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Recombinant DNA Technology

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I. Introduction

Where ignorance of local manners—and language—has led me astray, I ask due pardon from the local proprietors.

J. D. Bu'Lock (1966)

Read not to contradict and confute, nor to believe and take for granted . . . but to weigh and consider.

Francis Bacon (1597)

This article is addressed to those biologists who have knowledge of molecular genetics but would like to be familiar with the literature dealing with the rapidly advancing forefront of recombinant DNA technology. Genetic recombination that results from the breaking and rejoining of DNA molecules is subject to strong taxonomic constraints *in vivo*. Exchange of genetic material rarely occurs between unrelated organisms, and even similar bacterial species may not exchange chromosomal genes. Plasmids and viruses can incorporate small pieces of host DNA in their own chromosomes and act as vectors for transference of these genes to closely related bacterial species. These vectors bearing chromosomal genes can either become integrated into the chromosome of the recipient cell or exist as independently replicating units in the cytoplasm.

A certain degree of DNA sequence homology is a prerequisite for genetic recombination *in vivo*. However, recombination between DNA molecules *in vitro* is not subject to these taxonomic restrictions. Consequently, genetic material from organisms of quite diverse origins with little DNA sequence homology can now be attached into an appropriate vector and introduced into a host cell for propagation and expression. The possibility of cloning DNA of higher organisms into a genetically well-characterized haploid microbe such as *Escherichia coli* is an important recent development in applied microbiology. This new technology has paved the way for the propagation of lines of organisms that are genetically alike and that all contain identical recombinant DNA molecules. A variety of novel genetic combinations in microorganisms can thus be created.

This new technology also makes it possible to isolate defined genes and to prepare large quantities of homogeneous fractions of DNA from organisms whose genetics and biochemistry are not well known. Microorganisms that have been used to benefit man for centuries can now be genetically modified by implanting foreign genes into them. Furthermore, mammalian proteins and other useful products can be synthesized through the use of microorganisms genetically modified by the implantation of foreign genes. Ultimately, this new technology will surely accelerate the understanding of the regulatory biology of the organisms that have thus far been refractory to *in vitro* methods of genetic analysis (Dawid and Wahli, 1979; Sinsheimer, 1977; Setlow and Hollaender, 1979; Maniatis, 1980).

Recombinant DNA technology generally involves several steps:

1. DNA fragments coding for proteins of interest are synthesized chemically or isolated from an organism.
2. These DNA fragments are inserted in a restriction endonuclease cleavage site of the vector that does not inactivate any gene required for the vector's maintenance and selective marker.
3. The recombinant DNA molecules are then introduced into a host to replicate using the replication origin of the vector.
4. Recipient host cells that have acquired the recombinant DNA are selected. Selection pressure is applied to enrich bacteria with a selectable marker.
5. Desired clones are then characterized to ensure that they maintain true copies of the DNA segment that was originally cloned.

II. Plasmid DNA Purification

Plasmids are widely used as vectors and methods of their isolation can vary depending upon the host organism. In *E. coli*, many methods are used for isolating plasmid DNA free from chromosomal DNA. Plasmid-harboring *E. coli* is grown in any rich medium such as Typticase Soy broth or a minimal salt solution supplemented with casamino acids.

In certain plasmid isolations, such as ColE1 derivatives, where protein synthesis is not required to initiate plasmid DNA replication, 300 μg/ml of chloramphenicol is added in the log phase of growth. This selectively inhibits chromosome duplication and allows amplification of plasmid DNA so that 45% of total cellular DNA is plasmid (Clewell, 1972). Cells are centrifuged and suspended in 50 m*M* Tris buffer containing 25% sucrose. Lysozyme in the presence of EDTA is added to hydrolyze the peptidoglycan layer of the cell wall. Protoplasts thus formed are then lysed by addition of the anionic detergent sodium dodecyl sulfate so that the chromosome remains attached to the cell envelope. Sodium chloride is added to 1 *M* concentration to precipitate chromosomal DNA and cell debris. After centrifugation, the supernatant containing plasmid DNA is removed (Guerry *et al.*, 1973). Usually, phenol or pronase and ribonuclease are used to remove protein and RNA. The plasmid DNA is subsequently precipitated with ethanol or with polyethylene glycol (Johnson and Gunsalus, 1977). The DNA is then purified by CsCl density-gradient centrifugation in the presence of ethidium bromide (Martens and Clayton, 1977).

Other procedures are now available for isolating plasmids from *E. coli* (Ohlsson *et al.*, 1978; Colman *et al.*, 1979), pseudomonads (Johnson and Gunsalus, 1977; Palchaudhuri and Chakrabarty, 1976; Hansen and Olsen, 1978); *Bacillus subtilis* (Gryczan and Dubnau, 1978); *Serratia marcescens* (Timmis and Winkler, 1973); *Streptococcus* (Clewell *et al.*, 1974); *Streptomyces* (Bibb *et al.*, 1978; Malik, 1977, 1978; Schrempf *et al.*, 1975; Yagisawa *et al.*, 1978), *Agrobacterium* (Currier and Nester, 1976); yeast

(Zakian *et al.*, 1979); and other eukaryotic cells (Shoyab and Sen, 1978). It is extremely important that DNA be free from contaminating nucleases and RNA species. Sometimes DNA has to be purified by polyacrylamide gel electrophoresis, sucrose gradient centrifugation, or column chromatography before it can be further used (Hardies and Wells, 1976, 1979; Hardies *et al.*, 1979).

III. Isolation of Genes

Chromosomal location of DNA sequences of a gene affects the ease with which the genes are isolated. On the basis of phenotypic effect and organization in the chromosomes, genes can be subdivided into the following groups:

1. Simple genes. Their product is a single polypeptide or single RNA (e.g., ribosomal RNA, tRNA, insulin, histone, ovalbumin, or monomeric enzymes). Such genes are the easiest ones to isolate.

2. Complex genes. Many multimeric proteins are aggregates of several nonidentical polypeptide subunits. Some require cytochromes and specific cofactors for their biological activity. All of these genes coding for the various parts of the functional enzyme aggregate may not be clustered together on the chromosome, e.g., the genes that code for the tryptophan synthetase, fatty acid synthetase, mixed-function oxidase, hydroxylases, and enzymes that oxidize drugs but require cytochrome P-450.

3. Operons. Many genes specifying degradative and biosynthetic pathways are clustered adjacent to each other on the chromosome, and these gene clusters are called operons. Examples are *lac* operon and histidine operon (Miller and Reznikoff, 1978).

4. Regulon. Certain genes specifying a biosynthetic function such as arginine biosynthesis are organized in several minioperons widely scattered on chromosomes. However, all such genes are regulated coordinately and have been called regulons.

5. Multiple regulons. Many products, e.g., antibiotics, are assembled from precursors derived from several different pathways. For example, L-cysteine, L-valine, and L-α-aminoadipate are involved in the biosynthesis of β-lactam antibiotics (Malik, 1980). Multiple regulons participating in the synthesis and regulation of the biosynthesis of such complex molecules are probably scattered all over the chromosome. Isolation of all genes involved in antibiotic synthesis can therefore be a difficult task.

Since genes are composed of DNA stretches, to isolate genes one must first isolate DNA. Purified cytoplasmic organelles such as mitochondria and chloroplasts can be used to obtain their DNA. However, isolation of specific genes from total DNA is difficult and many properties of genes are used to advantage to isolate the genes. Human genome contains about 10^7 globin gene-sized DNA sequences, of which approximately 60% occur in one or a

few copies. To increase the probability of success in cloning a particular gene from such a highly complex genome, it helps to enrich DNA sequences before cloning into a vector. A combination of several methods could yield an enrichment of several thousandfold. Genes located on plasmids or phages are greatly enriched by purifying the plasmid or phage DNA. Many genes involved in aromatic and sterol metabolism (Chakrabarty, 1978), heavy metal transformations, and drug resistance are located on plasmids. Genes for the insecticide toxin produced by *Bacillus thuringiensis* may be located on a bacteriophage (Perlak *et al.*, 1979; Templeton *et al.*, 1979). After preparations that have been enriched for a desired gene, recombinant DNA technology is used to isolate the genes from such preparations.

A. Selection for Function

Many genes can be selected by their function if mutants of a host organism lacking that function are available. Cohen and Chang (1973) were the first to take advantage of the fact that certain genes determining resistance to antibiotics were located on plasmids. These genes were enriched by purifying plasmid DNA. Their isolation was therefore made easier by selecting clones that harbored genes responsible for resistance to antibiotics. These authors reported the *in vitro* construction of DNA molecules that combined genetic information from two different *E. coli* plasmids carrying drug-resistance markers. These recombined plasmids were inserted by transformation into drug-sensitive *E. coli*. The transformant clones were selected by directly plating cells on nutrient agar plates supplemented with antibiotics. On such media, only cells that acquired recombinant plasmids survived since the host was killed by antibiotic. Cohen and Chang were also the first to insert the penicillinase gene from a gram-positive bacterium *Staphylococcus* plasmid into an *E. coli* plasmid. The penicillinase gene was expressed and maintained when the chimeric plasmid was introduced into *E. coli*.

A thymidylate synthetase gene from the *B. subtilis* bacteriophage ϕ3T has also been cloned and expressed in *E. coli*. Phage ϕ3T DNA was digested with *Eco* RI and ligated to an *Eco*RI-digested pMB9 plasmid of *E. coli*. This recombinant DNA was used to transform a thy^- *E. coli*. The desired transformants had a thy^+ tet^R phenotype (Ehrlich *et al.*, 1976). These recombinant plasmids replicated in *E. coli*, utilizing the origin of DNA replication of the *E. coli* vector plasmid.

Such experiments did not tell whether genes of gram-positive organisms were being transcribed in *E. coli* from a promoter of the vector or from their own promoter. Base sequences of regulatory elements, e.g., promoters, could be very different in different organisms. The level of gene expression in a given organism could vary depending upon the efficiency of interaction between regulatory sequences and corresponding protein molecules.

1. *Genome Library*

Techniques for the construction and screening of a complete library of clones for any genome is a practical starting point for amplifying specific genes (Wensink *et al.*, 1974; Benton and Davis, 1977; Sternberg *et al.*, 1977; Maniatis *et al.*, 1978; Dodgson *et al.*, 1979; Gergen *et al.*, 1979). A complete library of genome fragments is a set of independent clones that, statistically, contains the entire genome among the recombinant DNA molecules (Clarke and Carbon, 1976). Complete sets of DNAs can be maintained if no segment is disruptive to the vector or host. If every DNA segment is transformed and maintained with equal efficiency, then the probability (P) of the presence of a desired DNA sequence in a genome bank is as follows:

$$P = 1 - \left[\left(1 - \frac{x}{L}\right) F\right]^N$$

where x = length of desired segment of DNA, L = average length of DNA fragment cloned, F = fraction of genome represented by an average fragment, and N = number of transformant colonies containing cloned DNA.

Clarke and Carbon calculated that 720 transformed colonies will give a probability of 0.90 that one *E. coli* gene was cloned with an average molecular weight of 8.5×10^6. However, the probability increases to 0.99 with a genome bank of twice this size.

A library of soybean genome (Breiner *et al.*, 1979) and chicken genome has been constructed in the vector λ charon 4A (Maniatis *et al.*, 1978). Chicken DNA was partially digested with restriction enzymes *Hae*III and *Alu*I. Fragments of 14–22 kb were purified by sucrose gradient fractionation. This DNA was methylated by *Eco*RI methylase, ligated to synthetic dodecameric linkers, digested with *Eco*RI restriction enzyme, and refractionated on sucrose gradients. These fragments were then ligated to purified charon 4A left and right arms joined at the λ cohesive end site. The ligated concatemeric DNA was treated with the *in vitro* packaging extracts. Packaged phages were purified by cesium chloride centrifugation. These phages were amplified by growth on plates at subconfluent densities. Several independent globin gene-containing recombinants were selected from the chicken genome library by screening with adult and embryonic globin cDNA as a probe (Dodgson *et al.*, 1979).

2. *Screening of Genome Library*

a. Auxotrophic Complementation. Many genes have been identified by transforming auxotrophs with recombinant molecules. For making genomic banks in *E. coli*, the recipient is usually F^+. This allows transfer of recom-

binant plasmids to other *E. coli* auxotrophs (Miller, 1972) and provides a rapid method for identifying a recombinant plasmid containing a specific gene from a genome bank of randomly cloned genome fragments (Clarke and Carbon, 1976; Latrou *et al.*, 1980). Whenever applicable, this procedure is extremely rapid, since a large number of colonies from a genome bank can be screened with little effort. Many individual colonies from the genome library can be grown in a grid on a master plate and replicated to a lawn of recipient auxotroph cells on the appropriate selective media. However, the only genes selected by this method are those for which a bacterial mutation exists and that are expressed in the bacterial host. The expression of the desired gene can vary depending on the vector, cloning site, and host cell; and such a selection may not produce the gene of interest even though the source of a gene is a bacterium related to the host organism.

Methods have been developed for the selection of DNA sequences that complement metabolic mutations and structural defects such as those involved in mutant flagellar genes (Silverman and Simon, 1977; Schell and Wilson, 1979; Walz *et al.*, 1978; Moore and James, 1979; Vapnek *et al.*, 1977; Chinault and Carbon, 1979; Gardner, 1979; Struhl *et al.*, 1976). Recombinant molecules carrying specific genes have been selected directly by using appropriate *E. coli* auxotrophs as host. Restrictionless strains of *E. coli* that are transformed with high efficiency are usually used as recipients. Genes that complement *trp*, *gal*, *lop*, *lacZ*, *ara*, *thyA*, *leu*, *bio*, and many others have been identified in this manner (Clarke and Carbon, 1976; Hershfield *et al.*, 1974; Berg *et al.*, 1976; Collins *et al.*, 1976; Cohen *et al.*, 1978).

A *Saccharomyces cerevisiae* DNA segment of 4700 base pairs containing the galactokinase gene (*gal-1*) has been cloned in pBR322 (*Hin*dIII site) and maintained in an *E. coli* strain that carries a deletion in its own galactokinase gene (*galK*). The yeast gene was shown to be present (1) by complementation of the *E. coli* galactokinase deletion, (2) by hybridization of the cloned DNA fragment to the restriction enzyme digest of total yeast DNA, and (3) by demonstration of yeast galactokinase, a monomeric protein, in cell-free extracts of the *E. coli* harboring the plasmic that contains the yeast galactokinase gene. The yeast galactokinase activity in *E. coli* extracts is 0.7% of the bacterial galactokinase activity present in wild-type *E. coli* cells fully induced with fucose. *E. coli* cells lacking a functional galactokinase gene but harboring the yeast galactokinase gene grow slowly in minimal medium containing galactose as the sole carbon source. Such cells have a generation time of 14.3 hours (Citron *et al.*, 1979). The replication of pBR322 is under relaxed control and 50–100 copies per cell of the hybrid plasmid containing the yeast galactokinase gene could be expected. These multiple copies of the gene per cell should allow production of galactokinase enzyme in sufficient

amount for good growth of the host, considering that turnover numbers *in vivo* of the *E. coli* and yeast galactokinase enzymes are similar. The yeast galactokinase gene is expressed in *E. coli* very poorly. This decreased expression of yeast gene in *E. coli* could be due to inefficient transcription, translation, posttranscriptional or posttranslational modification of galactokinase, or a combination of all. St. John and Davis (1979) have also reported the cloning in *E. coli* of yeast galactose-inducible sequences selected by another technique. The similarity between sequences cloned by St. John and Davis (1979) and Citron *et al.* (1979) cannot be assessed by the data given. *Saccharomyces cerevisiae* genes coding for β-galactosidase (Dickson and Martin, 1978) and several genes involved in other biosynthetic pathways (Struhl *et al.*, 1976; Carbon *et al.*, 1977; Ratzkin and Carbon, 1977; Struhl and Davis, 1977; Clark and Carbon, 1978; Bach *et al.*, 1979) are expressed when cloned in *E. coli*. Several yeast genes, for example, *leu-2*, *his-3*, *ura-3*, *trpG*, and *adh-2*, have been isolated by complementation test (Williamson *et al.*, 1980). Yeast cells carrying an *ampR* gene of *E. coli* plasmid ligated to a yeast replicon produce penicillinase (Chevallier and Aigle, 1979).

b. In Situ Hybridization. Colonies or phage plaques with the desired gene can be identified *in situ* by hybridization to a radioactively labeled RNA probe complementary to the desired gene (Grunstein and Hogness, 1975). This procedure is used when the desired clones cannot be assayed for the function of the gene of interest. Under such circumstances, DNA sequences for the gene of interest are detected by taking advantage of the complimentarity of the RNA or DNA probe.

For each screening of the library, petri dishes (150 mm) with up to 10 plaque-forming units (PFU) or colonies per plate can be used. From each petri plate, viral plaque DNA or colonies are fixed to the nitrocellulose filters and lysed, and the DNA is denaturated by alkali treatment (Benton and Davis, 1977). Colonies or plaques that hybridize with ^{32}P-labeled probe RNA or cDNA of the genes of interest are located by autoradiography or fluorography. Duplicate positive plaques or clones can be purified through several further platings at low plaque density. Colony hybridization has been used widely. Similar procedures have been developed for SV40 plaques (Villarreal and Berg, 1977), and have been used to isolate *Drosophila* tRNA genes (Dunn *et al.*, 1979) and ribosomal DNA of *B. subtilis* (Moran and Bott, 1979), soybean (Breiner *et al.*, 1979), and *Dictyostelium* (Maizels, 1976)

c. In Situ Immunoassays. Radioisotopes such as ^{125}I have been used for many years as labels for monitoring the distribution of reagents in immunological assay systems. Nonradioactive labels such as bacteriophages, enzymes, stable free radicals, and fluorescent and chemiluminescent groups

have also been used (Simpson *et al.*, 1979; O'Sullivan *et al.*, 1979; Harris *et al.*, 1979). Radioimmunoassays have been used to identify genes for which *E. coli* mutants do not exist (Henning *et al.*, 1979). The method depends on the presence of the gene product and requires expression of the cloned gene in the host. Both viral and phage vectors can be used, since the technique is applicable to phage plaques as well as colonies.

Broome and Gilbert (1978) developed a very sensitive solid-phase radioimmunoassay capable of detecting picograms of specific antigens. This pioneering methodology was first used to select *E. coli* clones that synthesized rat proinsulin (Villa-Komaroff *et al.*, 1978). Such assay can be used to screen clones for the presence and expression of specific gene sequences. Because of the extreme sensitivity of this assay, it can detect even the very low level of expression resulting in the synthesis of only a few molecules per cell. Hybridoma technology can be used to make specific monoclonal antibody (F. Melchers *et al.*, 1978). The combination of radioimmunoassays with gel electrophoresis can be used to characterize immunoreactive proteins (Secher and Berke, 1980).

Enzymes involved in the biosynthesis of peptide antibiotics have been purified to homogeneity (Kleinkauf and Koischvitz, 1980). Such pure enzymes could now be used to make specific antibodies needed for a radioimmunoassay to isolate the genes involved in peptide antibiotic synthesis.

d. Sib Selection. This method can be used to isolate clones from a genome library. The whole genome library is divided into pools, each containing many different clones. Each pool is tested by the pertinent assay procedure for the presence of a desired gene. That pool containing the gene of interest is subdivided and retested until the individual clone is identified. Sea urchin histone genes and human interferon genes have been isolated in this way (Kedes *et al.*, 1975; Bylinsky, 1980; Gilbert and Villa-Komaroff, 1980).

B. Physical Differences

1. Density

If the gene differs significantly in base composition from the total cellular DNA and is present in multiple copies per genome, then the repeated sequences band in CsCl gradient as a satellite in addition to the main chromosomal band. Ag^+ or Hg^+ increases such density differences. Ribosomal RNA genes of *Xenopus laevis* were isolated on the basis of density differences and were the first eukaryotic genes to be cloned in *E. coli* (Morrow *et al.*, 1974).

Amplified rRNA genes of *Tetrahymena* are free from chromosomes in the macronucleus of *Tetrahymena* (Din and Engberg, 1979) and can be easily separated on sucrose gradient or by agarose gel electrophoresis.

An *Eco*RI-generated fragment of plasmid pSC122 was purified by equilibrium centrifugation in cesium chloride (Timmis *et al.*, 1975). This fragment coding for ampicillin resistance had a buoyant density of 1.692 gm/cm^3 whereas the remaining DNA fragment banded at a buoyant density of 1.710 gm/cm^3.

Firtell *et al.* (1976) enriched recombinant DNA molecules of *Dictyostelium discoideum* (22% GC) and pSC101 (50% GC) by fractionation on a CsCl density gradient. The region between 30 and 40% GC yielded about 85% hybrid molecules, whereas the initial population contained 2–5% hybrids. The approach used by Firtell *et al.* (1976) could be used to enrich recombinant molecules with pieces of *Streptomyces* genome (70% GC) if they could be ligated with homopolymer (dGidC) tailing into a vector with low GC content.

2. *Size*

Many enrichment procedures utilize information about the size of the DNA fragment required for the desired gene.

a. Gel Electrophoresis. Restriction enzyme-generated fragments are separated according to size by gel electrophoresis (Southern, 1979a). Bands containing a gene can be designated (Helling *et al.*, 1974) when the fragment order of a simple genome such as that of a plasmid or phage is known (Table I). For organisms with a high efficiency of transformation, the bands of endonuclease-digested DNA can be isolated after electrophoresis and correlated with specific genes after transformation (Young *et al.*, 1977; Brown and Carlton, 1980; Mazza and Zalizzi, 1978).

Gel electrophoresis is routinely used for determining the fragment composition of recombinant DNA molecules constructed by ligation of cohesive ends generated by restriction enzymes. Digestion by the same restriction enzyme regenerates the original DNA fragments and such fragments are easily identified by gel electrophoresis. As little as 10 μg of DNA per band can be visualized by staining the gel with ethidium bromide, which fluoresces under UV light (Helling *et al.*, 1974). DNA can be extracted from these resolved bands and used for further analysis with various restriction enzymes, nucleotide sequencing, or transformation. Agarose gel electrophoresis is best suited for characterizing fragments above 1.5 kb. For smaller fragments, polyacrylamide gels are used. Standards of λ and ϕX174 DNA already cleaved with restriction enzymes are commercially available

TABLE I
FRAGMENT SIZES[a] PRODUCED BY RESTRICTION ENDONUCLEASE DIGESTION OF λ AND φX174 GENOMES

	λ			φX174		
Fragment number	*Hin*dIII	*Eco*RI	*Bam*HI	*Hae*III	*Hae*II	*Taq*I
1	23,720	21,800	17,400	1,353	2,314	2,914
2	9,460	7,550	7,300	1,078	1,565	1,175
3	6,670	5,930	6,760	872	783	404
4	4,260	5,540	6,470	603	269	327
5	2,250	4,800	5,540	310	185	231
6	1,960	3,380	5,540	281	123	141
7	590			271	93	87
8	100			234	54	54
9				194		33
10				118		20
11				72		

[a] In base pairs.

and can be used as markers for determining size of unknown DNA fragments.

Digestion of *E. coli* DNA with *Eco*RI restriction enzyme yields about 500–600 fragments differing in molecular weight, base composition, and sequence. When this digest is examined by agarose gel electrophoresis, a featureless pattern is obtained. However, Fischer and Lerman (1979) have developed a two-dimensional electrophoretic method that resolves the large number of components of an *Eco*RI-digested *E. coli* chromosome into 350 discernible spots. The significance of this technique is demonstrated by the detection of four spots unique to a digest of *E. coli* lysogenized by λ. These four spots were not observed in uninfected *E. coli*, but two of these spots were due to λ phage DNA alone. Creighton (1979) used a similar procedure in a study of protein denaturation.

Rapid and sensitive procedures for detecting plasmids by agarose gel electrophoresis have been developed (LeBlanc and Lee, 1979). Plasmids present in one copy per cell with a molecular weight of up to 1.5×10^8 can be identified. One colony is sufficient to obtain a fragment pattern of a recombinant plasmid after restriction endonuclease cleavage (Eckhardt, 1978; Klein *et al.*, 1980).

Southern (1975) developed the technology for studying gene arrangement directly. High-molecular-weight DNA is cleaved with a restriction enzyme and separated into fragments by agarose gel electrophoresis. The DNA is