Restriction Enzymes: New Tools for Studying DNA

Bacteria, despite their small size, have an effective mechanism for defense against invading DNA's. Restriction enzymes—enzymes that can recognize and cleave foreign DNA—are part of this defense mechanism. These enzymes are extremely specific; each one cuts only within certain well-defined base sequences in double-stranded DNA molecules. Because of this specificity, restriction enzymes are now proving to be advantageous not just to bacteria but also to investigators who are using them to attack fundamental problems of DNA structure and function.

Not all DNA's are recognized as foreign by a restriction enzyme in a particular bacterial strain. For example, the restriction endonucleases (restriction enzymes are endonucleases because they cut DNA strands in the interior of the molecule) must not attack the cell's own DNA, for this would be equivalent to suicide. Moreover, the DNA from a virus previously grown in one strain can infect and reproduce in other cells of the same strain without being degraded by a restriction enzyme.

A DNA molecule is protected from restriction enzymes by the activity of another type of enzyme called a modification methylase. The methylase attaches methyl groups to specific sites on the DNA—apparently the same sites recognized and attacked by the corresponding endonuclease. The presence of the methyl groups prevents the restriction enzyme from cutting the DNA. There are several restriction-modification systems differing in their specificities for the nucleotide sequence they recognize. A DNA modified by the methylase of one system is protected from the endonuclease of that system but it is still susceptible to a restriction enzyme with a different specificity.

Restriction enzymes are widely distributed throughout the bacterial world; however, the enzymes of *Escherichia coli* and *Hemophilus influenzae* are the best characterized thus far. These enzymes can be subdivided into two classes depending on whether they require the cofactors adenosine triphosphate, *S*-adenosyl methionine, and magnesium ions for their activity (type I enzymes), or whether they require only magnesium ions (type II enzymes).

The restriction endonucleases from *E. coli* strains B and K are type I enzymes. Matthew Meselson and his colleagues at Harvard University, Cambridge, Massachusetts, isolated and characterized endonuclease K. This enzyme is large and complex in structure; it is composed of three kinds of subunits. Recently, Meselson found that a purified preparation of the restriction endonuclease also catalyzes the methylation reaction.

The modification and restriction system of *E. coli* strain B is genetically related to that of strain K. Stuart Linn and his associates at the University of California, Berkeley, have been investigating the subunit structures and mechanisms of these enzymes. According to Linn, the methylase consists of two kinds of subunits; the same two are also found in endonuclease B, plus a third subunit that presumably converts the methylase to the endonuclease.

Type II Restriction Enzymes

The type II enzymes are smaller and simpler in subunit composition than the type I enzymes. Moreover, they appear to be more specific in their cleavage sites. Norton Zinder and Kensuke Horiuchi at the Rockefeller University, New York, found that although endonuclease B attaches to DNA at specific sites—the same ones recognized by the methylase—it does not appear to cut the molecule there; the enzyme may move along the DNA before cleaving it. It is not known whether specific base sequences are required at the actual cleavage sites.

Hamilton Smith and Thomas Kelly at Johns Hopkins University School of Medicine, Baltimore, Maryland, determined the sequence of bases at the cleavage site for a type II restriction enzyme, endonuclease R isolated from *H. influenzae*. The sequence is shown in Fig. 1a. It consists of six bases, with ambiguity in the bases in the center of the sequence. Either purine (adenine or guanine) or pyrimidine (cytosine or thymine) may occupy the positions indicated. The outstanding characteristic of the base sequence is its symmetry; reading from the 5′ terminals, the base sequences of the two strands are identical. Smith thinks that this symmetry may be essential for the function of the enzyme—the cleavage at equivalent points of two DNA strands of opposite polarity.

In fact, the nucleotide sequences at the cleavage sites of two additional type II endonucleases are known and they possess the same kind of symmetry. Herbert W. Boyer and his colleagues at the University of California Medical Center, San Francisco, have determined the sequences recognized by these two restriction enzymes, both genetically controlled not by the *E. coli* chromosome but by drug resistance transfer factors. Drug resistance transfer factors are plasmids, DNA molecules that replicate independently of the bacterial chromosome; they may be transferred between bacterial cells. The substrate sequence for endonuclease RI is shown in Fig. 1b. This nucleotide sequence is also symmetrical but, unlike that of the endonuclease R substrate, the base ambiguity is at the ends of the sequence where either adenine or thymine are permissible.

Janet Mertz and Ronald Davis, working with Paul Berg at Stanford University Medical Center, Stanford, California, found that the cuts made by the RI enzyme are staggered in such a way that "sticky" or "cohesive" ends are formed. Since the single-stranded ends of the DNA fragments have complementary base sequences, the fragments, if separated, will reassociate under appropriate conditions. They can then be covalently linked by the enzyme DNA ligase. This means that any DNA fragments produced by the action of endonuclease RI can be joined together. According to Boyer, endonuclease RII also attacks a symmetrical sequence of nucleotides and generates cohesive ends.

Numerous investigators have recognized that the specificity, especially of the type II restriction enzymes, makes them valuable tools for studying DNA structure and function and for mapping the genetic and physical features of chromosomes. The importance of these theoretical and practical applications has in turn stimulated the search for new restriction enzymes with different specificities. Such enzymes are being found and characterized.

The use of restriction enzymes for the study of the chromosome of the simian virus SV40 illustrates some of their applications. The chromosome of

SV40 consists of a circular double-stranded DNA molecule containing about 5000 nucleotide pairs, enough to code for about eight proteins. This virus is a transforming virus; that is, its DNA can be incorporated into the genome of the cells of some animal species, and these cells then acquire properties characteristic of tumor cells.

Because SV40 DNA is circular, it lacks a reference point to which the structural and genetic features of the chromosome can be related. Recently, however, Carel Mulder and Hajo Delius at Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, and Paul Berg and his colleagues found that endonuclease RI cleaves the SV40 chromosome reproducibly at a unique site to yield a double-stranded linear molecule. The RI cleavage site may thus serve as a reference point.

Norman Salzman and his associates at the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, used endonuclease RI to cleave replicating molecules of SV40 DNA. They interpreted electron micrographs of the cleaved molecules as showing that there was a specific site for initiation of replication and that replication was bidirectional—progressing in both directions away from the initiation site. Salzman also showed that the initiation site was one-third the length of the SV40 genome distant from the RI reference site.

Restriction enzymes with different specificities generate fragments of viral DNA that have common regions. Such overlapping fragments are a prerequisite for mapping the genome and, ultimately, for determining the base sequences of genes. Daniel Nathans, Kathleen Danna, and their colleagues at Johns Hopkins University School of Medicine, found that endonuclease R from *H. influenzae* cleaved the SV40 chromosome into 11 fragments; a restriction enzyme isolated from *H. parainfluenzae* produced four fragments. They found that the site cleaved by the RI enzyme was within a particular fragment produced by the action of the *H. influenzae* enzyme. With this information, Nathans and Danna were able to construct a physical map of the arrangement of the fragments in the SV40 genome. Such a physical map is a preliminary step to developing a functional map showing the location of genes and punctuation and control sites on the chromosome.

For example, Nathans and Danna have identified the fragments containing the initiation and termination sites for replication of the SV40 chromosome. The location of the initiation site relative to the RI cleavage site found by them agrees with that found by Salzman. Nathans and Danna also concluded that replication is bidirectional, proceeding at the same rate in both directions until it terminates at a site 180 degrees from the initiation site. Since the fragments containing the start and stop signals for DNA replication are now known and can be isolated, sequencing of these punctuation sites may be possible. A direct method for the determination of the base sequence of such punctuation sites is necessary since they presumably produce no RNA or protein.

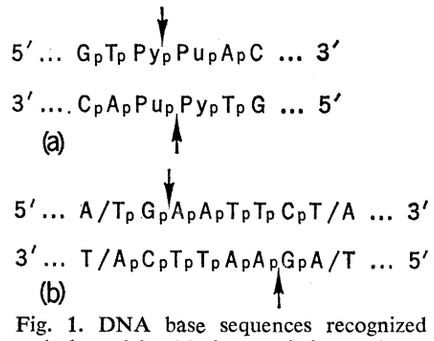


Fig. 1. DNA base sequences recognized and cleaved by (a) the restriction endonuclease of *Hemophilus influenzae* and (b) endonuclease RI from *Escherichia coli*. The arrows indicate the bonds cleaved; *A*, adenine; *T*, thymine; *G*, guanine; *C*, cytosine; *Pu*, purine; *Py*, pyrimidine; and *p*, phosphate.

Study of Transformation

The identification of the genetic functions required for transformation by a virus like SV40 is another important problem now being approached with the aid of restriction enzymes. Joseph Sambrook, Philip Sharp, and others at Cold Spring Harbor Laboratory are trying to determine whether the circular SV40 chromosome breaks at a specific point when it is integrated into the genome of a transformed cell. Cleavage of the integrated SV40 chromosome with endonuclease RI produces two SV40 fragments. (The enzyme cuts at the RI site within the SV40 chromosome and also cuts the host DNA on both sides of the integrated viral DNA.) If both the site of integration into the host chromosome and the site of SV40 breakage are specific, the sizes of the two pieces should remain constant in preparations from different transformed cell lines.

Adenovirus type 2 also transforms cultured cells in vitro, although it is not known to cause tumors in animals. The adenovirus 2 chromosome is a linear double-stranded DNA molecule. Sharp and Sambrook obtained six fragments when they used endonuclease RI to cleave this DNA molecule. They have been using these fragments to determine what portions of the adenovirus 2 genome are present in transformed cells. They have found that one line of transformed rat cells contained only about 40 percent of the viral genome. That 40 percent included one copy of each of three fragments and about one-third of another. The remainder of the genome was missing. Identification of the genes on the fragments incorporated into the rat cell genome could give some information about which genes are important for transformation and which are dispensable.

Determination of the actual sequences of the nucleotides in DNA should also be facilitated by the use of bacterial restriction enzymes. Because most of them cleave at specific base sequences, enzymes with different specificities can be employed to produce overlapping DNA fragments, just as proteolytic enzymes like trypsin and chymotrypsin have been employed for the analysis of amino acid sequences in proteins. Before such sequence analysis is accomplished, however, preliminary steps—the identification of interesting segments of DNA like production and control regions and the isolation of those segments—must be taken.

Although the genome of the bacteriophage ϕX174 is small—a single strand of only 5500 nucleotides—sequencing of a molecule of this size is not feasible. Marshall H. Edgell and Clyde A. Hutchison, III, at the University of North Carolina School of Medicine, Chapel Hill, are using restriction enzymes to prepare fragments of ϕX174 small enough to be sequenced. Since restriction enzymes do not cut single-stranded DNA, Edgell and Hutchison must employ the replicative form (RF) of the phage chromosome. (The RF is a double-stranded intermediate produced during replication.) Both the restriction endonuclease from *H. influenzae* and endonuclease Z, recently isolated from *H. aegyptius* in their laboratory, cleave the RF of ϕX174. Edgell and Hutchison had previously developed an assay that allows them to determine which genes are carried on a particular fragment of ϕX174. Since the order of the eight structural genes in the ϕX174 genome was already known, they are

now developing maps of the positions of the two types of fragments (those produced by endonuclease R and those produced by endonuclease Z) in the ϕX174 genome.

Edgell and Hutchison want to use the mapped fragments to locate interesting regions—sites of initiation or termination of RNA synthesis, for example—that are difficult or impossible to map by conventional genetic techniques. They now think that they have identified three promoter regions. (The promoter is the DNA region that binds RNA polymerase, the enzyme that catalyzes RNA synthesis.) One promoter appears to be located within a segment of the ϕX174 genome about 100 nucleotides long. It should be possible to sequence a DNA fragment of that size. Moreover, according to Edgell and Hutchison, simultaneous treatment of ϕX174 RF with both endonucleases R and Z should produce that fragment.

Eukaryotic chromosomes, because of their greater size and complexity, are much more difficult to study than the relatively simple viral chromosomes. However, several scientists are beginning to use bacterial restriction enzymes to probe the structure of these chromosomes. For example, Charles Thomas at Harvard Medical School, Boston, has been investigating the structure of the lampbrush chromosomes of newt oocytes (developing egg cells). These chromosomes have loops of temporarily unwound DNA projecting from them. Thomas finds that the enzyme from *H. influenzae* breaks some, but not all, of the loops. The patterns of breakage and the size distribution of the DNA fragments produced should give some information about gene arrangement in the chromosomes. It is also theoretically possible to isolate particular genes by cleaving chromosomes with the right combination of restriction enzymes.

The ability to isolate and combine DNA's from two or more different sources permits a new approach for studying control of gene expression. Endonucleases RI and RII, because they generate cohesive ends, should be particularly useful for the production of such combined DNA molecules. Boyer and his colleagues plan to use these enzymes to insert bacterial or even mammalian DNA into bacterial plasmids. The plasmids would then serve to introduce this new DNA into bacterial cells, where its expression could be studied.

Speaking of Science

Holography: Beginnings of a New Art Form

Paintings, statues, and many other art works are appreciated because of nuances of shape and color discernible by the human eye. Visual appeal is also the basis of most advertising and commercial displays. Not surprisingly, then, advances in optical science have found reflections in both pure and commercial art. Photography, for example, made possible a much wider acquaintance with the limited supply of ancient art treasures, and became a new art form in itself. Television provided a new advertising medium whose potential for viewer involvement is much higher than that of the printed page.

The most recent advances in optical science—lasers and the holographic techniques that they make possible—are still largely confined to industrial and university laboratories. But their potential for creating new visual forms and for enhancing our perception of old forms has not gone unnoticed. Indeed, because holographic images are three-dimensional and can be projected so as to appear free-standing, they have more dramatic possibilities than any two-dimensional medium. And recently, with development of high-powered lasers and pulsed-laser holography, the first tentative invasions of the commercial marketplace and of the art world have begun.

One of the more striking commercial displays is the image of a hand offering a diamond ring and bracelet (shown at left), which was suspended above the sidewalk on Fifth Avenue in New York during November 1972, stopping traffic and attracting attention to Cartier's, from whose front window the hand seemed to protrude. The image, which provoked one passerby to assail it with her umbrella and declare it to be "devil's work," was projected from a hologram produced by Holoconcepts Corporation of America and McDonnell Douglas Electronics Company. Holoconcepts is one of several firms now using holography to create promotional displays, and according to its president, Robert Schinella, this medium makes possible "a new world of selling and marketing products."

Making holograms of life-size objects requires powerful lasers to illuminate them. McDonnell Douglas Electronics, for example, uses a 10-joule ruby laser to record holograms of objects as large as 3 by 3 by 2½ meters. Moreover, the coherence length of the light must be as great as the depth of the object or scene. Only in the last few years have lasers whose emissions have coherence lengths of several meters been developed by R. Wuerker and his colleagues at TRW in Redondo Beach, California, and others. Light pulses of very short duration have also been achieved, making possible holographic "snapshots" of living subjects. A firm that is now part of McDonnell Douglas Electronics, for example, made a holographic portrait of Dennis Gabor, the inventor of holography, who in 1971 was awarded the Nobel Prize in Physics for his work. More recently, Salvador Dali has reportedly created a three-dimensional portrait of the controversial rock star Alice Cooper in what is perhaps the first use of the holographic medium by a well-known artist.

Because of the three-dimensional nature of holography, this technique would seem a natural for preserving copies of decaying statuary. This idea, which curiously enough arose out of a 1971 summer study of lasers and their economic implications by the Jason group of scientists, led Walter Munk of the University of California

Another way to introduce new, functional DNA into cells—mammalian cells in this case—is to combine it with the genome of a transforming virus like SV40. Paul Berg and his colleagues have achieved just that; they were able to insert the *E. coli* galactose operon—the gene sequence required for galactose metabolism—into the SV40 chromosome. They used endonuclease RI to open both the circular SV40 DNA molecule and also a circular plasmid carrying the galactose operon. They then generated cohesive ends on these linear molecules by enzymatically adding a chain of adenine nucleotides to the 3′ ends of the SV40 DNA and a chain of thymine nucleotides to the 3′ ends of the DNA containing the galactose operon. (This step ensures that the two different molecules will combine.) Mixture of the two molecules, followed by appropriate enzyme treatment to complete and covalently join them together, yields a new circular molecule containing both sets of genes.

The next step is to transform cells with these molecules. The enzymes of *E. coli* and of mammalian cells are probably sufficiently different to be distinguishable. Thus, if the *E. coli* galactose genes function in the transformed cells, this should be a good system for studying the regulation in mammalian cells of a gene system whose function in *E. coli* is reasonally well understood.

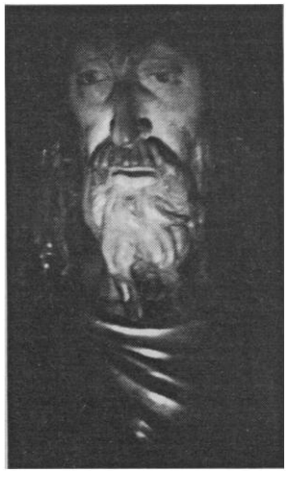
The ability to introduce functioning genes into mammalian cells has obvious implications for genetic engineering. At present, however, Berg emphasizes that his main interest is the study of gene regulation in mammalian cell cultures. He believes that the prospect of gene replacement therapy by this mechanism is remote and fraught with difficulty. An oncogenic virus, like SV40, is not an appropriate vehicle for introducing new genetic material into the cells of living organisms. Moreover, he thinks that it will be most difficult to design a gene carrier that will produce only the desired effect without also causing harmful side effects. Nevertheless, the prospect of such genetic modification, however distant and remote, exists.—JEAN L. MARX

Additional Readings

1. K. Danna and D. Nathans, *Proc. Nat. Acad. Sci. U.S.A.* **68**, 2193 (1971).
2. D. A. Jackson, R. H. Symons, P. Berg, *ibid.* **69**, 2904 (1972).
3. M. Meselson, R. Yuan, J. Heywood, *Annu. Rev. Biochem.* **41**, 447 (1972).
4. M. Sclair, M. H. Edgell, C. A. Hutchison, III, *J. Virol.* **11**, 378 (1973).

or At Least of an Advertising Bonanza

at San Diego (UCSD) to propose a series of experiments that were carried out by John Asmus of Science Applications Incorporated in Albuquerque, New Mexico, and Wuerker from TRW. Working in cooperation with F. Valcanover, superintendent of galleries in Venice, Italy, they successfully produced holograms of several Venetian statues (see photograph of the holographic image of a 15th-century woodcarving of St. John the Baptist by Donatello, at left). According to Asmus, these first holograms were crude but nonetheless produced images that were esthetically satisfying to see. Potentially, holograms could be used to build an archival library of the world's statuary, storing information that could someday be used to reproduce damaged objects. Since 35 percent of Venetian art is now severely damaged, according to Valcanover, and that in many other cities showing decay as well, the matter is of no little urgency.



The Venice experiment also yielded a hopeful means of reversing the decay of art works. More or less by accident, the investigators found that a laser beam is an excellent way to clean marble surfaces blackened by weathering and air pollution. Since then, Asmus has experimented with other artifacts—from stained manuscripts to encrusted bronzes—and finds that by varying the pulse length and energy of his laser he can clean nearly anything; he is consequently being deluged with requests from art restoration centers around the world.

Reproduction of art objects is an unpopular concept among artists and those who believe that pirated copies will diminish the value of the original, and that aspect of holographic copying of statues has yet to be faced. Nonetheless, according to Munk, holographic images would preserve for posterity many pieces of art that are now rapidly decaying under the combined onslaughts of weather, corrosive organic pollutants, and acts of vandalism, such as the damaging of the "Pietà"—in effect, the holograms would serve as a uniquely accurate template for reproduction or repair. Comparisons of two holograms taken in rapid succession gives an interference pattern that can identify points of stress, submicroscopic cracks, and other information that would aid in restoration. To investigate these and other possibilities, a new institute for science in art is in the process of being formed at UCSD.

Only a few artists are actively doing holographic art, and in some quarters the medium is receiving the sneers that originally greeted computer-generated music. Means of physically reproducing art forms in ways that can take advantage of the accuracy of the information stored in a hologram are not yet available, so that copies of statuary are not an immediate prospect. But holographic images of statues could be displayed, making these art objects accessible to a far greater audience and thus perhaps helping to alleviate the conflict between the need for greater physical security in museums and the desire to allow more people to enjoy their contents. Nonetheless, any major impact of holographic techniques on the art world, which is more conservative in its acceptance of new techniques than the commercial art community, seems to be some time off. But look for bolder, brighter, three-dimensional advertisements in the near future.—ALLEN L. HAMMOND