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GENETIC ENGINEERING

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Summary

The field of genetic engineering is reviewed with a special emphasis on in vitro DNA recombinant technology. The basic principles of the biochemistry of DNA splicing and of gene transfer are described. An important distinction is made between the insertion and cloning of genes derived from genomic DNA ("natural" genes) and of DNA synthetized in vitro ("synthetic" genes). Cloning of genes synthetized from mRNA has provided the probes necessary for the identification of genomic clones, and recently it has made possible the synthesis of specific mammalian proteins in bacteria.

Zusammenfassung

Die Möglichkeiten der Genmanipulation werden rezensiert, wobei besonders auf die in vitro DNS-Rekombinationstechnologie eingegangen wird. Ferner werden die biochemischen Grundlagen der Zusammenfügung von DNS-Molekülen und der Genübertragung beschrieben. Besondere Aufmerksamkeit gilt der Unterscheidung der Insertion und Klonierung einerseits von Genen, welche von genomischer DNS (natürliche Gene) stammen, und andererseits solchen, welche in vitro synthetisiert (synthetische Gene) wurden. Das Klonieren von Genen, welche mit mRNS als Matrize synthetisiert wurden, liefert die notwendigen Proben für die Identifikation der Gene im Erbgut und neulich für die Synthese von spezifischen Säugerproteinen in Bakterien.

Introduction

What has been recently known as "genetic engineering" is a relatively new field of experimental genetics which relies on the possibility of cutting and joining fragments of DNA and of introducing these into circular vector DNA molecules, such as plasmids or

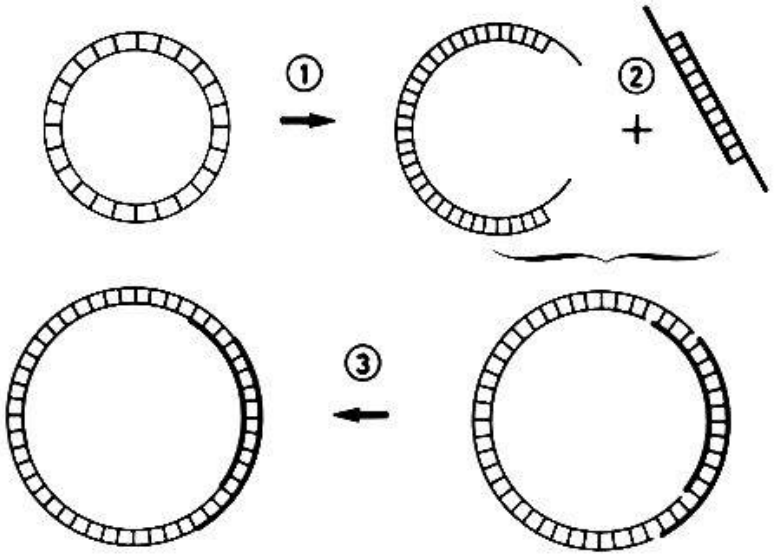

Fig. 1. Schematic representation of the use of a circular vector molecule (plasmid or viral DNA) for the insertion of a segment of foreign DNA. See text for explanation on the joining reaction by complementary ends (2) and ligation (3).

viral DNA. These "transplanted" genes can then replicate within a bacterial or animal cell together with the vector molecules. Other procedures such as cell fusion or nuclear transplantation also imply some form of genetic engineering, especially when applied to experimental embryology, but this paper will deal with DNA recombination in vitro. We will discuss what kind of gene manipulations are now possible, how these are performed and finally why the implications of this new technology are important.

I. Biochemistry of gene joining

There are two basic principles on which all of the DNA recombination technologies are based:

1. End to end joining of a DNA fragment to each of the two ends of another DNA molecule. This results in the formation of a circular hybrid DNA molecule.
2. One of the two DNA partners in this joining reaction is a self replicating DNA, normally circular, and capable of replication and propagation within a host cell (cf. virus). This molecule assumes the role of a vector.

The general scheme used for most of these manipulations is illustrated in Figure 1. A circular vector molecule is first made linear (1) and then linked to another DNA molecule (2). The new circular molecule formed (3) consists of the original vector plus a segment of foreign DNA inserted within the vector.

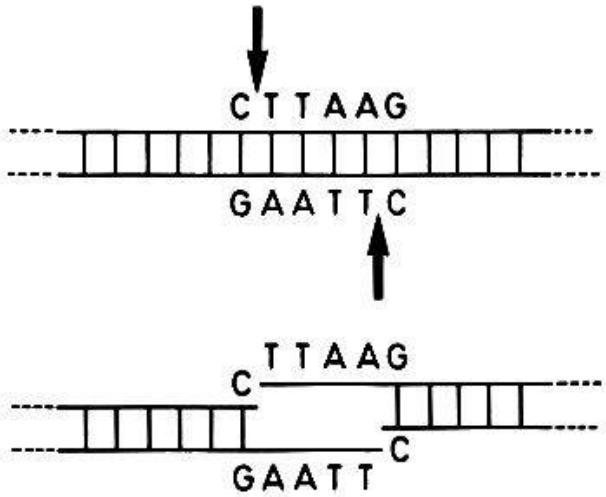

Fig. 2. The cleavage site of the restriction endonuclease EcoRI. The two arrows indicate the two specific simple strand breaks introduced by that particular enzyme. The resulting DNA fragments have complementary ends.

The joining of two DNA molecules can be performed by two general procedures.

1. Joining by pairing of complementary single stranded ends ("cohesive ends").

   There are two ways by which single stranded ends can be generated in a DNA molecule.

   a) Homopolymeric "tails" can be added by an enzymatic elongation on the 3' end of each of the two DNA strands. If one preparation of DNA is thus "tailed" with a stretch of poly-A and another with a stretch of poly-T, for instance, the two different DNAs will be able to anneal. The same applies to DNAs prepared with poly-G and poly-C (1, 2, 3).

   b) Certain restriction endonucleases (4) cut the DNA in staggered way and generate on each side of the cleavage point a short single strand sequence. These are obviously complementary. Thus DNA fragments resulting from such type of cleavage can be linked by the complementarity of their single stranded ends and ligated to reform a continous molecule. An example of such a staggered cut is illustrated in Figure 2. The two arrows indicate each of the cleavages produced by the enzyme EcoRI, and the complementarity of the two ends produced.

2. Joining by "blunt-end" ligation

   In addition to the linkage procedures described above, and which are based on the complementarity of short single strand extensions, it is possible to simply join two DNA molecules end to end with an enzymatic ligation. As an extension of that procedure, it has been possible to join to a given DNA preparation short DNA segments, also by blunt

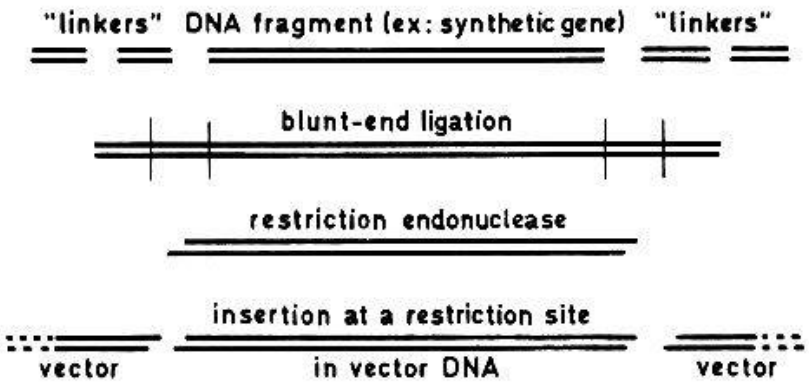

Fig. 3. Schematic representation of the use of specific DNA "linker" molecules for the insertion of a DNA fragment at a given endonuclease restriction site. See text for explanations.

end ligation. If these short segments, referred to as "linkers", contain a specific site for a restriction endonuclease (see 1b above and Figure 2), then digestion with that enzyme will generate the specific single strand extensions (5). Consequently the original DNA molecule can be inserted at the position of that restriction site in a vector molecule such as a plasmid. This rather complex but very useful procedure is schematically illustrated in Figure 3.

It is important to realize that any of these different methods for joining DNA can be applied to any DNA and that DNAs from any possible source can thus be joined. At the level of the naked DNA, and in particular for these joining reactions, there is no influence of the origin of the DNA and any kind of hybrid molecules can be formed in vitro.

II. The vectors

We have seen that one of the two partners in the joining reaction is a vector molecule, capable of replication in its host cell. These vectors are either the DNA of bacteriophages or plasmids, or the DNA of animal viruses, depending in which types of cells the foreign inserted gene is to be multiplied and studied. The vector molecules are chosen according to a number of criteria. The DNA of these vectors should be capable of autonomous replication in its host cell. The insertion of a fragment of foreign DNA should not abolish the capacity of the vector molecule to replicate. Infection or transformation of the host cells should be possible with naked DNA. The circular vector molecule should be easily made linear by cleavage with a specific endonuclease. Another important feature is the presence in the vector of a gene which product will confer a particular phenotype on the infected cells such as resistance to an antibiotic. This vector-specific phenotype will permit the selection or screening of vector-containing cells.

The vectors most employed for the transfer of genes into bacterial cells have been bacterial plasmids and bacteriphages, in particular bacteriophage lambda. Many new strains of plasmids and of lambda have recently been constructed by genetic and biochemical recombination in order to produce the most favourable vectors for DNA cloning. Such features as the maximum size of the DNA segment incorporated, the possibility of selecting or screening for recombinant molecules, the amplification of vector molecules in the host cells and the crippling of the vector to make it unviable outside the laboratory environment have been given particular consideration.

More recently vectors have also been produced which can transfer and replicate DNA into animal cells. The virus most commonly used is the transforming virus SV40, of which only a segment, containing the sequences necessary for replication, is sufficient for this purpose.

III. Source and specificity of the genes inserted

Two very different situations exist depending on the source and the kind of DNA to be inserted into a vector.

1. Genes from cellular or genomic DNA

Because a given gene represents in the case of complex genomes (such as mammalian DNA) one part in one to ten millions, the cloning of unique genes confronts us with a difficult problem of specificity and selection. It was believed initially that the only possible approach consisted in the purification of DNA fragments specific for a given gene followed by its insertion into a plasmid or bacteriophage vector. Considerable efforts have indeed been applied to the difficult problem of gene purification, using various physicochemical fractionations, including preparative hybridisation. More recently, however, it has become evident that the effort should be concentrated in the screening and identification of recombinants rather than in gene purification. Indeed, very efficient in situ hybridisation procedures have become available (6), which enable to identify recombinants carrying a specific gene among several thousands of others. Consequently, the present strategy for the cloning of specific genes from genomic DNA involves only minimal enrichment for that gene in the DNA preparation, random insertion in a lambda bacteriophage vector ("shotgun"), and then screening by in situ hybridisation to phage DNA transferred onto sheets of nitrocellulose filters. This procedure can in principle allow the cloning and the identification of any gene for which a specific probe is available for the hybridisation step. This need for a pure probe is an important limitation and one way of constructing these will be mentioned below in connection with the cloning of "synthetic genes".

It is obvious that the cloning of genomic DNA will be the only way to study the organisation and structure of genes as they are arranged in the genome. It is therefore the procedure of choice when this is the objective. On the other hand, if the study is directed towards the expression of a given eukaryotic gene in a bacterial cell, it is likely that cloned genomic genes will in many instances not be useful for that purpose. The discovery of the discontinuity of most eukaryotic genes studied so far (7, 8, 9) makes it indeed necessary to envisage an alternative approach allowing the insertion of a continuous gene and its subsequent expression into the protein specified by that gene.

2. Genes synthetized in vitro ("synthetic genes")

a) As we have just seen, the difficult problem of identification of a unique gene requires the availability of a specific probe. To circumvent this complexity problem, which cannot be solved at the level of cellular DNA, it was envisaged to make use of messenger RNA, frequently available with a much lower degree of complexity, and in some cases even with considerable homogeneity. Attempts were therefore made to utilize the sequence information of mRNA for the enzymatic synthesis of a "gene", corresponding to that mRNA. It was possible to use complementary DNA (cDNA), made with reverse transcriptase, as the template for the synthesis of the second strand of DNA. Several procedures made that synthesis of doublestranded DNA in vitro possible (10). The synthetic DNA could then be inserted into an appropriate vector (generally a plasmid) by homopolymeric "tailing" method mentioned above (1, 1a). Alternatively, the use of "linkers", as described in 1, 2. above, has also been used for the joining of synthetic DNA to a plasmid vector. The possibility of synthesizing a specific DNA molecule in vitro from mRNA and to insert it into a plasmid has allowed the first cloning of a specific gene segment in the case of rabbit globin (3, 11), which was also the first cloning of a mammalian gene sequence. Since then, that procedure has been used for many other cases such as other globin genes (12, 13, 14), immunoglobulins (15), ovalbumin (16), insulin (5) and various other polypeptide hormones (17). It is generally applicable whenever a mRNA is available. If an appropriate identification procedure exists, the cloning procedure can even be successful with a heterogenous mRNA preparation as starting material.
Figure 4 (11) illustrates, in the case of a globin mRNA, the series of steps involved in the construction of "cDNA plasmids". On the left are indicated the reactions leading to the synthesis of double-stranded DNA from mRNA and the subsequent addition of poly-dG tails. On the right, a circular plasmid DNA molecule which carries a gene for resistance to Kanamycin, is schematized. Following cleavage by the restriction endonuclease EcoRI,

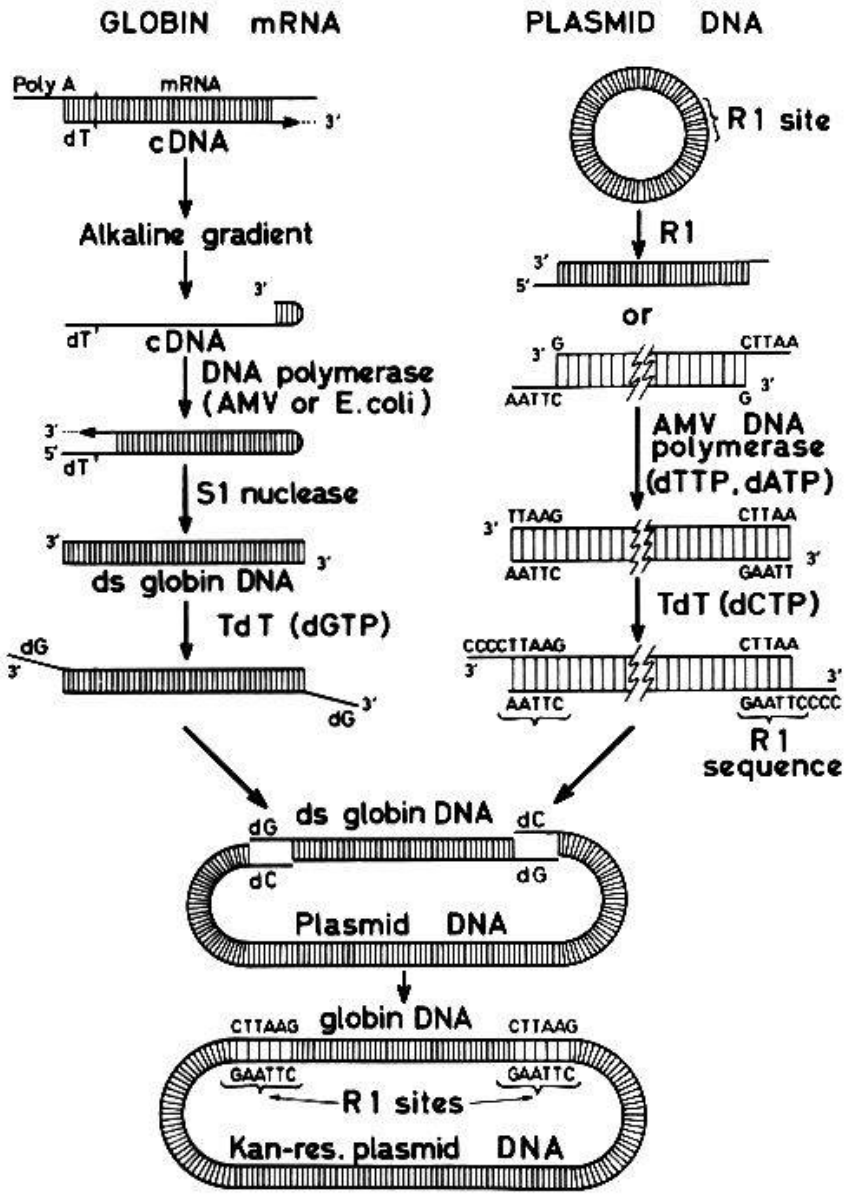

Fig. 4. Schematic representation of the steps involved in the enzymatic synthesis of a "synthetic globin gene" and in its insertion into a bacterial plasmid. TdT is terminal transferase. The plasmid used carries a gene for the resistance to Kanamycin. R1 is EcoR1 endonuclease.

and repair of the two ends of the linear molecule, elongation is performed, also with the enzyme terminal transferase (=TdT) but with poly-dC. Following annealing of synthetic DNA-dG and plasmid DNA-dC, the hybrid DNA molecules are used to transform sensitized E. coli cells and colonies resistant to the antibiotic Kanamycin are selected. Circular plasmid molecules can only be generated by the insertion of the dG-containing synthetic DNA.

b) As an alternative to the enzymatic synthesis of a gene from mRNA, the possibility also exists to rely on the chemical synthesis of a DNA segment. This technique is limited to relatively short DNA sequences (up to about 100 base pairs), but because the sequence is simply deduced from the amino acid sequence of a given polypeptide, the DNA cloned will be specific and the method does not require mRNA. This chemical synthesis has been utilized for the cloning of the hormone somatostatin (18) and of the A and the B chains of human insulin.

In general, the problem of the identification of clones carrying a specific gene following cloning is done a) by hybridisation in situ with a purified or semi-purified probe (for instance mRNA) (19), b) by the use of DNA from different recombinant plasmids for the inhibition of the translation in vitro of a given protein from mRNA (20), or c) by the identification of the specific protein product of the inserted gene in the bacterial cell, either by immunological (21) or by biological tests.

IV. Expression of foreign genes inserted in host cells

When the objective of the cloning experiment is that the inserted foreign gene be expressed into its mRNA and protein product in the host cell, a number of problems have to be dealt with at all the levels of the expression phenomenon.

1. The inserted gene must be under the control of a strong promotor of the plasmid or phage vector. This can be achieved by an appropriate choice of the vector and of the insertion site.
2. For the initiation of translation, a ribosomal binding site and an initiation triplet must be present. Rather than relying for these signals on the sequences of the inserted foreign gene itself, one has generally preferred to use the initiation sequences of an endogenous bacterial protein, next to which the foreign gene was deliberately inserted.
3. This later approach requires that the foreign structural gene sequence be joined in phase with the endogenous initiation triplet.
4. There are cases where a foreign protein is rapidly digested by bacterial proteases. This instability has seriously limited the successful expression of specific proteins in several cases. One way to deal with this problem is to select protease-free bacterial mutants; another is to attempt the "stabilisation" of the foreign polypeptide by a bacterial protein. To this effect, one can deliberately insert a foreign gene within the structural gene for a bacterial protein. The final product will consist of the initial (NH_2) portion of the bacterial protein followed by the polypeptide sequence specified by the inserted gene.

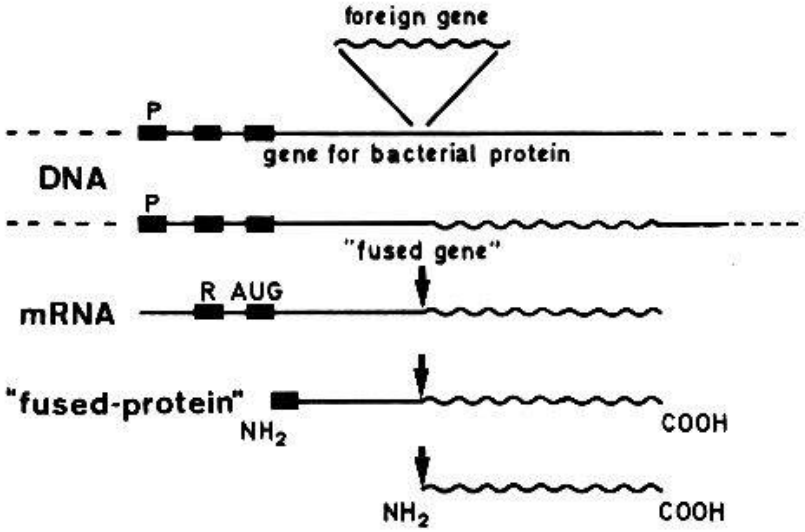

Fig. 5. Diagram illustrating the insertion of a foreign gene within a bacterial structural gene (fused gene). P, R and AUG refer to the bacterial promotor, ribosomal binding site and initiation triplet respectively. See text for explanations.

Fused genes produce "fused proteins" as depicted schematically in Figure 5. Much work will still have to be done to allow the recovery of the specific foreign protein from such "fused proteins".

5. Finally, an ingenious extention of the situation just described (see Figure 5) consists in the use of a bacterial protein which is normally secreted in the periplasmic space and out of the bacterial cell. Thus the amino end of the secreted bacterial protein serves as a "leader sequence" for the secretion of the foreign polypeptide chain as well. The first illustration of that mechanism is the secretion of insulin after the insertion of that gene within the β lactamase gene of an E. coly plasmid (22).

The rapid progress in the various levels of "expression" problem allows great hopes that efficient expression of many different inserted foreign genes will be obtained in bacteria.

This brief review has outlined the different steps involved in the cloning of specific genes with the use of appropriate vectors. It has stressed the basic difference between the cloning of genomic DNA (necessary for a certain kind of studies and almost impossible without an available probe) and the cloning of synthetic genes. The later can precisely provide the probe needed for the identification of specific cellular DNA clones. It is also the procedure of choice to achieve the expression of a foreign gene in the host cell, a technique which carries great promise for the future.

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