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Review Articles

Molecular Cloning of DNA

An Introduction Into Techniques and Problems

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Contents

Summary. Biochemical, biophysical and genetic studies of DNA segments of complex genomes are greatly facilitated by a variety of techniques, called molecular cloning of DNA, which permit propagation of single DNA segments of virtually any origin in bacterial cells. Molecular cloning requires in vitro recombination of DNA fragments with a prokaryotic genetic element (a plasmid or a bacteriophage DNA) which serves as replication vehicle (also called vector) in the bacterial host. The experimental conditions allow the production of bacterial clones each harboring a single fragment of exogeneous DNA out of an initially highly heterogeneous mixture of fragments. The following steps are involved: foreign DNA is first dissected into small pieces of up to some 17000 basepairs in length. The fragments are then joined in vitro to the vector molecules by means of well characterized DNA enzymes. The resulting recombinant molecules are introduced into the host cells by a

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transformation or transfection step. Among the progeny of transformed or transfected cells those clones are selected which carry a fragment of interest. Selection is in most cases accomplished by a combination of genetic and physical methods and is based on properties of the vectors as well as on attributes of the cloned foreign DNA. It is anticipated that bacterial host cells are not only suitable for amplifying DNA but also for the expression of useful functions which originate from other, preferably higher organisms. Two questions cannot be answered conclusively at present: first, is functional expression of genes generally possible in a heterologous cellular environment, and second, if it is possible, is it always harmless or does it create, at least occasionally, a biological hazard. Besides a detailed description of the techniques developed for molecular cloning, problems connected with functional expression and biohazards are discussed. In addition, results are presented which were obtained in the recent past by applying DNA cloning procedures.

Introduction

New experiences in molecular genetics and in biochemistry of nucleic acids have initiated experiments of possibly far reaching influence on scientific knowledge and technical developments in different areas. It has to a certain degree become feasible to recombine genes or parts of genes in vitro in arrangements which do not naturally exist, and to introduce them into living cells in such a way that the DNA is correctly replicated in the host. These recombination experiments have become known as "molecular cloning of DNA" or "recombinant DNA research".

Most of the experiments dealing with recombinant DNA are, at present, performed on the level of prokaryotic organisms. These organisms participate in DNA exchange and recombination events in vivo. Among the most powerful molecular tools in recombinant DNA research are bacterial plasmids and bacteriophages. Both are known for their ability to promote gene transfer and recombination in prokaryotes.

DNA recombination in prokaryotic organisms is not yet fully understood. It appears that a variety of mechanisms exist. One of them, designated general recombination, operates on the basis of nucleotide homology and requires in *E. coli* the function of the *rec*A gene (see Clark, 1973; Hotchiss, 1974). In addition, one or more *rec*A-independent systems are known which are involved in non-homologous or "illegitimate" recombination. The latter category operates with a variety of discrete DNA sequences which can be inserted into a bacterial genome at many sites. Since these sequences are able to move from one position in a genome to another one or even to another genome, they were designated translocatable (or transposable) genetic elements. Host genomes, plasmids and bacteriophage DNAs participate in the translocation of DNA in prokaryotes. At least three kinds of transposable elements have been identified: insertion sequences (IS), transposons (Tn) and certain bacteriophages (for recent reviews see Starlinger and Saedler, 1976; Kleckner, 1977). The bacteriophage lambda which is in use as cloning vector may itself be considered a complex translocatable

element (Kleckner, 1977). Transposons enclose genes for antibiotic resistance. They play a major role as selection markers for DNA cloning with plasmids. Insertion sequences are known to cause mutations in bacterial genomes through insertion and excision, events of low specificity with respect to their target sites in the genomes. In this article insertion sequences are not considered further.

DNA cloning is an experimental procedure which extends the "host range" for DNA of unrelated species in a very significant way. Exchange of genetic material between cells occurs in vivo, occasionally even between cells of different species. Some well known cases of plasmid mediated gene transfer cover a broad range of distantly related or even unrelated prokaryotic organisms (Datta et al., 1971). Cloning of DNA, however, goes beyond the limits of biological systems. It makes "gene transfer" possible between cells of species which most probably do not exchange genetic material in normal life. The stable maintenance of an eukaryotic gene in a prokaryotic organism is, at least to our knowledge, without precedence in nature.

In most cloning experiments bacteria are the hosts for foreign DNA. With respect to their biological characteristics these microorganisms are sometimes referred to as "hybrid organisms" or even as new "biotypes". These qualifications may not be regarded adequate. An *E. coli* cell which harbors a plasmid to which a piece of foreign DNA is covalently connected adds probably less than one thousandth to its own genetic complexity and in many cases the additional information will be more or less silent. Therefore the *E. coli* cell will at best acquire one or a few new properties, but this should not affect its identity as a bacterial organism or its forming part of a particular species. It is more appropriate to refer to the recombinant plasmids and phage DNAs as "chimeric" or "hybrid" DNA since the contribution of the foreign DNA segment to the vector genome, which promotes replication in the host cell, is significant at least with respect to the amount of DNA added.

Amplification and purification of DNA segments from complex genomes are the most noticeable results obtained so far with molecular cloning of DNA. The benefits of these results are obvious. A more detailed understanding of the structure and organisation of genes and genomes of all living organisms will be possible. Many still open questions related to replication of chromosomes, activation or repression of genes during growth, development and differentiation of cells and organs will probably be answered in the near future. The advantages of molecular cloning will eventually contribute to the understanding of pathological conditions, including hereditary diseases.

The prospects go, however, beyond the advancement of theoretical knowledge. The "implantation" of favorable hereditable properties into the genomes of higher (or lower) organisms are seriously considered together with its use for medicine or in areas of agriculture and industry. Some possibilities of useful applications will be discussed in the last chapter.

The general discussion about the consequences of recombinant DNA research includes the biological risks which may accompany the expected benefits. It should be pointed out and will be discussed in some detail later, that at present it is unknown what the risks are and whether there is a real basis for the assumption that potentially hazardous organisms could be created in vitro.

Molecular cloning of DNA should be distinguished from two other experimental designs both dealing with gene transfer initiated by in vitro manipulations and bypassing natural transfer barriers: the physical injection of either intact nuclei into enucleated eggs from a frog (Gurdon, 1968) or of DNA into the nuclei of intact oocytes (Mertz and Gurdon, 1977), and the biochemical transformation of animal tissue culture cells by the uptake of defined DNA segments or genes of viral origin (e.g. Bacchetti and Graham, 1977). In the case of injected nuclei no selection with respect to a distinct part of the genome is intended. In the two other cases no vector is involved and the transferred DNA is neither amplified nor recovered. The goal in these experiments is the study of functional expression of extraneous DNA by investigating the development of a whole organism or isolated transcription and translation events, or the transformation of a genetically defect cell line. These approaches point to useful extensions of molecular cloning, but they are not part of it.

Recombinant DNA research is a young discipline and moving fast. New and important results are coming up almost every month and the number of investigators in this area is still growing. The emphasis of the author, therefore, is not to present a complete survey of the results obtained with DNA cloning to date, but rather to provide first, a detailed description of the technical and procedural prerequisites of cloning experiments and, secondly, the discussion of some essential aspects related to functional expression of cloned genes and, in addition, biohazard problems. Since the new techniques are interesting not per se but for the results which were not available without them, a separate chapter is enclosed which attempts to give a representative view on existing results. This survey is presented in the form of tables of the genes and DNA fragments reported as cloned. For a variety of results additional information is given about the goals of individual cloning experiments. The purpose of this presentation is to provide a summarizing outline about what kind of experiment can be done with molecular cloning and what type of result may be expected in the near future.

The Principles of DNA Cloning Experiments

Amplification and cloning of DNA fragments are the result of an experimental procedure in the course of which pieces of DNA of different origin are joined together in vitro, to composite molecules. These "hybrid" DNA molecules are able to infect some host cell and to replicate autonomously within the host after infection. Such an experiment is, in the most general terms, characterized by the transfer of genetic material from an environment of high complexity into one of low complexity. The genome of an *E. coli* cell is estimated to contain up to 5000 different genes. Most corresponding estimates for higher cells range somewhere between 5000 and 50,000 or even more genes. Thus a DNA segment from the genome of any living cell containing a gene or at most a couple of genes comprises a very small part of the total genetic information. Plasmids or viral DNAs to which a DNA segment may be transferred are small, yet autonomous, genetic elements containing not more than approximately 30 genes, in many cases

even less. This transfer, depending on the plasmid or viral DNA used, is accompanied by a very significant increase in the number of copies per cell. This increase is the amplification component in the experiments to be described. The second component, the cloning, is concerned with the purification of one DNA segment out of a mixture containing a large number of different segments. Cloning results from the fact that under suitable conditions an infected cell contains only one composite DNA molecule and, hence, amplifies only that DNA segment which is part of the infecting small "hybrid" genome. Since single bacterial clones are easily detected and grown to large quantities purification and amplification may be considered as two results from one experiment. In effect, bacterial cells perform what cannot be achieved in vitro, they produce large numbers of homogeneous copies of DNA segments of virtually any origin.

Two different DNA elements are brought together in these experiments, the vector (or vehicle) providing the apparatus for autonomous replication and some genetic marker facilitating detection of amplified DNA clones in a population of infected host cells, and the DNA segment of interest. Small DNA molecules serve as vectors, as for instance bacterial plasmids or the DNA of the bacteriophage λ. The vectors are either covalently closed circular DNA molecules (plasmid) or linear DNA molecules (λ DNA). Insertion of a piece of foreign DNA into a vector genome requires cleavage of the latter at a site where insertion destroys neither the capacity to replicate autonomously nor the function which serves later as selective marker. The foreign DNA segments which are to be cloned are obtained after isolation from their natural sources by controlled mechanical or enzymatical cleavage. Coupling to the vector molecules is in most experiments achieved by the spontaneous formation of hydrogen bonds between complementary single-stranded extensions at the 5′ ends of the cleaved vectors and the foreign DNA segments, followed by covalent joining through the action of DNA ligase. In the case of plasmids the hybrid vector (or recombinant DNA) results from the correct connection of two components forming a new circular DNA molecule. In the case of lambda DNA three fragments (two λ fragments at either side of the foreign DNA segment), constitute the recombinant DNA. Propagation of the composite DNA occurs in most experiments within cells of the same species from which the native vector was isolated. The procedure leading to the uptake of recombinant DNA by the host cells is called transformation for plasmids and transfection for bacteriophage DNA. The general scheme of a cloning experiment is illustrated in Figure 1.

Essentially three different possibilities exist for obtaining DNA fragments from complex genomes through cloning. The first approach starts with a DNA fragment which is homogeneous when it is coupled to the vector, i.e. purification is preceding amplification and cloning. The specific advantage of this approach is that only minor problems arise in selecting the amplified fragment in the host cell. It is limited, however, to the rare cases where a sufficiently purified DNA fragment is available. Examples are synthetic DNA or DNA fragments obtained after reversed transcription of highly purified mRNAs or DNA fragments which can easily be enriched biochemically due to their high copy numbers in the genome from which they are isolated. In this approach the capacity to amplify DNA fragments is emphasized over that to purify them.

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