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SCIENTIFIC AMERICAN OFFPRINTS

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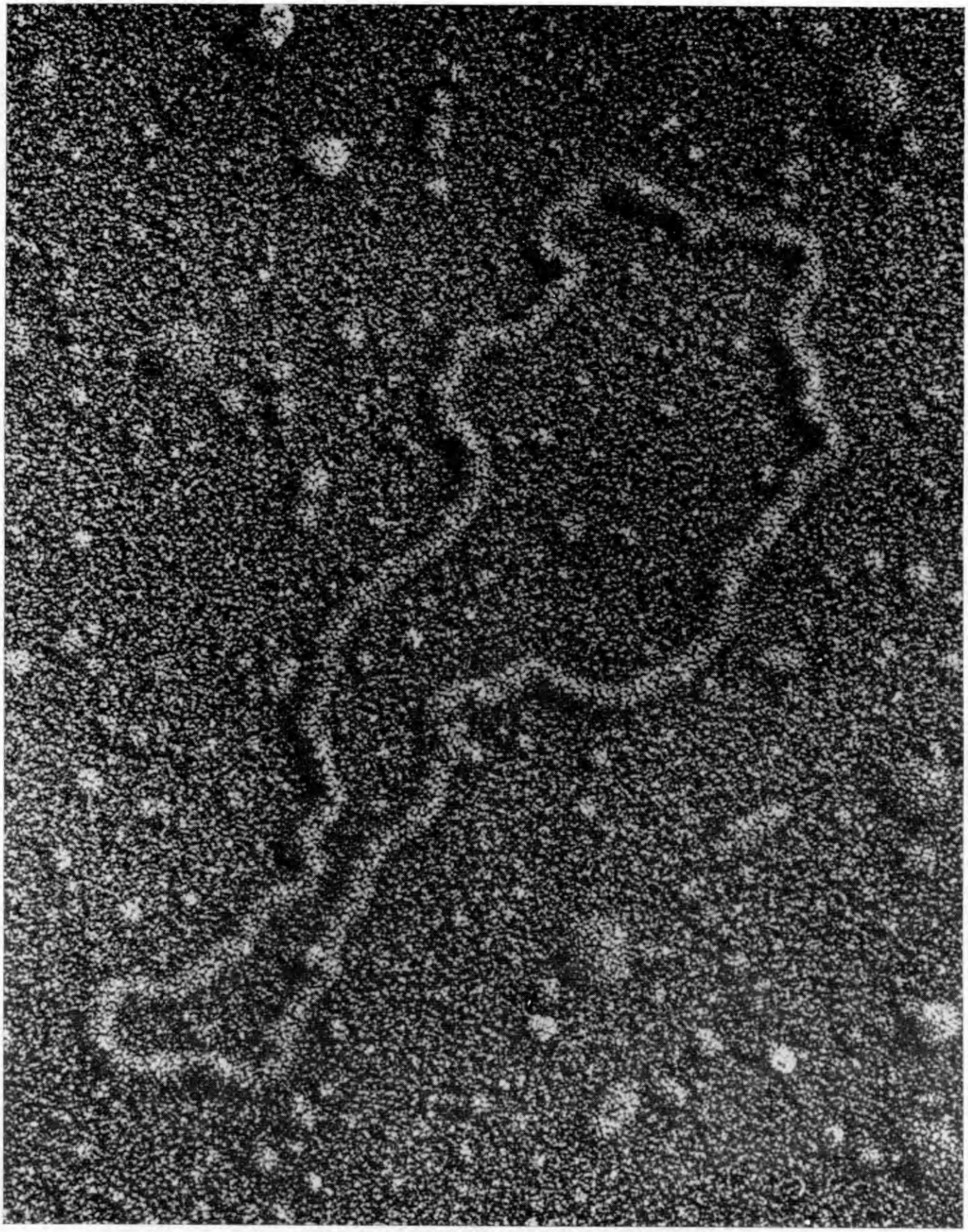
SCIENTIFIC AMERICAN
JULY 1975
VOL. 233, NO. 1 PP. 24-33

PUBLISHED BY **W. H. FREEMAN AND COMPANY** 660 MARKET STREET, SAN FRANCISCO, CALIFORNIA 94104



PLASMID *pSC*101 is shadowed with platinum-palladium and enlarged 230,000 diameters in an electron micrograph made by the author. A plasmid is a molecule of DNA that exists apart from the chromosome in a bacterium and replicates on its own, often carrying the genes for some supplementary activity such as resistance to antibiotics. This plasmid, a small one made by shearing a larger plasmid native to the bacterium *Escherichia coli*, is a circular, or closed-loop, molecule of DNA about three micrometers in circumference that carries the genetic information for replicating itself in *E. coli* and for conferring resistance to the antibiotic tetracycline. It was the "vehicle" for the first gene-manipulation experiments by the author and his colleagues. Foreign DNA was spliced to it and the plasmid was introduced into *E. coli*, where it replicated and expressed both its own and the foreign DNA's genetic information.

THE MANIPULATION OF GENES

Techniques for cleaving DNA and splicing it into a carrier molecule make it possible to transfer genetic information from one organism to an unrelated one. There the DNA replicates and expresses itself

by Stanley N. Cohen

Mythology is full of hybrid creatures such as the Sphinx, the Minotaur and the Chimera, but the real world is not; it is populated by organisms that have been shaped not by the union of characteristics derived from very dissimilar organisms but by evolution within species that retain their basic identity generation after generation. This is because there are natural barriers that normally prevent the exchange of genetic information between unrelated organisms. The barriers are still poorly understood, but they are of fundamental biological importance.

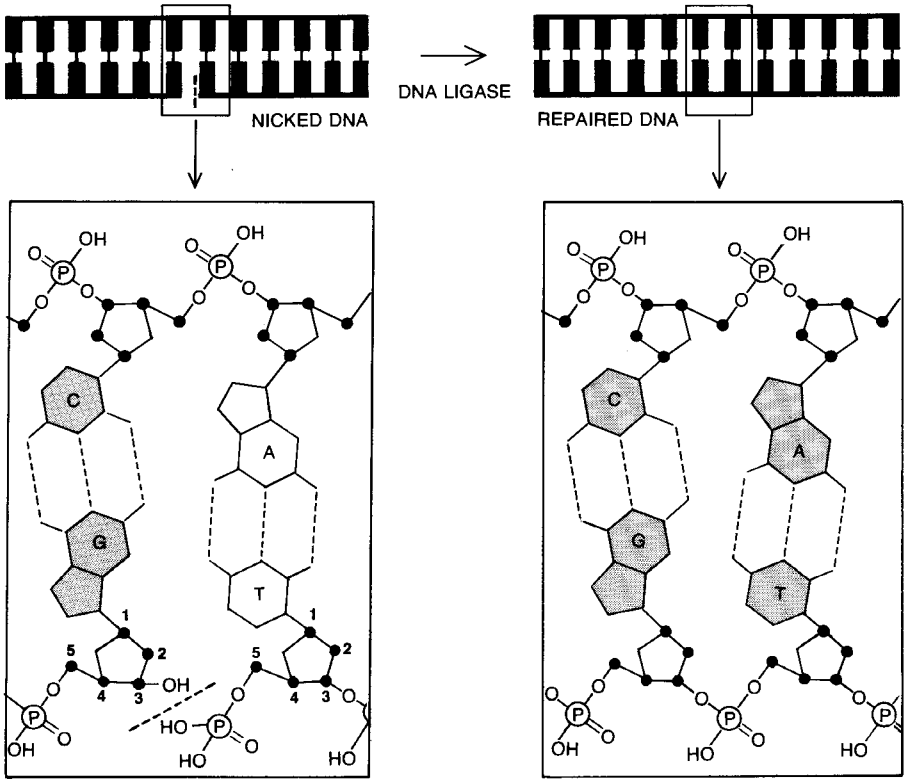
The basic unit of biological relatedness is the species, and in organisms that reproduce sexually species are defined by the ability of their members to breed with one another. Species are determined and defined by the genes they carry, so that in organisms that reproduce asexually the concept of species depends on nature's ability to prevent the biologically significant exchange of genetic material—the nucleic acid DNA—between unrelated groups.

The persistence of genetic uniqueness is perhaps most remarkable in simple organisms such as bacteria. Even when they occupy the same habitat most bacterial species do not exchange genetic information. Even rather similar species of bacteria do not ordinarily exchange the genes on their chromosomes, the structures that carry most of their genetic information. There are exceptions, however. There are bits of DNA, called plasmids, that exist apart from the chromosomes in some bacteria. Sometimes a plasmid can pick up a short segment of DNA from the chromosome of its own cell and transfer it to the cell of a related bacterial species, and sometimes the plasmid and the segment of chromosomal DNA can become integrated into the chromosome of the recipient cell. This transfer of genes between species by extrachromosomal elements has surely played some role in bacterial evolution, but apparently it has not been widespread in nature. Otherwise the characteristics of the common bacterial species would not have remained so largely intact over the huge number of bacterial generations that have existed during the era of modern bacteriology.

In 1973 Annie C. Y. Chang and I at the Stanford University School of Medicine and Herbert W. Boyer and Robert B. Helling at the University of California School of Medicine at San Francisco reported the construction in a test tube of biologically functional DNA molecules that combined genetic information from two different sources. We made the molecules by splicing together segments of two different plasmids found in the colon bacillus *Escherichia coli* and then inserting the composite DNA into *E. coli* cells, where it replicated itself and expressed the genetic information of both parent plasmids. Soon afterward we introduced plasmid genes from an unrelated bacterial species, *Staphylococcus aureus,* into *E. coli,* where they too expressed the biological properties they had displayed in their original host; then, applying the same procedures with John F. Morrow of Stanford and Howard M. Goodman in San Francisco, we were able to insert into *E. coli* some genes from an animal: the toad *Xenopus laevis.*

We called our composite molecules DNA chimeras because they were conceptually similar to the mythological Chimera (a creature with the head of a lion, the body of a goat and the tail of a serpent) and were the molecular counterparts of hybrid plant chimeras produced by agricultural grafting. The procedure we described has since been used and extended by workers in several laboratories. It has been called plasmid engineering, because it utilizes plasmids to introduce the foreign genes, and molecular cloning, because it provides a way to propagate a clone, or line of genetically alike organisms, all containing identical composite DNA molecules. Because of the method's potential for creating a wide variety of novel genetic combinations in microorganisms it is also known as genetic engineering and genetic manipulation. The procedure actually consists of several distinct biochemical and biological manipulations that were made possible by a series of independent discoveries made in rapid succession in the late 1960's and early 1970's. There are four essential elements: a method of breaking and joining DNA molecules derived from different sources; a suitable gene carrier that can replicate both itself and a foreign DNA segment linked to it; a means of introducing the composite DNA molecule, or chimera, into a functional bacterial cell, and a method of selecting from a large population of cells a clone of recipient cells that has acquired the molecular chimera.

In 1967 DNA ligases—enzymes that can repair breaks in DNA and under certain conditions can join together the loose ends of DNA strands—were discovered almost simultaneously in five laboratories. A DNA strand is a chain of nucleotides, each consisting of a deoxyribose sugar ring, a phosphate group and one of four organic bases: adenine, thymine, guanine and cytosine. The sugars and phosphates form the backbone of the strand, from which the bases project. The individual nucleotide building blocks are connected by phosphodiester bonds between the carbon atom at position No. 3 on one sugar and the carbon atom at position No. 5 on the adjacent sugar. Double-strand DNA, the form found in most organisms, consists of two



DNA LIGASE is an enzyme that repairs "nicks," or breaks in one strand of a double-strand molecule of DNA (*top*). A strand of DNA is a chain of nucleotides (*bottom*), each consisting of a deoxyribose sugar and a phosphate group and one of four organic bases: adenine (*A*), thymine (*T*), guanine (*G*) and cytosine (*C*). The sugars and phosphates constitute the backbone of the strand, and paired bases, linked by hydrogen bonds (*broken black lines*), connect two strands. The ligase catalyzes synthesis of a bond at the site of the break (*broken colored line*) between the phosphate of one nucleotide and the sugar of the next nucleotide.

chains of nucleotides linked by hydrogen bonds between their projecting bases. The bases are complementary: adenine (*A*) is always opposite thymine (*T*), and guanine (*G*) is always opposite cytosine (*C*). The function of the ligase is to repair "nicks," or breaks in single DNA strands, by synthesizing a phosphodiester bond between adjoining nucleotides [*see illustration above*].

In 1970 a group working in the laboratory of H. Gobind Khorana, who was then at the University of Wisconsin, found that the ligase produced by the bacterial virus T4 could sometimes catalyze the end-to-end linkage of completely separated double-strand DNA segments. The reaction required that the ends of two segments be able to find each other; such positioning of two DNA molecules was a matter of chance, and so the reaction was inefficient. It was clear that efficient joining of DNA molecules required a mechanism for holding the two DNA ends together so that the ligase could act.

An ingenious way of accomplishing this was developed and tested independently in two laboratories at Stanford: by Peter Lobban and A. Dale Kaiser and by David Jackson, Robert Symons and Paul Berg. Earlier work by others had shown that the ends of the DNA molecules of certain bacterial viruses can be joined by base-pairing between complementary sequences of nucleotides that are naturally present on single-strand segments projecting from the ends of those molecules: *A*'s pair with *T*'s, *G*'s pair with *C*'s and the molecules are held together by hydrogen bonds that form between the pairs. The principle of linking DNA molecules by means of the single-strand projections had been exploited in Khorana's laboratory for joining short synthetic sequences of nucleotides into longer segments of DNA.

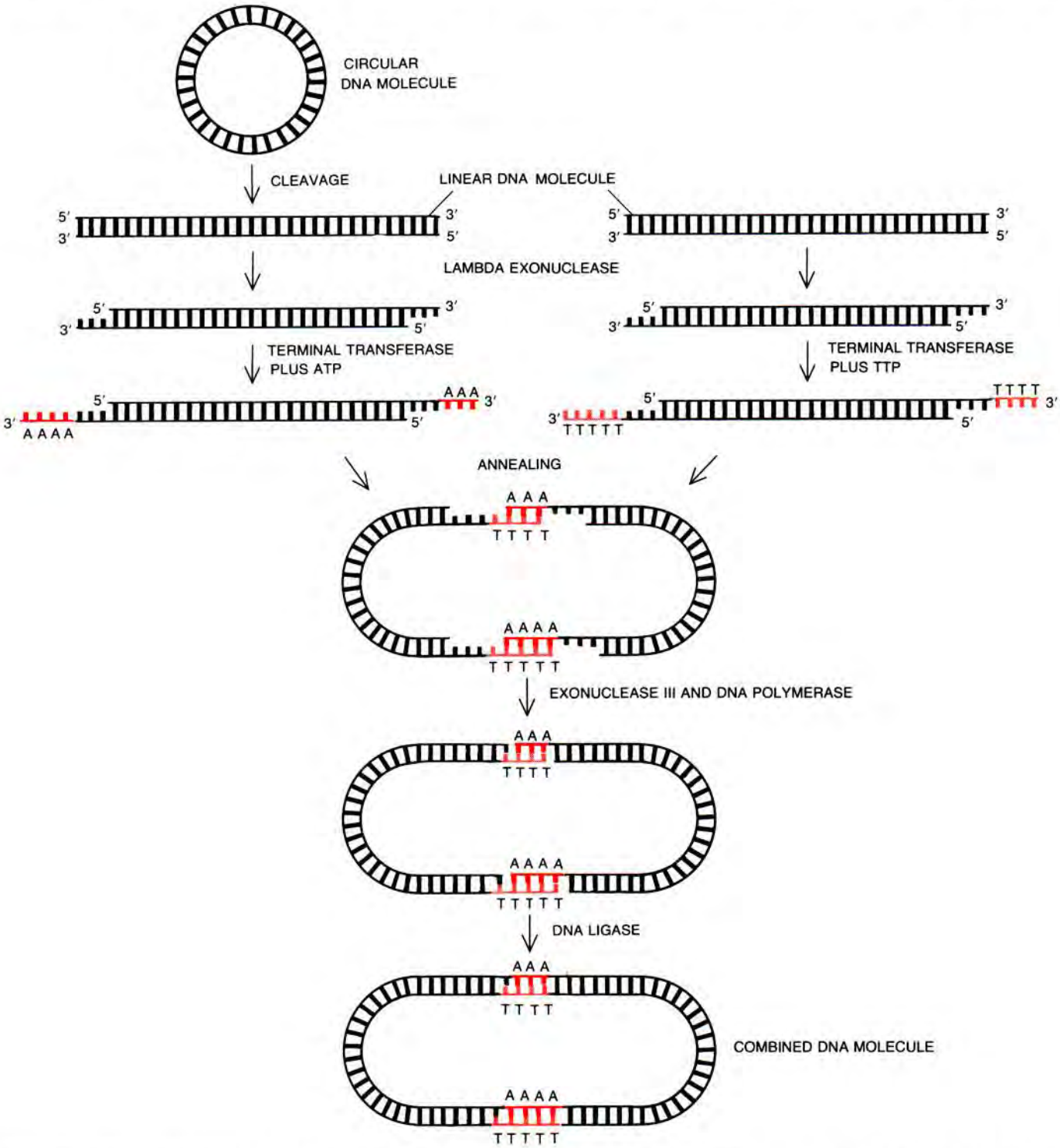
The Stanford groups knew too that an enzyme, terminal transferase, would catalyze the stepwise addition, specifically at what are called the 3′ ends of single strands of DNA, of a series of identical nucleotides. If the enzyme worked also with double-strand DNA, then a block of identical nucleotides could be added to one population of DNA molecules and a block of the complementary nucleotides could be added to another population from another source. Molecules of the two populations could then be annealed by hydrogen bonding and sealed together by DNA ligase. The method was potentially capable of joining any two species of DNA. While Lobban and Kaiser tested the terminal-transferase procedure with the DNA of the bacterial virus P22, Jackson, Symons and Berg applied the procedure to link the DNA of the animal virus SV40 to bacterial-virus DNA.

The SV40 and bacterial-virus DNA molecules Berg's group worked with are closed loops, and the loops had first to be cleaved to provide linear molecules with free ends for further processing and linkage [*see illustration on opposite page*]. (As it happened, the particular enzyme chosen to cleave the loops was the *Eco RI* endonuclease, which was later to be used in a different procedure for making the first biologically functional gene combinations. At the time, however, the enzyme's special property of producing complementary single-strand ends all by itself had not yet been discovered.)

The cleaved linear molecules were treated with an enzyme, produced by the bacterial virus lambda, called an exonuclease because it operates by cutting off nucleotides at the end of a DNA molecule. The lambda exonuclease chewed back the 5′ ends of DNA molecules and thus left projecting single-strand ends that had 3′ termini to which the blocks of complementary nucleotides could be added. The next step was to add, with the help of terminal transferase, a block of *A*'s at the 3′ end of one of the two DNA species to be linked and a block of *T*'s at the 3′ ends of the other species. The species were mixed together. Fragments having complementary blocks at their ends could find each other, line up and become annealed by hydrogen bonding, thus forming combined molecules. To fill the gaps at the 5′ ends of the original segments the investigators supplied nucleotides and two more enzymes: exonuclease III and DNA polymerase. Finally the nicks in the molecules were sealed with DNA ligase.

The method of making cohesive termini for joining DNA molecules in the first successful genetic-manipulation experiments was conceptually and operationally different from the terminal-transferase procedure. It was also much simpler. It depended on the ability of one of a group of enzymes called restriction endonucleases to make complementary-ended fragments during the cleavage of DNA at a site within the molecule, instead of requiring the addition of new blocks of complementary nucleotides to DNA termini.

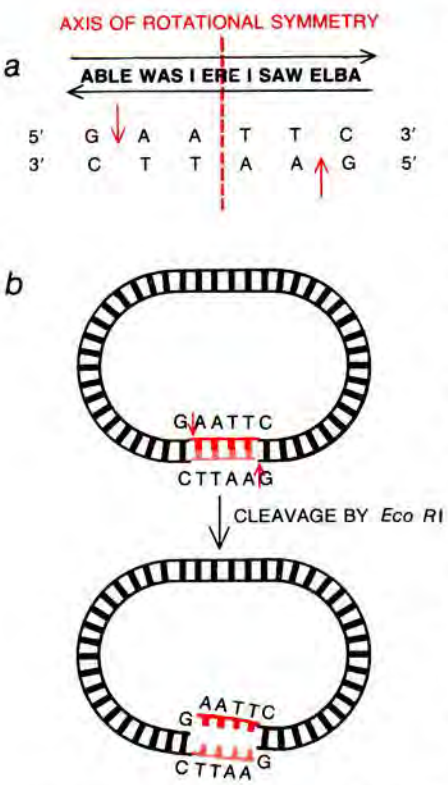
Viruses grown on certain strains of *E. coli* were known to be restricted in their ability to grow subsequently on other strains. Investigations had shown that this restriction was due to bacterial enzymes that recognize specific sites on a "foreign" viral DNA and cleave that DNA. (To protect its own DNA the bacterial cell makes a modification enzyme that adds methyl groups to nucleotides constituting the recognition sites for the restriction endonuclease, making them resistant to cleavage.) Restriction endonucleases (and modification methylases) are widespread in microorganisms; genes for making them were found on viral chromosomes and extrachromosomal plasmid DNA as well as on many bacterial chromosomes. During the early 1970's the nucleotide sequences at the cleavage sites recognized by several re-



TERMINAL-TRANSFERASE procedure for joining DNA molecules involves a number of steps, each dependent on a different enzyme. If one of the molecules to be joined is a closed loop, it must first be cleaved. The linear molecules are treated with lambda exonuclease, an enzyme that cuts nucleotides off the 5′ end of DNA strands (the end with a phosphate group on the No. 5 carbon). Then specific nucleotides are added to the 3′ end (the end with an OH group on the No. 3 carbon) by the action of the enzyme terminal transferase. One DNA species is supplied with adenosine triphosphate (ATP), the other with thymidine triphosphate (TTP), so that *A* nucleotides are added to one species and complementary *T* nucleotides to the other. When the two species are mixed, the complementary bases pair up, annealing the molecules. Nucleotides and the enzymes DNA polymerase and exonuclease III are added to fill gaps and DNA ligase is added to seal the DNA backbones. The result is a double molecule composed of two separate DNA segments.

striction endonucleases were identified. In every instance, it developed, the cleavage was at or near an axis of rotational symmetry: a palindrome where the nucleotide base sequences read the same on both strands in the 5′-to-3′ direction [*see illustration below*].

In some instances the breaks in the DNA strands made by restriction enzymes were opposite each other. One particular endonuclease, however, the *Eco RI* enzyme isolated by Robert N. Yoshimori in Boyer's laboratory in San Francisco, had a property that was of special interest. Unlike the other nucleases known at the time, this enzyme introduced breaks in the two DNA strands that were separated by several nucleotides. Because of the symmetrical, palindromic arrangement of the nucleotides in the region of cleavage this separation of the cleavage points on the two strands yielded DNA termini with projecting complementary nucleotide sequences: "sticky" mortise-and-tenon termini. The *Eco RI* enzyme thus produced in one step DNA molecules that were functionally equivalent to the cohesive-end molecules produced by the complicated terminal-transferase procedure.



RESTRICTION ENDONUCLEASES cleave DNA at sites where complementary nucleotides are arranged in rotational symmetry: a palindrome, comparable to a word palindrome (*a*). The endonuclease *Eco RI* has the additional property of cleaving complementary strands of DNA at sites (*colored arrows*) four nucleotides apart. Such cleavage (*b*) yields DNA fragments with complementary, overlapping single-strand ends. As a result the end of any DNA fragment produced by *Eco RI* cleavage can anneal with any other fragment produced by the enzyme.

The experiments that led to the discovery of the capabilities of *Eco RI* were reported independently and simultaneously in November, 1972, by Janet Mertz and Ronald W. Davis of Stanford and by another Stanford investigator, Vittorio Sgaramella. Sgaramella found that molecules of the bacterial virus P22 could be cleaved with *Eco RI* and would then link up end to end to form DNA segments equal in length to two or more viral-DNA molecules. Mertz and Davis observed that closed-loop SV40-DNA molecules cleaved by *Eco RI* would reform themselves into circular molecules by hydrogen bonding and could be sealed with DNA ligase; the reconstituted molecules were infectious in animal cells growing in tissue culture. Boyer and his colleagues analyzed the nucleotide sequences at the DNA termini produced by *Eco RI*, and their evidence confirmed the complementary nature of the termini, which accounted for their cohesive activity.

In late 1972, then, several methods were available by which one could join double-strand molecules of DNA. That was a major step in the development of a system for manipulating genes. More was necessary, however. Most segments of DNA do not have an inherent capacity for self-replication; in order to reproduce themselves in a biological system they need to be integrated into DNA molecules that can replicate in the particular system. Even a DNA segment that can replicate in its original host was not likely to have the specific genetic signals required for replication in a different environment. If foreign DNA was to be propagated in bacteria, as had long been proposed in speculative scenarios of genetic engineering, a suitable vehicle, or carrier, was required. A composite DNA molecule consisting of the vehicle and the desired foreign DNA would have to be introduced into a population of functional host bacteria. Finally, it would be necessary to select, or identify, those cells in the bacterial population that took up the DNA chimeras. In 1972 it still seemed possible that the genetic information on totally foreign DNA molecules might produce an aberrant situation that would prevent the propagation of hybrid molecules in a new host.

Molecular biologists had focused for many years on viruses and their relations with bacteria, and so it was natural that bacterial viruses were thought of as the most likely vehicles for genetic manipulation. For some time there had been speculation and discussion about using viruses, such as lambda, that occasionally acquire bits of the *E. coli* chromosome by natural recombination mechanisms for cloning DNA from foreign sources. It was not a virus, however, but a plasmid that first served as a vehicle for introducing foreign genes into a bacterium and that provided a mechanism for the replication and selection of the foreign DNA.

A ubiquitous group of plasmids that confer on their host bacteria the ability to resist a number of antibiotics had been studied intensively for more than a decade. Antibiotic-resistant *E. coli* isolated in many parts of the world, for example, were found to contain plasmids, designated *R* factors (for "resistance"), carrying the genetic information for products that in one way or another could interfere with the action of specific antibiotics [see "Infectious Drug Resistance," by Tsutomu Watanabe; SCIENTIFIC AMERICAN, December, 1967]. Double-strand circular molecules of *R*-factor DNA had been separated from bacterial chromosomal DNA by centrifugation in density gradients and had been characterized by biochemical and physical techniques [see "The Molecule of Infectious Drug Resistance," by Royston C. Clowes; SCIENTIFIC AMERICAN, April, 1973].

In 1970 Morton Mandel and A. Higa of the University of Hawaii School of Medicine had discovered that treatment of *E. coli* with calcium salts enabled the bacteria to take up viral DNA. At Stanford, Chang and I, with Leslie Hsu, found that if we made the cell membranes of *E. coli* permeable by treating them with calcium chloride, purified *R*-factor DNA could be introduced into them [*see illustration on opposite page*]. The *R*-factor DNA is taken up in this transformation process by only about one bacterial cell in a million, but those few cells can be selected because they live and multiply in the presence of the antibiotics to which the *R* factor confers resistance, whereas other cells die. Each transformed cell gives rise to a clone that contains exact replicas of the parent plasmid DNA molecules, and so we reasoned that plasmids might serve as vehicles for propagating new genetic information in a line of *E. coli* cells.

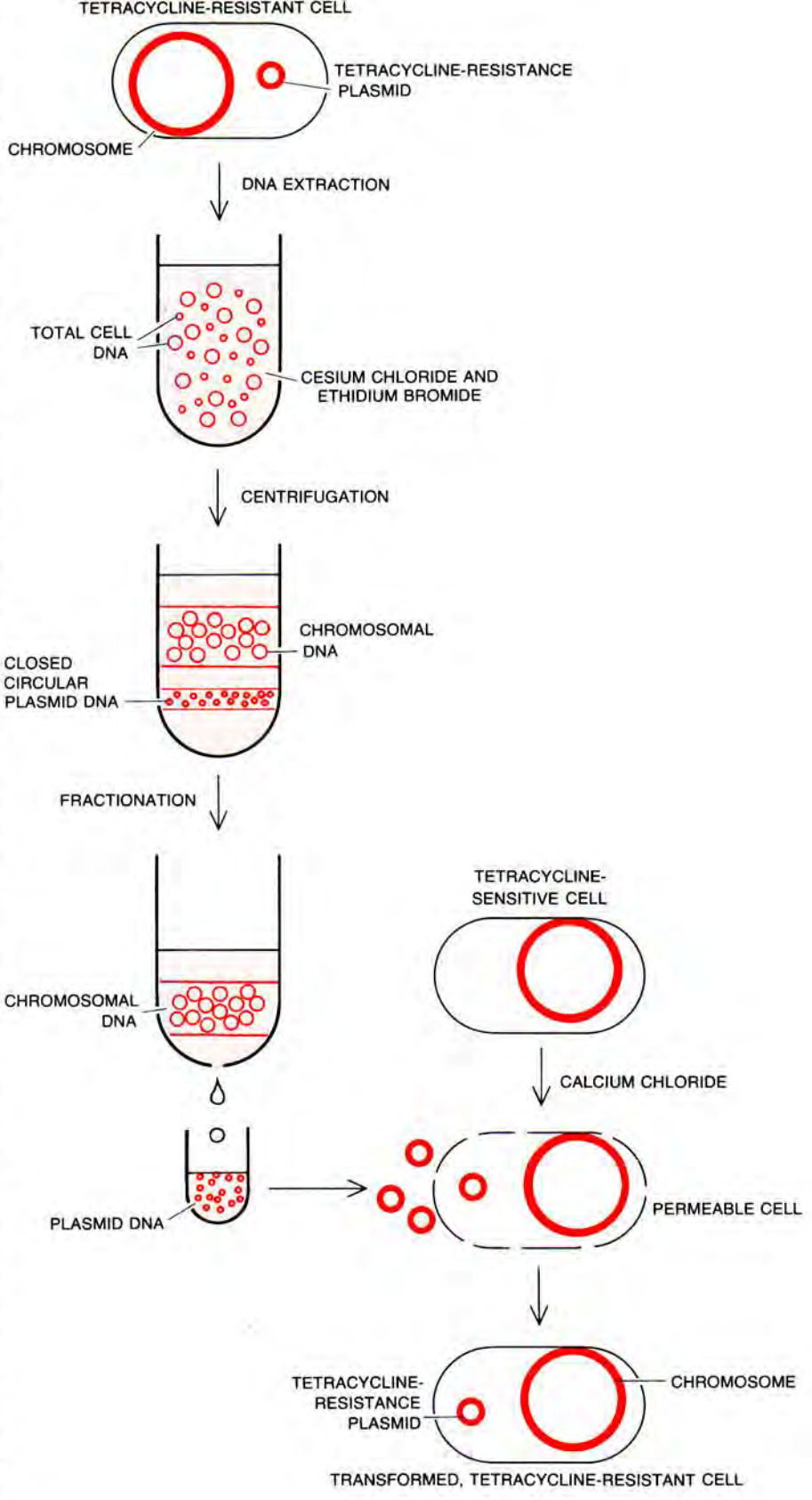
In an effort to explore the genetic and molecular properties of various regions of the *R*-factor DNA we had begun to take plasmids apart by shearing their DNA mechanically and then transforming *E. coli* with the resulting

fragments. Soon afterward we began to cleave the plasmids with the *Eco* RI enzyme, which had been shown to produce multiple site-specific breaks in several viruses. It might therefore be counted on to cleave all molecules of a bacterial plasmid in the same way, so that any particular species of DNA would yield a specific set of cleavage fragments, and do so reproducibly. The fragments could then be separated and identified according to the different rates at which they would migrate through a gel under the influence of an electric current.

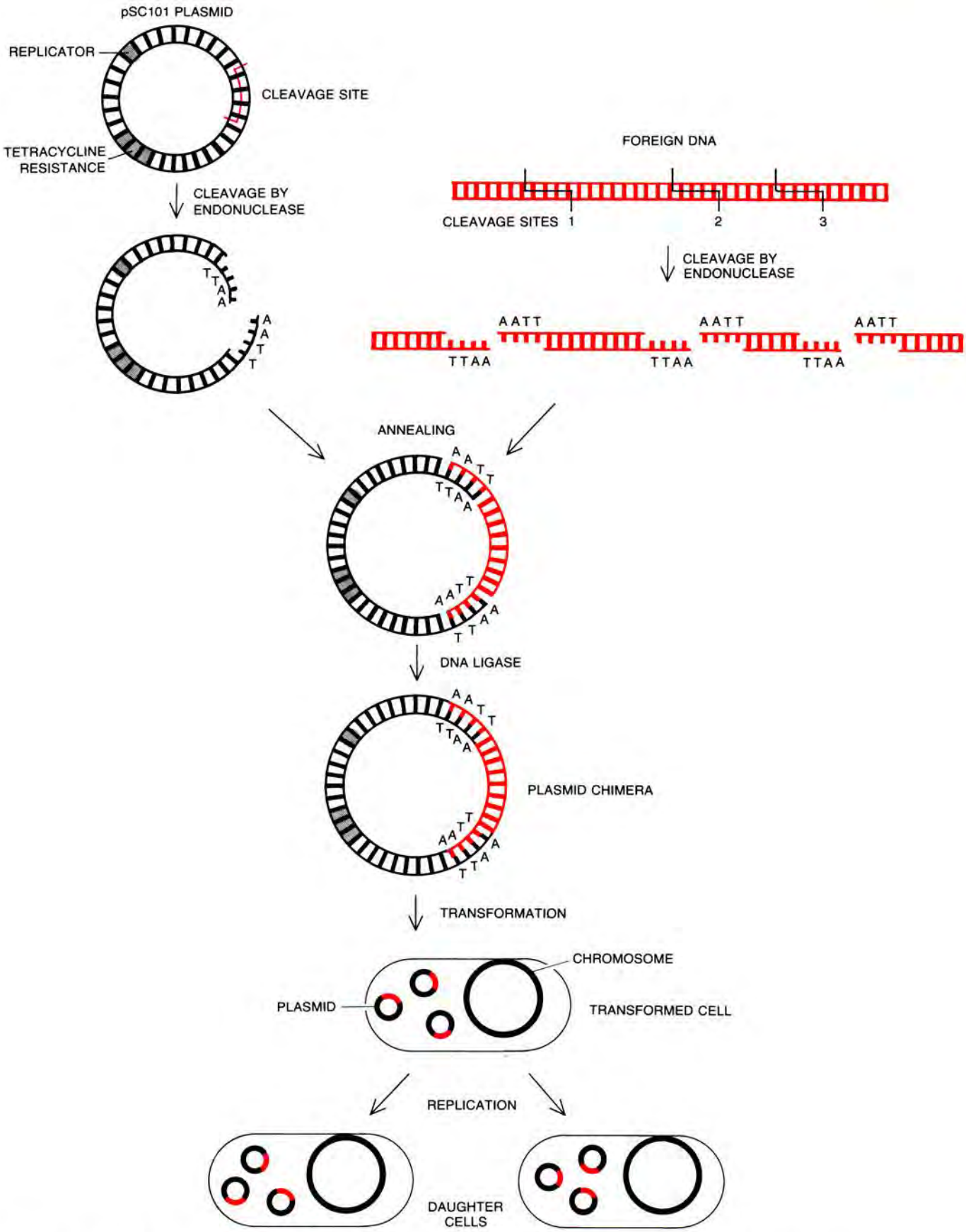
When the DNA termini produced by *Eco* RI endonuclease were found to be cohesive, Chang and I, in collaboration with Boyer and Helling in San Francisco, proceeded to search for a plasmid that the enzyme would cleave without affecting the plasmid's ability to replicate or to confer antibiotic resistance. We hoped that if such a plasmid could be found, we could insert a segment of foreign DNA at the *Eco* RI cleavage site, and that it might be possible to propagate the foreign DNA in *E. coli*.

In our collection at Stanford there was a small plasmid, *pSC*101, that had been isolated following the mechanical shearing of a large plasmid bearing genes for multiple antibiotic resistance. It was less than a twelfth as long as the parent plasmid, but it did retain the genetic information for its replication in *E. coli* and for conferring resistance to one antibiotic, tetracycline. When we subjected *pSC*101 DNA to cleavage by *Eco* RI and analyzed the products by gel electrophoresis, we found that the enzyme had cut the plasmid molecule in only one place, producing a single linear fragment. We were able to join the ends of that fragment again by hydrogen bonding and reseal them with DNA ligase, and when we introduced the reconstituted circular DNA molecules into *E. coli* by transformation, they were biologically functional plasmids: they replicated and conferred tetracycline resistance.

The next step was to see if a fragment of foreign DNA could be inserted at the cleavage site without interfering with replication or expression of tetracycline resistance and thus destroying the plasmid's ability to serve as a cloning vehicle. We mixed the DNA of another *E. coli* plasmid, which carried resistance to the antibiotic kanamycin, with the *pSC*101 DNA. We subjected the mixed DNA to cleavage by *Eco* RI and then to ligation, transformed *E. coli* with the resulting DNA and found that some of the transformed bacteria were indeed resist-



PLASMID DNA can be introduced into a bacterial cell by the procedure called transformation. Plasmids carrying genes for resistance to the antibiotic tetracycline (*top left*) are separated from bacterial chromosomal DNA. Because differential binding of ethidium bromide by the two DNA species makes the circular plasmid DNA denser than the chromosomal DNA, the plasmids form a distinct band on centrifugation in a cesium chloride gradient and can be separated (*bottom left*). The plasmid DNA is mixed with bacterial cells that are not resistant to tetracycline and that have been made permeable by treatment with a calcium salt. The DNA enters the cells, replicates there and makes the cells resistant to tetracycline.



FOREIGN DNA is spliced into the *pSC*101 plasmid and introduced with the plasmid into the bacterium *Escherichia coli*. The plasmid is cleaved by the endonuclease *Eco* RI at a single site that does not interfere with the plasmid's genes for replication or for resistance to tetracycline (*top left*). The nucleotide sequence recognized by *Eco* RI is present also in other DNA, so that a foreign DNA exposed to the endonuclease is cleaved about once in every 4,000 to 16,000 nucleotide pairs on a random basis (*top right*). Fragments of cleaved foreign DNA are annealed to the plasmid DNA by hydrogen bonding of the complementary base pairs, and the new composite molecules are sealed by DNA ligase. The DNA chimeras, each consisting of the entire plasmid and a foreign DNA fragment, are introduced into *E. coli* by transformation, and the foreign DNA is replicated by virtue of the replication functions of the plasmid.

ant to both tetracycline and kanamycin. The plasmids isolated from such transformants contained the entire *pSC*101 DNA segment and also a second DNA fragment that carried the information for kanamycin resistance, although it lacked replication functions of its own. The results meant that the *pSC*101 could serve as a cloning vehicle for introducing at least a nonreplicating segment of a related DNA into *E. coli*. And the procedure was extraordinarily simple.

Could genes from other species be introduced into *E. coli* plasmids, however? There might be genetic signals on foreign DNA that would prevent its propagation or expression in *E. coli*. We decided to try to combine DNA from a plasmid of another bacterium, the *pI*258 plasmid of *Staphylococcus aureus*, with our original *E. coli* plasmid. The staphylococcal plasmid had already been studied in several laboratories; we had found that it was cleaved into four DNA fragments by *Eco RI*. Since *pI*258 was not native to *E. coli* or to related bacteria, it could not on its own propagate in an *E. coli* host. And it was known to carry a gene for resistance to still another antibiotic, penicillin, that would serve as a marker for selecting any transformed clones. (Penicillin resistance, like combined resistance to tetracycline and kanamycin, was already widespread among *E. coli* strains in nature. That was important; if genes from a bacterial species that cannot normally exchange genetic information with the colon bacillus were to be introduced into it, it was essential that they carry only antibiotic-resistance traits that were already prevalent in *E. coli*. Otherwise we would be extending the species' antibiotic-resistance capabilities.)

Chang and I repeated the experiment that had been successful with two kinds of *E. coli* plasmids, but this time we did it with a mixture of the *E. coli*'s *pSC*-101 and the staphylococcal *pI*258: we cleaved the mixed plasmids with *Eco RI* endonuclease, treated them with ligase and then transformed *E. coli*. Next we isolated transformed bacteria that expressed the penicillin resistance coded for by the *S. aureus* plasmid as well as the tetracycline resistance of the *E. coli* plasmid. These doubly resistant cells were found to contain a new DNA species that had the molecular characteristics of the staphylococcal plasmid DNA as well as the characteristics of *pSC*101.

The replication and expression in *E. coli* of genes derived from an organism ordinarily quite unable to exchange genes with *E. coli* represented a breach in the barriers that normally separate biological species. The bulk of the genetic information expressed in the transformed bacteria defined it as *E. coli*, but the transformed cells also carried replicating DNA molecules that had molecular and biological characteristics derived from an unrelated species, *S. aureus*. The fact that the foreign genes were on a plasmid meant that they would be easy to isolate and purify in large quantities for further study. Moreover, there was a possibility that one might introduce genes into the easy-to-grow *E. coli* that specify a wide variety of metabolic or synthesizing functions (such as photosynthesis or antibiotic production) and that are indigenous to other biological classes. Potentially the *pSC*101 plasmid and the molecular-cloning procedure could serve to introduce DNA molecules from complex higher organisms into bacterial hosts, making it possible to apply relatively simple bacterial genetic and biochemical techniques to the study of animal-cell genes.

Could animal-cell genes in fact be introduced into bacteria, and would they replicate there? Boyer, Chang, Helling and I, together with Morrow and Goodman, immediately undertook to find out. We picked certain genes that had been well studied and characterized and were available, purified, in quantity: the genes that code for a precursor of the ribosomes (the structure on which proteins are synthesized) in the toad *Xenopus laevis*. The genes had properties that would enable us to identify them if we succeeded in getting them to propagate in bacteria. The toad DNA was suitable for another reason: although we would be constructing a novel biological combination containing genes from both animal cells and bacteria, we and others expected that no hazard would result from transplanting the highly purified ribosomal genes of a toad.

Unlike the foreign DNA's of our earlier experiments, the toad genes did not express traits (such as antibiotic resistance) that could help us to select bacteria carrying plasmid chimeras. The tetracycline resistance conferred by *pSC*101 would make it possible to select transformed clones, however, and we could then proceed to examine the DNA isolated from such clones to see if any clones contained a foreign DNA having the molecular properties of toad ribosomal DNA. The endonuclease-generated fragments of toad ribosomal DNA have characteristic sizes and base compositions; DNA from the transformed cells could be tested for those characteristics. The genes propagated in bacteria could also be tested for nucleotide-sequence homology with DNA isolated directly from the toad.

When we did the experiment and analyzed the resulting transformed cells, we found that the animal-cell genes were indeed reproducing themselves in generation after generation of bacteria by means of the plasmid's replication functions. In addition, the nucleotide sequences of the toad DNA were being transcribed into an RNA product in the bacterial cells.

Within a very few months after the first DNA-cloning experiments the procedure was being used in a number of laboratories to clone bacterial and animal-cell DNA from a variety of sources. Soon two plasmids other than *pSC*101 were discovered that have a single *Eco RI* cleavage site at a location that does not interfere with essential genes. One of these plasmids is present in many copies in the bacterial cell, making it possible to "amplify," or multiply many times, any DNA fragments linked to it. Investigators at the University of Edinburgh and at Stanford went on to develop mutants of the virus lambda (which ordinarily infects *E. coli*) that made the virus too an effective cloning vehicle. Other restriction endonucleases were discovered that also make cohesive termini but that cleave DNA at different sites from the *Eco RI* enzymes, so that chromosomes can now be taken apart and put together in various ways.

The investigative possibilities of DNA cloning are already being explored intensively. Some workers have isolated from complex chromosomes certain regions that are implicated in particular functions such as replication. Others are making plasmids to order with specific properties that should clarify aspects of extrachromosomal-DNA biology that have been hard to study. The organization of complex chromosomes, such as those of the fruit fly *Drosophila*, is being studied by cloning the animal genes in bacteria. Within the past few months methods have been developed for selectively cloning specific genes of higher organisms through the use of radioactively labeled RNA probes: instead of purifying the genes to be studied before introducing them into bacteria, one can transform bacteria with a heterogeneous population of animal-cell DNA and then isolate those genes that produce a particular species of RNA. It is also possible to isolate groups of genes that are expressed concurrently at a particular stage in the animal's development.

The potential seems to be even broader. Gene manipulation opens the pros-

pect of constructing bacterial cells, which can be grown easily and inexpensively, that will synthesize a variety of biologically produced substances such as antibiotics and hormones, or enzymes that can convert sunlight directly into food substances or usable energy. Perhaps it even provides an experimental basis for introducing new genetic information into plant or animal cells.

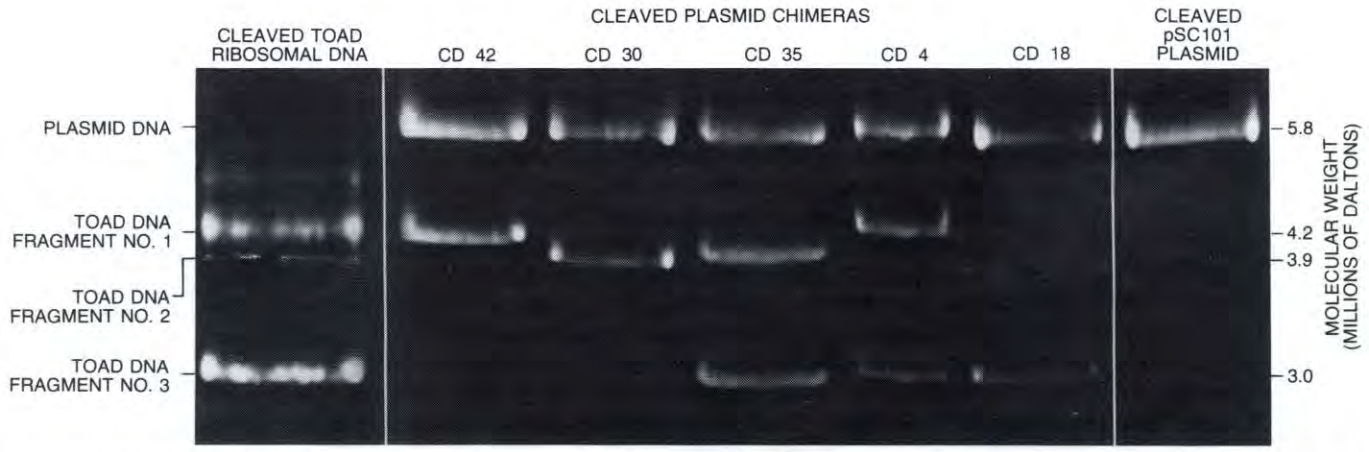
It has been clear from the beginning of experimentation in molecular cloning that the construction of some kinds of novel gene combinations may have a potential for biological hazard, and the scientific community has moved quickly to make certain that research in genetic manipulation would not endanger the public. For a time after our initial experiments the *pSC*101 plasmid was the only vehicle known to be suitable for cloning foreign DNA in *E. coli*, and our colleagues asked for supplies with which to pursue studies we knew were of major scientific and medical importance. Investigators normally facilitate the free exchange of bacteria and other experimental strains they have isolated or developed, but Chang and I were concerned that manipulation of certain genes could give rise to novel organisms whose infectious properties and ecological effects could not be predicted. In agreeing to provide the plasmid we therefore asked for assurance that our colleagues would neither introduce tumor viruses into bacteria nor create antibiotic-resistance combinations that were not already present in nature; we also asked the recipients not to send the plasmid on to other laboratories, so that we could keep track of its distribution.

When still other cloning vehicles were discovered, it became apparent that a more general mechanism for ensuring experimental safety in gene-manipulation research was advisable. The groundwork for such control had been established earlier: the National Academy of Sciences had been urged to consider the "possibility that potentially biohazardous consequences might result from widespread or injudicious use" of these techniques and had asked Paul Berg to form an advisory committee that would consider the issue. Berg too had been concerned about the potential hazards of certain kinds of experimentation for some years, and had himself decided to abandon plans to try to introduce genes from the tumor virus SV40 into bacteria because of the possible danger if the experiment were successful.

Berg brought together a number of investigators, including some who were then directly involved in molecular cloning, in the spring of 1974. In a report released in July and in a letter to leading professional journals the members of the committee expressed their "concern about the possible unfortunate consequences of indiscriminate application" of the techniques and formally asked all investigators to join them in voluntarily deferring two types of experiments (which had, as a matter of fact, been avoided by informal consensus up until that time). Experiments of Type I involved the construction of novel organisms containing combinations of toxin-producing capabilities or of antibiotic-resistance genes not found in nature. Type 2 experiments involved the introduction of DNA from tumor viruses or other animal viruses into bacteria; the committee noted that "such recombinant molecules might be more easily disseminated to bacterial populations in humans and other species, and might thus increase the incidence of cancer or other diseases."

The Academy committee was concerned largely because of our inability to assess the hazards of certain experiments accurately before the experiments were undertaken. Guidelines for safety had long been available in other areas of potentially hazardous research, such as studies involving known disease-causing bacteria and viruses, radioactive isotopes or toxic chemicals. Because of the newness of the microbial gene-manipulation methods, no such guidelines had yet been developed for work in this area, however; there was the possibility that potentially hazardous experiments might proceed before appropriate guidelines could be considered and implemented. We recognized that most work with the new methods did not and would not involve experiments of a hazardous nature but we recommended the deferral of Type I and Type II experiments until the hazards were more carefully assessed, until it was determined whether or not the work could be undertaken safely and until adequate safety precautions were available. The committee also proposed that an international meeting be held early in 1975 to consider the matter more fully.

Such a meeting was held in February at the Asilomar Conference Center near Pacific Grove, Calif. It brought together 86 American biologists and 53 investigators from 16 other countries, who spent three and a half days reviewing progress in the field of molecular cloning and formulating guidelines that would allow most types of new hereditary characteristics to be introduced into bacteria and

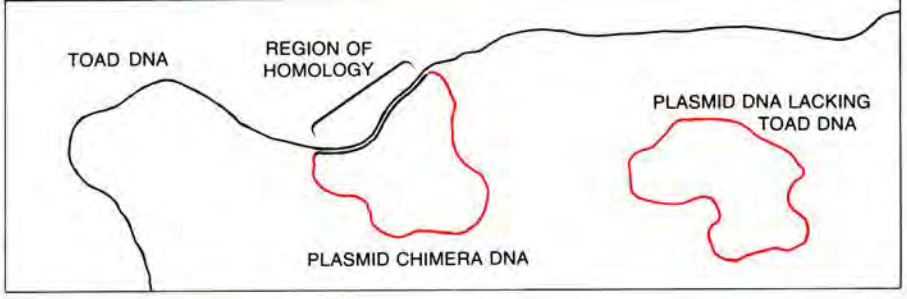


GEL ELECTROPHORESIS demonstrates the presence of toad DNA in chimeric plasmids. Fragments of DNA migrate through a gel at different rates under the influence of an electric current, depending on their size. Linear molecules of plasmid DNA (*right*) and the cleavage products of toad ribosomal DNA (*left*) therefore have characteristic sizes and migrate characteristic distances in a given time. The bands of DNA, visualized by a fluorescent dye, are photographed in ultraviolet. All five chimeric plasmids (*center*) contain a plasmid DNA molecule; in addition each chimera includes one or more fragments characteristic of original toad DNA.

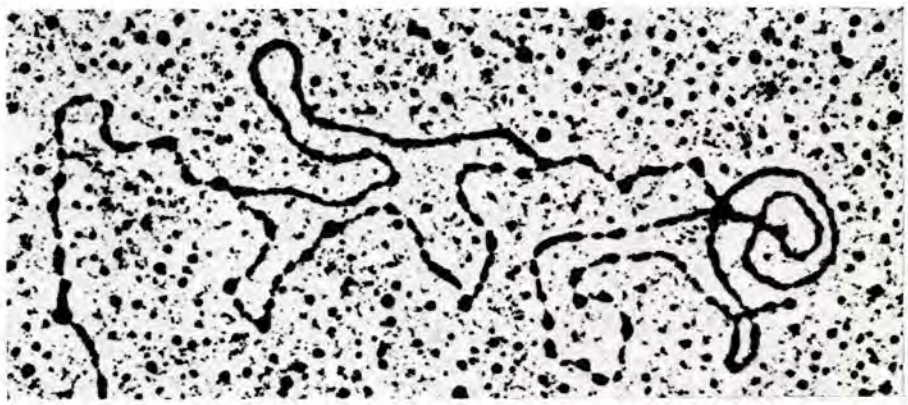
viruses safely. Invited nonscientists from the fields of law and ethics participated in the discussions and decisions at Asilomar, along with representatives of agencies that provide Federal funds for scientific research; the meetings were open to the press and were fully reported. The issues were complex and there were wide differences of opinion on many of them, but there was consensus on three major points. First, the newly developed cloning methods offer the prospect of dealing with a wide variety of important scientific and medical problems as well as other problems that trouble society, such as environmental pollution and food and energy shortages. Second, the accidental dissemination of certain novel biological combinations may present varying degrees of potential risk. The construction of such combinations should proceed only under a graded series of precautions, principally biological and physical barriers, adequate to prevent the escape of any hazardous organisms; the extent of the actual risk should be explored by experiments conducted under strict containment conditions. Third, some experiments are potentially too hazardous to be carried out for the present, even with the most careful containment. Future research and experience may show that many of the potential hazards considered at the meeting are less serious and less probable than we now suspect. Nevertheless, it was agreed that standards of protection should be high at the beginning and that they can be modified later if the assessment of risk changes.

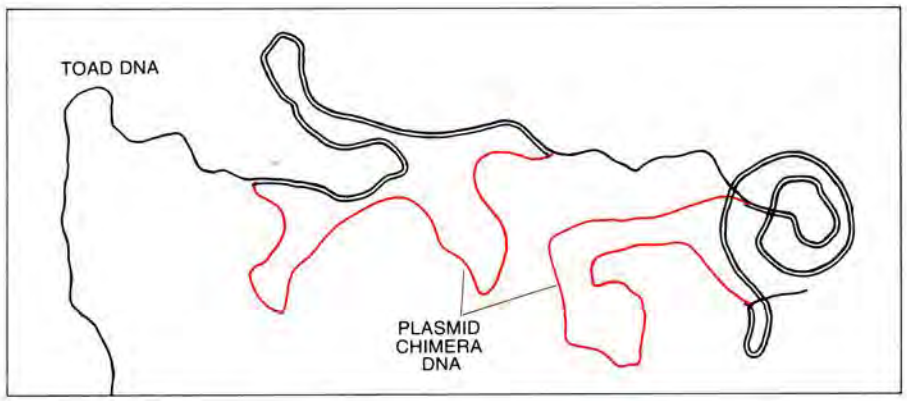
Physical containment barriers have long been used in the U.S. space-exploration program to minimize the possibility of contamination of the earth by extraterrestrial microbes. Containment procedures are also employed routinely to protect laboratory workers and the public from hazards associated with radioactive isotopes and toxic chemicals and in work with disease-causing bacteria and viruses. The Asilomar meeting formulated the additional concept of biological barriers, which involve fastidious cloning vehicles that are able to propagate only in specialized hosts and equally fastidious bacterial strains that are unable to live except under stringent laboratory conditions.

In the past the scientific community has commonly policed its own actions informally, responding to ethical concerns with self-imposed restraint. Usually, but not always, society at large has also considered the public well-being in determining how knowledge obtained by basic scientific research should be applied. Extensive public scrutiny and open discussion by scientists and nonscientists of the possible risks and benefits of a particular line of basic research has been rare, however, when (as in this case) the hazards in question are only potential and, for some experiments, even hypothetical. As this article is being written it is still too early to know what the long-range outcome of the public discussions initiated by scientists working in genetic manipulation will be. One can hope that the forthright approach and the rigorous standards that have been adopted for research in the cloning of recombinant DNA molecules will promote a sharper focus on other issues relevant to public and environmental safety.



HETERODUPLEX ANALYSIS identifies regions of a toad DNA (*black*) that have been incorporated in a chimeric plasmid DNA molecule. DNA isolated from toad eggs and the DNA of the chimera are denatured, that is, each natural double-strand molecule is split into two single strands of DNA, by alkali treatment. The toad and the chimeric DNA's are mixed together, and any complementary sequences are allowed to find each other. The toad DNA incorporated in the chimeras has nucleotide sequences that are complementary to sequences in the DNA taken directly from the animal source. Those homologous sequences anneal to form heteroduplex double-strand DNA that can be identified in electron micrographs.





PRESENCE OF TOAD DNA in two separate chimeric plasmid molecules is demonstrated by an electron micrograph made by John F. Morrow at the Stanford University School of Medicine. As is indicated in the drawing (*bottom*), there are DNA strands from two plasmids and a strand of toad DNA. The micrograph shows thickened regions of DNA where nucleotide sequences are homologous and two single strands have been annealed. The toad DNA in the chimeras codes for ribosomes, and the space between the two heteroduplex regions is compatible with the spacing of multiple ribosomal genes in toad DNA.

The Author

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PERSPECTIVE

DNA cloning: A personal view after 40 years

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Edited by Joseph L. Goldstein, University of Texas Southwestern Medical Center, Dallas, TX, and approved August 13, 2013 (received for review August 2, 2013)

In November 1973, my colleagues A. C. Y. Chang, H. W. Boyer, R. B. Helling, and I reported in PNAS that individual genes can be cloned and isolated by enzymatically cleaving DNA molecules into fragments, linking the fragments to an autonomously replicating plasmid, and introducing the resulting recombinant DNA molecules into bacteria. A few months later, Chang and I reported that genes from unrelated bacterial species can be combined and propagated using the same approach and that interspecies recombinant DNA molecules can produce a biologically functional protein in a foreign host. Soon afterward, Boyer's laboratory and mine published our collaborative discovery that even genes from animal cells can be cloned in bacteria. These three PNAS papers quickly led to the use of DNA cloning methods in multiple areas of the biological and chemical sciences. They also resulted in a highly public controversy about the potential hazards of laboratory manipulation of genetic material, a decision by Stanford University and the University of California to seek patents on the technology that Boyer and I had invented, and the application of DNA cloning methods for commercial purposes. In the 40 years that have passed since publication of our findings, use of DNA cloning has produced insights about the workings of genes and cells in health and disease and has altered the nature of the biotechnology and biopharmaceutical industries. Here, I provide a personal perspective of the events that led to, and followed, our report of DNA cloning.

restriction enzyme | pSC101 | *Eco*RI | genetic engineering | gene cloning

In a PNAS paper entitled "Construction of Biologically Functional Bacterial Plasmids *In Vitro*," my colleagues A. C. Y. Chang, H. W. Boyer, R. B. Helling, and I reported in November 1973 that individual genes can be cloned and isolated by enzymatically fragmenting DNA molecules, linking the pooled fragments to autonomously replicating circular bacterial genetic elements known as plasmids, and introducing the resulting recombinant DNA molecules into bacteria (1). Boyer and I were young faculty at the University of California, San Francisco (UCSF) and Stanford, respectively. Annie Chang was a Research Technician in my laboratory and Bob Helling was a University of Michigan professor on sabbatical leave in Boyer's laboratory. A few months later, Chang and I reported that genes from totally unrelated bacterial species can be combined and propagated using the same approach (2) and that interspecies recombinant DNA molecules can produce a biologically functional protein in a foreign host. Soon afterward, Boyer's laboratory and mine published collaborative experiments demonstrating that genes from eukaryotic cells can be cloned in bacteria (3).

Bacterial viruses and plasmids had been shown to pick up DNA from the chromosomes of their hosts (4); hybrid viruses from animal cells also had been reported (5, 6). However, it had long been known that only closely related species can interbreed and produce viable offspring, and hybrids displaying heritable characteristics of very different species exist only in mythology; thus, there was uncertainty about whether so-called "natural barriers created during evolution" (7, 8) would prevent propagation of genes across different biological domains. Stringent host range limitations to virus propagation had been observed, and, in some instances, impediments to survival of foreign DNA had been found even among subgroups of the same species (9). Supporting the notion that DNA was unlikely to survive in cells of an unrelated species was the finding that individual biological species maintain characteristic ratios of A+T to G+C base pairs (10, 11). Our discovery that DNA can be transplanted to, and propagated in, a different species, and even in a different biological kingdom, by attaching it to a vector indigenous to the recipient led to the realization that natural barriers to DNA survival are not so constraining after all, and that "genetic engineering"—at least at the cellular level—is possible (8). It also provided a protocol that enabled such engineering to be done by virtually any laboratory having modest genetic and biochemical capabilities.

Our DNA cloning experiments resulted from the pursuit of fundamental biological questions rather than goals that most observers might regard as practical or "translational." I was investigating mechanisms underlying the ability of plasmids to acquire genes conferring antibiotic resistance and to exist separately from bacterial chromosomes; Herb Boyer was studying enzymes that restrict and destroy foreign DNA. The PNAS publications resulting from these pursuits generated considerable scientific excitement—and work aimed at repeating and extending the findings was undertaken almost immediately by other researchers. The papers also prompted a highly public controversy about potential hazards of "genetic tinkering," a decision by Stanford University and the University of California to seek patents on the technology that Boyer and I had invented, and efforts by entrepreneurs and industry to implement DNA cloning methods for commercial purposes. In the 40 years that have now passed since publication of these PNAS papers, use of DNA cloning methods has produced important insights about the workings of genes and cells in health and disease and has profoundly altered the biotechnology and pharmaceutical industries. I provide here a personal perspective of these events.

Plasmids and Antibiotic Resistance

After the development of antimicrobial agents in the 1940s, the notion was prevalent that these drugs would end infectious diseases caused by bacteria. Of course that did not happen, and the reason was the occurrence of antibiotic resistance. Investigations carried out primarily in laboratories in Japan and the United Kingdom in the early 1960s showed that antibiotic resistance in bacteria commonly is associated with the acquisition of genes—often multiple genes—capable of destroying antibiotics or otherwise interfering

Author contributions: S.N.C. wrote the paper.

The author declares no conflict of interest.

This article is a PNAS Direct Submission.

This article was invited in recognition of the 40th anniversary of the November 1973 PNAS paper by S. N. Cohen, A. C. Y. Chang, H. W. Boyer, and R. B. Helling reporting a method for constructing and cloning biologically functional DNA molecules (1).

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with their actions. The resistance properties commonly did not map genetically to the bacterial chromosomes, suggesting that the genes encoding resistance were located on separate elements (some had called them episomes) analogous to the fertility factor (F-factor) discovered earlier (12). Like F-factors, resistance factors (R-factors) were capable of being transferred between bacteria by cell-to-cell contact (13, 14). In 1952, Joshua Lederberg had given the name "plasmids" to such extrachromosomal genetic elements (15). The antibiotic-inactivating genes carried by resistance plasmids provide a biological advantage to host bacteria in populations exposed to antimicrobial drugs, and, in barely a decade after the introduction of antibiotics to treat human infections, R-plasmid–mediated multidrug resistance had become a major medical problem as well as a scientific enigma.

The transfer of resistance properties between bacteria was found by centrifugation analysis to be sometimes associated with the acquisition of heterogeneous DNA bands (16, 17). However, the molecular nature of this DNA was controversial. Particularly uncertain was whether the resistance and transfer components of R-plasmids were carried by the same DNA molecule or were located on separate molecules that can interact transiently during interbacterial transfer (18, 19). Importantly, nothing was known about the genetic recombination mechanisms that had enabled the accumulation of multiple resistance genes on the same genetic element. Before moving to Stanford, I prepared National Institutes of Health (NIH) and National Science Foundation (NSF) research proposals aimed at addressing these questions. As a postdoctoral fellow in the laboratory of Jerard Hurwitz at the Albert Einstein College of Medicine, I had found that genes on different segments of DNA of bacteriophage λ were controlled temporally at the level of transcription during the production of virus particles (20), and I thought that some of the approaches I used in those experiments could be applied to the study of R-plasmids. I had been trained as both a physician and scientist and believed that an understanding of resistance plasmids was both medically and scientifically important. Bacteriophage λ was the most extensively and competitively investigated bacteriophage of that era, but the role of R-plasmids in antibiotic resistance was being studied in only a small number of microbiology laboratories; I liked the prospect of working in what was still a quiet backwater of scientific research.

Research in the burgeoning field of molecular biology during the 1960s focused on bacteriophages for an important reason: a bacterial cell infected by a virus generates thousands of identical copies—clones—of a single infecting genome during the normal viral life cycle. Thus, phenotypic effects can be correlated with the results of biochemical analyses. I realized that elucidation of how resistance genes function and how R-plasmids evolve required a way to clone individual plasmid DNA molecules and to isolate the resistance genes. Genetic mapping of R-plasmid properties had led to the prediction that bacterial plasmids exist as DNA circles (19, 21, 22), and I proposed to use circularity to isolate intact resistance plasmid DNA. If I could obtain R-plasmid circles—I reasoned—I could apply DNA fragmentation approaches I had used to study λ gene expression, together with ultracentrifugation analysis and DNA hybridization methods, to assess changes in circle size associated with the gain or loss of resistance phenotypes or transferability. Stanley Falkow, whose published investigations at Walter Reed Medical Center and Georgetown University had been instrumental in attracting me to plasmid biology, agreed to provide bacterial strains and plasmids for my initial experiments. However, Falkow also told me that he planned to stop working on plasmids to pursue other scientific interests, and the decision of an established expert to abandon a field that I had just decided to enter was disconcerting! However, there was also good news: in October 1967, Donald Helinski and his colleagues at the University of California, San Diego published the first molecular evidence for circular DNA forms of a transmissible plasmid (23), supporting the notion that R-factors, too, would be DNA circles.

I arrived at Stanford in March 1968, and, the following year, Christine Miller, who was then a newly hired laboratory technician, and I reported the purification of intact circular DNA of the large antibiotic resistance plasmid R1 (24). Several months later, I was invited to Caltech by Jerome Vinograd, a pioneer in the study of circular DNA of animal cells, to give a seminar talk about our plasmid results. Discussions with Norman Davidson and his graduate student Phillip Sharp during that visit initiated a collaboration between our laboratories aimed at using electron microscope-based heteroduplex analysis, which had been developed earlier in Davidson's laboratory (25) and also at the University of Wisconsin (26), to compare regions of sequence similarity on different plasmids: annealing of homologous DNAs results in smooth thickened DNA segments that are distinguishable visually from thinner, more kinky regions of single-stranded DNA. We expected that such experiments would provide information about the structural relationships between resistance genes that had been picked up by plasmids during their meandering through bacterial populations. The results of these experiments and also of separate investigations from Davidson's laboratory (27, 28) showed remarkable sequence conservation among large segments of different R-plasmids and, importantly, provided direct physical evidence that plasmid sequences associated with interbacterial DNA transfer had become linked covalently to resistance genes to form large circles of R-plasmid DNA. Sharp's electron microscopy also detected a phenomenon that we didn't yet understand the significance of: short inverted repeats of DNA sequences that bracket regions containing certain resistance genes—presaging by a couple of years the discovery of insertion sequence (IS) elements and antibiotic resistance transposons, which enter plasmids by illegitimate (i.e., nonhomologous) recombination and lead to rearrangements of plasmid structure (for reviews, see refs. 29 and 30).

Bacterial Transformation by Plasmid DNA

The methods for heteroduplex analysis that I learned from Sharp during our collaboration enabled me to use electron microscopy in my plasmid studies at Stanford when he and Davidson later moved on to other scientific interests. However, still missing from a growing collection of tools available to investigate the molecular biology of plasmids was a method for reintroducing plasmid DNA molecules into bacteria. Genetic transformation using naked DNA had been shown for pneumococcus, *Bacillus* species, and certain other bacteria, but not for *Escherichia coli* or other microbes that carry the R-plasmids I was studying. Quite fortuitously, a critical discovery by Morton Mandel and Akiko Higa reported in the October 1970 issue of the *Journal of Molecular Biology* (31) pointed the way toward such a method.

Mandel and Higa found that *E. coli* cells treated with calcium ions can take up DNA of the temperate bacteriophages λ and P22 and that such cells can release infective virus. The procedure is robust, and Peter Lobban, a graduate student working with A. Dale Kaiser in the Stanford Department of Biochemistry, had begun using it to introduce phage P22 DNA into *Salmonella typhimurium,* a close relative of *E. coli*. Mandel and Higa had reported an attempt to genetically transform *E. coli* to express an antibiotic resistance gene

present on the bacterial chromosome and had met with failure. However, plasmids, like phages, are extrachromosomal elements; if R-plasmid DNA could be taken up by *E. coli* at even a low frequency, and if the antibiotic resistance genes carried by circular R-plasmid replicons were expressed in these cells, colonies of bacteria that acquire plasmids could be selected using culture media containing appropriate antibiotics. Such selection might enable cloning of single molecules of plasmid DNA. I asked Leslie Hsu, a first-year medical student who had come to my laboratory for research training, to undertake such experiments.

By late 1971, Leslie, with the help of Annie Chang, had shown that bacterial clones containing autonomously replicating plasmid molecules can be obtained using a modification of the Mandel and Higa procedure. Later work by others (32) indicated that the failure of chromosomal DNA to transform calcium chloride-treated *E. coli* in Mandel and Higa's earlier experiments had resulted from exonucleolytic digestion of fragmented chromosomal DNA by a bacterial enzyme. Luckily, the circularity of R-plasmid DNA molecules had avoided this pitfall. The resulting *E. coli* colonies each contained a circular DNA species having the resistance, fertility, and sedimentation properties of the parental genetic element. Publication of our paper reporting these findings in August 1972 (33) interested plasmid researchers but, so far as I could determine, was hardly noticed by others. There was scant awareness in the phage-oriented world of 1972 molecular biology of the implications of being able to clone plasmid DNA molecules, and our report did nothing to alter the backwater nature of the field of plasmid biology. That was fine with me: I was a junior scientist whose laboratory included just a few students and postdocs, plus two research assistants. My primary academic appointment at Stanford was then in the Department of Medicine, and my clinical teaching responsibilities affected the time I had available for laboratory research; the quiet reception that our paper received allowed me to proceed with less pressure to undertake the experiments I had long been planning.

In May 1972, Annie and I began to break apart molecules of the large multidrug resistance plasmids R6 and R6-5 using the mechanical shearing procedure I had used 6 years earlier to separate and study the two halves of the bacteriophage λ genome (20). The fragmented plasmid DNA was introduced into calcium chloride-treated bacteria, and transformants were screened for cells that acquired individual resistance determinants. I knew from the heteroduplex experiments carried out with Sharp and Davidson that R6 and R6-5 contained repeats of some DNA sequences and hoped that ordinary genetic recombination between these homologous segments would lead to circularization of shear-generated fragments in calcium chloride-treated cells—and perhaps even the formation of novel plasmids lacking some of the original DNA resistance determinants. If such recombinants occurred even infrequently, antibiotic selection might identify colonies of bacteria acquiring them. We did in fact identify bacteria containing a small plasmid that expressed only tetracycline resistance and that we thought had been derived from R-5 (34). However, our later investigations (35) indicated that this was a natural plasmid that had contaminated the DNA preparations; we had underestimated the power of antibiotic selection to identify rare bacterial cells transformed by resistance plasmids. Whereas our efforts in mid-1972 to clone resistance genes from plasmids used largely the same strategy that Boyer and I and our colleagues used successfully less than a year later, they lacked a key ingredient of the later experiments: an enzyme that cuts each plasmid DNA molecule identically and produces complementary sequences at the ends of DNA fragments it generates—i.e., the restriction endonuclease *Eco*RI.

Restriction Endonucleases

The ability of bacteria to restrict the growth of phage that had been propagated on other strains had been known since the late 1930s, but work aimed at understanding the mechanism underlying this phenomenon didn't begin for another 20 years. Much of that work was carried out by the Swiss microbiologist and geneticist Werner Arber and his student Daisy Dussoix, who showed that the DNA of restricted phage is enzymatically degraded (9). In 1970, Hamilton Smith and his colleagues at Johns Hopkins University reported that a restriction enzyme they named *Hin*dII—a protein isolated from the bacterial pathogen, *Haemophilus influenzae*—recognizes particular nucleotide sequences in DNA and cuts duplex DNA site-specifically at these sequences (36). The following year, Karen Danna and Daniel Nathans found that the *Hin*dII endonuclease cleaves DNA of the mammalian tumor virus SV40 into 11 fragments that can be separated by acrylamide gel electrophoresis, demonstrating the utility of restriction endonucleases for DNA analysis (37). Arber, Nathans, and Smith received the 1978 Nobel Prize in Physiology or Medicine for these accomplishments.

Herb Boyer was studying restriction endonucleases in his laboratory at UCSF. Collaborating with him was Assistant Professor in Residence Daisy Dussoix (then Roulland-Dussoix), who as a student had participated with Arber in early investigations of the restriction/modification phenomenon. Some plasmids had been found to encode restriction enzymes (38, 39), and the Ph.D. thesis project of Robert Yoshimori, a graduate student working with Boyer and Roulland-Dussoix, was to identify new restriction enzymes that might be encoded by *E. coli* plasmids. He found two such enzymes (40), and Boyer and his colleagues proceeded to purify them and investigate their properties. One of the enzymes was *Eco*RI (*E. coli* Restriction endonuclease I). Like *Hin*dII, *Eco*RI cleaved DNA site-specifically, and Boyer set out to determine the nucleotide sequence attacked by the enzyme. Boyer also provided a sample of *Eco*RI to Norman Salzman at the NIH, whose laboratory found that the oncogenic virus SV40 is cleaved once by this endonuclease and used the cleavage site to map the origin and direction of SV40 DNA replication (41). John Morrow, a Stanford graduate student of Paul Berg, who also received *Eco*RI from Boyer for testing on SV40 DNA, made the same observation (42). Additional experiments with *Eco*RI samples that Boyer had given to others yielded an unexpected dividend: evidence that *Eco*RI, unlike *Hin*dII, cleaves the DNA sequence it recognizes asymmetrically, generating single-strand extensions that contain nucleotides complementary to those present at the ends of other *Eco*RI-generated fragments.

Hydrogen bonding between dA and dT deoxynucleotides and between dGs and dCs had been known for a decade to be able to hold DNA strands together. Alfred Hershey and his colleagues at the Cold Spring Harbor Laboratory had reported in 1963 that bacteriophage λ DNA contains complementary single-strand segments at its ends, enabling linear DNA that had been packaged in a viral particle to become circular and insert into the bacterial chromosome (43). Cohesive ends on λ DNA molecules were used as substrate by Martin Gellert (44) and others (45) to isolate an enzyme, DNA ligase, that covalently joins λ DNA segments held together by complementary ends. Complementary ends were thus well recognized as a device for joining together DNA molecules (46). Attribution of credit for who first made the observation that cleavage of duplex DNA by *Eco*RI generates fragments that have complementary cohesive termini has been controversial, but what is shown by the published record is that three separate

papers simultaneously reporting this finding appeared in the November 1972 issue of PNAS: the papers were authored by Janet E. Mertz and Ronald W. Davis of the Stanford Department of Biochemistry (47), by Vittorio Sgaramella of the Stanford Department of Genetics (48), and by Boyer and his coworkers Howard Goodman and Joe Hedgpeth (49) at UCSF.

The Hawaii Meeting and the Initial DNA Cloning Experiments

In early 1972, I agreed to join Tsutomu Watanabe, a Keio University microbiologist who was a pioneer in studies of bacterial antibiotic resistance, and Donald Helinski in organizing a United States–Japan conference on plasmids later that year. A few weeks before the meeting, which was held in mid-November at the University of Hawaii in Honolulu, Don contacted me to suggest that Herb Boyer, whose work on the plasmid-encoded *Eco*RI enzyme he had just learned about, be added to the list of speakers. I telephoned our invitation to Herb and thus began a scientific interaction that less than 6 months later resulted in the cloning of antibiotic resistance genes from plasmids.

The actual collaboration began during a long walk near Honolulu's Waikiki Beach in search of a sandwich shop to have a late evening snack. Boyer and I were joined by Stanley Falkow, who recently had moved his laboratory to the University of Washington, Charles Brinton, a microbiologist from the University of Pittsburgh, and Charles's wife, Ginger. During that walk, Herb and I discussed recent results from our laboratories. I described our experiments showing that *E. coli* could be transformed genetically with naked plasmid DNA, and our plasmid DNA shearing experiments, which had not yet been published, and Herb described the similarly unpublished sequencing data that he, Joe Hedgpeth, and Howard Goodman had obtained for the *Eco*RI cleavage site. As Herb and I talked, I realized that *Eco*RI was the missing ingredient needed for molecular analysis of antibiotic resistance plasmids. Large plasmids would be cut specifically and reproducibly by the enzyme, and this method of cleavage would surely be better than the haphazard mechanical shearing methods I had been using for fragmentation of plasmid DNA circles. Because *Eco*RI recognizes a six base pair sequence, cleavage sites on duplex DNA would be on average about 4,100 base pairs apart, and each of the DNA fragments produced would likely contain only a few genes. The number of fragments would be few enough to separate them by centrifugation, enabling their use in DNA–DNA hybridization experiments. Because of the asymmetry of cleavage of the *Eco*RI recognition sequence, the ends of the multiple plasmid DNA fragments generated by *Eco*RI would be complementary—and under the right conditions individual plasmid DNA fragments in the mixture could join to each other in different combinations. If cleavage by *Eco*RI left the replication function of the plasmid intact, the region encoding this function might join randomly to different combinations of antibiotic resistance genes in the fragment mixture, forming DNA circles that could be sealed using DNA ligase, as had been shown for the complementary extensions at the ends of λ DNA (44, 45). And the plasmid DNA transformation procedure would enable us to select for, and hopefully to separately clone, specific resistance genes using appropriate combinations of antibiotics on culture plates.

Herb initially didn't appear to be especially interested in studying R-plasmid DNA structure and offered simply to provide the enzyme that he generously had given to others. However, he was excited about the use of autonomously replicating R-plasmids to clone *Eco*RI-generated DNA fragments. By the time we encountered a small delicatessen having an enticing window sign that read, "Shalom," in place of the ubiquitous "Aloha," we had decided to proceed collaboratively and agreed on the basic design of the project that our laboratories would jointly carry out. We would target the R6-5 plasmid, which Sharp, Davidson, and I had learned much about from heteroduplex analysis, and which Chang and I had been shearing using a mechanical stirring device and metal blades, in our initial experiments. A few minutes later, over warm corned beef sandwiches and cold beer (Fig. 1), Herb and I sketched out an experimental plan on napkins taken from the dispenser at our table.

Our strategy was straightforward (Fig. 2), but there was no assurance that it would work. Yes, we knew that we could genetically transform *E. coli* with plasmid DNA and use antibiotic resistance genes to identify cells that acquire plasmids, and we expected from the nucleotide sequence at the *Eco*RI cleavage site that the restriction enzyme would cut the DNA of our large plasmids reproducibly into multiple fragments. We knew from published earlier results that DNAs having complementary ends would link together by base pairing: Khorana and his colleagues had joined together double-stranded fragments of synthetic DNAs in vitro by chemically adding complementary nucleotides to them one at a time (50)—

Fig. 1. Cartoon by D. Adair in the *Honolulu Advertiser* newspaper, September 26, 1988 accompanying an article reporting demolition of the Waikiki beach delicatessen where the initial DNA cloning experiments were planned. The persons depicted clockwise are presumed to be H. Boyer (12 o'clock), S. Cohen, G. Brinton, C. Brinton, and S. Falkow. Reprinted by permission.

demonstrating that such recombination is independent of the sequence of the duplex segments being joined. Jensen et al. (51) had used the strategy of bringing natural DNAs together by enzymatically adding stretches of complementary nucleotides to their termini, and Peter Lobban in Dale Kaiser's laboratory and David Jackson in Paul Berg's laboratory showed that disparate fragments from either the same genome or different genomes that were held together by enzymatically installed complementary single-strand segments can be ligated to create covalently bonded junctions (52, 53). Berg later commented: "it doesn't take a genius to figure out that if you can create artificial ends that are complementary to each other, the two DNA molecules will come together" (54). Moreover, results obtained by Mertz and Davis (47) and by Sgaramella (48) and published during the month of the Hawaii meeting showed that the four nucleotide single-strand extensions generated by *Eco*RI are sufficient in length to enable DNA fragments to be spliced together in vitro.

However, notwithstanding our expectation that we would be able to biochemically join the complementary ends of *Eco*RI-generated fragments of plasmid DNA, there were important biological unknowns in the experiments that Boyer and I planned. Would cleavage of R6-5 with *Eco*RI disrupt regions needed for plasmid DNA replication or expression of antibiotic resistance? And would recombinant DNA molecules created in the laboratory be reproduced and transcribed in bacterial cells? DNA junctions formed during legitimate genetic recombination in cells are generated by a process that has resulted from billions of years of evolution; would the random joining of DNAs by artificial means create anomalous chromatin conformations

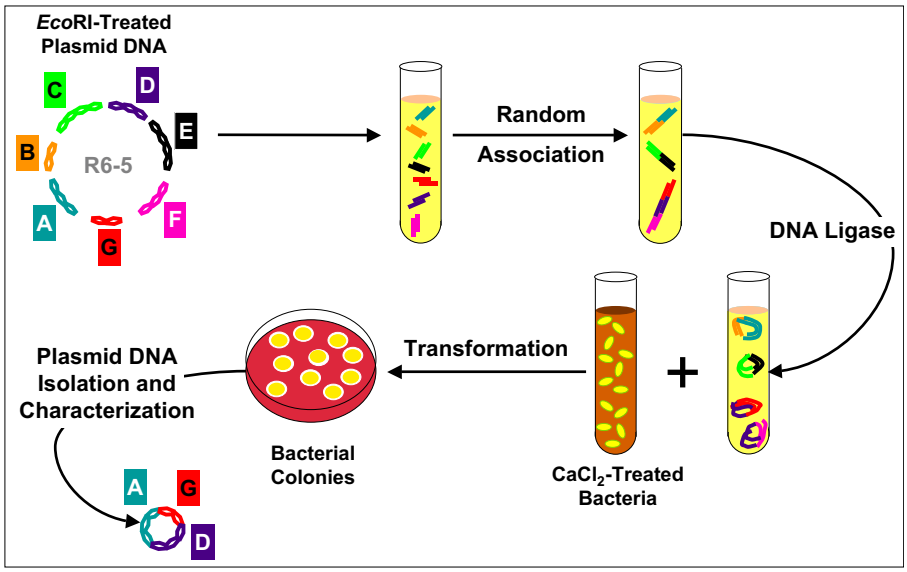

Fig. 2. Schematic diagram of the strategy used for construction of biologically functional plasmids (1). R6-5 plasmid DNA fragments generated by cleavage using the *Eco*RI endonuclease were allowed to associate randomly in vitro and were then covalently joined by DNA ligase. DNA in the resulting mixture was introduced into calcium chloride-treated *E. coli*, and bacterial colonies expressing individual antibiotic resistance phenotypes encoded by R6-5 were selected on media containing antibiotics. Plasmid constructs isolated from these *E. coli* clones contained DNA fragments carrying specific resistance genes.

that prevent propagation of the molecules? These multiple issues led Falkow, who together with Charles Brinton had participated in the discussion and who envisioned the possibility of isolating an enteric bacterial toxin gene he had been studying using the procedure that Boyer and I had just sketched out, to remark, "If it works, let me know" (55). A senior Stanford colleague whom I spoke with after my return to Palo Alto was considerably less sanguine, proffering the opinion that nothing interpretable was likely to come from the "messy" experimental design.

We began the experiments shortly after the new year. They went more smoothly than we could have hoped, and by March 1973 we had demonstrated the feasibility of the DNA cloning approach that Boyer and I had outlined a few months earlier on delicatessen napkins. During a visit to the Cold Spring Harbor Laboratory that winter to give a seminar talk, Herb learned about the still unpublished agarose gel electrophoresis/DNA staining method that Phillip Sharp, Bill Sugden, and Joseph Sambrook had developed to separate and visualize fragments of DNA generated by restriction enzymes (56); this advance offered a hugely important addition to the centrifugation and heteroduplex methods we were using to analyze plasmids. In the collaboration, Herb's laboratory purified the restriction endonuclease we used and characterized plasmid DNA in ethidium bromide-stained agarose gels. My laboratory isolated and purified plasmid DNA, did bacterial transformations and selection, and characterized the products by heteroduplex analysis and ultracentrifugation in gradients. Data were analyzed at both places, and results were discussed between laboratories almost daily. I'd arrive in the laboratory early in the morning to look at the culture plates when colonies produced by cells plated late the previous evening were still tiny. I often wished that the bacteria would grow faster so that we could obtain results sooner. Annie lived in San Francisco and carried materials between Stanford and UCSF. We'd hurry to isolate plasmid DNA so that she could carry some of it to Herb's laboratory for gel analysis the next day. It was an extraordinarily exciting time for all of us.

By introducing a mixture of ligated *Eco*RI-generated R6-5 DNA fragments into *E. coli*, we recovered a plasmid that expressed kanamycin resistance but not the other resistance genes of R6-5. This replicon included only three of the DNA fragments characteristic of the parental plasmid (Fig. 3, *Top*, lanes a and b). Further analysis indicated that one of these fragments encoded functions and sites necessary for autonomous DNA replication but contained no detectable resistance gene; a second fragment lacking the capability for autonomous replication, but carrying a kanamycin resistance gene, had been attached in the fragment mixture to the replication region, and, during bacterial transformation and selection of kanamycin resistant cells, the gene had been cloned. We were absolutely elated but knew that robust DNA cloning would require a plasmid vector that includes both a selectable gene and replication capabilities on a single *Eco*RI-generated fragment. We found that the tetracycline resistance plasmid that Chang and I had isolated during our mechanical shearing experiments—a replicon we named pSC101 (Fig. 3, *Top*, lane c and Fig. 3, *Middle*)—had exactly these properties. Using pSC101 as a vector, we were able to identify the specific R6-5 DNA fragment that carries the kanamycin resistance gene (Fig. 3, *Bottom*).

By early May, we had shown that our cloning results were reproducible, and we met to decide on the figures for the manuscript we would be preparing. I outlined the paper's format in a notebook that sometimes has been referred to as my "laboratory notebook" (57) but which was used for jotting down ideas and future plans rather than for recording experimental data. The paper (1) was completed in early June and, after being modified to address small points raised by

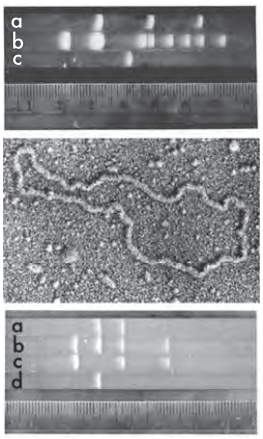

Fig. 3. DNA analysis in the initial DNA cloning experiments. *(Top)* Agarose-gel electrophoresis of (lane a) the pSC102 plasmid containing three of the multiple *Eco*RI-generated fragments of R6-5 DNA (lane b). Lane c shows that *Eco*RI cleavage of the pSC101 vector produces a single DNA fragment of the expected size. (*Middle*) Electron photomicrograph of pSC101, the first plasmid used successfully as a vector for DNA cloning. (*Bottom*) Agarose gel electrophoresis showing cloning of the kanamycin resistance gene of R6-5: (lane d) *Eco*RI-cleaved DNA of the pSC101 plasmid vector, (lane c) *Eco*RI-generated fragments of a novel plasmid (pSC102) that had been constructed from R6-5 (see *Top*) and that expresses the kanamycin resistance determinant of the parental R6-5 replicon, (lane b) mixture of the DNAs shown in lanes c and d, and (lane a) *Eco*RI-generated fragments of a novel plasmid (pSC105) expressing both the tetracycline resistance gene of the pSC101 vector and the kanamycin resistance gene, which had been cloned from pSC102 by attaching it to pSC101. *Top* and *Bottom* are from ref. 1.

reviewers, was communicated to PNAS by Academy member Norman Davidson.

Herb and I had recognized that small antibiotic resistance plasmids such as pSC101 might enable the cloning of eukaryotic cell genes in *E. coli* and included this statement in our paper's Discussion section. However, which eukaryotic DNA should be used to test the notion? We couldn't specifically select bacteria containing cloned eukaryotic DNA, and only a few cellular genes of eukaryotes had been purified and characterized well enough to identify them unambiguously in bacterial isolates. Besides, there were other experiments that each of us wanted to do, and our priorities were different. Herb was eager to use a rapidly expanding collection of restriction enzymes to construct high copy-number vectors that offer increased flexibility of cloning sites. The most pressing issue for me was to learn whether DNA hybrids containing very different components derived from unrelated species could be propagated and cloned. Our laboratories exchanged experimental tools and set out separately to address our different priorities.

Testing of "Interspecies Barriers"

Design of specific experiments that I believed could provide an initial test of the hypothesized barriers to interspecies gene transfer began after completion of the Cohen, Chang, Boyer, and Helling manuscript. Richard Novick of the Public Health Research Institute in New York and his colleagues had described an 18-kb plasmid named pI258 (58) that replicates autonomously in *Staphyloccus aureus*, but not in *E. coli*. pI258 had been shown to carry a β-lactamase gene encoding resistance to penicillins, and such resistance might be used to select *E. coli* transformants carrying hybrid plasmids expressing β-lactamase. Whether DNAs known to be highly disparate in nucleotide composition (11) and taken from microbes as different as the Gram-positive coccus *S. aureus* and the Gram-negative rod-shaped *E. coli* could be propagated as part of the same replicon and whether the staphylococcal gene would be expressed in the new host was questionable. However, if these events occurred, the density gradient analysis methods that Miller and I had used earlier (24) would aid in establishing the origin of DNA segments that differ in A+T/G+C ratio. *E. coli* cells resistant to both penicillin/ampicillin and tetracycline were already highly prevalent, so combining pI258 and pSC101 DNAs would not produce a novel resistance combination.

The experiments themselves were not complicated and the results were conclusive. We cleaved pI258 DNA and pSC101 DNA using the *Eco*RI enzyme, characterized, and then combined, the DNA fragments, introduced the ligated mixture into calcium chloride-treated *E. coli*, and selected bacterial colonies that expressed both the ampicillin resistance of pI258 and the tetracycline resistance encoded by pSC101 (Fig. 4, *Upper*). Buoyant density ultracentrifugation (Fig. 4, *Lower*), agarose gel electrophoresis, and electron microscope heteroduplex analysis showed that the *E. coli* colonies contained plasmids that included DNA sequences from the two species (2).

Gordon Conference Discussions About Biohazard Concerns and Cloning of Eukaryotic DNA in Bacteria

Sometime in late May or early June 1973, Boyer received an invitation to give an informal talk at the Gordon Research Conference on Nucleic Acids scheduled for mid-June. He described his laboratory's work at a session on "Bacterial Enzymes in the Analysis of DNA" and, near the end of his presentation, reported results from our collaborative experiments, which had not yet been accepted for publication. His presentation prompted Bill Sugden, one of the inventors of agarose gel electrophoresis attending the Gordon Conference to comment, "well, now we can put together any DNAs we want to" (59). The following morning on the last day of the meeting, a special session was called by cochairs Maxine Singer and Dieter Söll to discuss the implications of the data that Boyer had shown. Seven months earlier, the ability of *Eco*RI to generate cohesive DNA ends that could be used for joining DNAs biochemically had been reported; however, now presented with evidence that *Eco*RI-generated, ordinarily nonreplicating DNA fragments can actually be propagated in bacteria by attaching them to plasmid DNA—and that hybrid DNAs created in this way are biologically functional—the attendees were concerned that hybrids "with biological activity of unpredictable nature may eventually be created" (60). They voted to communicate this concern to the presidents of the National Academy of Sciences (NAS) and its Institute of Medicine, suggesting that a study committee be established to consider the issue and to "recommend specific actions or guidelines."

A discussion at the same Gordon Conference between Boyer and John Morrow, who had completed his Ph.D. thesis project in Paul Berg's laboratory at Stanford but had not yet moved to a postdoctoral position with Donald Brown at the Carnegie Institution of Washington laboratory in Baltimore, MD, led to a second collaboration between Boyer's laboratory and mine. Brown had purified and characterized the ribosomal genes of the African frog, *Xenopus laevis*, and Morrow had found that this DNA was cleaved by the *Eco*RI enzyme preparation that Boyer had provided for analysis of SV40 viral DNA. Morrow and Boyer discussed trying to clone *Eco*RI-generated fragments of frog ribosomal DNA using the approach that Boyer's lab and mine had employed to clone plasmid DNA fragments. Brown agreed to allow the DNA he had given to Morrow to be used for the attempt. However, how to identify cloned ribosomal RNA? When Herb returned to UCSF, he phoned me to discuss this question and to invite my participation in the proposed project. We agreed that multiple parameters would be needed to show unambiguously that DNA from another biological kingdom was being propagated in bacteria. Although there were no phenotypic properties that would enable bacterial colonies that acquired plasmids carrying ribosomal DNA inserts to be selected, ribosomal genes, which were known to be extraordinarily rich in G+C base pairs (61), could be distinguished from *E. coli*

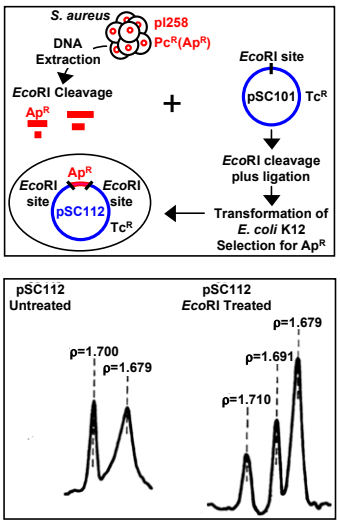

Fig. 4. Cloning of *S. aureus* plasmid DNA in *E. coli*. (*Upper*) Schematic diagram of strategy used for testing the viability of interspecies DNA hybrids (2). DNA of the pI258 plasmid, which carries a β lactamase gene encoding resistance to penicillins in *S. aureus* was cleaved by *Eco*RI endonuclease and mixed with similarly cleaved DNA of the pSC101 vector encoding tetracycline resistance. After ligation, the mixture was introduced into *E. coli* cells, and colonies that expressed both resistance phenotypes were identified. (*Lower*) Centrifugation analysis in isopycnic density gradient of plasmid DNA (pSC112) isolated from an *E. coli* clone expressing both resistances and showing DNA species that band at buoyant densities characteristic of *E. coli* (ρ = 1.710) and *S. aureus* (ρ = 1.68–1.69) DNAs and reflect the distinctly different A+T/G+C nucleotide ratios of these unrelated bacterial species. *Lower* is from ref. 2.

DNA by buoyant density differences during isopycnic centrifugation, as well as by fragment size during agarose gel analysis and by electron microscopy heteroduplex analysis. I thought that it might be necessary to screen a large number of individual bacterial colonies to find recombinant plasmids, but it was worth a try.

Morrow and Chang carried out the bulk of the experiments, which were done largely in my laboratory at Stanford during the late summer and early fall of 1973—still several months before the November publication of results from the initial collaboration with Boyer. As data began to accumulate to support the conclusion that eukaryotic DNA can actually be propagated in bacteria, our examination of grids prepared for electron microscope heteroduplex analysis removed any remaining uncertainty (Fig. 5): heteroduplexes formed between the ribosomal RNA taken from frogs and recombinant plasmids isolated from *E. coli* revealed homology at two locations that were spaced at the distance that Brown's laboratory had shown to be the spacing between sequence repeats present on ribosomal RNA genes (3). And additional DNA/RNA hybridization experiments indicated that transcripts capable of interacting with ribosomal RNA genes of the frog were produced in *E. coli*.

The news that eukaryotic DNA can be cloned and amplified in bacteria spread immediately in the scientific community, and requests for the pSC01 plasmid began to arrive at my laboratory. The first to receive pSC101 was David Hogness, a distinguished Stanford Department of Biochemistry professor who had been attempting unsuccessfully to clone *Drosophila melanogaster* DNA by using λdv, a nonlytic phage variant that Kenichi Matsubara and Dale Kaiser had shown can replicate autonomously in *E. coli* (62). Attaching the *Drosophila* DNA to the pSC101 plasmid enabled Hogness and his coworkers to confirm in late 1974 the cloning of eukaryotic cell genes in bacteria and to use DNA cloning to map sequences in *Drosophila* chromosomes (63). Concurrently, other laboratories focused on constructing new bacteriophage λ variants able to produce viable molecular hybrids (64, 65), and, in November 1974, Ron Davis and his coworkers at Stanford reported that such hybrids can be propagated as plaque-forming phage (66).

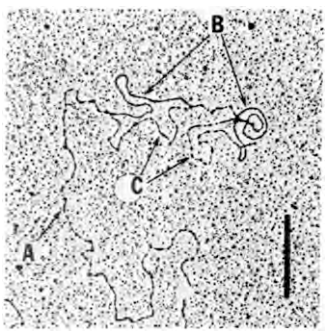

Fig. 5. Electric photomicrograph of heteroduplex showing homology between DNA isolated from *X. laevis* oocytes and plasmid DNA isolated from bacteria and containing fragments of ribosomal RNA genes that had been cloned by attaching the eukaryotic cell DNA to the pSC101 vector. (*A*) Single strand of *X. laevis* rDNA. (*B*) Double-stranded regions of homology between *X. laevis* rDNA and plasmid DNA isolated from *E. coli*. (*C*), Single-strand DNA regions corresponding in length to the pSC101 vector, which shares no homology with *X. laevis* rDNA. (Scale bar: 1 μm.) Figure is from ref. 3.

Biohazard Speculations Mount

In response to the vote of attendees at the nucleic acids Gordon Conference and the consequent letter from Singer and Söll, NAS President Phillip Handler chose Paul Berg to form a committee to evaluate possible biohazardous consequences of constructing hybrid DNAs. Berg had thought deeply about this issue and was a perfect choice: using complementary ends installed onto DNA and further manipulations that Berg and his postdoctoral fellows David Jackson and Robert Symons credited to Dale Kaiser's graduate student Peter Lobban, Berg's laboratory had biochemically attached DNA of the SV40 tumor virus to a version of the bacteriophage λdv replicon that includes the galactose (*gal)* operon of *E. coli* (52). Berg later received the 1980 Nobel Prize in Chemistry in recognition of "his fundamental studies of the biochemistry of nucleic acids, with particular regard to recombinant DNA." Berg and his graduate student Janet Mertz planned to introduce these SV40-λdv*gal* hybrid DNA molecules into mammalian cells to determine whether the bacterial gene would function there (54, 67), and he and Mertz have written that they also wished to propagate the SV40-λdv*gal* hybrid DNA molecules in *E. coli* (67,68). However, at a Cold Spring Harbor Laboratory summer course in 1971 where Mertz described her proposed experiments, biologist Robert Pollack raised biohazard concerns about the possibility of creating oncogenic *E. coli* by such experiments, and Berg was persuaded to forego attempts at cloning the biochemically spliced SV40-λdv*gal* DNA molecules in either eukaryotic cells or bacteria (54, 68). Ironically with regard to Pollack's scenario, Mertz's 1975 PhD dissertation (67) stated that "scientific problems have been encountered during attempts to use λdv*gal* as a vector for replicating other DNA molecules" and that "the plasmid is unstable and readily lost from its *E. coli* host" (67). It was later learned that insertion of foreign DNA into the λdv*gal* site that the Berg team had used for construction of hybrid DNA molecules (52, 54) disrupts a gene essential for λdv*gal* replication in bacteria (54, 66, 69), possibly explaining the lack of success of the DNA cloning attempts reported in Mertz's dissertation (67). But Pollack's concerns and Berg's decision had importantly raised awareness about possible biohazardous consequences of creating novel DNA combinations (54).

The committee that Berg formed to address the biohazard concerns of the Gordon Conference participants consisted mostly of experts on oncogenic viruses, and it initially focused on issues related to the introduction of mammalian cell virus sequences into bacteria. However, during discussions by the group, its focus expanded to address the possible construction of novel resistance-gene combinations, and Herb Boyer, David Hogness, Ron Davis, and I were invited to participate in the formulation of the committee's final recommendations. These recommendations, which were published concurrently in July 1974 in PNAS, *Science*, and *Nature* as a letter entitled, "Potential Biohazards of Recombinant DNA Molecules," proposed a voluntary moratorium on the introduction of resistance genes into bacterial species that do not already express that type of resistance and on the linkage of animal virus genes to plasmids (70–72).

An article by *New York Times* journalist Victor McElheny, who learned about our DNA cloning experiments from Berg et al. committee member David Baltimore, appeared in the *Times* a few weeks before release of the committee's moratorium proposal (73). In this article, which was headlined, "Animal Gene Shifted to Bacteria; Aid Seen to Medicine and Farm," McElheny and the scientists he interviewed spoke optimistically about the potential benefits of DNA cloning, which was by then increasingly referred to as "recombinant DNA technology." However, a press conference arranged by the NAS to announce the moratorium proposed by the Berg et al. letter resulted in an abrupt shift of public focus to biohazard issues. The notion that prompted the shift: "if the researchers themselves are concerned, then the dangers must be truly horrific." The unprecedented effort of scientists to restrict their own research in order to guard against hazards that were not known to exist was so novel that this effort was widely interpreted as implying that danger was likely. A more extensive personal perspective on the Berg et al. letter,

the Asilomar Conference that it led to, and the post-Asilomar period of interaction between scientists and legislators is provided in the oral history I recorded with University of California Berkeley historian Sally Smith Hughes for the Bancroft Library in the mid-1990s (74).

Stanford/UCSF DNA Cloning Patents

McElheny's upbeat article in May 1974 was read by Niels Reimers, whose job as Director of the Office of Technology Licensing at Stanford was to help fund the university's academic programs by promoting the licensing of inventions made at the university. The day after the article appeared, I received a telephone call from Reimers indicating that he wanted to discuss patenting the technology that Boyer and I had invented. My first reaction was quite negative. Could findings of basic research funded by the public be patented, and should they be? I told him that our work depended on years of fundamental research on plasmid biology by many laboratories and on properties of DNA, DNA ligase, and restriction enzymes that had been discovered by others. And would a patent adversely affect advancement of the science? Reimers pointed out that prior knowledge is a pillar for every invention and that a well-honed legal process determines whether a particular advance is novel and patentable, as well as the validity of the inventorship claimed in the application. He explained that only commercial entities would pay royalties, that a patent would not impede noncommercial use of DNA cloning methods, and that funds received by Stanford and UCSF would aid research programs at these institutions. I discussed Reimer's proposal with Herb, and together we agreed to let our universities proceed with applications for patents that eventually had 461 licensees before their expiration in 1997. Reimers' oral history is a source of further information about the events that led to these patents (75).

Converting Promise into Reality

As experiments using DNA cloning procedures proceeded without adverse incident in laboratories around the world, biohazard fears dissipated (74). And multiple scientific advances in these laboratories began to turn the early promise of DNA cloning for producing fundamental knowledge and practical applications into reality. Better strategies for detecting cloned genes and their products were devised (76, 77), and methods were soon developed for cloning enzymatically generated DNA sequences complementary to mRNA (78). The strategies we had used for cloning DNA in *E. coli* were shown to be applicable to multiple eukaryotic and prokaryotic hosts. Immunologically reactive (79–81) and then biologically functional (82) eukaryotic proteins were produced in bacteria. Specialized vectors were developed by academic and industrial laboratories to express human proteins such as insulin and growth hormone in *E. coli*, to produce vaccines containing antigens expressed from cloned genes, and to identify genetic regulatory signals using reporter genes. Together, these advances provided a foundation for the creation of biotechnology companies. Herb Boyer and entrepreneur Robert A. Swanson founded an enterprise that became preeminent among these companies: Genentech. Efficient DNA sequencing methods invented by Allan Maxam and Walter Gilbert (83) and by Frederick Sanger and his colleagues (84) dramatically facilitated analysis of cloned DNA, and, together with the invention of the PCR by Kary Mullis (85), information that DNA sequencing yielded about the structure and function of cloned genes (86) led to the birth of the field of genomics. The number and breadth of the scientific discoveries that have occurred during a four-decade time frame seem unprecedented, and the consequent growth of knowledge in biology and chemistry has been almost logarithmic. However, although this article has been retrospective, in reality, the accelerated scientific journey that has resulted from the ability to clone DNA has only begun.

ACKNOWLEDGMENTS. I thank Herb Boyer, Annie Chang, and Bob Helling for their partnership in the scientific collaboration that resulted in successful DNA cloning, and both Annie Chang and Christine Miller for the other contributions they made to my laboratory and to science over decades. I also thank the many scientists whose prior research made possible the experiments I've described, especially plasmid biologists/microbiologists Stanley Falkow, Donald Helinski, and Richard Novick; biochemists Martin Gellert, Robert Lehman, Jerard Hurwitz, and Phillip Sharp; geneticists/microbiologists Dale Kaiser, Peter Lobban, Ronald Davis, and Vittorio Sgaramella; and biologist James D. Watson. The contributions and support of now-deceased friends and colleagues, especially Joshua Lederberg, Norton Zinder, Jerome Vinograd, and Norman Davidson, have also been of major importance to me, both scientifically and personally. I apologize to those whose relevant contributions have not been specifically mentioned. For more extensive information about my perspective of the events I've related here, I refer readers to my Bancroft Library oral history (74), and for the perspectives of others, to the Bancroft Library oral histories of Herbert W. Boyer (87), Paul Berg (54), and Niels Reimers (75).

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SCIENTIFIC AMERICAN OFFPRINTS

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by Clifford Grobstein

SCIENTIFIC AMERICAN
JULY 1977
VOL. 237, NO. 1 PP. 22–33

PUBLISHED BY **W. H. FREEMAN AND COMPANY** 660 MARKET STREET, SAN FRANCISCO, CALIFORNIA 94104

The Recombinant-DNA Debate

The four-year-old controversy over the potential biohazards presented by the gene-splicing method and the effectiveness of plans for their containment is viewed in a broader context

by Clifford Grobstein

The guidelines for research involving recombinant-DNA molecules issued a year ago by the National Institutes of Health were the culmination of an extraordinary effort at self-regulation on the part of the scientific community. Yet the policy debate over recombinant-DNA research was clearly not laid to rest by the appearance of the NIH guidelines. Instead the debate has escalated in recent months both in intensity and in the range of public involvement. A watershed of sorts was reached in March at a public forum held by the National Academy of Sciences in Washington. The forum was in part a repeat performance by scientists arguing fixed positions that were established early in the debate. There were, however, new participants on the scene, and they presented a varied and rapidly shifting agenda. They made it clear that research with recombinant DNA had become a political issue. As one speaker remarked, the Academy forum may have been the last major public discussion of recombinant DNA arranged by the scientists involved in the research. Nonscientists at the forum, by word and deed, reiterated the theme that science has become too consequential either to be left to the self-regulation of scientists or to be allowed to wear a veil of political chastity.

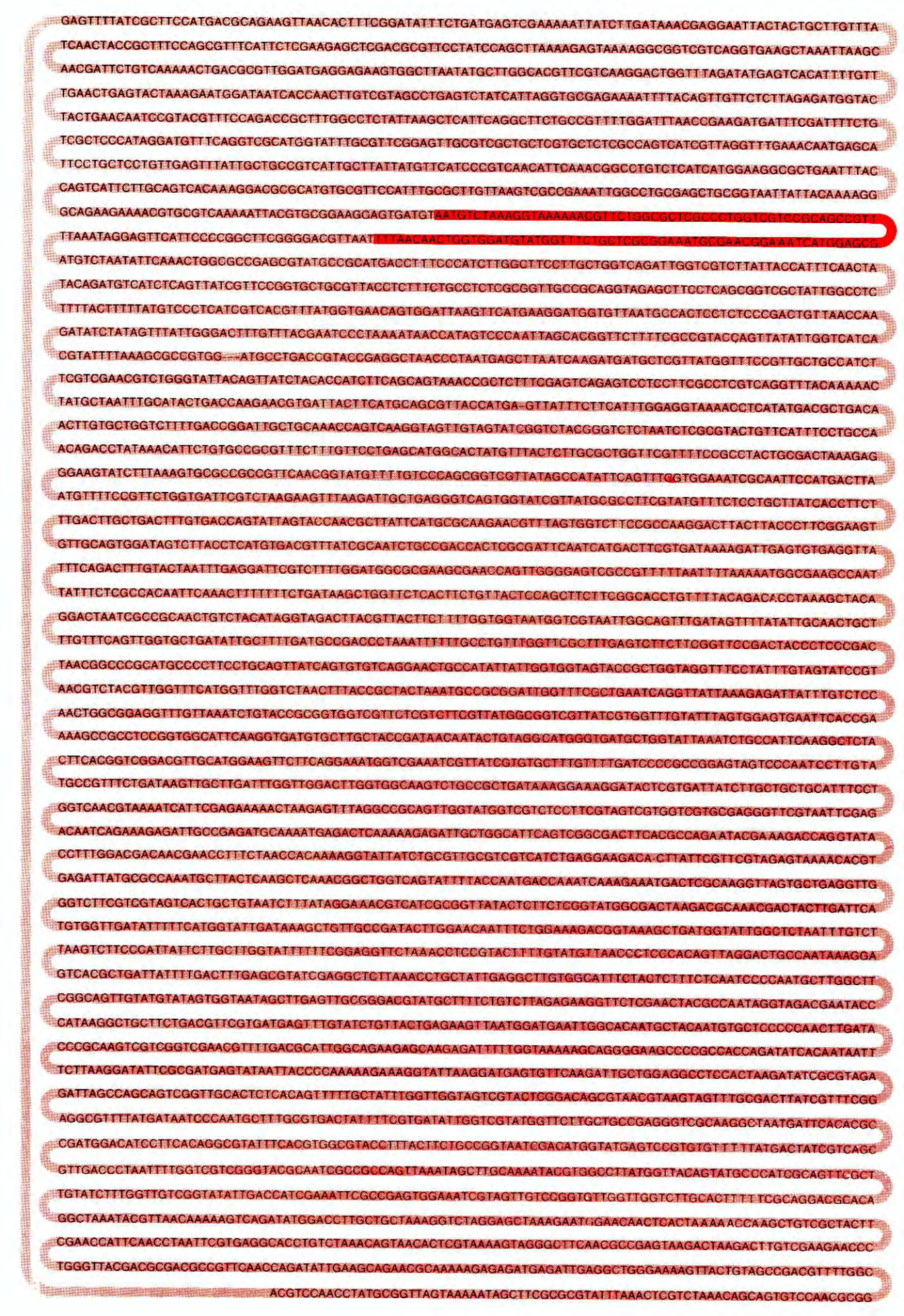
Science of course is crucially consequential to society, precisely because it is an intensifying source of both benefits and risks. Research with recombinant DNA may provide major new social benefits of uncertain magnitude: more effective and cheaper pharmaceutical products; better understanding of the causes of cancer; more abundant food crops; even new approaches to the energy problem. These and other possible outcomes are envisioned in "best-case scenarios" for the future application of recombinant-DNA technology. "Worst-case scenarios" can also be conceived: worldwide epidemics caused by newly created pathogens; the triggering of catastrophic ecological imbalances; new tools for militarists and terrorists; the power to dominate and control the human spirit.

Both the best-case and worst-case scenarios are largely speculative; the gap between them symbolizes the large degree of uncertainty that surrounds this major step forward in molecular genetics. The material basis of biological heredity has been broken into in the past two decades, and it seems as though each of the fragments has acquired a life of its own. In this resulting period of instability fear threatens to override wonder as the implications of the research diffuse more widely. The fear is not so much of any clear and present danger as it is of imagined future hazards. The classic response to such fears is rigid containment: the Great Wall, the Maginot Line, the cold war. All are manifestations of the effort to provide absolute security against unpredictable risks, and yet each generates its own risk. The escalation of the recombinant-DNA debate has a component of this kind of behavior, but there is a more rational component as well.

The first round of the fateful debate began in 1974, when investigators at the leading edge of work in this field declared a voluntary moratorium on several types of experiment judged to be conceivably risky. A set of techniques had been developed that made it possible to cut the long, threadlike molecules of DNA into pieces with the aid of certain enzymes, to recombine the resulting segments of DNA with the DNA of a suitable vector, or carrier, and to reinsert the recombinant into an appropriate host cell to propagate and possibly to function.

The significance of the new developments is rooted in the central biological role of DNA as the transmitter of genetic information between generations. The transmission of the encoded genetic message depends on the ability of a cell to generate exact replicas of the parental DNA and to allocate the replicas among the offspring. In addition the success of genetic transmission depends on the ability of the offspring to "express" the

GENETIC CODE of an extremely small bacterial virus, the bacteriophage designated ϕX174, is given by the sequence of letters on the opposite page. The letters stand for the four nucleotides cytosine, guanine, adenine and thymine, which are linked end to end to make up each strand of the normally double-strand DNA molecule. The genetic message embodied in each strand of DNA is represented by the particular sequence of nucleotides, any one of which may follow any other. In the ϕX174 virus the DNA molecule, which has only a single circular strand for part of its life cycle, consists of approximately 5,375 nucleotides; the nucleotides are grouped into nine known genes, which are responsible in turn for coding the amino acid sequences of nine different proteins. For example, the dark-color segment of the molecule, called gene *J*, codes for a small protein that is part of the virus; this segment also happens to be the shortest gene in the ϕX174 genome. The complete nucleotide sequence for the DNA in ϕX174 was worked out recently by Frederick Sanger and his colleagues at the British Medical Research Council Laboratory of Molecular Biology in Cambridge. About 2,000 pages of this type would be required to show the nucleotide sequence for the DNA in the chromosome of a typical single-cell bacterium; roughly a million pages would be needed to similarly display the genetic code embodied in DNA molecules that make up chromosomes of a mammalian cell.



encoded information properly by referring to it to control essential life processes. The mechanism of genetic expression in higher organisms is at present only dimly understood, and the discovery of the new recombinant-DNA techniques seemed immediately to open a broad new avenue to increased knowledge in this field.

The detailed mechanisms of genetic replication and expression are enormously complex. The essence of the matter, however, is found in the famous "double helix" structure of DNA. Both of the two long, interwound and complementary strands of the DNA molecule are made up of four kinds of nucleotides, cytosine, guanine, adenine and thymine (abbreviated *C, G, A* and *T*), which are linked end to end like a train of boxcars. The genetic message of each strand is embodied in the particular sequence of nucleotides, any one of which may follow any other. For example, the sequence *CATTACTAG* contains five identifiable English words: *CAT, AT, TACT, ACT* and *TAG*. The genetic message, however, is "written" in triplets: *CAT, TAC* and *TAG*. In general each triplet "codon" determines, through a series of intermediate steps, the position of a specific amino acid in a protein molecule.

Proteins, like nucleic acids, can be visualized as long trains of boxcars coupled end to end; here, however, the subunits are amino acids rather than nucleotides. The sequence of nucleotides in a given DNA molecule determines the sequence of amino acids in a particular protein, with each triple-nucleotide codon placing one of 20 possible amino acids at each successive position in the protein chain. The sequence of amino acids in turn specifically establishes both the structure and the function of the protein. Thus the nucleotide sequence of DNA precisely specifies the protein-building properties of the organism. Moreover, virtually every property of the organism, from enzymatic action to eye color, depends on protein structure in one way or another.

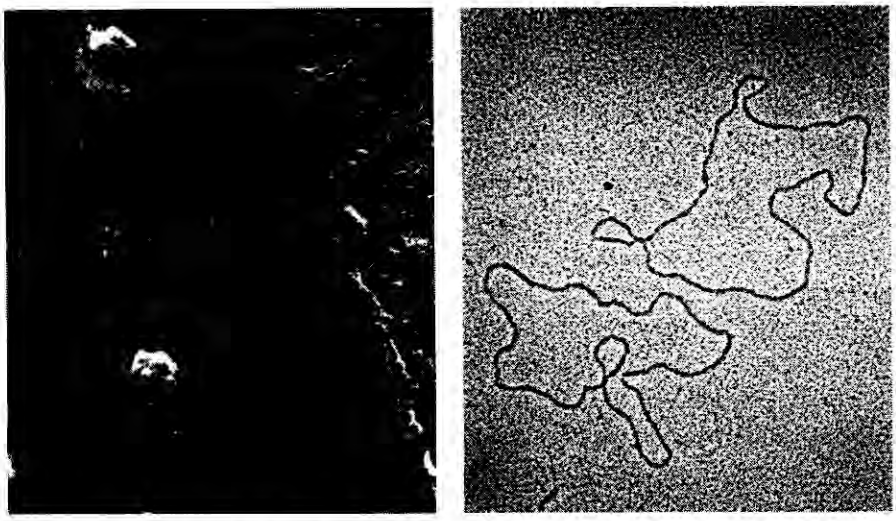
The transmission of the essential genetic information between generations depends on the precise replication of the nucleotide sequences of DNA. The mechanism for replication stems from the complementary relation between the two strands of the DNA molecule. A sequence on one strand (for example *CATTACTAG*) lies immediately opposite a complementary sequence (*GTAATGATC*) on the other strand. The strands are complementary because *C* and *G* are always opposite on the intercoiled strands, as are *A* and *T*. Complementarity depends on the special chemical affinity, or binding, between *C* and *G* on the one hand and *A* and *T* on the other. The sum of these bonds, repeating along the length of the strands, is what holds the strands together in the double helix. Under appropriate conditions affinity is reduced and the two strands can unwind and separate. The single strands can again pair and rewind when conditions for high affinity are restored.

Double-strand DNA replicates by means of an extension of these properties. The unwinding and separation of the strands begins at a localized site along the DNA molecule. In the presence of suitable enzymes and free nucleotides a new chain is formed next to the exposed portion of each unpaired older chain. Each nucleotide lines up next to its opposite number (*C* next to *G*, *A* next to *T*). The complementary sequence thus established is then linked end to end by an enzyme that closes the nucleotide couplings. When the replication process has traveled along the entire length of the original double helix, two new helixes identical with the first one have been formed. The replication of DNA is the most fundamental chemical reaction in the living world. It fully accounts for the classical first principle of heredity: like begets like.

If DNA replication always worked without error, life would be far more homogeneous than it is. Here, however, a second classical principle of heredity intervenes: the principle of mutational variation, or the appearance in the offspring of new hereditary characteristics not present in the progenitors. Mutations arise through error, at least partly in the replication process. For example, the substitution of one nucleotide by another changes the triplet codon and puts a different amino acid in the corresponding position in the resulting protein. Single-nucleotide errors lead to single-amino-acid errors. Thus, a single-nucleotide error is responsible for the human disease sickle-cell anemia. Most mutations are not such simple, single-nucleotide exchanges; nevertheless, they correlate directly with altered, transposed or deleted nucleotide sequences in DNA. When these changes appear in a gene (that is, a segment of DNA that codes the amino acid sequence of a particular protein), a change in the protein and hence in the hereditary properties it controls is the result.

Therein lies the crux of recombinant-DNA technology. It makes possible for the first time the direct manipulation of nucleotide sequences. Changes in nucleotide sequence that are produced by "natural" errors are random, even when their overall frequency is artificially increased. In natural populations Darwinian selection "chooses" among the random errors, increasing the representation in breeding populations of those errors that lead to more offspring in particular environments. Artificial selection, practiced by human beings for millenniums, favors errors that meet human needs (agricultural breeding) or whims (exotic-pet breeding). The success of both natural and artificial selection, however, is dependent on the random occurrence of desirable mutations. There was no way to direct genetic change itself until recombinant-DNA techniques came along. The new techniques enable one to deliberately introduce known and successful nucleotide sequences from one strain or species into another, thereby conferring a desired property.

The recombinant-DNA approach involves experimental ingenuity and detailed knowledge of the DNA molecule. It begins with an attack on DNA by the proteins called restriction enzymes, which are isolated from bacteria. The enzyme attack breaks the double chain

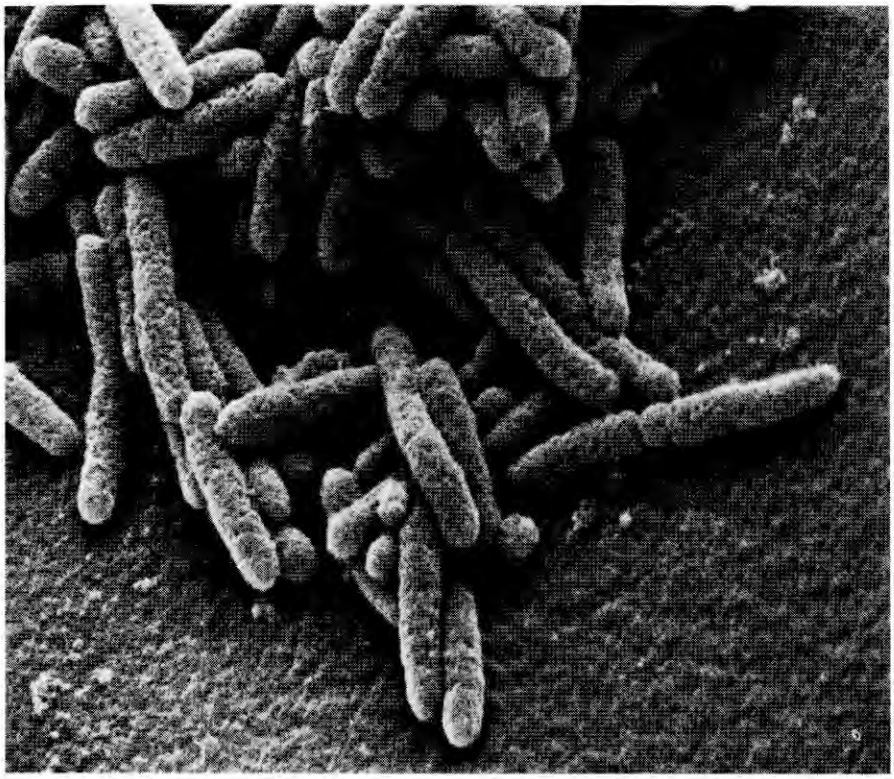


BACTERIOPHAGE ΦX174 AND ITS DNA are portrayed in this pair of electron micrographs. The virus infects the common intestinal bacterium *Escherichia coli*. In the micrograph at left, made by Jack D. Griffith of the Stanford University School of Medicine and Andrew Stachelin of the University of Colorado, two ϕX174 particles are seen attached to surface of an *E. coli* cell. In micrograph at right, made by Griffith, the DNA molecules of two ϕX174 viruses are seen in their double-strand form; each molecule is about 18,000 angstroms long.

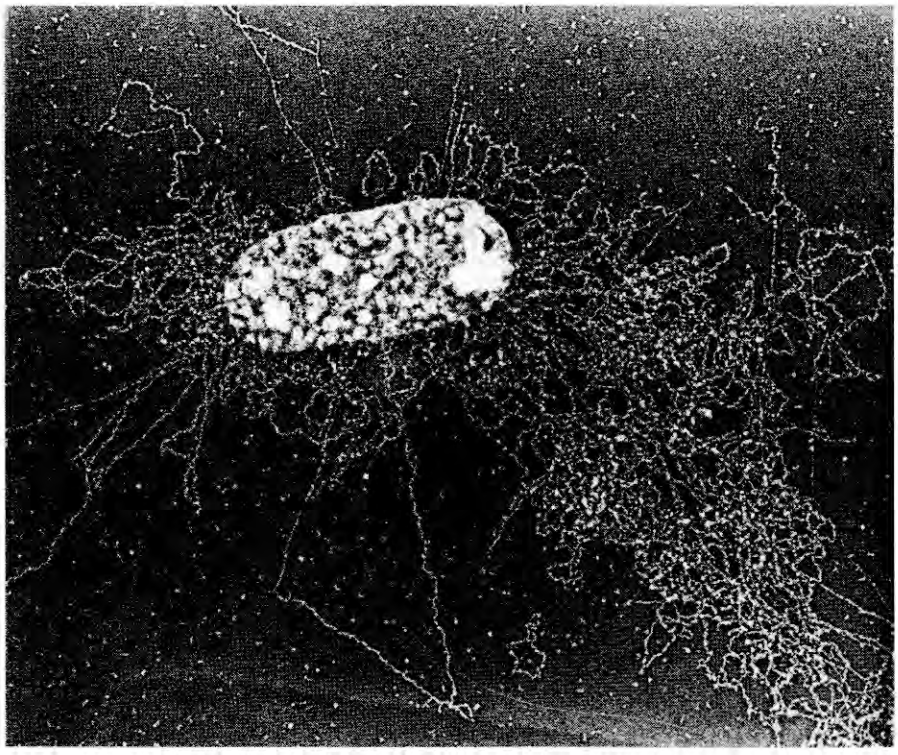
of DNA at particular sequences, say at the sequence *CATTAC*, which is opposite the complementary sequence *GTAATG*. The break does not always occur at the same point on the two strands. It may, for example, be between the two *T*'s in the first strand but just to the right of the *ATG* in the second strand. On separation one piece therefore ends in *TAC*, whereas the other ends in *ATG*. Since the single-strand ends are complementary, they will under suitable conditions stick side by side, and they can then be coupled together end to end. If the same restriction enzyme is used on the DNA from two different sources, both of which have the appropriate target sequence, then sequences with the same "sticky" ends will result. By taking advantage of this stickiness two sequences from any source can be recombined into a single DNA molecule.

The only further step necessary is to put the recombinant DNA into a suitable host organism. The recombinant must have the ability to penetrate the host and become part of its genetic system. An effective way to accomplish this has been developed for the common intestinal bacterium *Escherichia coli*. In addition to its single large circular chromosome the *E. coli* bacterium may have one or more independently replicating, smaller loops of DNA known as plasmids. The plasmids can be isolated from the bacteria, broken open by restriction enzymes and used as one component of a recombinant. After linking up the plasmid DNA with the "foreign" DNA the circular form of the plasmid can be restored and the structure returned to a whole cell. There it can resume replication, duplicating not only its own native sequence but also the foreign one. A strain of bacteria is thus obtained that will yield an indefinite number of copies of the inserted nucleotide sequence from the foreign source.

Standing alone, none of this appears to be particularly momentous or threatening; it is only a new and intriguing kind of chemistry applied to living organisms. Given the complexity of living organisms and the still more complex world of social phenomena, however, this new chemistry quickly builds into varied new potentials, both speculative and real. Suppose, for example, one were to isolate the nucleotide sequence necessary to produce a potent toxin and to transfer it to *E. coli*, usually a harmless inhabitant of every human intestinal tract. Would a dangerous new pathogen be created? Would the transformed *E. coli* release a toxin in the human gut? Might such a new pathogen escape from control and induce epidemics? Questions of this kind have answers, but they take time to find. To gain some time for reflection investigators in 1974 called for a partial and temporary moratorium on those experi-



PILE OF E. COLI CELLS appears in this scanning electron micrograph made by David Scharf. Some of the cells have been caught in the act of asexual reproduction (cell division); a few appear to be transferring their DNA by means of the threadlike connection characteristic of the process known as conjugation. *E. coli* bacteria are considered by most investigators to be most suitable host cells for recombinant-DNA experiments. Magnification is 11,000 diameters.



E. COLI SPEWS OUT DNA through its chemically disrupted cell wall in this electron micrograph by Griffith. Most of the DNA is in the form of a single large molecule of double-strand DNA, which constitutes the chromosome of this simple prokaryotic organism. In addition the *E. coli* bacterium may have one or more of the independently replicating loops of DNA known as plasmids; one of these smaller extrachromosomal DNA molecules can be seen near the bottom. Plasmids derived from *E. coli* cells play an important role in recombinant-DNA research, since they form one class of vectors, or carriers, into which segments of "foreign" DNA can be spliced prior to their being reinserted into an appropriate host cell to propagate, thereby duplicating not only their own native nucleotide sequence but also the foreign sequence.

ments thought to be potentially the riskiest. The separation of the certainly safe experiments from the less certainly safe ones became the chief function of the guidelines released by the NIH in June, 1976. The guidelines, which replaced the temporary moratorium, were derived from worst-case analyses of various kinds of experiments; the object was to evaluate the possible range of hazards and to prescribe appropriate matching safeguards in order to minimize the unknown risks. The guidelines assigned heavy responsibility to individual investigators, and they buttressed this responsibility with special monitoring committees in the sponsoring institutions and in the funding agency.

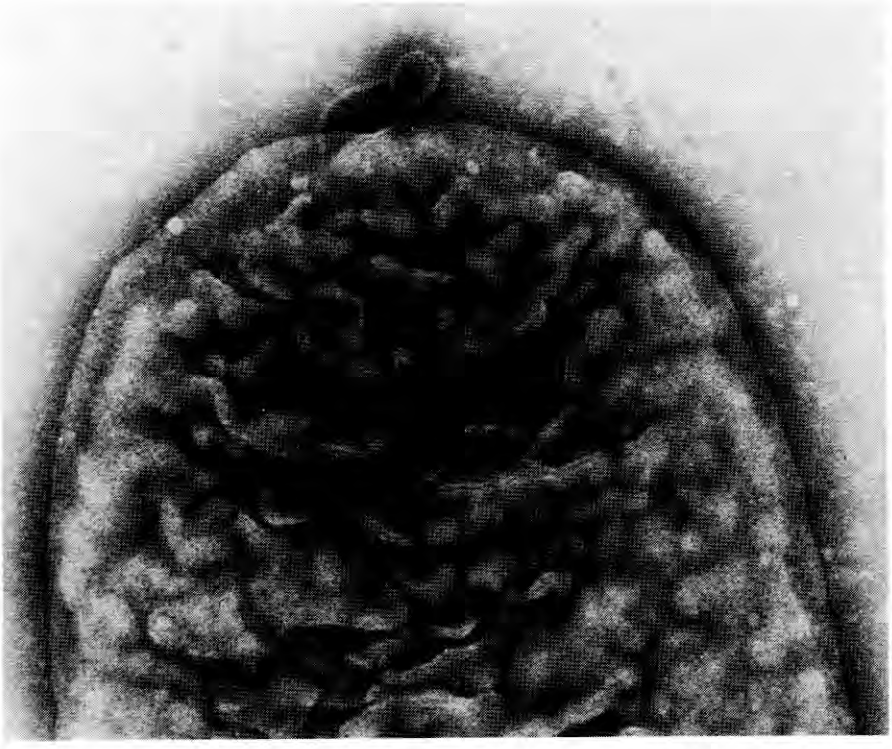
If such regulations have been adopted, why is debate continuing? Briefly, it is because the matching of estimated risk and prescribed containment adopted by the guidelines is regarded by critics as being inadequate in dealing with potential biohazards and incomplete in failing to address other important issues. The most vocal critics have presented their own worst-case analyses in the scientific and general press. These accounts have led to widespread alarm and to public-policy deliberations at the level of local communities, states and the Federal Government. The expressed concerns of the critics have generated a revised agenda for what is now emerging as a broadened second round of policymaking.

Potential biohazards and estimated degrees of risk continue to dominate the debate. The NIH guidelines balance the estimated risk of a given experiment and recommend specific measures for containing the risks. (Risk, it must be remembered, means possible danger, not demonstrated danger.) Those experiments judged to present an excessive risk are entirely proscribed. At the other end of the spectrum experiments judged to present an insignificant risk require only the safeguards of good laboratory practice. Between these extremes the guidelines establish various levels of estimated risk and prescribe combinations of suitably increasing physical and biological containment. The release into the environment of any recombinant organisms is forbidden.

Unfortunately, given the growing but still limited state of knowledge, wide disagreement is possible, both as to estimated degrees of risk and as to the efficacy of the proposed containment. Some critics project fragmentary information into the inevitable spread of dangerous, newly created organisms, threatening both the public health and the environment. Some defenders project the same fragmentary information to the conclusion that the NIH guidelines are already overly cautious. They believe the actual hazard under existing precautions will turn out to be no greater than that routinely faced in the use of automobiles, jet aircraft and other accepted technologies. The wide range of estimates is possible because of the multiplicity of conceivable experiments and because experience and critical data are inadequate for certainty on many points. One fact that is certain is that no known untoward event has yet resulted from recombinant-DNA research.

What emerges on the new policy agenda, then, is the need for effective policy-oriented research to reduce the current uncertainty as to the risk of particular kinds of experiments. For example, there is dispute over the use of *E. coli* as a host for recombinant DNA. One side argues that scientists must be mad to pick a normal human inhabitant (and a sometime human pathogen) to serve as a host for recombinant DNA. This view, in extreme form, demands the suspension of all recombinant-DNA research until an organism safer than *E. coli* can be found. The other side argues (1) that the vast amount of information available on *E. coli* makes it invaluable, (2) that the *K*-12 strain of *E. coli* actually used in laboratory research has been so modified genetically in adapting to laboratory conditions that it survives only with difficulty in the human intestine and (3) that new strains of *K*-12 have been developed with additional genetic deficiencies that will make survival outside of laboratory conditions essentially impossible. The use of such genetically deficient strains is what is meant by the term "biological containment." The concept is supported by proponents of the research as an efficacious new approach to safety and derided by critics as likely to be circumvented by natural recombination.

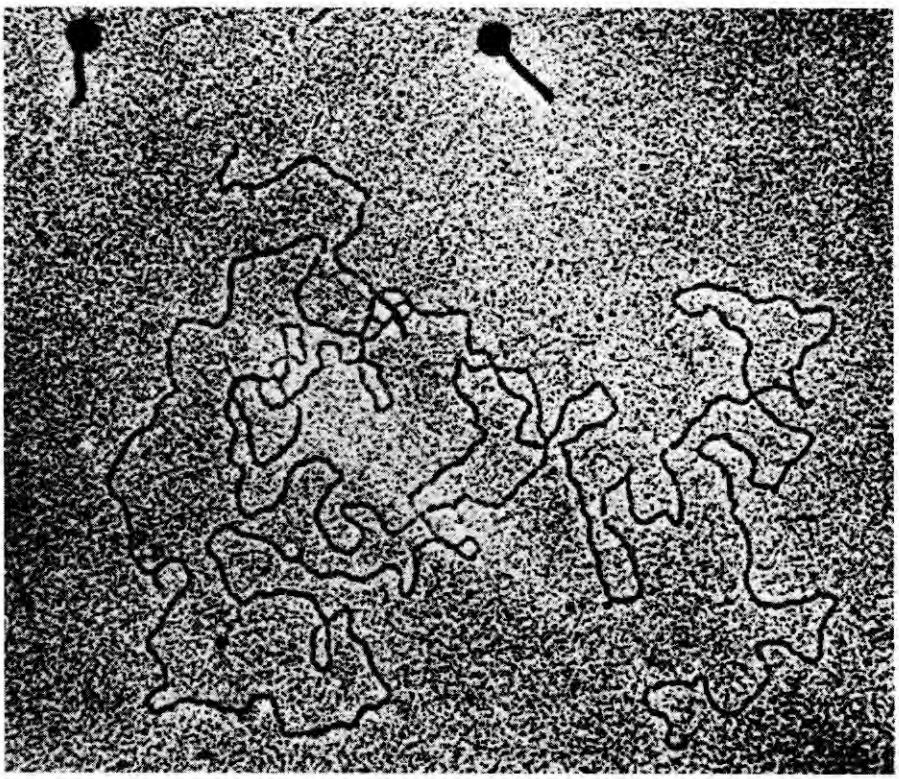
Such differences of opinion are normally reduced by scientists to experimental questions. For example, the suitability of the *K*-12 strain of *E. coli* as an experimental organism can be judged only from the effect of recombinant genes on the ecological relations of *E. coli* within the human intestine, including the degree of success of recombinant strains in competing with other strains of *E. coli* and with other organisms. Information on these matters is growing. Such questions, however, are not normally subjects of profound scientific interest. They have recently become matters of priority only because they may provide information that would be useful in arriving at a policy decision. Research on policy-oriented questions has never had a very high status among scientists engaged in basic research or even among those engaged in applied research. Therefore policy-oriented research must be encouraged through special funding mechanisms and through suitable new institutional arrangements. A regulatory agency for recombinant-DNA research and other conceivably hazardous kinds of research is urgently needed outside the NIH, and it should include a research component. The Center for Disease Control and its National Institute for Occupational Safety and Health come



BACTERIAL VIRUS IS ATTACHED to the wall of an *E. coli* cell in this electron micrograph made by Maria Schnoss of the Stanford School of Medicine. This particular virus, named bacteriophage lambda, normally infects the bacterium by injecting its DNA into the host cell through a long taillike appendage. The magnification is approximately 140,000 diameters.

to mind as possible models for such a dual-purpose agency.

Also related to the question of biohazards is a controversy over the desirability of centralizing recombinant-DNA research facilities. Some of those who fear severe dangers from recombinant organisms have urged that the potentially more hazardous research be concentrated in remote places with extremely stringent containment procedures. Those who minimize the hazard are opposed to the concentration concept because it would tend to separate the research from the intellectual mainstream and would be unnecessarily expensive in facilities. The argument has been particularly strenuous with respect to experiments requiring *P*3 facilities, which are defined as those necessary to contain "moderate risk" experiments. *P*4 facilities for "high risk" experimentation are expected to be fewer in number because of their high cost; generally speaking they are likely also to be comparatively isolated. The current NIH guidelines provide little direction in these matters. A decision on a firmer policy belongs on the discussion agenda. Particularly urgent is careful consideration of such intermediate possibilities as the use of centralized, high-risk facilities for making particular recombinations for the first time. These activities, together with preliminary testing of new recombinants for possible hazards, might also be carried out by the proposed new regulatory agency.



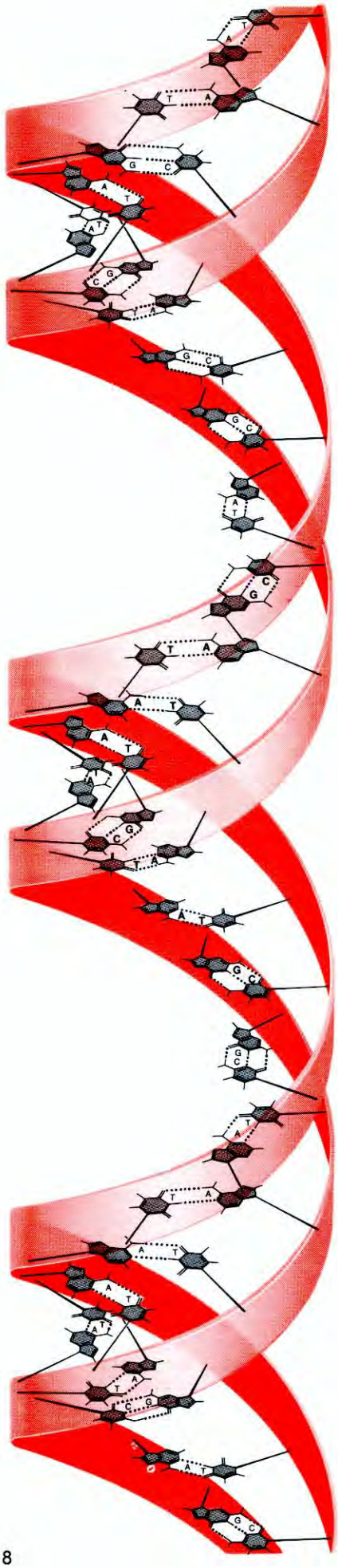
BACTERIOPHAGE LAMBDA AND ITS DNA are both represented in this electron micrograph provided by Griffith. Two complete lambda viruses are at the top; the long double-strand DNA molecule of a disrupted lambda is below them. DNA from bacteriophage lambda can also serve as a vector for recombinant-DNA experiments involving *E. coli* host cells.

A special case that emphasizes the advantages of initial testing in a central facility is provided by what are called "shotgun" experiments. These experiments, which offer special advantages to the investigator, may also present special hazards. Shotgun experiments involve exposing the total DNA of a given organism to restriction enzymes in order to obtain many DNA fragments. The fragments are then each recombined with DNA from a suitable vector and the recombinants are randomly reinserted into *E. coli* host cells. The next step is to spread the *E. coli* cells on a nutrient substrate so that each recipient cell, containing a particular inserted foreign sequence, grows into a colony. If the experiment is successful, the yield is a "library" of all the nucleotide sequences of a particular organism, each sequence growing in a separate strain and accessible to manipulation and cross-combination at will.

This experimental approach is laborious but far less so than anything else available for the exploration of the complex genetic systems of higher organisms. There is, however, a risk of unknown magnitude that portions of the DNA with unknown or repressed functions might duplicate and create unanticipated hazards. The result might be particularly unfortunate if the original DNA preparation were to contain genetic material from parasites or from viruses associated with the species under study. Under the NIH guidelines, therefore, shotgun experiments are regarded as being more dangerous than those involving purified and characterized DNA. Experiments in this category are treated as being increasingly more dangerous as the test organism under study is biologically more like the human organism. Thus experiments with primate DNA are considered to be more dangerous than experiments with mouse DNA. This approach appears to represent a reasonable precaution with respect to human health hazards, but it is less reasonable with respect to potential ecological effects. For example, shotgun recombinants involving DNA from plant sources could conceivably lead to ecologically dangerous effects if they were to escape into the environment. Shotgun procedures might therefore be best conducted first in special centralized facilities that could also act as storage and distribution centers for the recombinant products once they had been tested for safety.

These examples suggest several advantages for the creation of a Center for Genetic Resources. The center might not only carry out DNA recombinations suspected to be hazardous but also function to preserve genetic information contained in threatened natural species and in special strains of cells or organisms developed for research and other purposes. Stored genetic information can be expected to be increasingly important in the future. For example, new genetic infusions into domesticated stocks of plants and animals from their wild progenitors have long been used to strengthen the response of the domesticated stocks to changing conditions of husbandry. The sources of wild progenitors are threatened by the reduction of wild habitats all over the world.

The possibility of a biohazard need not arise only as a by-product of basic research. The practical applications of recombinant-DNA techniques, together with the applied research and development leading to them, are at least equally likely sources. For example, recombinant techniques may enormously expand the use of bacteria (and other microorganisms) for the production of certain proteins and other pharmacological products. Microorganisms have long played an essential role in the food, beverage, pharmaceutical and chemical industries, and more precise genetic control of their characteristics has already yielded large benefits. The recombinant-DNA techniques not only offer advances on current practice but also suggest a new realm of "bacterifacture" in which the rapid, controlled growth of microorganisms is coupled to the pro-



duction of specific products normally made only by higher organisms. Included among the possibilities are the production of insulin, blood-clotting factors and immunological agents. The probability of those possibilities ever being realized is no more easily assessed than the risks, but success in realizing them clearly could provide substantial economic and social benefits. Accordingly entrepreneurial interests have been aroused.

The NIH guidelines are silent on the matter of commercial applications other than stipulating that large-scale experiments (beyond production batches of 10 liters) with recombinants "known to make harmful products" be prohibited unless specially sanctioned. The guidelines also require detailed reporting of proposed recombinant-DNA experiments, a provision that runs counter to the protection of proprietary interest. There have been discussions of these matters between the NIH and representatives of industry. In addition industry spokesmen have testified at Congressional hearings. It is known that some industrial research already is under way and that representatives of industry generally endorse the precautionary approach of the NIH guidelines, but they are resistant to limitations on proprietary rights and on the size of batch production. Moreover, patent policy has come up as an issue and there has been some uncertainty in the Department of Commerce as to how it should be handled. Indeed, the possible commercial applications of recombinant-DNA techniques have yet to be publicly evaluated as a serious policy question, and they must be high on the agenda of the next round of discussions.

The problems of commercial applications lead from immediate issues to broader ones and to a larger time frame. Recombinant-DNA techniques have revived the debate over "genetic engineering" and have once again raised questions about the applications of fundamental biomedical research to technology, to the quality of life and to the future of society. Recombinant DNA has now joined nuclear fission, overpopulation, famine and resource shortages in the doomsday scenarios of "creative pessimism." These issues are even more difficult to deal with objectively than those related to potential biohazards, but they are plainly apparent in the general public discussion and in the public statements of respected scientists.

For example, Robert L. Sinsheimer of the California Institute of Technology has persistently raised issues that are in part practical and in part philosophical. Along with George Wald of Harvard University and Erwin H. Chargaff of the Columbia University College of Physicians and Surgeons, he suggests that the entire recombinant-DNA approach to gaining an understanding of the complexities of higher genetic systems is misbegotten. The argument is not that the approach may not work but that its alleged huge risks are unnecessary because less risky, although slower, means are available. Sinsheimer emphasizes the fundamental difference between simple prokaryotic organisms such as bacteria and complex eukaryotic organisms, including human beings. Prokaryotes, typically one-cell organisms, have a single, comparatively simple chromosome floating freely within the cell body, whereas eukaryotic cells have a nucleus that is bounded by a membrane and contains a number of far more complex chromosomes. The paleontological record suggests that prokaryotes existed on the earth for a billion or more years before the more complex eukaryotes arrived on the scene. Sinsheimer proposes that throughout the evolution of the eukaryotes there has been a genetic barrier between them and the prokaryotes, behind which eukaryotes have developed their more complex mechanisms of genetic control. To transfer these mechanisms, which are possibly the key to the evolutionary success and enormous diversity of eukaryotes, to prokaryotes may introduce, he says, incalculable evolutionary dangers. The prokaryotes may be made far more effective, both as competitors and as parasites, negating an ancient evolutionary strategy.

Sinsheimer's argument has won only a few vocal adherents among biologists, and he himself concedes that it is speculative. Nevertheless, his argument

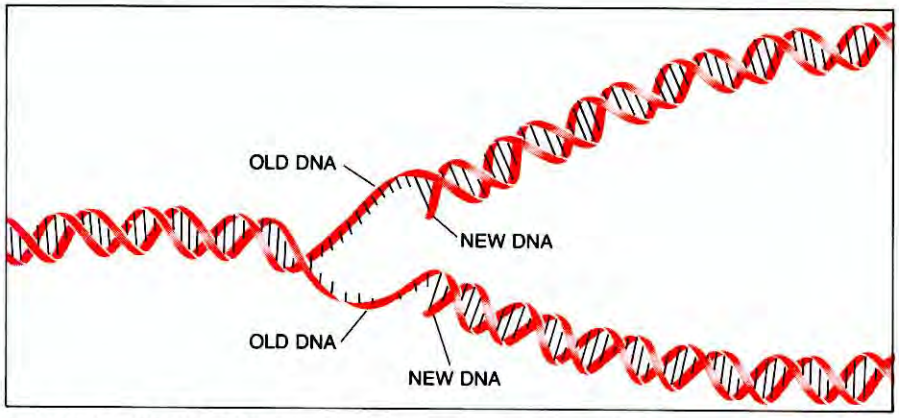
DOUBLE-HELIX STRUCTURE OF DNA is evident in this simplified diagram of a short segment of the deoxyribonucleic acid (DNA) molecule. The sugar and phosphate groups that are linked end to end to form the outer structural "backbones" of the double-strand molecule are represented schematically here by the two helical colored bands. The inner portion of each polynucleotide chain, drawn in somewhat greater detail, consists of a variable sequence of four kinds of bases: two purines (adenine and guanine, or *A* and *G*) and two pyrimidines (thymine and cytosine, or *T* and *C*). The two chains, which run in opposite directions, are held together by hydrogen bonds (*dotted black lines*) between pairs of bases. Adenine is always paired with thymine, and guanine is always paired with cytosine. The planes of the bases are perpendicular to the common axis of the two helixes. The diameter of the double helix is 20 angstroms. Adjacent bases are separated by 3.4 angstroms along the axis and are displaced successively around the axis by an angle of 36 degrees. The structure therefore repeats after 10 bases on each chain (360 degrees), or at intervals of 34 angstroms. The genetic information is stored in the sequence of bases along each chain. In this case the sequence *CATTACTAG* on one strand is identified in boldface type opposite complementary sequence *GTAATGATC* on other strand.

has attracted significant public attention, and it is widely cited to support opposition to continued recombinant-DNA research. Bernard D. Davis, a Harvard Medical School microbiologist, has provided a rebuttal, particularly with respect to the concept of a genetic barrier between prokaryotes and eukaryotes. He believes there has been an ample and continuous opportunity for the exchange of DNA between the two groups. He points out that bacteria can take up naked DNA from their immediate environment and that *E. coli* would be exposed to such DNA arising from dead human cells in the human intestine. Microorganisms might similarly take up DNA in the process of decomposing dead animals. Therefore, Davis argues, most recombinants probably have already been tried in the natural evolutionary arena and have been found wanting. Reasoning on analogy with extensive information on pathogenic bacteria, Davis concludes that under the existing NIH guidelines the probability for survival in nature of laboratory-produced prokaryote-eukaryote recombinants is vanishingly small.

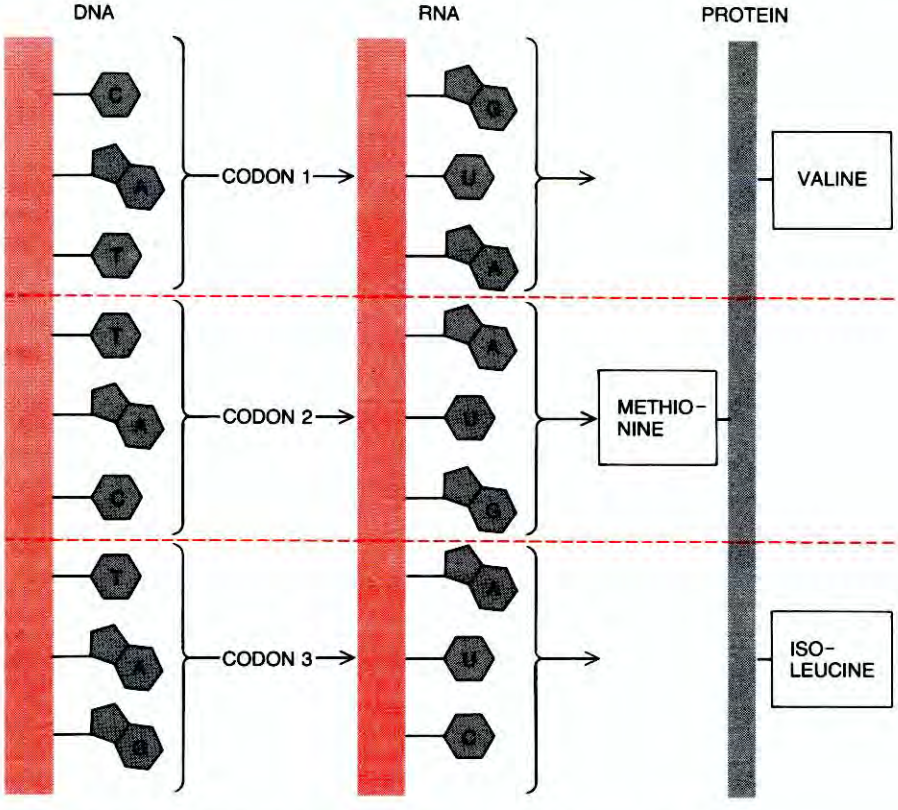
This clash of opinion on a major biological issue illustrates the difficulty of assessment of even comparatively value-free questions when critical information is fragmentary. The controversy over the risk-benefit ratio becomes even more intense when issues involve substantial value judgments as well. Here again a concern of skeptics and opponents of recombinant techniques is sharply articulated by Sinsheimer. He asks: "Do we want to assume the basic responsibility for life on this planet? To develop new living forms for our own purposes? Shall we take into our own hands our own future evolution?" Since the questions include such concepts as responsibility, purpose and control of the future, they clearly involve considerations beyond science alone.

The human species has, of course, been altering life on this planet from the beginnings of human culture. When hunting and gathering gave rise to animal husbandry and agriculture, human choice and purpose began to influence the evolution of selected species. Unconscious human selection was replaced by deliberate plant and animal breeding, and the further development of human culture is now clearly altering the entire ecosystem. Moreover, the biocultural progression of the human species, based partly on human purpose, is undoubtedly altering the human gene pool and will slowly modify the species in unpredictable ways. Nevertheless, the advent of recombinant-DNA techniques has obviously enhanced the prospects for genetic engineering and has restressed the need to assess its implications. Can it be assumed that success in introducing recombinant DNA's into *E. coli* means that there will be similar success in introducing them into the human species? If it can, what is the probable time frame for applying the technique to the human species? Is it accurate and responsible to suggest that we have almost in hand control of "our own future evolution"? These certainly are questions for scientific assessment, and they should have a prominent place on the new policy agenda.

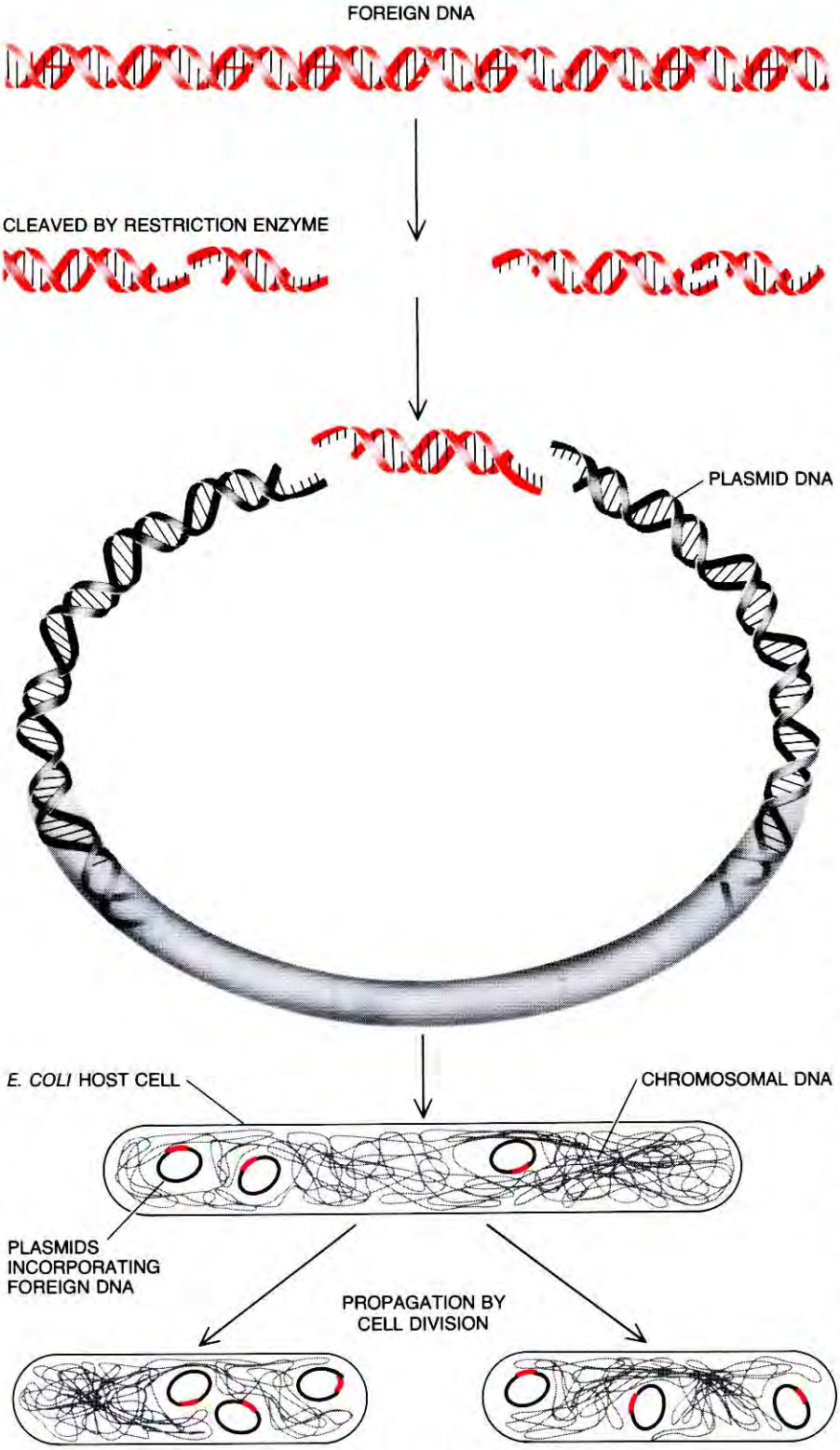
Sinsheimer has gone into still another controversial area, not only for the scientific community but also for the entire



REPLICATION OF DNA depends on the complementary relation between the nucleotide sequences on the two strands of the DNA molecule. Under appropriate chemical conditions the hydrogen bonds between the bases are weakened and the two strands can unwind and separate. In the presence of suitable enzymes and free nucleotides a new chain can be formed next to the exposed portion of each unpaired older chain. The complementary sequence that is formed by each nucleotide lining up next to its opposite is then linked end to end by an enzyme that "zips up" the nucleotide couplings. In this way two new helixes identical with the first can be formed.



ROLE OF DNA IN PROTEIN SYNTHESIS is suggested by this highly schematic diagram. The genetic message contained in the nucleotide sequence *CATTACTAG*, for example, is "written" in the form of the triplet "codons" *CAT*, *TAC* and *TAG*. Each codon determines, through a series of intermediate steps involving a molecule of ribonucleic acid (RNA), the position of a specific amino acid in a protein molecule. Thus the sequence of nucleotides in a given DNA molecule specifies the corresponding sequence of amino acids in a particular protein, with each triple-nucleotide codon placing one of 20 possible amino acids at each successive position in the protein chain. Since the sequence of amino acids in turn establishes both the structure and the function of the protein, the nucleotide sequence of DNA determines virtually every property of organism. Letter *U* stands for the pyrimidine uracil, a constituent of RNA.



RECOMBINANT-DNA TECHNIQUE makes it possible for the first time to deliberately introduce nucleotide sequences from the DNA of one strain or species of organism into the DNA of another. The DNA of the "foreign" organism is first treated with restriction enzymes, which cleave the double-strand molecule at particular nucleotide sequences (typically thousands of base pairs apart) on a random basis. The same enzyme is then used to cleave the DNA of a suitable vector, in this case a plasmid isolated from *E. coli* bacteria. Since the break caused by the enzyme does not occur at the same point on both strands, the chemical treatment results in a mixture of DNA segments that have complementary single-strand ends. Under suitable conditions the "sticky" ends of two different sequences can be coupled to form a single DNA molecule. For example, after recombining the foreign DNA with the plasmid DNA the circular form of the plasmid can be restored and the structure can be inserted into a suitable host cell (in this case *E. coli*), where the plasmid can resume replication, thereby propagating an indefinite number of "cloned" copies of the inserted nucleotide sequence from the foreign source.

society. Arguing that time may be needed to "pace" new genetic knowledge to human capacities for putting nature to intelligent use, he wonders whether "there are certain matters best left unknown, at least for a time." This is high heresy in the scientific community, whose fundamental premise is that the growth of knowledge is the driver and not the captive of other values. The rejection of the concept of "forbidden knowledge" was part of the heroic period at the beginning of modern science, when it included willingness to face the Inquisition and the stake. Having been seared by the nuclear flame and now confronting the more subtle implications of the innermost language of life, 20th-century scientists fear not the stake but the judgment of history. Chargaff, a pioneer in the investigations that led to the decipherment of the genetic language, says: "My generation, or perhaps the one preceding mine, has been the first to engage, under the leadership of the exact sciences, in a destructive colonial warfare against nature. The future will curse us for it."

Sinsheimer and Chargaff, along with a number of philosophers, historians and sociologists of science, are clearly suggesting that the possible consequences of knowing must be consciously included in decisions about the directions of the search for knowledge itself. No issue cuts more deeply to the core of modern science. The self-doubt expressed by some scientists reflects a general questioning in the U.S. of the net benefits of science and technology. Cost-benefit analysis is a current preoccupation, and it is being increasingly applied to the generation of knowledge itself. It is hard enough to assess what we may gain or lose from particular new knowledge; it is even harder to assess the costs of not having it. This problem is epitomized by the recombinant-DNA controversy. The rise of molecular genetics in the U.S. is the direct product of a series of decisions made after World War II that provided funds for biomedical research. The objective was the conquest of the "killer" diseases: cancer, heart disease and stroke. Those diseases are still much with us, although they are better understood and cared for. Meanwhile, out of Federally supported research also came the impetus that led to the discovery of the double helix, the genetic code, the structure of proteins and recombinant DNA. In a classic "double take" the public is now asking whether it has been buying health and well-being or chimeric monsters. Is molecular genetics and all biomedical technology a sorcerer's apprentice? Are we increasing rather than lessening our burden of pain and anxiety?

The last question leads to yet another issue. Biohazard and ecohazard may arise inadvertently, but "sociohazard" may be the product of deliberate malev-

olence. The U.S. is a signatory to an international legal convention that has renounced biological warfare, including research to produce the necessary agents. Not all countries have taken this step, and public renunciation without adequate inspection cannot ensure that covert activities do not exist. Opponents of recombinant-DNA research see its techniques as being ideally suited to serve malevolent purposes, either as agents of organized warfare or of sabotage and terrorism. The techniques do not require large installations or highly sophisticated instrumentation. Contrary views have not denied this but have noted that recombinant-DNA techniques would not be the first technology to have potential malevolent applications. Explosives have such applications, but society does not completely ban them; it takes prudent precautions against their misuse.

Nevertheless, the issue of the possible misuse of recombinant-DNA technology deserves a place on the policy agenda, because it emphasizes the need for international discussion of the implications and management of recombinant-DNA research and recombinant-DNA applications. It can be argued that the U.S. is not ready for such discussion until its own policies are in better order. It is not too early, however, to begin the internal consideration of how best to approach the international arena.

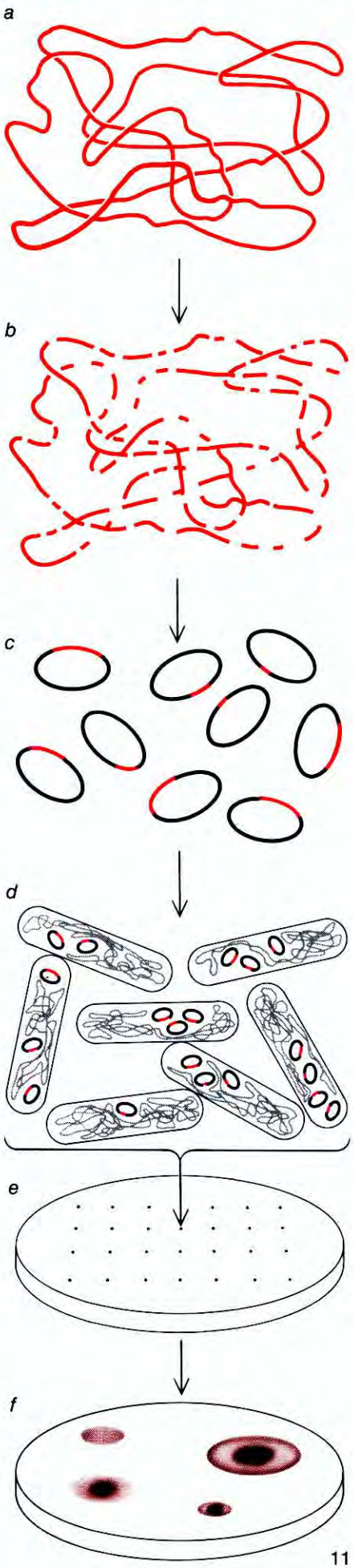
These are the chief issues that have emerged from the policy debate so far. It is a not inconsiderable list. The debate has not been raging on every street corner, but it became strenuous enough in Cambridge last summer to have repercussions across the continent. For example, an evaluation presented by a panel of nonscientists to the Cambridge City Council was not too different in content from one produced by a task force of the Quality of Life Board of the City of San Diego, where I live. Both groups accepted within their community the continuance of recombinant-DNA research requiring *P*3 facilities but sought somewhat greater assurances of safety than those provided by the NIH guidelines. Meanwhile the Attorney General of the State of New York held a public hearing, and a joint hearing was conducted by two committees of the Assembly of the State of California Legislature. Legislation regulating DNA research was later introduced in the California Assembly, and it is still under consideration. Congress has also held several hearings and various items of regulatory legislation have been introduced in both the Senate and the House of Representatives. These local, state and Federal initiatives emphasize the necessity to get on with the policy agenda.

The agenda should be viewed in at least two time frames: immediate and longer range. A consensus has been growing that there is an immediate need to give the quasi regulation represented by the NIH guidelines a statutory base. In particular, regulation must be extended to activities not supported by Federal agencies, especially in the industrial sector. However this is to be done, it is important to maintain flexibility, since the problems to be dealt with will change as greater knowledge and experience are acquired.

Moreover, given the complexity of the longer-term issues, immediate legislation probably should be provisional and limited. A mechanism should be included, however, that actively leads toward a more definitive future policy. This requires provision for a new, comprehensive assessment of all the issues raised by recombinant-DNA research, including the probable effectiveness of the regulatory devices put in place under the NIH guidelines.

The need for such a new national assessment is demonstrated by the nature of the critical challenge to the product of the earlier assessment. First, it has been alleged that the 1975 Asilomar conference establishing the pattern for the NIH guidelines was dominated by scientists involved in the research, and therefore it could not yield a broad enough perspective. Second, it is argued that the earlier assessment was devoted primarily to the question of potential biohazards and did not address in any depth other gravely important questions. The passage of time has added several more points: that circumstances already have changed as research has progressed, that experience has grown and that a wider range of opinion has come to bear on the issue. Whatever format is adopted for the reappraisal of the recombinant-DNA issue, the public must be assured that the process is a comprehensive and objective one.

Whoever undertakes this new national review should first carefully examine the current situation, including the actual effectiveness of the regulatory mecha-



"SHOTGUN" EXPERIMENT is a type of recombinant-DNA experiment in which the total DNA of an organism (*a*) is exposed to restriction enzymes in order to yield many fragments (*b*), which are then recombined with the DNA from a suitable vector (*c*) and randomly reinserted with the vector into the host cells (*d*). The *E. coli* hosts are next spread on a nutrient substrate (*e*) so that each recipient cell, containing a particular inserted foreign nucleotide sequence, can grow into a colony (*f*). The result, if the experiment is successful, is a "library" of all the nucleotide sequences of the organism. Under the guidelines issued by the National Institutes of Health last year shotgun experiments are regarded as being potentially more hazardous than those involving purified and characterized DNA, since it is not known whether portions of the DNA with unknown or repressed functions might cause unexpected problems.

nisms provided by the NIH guidelines. Particular attention needs to be paid to the local institutional biohazards committees mandated by the NIH guidelines. Beyond the responsibility assigned to the principal investigator these committees are the only source of local surveillance and standard-setting. Their composition and charge are unique, yet their authority and procedures are stipulated only generally in the NIH guidelines. They may well need the stimulus and support of external interests to carry out their important task. Moreover, no provision has been made for budgeting what may turn out to be their considerable cost for technical surveillance, personnel training and medical monitoring. Like all insurance, security against biohazard must be bought. The cost should be borne as an additional expense of the research, not as a competitor for existing funds.

Similarly, the actual performance of the NIH study sections, which are mandated by the guidelines to be independent evaluators of biohazards and containment, needs to be examined. Study sections are already heavily overloaded with the job of evaluating scientific quality. Yet these part-time peer groups are asked to assume another difficult function. If the responsibility is to be taken seriously, it too will entail additional costs.

Of special importance for early attention is an effective monitoring system for following the actual directions of recombinant-DNA research. The techniques involved are so rich in possibilities, whether for fundamental research or applications, for benefit or risk, that "early warning" is essential. Systematic following of the directions of investigators' interests, from applications for support through informal communication to formal publication, is essential to the early detection and assessment of either risks or opportunities. Needless to say, monitoring is particularly difficult in industrial research. It might therefore be desirable to limit or postpone certain development efforts pending closer study and greater knowledge of the underlying problems.

Equally urgent is a determined effort toward a more effective assessment of risks and their limitation. The specific assignment of responsibility for this kind of policy-oriented research should be an early recommendation of the body undertaking the reassessment. Given the differing perspectives required by regulation and the NIH mission to promote health-related research, the regulatory function probably belongs elsewhere in the long run. On the other hand, given the need for careful study of the implications of relying on existing agencies or of establishing a new one, the temporary continued assignment of this responsibility to the NIH may be desirable. This interim solution, if it is adopted, must be accompanied by additional funding to carry it out effectively.

Considerations of biohazards and physical and biological containment have necessarily had a high priority in this early phase of recombinant-DNA research. Many informed observers believe, however, that these concerns will decline in importance as research continues and experience grows. Therefore although the current furor makes a rational approach to the biohazards question an essential part of any successful recombinant-DNA policy, this approach does not exhaust the longer-term requirements and may even distort them. More crucial in the long run may be several other issues that have been raised directly or indirectly.

For example, in investing in fundamental genetic research that can profit from recombinant-DNA techniques, what relative priorities should be assigned to potential applications? In the past the national strategy in biomedical research has been to invest directly in basic research, without declared objectives, while also investing in specific objectives, allowing some of the latter support to "trickle down" to basic research. Thus an investigator of the interaction of viruses and cells, say, might be alternatively or simultaneously supported by funds for fundamental investigation and by funds intended for promoting the development of an effective therapy for cancer. What should be the priorities among possible practical applications of molecular genetics? Competing lines of inquiry include the microbiological synthesis of drugs, specific human gene therapies, the improved efficiency of photosynthesis, nitrogen fixation by food crops, enhanced agricultural production and so on. There are quite different potential risks and benefits in each of these directions, and all are unlikely to be maximally supported at once. In the new areas that are opening up is a new research strategy called for? If it is, by what procedures should it be formulated and how should it be implemented?

It is widely recognized that there is a logical continuum running from basic research through applied research and development to technological application. It is also recognized that movement along this continuum is neither smooth nor fully predictable and that varying motivations and institutional arrangements operate along its length. Recombinant-DNA techniques are the product of fundamental investigation, supported almost entirely by the partnership of the Federal Government and the universities. For the moment, at least, the techniques are likely to remain useful primarily in that area. The techniques may also be useful for various industrial purposes, however. Given the nature of the original investment as well as the complex issues raised, should technological uses, at least for a time, be kept under Federal control? Should some of the return from successful applications be employed to recycle the original investment of Federal resources? Should this promising new technology be a prototype for establishing a revolving capital fund to support a more stably financed basic-research effort?

The possibilities of genetic engineering and evolutionary control illustrate the fundamental dilemmas raised by the new capabilities conferred by scientific knowledge. Society has entered an age of intervention, in which the automatic operation of natural processes is increasingly, through informed intervention, brought consciously into the orbit of human purpose. Many events that humanity formerly could regard only as a boon or a scourge—an act of God or of nature—are now the partial product of human decision and intervention. If human beings do not have the capability today to invent new organisms or to initiate life itself, they may soon have that capability. If they cannot today consciously and fully control the behavior of large ecosystems, that power is not far beyond what has already been achieved. The humility of individuals understandably shrinks from awesome powers that were earlier assigned to divine will. It was not, however, the humility of individuals that conferred these emerging capabilities or is called on to control them today. It was the social interaction of individuals, operating through social institutions, that brought us to the present fateful decision making. Imperfect though they are, our social institutions built the platform for the age of intervention.

The policy challenge we face, refracted in the exquisite structure and potential of the double helix, is whether we can create institutions able to transform the fruits of an age of reason into the achievements of an age of intervention. There are voices today urging us not only to eschew conscious intervention but also to distrust and limit the uses and consequences of reason itself. Perhaps it needs to be restated that it was, after all, natural selection that evoked the double helix and all it conveys. Included among the products are human knowledge and judgment, to which has now passed the duty of designing social processes and structures that can cope with the manipulability of the double helix itself.

The concept and control of the double helix signal a new frontier of biocultural progression. A stereoscopic vision that includes both "creative pessimism" and "creative optimism" is now required. Neither alone can do justice to the profound revelations human beings have recently experienced. A single eye is particularly limited in yielding depth and perspective. For the age of intervention at least two are needed.

PHYSICAL CONTAINMENT	BIOLOGICAL CONTAINMENT (FOR *E. COLI* HOST SYSTEMS ONLY)		
	*EK*1	*EK*2	*EK*3
*P*1	DNA from nonpathogenic prokaryotes that naturally exchange genes with *E. coli* Plasmid or bacteriophage DNA from host cells that naturally exchange genes with *E. coli*. (If plasmid or bacteriophage genome contains harmful genes or if DNA segment is less than 99 percent pure and characterized, higher levels of containment are required.)		
*P*2	DNA from embryonic or germ-line cells of cold-blooded vertebrates DNA from other cold-blooded animals and lower eukaryotes (except insects maintained in the laboratory for fewer than 10 generations) DNA from plants (except plants containing known pathogens or producing known toxins) DNA from low-risk pathogenic prokaryotes that naturally exchange genes with *E. coli* Organelle DNA from nonprimate eukaryotes. (For organelle DNA that is less than 99 percent pure higher levels of containment are required.)	DNA from nonembryonic cold-blooded vertebrates DNA from moderate-risk pathogenic prokaryotes that naturally exchange genes with *E. coli* DNA from nonpathogenic prokaryotes that do not naturally exchange genes with *E. coli* DNA from plant viruses Organelle DNA from primates. (For organelle DNA that is less than 99 percent pure higher levels of containment are required.) Plasmid or bacteriophage DNA from host cells that do not naturally exchange genes with *E. coli*. (If there is a risk that recombinant will increase pathogenicity or ecological potential of host, higher levels of containment are required.)	
*P*3	DNA from nonpathogenic prokaryotes that do not naturally exchange genes with *E. coli* DNA from plant viruses Plasmid or bacteriophage DNA from host cells that do not naturally exchange genes with *E. coli*. (If there is a risk that recombinant will increase pathogenicity or ecological potential of host, higher levels of containment are required.)	DNA from embryonic primate-tissue or germ-line cells DNA from other mammalian cells DNA from birds DNA from embryonic, nonembryonic or germ-line vertebrate cells (if vertebrate produces a toxin) DNA from moderate-risk pathogenic prokaryotes that do not naturally exchange genes with *E. coli* DNA from animal viruses (if cloned DNA does not contain harmful genes)	DNA from nonembryonic primate tissue DNA from animal viruses (if cloned DNA contains harmful genes)
*P*4		DNA from nonembryonic primate tissue DNA from animal viruses (if cloned DNA contains harmful genes)	

"SHOTGUN" EXPERIMENTS USING *E. COLI K*-12 OR ITS DERIVATIVES AS THE HOST CELL AND PLASMIDS, BACTERIOPHAGES OR OTHER VIRUSES AS THE CLONING VECTORS

EXPERIMENTS IN WHICH PURE, CHARACTERIZED "FOREIGN" GENES CARRIED BY PLASMIDS, BACTERIOPHAGES OR OTHER VIRUSES ARE CLONED IN *E. COLI K*-12 OR ITS DERIVATIVES

SOME EXAMPLES of the physical and biological containment requirements set forth in the NIH guidelines for research involving recombinant-DNA molecules, issued in June, 1976, are given in this table. The guidelines, which replaced the partial moratorium that limited such research for the preceding two years, are based on "worst case" estimates of the potential risks associated with various classes of recombinant-DNA experiments. Certain experiments are banned, such as those involving DNA from known high-risk pathogens; other experiments, such as those involving DNA from organisms that are known to exchange genes with *E. coli* in nature, require only the safeguards of good laboratory practice (physical-containment level *P*1) and the use of the standard *K*-12 laboratory strain of *E. coli* (biological-containment level *EK*1). Between these extremes the NIH guidelines prescribe appropriate combinations of increasing physical and biological containment for increasing levels of estimated risk. (In this table containment increases from upper left to lower right.)

Thus physical-containment levels *P*2, *P*3 and *P*4 correspond respectively to minimum isolation, moderate isolation and maximum isolation. Biological-containment level *EK*2 refers to the use of new "crippled" strains of *K*-12 incorporating various genetic defects designed to make the cells' survival outside of laboratory conditions essentially impossible. Level *EK*3 is reserved for an *EK*2-level host-vector system that has successfully passed additional field-testing. Because of the very limited availability of *P*4 facilities and because no bacterial host-vector system has yet been certified by the NIH as satisfying the *EK*3 criteria, the recombinant-DNA experiments now in progress in the U.S. with *E. coli* host systems are with a few exceptions limited to those in the unshaded boxes. Experiments with animal-virus host systems (currently only the polyoma and SV40 viruses) require either the *P*3 or the *P*4 level of physical containment. Experiments with plant-virus host systems have special physical-containment requirements that are analogous to the *P*1-to-*P*4 system.

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