Genetic engineering and plasmids

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I. *Introduction*

We shall first define what is meant by genetic engineering, and then spend some time describing how these genetic manipulations are performed. Finally we will discuss some of the implications of this new field and outline the possible developments which can be expected.

Genetic engineering in the broad sense of the term is not new, and for many years geneticists have been able to modifiy the genome of viruses and bacteria, and in some cases of higher organisms as well, as the result of mutations and selection. More recently, fusion of cells from higher organisms, and nuclear transfer have also allowed the creation of genetically different cell lines. In fact, what is meant by genetic engineering in the present context, are the newly developped techniques which permit the transfer in vitro of genes, groups of genes, or portion of genes, from one organism to another, in particular across species barriers.

Such transfer of genes can be achieved by biochemical techniques which involve the slicing of DNA segments and their 'transplantation' into the DNA of another organism. These experiments result in the creation, in vitro, of new genomes, carring and perpetueting genes belonging to another species[1]. These developments have important implications for biological research as a whole, and in particular for the study of gene structure, function and regulation in the case of higher organisms. They may have practical implications as well for such problems as the large scale production of specific proteins.

Depending on the source of the DNA and the nature of the receptor, into which it is inserted, one should distinguish three broad categories of experiments. The first consists of transfer of *prokaryotic* DNA into the genome of another *prokaryote*, for instance the insertion of bacteriophage DNA into the DNA of a bacterial plasmid. The second group concerns the transfer of genes from an *eukaryote* into a *prokaryote* receptor DNA. Such experiments could involve, for instance, the introduction of a mammalian gene into a bacterial plasmid or bacteriophage. Thirdly, one can envisage the insertion of genes into the DNA of *eukaryotes*, with the use of a viral vector.

II. *The technology of genetic engineering*

One is confronted with two different sets of problems which both require the development of new technologies: on the one hand, the handling of DNA fragments, their excision and their reinsertion into other DNA (the biochemistry of gene transfer), and on the other hand, the question of gene specificity, which includes selection, screening, etc., an especially difficult question in the cases of complexe genomes.

A) *The biochemistry of gene transfer*

Most of these experiments are based on the principle of molecular hybridization of DNA strands, which results from the complementarity of specific nucleotide base pairs. One can formulate two basic principles for these experiments: 1. The different DNA molecules which are to be joined are first prepared with single-strand tails (cohesive ends) of such structure that the single-strand tail of one DNA molecule is complementary to the single-strand tail of another DNA molecule. Joining of DNA segments occurs, therefore, by the pairing of short complementary single-strand stretches on the two DNA fragments.

2. One of the two partners in this DNA joining reaction is a self-replicating, autonomous DNA, capable of infecting a bacterial host and of replicating either autonomously or following integration into the bacterial chromosome. One of the two DNA participating in the joining reaction is therefore a receptor and also a vector, such as a bacterial phage or a plasmid.

What is meant by DNA transfer experiments consists therefore in the transfer of a fragment of foreign DNA into the circular DNA of a bacteriophage or plasmid vector. There are two biochemical mechanisms to achieve this end-to-end joining of different DNA molecules: a) Joining by *homopolymeric tails*. It is possible with an enzyme prepared from calf thymus (terminal transferase) to polymerize deoxynucleotides from the 3′ end of a DNA strand. With double-stranded DNA, this enzyme will also polymerize nucleotides if the 3′ end of each DNA strand is freely available. In this reaction, if only one of the four triphosphates is provided, terminal transferase can then add homopolymeric tails (single-strand) to each side of a DNA molecule. For instance, a fragment of DNA from the bacteriophage lambda can be 'tailed' with stretches of poly dA. Independently, an unrelated DNA such as DNA from the virus SV 40, can be elongated with a similar procedure, but with homopolymeric tails of poly dT. When the two unrelated DNA molecules are mixed, pairing of the poly dA and poly dT stretches takes place and circular hybride

[1] S. N. COHEN and A. C. Y. CHANG, Proc. natn. Acad. Sci. USA *71*, 1030 (1974).

molecules are formed containing DNA from both SV 40 and lambda bacteriophage (ref. [2]). This principle therefore involves the enzymatic synthesis of artificial 'sticky ends'. It is generally applicable to the joining of any DNA fragments.

b) Joining by the 'sticky ends' produced by *restriction endonucleases*. Restriction DNA endonucleases are a group of enzymes capable of cleaving DNA at precise locations. The microorganisms which produce a given restriction nuclease protects its own DNA in modifying those specific target sites for the restriction enzyme. In the case of certain restriction endonucleases, the two strands of the DNA are cleaved at exactly the same position, generating therefore 'even breaks'. In the case of other restriction endonucleases, however, the enzyme cleaves the two DNA strands at distinct positions, distant from one another by a few nucleotides (about 3 to 5). These enzymes therefore generate 'staggered breaks'. The crucial feature of this type of cleavage is that the ends of DNA fragment produced by a given restriction endonuclease of this kind will have short single-strand projections complementary to each other (reviews [3,4]). As a consequence, the ends of such DNA fragments can anneal to each other by complementary base pairing, with the possibility of forming circular DNA molecules containing DNA from two different origins. It is of interest that different restriction endonucleases produce different single-strand projections (different nucleotide sequences) but that a given enzyme will generate the same structure no matter what the source of the DNA, providing therefore a general mechanism for the joining of DNA from unrelated species.

The vector DNA used as receptor molecule for the insertion of genes, is chosen according to a number of criteria. The DNA of the receptor molecule should be capable of autonomous replication in its host cell. 'Infection' of the host cell should be possible with the naked DNA (transformation, transfection). The circular vector DNA should be easily made linear by cleavage at a single point. The insertion of a fragment of foreign DNA should not abolish the capacity of the vector molecule to replicate in its host cell. And finally, for practical purposes, the vector DNA should carry a gene whose product will confer a particular phenotype on the infected cell, such as resistance to an antibiotic. This vector-dependent phenotype will permit the selection or screening of those host cell colonies into which the vector molecule has replicated. For very obvious reasons, in the last few years experiments on gene transfer have relied heavily on plasmid DNA and on bacteriophage DNA as vector molecules.

a) *Plasmids* (see review [5]). These are circular DNA molecules which, when appropriately chosen, meet all the requirements indicated above for a gene transfer vector. So far, plasmids have mainly been known to microbiologists as carriers of genes for the resistance to antibiotics, and especially for their capacity to transmit these resistances to other bacteria. The microbiological properties of plasmids are now making them very useful tools in the field of genetic engineering. For this purpose, certain plasmids were selected which are cleaved at a single point by a given restriction endonuclease enzyme without affecting the antibiotic resistant gene. It was shown that the introduction of even very large pieces of foreign DNA followed by recircularization of the plasmid molecule did not affect the subsequent replication and growth of the hybride plasmid in its host cell. Another interest in plasmid DNA as vector for transfer experiments is that, in certain cases, plasmids can be amplified in their host cells environment, simply by an inhibition of host cell DNA replication. This can result in bacterial cultures with several thousand plasmids DNA molecules per bacteria. Several laboratories, and in particular that of COHEN[1,6], are actively involved in the selection and construction of plasmids which are well suited for gene transfer experiments.

b) *Bacteriophage lambda*. Bacteriophage lambda has proved to be an excellent vector molecule, mainly because it can be used easily in transfection experiments. Because of the very well known genetics of lambda, several possible selection or screening procedures can be devised. Contrary to the case of plasmid DNA, there is here a strict limitation concerning the size of foreign DNA which can be accomodated by the bacteriophage. The size of lambda DNA is about 30 millions daltons and lambda DNA hybrids larger than 10% of the normal value are usually not viable. However, since a large percentage of lambda DNA itself is non-essential for growth, several tricks have been devised to start with deleted lambda molecules, containing for instance only 3/4 of the lambda DNA mass, and into which therefore a much larger fragment of foreign DNA can be inserted (see ref. [7]).

In summary, the different steps involved in a DNA transfer experiment are as follows: 1. Preparation of the DNA fragment (foreign DNA to be inserted) and of the vector DNA. This involves the construction of cohesive ends either as homopolymeric tails (terminal transferase) or as the staggered ends generated by restriction endonuclease.

2. Annealing of vector DNA and foreign DNA by virtue of the complementarity of their cohesive ends.

[2] D. A. JACKSON, R. H. SYMONS and P. BERG, Proc. natn. Acad. Sci. USA *69*, 2904 (1972).
[3] D. NATHANS and H. O. SMITH, A. Rev. Biochem. *44*, 273 (1975).
[4] W. ARBER Progr. nucl. Acid Res. molec. Biol. *14*, 1 (1974).
[5] D. J. SHERATT, Cell. *3*, 189 (1974).
[6] A. C. Y. CHANG, R. A. LANSMAN, D. A. CLAYTON and S. N. COHEN, Cell *6*, 231 (1975).
[7] K. MURRAY, N. E. MURRAY and W. J. BRAMMAR, Proc. 10th FEBS Meeting *38*, 193 (1975).

3. Ligation with the enzyme polynucleotide ligase (prepared from T4 bacteriophage-infected *E. coli*) and formation of covalent circles consisting of the vector molecule plus a fragment of foreign DNA.

4. Introduction of the hybrid, or chimeric vector into its host cell with replication, and possibly amplification of the foreign gene.

B) *Selection and specificity*

There are two levels at which an important selection problem occurs. First, when a DNA insertion experiment is carried out, with plasmid DNA or bacteriophage DNA as vector, the resulting molecules consist of recombinant DNA (containing foreign DNA fragment) as well as plasmid or bacteriophage which has circulated without exogenous DNA. Techniques have therefore been evolved for the selection or for the screening for recombinant DNA molecules. When the joining reaction is carried out with DNA previously elongated with appropriate homopolymeric tails, the vector molecule cannot circulate by itself and can only do so by pairing with a fragment of foreign DNA containing complementary homopolymeric tails. In such favorable circumstances, therefore, the resulting viable plasmids (or bacteriophages) will all be recombinants, carring a fragment of foreign DNA. When joining is performed by the cohesive ends generated by restriction endonucleases, on the other hand, selection or screening for recombinants is necessary. For instance, selection for bacteriophage lambda recombinants can be obtained if the starting lambda DNA molecule is too small to produce viable bacteriophage. The insertion of DNA fragment will restore the appropriate DNA size compatible with lambda bacteriophage viability. Screening for recombinant molecules can be achieved if the insertion of a fragment of foreign DNA has modified a given phenotype in the receptor plasmid or bacteriophage. Several such systems have now been developed, in particular in the case of bacteriophage lambda (ref. [7]) and recombinants can easily be identified, even among several thousands of plaques or colonies.

More important, and also more difficult, is the general problem of the specificity of a given recombinant for a given gene. This is a crucial aspect of genetic engineering, one which is especially difficult to deal with in the case of higher organisms with complex genomes and where any given specific DNA fragment represents less than one part in a million of the total DNA. So far, four different approaches have been envisaged to cope with this problem of gene specificity in DNA transfer experiments.

1. In certain exceptional cases, a gene, or a group of genes might have an overall chemical composition which results in a particular physico-chemical behaviour. The unusual guanosine-cytosine (GC) content of the genes for ribosomal RNA and of the histone genes has thus allowed their enrichment from the bulk of the DNA, on the basis of a difference in density. Purification of genes on the basis of density differences is not, however, a generally applicable principle.

2. One approach to the specificity problem consists in the *random insertion* of a large mixture of DNA fragments, for instance unfractionated DNA from the entire genome, followed by growth of all the recombinants in bacteria and selection or screening for recombinants carrying a specific gene. The feasibility of this so-called *shotgun* approach depends greatly on the availability of selection and screening techniques. In theory, selection could be envisaged if a newly inserted gene were expressed into a product, allowing selection among recombinants. Screening methodology has been mainly based on the identification of colonies carrying a given gene by molecular hybridization, usually of DNA immobilized on nitrocellulose membrane filters. These techniques are applicable to the screening of several thousands of recombinants. Gene insertion with unfractionated DNA appears, however, to be an unpractical approach in the case of organisms with complexe DNA, a situation which would require screening among several millions of recombinants.

3. Much more sophisticated, and also promizing, is the strategy consisting in the *purification of a given gene* (or its enrichment) followed by the deliberate insertion of a given gene into the appropriate receptor plasmid or bacteriophage. Methods for the purification of specific genes rely mostly on the technique of hybridization to a specific probe (messenger RNA or complementary DNA). Purification by hybridization can be attempted either with fully denatured DNA fragments or with DNA which is only partially denatured. Progress in this important field of research depends to a great extent on the availability of large amounts of pure nucleic acid probes, and it is likely that plasmids carrying specific genes and constructed by another approach (see 4 below) will prove very usuful in this respect.

4. Messenger RNA-directed insertion of specific gene sequences. If one can synthesize double-stranded DNA using as a template messenger RNA coding for a given protein, one might be in a position to insert selectively the gene sequence for that given protein. Experiments based on that principle will be described in the next section.

C) *Messenger RNA-directed insertion of specific gene sequences*

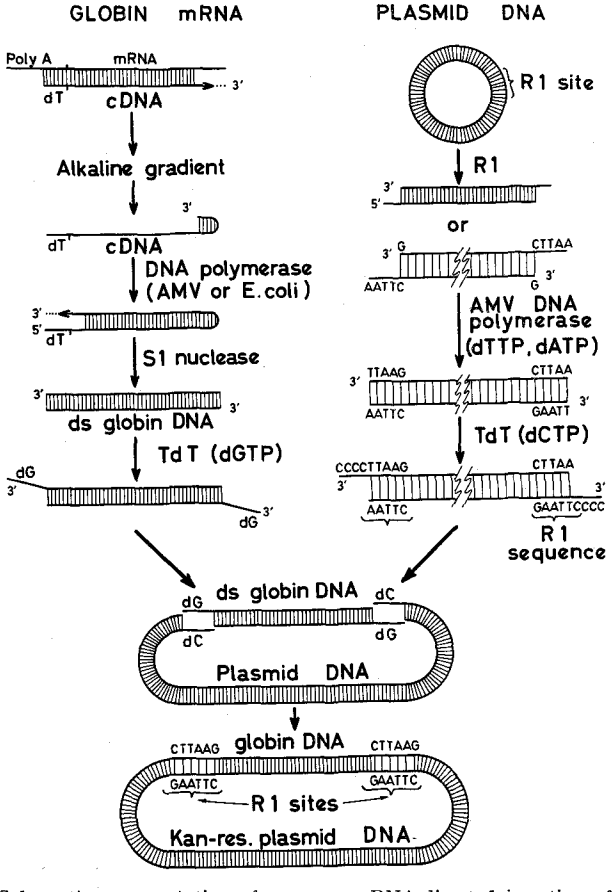
It is known that messenger RNA can be used as a template for the synthesis of single-strand complementary DNA (cDNA). It was observed recently (ref. [8,9])

[8] F. Rougeon, P. Kourilsky and B. Mach, Nucl. Acids Res. *2*, 2365 (1975).

[9] F. Rougeon and B. Mach, Proc. natn. Acad. Sci. USA, *73*, 3418 (1976).

that cDNA, once isolated, can function as a template for the synthesis of a second strand of DNA, with the enzyme DNA polymerase from *E. coli* or in certain special cases with avian myeloblastosis virus DNA polymerase. It was therefore possible, starting from messenger RNA, to obtain a double-stranded DNA molecule of the same sequence. These experiments are illustrated schematically in the Figure for the case of rabbit globin messenger RNA. Globin DNA could then be elongated with a homopolymeric tail of poly dG and, independently, a vector DNA molecule was prepared with homopolymeric tails of poly dC. The vector chosen for these experiments is the *E. coli* plasmid pCR1, which can be opened (converted to a linear form) at a single site by the enzyme EcoR1. This plasmid carries a gene conferring resistance to the antibiotic kanamycin, and therefore bacteria successfully infected by pCR1 can be selected as kanamycin-resistant colonies.

As indicated in the Figure, globin-specific DNA and linear plasmid DNA, carrying respectively poly dG and poly dC homopolymeric tails, were hybridized, and the hybrid molecules were used directly to transform *E. coli* cells to kanamycin resistance. In these experiments, it was not found necessary to use polynucleotide ligase to obtain covalent circles. DNA was prepared from the kanamycin-resistant colonies and tested for the presence for globin specific sequences. These assays were performed by hybridization either on millipore filter or in liquid, using as probes radioactive complementary DNA synthesized from rabbit globin messenger RNA, of the α- or β-globin specificity. A large number of bacterial clones carrying either α- or β-globin gene sequences were thus obtained and plasmide DNA was prepared from a number of these clones[10]. Detailed studies of these globin-specific chimeric plasmids revealed that they carry either α- or β-globin gene sequences but never both, as expected from the cloning experiment. The length of the globin-specific inserted sequence varied from about 400 to over 600 base pairs. In the case of the β-globin-specific plasmids in particular, clones were obtained which carried the entire structural gene for globin, as well as extensive portions of the non-translated region on either side of the structural sequence. Finally it was observed that when certain globin-specific plasmids were digested with the restriction endonuclease EcoR1, the globin-specific gene sequence could be excized and the easily purified by gel electrophoresis. This last experiment indicated that, as predicted from the scheme on the Figure, EcoR1-specific sites had indeed been reconstructed on either side of the inserted globin sequence[10].



Schematic representation of messenger RNA-directed insertion of globine gene sequences into an *E. coli* plasmid. For experimental details see ref. [8–10].

The methodology developed in the case of rabbit globin genes, and just described above, has also allowed us to construct recombinant plasmids carrying mouse α- or β-globin genes and more recently mouse immunoglobulin light chain gene sequences. In principle, the procedure should be applicable to any messenger RNA available with a sufficient degree of purity. These globin-specific or immunoglobulin-specific plasmids should prove to be very useful tools, in particular for the assay of gene-specific sequences (either messenger RNA or DNA) under various biological conditions, and for the purification (by hybridization) of specific genes from cellular DNA.

III. *Prospects and possible uses for genetic engineering*

Using the biochemical and biological techniques outlined in the previous sections, as well as others, it has been possible in the last few years to achieve first the transfer of DNA from a bacteriophage or a plasmid into another phage or plasmid. Then, DNA of higher organisms, such as the genes coding for ribosomal RNA of *Xenopus laevis* were transferred in plasmids growing in *E. coli* (ref. [11]), and the same experiment was later on performed with the genes coding for histones, also of *Xenopus laevis* (ref. [12]). In the case of mammalian genes, rabbit and mouse globin, and mouse immunoglobulin gene sequences have been inserted into plasmid DNA (ref. [8]) but these

experiments are limited to the portions coded for by the messenger RNA.

One of the interesting observation that can be made in the light of all these different experiments is that although nature has always devised rather strict barriers, preventing the exchange of genetic information from among different species, one finds that DNA molecules in vitro are remarquably susceptible to exchange, no matter what the source of DNA. This complete 'neutrality' of DNA in vitro with respects to its origin is in remarkable contrast with the strict species-specificity maintained during evolution.

There are three obvious levels at which genetic engineering experiments might prove useful: 1. The cloning of specific genes in bacterial vector is likely to represent a major advance for the different areas of biology dealing with the study of gene structure, gene regulation and of their expression. This new technology represents, therefore a decisive tool and a major contribution to modern biological research.

2. An obvious interest in recombinant DNA molecules lies in the possibility of the expression of the foreign gene. Independently of the biological interest of such a phenomenon, it raises the possibility of utilizing recombinant plasmids or bacteriophages for the large scale production of specific proteins. This second aspect of genetic engineering is at this stage only hypothetical, but nevertheless it is the subject of extensive research. One can certainly envisage the possibility of growing bacteria capable of synthesizing large amounts of such proteins as pro-insulin, or viral antigens (which could be used as vaccines) as well as a number of pharmacologically interesting proteins. One could even envisage the possibility of synthesizing, in a bacterial culture, both the heavy and the light chain of a specific immunoglobulin molecule with antibody activity.

3. The third aspect of genetic engineering concerns the possibility, even more hypothetical, of transferring specific genes from a viral vector into chromosomes of higher organisms, including man. Since the technology for introducing any DNA fragment into a circular DNA molecule, such as SV 40 DNA for instance is already available, work in this field is concentrating on the development of safe viral vectors, capable nevertheless of integrating their DNA into the genome of their host cells. If DNA transfer experiments of that type concern somatic cells, the possible effects of such a gene transfer will obviously be limited to the life span of that cell. If however, one envisaged to perform such a viral-mediated gene transfer experiment with germ line cells, one could, at least in theory, consider attacking the fundamental question of the correction of genetic defects by genetic engineering. It is important to stress that at the present state of our technology this third aspect of genetic engineering is rather hypothetical.

In conclusion, whether or not the recent progress in genetic engineering will revolutionize biology, and possibly medicine, is not yet known. What is known, however, is that all progress in this field of biology is intimately dependent on the progress of research in microbiology. It is in fact amusing to observe that a number of important research laboratories which had not so long ago moved away from the study of bacteria or viruses to approach the study of higher organisms, are now, with the advent of genetic engineering, rapidly going back to bacteria and viruses which become once more their best tools. The fact that a problem as intriguing and important as the genetic control of the diversity of antibodies might well be solved one day by looking at the DNA of an *E. coli* plasmid or by analyzing recombinant bacteriophages, is a good illustration of the unity and the importance of microbiology.

[10] F. Rougeon and B. Mach, J. biol. Chem., in press (1976).
[11] J. Morrow, S. N. Cohen, A. C. Y. Chang, H. W. Boyer, H. W. Goodman and R. Helling, Proc. natn. Acad. Sci. USA *71*, 1743 (1974).
[12] L. H. Kedes, S. N. Cohen, D. Houseman and A. C. Y. Chang, Nature, Lond. *255*, 533 (1975).