close coordination between our ethical and scientific contributions continued through the period when the preliminary work was completed, and embryos were first replaced into the uterus, and through frustrating years when few clinical advances were made (Edwards and Steptoe, 1980). During this period we made numerous calls for investigations, committees, if necessary for legislation to clarify the status of embryos, patients and doctors, without any response. It is worthwhile

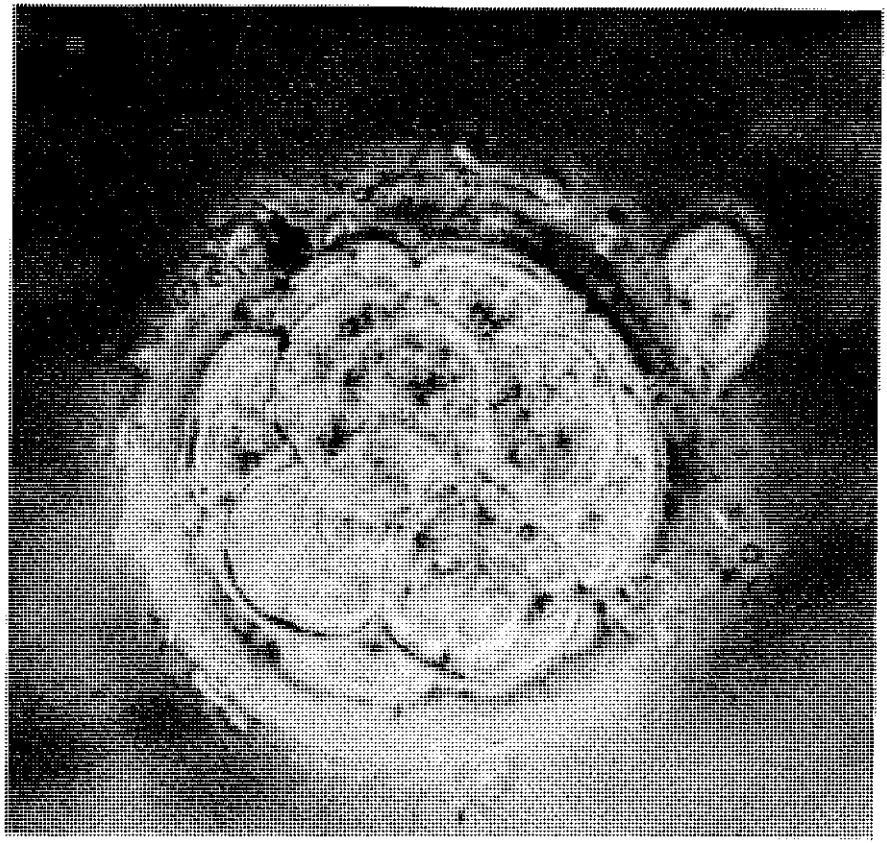

FIG. 1b. Human embryos *in vitro*. 16-32 cell embryo.

recording some of these statements, for example, from Edwards and Sharpe (1971):

" . . . we believe that it is important at this stage to elaborate the emerging issues in order to give time for defining and evolving social attitudes on which to base rules of conduct for scientists in society . . . to offer comments and suggestions on ways of helping society, science and law to live more safely, harmoniously and with greater confidence in keeping pace with advances in human embryology and other disciplines."

From the same paper:

"In human embryology as in other areas of swift scientific advance, the achievements of science catch unprepared society that lacks either ready-made

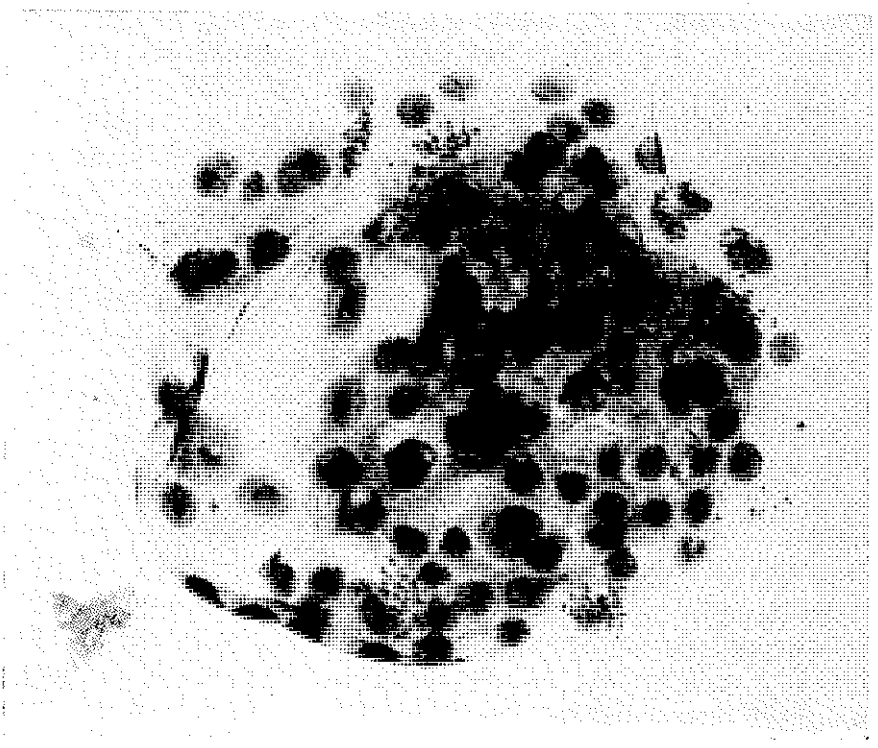

FIG. 1c. Human embryos *in vitro.* Stained human blastocyst, with numerous nuclei and chromosomes.

attitudes or institutional means of forming new ones . . . When scientists clearly foresee potential conflicts with existing rules of society arising from their work parodoxically both human progress and scientific freedom may hang on their activism in arenas generally regarded as social or political. Scientists may have to make disclosures of their work and its consequences that run against their immediate interests; they may have to stir up public opinion, even lobby for laws before legislatures, in the hope that the attitudes of society as evidenced in its laws will mature at a rate not too far behind the transition of scientific discovery into technological achievement."

Again, quotations from Edwards (1975):

"A significant number . . . of new moral and ethical issues . . . also arise from medical and technical advances in specific areas of research, which challenge established ideas or open new possibilities of choice . . . While the excessive publicity lavished on these advances is to be deplored, the attention they attract should be welcomed by those concerned in discussing and establishing ethical

and social standards or social awareness, for the resulting discussions often concern people in many different professions and with widely diverging views."

and again:

"I find great difficulty in accepting the notion that conception is divine as the reason or solace for withholding help for infertile couples yearning for their own children, or that the sacredness of life is sufficient reason to avoid aborting all fetuses with severe genetic defects. The relevant point in this connection has been made may times over; if God gave us our intelligence and ability, we were obviously meant to use them. A 'head-in-sand' policy of adhering to pre-conceived notions in the face of new ideas and approaches is a short-cut to irrelevancy."

Many ethical debates were held during these years, with papers written by scientists, theologians, lawyers and others. Some debates were somewhat fierce, e.g. the Kennedy Institute in Washington in 1971 where a scientist (Watson, 1971) was outspoken about the potential dangers of cloning and the possibility of the birth of abnormal babies, despite the evidence in animals showing the pre-implantation embryo is resistant to malformations. A theologian (Ramsey, 1972) felt that conception should remain as it is, divine and unchanging, and an "ethicist" felt that fertilization *in vitro* represented immoral experiments on the unborn, and feared the future consequences of the work, i.e. the "camel's nose" or "thin end of the wedge" argument (Kass, 1971). I shall return to these debates later in this review.

The first children conceived in vitro

The months and years preceding the birth of Louise Brown were, in retrospect, quite remarkable. While individual ethicists were making their charges, many scientists were openly sceptical of the results of work on the fertilization of human eggs *in vitro*, constantly pointing out how observations on animals offered little chance of success. Indeed, "experts" had expressed reservations about the validity of virtually every stage of our work on human conception. Even the Australian group working on *in vitro* fertilization became discouraged at this time, and literally abandoned their work just before Louise Brown was born. Perhaps this widespread scepticism was the cause of the lack of urgency about establishing enquiries into the ethics of human conception *in vitro*, the widespread doubts questioning whether any birth would occur at all.

Even after 1978, after Louise and other babies had been born, and during the period when we had no facilities for work, the doubts and problems remained. One baby was indeed born in Australia, after conception *in vitro*, but the clinicians responsible appeared to have massive problems in repeating the work (Lopata, 1980). The problems were largely of their own making, because their failure to employ an embryologist to solve their problems led to an increasing disillusion. Pregnancies and births were soon gained when such a scientist was appointed, and the work in Oldham could be confirmed (Trounson *et al.*, 1981; Wood *et al.*, 1981). During this period, we continued to write papers stressing the ethics of *in vitro* fertilization (Edwards, 1975, 1980), but a response arose in only one country as far as I am aware: the USA, where a report, prepared after a large consultative meeting, was apparently lying in a pigeon hole ever since! (Federal Register, 1979).

A rapid expansion of work on *in vitro* fertilization began in 1980. The Australian team separated into two groups, and one of them reported the establishment of several pregnancies (Wood *et al.*, 1981). Clinics were opened in the USA (Jones *et al.*, 1982), and in other countries (Edwards and Purdy, 1982). We resumed our work in Bourn Hall in October, 1980, establishing some pregnancies before Christmas, and others afterwards (Edwards, 1981; Edwards *et al.*, 1981). Despite this increased activity, the ethical debates did not begin in earnest until late in 1981 and in 1982, when world-wide discussion began — especially in those countries where clinics were opening — almost 15 years after human eggs were first fertilized *in vitro*! This curious and delayed over-reaction should ultimately provide some answers to the ethical debates in the U.K., and similar commissions are now judging or have judged the ethical issues in other countries (National Health and Medical Research Council, Australia, 1982).

Having sketched briefly tbe history of work on human conception *in vitro* until the present day, I should like now to describe the current clinical situation, and then turn to the ethics relating to children conceived *in vitro*.

2. The current clinical situation

I will describe current work on *in-vitro* fertilization very briefly, since numerous reports have appeared in recent months (Edwards, 1981; Ed-

wards and Fishel, 1982; Edwards and Purdy, 1982; Edwards *et al.*, 1981, 1983; Feichtinger *et al.*, 1982; Fishel and Edwards, 1982; Fishel *et al.*, 1983a,b; Johnston *et al.*, 1981; Jones *et al.*, 1982; Lopata, 1980, 1982, 1983; Steptoe *et al.*, 1983; Steptoe and Webster, 1982, 1983; Trounson, 1982a; Trounson *et al.*, 1981a,b; 1983; Wood *et al.*, 1981, 1982).

Most clinics now use the controlled ovulatory cycle, using clomiphene or HMG to induce follicle growth and HCG to induce ovulation (Edwards and Steptoe, 1975; Wood *et al.*, 1981; Jones *et al.*, 1982); we are still monitoring the natural cycle of some patients to compare with stimulated cycles (Edwards and Purdy, 1982) (Figure 2). Some of the difficulties associated with the use of stimulants have been recognised e.g. the temporary impairment by clomiphene of the response of oestrogen-sensitive tissues to oestrogens, such as the ovary or pituitary gland, and the risks of a short luteal phase after the use of HMG in cyclic women.

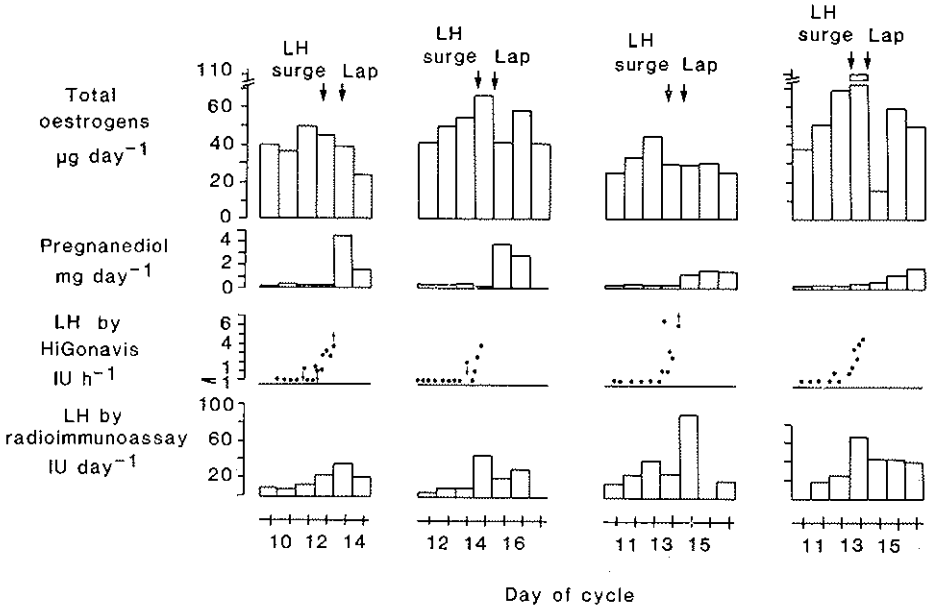

FIG. 2a. Endocrinological assessment of follicle growth and the beginning of ovulation in women. Monitoring the natural menstrual cycle in four patients. Twenty-four hour urinary oestrogens were used to monitor follicular growth, and urinary LH was assayed to detect the surge which indicated the onset of follicular maturation. Laparoscopy was usually performed 24-26 hours after the surge of LH. Rising levels of urinary pregnanediol showed that luteinization had occurred (Edwards *et al.*, 1980).

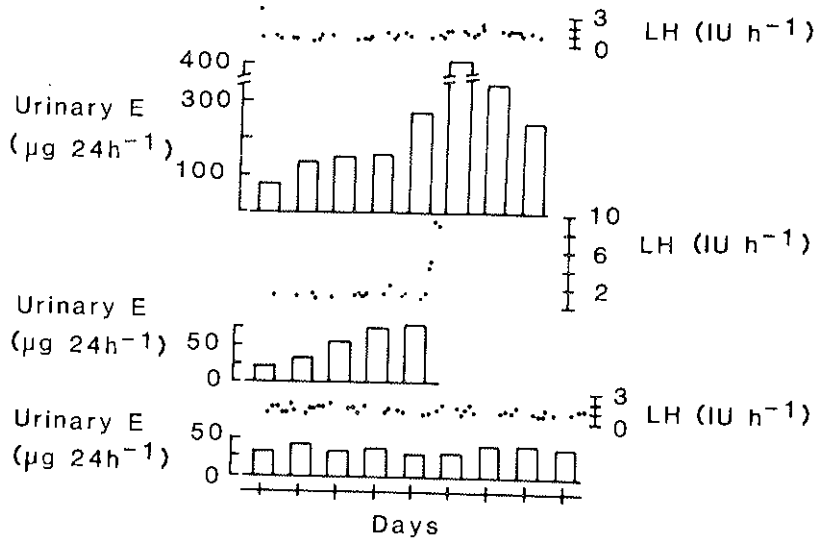

FIG. 2b. Endocrinological assessment of follicle growth and the beginning of ovulation in women. Response of three patients to clomiphene (Edwards, 1981). *Upper*: A considerable rise in levels of urinary oestrogens was not followed by an LH surge. The pituitary response to oestrogens had evidently been impaired, perhaps by the anti-oestrogenic action of clomiphene. The levels of urinary oestrogens declined as the follicles became atretic. *Middle*: A weak rise in urinary oestrogens was followed by an LH surge. *Lower*: No distinct rise in levels of urinary oestrogens, and no LH surge occurred in this patient.

Clomiphene can be combined with HMG to stimulate follicle growth, and is proving very effective.

Follicular growth is monitored by ultrasound and by measurements of plasma or urinary oestrogens. A combination of the two methods is preferable. A good follicular response, with satisfactory levels of oestrogens, is a benefit in establishing pregnancy.

Follicular and oocyte maturation is induced by an injection of HCG or by an endogenous surge of LH. Some clinics monitor the hourly levels of LH in patients using several assays spread at intervals throughout the day; others discharge patients if an endogenous LH surge intervenes before HCG can be given. With experience, the correct time to inject HCG can be simply assessed and laparoscopy for oocyte recovery is preferentially performed between 33 and 36 hours later. If the natural LH surge is monitored, the interval between its beginning in plasma and laparoscopy is also 33-36 hours, whereas if urinary LH is assayed the interval is 24-26

hours; ovulation occurs 37 hr. after an injection of HCG or the initial rise in plasma LH (Table 2).

One or more oocytes are aspirated in more than 90% of patients provided there are no complications such as adhesions or endometriosis (Table 3). Some clinics now aspirate follicles by sonography and avoid laparoscopy (Hamberger, 1982; Lenz and Lauritsen, 1982). There has been some debate about the medium used for flushing follicles, the gas phase to establish the pneumoperitoneum, and the possibility of impairing the luteal phase if too many granulosa cells are aspirated. Preovulatory oocytes can be identified quickly, and their stage of maturation assessed by the appearance of the surrounding cumulus cells. "Under-ripe" oocytes can be cultured *in vitro* to complete their maturation before fertilization *in vitro* (Table 4).

Various media can be used for fertilization *in vitro*, e.g. Earles saline, Ham's F10, Tyrodes medium, etc. Pyruvate and serum or serum albumin are added. High rates of fertilization are attained — 90% or more of the preovulatory oocytes if the spermatozoa are of "good" quality (Table 5).

TAB. 2 - *Timing of ovulation following the beginning of the LH surge in plasma or following on injection of HCG. The dotted line indicates the timing of ovulation, which appears to be slightly delayed after the use of clomiphene or HMG as compared with the natural cycle.*

Time (hr and min) after beginning of LH surge	Proportion of women who had ovulated [a]		Time (hr and min) after injection of HCG	Proportion of women who had ovulated [b] HMG or clomiphene stimulation
	Natural cycle	Clomiphene stimulation		
Before 34	0/15	0/7	29 - 31	1/59
34 - 34.55	0/20	2/15	32 - 35	2?/58
35 - 35.55	1/10	0/23	35 - 37	1/5
36 - 36.55	0/7	0/14		
37 - 37.55	5/5	3/12	37 - 38.30	3/7
38 - 38.55	1/1	2/4		
39 - 47	2/3	1/1	40 - 44	4/6

[a] Modified from Testart and Frydman, 1981.

[b] From Edwards and Steptoe, 1975.

TAB. 3 - *Assessment of laparoscopy.* 30 September, 1981 - 14 January, 1982 (Edwards *et al.*, 1982).

Patients	No.
Laparoscopies	166
Failed laparoscopies	21
Oocyte recovery	145 (87%)
No. of ruptured follicles	3 (2%)
Method failure	5 (3%)
Oocyte recovery without laparoscopic complications *	153 (95%)

* Complications include encephaloid ovaries (1), substantial adhesions (7), cystic response to stimulation (2), severe endometriosis (1), many tiny follicles (2).

Severe agglutination or clumping of spermatozoa and inflammation in the male tract reduce the chance of fertilization to 50% or less. Very few spermatozoa are needed for fertilization *in vitro*, and it can be achieved with oligospermic men even if the numbers of spermatozoa are very few indeed. One of our patients with only 600,000 spermatozoa altogether now has his own child, conceived *in vitro*. This observation raises an interesting ethical aspect of AID, because many couples now having to accept this method have a chance of obtaining their own child by *in-vitro* fertilization. I shall return to this point later in this article.

Fertilization occurs rapidly *in vitro*, and the vast majority of eggs are monospermic. Simple or complex media such as Earles, Ham's F10 or others, containing pyruvate and serum, sustain the growth of embryos to blastocysts. The vast majority of embryos cleave normally and can be replaced in their mother. Embryonic growth is regular, and almost all embryos reach the blastocyst stage of development and expand, although many do not "hatch" from the zona pellucida *in vitro*. Embryos replaced at all stages from the 1-cell to blastocysts have resulted in pregnancies. Delayed growth or cytoplasmic fragments are not incompatible with pregnancy although most success is obtained with normally-cleaving embryos. At present, no successful pregnancies has been achieved using embryos which had been frozen-stored, but this step should only be a matter of time.

In virtually all clinics, the embryos are replaced by means of fine catheters passed through the cervical canal. A small amount of medium

TAB. 4 - *Effects of the duration of oocyte culture in rates of fertilization and pregnancy.*

1. *Fertilization*

Period of oocyte incubation (hr) [a]	No and % (brackets) of oocytes fertilised [a]	Period of oocyte incubation (hr) [b]	No and % (brackets) of oocytes fertilised [b]
0	20/42 (48)	½ - 5	61/67 (91)
4-4½	3/4 (75)	5½ - 10	1/1 (—)
5-5½	34/37 (92)	11½ - 15	13/20 (65)
6-6½	28/36 (78)	15½ - 20	9/16 (56)
		> 20½	15/18 (83)

2. *Pregnancy*

Period of oocyte incubation (hr) [a]	No and % (brackets) of patients becoming pregnant after embryo replacement [a]	Period of oocyte incubation (hr) [c]	No and % (brackets) of patients becoming pregnant after embryo replacement [c]
0	0/8 (0)	½ - 2	4/18 (22)
4-4½	0/2 (0)	3 - 5	17/85 (20)
5-5½	4/22 (18)	6 - 8	16/48 (33)
6-6½	3/18 (17)	> 9	5/21 (24)

[a] Data from Trounson *et al.*, 1982.
[b] Data from Edwards *et al.*, 1983.
[c] Data from Fishel *et al.*, 1983b.

TAB. 5 - *Fertilization Rates* in vitro (Edwards *et al.*, 1982).

Spermatozoa	Total no. of patients	No. with ⩾ 1 fertilised oocyte	% Oocytes fertilised
Satisfactory	95	87 (92%)	85%
Head clumps, viscous seminal plasma	11	10 (90%)	95%
Some cells/debris	25	20 (80%)	70%
Many immotile sluggish/erratic	20	12 (60%)	50%
Massive clumping	10	5 (50%)	45%
Tail agglutination, many immotile	12	5 (41%)	41%
Massive cells/debris	7	2 (30%)	30%

is used, and anaesthetics are not required. Two embryos are replaced by most clinics, provided they are available, because the chance of pregnancy may be greater. Three may be replaced in a younger patient, and four are replaced very rarely. Four embryos are seldom available, and many doctors have doubts about the risks of a quadruple pregnancy. The greater chance of pregnancy after replacing two or more embryos might be due to better endocrine conditions during the luteal phase in patients with two or more follicles.

The chances of a clinical pregnancy after embryo replacement are approximately one-in-four in some clinics, but far less in others (Table 6). Some pregnancies are "biochemical", detected by a slight rise in levels of HCGβ, and a delay of two days or slightly longer in the return to menstruation. Similar short-lived pregnancies have also been detected after conception *in vivo*.

Many children have now been born after conception *in vitro*, perhaps more than 120, including 70 from Bourn Hall. Approximately 30% of the clinical pregnancies terminate in abortion, and chromosomal imbalance has been found in a few abortuses, just as in natural conception. One major defect has been reported in the children horn so far, involving the heart vessels, and this defect also arises in children conceived *in vivo* although rarely. There are reasonable grounds to conclude that there are no additional risks of malformations following *in-vitro* fertilization above those found after conception *in vivo*.

Fertilizing human eggs *in vitro* raises many ethical issues. Some of them concern the children conceived *in vitro*; others arise over the use of human embryos for research purposes. Before turning to these issues, it is necessary to outline the background to ethical standards and decision-making in our society, in order to clarify the roles of individuals, professional organizations and the law in establishing standards that are widely accepted.

3. Ethics today

Debates on ethical issues are a commonplace today, on television, in newspapers, journals, and numerous other forms of communication. Many people, professionals and others, give their opinion on the numerous complex issues which arise in modern society, a situation obviously very different from earlier times when standards were set by church or state. Some ethical issues have been discussed over centuries, including several

TAB. 6a - *Overall success rates using the natural menstrual cycle* (Edwards *et al.*, 1982).

Patients	1.1.81 - 14.1.82	1.10.81 - 14.1.82
Laparoscopies	382	35
With > 1 oocyte	312	29
With > 1 fertilized	236	25
Replacements	232	24
Clinical pregnancies	37 (16%) *	6 (25%) *
Elevated HCGβ	5+	0

* % of replacements.

TAB. 6b - *Overall success rates with clomiphene and a natural LH surge* (Edwards *et al.*, 1982).

Patients	1.1.81 - 14.1.82	1.10.81 - 14.1.82
Laparoscopies	144	61
With > 1 oocyte	113	50
With > 1 fertilized	85	40
Replacements	83	40
Clinical pregnancies	18 (22%) *	9 (23%) *
Elevated HCGβ	1+	1+

* % of replacements.

TAB. 6c - *Overall success rates with clomiphene and HCG* (Edwards *et al.*, 1982).

Patients	1.1.81 - 14.1.82	1.10.81 - 14.1.82
Laparoscopies	123	70
With > 1 oocyte	113	66
With > 1 fertilized	99	62
Replacements	99	62
Clinical pregnancies	21 (21%) *	13 (21%) *
Elevated HCGβ	6+	1+

* % of replacements.

concerned with human reproduction. In those earlier times, ethical decisions were taken by a few and imposed on the majority by state or ecclesiastical law, sometimes exceedingly harshly. Since Victorian times, the "received" ethic of the church has gradually been eroded, and modified by the adoption of a widespread pragmatism based on the impact of new scientific, medical and industrial advances. Papal pronouncements, for example, do not carry the same weight as before, even among Catholics.

In a sense, debates on the fertilization of human eggs *in vitro* are a continuation of earlier ethical debates on contraception and abortion. Conception is perhaps the last area of human reproductive life to be brought into the province of science and medicine, and debates on it will presumably reflect the viewpoints expressed on other aspects of human reproduction. There was not much to debate when conception could occur only *in vivo*, deep within the oviduct, out of reach of both scientific and medical care. The situation was simply accepted as established and unchanging: a new baby was born a result of a random union between spermatozoon and egg, for better or worse. A sharp reaction was perhaps to be expected once it became clear that human eggs could be fertilized *in vitro*, and human embryos grown for several days in culture before being replaced in the mother. Indeed, a sharp reaction is to be welcomed, because the conception of its children must be an important aspect of any society, and it would be far more worrying if there were no reaction at all! A frontier to medicine and ethics had moved, as the pace of the scientific work, painfully slow to the scientist but probably very fast to the onlooker, stimulated a new field of ethical debate.

Initially, debates on the ethics of fertilization *in vitro* were left largely to individuals in various professions; governments and other organizations entered the fray very late in the day, and only after many children conceived *in vitro* had been born. This deficiency has now been more than rectified, with six or more commissions currently investigating *in vitro* fertilization in the United Kingdom alone!

What are the foundations of today's ethics? Some cynics contend when the basis is simple: what can be done will be done by someone, somewhere. This is a great over-simplification of the situation. There is no doubt that the decline of established authority in ethics could open the way for individual scientists or doctors to take their own individual decisions on what is right or otherwise. In practice, there are many constraints to such an unfettered liberty. Even if the fount of ethical authority has changed, the fundamentals remain as they were, based on standards learned

and practised in society by its individual members, and these values are based on Greek and Judaean-Christian beliefs just as earlier. The principles remain as relevant as before, even though practised by large numbers of people as opposed to the few theologians and moral philosophers of earlier times.

Taking ethical decisions has become more complex in one respect. Church and state have discussed abortion, infanticide, contraception, sexuality and other issues for centuries, and have established a firm set of principles and knowledge. Even so, there has been a sharp division of opinion between them and lay members of society on these topics. It is much more difficult to find answers in the Bible or other traditional sources to the level ethical issues raised by scientific advance in todays society: conception *in vitro*, genetic engineering or nuclear weapons. These issues are novel, arising suddenly and being applied or extended quickly; they are also highly technical.

Since these issues are so novel, it is impossible to refer to an earlier debate or stance, and ethical decisions have to be based on the framework and belief in a society, pooling all available knowledge and attitudes in an attempt to reach a satisfactory moral conclusion. Participants in such debates must have a considerable knowledge of the facts, an awareness of the implications of the work, and a facility to place such new issues into the correct social framework of belief and acceptance. Many traditional purveyors of ethics do not satisfy these conditions, being untrained either in obtaining the knowledge or grasping its implications. Debate and decisions are therefore left in the care of professional men such as scientists, doctors, lawyers, journalists and others.

The basis of today's ethics appears to be undoubtedly utilitarian, tempered by the observance of values such as "do no harm". The State enters into the debate, and many complex issues become codified in law. Codes of behaviour are also established by convention (see Dunstan, 1978), and members of a particular society are expected to live by them.

There are great differences in the ethical duties imposed on scientists and doctors. Scientists are given considerable freedom to research, and their findings can have a considerable impact at many levels of society. Fortunately, the great majority accept and practice their responsibility to society, if only to retain their freedom of enquiry. I know of no form of supervision over their work other than those established by finance or facilities, at least in most western nations. Doctors are in a different situation, being held responsible to their own professional or specialist

organizations, which establish and enforce broad ethical standards. This explicit self-regulation within the profession, and the existence of a consultative machinery when new issues arise, are of great help to individual doctors. They also sustain widespread support by society, because such professional organizations are seen to reach decisions and present them as collective expert opinion, so carrying a great weight of authority. Perhaps this situation is changing nowadays, because decisions previously reserved to doctors, and including questions of life or death for the newborn or for the aged and infirm, are now being brought before a wider society.

I would now like to discuss the ethics of *in-vitro* fertilization, and consider first the issues raised by the conception of children *in vitro*, and then debate the issues arising over research on human embryos growing in culture.

4. The ethics of children conceived in vitro

The primary ethic of *in-vitro* fertilization is clear. Any child born after conception *in vitro* must be normal, and delivered into a loving family. This rule must apply to the alleviation of infertility, where embryos are grown undisturbed *in vitro*, and also to potential work such as averting the birth of handicapped children by typing embryos carrying deleterious genes. I have discussed various aspects of the current ethical situation elsewhere (Edwards, 1980, 1982, 1983a), and will give a condensed version here.

Treatment of the infertile

I would like to describe first some of the earlier debates on the ethics of *in-vitro* fertilization for the cure of infertility, which were mentioned in the introduction to this review. They reveal some interesting attitudes to conception *in vitro*. One early argument held that conception *in vitro* was not a "cure" of infertility, because the original derangement remained in the oviduct (Kass, 1971; Ramsey, 1972): the only cure was to repair the oviduct. Treating infertility by replacing embryos growing *in vitro* was held to be synonymous with treating a symptom, a "desire" to have children. Surprisingly, this view has been repeated recently (Daniel, 1982). This argument always seemed indefensible to me (Edwards, 1974), since

much of medicine traditionally treats symptoms and conditions, using by-pass operations or remedies such as false teeth and spectacles. Presumably, the use of pacemakers, or ectopic grafts of pancreatic tissue for the treatment of diabetes is merely satisfying the "desire" of those patients to live! It is doubtful just how many medical or surgical treatments actually "cure" a defect, i.e. leave it as it was before the disease, and *in-vitro* fertilization at least involves a partial cure, because the uterus is "cured" and can now accept a baby, even if the oviduct remains damaged. Such debates are red herrings, diverting attention from serious debate, substituting semantics for genuine issues.

Another scarcely credible argument of those years criticised the cure of infertility by fertilization *in vitro* because the future child could not consent to such "experimental" procedures (Ramsey, 1972). Once again, this semantic argument has also been revived recently (Walters and Singer, 1982). Its implications are considerable. The fetus is not consulted about every drug, pill, body activity etc. undertaken by a pregnant mother, yet every medicament offered during pregnancy, and perhaps even before conception, could be potentially damaging. The argument misunderstands the nature of medicine itself, because virtually all treatments — of adults, children and fetuses alike — carry some hint of the unknown, some degree of experimentation. Nor is the fetus asked about AID, a massive intrusion into legitimacy, yet the proposer of this argument apparently believed that A.I.D. is acceptable, providing the recipient simultaneously performs sexual intercourse with her husband (Ramsey, 1970)!

In earlier days, there was also a belief that "natural" conception was superior to "artificial" conception *in vitro*, because it was expressed in a loving relationship between the couple, and was in some way ordained divinely. "Artificial" conception would "dehumanise" mankind, according to some commentators (McCormick, 1972). This viewpoint was challenged by, amongst others, several theologians (Fletcher, 1971), pointing out that interference with conception for good ends is more acceptable than accepting the *status quo*. A glance at some of the unfortunate products of natural conception — triploid and trisomic children, genetic lesions, disordered growths in the mother's body, is sufficient to demand that some sort of intervention could be extremely beneficial. And loving relationships between couples are perhaps expressed in greater measure in response to the clinical necessities of *in-vitro* fertilization than during conception *in vivo*. There is no guarantee of a loving relationship in "natural" conception, merely a sexual relationship.

Earlier debates on the outlandish consequences of fertilization *in vitro*, on cloning an individual, establishing a master race, man-animal hybrids, have been presented elsewhere (Edwards, 1974, 1980). There were short-sighted and specious arguments that the infertile should not be treated because too many children are born already, or that adoption was a better alternative than *in-vitro* fertilization. Despite these and other debates on the ethics of fertilization *in vitro*, no law and virtually no standards were established in those early days, except for one Act in the U.K., the Congenital Disabilities (Civil Liabilities) Act, which was not designed in any way to deal with human conception *in vitro*.

Alleviation of infertility: current debates

There has been one major change in the situation in recent years. Many babies conceived *in vitro* have now been born to infertile couples, and many more are on the way. Numerous clinics are opening around the world. Ethical Commissions have been established in six or more countries, and their recommendations should provide a fascinating set of documents to compare one with another. The massive increase in the clinical application of work on fertilization *in vitro* has apparently stimulated ethical arguments of a more practical and relevant nature, to deal with particular situations stemming from its application.

There can be no objections to human conception *in vitro* where, an infertile couple desire their own child. Extensive research in animals and man has revealed the safety of the method for parents and children, and full, frequent and open discussions have been held amongst all sections of the community. Embryonic growth *in vitro* is normal, and the embryos are resistant to malformation, e.g. they tolerate division into halves or even quarters, the removal of a pronucleus, and exposure to various drugs or chemicals. There is no evidence of an increased chance of anomalies in the children although careful follow-up studies are needed. The size of the problem is considerable: potential patients include those with tubal disorders, oligospermia, idiopathic infertility, immunological infertility and others — perhaps more than one half of all infertile couples.

Many of the rights of patients and doctors are similar to those associated with other forms of medicine, but some novel situations do arise. The use of superovulation treatments can result in several eggs being fertilized *in vitro*. A request from a patient for four or five embryos may be unacceptable to the embryologist or doctor, who wish to avoid the complications

of multipregnancy. Yet the embryos presumably belong to the parents, who must have a major claim to the number replaced. Careful counselling of the patients is essential, but so too is a clear understanding of the nature of the legal "ownership" of an embryo by the parents, i.e. if it is merely their "property" or "possession", or if it is given the status of a fetus (Revillard, 1983).

Similar differences of opinion between scientists, doctors and patients might involve embryos which are cleaving erratically or slowly. The embryologist has a unique responsibility to patients. He must ensure the correct parentage of an embryo, which demands strict discipline in identifying the parents, the gametes and the embryo. He must also ensure the conditions of growth are optimal, and attempt to identify any anomalous development. The resolution of such issues is perhaps best left to local ethical committees, who can have a responsible role in such matters.

There are some ethical challenges in *in-vitro* fertilization. A.I.D. is presently offered to couples where the husband has severe oligospermia, but fertilization *in vitro* can be successful even with very few spermatozoa. Doctors treating oligospermia now face an ethical decision: to use A.I.D., which is undoubtedly easier, but where the baby is genetically unrelated to the husband, or conception *in vitro*. The importance of paternity within a marriage is being debated.

Prenatal adoption of embryos, and surrogate mothers

It has been possible to donate oocytes or embryos to recipients since 1970, when the first blastocysts were grown *in vitro*. Couples in which one, or both partners have problems in gametogenesis or the liberation of gametes, and who otherwise have no chance of pregnancy, could thereby have their own family by "prenatal adoption" of an oocyte or embryo. Should these steps be taken? The children will be related genetically to only one or neither of the infertile couple. Nevertheless, similar familial and ethical issues arise after A.I.D., adoption, and with step-mothers and step-fathers; oocyte or embryo donation would raise no problems other than those already faced by many adopting or fostering couples. Indeed some of the ethical problems with oocyte or embryo donation may be less than with adoption because the recipients must gestate the embryos and all the normal forms of biological identity, and the psychological and emotional interactions between parents and child can be fulfilled during pregnancy and delivery.

Surrogate mothers who carry a baby for a couple who do not wish to, or cannot gestate a baby, raise different issues. A surrogate could gestate a transferred embryo for another couple, and would have no genetic relationship with the baby. Surrogate mothers have been known to accept A.I.D., with the intention of giving the newborn child to the donor, a situation more extreme than embryo transfer, because the surrogate surrenders her own genetic child at birth.

Surrogate motherhood by embryo transfer is not a step to be undertaken lightly. The surrogate is transitory, unrelated, a temporary incubator, without medical reason for her treatment. She is exposed to the dangers of pregnancy and delivery. The fetus is exposed to her manner of life, and could be damaged if she smokes heavily or takes drugs. I understand she has a legal right in some countries to change her mind, abort the child, or claim it at birth. Problems could arise of a legal, familial, psychological and emotional nature for all concerned, including the doctors. Surrogate mothers should not be introduced into programmes of *in-vitro* fertilization until careful analyses have been made of these complex aspects of the treatment.

Dividing, typing and modifying embryos before replacement

A potential ethical issue concerns the birth of twins or triplets. At present, several sets of non-identical twins have been born through the replacement of two or more embryos. Identical twins could also be born in pregnancies of *in-vitro* fertilization. Theoretically, a human embryo could be divided into two, to produce identical twins, and this procedure could help the parents considerably because replacing two or more embryos apparently gives a higher incidence of implantation than replacing a singleton. There should be no ethical objection to identical twins, because enough occur naturally. But how about quadruplets? One limit would be the willingness of the doctor to initiate a quandruple pregnancy, with its attendant risks of abortion and maternal morbidity. Again theoretically, two of the embryos could be frozen-stored, and replaced later in the mother, to produce a second set of identical twins which were identical to the first set. Some sort of guide seems to be needed here, perhaps embodied in legislation, to decide on how far such treatments should be carried, because they are already practised in veterinary medicine.

There is another compelling reason for dividing embryos into two. One half could be examined to type the other, especially in families known

to be carrying inherited defects. The excision of pieces of trophoblast might provide an alternative method of typing embryos. Embryos carrying a defective gene could then be "aborted in vitro" at 5 days of gestation, and a non-defective embryo replaced in the mother. This approach would surely be preferable to the current method of aborting fetuses later in pregnancy; it will demand the use of gene probes effective in minute pieces of tissue, although one is apparently already available which could identify sex (Singh and Jones, 1982); I will discuss such work later in this article. It is perhaps preferable also to await higher rates of pregnancy after fertilization *in vitro* to make this approach to genetic screening more acceptable. The birth of handicapped children could be avoided, but an ethical decision will have to be made between the rights of a child to a normal birth against those of the use of a blastocyst growing *in vitro* for typing. I believe the rights of the child are incomparably greater than those of the blastocyst. The ethical situation changes as soon as abnormal conditions in embryos can be diagnosed because there will be a choice for medical intervention: the present choice between pregnancy or abortion will be extended to include scientific intervention to reduce the number of abnormal pregnancies occurring in the first place.

Should gene transfer be applied to embryos destined to grow to full term? Transferred DNA can be incorporated into the recipient's genome and transmitted to offspring, but there have been varied reports about the nature of the expression of the gene in the recipient. The transferred gene is not regulated by the usual developmental mechanisms because it is incorporated in the "wrong" place on the genome. Nevertheless it can function effectively as shown by recent work on the transfer of genes into fertilised eggs. Rat genes for growth hormone and a promoter were injected into the male pronucleus of mouse eggs. Body size in the offspring was increased considerably even though the extra growth hormone was apparently synthesized in the liver rather than the pituitary gland (Palmiter *et al.*, 1982). Such techniques might one day be applicable to human embryos, to avert the expression of a recessive mutant gene, although I believe it would be preferable to identify and discard the anomalous embryos. There will be major difficulties in technique, and, more importantly, in selecting the correct embryo for treatment. It also seems highly doubtful that such techniques will be used to modify the multifactorial quantitative characteristics of human beings such as intelligence, aptitude, ability, etc. for many years to come.

Freezing embryos before replacement in the uterus

Another ethical issue concerns the frozen-storage of embryos, which could be invaluable in establishing a pregnancy between a husband and wife by storing spare embryos for later replacement. This procedure must be ethically acceptable. Frozen embryos could also be replaced into a recipient mother, a simpler procedure than with fresh embryos, because there is no need to co-ordinate the menstrual cycle of donor and recipient. What is novel about freezing, what questions and doubts arise that have not already arisen with the use of fresh embryos? In my opinion, there are very few ethically novel situations: many of the issues are already raised by the use of fresh embryos. Freezing merely makes some approaches easier; a frozen embryo, when thawed, is like a fresh embryo.

Nevertheless, some bizarre situations could arise. An embryo stored for many years before replacement into its mother might be born after she had given birth to a sibling which was ovulated later. Would the date of fertilization be important in the question of inheritance, or the date of birth? Birthdate or time is used today, e.g. when the sequence of conception of two twins cannot be determined. Would this ruling still apply when the exact date of fertilization was known? The child, when adult, could perhaps claim an earlier conception as a right of inheritance. Here is an interesting conundrum for the legal profession!

The potential consequences arising through the death of one parent or of divorce before the frozen embryos were replaced will have to be considered, perhaps in law. Children could be born to a parent who had died, or to a parent who had divorced his partner and was unaware of the replacement of his embryo into a recipient. In a sense, many of these issues are already raised by the use of fresh embryos, where a parent might die between fertilization and replacement of the embryo or frozen spermatozoa may be used from a deceased busband. Such ethical problems are obviously magnified considerably by the frozen-storage of embryos, and it could prove necessary to arrange a time limit to storage and ensure that both parents and recipients give consent to the replacement of an embryo into any uterus.

My remarks on the ethics of frozen embryos have been concerned with their use in the alleviation of infertility. Their use in research raises different ethical problems. Yet, in a sense, the disposal of banks of frozen embryos for research work represents the culmination of a set of earlier decisions to freeze single embryos. The frozen embryo is virtually fresh

when thawed, and the sharpest ethical questions concern their subsequent use in research, just as if they were fresh embryos. I will now describe the potential value of human embryos in studies on early embryology, and then discuss the attendant ethical issues.

5. Research on human embryos growing in vitro

Some sharp ethical decisions relate to the study of human embryos *in vitro*. A great deal of fundamental knowledge on embryonic growth, the origin of human malformations and some potential clinical methods could be gained, but such research involves studying human embryos during their growth *in vitro*. The embryos would be grown without any intention of their being replaced in the mother, and such work would be unacceptable to many people who would object on ethical grounds to examining early stages of human development.

There is a need to describe the potential advantages of such work before discussing the ethical situation. I shall not deal with studies on human reproductive physiology which do not involve fertilizing eggs *in vitro* or culturing embryos, such as the growth of follicles, the timing of ovulation in women, and other topics. Such studies have been described in detail elsewhere (Edwards, 1974, 1980, 1982, 1983a,b); some of them will be of great value in understanding the rhythm method and other methods of contraception and disorders in reproduction. I will first describe the source of embryos which could be used for research, then provide some examples of the research topics, and finally discuss the underlying ethical situation.

The "spare" embryo

In many programmes of *in-vitro* fertilization, ovarian stimulants are used to produce three or four oocytes for fertilization, in attempts to give infertile couples the best chance of establishing a pregnancy. Spare embryos could be avoided by limiting the number of oocytes removed from the ovary, or the number inseminated but this decision could reduce the chance of establishing pregnancy. There is a mixed population of follicles growing after ovarian stimulation, which are in various stages of their maturation (Fowler *et al.*, 1978), and each of them must he aspirated to ensure that the ripest oocytes are harvested. All of the oocytes must be inseminated,

because only one or two may be fertilized even though more are harvested.

Inevitably, four or perhaps more embryos will be obtained from some patients. Only two or three, should be replaced, depending on the age of the mother. What should happen to the others? They could be frozen for later replacement into the mother but many clinics do not have this facility. Should all the embryos be replaced in the mother, even if there are five or six? There is no doubt that many gynaecologists would roundly condemn such a practice; indeed, the risks of multipregnancy could raise more serious ethical issues than the use of spare embryos in research (Dunstan, 1983). In effect, therefore, spare embryos will inevitably arise in clinics practising *in vitro* fertilization.

Working with spare embryos might be avoided if oocytes and spermatozoa could be placed in the uterus for fertilization and cleavage to occur *in situ.* Alternatively, the earliest stages of fertilization could be attained *in vitro*, and the 1-cell eggs then replaced in the mother's uterus for cleavage to occur. Pronucleate eggs of the rhesus monkey will develop in the uterus, implant, and develop normally (Marston *et al.*, 1977), and two human pregnancies have resulted during similar clinical studies (Craft *et al.*, 1982). In a sense, this approach is similar to that involved in Estes' operation on infertile patients with tubal occlusion, where the ovary is transferred to the uterine wall so that ovulation and fertilization can occur into the uterus. Pregnancies have very rarely, if ever, been reported in laboratory animals by transferring 1-cell eggs into the uterus, and Estes operation has been extremely unsuccessful in achieving human pregnancy (Adams, 1979). The human uterus might be more receptive to 1-cell embryos than in animals, because the long period between the LH surge and ovulation (37 hours), does give time for some degree of luteinization and for secretory changes to begin in the endometrium. The situation differs in mice and rabbits, where the corresponding interval is 12 hours approximately, so that luteinization would hardly have begun when the 1-cell eggs were replaced. Theoretically therefore, there is a possibility that the replacement of 1-cell human eggs is an alternative of replacing cleaving embryos, but in practice the incidence of pregnancy seems to be much lower, and the method is unlikely to be used widely.

The number of spare embryos in infertility clinics could be reduced by monitoring the natural cycle, and harvesting the single — or rarely two ripening oocytes just before ovulation. This approach was initially successful in Oldham (Edwards *et al.*, 1980; Steptoe *et al.*, 1980), and we are still testing this method in Bourn Hall for comparison with ovarian stimula-

tion (Edwards and Purdy, 1982; Edwards *et al.*, 1982; Fishel *et al.*, 1983a,b). All the products of fertilization *in vitro* can be replaced in the mother since only one or two embryos are obtained from virtually all patients, and this situation eases the ethical dilemma. At the present time, however, most clinics depend on ovarian stimulation to avoid the difficulties of working with the natural menstrual cycle, and with the intention of obtaining two or more embryos for replacement in order to raise the chance of pregnancy.

In some laboratories, preovulatory oocytes are being harvested from consenting women who are not infertile. Thees oocytes are being collected and fertilized *in vitro* with no intention of replacing the embryos into the uterus — they are to be used for research purposes only, for observational or experimental studies. These embryos are not the "spare" embryos arising in clinics treating infertility by conception *in vitro*, because they will be used in a manner similar to animal embryos used in research. In the U.K., the Medical Research Council has sanctioned this practice (Medical Research Council, 1982), and the study of embryos growing *in vitro* was also acceptable to an ethical committee in the USA (Federal Register, 1979).

There are, therefore, two major sources of embryos for research: the "additional" or "spare" embryos arising in infertility clinics, and those arising as a deliberate decision to fertilize eggs for purposes of research. A few embryos may also be recovered from the female reproductive tract of consenting patients after fertilization *in vivo*, and similar ethical situations will arise.

Improving methods to alleviate infertility

The successful introduction of *in-vitro* fertilization into a hospital involves an initial stage when methods must be established for successfully culturing embryos. There is no intention of replacing the embryo into the mother; they have to be grown and examined to establish standards of culture compatible with the introduction of a clinical programme for alleviating infertility. Even when many pregnancies have been established, the growth of some embryos must be monitored to ensure that conditions remain sufficiently reliable to fully sustain the growth of those embryos which will be replaced in the mother.

Most data on the growth of human embryos *in vitro* is empirical, gained by culturing embryos in various media, and adding serum albumin, pyruvate, and otber compounds. No knowledge exists on fundamentals

such as intermediary metabolism, or the synthesis of macromolecules in human embryos. There are obviously reservations about using human embryos for biochemical studies, but some minimal information is essential on these early stages of growth if the methods of culture are to be improved. Tests are needed on such variations in culture media as pH, osmotic pressure, the addition of increased amounts of proteins during later cleavage. The overall pattern of growth appears to be similar in most mammalian species. Nevertheless differences exist between animal species, e.g. between rat, mouse and monkey (Brinster, 1972), hence the "correct" conditions for human embryos cannot be gained by studying animal embryos. Even today, we do not know if replacements at 4½ or 5 days give better results than at 2-3 days, because most groups replace embryos in early cleavage.

The conditions of culture, and the equipment being used are constantly tested in Bourn Hall. Mouse embryos are used in preliminary tests; interestingly, hamster embryos do not grow in the media designed for human embryos, illustrating the considerable species variations in early embryology. Mouse embryos alone are not a sufficient test, and we use any human embryos not replaced in the mother to complete the test. These embryos should develop into blastocysts, and also expand slightly; few of them "hatch" from their *zona pellucida.* The development of these embryos to blastocysts is considered to show that the methods of embryo culture are satisfactory. The embryos can also be assessed for any indication of fragmentation, uneven cleavage, etc., for these conditions could be the cause of any impaired growth of embryos replaced in the uterus.

Origin of chromosomal anomalies in human embryos

More than one half of spontaneously aborted fetuses during the first trimester of human pregnancy have a chromosomal anomaly, including autosomal and sex chromosome monosomics and trisomics, triploids, tetraploids and mosaics. I shall discuss only the commonly-occurring anomalies in this article. Chromosomally imbalanced abortuses have been identified after *in-vitro* fertilization, including a triploid (Steptoe and Edwards, 1979; Steptoe *et al.*, 1980), and monosomic and trisomic fetuses (Frydman, 1982). Everything possible must be done to understand the causes of such debilitating conditions in fetuses and children, especially by those working on *in vitro* fertilization.

Trisomic fetuses are the most common chromosomal anomaly, account-

ing for more than one-half of the total. Trisomies have been identified for each chromosome except number one, and trisomy-16 is very common in fetuses, accounting for one-third of all trisomic abortions but so far not found in any liveborn children (Table 7) (Lauritsen, 1982). The varying incidence of the different trisomies may be due to their death at various stages of early gestation, some before tissue can be obtained for typing, and many types of imbalance might be found in pre-implantation embryos.

The incidence of trisomies rises from 1.6% to 25% in mothers between the ages of 35 and 49 (Tsuji and Nakano, 1978; Hassold *et al.*, 1980). The great majority evidently arise through nondisjunction in the first meiotic division of the oocyte, rarely in the second meiotic division or in the testis, and very infrequently during a cleavage division of the

TAB. 7 - *Types and frequencies of autosomal trisomies in spontaneous human abortions* (Lauritsen, 1982).

Chromosome group	Trisomy	No	%
A	1	—	—
	2	33	4.9
B	3	4	0.6
	4	17	2.5
C	5	1	0.2
	6	3	0.5
	7	27	4.0
	8	26	3.9
	9	18	2.7
	10	13	2.0
	11	2	0.3
	12	7	1.0
D	13	31	4.6
	14	31	4.6
	15	52	7.7
E	16	216	32.3
	17	4	0.6
	18	34	5.1
F	19	1	0.2
	20	18	2.7
G	21	63	9.4
	22	68	10.2
TOTAL		669	100.0

embryo (Hassold and Matsuyama, 1978; Niikawa *et al.*, 1977; Lauritsen and Friedrich, 1976; Kajii *et al.*, 1980; Mikkelsen *et al.*, 1980). Monosomy and trisomy for the sex chromosomes can also arise in spermatogenic cells (Polani, 1981).

Non disjunction could be due to the separation of homologous chromosomes during the long dictyotene period (desynapsis), resulting in the disordered segregation of chromosomes into the first polar body. The chances of non-disjunction might be greater when the dictyate stage is extended, so explaining the high incidence of trisomies in older mothers. Environmental conditions might increase the chance of non-disjunction, illustrated by the effect of low doses of X-rays on mouse oocytes in diakinesis (Tease, 1982).

Non-disjunction could equally arise through the failure of homologous chromosomes to pair during organogenesis in the fetal ovary (asynapsis), as oocytes are formed successively (Henderson and Edwards, 1968; Jagiello and Fang, 1979). A low number of chiasmata would then be expected in oocytes, and was detected in the oocytes of older mothers (Henderson and Edwards, 1968; Edwards, 1980). Oocytes with few chiasmata were evidently conserved until late in reproductive life, so offering another explanation for the high frequency of trisomy in fetuses of older mothers. It is important to decide if asynapsis or desynapsis is responsible for the origin of monosomies and trisomies. If asynapsis is involved, then the effect of X-rays or drugs, or any extra chance of nondisjunction arising during conception *in vitro* would be slight. If desynapsis is responsible, the agents causing non-disjunction could act at any stage from the formation of oocytes in the foetus until immediately before ovulation. Factors affecting follicular growth or ovulation, e.g. the ovarian stimulants used in the treatment of infertility, high levels of oestrogens, oocyte aspiration or ultrasonography could cause weakly-paired homologous chromosomes to separate, e.g., by causing the chiasmata to terminalise and so raise the chances of uneven chromosomal segregation into the first polar body. The increased risks of non-disjunction during *in-vitro* fertilization seem to be slight, but tests should be made on animals, or chromosomal analyses carried out on monosomic or trisomic human blastocysts arising after fertilization *in vitro*, to identify any trisomies and to determine the origin of the extra chromosome by banding techniques.

Between 5% and 8% of the trisomics which are aborted spontaneously are autosomal mosaics, which might represent a high proportion of the few trisomic fetuses surviving to birth. Mosaics were thought to arise

through non-disjunction during a cleavage division in the embryo, a mistake firmly corrected by Hassold (1982). They evidently arise from a uniform trisomic embryo which reverts to partial diploidy through the loss of the extra chromosome in some cells, and they are more frequent in older mothers. Trisomies involving small chromosomes might be able to revert to diploids because these chromosomes are more easily displaced from mitotic spindles than the large chromosomes (Ford and Lester, 1982). There is no information on chromosome shedding during cleavage *in vitro* or *in vivo*, which is an important reason for studying the chromosome complement of pre-implantation and post-implantation embryos; perhaps the presence of small nuclei in some embryos indicates the loss of these extra chromosomes.

Triploidy accounts for 20% of chromosmally unbalanced embryos and arises *in vivo* mostly through dispermy or fertilization involving a diploid spermatozoon (Jacobs *et al.*, 1978). Many active spermatozoa in the insemination droplet, or disorders in the first or second meiotic divisions *in vitro* could dispose to triploidy, and eggs with three pronuclei have been found after fertilization *in vitro*, especially in oocytes inseminated immediately or long after harvesting from the ovary (Trounson *et al.*, 1982). We find very few of these eggs (1-2%), and no triploid embryos growing *in vitro* (Edwards, 1980; Edwards *et al.*, 1981), although a triploid fetus aborted early in gestation after the replacement of an embryo (Steptoe *et al.*, 1980). Tetraploids account for 5% of chromosomally-imbalanced human abortuses, and presumably arise through a suppression of syngamy or the first cleavage division.

An unusual chromosomal imbalance associated with morphological degeneration in the placenta is the complete hydatidiform male, arising in embryos which are androgenetic in origin (Kajii and Ohama, 1977). Choriocarcinoma can be a sequel to the hydatidiform mole. Fertilization is evidently dispermic, or involves a diploid spermatozoon, and the pronucleate egg is initially triploid. The female pronucleus is apparently excluded at syngamy, and the embryo develops with a diploid set of paternal chromosomes (Fig. 3). Observations *in vitro* tend to confirm that androgenones arise through the premature growth of a male pronucleus (Edwards, 1981) or the extrusion of a female pronucleus in a tripronucleate egg (Trounson, 1982b). Some tripronucleate eggs could revert to normal diploids by the exclusion of a male pronucleus at syngamy (Fig. 3). Gynogenetic human fetuses have not been identified although they presumably exist: they may be very rare or develop normally and so escape detection. Partial hydatidi-

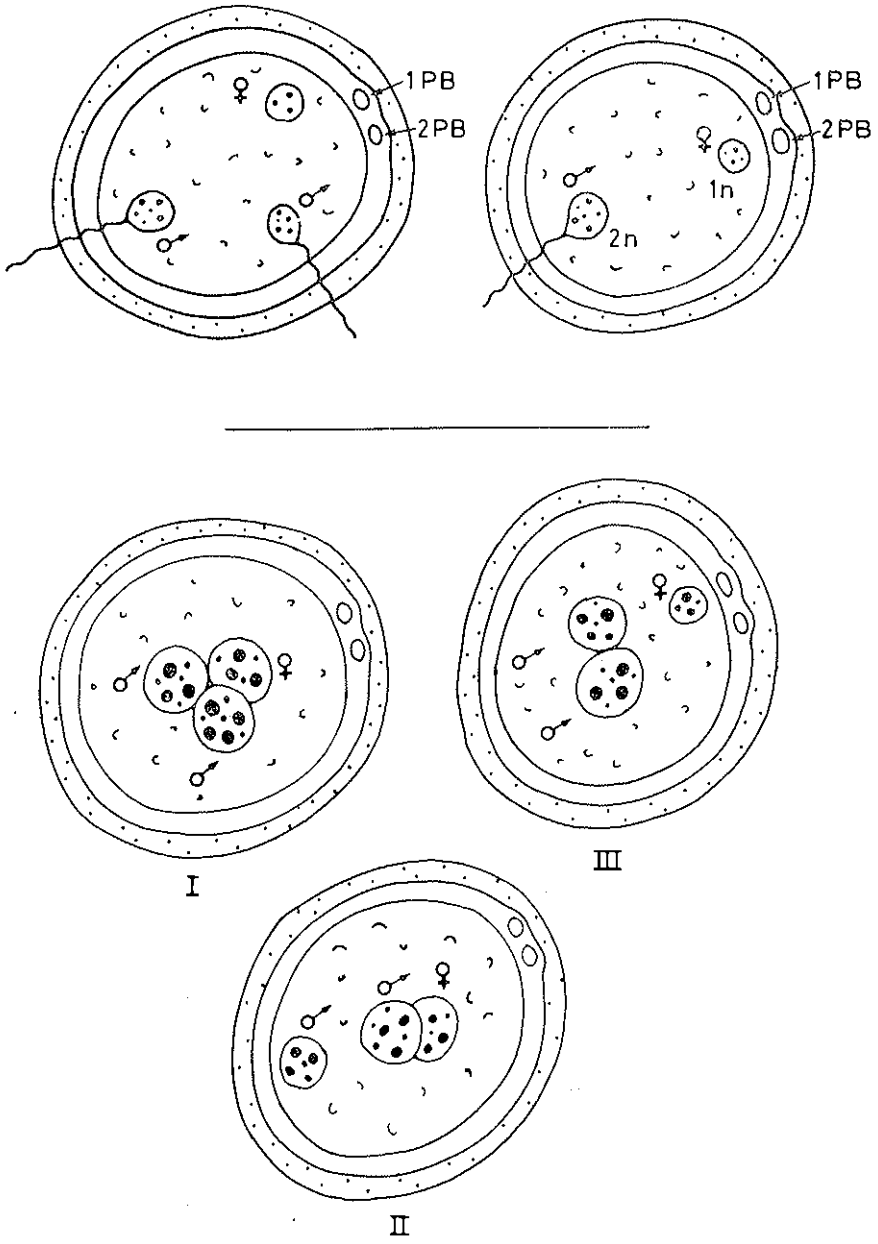

FIG. 3. Diagram to show the factors causing androgenesis, triploidy, and a potential reversion to diploidy at fertilization and syngamy in human eggs. The upper illustration shows the two postulated causes of diandry. Fertilization can occur through dispermy (left) or through fertilization by a diploid spermatozoon (right). A third mechanism may be involved, involving a premature DNA synthesis by the male pronucleus. The lower illustration shows three consequences of dispermy at syngamy. The three pronuclei may come together, and all enter syngamy, to result in a triploid embryo. Alternatively, a male pronucleus may be excluded, so that the embryo reverts to a normal diploid with one set of maternal and one set of paternal chromosomes. Thirdly, the female pronucleus may be excluded, and this leads to syngamy involving two male pronuclei and the establishment of an androgenetic embryo.

form changes are observed in some triploid fetuses, mainly those containing two paternal sets of chromosomes (Jacobs *et al.*, 1982).

Information gained on human chromosomal anomalies could avert the birth of afflicted children. We need to know the causes of non-disjunction, of polyspermy, about the regulation of syngamy, why some trisomic embryos revert to diploids, and why cleavage fails so that diploid embryos become tetraploid Considerable amounts of this knowledge could be gained through fertilization and embryonic growth *in vitro* by examining chromosomes in pre- and post-implantation embryos. Such knowledge is urgently needed.

Studies on pre-implantation embryos

It is impossible to review the numerous studies of considerable clinical value now possible on human pre-implantation growth. Virtually the whole of experimental embryology on animal embryos, plus some other studies, can now be applied to human embryos. Should studies on human embryos be carried out at all? Should they be restricted to those of more immediate clinical importance, bearing in mind that almost any piece of research on pre-implantation embryos could find some clinical justification (Edwards, 1983a,b)?

A glance at a very arbitrary list of some of the most recent publications on animal embryos shows the wealth of potential studies: the causes of parthenogenesis (Eppig, 1982), the separation of blastomeres to measure their developmental potential in the production of twins or quadruplets (Willadsen, 1981), the synthesis of macromolecules at various times after fertilization (Kidder and Pedersen, 1982), the expression of positional information and cell allocation in cleaving embryos (Johnson *et al.*, 1981; Johnson and Ziomek, 1982; Kimber *et al.*, 1982; Spindle, 1982), the content of viral particles in embryos (Huang and Calarco, 1981), the expression and functions of oncofoetal antigens on embryos (Edidin *et al.*, 1982), the immunological aspects of choriocarcinoma (Brewer *et al.*, 1978), and the secretion of glycoproteins and hormones by embryos (Fishel and Surani, 1980). Some studies have already been carried out on human embryos, e.g. the ultrastructural analysis of fertilized eggs (Soupart and Strong, 1974; Sathananthan and Trounson, 1982), cleaving embryos Edwards, 1980; Sundstrom *et al.*, 1981; Dvorak *et al.*, 1982) and newly-hatched blastocysts (Edwards, 1980; Mohr and Trounson, 1982).

Some topics are exciting scientifically, e.g. the role of glycoproteins

and glycosidases in so many aspects of reproduction and embryology. These include capacitation and sperm-egg recognition (Ahuja, 1982), and the patching of gonadotrophic and other hormones as they interact with membranes of target cells (Amsterdam *et al.*, 1980; Kalyan *et al.*, 1982). Some potential studies on fertilization are academic, e.g. how sperm motility or sperm enzymes help in traversing the *zona pellucida* (Saling, 1981; Schatten, 1982) and others more practical, e.g. the value of the "hamster" test in assessing male infertility or the separation of human X- and Y-spermatozoa (Miyake *et al.*, 1982; Beerninck and Ericsson, 1982; Daniell *et al.*, 1982).

Likewise, many studies on embryos are primarily scientific, although their clinical application could be close. These include the role of glycoproteins in implantation and embryonic differentiation (Chavez and Enders, 1982; Feizi, 1981; Knowles *et al.*, 1982; Schechter *et al.*, 1979; Zieske and Bernstein, 1982), the role of embryonic and histocompatibility antigens in cytodifferentiation as a code for cellular movement and differentiation (Hood *et al.*, 1977), or as an anchoring membranal component involved in differentiation (Ohno, 1977; see also Edidin *et al.*, 1982). Multispecific H-2 antigens expressed on mouse blastocysts might be "precursors" of haplotype-specific H-2 antigens on adult tissues (Conzad and Warner, 1981). Other fundamental studies include the identification of embryonic secretions, e.g. early pregnancy factor released from fertilized eggs (Cavanagh *et al.*, 1982; Smart *et al.*, 1981), the time of X-inactivation in human embryos by cytological methods, as in mouse embryos (Rastan, 1982), to discover how and why the paternal X chromosome is selectively inactivated in trophectoderm, extraembryonic ectoderm and primary endoderm (Takagi and Sasaki, 1975; Harper *et al.*, 1982), and several studies on implantation such as the role of prostaglandins in nidation (Hurst and McFarlane, 1982; Hyland *et al.*, 1982; Kennedy and Lukash, 1982), the possibility of delayed implantation in women, as shown by delayed rises in the secretion of HCG (Fig. 4) (Batzer *et al.*, 1981; Edwards, 1983a,b), and the role of decidual cells in synthesising prolactin and other hormones (Rosenberg *et al.*, 1980).

Some of these studies are obviously of more immediate relevance than others to the clinical application of *in-vitro* fertilization. Making identical twins, for example, could help in the treatment of infertility because twin transfers give higher rates of implantation than single transfers, as described earlier in this review. I have listed these studies in some detail to illustrate the wealth of research now possible on human

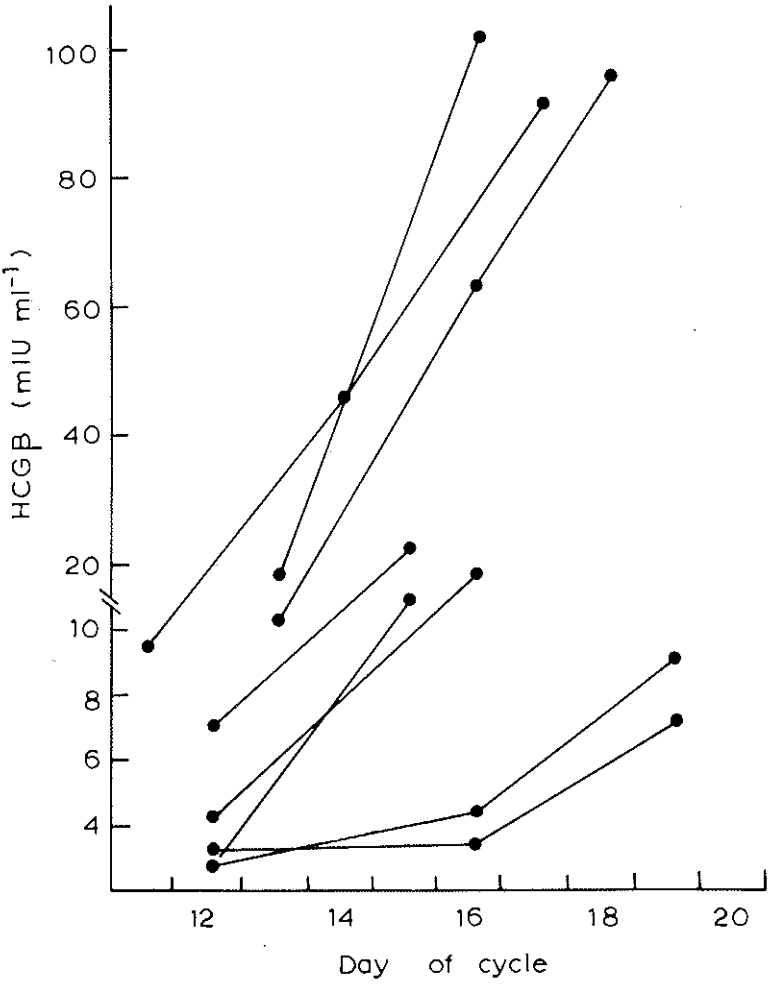

FIG. 4. Rising levels of urinary HCGβ in early pregnancy in patients in Bourn Hall. Notice that the rise of HCGβ was delayed in two patients, who have since given birth to a child (Fishel *et al.*, 1983).

embryos in their pre-implantation stages of growth. Each of these studies could provide valuable information on normal and abnormal growth gene action, cancer and other metabolic diseases. When contemplating such possibilities, I am tempted to ask if we can afford *not* to do these studies, because of their considerable implications in so many areas of medicine.

Typing embryos

One priority that should be accorded to research on human pre-implantation embryos is the prevention or alleviation of inherited birth defects. The identification of abnormal embryos and their "abortion *in vitro*" would be preferable to amniocentesis and abortion in the first or second trimester (Williamson *et al.*, 1981), or to attempts at repairing defects by genetic engineering (Edwards, 1982; Williamson, 1982) even though this rapidly-moving field of research has now culminated in the enhanced growth of mice following the transfer of the gene for growth hormone and a promotor into the male pronucleus (Palmiter *et al.*, 1982).

Excised pieces of trophoblast, or duplicate embryos prepared for the purpose could be used to identify genes or gene products in embryos. Sex could be identified by means of sex chromatin, sex chromosomes, the Y body or the H-Y antigen, and some enzyme deficiencies and chromosomal anomalies might also be detected. The most exciting prospects involve the use of DNA probes to identify specific genes, and the potential of this approach has been illustrated by studies on a probe for the sex-determining gene in mice (Singh and Jones, 1982; Hansmann, 1982). It is just possible that homozygous and heterozygous (carrier) embryos with deleterious genes might be identified using specific gene probes. There are many problems, especially in relation to the small amount of tissue available; perhaps the amount of tissue could be increased by the use of outgrowths from blastocysts, storing the replicate embryo at low temperatures during the typing procedure. Improvements are perhaps needed first in the rate of implantation after embryo replacement, as discussed earlier in this review.

Alternative methods for typing fetuses could come more quickly in other areas of medicine, e.g. during the second month of pregnancy using excised pieces of trophoblast or chorionic villi, a method evidently introduced in China and the U.S.S.R. to predict fetal sex by detecting sex chromatin (Department of Obstetrics and Gynaecology, Anchan; Kazy *et al.*, 1982). This method is now used in combination with restriction endonuclease analysis of fetal DNA to identify the various haemoglobinopathies, the fetuses being classified at much earlier ages than by the use of amniocentesis (Williamson *et al.*, 1981; Gosden *et al.*, 1982; Old *et al.*, 1982).

Post-implantation embryos and tissues in vitro

Human embryos are now being grown to blastocysts in many clinics and could develop through the stages of implantation and into the period

of cytodifferentiation and early morphogenesis *in vitro*. To the best of my knowledge, only one report has described the growth of a human embryo *in vitro* through these stages, until Stage 5a on day 9 (Edwards and Surani, 1978; Edwards, 1980). Should embryos be grown through these stages of development? In my view, such studies should be carried out because the advantages in science and medicine outweigh any objections to growing embryos to day 14 (Edwards, 1982, 1983a,b). An Ethical Committee in the U.S.A. suggested that the limit should be 14 days (Federal Register, 1979), and recently, the guidelines of the Medical Research Council in the U.K. also appeared to embrace this limit (Medical Research Council, 1982).

Is it feasible to grow human embryos to day 14? Animal embryos can be grown *in vitro* for limited periods of time through their post-implantation stages of growth, hut there are major difficulties between the blastocyst and headfold stages (New, 1978, 1983). Blastocysts of several species will hatch from the *zona pellucida in vitro*, but their growth then becomes disorganized, as they attach to the culture vessel and form monolayers or multilayers of differentiating cells. Rodent embryos grow best in medium such as CMRL after the correct preparation of serum (Hsu, 1980; Tam and Snow, 1980), and provided the inner cell mass is oriented correctly (Wu *et al.*, 1981). Some cleaving primate embryos flushed from the oviduct grow *in vitro* and develop a yolk sac and primitive streak (Hearn, 1982; Pope *et al.*, 1982). Rat embryos explanted at 9 or 10 days of gestation display their best growth during the 2-3 days between the head-fold and early limb-bud stages, comparable to human embryos aged 2-5 weeks, when development in vitro is virtually indistinguishable from growth in utero (New, 1983). The large yolk sac of rodent embryos might help *in vitro* by performing respiration and nutritional functions as in utero, whereas the small yolk sac of primate embryos might be less effective.

Culturing human embryos through their post-implantation stages of growth could yield much information on the factors involved in the initial stages of organogenesis normal and abnormal differentiation of organs, e.g. the growth of the neural plate and tube (Jacobson, 1978) and the haemopoietic system (Metcalfe, 1977). Haemopoietic tissue appears in stages 5 and 6, the primitive streak in stage 6, angiogenesis, the notochord and the neural groove in stage 7, i.e. by 2 weeks of age or slightly later (O'Rahilly, 1973). Other studies of considerable potential value include the effect of genes on organ formation, the causes of hydatidiform degeneration, the incidence and effects of various forms of chromosomal imbalance, the effect of teratogens or different nutrients on embryonic

growth, the expression of histocompatibility and oncofetal antigens — the list is almost endless Each of these studies promises its own special contribution to medicine, just as with pre-implantation embryos, and each will have to be judged as ethically acceptable or otherwise.

Embryonic cells and tissue and grafting into adults

Unique opportunities could arise from the study of stem cells of differentiating embryonic tissues. Stem cells cease dividing, or do not persist in adult organs such as brain, myocardium, pancreatic Langerhans cells and ovary, hence natural repair is limited. Grafting embryonic stem cells into these tissues after they were damaged might encourage their natural repair, a concept opening new forms of treatment of some fundamental disorders in children and adults. Relatively undifferentiated cells would be obtained from 5-day blastocysts and these might have a wide spectrum of differentiation (Cole *et al.*, 1966; Evans and Kaufman, 1981). Stem cells specific for particular tissues would be obtained from human embryos aged up to 14 days post-fertilization or a little later, and my discussion will be concerned with these cells since they might be more valuable for grafting into specific organs.

If the therapeutic opportunities are theoretically considerable, so are the scientific and clinical problems. Introducing stem cells to the brain, heart, pancreas, kidney, etc., could raise intractible difficulties. The grafted cells might not divide and differentiate normally after colonisation, although ectopic sites might be useful for some types of graft. Rejection could raise problems: some fetal tissues, e.g. pancreas, are rejected like adult tissue (Sutherland, 1980). Embryological methods are available to make an embryo resemble one parent, including the induction of parthenogenesis, androgenesis and gynogenesis, and perhaps even cloning. Grafts taken from such embryos would be accepted by the parent since there would be no genetic incompatibility between them (Edwards, 1980; 1982; 1983a,b).

Would embryonic stem cells colonise and repair tissues? In many tissues, the clonal development of a few stem cells results in the formation of secondary stem cells and in large numbers of differentiating cells, e.g. the testis (Huckins, 1971, Oakberg, 1971) gut epithelium, hemopoietic tissue (Burton *et al.*, 1982; see Kay, 1965). The division of stem cells appears to be tightly programmed in some tissues, occurring in succession to produce groups of clones in a regular sequence (Edwards, 1982, 1983b; Burton *et al.*, 1982). These concepts on stem cells and their clonal develop-

ment show how the introduction of a few of them into a deficient tissue might result in the repopulation of large areas of it.

This fanciful use of embryonic cells for grafting into adults is perhaps the ultimate application of *in-vitro* fertilization. Its potential has been indicated in some recent studies. Grafts of donor bone marrow are now widely used, e.g. in the treatment of chronic granulocytic leukaemia and some inherited diseases, e.g. after ablative chemoradiotherapy. The availability of tissue from an identical twin avoids graft rejection and the problems of finding matching donors (Storb *et al.*, 1977; Fefer *et al.*, 1982; Goldman *et al.*, 1982). Embryos could be made genetically similar to victims of these disorders, by the methods described above, and their stem cells extracted for grafting. Few stem cells will be available, they may not differentiate or divide and might become malignant in culture, and they could have a restricted developmental potential as compared with cells taken from adult bone marrow (Metcalfe, 1977). Androgenetic embryos might even develop characteristics of hydatidiform moles (Kajii and Ohama, 1977). Moreover, the need for such grafts may be less urgent now that good rates of survival are obtained in patients given allogeneic bone marrow, or after autotransplants of bone marrow cleared of malignant cells by means of monoclonal antibodies (e.g. Good, 1982; Buckman *et al.*, 1982).

Nevertheless, hemopoetic cells extracted from the yolk sac of mouse embryos increase many fold in culture (Dexter *et al.*, 1977, 1980; Moore and Dexter, 1978), and they will colonise bone marrow in lethally irradiated adult mice (Moore and Metcalfe, 1970). Fewer foetal than adult cells may be required for successful grafting (Buckley *et al.*, 1976; Rieger *et al.*, 1977; O'Reilly *et al.*, 1978), and syngeneic cells are simpler to use because rejection is avoided and they could colonise hosts rapidly and minimise post-transplantation immunodeficiency (O'Reilly *et al.*, 1978). Bone marrow stem cells from the yolk sac would be accepted by allogeneic hosts, because fully competent T lymphocytes are not yet differentiated: hemopoetic cells from the liver of older fetuses have been used for similar purposes, especially after the unwanted T lymphocytes are removed (Good, 1982). Grafts of fetal liver cells and thymus tissue have corrected severe immunodeficiencies in children, although graft versus host responses could occur even with tissue taken from fetuses aged as little as 5 weeks (O'Reilly *et al.*, 1978; Good, 1982).

Tissue grafting is already being applied to several other organs, e.g. pancreas (Sutherland, 1980) and brain. Grafts of brain tissue reverse

behavioural defects induced in rats by lesions in the striatal complex or septohippocampal region (Bjorklund *et al.*, 1980; Low *et al.*, 1982), and repair an inherited deficiency of LH-RH neurones in the hypothalamus of adult mice (Krieger *et al.*, 1982). Neurones growing from the graft apparently form synaptic connections in the recipient's brain although there is a possibility that host neurones might have innervated the graft (Low *et al.*, 1982). The brain accepts allogeneic tissue, and appears to be an immunologically privileged site (Bjorklund *et al.*, 1982). Human fetal liver cells can migrate to the liver of a recipient human abortus when injected into the capillaries of the placenta (Gustavii *et al.*, 1982).

I believe there is a case for analysing the properties of embryonic stem cells *in vitro*, and considering their use in grafting. The clinical rewards could be great, perhaps even wider than those discussed so far, because grafting could be combined with the transfer of genes into embryos in order to repair genetic defects. The expression of the transferred gene would then be restricted to the grafted tissue in the recipient. Are these ideas so fanciful that they will never be introduced? Perhaps, perhaps not. After all, test-tube babies seemed to be an impossibility only a few years ago.

6. Ethics of research on human embryos in vitro

There can be little doubt that research on human embryos growing *in vitro* for some days after fertilization could be of considerable benefit in several areas of medicine as described above. What are the ethical reservations about such work? Many people would perhaps accept the need for research, if embryos were not grown until they reached advanced foetal stages, and provided they were not replaced in the mother. Many societies have sanctioned the use of I.U.Ds and post-coital contraceptives which destroy embryos probably aged up to 7 or more days after fertilization, and have also accepted abortion in the second trimester as a means of controlling fertility. Why, then, is there any concern over using the tiny human embryos for research? This question begs a persuasive answer, and defines attitudes accepted in society at large, but it does not help to formulate a personal ethic to embryo research. There is no ethical justification in doing something because others are already doing it, nor in taking instructions from above. Individual scientists and doctors should surely frame and act on their own ethical standards about research on human

embryos, and their responsibility can hardly be delegated to committees or organisations which may have considerable ethical authority, but which merely define the limits of research.

Some people will be unable to accept the thought of research on human embryos growing *in vitro*. They believe that fertilization is the beginning of life, that the right to life is absolute from this moment. Their viewpoint is not generally accepted, in view of the widespread use of post-coital contraception and abortion. Nevertheless, their stance does provide a basis for debate, and a first need in formulating an ethical viewpoint is to decide if human embryos do have any "rights", especially those conferring an absolute prohibition on their use for research.

"Rights" of the embryo in vitro

What rights can be conferred on an embryo up to, for example, 14 days post-fertilization, still the size of a pinhead and hardly beginning to differentiate? This question raises difficult questions about when life begins and the rights and duty of scientists and doctors to study embryos. Who can grant permission to a scientist or anyone else to study an embryo which will not survive as the result of his work? Even parents may be unable to give such consent: they can give permission for beneficial procedures on their children, but the situation is very different with studies on embryos, where benefits accrue to others and certainly not to the embryo itself. Parents may have the right to "give away" their embryos as a gift for a recipient to carry to birth, but hardly to carry out research on an embryo which will not survive. Such consent could only be given if the embryo was a "possession" of the parents, rather than a life in its own right. A similar argument about research on mid-term foetuses was advanced by Tiefel (1976).

What, then, are the roles of individuals, professional bodies and research institutions in granting permission or carrying out analyses on human embryos, even those that are "spare" in a clinic practising *in-vitro* fertilization? The situation is more extreme if fertilization was carried out without any intention to replace the embryo in its mother, where a deliberate decision was first taken to collect eggs and spermatozoa, and produce an embryo for research. Who has the right to do this? My own viewpoint is unchanged (Edwards, 1974):

"Some laboratories are prepared to develop programmes on fertilization *in vitro* and early growth of the embryo as a scientific study, thus accepting the

view that human embryos can be deliberately initiated in the laboratory and then destroyed later. The situation is very different from that occurring in clinical studies where the embryos are used in attempts to cure infertility, and it cannot be justified by reference to current social practices where embryos or foetuses conceived accidentally are aborted, for example by the use of I.U.D. or menstrual aspiration for birth control. Accepting fertilization *in vitro* as a laboratory study in its own right can thus lead to the establishment of values about early human growth, including the assumption that these stages of life are expendable for scientific purposes."

Committees, including ethical committees, can sanction the use of embryos for research, and help to share the responsibility for decision making. They should formulate their own ethical standards in reaching such decisions; it is barely enough for them to simply decide that the work is "ethically acceptable" (as stated by the Medical Research Council, 1982) without explaining the ethical basis of their decision. The individual researcher, too, will have to decide for himself the need for research. Can spare embryos be avoided, so that the ethical problems do not arise? A restriction on the number of oocytes aspirated from the stimulated ovary to one or two, or using the menstrual cycle, would avert most "spare" embryos, but not all of them. Fertilised eggs with 3 pronuclei, for example, could not possibly be replaced in the mother, since they would grow as triploid foetuses or hydatidiform moles, hence they would remain as "spare" embryos *in vitro*.

Are the tiny embryos growing *in vitro* a « bundle of cells », or a life to be protected at all costs? One approach to finding a solution is to critically examine the various facets of the absolutist's case to decide if they are scientifically correct. One of the pillars of this belief is that life begins at fertilization. This cannot be true. Life is continuous and patently present in the unfertilised oocyte and spermatozoon. This argument is also compromised by parthenogenesis, where oocytes are activated without fertilization and develop into advanced foetal stages.

Another argument offered by absolutists insists that something is intrinsically valuable in an embryo being human. Is this true? The embryos have a human karyotype and metabolism, but do not possess any of the higher functions or senses of older foetuses, and can only make a biochemical response to other biochemical stimuli. Animal embryos make similar responses.

Another defence offered by absolutists is the need to respect the individual genotype as established at fertilization, and so protect the basis of individuality. This argument is also unacceptable, because the genotypes

of a surprisingly large number of embryos might be established long after fertilization, for example in twins, mosaics and chimaeras. Mosaic trisomic embryos are reverting partially to diploid long after fertilization (Hassold, 1982). Androgenetic embryos are originally triploid at conception (Fig. 3). A hydatidiform mole or a choriocarcinoma has a unique human genotype, but no-one could give any value to such an embryo, and nobody should replace such an embryo into its mother. I will return to a discussion on the origin of human genotypes in the last section of this review.

The viewpoint of absolutists is consistent but arbitrary, selecting a partciular point in time (fertilization) to make a sudden transition from no rights to full rights. Any reasoning of the balance between benefits and problems is rejected, yet similar arguments about the value and acceptability of experimental studies apply throughout all ages of life, e.g. in the introduction of new drugs or surgical methods. Many difficult ethical problems about the value of experimental work on human beings apply to adults, to the sick or the very old. Embryo research does not involve the same depth of ethical decision as working with the later stages of life. An embryo is an embryo; it might be referred to as an embryonic or microscopic human being, but it is not a foetus or a child, and it cannot necessarily share the rights accorded to children or adults. We all know that many civil rights mature throughout life until old age, where they might be withdrawn, e.g. a driving licence. Equal rights cannot be given to a pre-implantation embryo and a child, and what can be done with an embryo could not possibly be acceptable if applied to other stages of life. Absolutists seem to stress the potential rather than the actual value of an embryo, to attribute "humanness" to embryos long before this is apparent.

I also suspect that another motivation behind absolutists is the fear that once fertilization is breached, then there is no obvious point to defend during later embryology: that studying human foetus undermines the values of human life or leads to abuses in embryos used for research or replacement in the mother. This "camel's nose" argument applies to virtually every human activity, not only fertilization *in vitro*. There must be an agreed limit to the application of research in embryos, especially those which grow into children, e.g. the exclusion of man/animal hybrids. But the study of human embryos for medical research is different, and, I believe, acceptable. It is necessary to be vigilant about the research and to try to ensure that any new procedure is beneficial to medicine. Many people have groundless fears about the nature of the research, perhaps partly due to a psychological remnant of the centuries of bitter debate, of

the distressing ecclesiastical and state laws and penalties associated with contraception, abortion, or adultery.

Ethical basis of research on embryos

What, then, is the justification of the research on these minute human embryos which are not sentient, and are virtually undifferentiated? I can only offer the balance between the sheer necessity of acquiring knowledge and the value placed on embryos before any of their senses or the central nervous system are differentiated. Some commentators argue that this is a utilitarian viewpoint. But, in a sense, embryo research involves a more extreme position. Proposing the "greatest good for the greatest number" hardly implies destroying the minority, at least not without some careful reasoning first! I find many of the alternatives discussed by moralists to make ethical judgements to be unacceptable and unhelpful. Some have suggested that "comprehension" or "understanding" should be a guide limiting full acceptance of a human being, but these characteristics are acquired long after birth. This point is obviously much too late in time to confer absolute protection on a human being. Likewise, notions that pain is not a good ethical guide because we inflict it on animals, are equally unsatisfactory. Human pain must be accepted as different from animal pain, since we are carnivorous and are prepared to use the results of animal research for our own benefit. Many people would strongly object to growing embryos *in vitro* after the neural and sensory systems had formed, and it is fortunate that decisions on the exact length of time to culture embryos can be left in abeyance, since it is virtually certain that human embryos will not easily grow *in vitro* beyond the period of allantochorionic placentation.

My stance confers very few rights, if any, on the early embryo. It differs totally from the absolutist argument, which is superficially attractive and sets a high standard of behaviour on its practitioners, but which is founded on indefensible scientific principles. Absolutism is a full stop to debate, restricting enquiry into causes of human disasters and their alleviation. There might be some value in licensing particular forms of study, giving some, but not total freedom for non-clinical research on human embryos, and concentrating on studies of more immediate clinical value. This hope is unlikely to become a reality, because no-one can foretell the application of the scientific knowledge, and highly abstract pieces of research might prove to have unforeseen clinical advantages. In a sense, accepting the need for research on a few embryos opens the floodgates to research on

many embryos, raising arguments about the ethics of quantity: that once one embryo is studied, then there is no limit to the number that can be studied for almost any purpose. This situation will have to be faced as the results of the research become increasingly apparent over the coming years.

I believe the benefits to be gained considerably outweigh any objections to the study of embryos *in vitro*. This decision cannot be justified by quoting the use of I.U.Ds., post-coital contraception, or abortion, although those accepting or practising such methods cannot criticise my stance. Nor does the argument that many embryos conceived *in vitro* are doomed to die anyway appeal to my sense of ethics, since ethical stances cannot be justified by referring to "Nature's mistakes". There is a recognised clash of principles, and an example might help to clarify the situation. Most of us would accept the injunction to "Love thy neighbour", but not if he was threatening injury or death to us, when the principle of self defence could become paramount. We are in a similar position, and I believe that the need for knowledge is greater than the respect to be accorded to an early embryo.

Beginning and ending life

Debates on "spare" embryos raise issues about the beginning and ending of human life. Such fundamental issues must be set in a wide perspective, relating embryonic studies to other areas of human activity involving similar problems.

Life is ended for many and varied reasons (Table 8). It is ended legally, if society condones it, and illegally. It is ended culpably or otherwise through negligence, carelessness, failure to take safety precautions against known dangers, and a delay in introducing new methods of treatment. Life can be deliberately ended medically, when there is no need to do so. Such decisions are taken on the premise that life is no longer worth having, e.g. after brain death or with severely deformed babies, and it may be taken on these occasions partly for the benefit of relatives or parents. Cost effectiveness may cost lives by withholding life-saving equipment.

Many of these situations involve a decision about the value of life to young, adult, and old human beings. Life is obviously not sacrosanct even when highly developed and organised, and examining minute embryos for research has minor ethical implications when compared with such devastating circumstances.

If ending life involves complex decisions so, too, does defining its

TAB. 8 - *Ending Life.*

Ending Life

LEGAL	ILLEGAL
War	Murder
Appropriate self-defence	Manslaughter
Capital punishment	Euthanasia
	Suicide?

NEGLIGENCE

Carelessness
Commercial gains outweight health considerations
Delay in using new methods

MEDICAL

Brain death - end life support
Severely deformed baby - no life support
Labour - baby threatening mother's life
Abortion for social or eugenic purposes
Medico-economic - "cost effectiveness"
Post-coital contraception, IUDs, *in-vitro* fertilization

PERSONAL BELIEF

Risk to oneself	*Risk to dependents*
Refusing life-saving help	Passing by
Calculated risk to save another	Withholding life-saving help
Calculated risk (e.g. Army)	

beginning. It does not begin suddenly and mysteriously at fertilization or at any other time (Table 9). A succession of stages are involved in establishing the genotype of an individual. Normal embryonic growth demands that gametes are normal, fertilization is monospermic, and embryonic growth is either uneventful or tolerable as in twinning or the formation of fusion chimaeras. Normal growth could even occur after dispermic fertilization if one of the two male pronuclei was shed at syngamy (Fig. 3). Genotypes become distorted at various stages of development:

TAB. 9 - *Beginning life. Life does not arise from non-living matter. Man cannot create human life.*

NORMAL EMBRYONIC GROWTH

Fertilization	*Embryology*	*Outcome*
One normal egg, one normal spermatozoon	Uneventful	Normal single child
Two or more normal eggs, two or more normal spermatozoa	Uneventful	Normal multiple children
One normal egg, one normal spermatozoon	Bisection, trisection	Identical twins, triplets, etc.
Two normal eggs, two normal spermatozoa	Fusion of embryos or tissues	Normal chimaeric child
One normal egg, two normal spermatozoa (dispermy)	Shedding of one male pronucleus at syngamy	Normal child *

ABNORMAL EMBRYONIC GROWTH

Fertilization	*Embryology*	*Outcome*
Abnormal egg or spermatozoon	Uneventful	Uniform monosomy, trisomy, etc. in abortus or child
Abnormal egg or spermatozoon	Shedding of extra chromosome from cells during cleavage	Mosaic trisomic abortus or child
One normal egg, one normal spermatozoon	Non-disjunction in some embryonic cells	Mosaic trisomic abortus or child
One normal egg, two normal spermatozoa (dispermy)	Uneventful	Triploid abortus or child
One normal egg, two normal spermatozoa (dispermy)	Shedding of female pronucleus at syngamy	Hydatidiform mole
One normal egg, one normal spermatozoon	Suppression of one cleavage	Uniform or mosaic tetraploid abortus or child
One unfertilised egg	Uneventful	Parthenogenetic embryos
Ovarian or testicular tumour	?	Embryonic tissue in gonad

* Still theoretical.

with chromosomally-unbalanced eggs or spermatozoa, dispermy and other disorders in fertilization, abnormal cleavage, parthenogenesis, and in ovarian or testicular tumours.

It is not easy from a study of genotypes to decide when an embryo becomes a characteristic individual. These complex decisions about genotypes are succeeded by difficulties in recognising the emergence of an individual in embryological terms. Implantation begins around day 7, neural tissue is found a few days later, and the telencephalon and optic vesicles form at 30 days. Which stage, if any, is decisive in deciding that a new life is established?

Perhaps we should envy the absolutists. Their logic points unerringly to fertilization as the beginning of life. Decisions are more complex for the rest of us.

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CONCLUDING STATEMENT

MAXIME SINGER

Like all scholars, we seek in our daily lives to enrich man's understanding of himself and the world about him. Without opportunities to share our knowledge, to have it inform the human condition, our work would be without significance. Therefore we are all grateful for the opportunity to share with the Vatican, the Pope and the Church, through the Pontifical Academy of Sciences, the results of our efforts.

Also, for all of us, I express our personal thanks for the gracious hospitality and for the rare privilege of working in this beautiful building. We especially thank the Academy personnel for their kind attention.

Finally, Professor Chagas, each of us counts it a special fortune to have met and worked with you. We admire your knowledge, your wisdom, and your polite way of prodding us. More, we all admire and applaud your vision and hard work in shaping the Pontifical Academy of Sciences to its present unique and critical role. Too often, science and religion stand at odds rather than recognizing their common roots in humanity. Under your leadership, this special place has become a quiet meeting ground.

Because this is a quiet and private meeting ground, it is appropriate that I make one divergence from my charge. The point I will raise should probably have been discussed yesterday during our consideration of questions of ethics and morality. It was in fact Father Wisser's remarks that reminded me of the issue. He described to us the process, within the Church, by which positions on moral questions are reached. It is self-evident that the ethical quality of the decision process itself is critical to the world-wide perception and evaluation of any final position. With great respect then I would point out a severe difficulty in that process, as it relates to questions of human reproduction. Without full and equal participation of women at every step of the process, the

Church's position on these matters will continue to be seen as ethically compromised by me and by millions of others, Roman Catholics and non-Catholics alike.

Dr. Lejeune and I agreed that to summarize the week's proceedings was pointless. It did however appear useful to try, in a non-technical way, to highlight what we have learned about Modern Biological Experimentation. And according to our conversation, I will emphasize the molecular aspects while Dr. Lejeune will emphasize the cellular aspects.

Dan Nathans made the point yesterday that because of recombinant DNA techniques we now approach biological experimentation in a way that was unimaginable before 1974. To recombinant DNA techniques themselves, I would add the methods which allow investigating genetic molecules on extremely small samples as well as techniques for determining nucleotide structure, synthesizing DNA molecules and carrying out specific mutations at will. Together these procedures have had profound effects and one of the most important is that previously distinct disciplines are merging. The phenomenological approach to biological problems is disappearing as we try to understand living systems as expressions of their fundamental genetic programs. We can do this because we are able to manipulate biological systems to yield precise answers to carefully stated questions. John Carbon's report on the chemical structure of functional yeast centromeres is a dramatic example of this. We saw the same principles at work in Beatrice Mintz's experiments with mouse embryo cells, and at yet another level in Dan Nathan's efforts to understand the complex interaction between T-antigen and the SV40 genome. We also learned from Gunter Hammerling how these techniques are helping to sort out the complexities of the histocompatibility system.

The manipulation of biological systems depends on the availability of cloned and characterized genes and cDNAs. Increasingly powerful variations of recombinant DNA techniques, some of which were described by Ron Davis, are leading to the point where essentially any gene of interest will be obtainable. In addition to their use in fundamental investigations of the regulation and modulation of gene expression, some of these genes will supply important therapeutic and industrial proteins. As Michel Revel stressed in his talk about interferon, newly designed bacterial host-vector systems increase the efficiency of eukaryote gene expression thereby bringing closer the realization of commercially feasible methods. Also, recent and continuing redesigns of eukaryotic vector

systems are improving experiments aimed at understanding the control of gene expression — an essential step toward understanding development and differentiation.

Major advances in scientific understanding are almost always related to new methods. Certainly the last decade stands out as a time of remarkable progress because there have been two major technical innovations. One is recombinant DNA. The other is the development of monoclonal antibodies. As with recombinant DNA, monoclonal antibodies are pleiotropic tools, useful in many kinds of investigations. George Köhler did the amateurs in our group a great service by explaining both advantages and problems associated with their use.

One of the most remarkable insights of the past few years is the growing appreciation of the flexibility of biological systems. Dr. Mintz stressed the flexibility of embryos and embryonic cells. Others talked about the flexibility of genomes themselves. Classical homologous crossing-over is only one of the ways by which genetic information is rearranged. Ron Davis showed us the many ways in which recombination occurs in yeast and how they could be manipulated to alter yeast genes. Ernest Winocour introduced us to the surprisingly high level of non-homologous recombination in mammalian cells and suggested that the reactions are dependent on specific nucleotide sequences. My own report tried to emphasize the surprising plasticity of some mammalian DNA sequences both in contemporary and evolutionary time. The construction of immunoglobulin genes from dispersed DNA segments was reviewed briefly by George Köhler (reminding us that genome flexibility is not only a random process but is specifically used in differentiation. And we had various hints that genome rearrangement is likely to have played a critical role in evolution.

The importance of genome reorganization and exchange in natural processes was dramatically demonstrated by Jeff Schell's description of the intimate relation between the Ti plasmid of *Agrobacterium tumefaciens* and infected plant cells. And this system also suggests a way to exploit natural recombination for the design of desirable plants. While not specifically covered during our meeting, I would point out that the rapidly developing work on the retroviruses of vertebrates also concerns genomic rearrangements and exchanges, and implicates such reactions in the evolution of the viruses and in carcinogenesis itself.

It is worth remembering that one of the fears frequently expressed during the height of the recombinant DNA debates concerned "tinkering"

with genomes. It was, some said, "unnatural". Clearly, it is not unnatural but is a fundamental property of genetic systems. Genomes are not fixed — and they evolve in many complex ways besides simple mutation by base pair changes. It is important to remember the inherent changeability of genomes in future, when we confront specific ethical issues in relation to alteration of human genomes.

In this brief week, many aspects of modern biological research have been described. The experience of hearing such a diverse program was a highly instructive one and quite different from our normally more specialized meetings. We come away with a sense of the great accomplishments and of high expectation for the future. But a meeting like this also reminds us of our ignorance. In expressing our enthusiasm for the accomplishments of biological research to non-scientists, it is essential that we also state what is unknown. Otherwise we risk serious misunderstanding of our endeavors.

CONCLUDING REMARKS

JÉRÔME LEJEUNE

After the summary so aptly given by Mrs. Singer, it seems difficult to continue the analysis of our discussions. With your permission I will try, Mr. Chairman, to express the feelings left in us by this week of working together.

Basic information: As already said by Mrs. Singer, we have witnessed the progressive unraveling of the coded message carried by macromolecules, DNA and RNA — copy, repeat, include, delete, transfer, invert, replace by, insert, and so on . . . — all these instructions look very much like the tricks used by an author editing a manuscript and then correcting the galley-proofs. We now have librairies of DNA segments, and dictionaries to check where some chapters do belong and what is their meaning. Really the book of life is of a fascinating richness.

But other instructions like "go to", "split", "pause", "come back", "if flag" and so on are more akin to the language of computers. Indeed, the tables of the laws of life are very complex multiplication tables.

Cooperation: At the other end of the size scale we find the whole cell, in which so much informations is imbedded. Its structural complexity would need more descriptive parameters than astrophysicists would use to figure out a galaxy.

Thanks to the experiments of Mrs. Mintz, we know now that this miniaturization of molecular subtleties confers on the primordial cells a kind of wisdom. The few active cells of the early blastocyst are able to educate and so to speak domesticate teratoma cells, gone wild in their unlimited growth. Apparently the blastocyst's cells know something more than these selfishly dividing tumor cells. By means yet unknown, they teach

them how to cooperate harmoniously in the make-up of a new individual. A beautiful lesson!

Among the wealth of new knowledge brought to us, I would thank especially Mr. Illmensee for his rehabilitation of the fecundation process. We geneticists have been lazily thinking that spermatozoa were mere vehicles of the chromosomes, their only payload being the DNA tapes. But nature is more refined. Sperm does carry some information to the cytoplasm as well, a domain previously restricted only to tbe female contribution. Maybe the rest of the sperm is not only a dispensable re-entry vehicle after all.

With Dr. Edwards, we witnessed the extraordinary achievement of the early blastocyst. Even in the conditions realized in extracorporeal fecundation, very inhabitual for our species, it tends to its own goal, the blossoming of a new member of our kind. It can withstand many aggressions. It can eventually be killed by them. But if it survives, it regulates, compensates with a stupendous stubbornness, and expresses the individual it is bound to by its genetic make-up.

All these data point to the fact that one cell alone cannot read the book of life of complex living beings. In order to exhaust the whole significance, many different cells have to cooperate, helping each other, in this deciphering and its complete expression. As algebrists would do, expanding a formula, reduced to its single expressions, the genetic information of the fecundated egg has to be progressively unfolded. Apparently various specialists are required in this interdisciplinary adventure.

Signalling: Obviously these early cells, these would-be specialists have to get committed to their particular way of reading, through some messages they receive from each other.

Dr. Schell showed us a part of their strategy, thanks to the ring of DNA responsible for the crown gall modification. This circular jewel reminds me of the famous ring of Gyges which if twisted in a given way, rendered its bearer invisible. Depending upon which segments of the ring are operating, it renders invisible the roots, the leaves or other parts of the plant. But proper procedures make them reappear at will.

Besides this quite mythological property, Dr. Schell's ring will possibly provide a vast field for the genetic manipulation of plants. Besides the "beer factories" predicted by the breeders of bacteria and

molds, we will some day have crops furnishing us very precious drugs and medicaments. A fate to which plants are eminently prone, if we remember that apart from the antibiotics, most of the molecules used in medicine are plant produced.

The external signalling between cells has been exposed in its very different aspects by Drs. Köhler, Hämmerling, Scharfstein and Koprowski. Here we deal with recognition, enhancement, competition, advertising or disparagement, exactly as we observe in the "République des lettres", the interactions between authors, readers and critics. Another example of a cooperative race.

Maybe the work of Dr. Saxén and of Prof. Ranzi affords us a first glimpse of this system of emission and reception of signals and of their significance. In the embryological development, the commitment to differentiate is transmitted by non-informative molecules, I mean by end products of the long chain of transfer of information from DNA to proteins and from them (through purely enzymatic processes) to glycopeptides. Of course we already know that cellular membranes are sensitive to this kind of molecules. But how is the reception of this information transmitted to the nucleus and how does it modify the way of reading the genetic message? That remains to be discovered, a fascinating endeavor.

It is absolutely necessary that the flow of information from DNA to cytoplasm must be in some way fed back to the blue point lecturing machine. As we have already seen, the message in higher organisms is too important to be read by one reader only (as in microorganisms); specialized readers must interchange their discoveries so that differentiation still leads to a common goal!

Thus we have observed two levels, the molecular one of the coded molecules, and the cellular one, of the supercoded organization of coded molecules. But what about the intermediate size, I mean the DNA tape *and* its cartridge, the chromosome?

Three of us addressed ourselves to this intermediate size. Dr. Carbon deciphered the DNA sequence of the anchoring point, the centromere. Dr. Davis manipulated chromosomes of Saccharomyces like an editor of a movie giving orders to a cutter for a new presentation of already filmed sequences.

I addressed myself to another gap of our knowledge, a tiny gap of the x chromosome, responsible for a terrible gap in mental development of affected children.

But these examples do not exhaust the future of chromosome mani-

pulations. Compared to our ability of manipulating the DNA molecule, we know practically nothing about directed modifications of the chromosomes. But Nature does.

Species Constitution: With the use of precise techniques the banding pattern of the chromosomes defines precisely the structure of the caryotype. The simplest observation being that each species, whether a whale or a mouse, can be defined by its own caryotype. As was noted in the study group "on the evolution of primates" which preceded this one, the genic differences are seemingly less striking than the chromosomal re-arrangements in telling a party related species. With minute variations the words seem common to all of them but the structure of the phrases and the length of the chapters make most of the difference. As Buffon said: "le style c'est l'homme". A quite prophetic thought!

We are still confronted with the task of understanding the significance of the distribution of the message in the various volumes of the encyclopedia of life.

Preferential translocations: Nobody knows nowadays why a given gene is carried by a particular chromosome in one species and is located in another place in another species. But already pathology teaches us that this allocation must have some functional meaning. For example, some malignancies are related to a single translocation (like the rearrangement of chromosomes 22 and chromosome 9 in chronic granulocytic leukemia). Even in some lymphomas, by looking at the rearranged chromosomes it can be safely predicted what kind of immunoglobulin will be expressed by the tumor cells.

Also, specificity of small deletions (retinoblastoma) or of reciprocal translocations (ataxia telangectasia) is well established. A kind of regulation of position effect is no doubt of extreme importance.

A tridimensional structure: All these facts point to the notion that uncoiled active chromosomes cannot be distributed at random inside the nucleus, like spaghetti floating in a bowl of soup. On the contrary they must be anchored by special points to the pores of the nucleus membrane. In this context, may I suggest that the dispersed repetitive sequences of DNA could be candidates for this function, and could also provide some specificity for accidental translocations. Be that as it may, the resting, active nucleus is surely a tridimensional network of precisely

interrelated chromatin fibers, a structure very reminiscent of the informative network of a fantastically miniaturized computer.

Future applications: If we could understand, even partially, how these phenomena are achieved we would possibly be much more efficient in fighting chromosomal diseases. For example, we know that during meiosis, especially for the maturation of the ovule, the chromosomal mechanism is directly geared to the hormonal regulation. The older the mother, the greater the risk that some "give" will happen between the two systems, and the higher the risk of malsegregation of a chromosome.

If this "give" could be corrected, a true prevention would ensue.

Even, in case of translocations, we know that the risk of malsegregation is much more severe if the translocation is carried by the mother than by the father. A kind of sieve, selecting against the unbalanced gametes, seems to protect partly the male production line. Here also, a means of prevention is awaiting discovery.

In the regulation of a whole chromosome, we know quite well, that in females, one of the X's is turned off, producing the Bar body. Hence the relative innocuity of the excess of the X chromosome, like in XXX conditions. The inactivation of two of the X's leaves only one fully active, a situation very close to the normal equilibrium.

If we could understand how to turn off and turn on whole segments of chromosomes, we would possess a powerful tool for compensating trisomies or monosomies.

Classical biochemistry: If the DNA, the genes and the chromosomes are like commanders, generals and staff headquarters of the struggle for life, what about the infantry men, the non-commissioned, the non-informative molecules?

It could very well be that the blossoming of molecular biology is masking the interest, still enormous, of classical biochemistry (like vitamins). In many instances we can either add small molecules or exclude them from the regimen (like phenylalanine in P.K.W.) and effectively protect the soma from inborn errors of the genome. The possibility remains open that curative medicine can do much to prevent the disastrous effects of a blurred genetic message, even without repairing the abnormal genes. This necessary approach is not as fashionable as genic manipulation but is possibly unjustly neglected.

Two conclusions: At the end of these reflections many questions have been raised, but "concluding remarks" were asked for by the program. I feel that conclusions are straightforward.

During this week, we scientists developed our work in the manner of a fully integrated organism. We were offered basic information, shared this in true cooperation, thanks to dependable signaling (turning off and on of the information was even materialized by the microphonic devices). We attained some wisdom thanks to the participants. More than a crude analogy, this week was a demonstration that the deciphering of life processes requires the cooperation and, so to speak, the coming together of various specialists. And we fully appreciated it.

The second one is that each of us, no matter whether he uses a pipette, a microscope, or an electrophoretic system, is a lover of life. We observe it as exactly as we can, but we also admire it, and admiration is really the highest human activity. Never a dog, however cheerful, smelled the perfume of a rose. Never a chimpanzee, however artful, gazed at the sunset or at a starry sky.

And it was very proper for our admiration of life that this meeting took place in this marvelous room of the Casina Pio IV. During our meetings I often stared in wonder at this ceiling's frescoes, which protected our discussions like a shell of beauty, And if you look at the four corners, you will understand why. In the rear at right, Veritas is depicted and indeed truth is our only goal. At the left is Concordia and it was our manner of exchanging ideas. Near the entrance, at the left, is Tranquillitas and we can pursue our endeavor in full tranquillity as long as admiration is our guide and guards us from pride and its dangers. Finally at the right is Letitia, and our joint efforts in unraveling some of the wonders of life, gave us indeed a profound joy.