Specialty Conference

Recombinant DNA in Medicine

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Studies in bacteria and bacterial viruses have led to methods to manipulate and recombine DNA in unique and reproducible ways and to amplify these recombined molecules millions of times. Once properly identified, the recombinant DNA molecules can be used in various ways useful in medicine and human biology. There are many applications for recombinant DNA technology. Cloned complementary DNA has been used to produce various human proteins in microorganisms. Insulin and growth hormone have been extensively and successfully tested in humans and insulin has been licensed for sale. Mass production of bacterial and viral antigens with recombinant DNA technology is likely to provide safe and effective vaccines for some disorders for which there is no prevention. The cloned probes for the human α- and β-globin loci, for specific disease genes, such as the Z allele of α-antitrypsin, and for random genomic sequences are proving useful for prenatally diagnosing human genetic disorders and preventing their clinical consequences.

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STEPHEN D. CEDERBAUM, MD:* Seldom has a scientific or biomedical breakthrough evoked the awe, controversy or sheer incredulity that has accompanied the snowballing developments in the field of recombinant DNA technology or, more popularly, gene cloning and genetic engineering. Little more than one generation after Avery, McCloud and McCarty[1] showed that genes were encoded in DNA and Watson and Crick[2] interpreted the structure of these molecules, genes are being cut, manipulated and recombined to produce unprecedented insights in genetics and molecular biology and in the prospect of gene therapy. These developments have occurred not without anxiety to both scientists and nonscientists. Theologians and philosophers have discussed the dangers of altering human genetic makeup and even of producing human life in laboratories; environmentalists and politicians have raised fears of epidemics decimating the human race. Neither the most apocalyptic fears nor the most optimistic dreams appear to be imminent though neither is out of the question. Recombinant DNA technology has already had great impact in the fields of genetics,

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ABBREVIATIONS USED IN TEXT

AAT = α_1-antitrypsin
ADH = alcohol dehydrogenase
cDNA = complementary DNA
kb = kilobases

hematology, endocrinology, immunology and rheumatology, and shortly will forcefully impinge on the study of infectious diseases, neurology, orthopedics, oncology, cardiology and virtually every other area of medicine and biology.

The successful manipulation of genes requires that the following distinct processes be successfully accomplished[3]: (1) that DNA be cut in a predictable and reproducible way; (2) that two distinct pieces of DNA be combined in a controlled manner; (3) that an isolated piece of DNA be amplified millions of times, and (4) that the cloned gene or genetic material be identified.

A restricted host range for bacterial viruses has been known for many years. Beginning in 1952 several groups of investigators described the phenomenon of host-induced modification of the viral genome wherein the host range was dependent on the strain in which the virus was propagated.[4] In the early 1960s, Arber[4] showed that successful replication was accompanied by maintenance of the DNA in its intact macromolecular form, whereas failure of infection was characterized by the destruction of the intact viral genome. He predicted the existence of enzymes that hydrolyzed foreign nucleic acids after the recognizable host sequences were modified to escape attack by these proteins. In the early 1970s, Smith and Wilcox[5] described bacterial restriction endonucleases that destroyed foreign DNA but not that of the host *Hemophilus influenzae*. They and others have purified and described more than 200 such enzymes, most of which have absolute sequence specificity. One of these, Eco RI, an enzyme derived from an *Escherichia coli* R strain, recognizes a specific hexanucleotide sequence (Figure 1) and causes cleavage of the double helix in an unequal manner, leaving two 4-base fragments without their complementary, hydrogen-bonded partners. A given genome, or fragment thereof, will be cut into identical pieces each time it is subjected to the hydrolytic action of Eco RI or any other specific restriction endonuclease. The host genome is protected from the action of this enzyme by modifying the bases—for example, by methylation—so that the enzyme fails to recognize its normal sequence.

Figure 1 underscores the second feature of many restriction endonucleases that is vital to the task of genetic recombination, the generation of free single-chain base sequences that tend to form active hydrogen bonds with their complements. Two different DNA fragments can be hydrolyzed with Eco RI and, when mixed together and ligated enzymatically, form a new DNA molecule (Figure 2).

The third vital feature of a successful gene cloning is the duplication or cloning of an individual isolated piece of DNA, which is accomplished by inserting the foreign DNA into a bacteriophage or an autonomously replicating bacterial plasmid.

The existence of sexuality or the transfer of genetic information between different bacterial strains has been known and studied for more than 30 years.[6] In the 1960s, the rapid emergence of multiple drug resistance in bacteria led to the discovery of R factors, or autonomously replicating plasmids that can live with a bac-

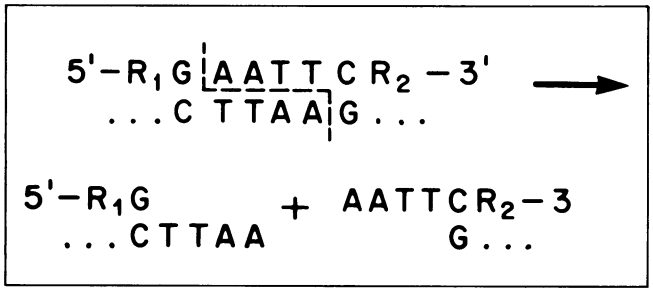

Figure 1.—The specific hexanucleotide sequence recognized by the restriction endonuclease Eco RI. R_1 and R_2 indicate the unknown flanking base sequences. The dashed line indicates the line cleavage of the double helix and the products are shown below, each with the 5′ cohesive end.

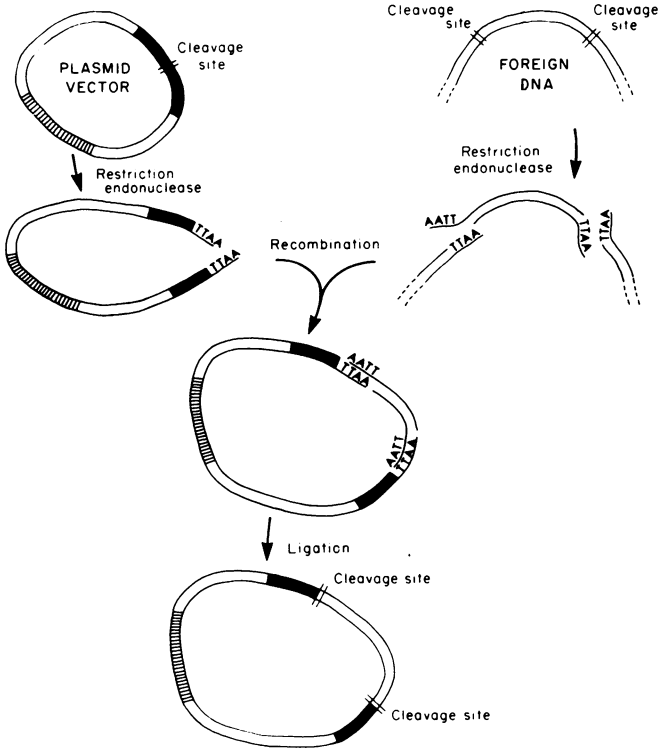

Figure 2.—Creation of a recombinant plasmid. The hatched areas represent a gene for tetracycline resistance and the dark areas for ampicillin resistance. After cleavage with Eco RI and reannealing, the foreign DNA is intercalated into the gene for ampicillin resistance.

terial cell and, in some instances, be induced to produce numerous copies.[7] This class of plasmids has been exploited as vectors for propagating unique pieces of DNA within bacterial cells (Figure 2). The plasmid may be cut with a specific restriction endonuclease and reconstructed with the foreign DNA inserted within the ampicillin-resistant locus, leaving the tetracycline-resistant gene intact. The recombinant vector is then introduced into the bacterial cell and the recipient selected by growth in the presence of an antibiotic. The recombinant plasmid may be identified by the loss of resistance to the second antibiotic, in this case ampicillin.

The gene-cloning procedure thus far described is not technically complex. In principle, one could produce a "library" of human DNA clones large enough to encompass the entire human genome. The library of individual clones would, however, be virtually useless. We would not have the faintest idea of the content of these clones and they could be used for little else save species identification, assuming they were reasonably species-specific.

A second limitation of the approach involves the nature of human genes and the use of cloned genes for protein production in bacteria. Genes for human proteins contain not only the DNA sequences that encode the amino acid sequence but also intervening sequences (introns), or base sequences intercalated in the coding regions, but not specifying a functional amino acid sequence. The introns are transcribed when messenger RNA is produced but must be excised before translation of mature RNA by the protein synthetic machinery.[8] Microorganisms do not have introns in their structural genes and lack the ability to excise them. Therefore, it is the sequence of mature message and not the gene that corresponds to the amino acid structural sequence of the protein product. To circumvent this problem, the DNA used for cloning is derived from messenger RNA or only the RNA actually transcribed in a specific tissue. The complementary DNA (cDNA) clone for phenylalanine hydroxylase would be found in a library from liver and not from spleen or pancreas.

The general scheme for cloning is shown in Figure 3. The messenger RNA is isolated from total RNA because of its polyadenylate tail and a complementary or cDNA chain is synthesized with the help of a reverse transcriptase. After separating the RNA-DNA hybrid, the second DNA chain is synthesized and, after suitable manipulation, is ready for incorporation into the plasmid vector.[9]

Isolating and identifying specific clones may be accomplished in various ways. This is the most difficult part of the cloning process and could, in some instances, require years. Various procedures have proved useful. The particular sequence in question can be enriched. Genes of the X chromosome can be obtained by isolating X chromosomes by differential cell sorting or by isolating a human X chromosome in a rodent host after cell hybridization.[10,11] Hemoglobin is isolated by using messenger RNA from reticulocytes, of which those for the α- and β-chain make up 90% of the total.[12] Rare messages can be enriched to near homogeneity using specific immunoprecipitation of ribosomes actively translating the protein.[13] The antibody recognizes the nascent chain and binds those ribosomes. The cDNA from these pools is greatly enriched for the desired protein. The metallothionein message may be greatly increased by growing cells either in culture or in animals in its heavy metal ligand, and the message further purified by size separation on a sucrose density gradient.[14] There are also strategies using principles similar to those noted.

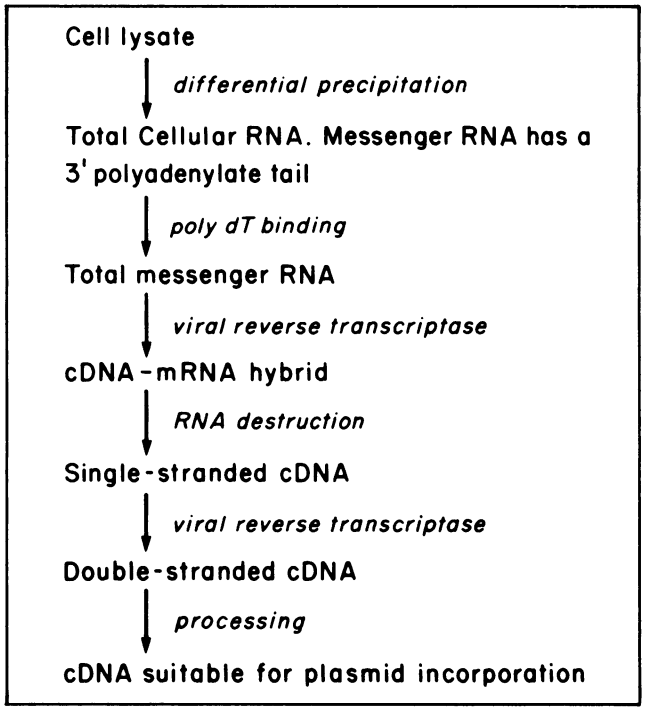

Figure 3.—Scheme of the important steps in cDNA production. cDNA = complementary DNA, mRNA = messenger RNA

The identity of a clone is proved ultimately by the congruence of its sequence and that of the RNA molecule or protein for which it codes. Because protein sequence information is laborious to obtain, indirect criteria are often applied. These generally require the availability of a monospecific or monoclonal antibody to the protein in question. Advantage is taken of the ability to translate messenger RNA in vitro, using most commonly a rabbit reticulocyte lysate. After antibody precipitation and polyacrylamide gel electrophoresis with sodium dodecyl sulfate, a radioactive band in the position of the molecular weight of the monomer of the protein in question is seen and is obliterated if excess cold antigen is added. The correct clone for the gene in question when denatured hybridizes with the complementary messenger RNA. This is assessed either by following the disappearance of the electrophoretic band from a whole cell messenger RNA preparation or

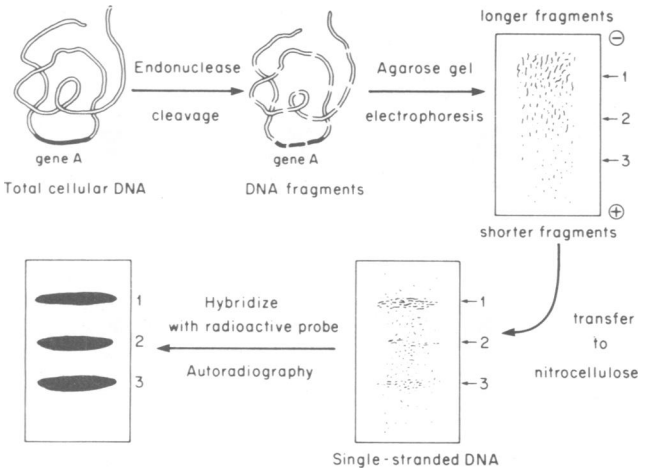

Figure 4.—Principles of the Southern blotting technique. After endonuclease cleavage and agarose gel eletrophoresis, the fragments of gene A are located at positions 1, 2 and 3, but cannot be seen against the background of random DNA fragments with a continuous size distribution. They are visualized with a complementary DNA probe rendered radioactive with ^{32}P, after hybridization and subjecting the nitrocellulose to autoradiography.

by its appearance after elution from the DNA and subsequent translation.[15]

Figure 4 shows the powerful technique by which genes complementary to a specific probe are recognized among random fragments of DNA. We now have a probe for human β-globin. To be useful, it must be able to identify and describe specific fragments of human DNA to which it is complementary. This is accomplished by obtaining whole cell DNA and cutting with one or another restriction endonuclease. When these random fragments are subjected to electrophoresis on agarose gel, they separate by size. When stained with ethidium bromide, a nonspecific DNA stain, we see a blur as the fragments distribute themselves continuously across the size scale. Dr Edwin Southern from Glasgow, Scotland, solved this problem.[16] The DNA is denatured and transferred from the agarose gel to nitrocellulose paper to which it is highly adherent. The probe rendered radioactive with ^{32}P is then hybridized to it. The radioactive band can then be visualized after autoradiography. A probe constructed chemically as a result of a known amino acid sequence can be used in this way to identify either fragments or clones on an agar plate.[17]

Protein Products of DNA Technology

GEORGE C. FAREED, MD:* Genetic engineering is a broad field encompassing classical types of genetic manipulation such as plant and animal breeding, human genetic counseling, conventional mutagenesis and somatic cell genetics. The techniques include chemical treatment of simple organisms such as bacteria, viruses, yeast and other lower organisms, selection of specific mutant phenotypes, fusion of cells in tissue culture to produce recombinant phenotypes, hybridoma antibodies and the dynamic field of recombinant DNA technology or gene cloning. Through recent advances in enzymology, bacterial genetics and nucleic acid chemistry, the DNA for specific genes can be chemically synthesized or physically isolated, such as those for peptide hormones and for antibodies and the interferons. It is possible to amplify them many thousandfold, determine their exact nucleotide sequences, locate entire structural genes and either chemically or biologically synthesize copies of these genes. Already, synthetic genes and cloned natural genes for many important substances, such as somatostatin, insulin, growth hormone, viral antigens and interferons, have been inserted into plasmids of bacteria and yeast that are capable of producing the functional product in abundant quantities.

Three broad classes of proteins are becoming products of the new biotechnologies: (1) enzymes; (2) hormones, such as growth hormone and insulin, which control various activities in different tissues of the body, and (3) some of the 100 or more molecules that make up the body's defense system against disease—such as interferon.

The dream of biotechnology is to turn the diversity of enzymes and other proteins to the benefit of humanity, designing an ideal microbe to make each desirable product. Many of these products can enhance the quality and length of our lives by their effects to counter infections, cancer, pain and aging. The specific goal of recombinant DNA for pharmacology is to produce in simple organisms such as bacteria and yeast, or possibly in farm animals, the multitude of human proteins and peptides that are physiologically beneficial. Most of these cannot be obtained for clinical use through other methods and their beneficial properties or uses cannot be evaluated until they are created through this technology.

Enzymes have evolved to catalyze the metabolism of virtually any raw material on earth. Enzymes turn sugars into alcohol and enable certain microbes to "mine" uranium and copper. There are 25,000 different species of microbe known, each with its own library of enzymes (though some enzymes are shared by many species).

Human Enzymes

The genes for several human enzymes or carrier proteins have been cloned and full-length cDNA recombined into bacterial or yeast plasmid vectors, permitting their synthesis in commercial amounts in the microbial cell. These include factors involved in blood clot formation or their dissolution, hydrolytic enzyme modifiers and albumin.

The potential usefulness of urokinase in fibrinolytic therapy for myocardial infarction and other conditions was recognized and studied more than a decade ago.[18] The plasminogen activators are present in small amounts in tissues and urine and the expense of puri-

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fication and their low abundance have made large-scale clinical studies difficult. Recently, investigators in the United States, Europe and Japan have cloned full-length cDNA for urokinase and other plasminogen activators and have achieved expression in microorganisms.[19,20] Genentech, Inc., is currently developing a clinical testing program for urokinase with its contractural partner, Grunenthal, Inc., in Germany. Commercially useful quantities of urokinase should be available by 1985.

Tissue plasminogen activator has become Genentech's fourth commercial product of genetic engineering technology, after interferon, insulin and human growth hormone. Dr Desire Collen of the University of Louvain, Belgium, purified small amounts of tissue plasminogen activator, an enzyme that dissolves fibrin and is produced naturally in the body; this led ultimately to its cloning and the creation of a bacterial expression vector for it.

Synthetic urokinase and tissue plasminogen activators will permit physicians to dissolve individual clots without causing the side effects common with anticoagulants such as heparin, which prevents the blood from clotting and can cause acute bleeding problems. This method of fibrinolysis is superior to that promoted by streptokinase as the proteolytic activity is restricted to the fibrin clot.

Liver proteins important in blood functions or transport of substances are being cloned and expressed in microorganisms. These include clotting factor IX,[21] fibrinogen,[22] albumin,[23,24] apolipoproteins[25] and α_1-antitrypsin. Among these, both albumin and α_1-antitrypsin have been produced in bacteria for clinical testing and large-scale production. The availability of "engineered" albumin will permit replacement with an osmotically active fluid without the risk of blood-derived products.

The recent cloning of the gene for human clotting factor IX[21] shows the power of recombinant DNA approaches to obtain the genes for proteins, which, compared with the genes for albumin, have few messenger RNAs. The production of complex proteins, such as clotting factors, which are normally glycosylated after their translation, may take place in yeast rather than in bacteria—which cannot do these posttranslational modifications—or in farm animals that have been genetically engineered to produce the human gene product.[26] Such progress raises the hopes that factor VIII may soon be produced and both factors VIII and IX become available in complete form for safe replacement therapy in cases of hemophilia A and B.

The gene for α_1-antitrypsin (AAT) has been cloned at the Zymos Corporation, Seattle, and its large-scale production was reported recently at the 11th International Conference on Yeast Genetics and Molecular Biology (Kyoto, Japan). α_1-Antitrypsin, normally produced in human liver and carried in the bloodstream, has been synthesized in ordinary bakers' yeast in a laboratory. α_1-Antitrypsin counteracts the effects of trypsin and perhaps other hydrolases produced by the body to destroy bacteria, air pollutants and cigarette smoke in the lungs. If insufficient AAT is produced by the body, the unneutralized enzymes may attack the lung tissue itself.

About 1 in 4,000 persons in the United States has severe, inherited AAT deficiency. These persons usually die prematurely of severe emphysema or liver disease.[27] About 1 in 20 persons in the United States is heterozygous and has a less severe deficiency. They too may be prone to pulmonary disease, especially if exposed to cigarette smoke or other unfavorable environmental pollutants.[28] The engineered product may be important in treating patients who have respiratory disease, whose bodies make insufficient quantities of the protein, and may prevent later disease if used preemptively.

The yeast cells selected for this genetic engineering ordinarily produce large quantities of alcohol dehydrogenase (ADH), an enzyme essential for normal growth. Zymos scientists have taken the promoter that triggers the production of ADH and attached it to the human DNA sequence coding for AAT. Thus, the yeast is tricked into producing the enzyme at any time it requires. The problems of safety will need to be examined in animals to assure that toxic or antigenic components of the yeast cell or growth media have been completely removed. Testing in animals to estimate the effective duration and inhibitory capacity of the protein also will be necessary. Long-term studies will be required to assure that the rate of deterioration in lung function has been stopped or substantially reduced. At present, no definitive treatment is known for illness caused by a genetic deficiency of AAT.

Although the initial clinical trials will be restricted to genetically deficient patients with emphysema, subsequent human trials may be extended to patients who have adult respiratory distress syndrome. Patients with this disease have abnormally low levels of AAT, and acute symptoms may be due partly to an excessive activity of an enzyme normally neutralized by AAT. Because adult respiratory distress syndrome is an acute rather than a chronic disease, the administration of AAT, if effective, would not require long-term studies to show its benefits.

Human Peptide Hormones

The second and most highly publicized family of products of recombinant DNA technology are the peptide hormones. These were the first substances produced in abundance and several have been subjected to successful clinical studies. The hormones include somatostatin (the first product of a synthetic gene),[29] human insulin,[30] growth hormone,[31] secretin,[32] calcitonin,[33] β-endorphin,[34] gastrin,[35] relaxin[36] and epidermal growth factor.[37] Small hypothalamic releasing factors, such as growth hormone releasing factor,[38] and newly discovered neuropeptides such as the calcitonin-related peptide important in limbic and cerebellar neural operations will be manufactured. Finally, many

growth and suppressor substances will become available and new therapies developed.

Somatostatin, the first biologically active human protein produced in bacteria, was made from a chemically synthesized cDNA, the sequence of which was deduced from the amino acid sequence, taking advantage of the universality of the genetic code.[29] Similarly, the first recombinant DNA product to be approved by the Food and Drug Administration, human insulin (Humulin) manufactured by Lilly, is produced in the same manner.[30] When the true sequence of the human gene was discovered, the functional artificial gene was found to differ in several codons for which degeneracy is present.

The bacterial production of insulin differs from that in humans in another important way. Whereas the insulin A and B chains are normally produced as a single molecule with excision of the connecting polypeptide after folding of the completed chain, the engineered A and B chains are produced in separate bacteria and combined in vitro. Human insulin is a little faster-acting than purified porcine insulin; in young patients who have diabetes a downward adjustment of the dose is necessary.[39,40] Immune reactions to human insulin have not been reported.

Bacterially produced human growth hormone also has been successfully tested in humans.[41] When commercially available in unlimited amounts, it will have a greater impact on medicine than insulin. Previously growth hormone was derived from human pituitary glands obtained at autopsy and was available in only limited amounts. Only patients with growth hormone deficiency could be treated and then often with doses that might have been suboptimal. The general availability of growth hormone also opens the door for possible abuse.

Protein Cellular Effectors

The final group of products produced in large amounts by recombinant DNA technology is the general class of proteins best described by the term cellular effectors. These include thymosin α_1,[42] the interferons and their hybrids[43,44] and, more recently, the prospect of producing numerous other lymphokines important for modulating the immune response, such as interleukin-2.[45]

The interferons have drawn the greatest amount of attention. They are small, heat-stable glycoproteins that have both antiviral and antitumor acivities.[46] When interferons are produced in bacteria, they are not glycosylated, but seem to retain biologic activity.[47] The results of the initial clinical trials with recombinant DNA interferon as an antiviral agent have been encouraging regarding the prophylactic treatment of rhinovirus infections and efficacy in hepatitis; cases of herpes infection are also being examined.[46] Although the antiviral activity of the interferons may prove most valuable clinically, they are also being examined for antitumor activity. In the first report[47] on a multidose phase I trial of leukocyte α-interferon from *E coli,* 81 patients with various refractory disseminated malignant lesions received injections of escalating amounts from 1 to 136 million units per injection. Toxic reactions were similar to those seen with nonrecombinant interferon—fever, chills, anorexia, nausea, vomiting, dose-dependent reversible leukopenia and serum aminotransferase elevations. Objective evidence of antitumor activity was seen in a small percentage of patients with tumor regression and remissions of as long as seven months, indicating that the genetically engineered product is biologically active. This tends to agree with smaller phase I trials for the treatment of osteosarcoma and Kaposi's sarcoma. Definitive assessment of the clinical efficacy of this and other interferons will await the results of phase II trials now in progress. Phase II cytoreductive studies in specific malignant neoplasms are under way to determine the frequency and duration of response with a maximum tolerated daily dose. Cloned interferons will probably be evaluated in many situations in which there is no therapy and a viral cause or immunologic disturbance has been noticed, such as in cases of multiple sclerosis and amyotrophic lateral sclerosis or even certain forms of arthritis.

When one looks at the contribution of DNA cloning technology to medical research on interferons, to other biologic response modifiers and to these various other protein products, all fields of medicine are certainly being profoundly influenced by this new evolving technology.

Recombinant DNA Products in Infectious Disease

MICHAEL A. LOVETT, MD:* The advances in antimicrobial therapy made in the past three decades have become the practical focus of the field of clinical infectious diseases. During the same three decades that followed the discovery of the double helical structure of DNA, advances in our basic understanding of microbial genetics have led to the creation of genetic engineering. Although recombinant DNA technology may increase the efficacy of industrially producing antimicrobial agents, it will be central to the next revolutions in the treatment of clinical infectious disease: new subunit vaccines and rapid diagnostic tools. In this review I will limit my discussion to the likely impact of recombinant DNA technology on preventing and diagnosing bacterial diseases. Similar considerations should be germane to viral, fungal and parasitic diseases.

The impetus for the use of recombinant DNA products in treating infectious disease arises from problems inherent in the use of native components of pathogens in diagnostic tests or vaccines. Bacteria may be difficult, or even impossible, to cultivate in vitro (such as *Mycobacterium leprae, Treponema pallidum*); the growth of large volumes of human pathogens (such as rickettsiae) can be dangerous; purification of microbial substances may be difficult, and the production of a desired substance by a pathogen may be unreliable. Contamination with undesirable pathogen-derived sub-

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TABLE 1.—*Likely Targets for Recombinant DNA-Based Vaccine*

Category	*Examples*	*Organism*
Cell surface proteins	Pili	*Neisseria gonorrhoeae, Escherichia coli*
	Outer membrane protein	Group B meningococci, *Hemophilus influenzae,* Group B streptococci
Toxins and virulence factors	IgA protease	*Neisseria, H influenzae*
	Enterotoxins	*E coli*
	Other toxins	*Clostridium difficile, Pseudomonas*
Polysaccharides	Kl capsular	*E coli*

stances is always a concern. Current recombinant DNA technology may overcome all the problems associated with pathogen-derived substances. Gene amplification and enhancement of gene expression in *E coli* or other bacterial hosts can cause the pathogen gene product to comprise a substantial fraction of the host cell mass, greatly facilitating purification and insuring stable production. It is safer to work with individual pathogenic gene products in *E coli* K12, in most cases, than with the native pathogenic bacterium.

Prospects for Recombinant Bacterial Vaccines

Human pathogenic bacteria are highly adapted to live in humans, and consequently the creation of effective antibacterial vaccines has been a formidable challenge. In some instances the ability of natural infection to confer long-lived immunity is in doubt (example: gonorrhea). Vaccines conferring reliable protection are available for only four pathogenic bacteria: meningococcal and pneumococcal vaccines consist of capsular polysaccharides and those for diphtheria and tetanus are inactivated bacterial toxins.

The study of pathogenesis has given us insight into the molecular basis of bacterial virulence factors. Recombinant DNA technology will not sufficiently control a bacterial disease unless key immunogens and virulence factors are defined and details of the host-parasite interaction are thoroughly understood.

Key bacterial surface proteins related to disease have generally shown great antigenic diversity. A classic example is the M protein of the group A streptococcus.[48] The M protein filaments protrude through the hyaluronic acid capsule and have an antiphagocytic activity that is critical to virulence. Immunization with an individual M protein confers lasting immunity. However, there are 55 antigenically distinct M protein types. This degree of antigenic diversity presents a difficult, but by no means insurmountable, challenge to recombinant DNA technology, as will be clear later in this discussion. Bacterial surface polysaccharides may also show antigenic diversity within a species (for example, pneumococcal capsular polysaccharides) and are often critical virulence factors or protective immunogens, or both.[49] Recombinant DNA technology has been most adept at enhancing expression of protein.

Targets for Recombinant Bacterial Vaccines

Likely targets for recombinant bacterial vaccines are listed in Table 1. The first group are the several varieties of bacterial surface proteins, among them the pili. Bacterial pili are filamentous, surface-located appendages that are polymers of polypeptide subunits. Pili are highly evolved to mediate attachment of bacteria to receptors on specific human tissues. In certain bacterial diseases, the presence of a special pilus-mediated attachment to a target tissue is critical to the virulence of the organism.[50]

To appreciate the potential of recombinant DNA to produce pili for human immunization, we must first consider important work with animals. K88 and K99 are designations for pili that mediate attachment of enterotoxic *E coli* to the small bowel of pigs and cattle, causing severe diarrhea. The genes for the pili have been cloned and expressed in *E coli* K12.[51] Expression of the cloned genes has been optimized and purified K88 and K99 subunit vaccines prevent diarrhea in animals (S. Falkow, PhD, verbal communication, 1982). This work is exciting for several reasons: It represents the first successful use of a recombinant DNA vaccine to control a bacterial disease. It also shows dramatically that parenteral vaccination with a subunit vaccine can result in effective immunity at a mucosal surface.

Enterotoxic *E coli* strains causing "traveler's diarrhea" possess special pili, designated CFA I and CFA II (for colonization factor antigens), that are critical virulence factors mediating attachment to human small bowel mucosa.[52] The CFA I and CFA II are therefore the analogues of K88 and K99 for human disease. It is reasonable to hope that the effective recombinant subunit vaccines based on CFA I and II may be created.

Bacterial strains causing pyelonephritis have special pili, called P pili, that bind to specific receptors of uroepithelial cells. These P pili have been cloned (G. Schoolnik, MD, and S. Falkow, PhD, verbal communication, 1982) and it should be feasible to learn whether or not P pili can form the basis of a human pyelonephritis vaccine.

Gonococcal pili mediate attachment to various human tissues and only piliated gonococci are virulent. The number of antigenically distinct gonococcal pili approaches that of group A streptococcal M protein types.[53] At present, the role of pili in immunity to the gonococcus is uncertain, but may be elucidated by structure-function studies of these pili (G. Schoolnik, MD, verbal communication, 1982). The gene for one serotype of gonococcal pili has recently been cloned in *E coli*.[53] As noted above, antigenic diversity of bacterial surface components critical to virulence is a problem that recombinant DNA technology must address. One

possible approach would combine the gene products of well-defined antigenically diverse determinants of type-specific immunity together into a polyvalent vaccine, much as may now be done using conventional approaches.

Other surface proteins in addition to pili are under consideration as candidates for experimental vaccines. Outer membrane protein of group B meningococci, type b *Hemophilus influenzae* and group B streptococci will be studied as adjuncts or alternatives to capsular polysaccharide.[54] Gonococcal[55] and chlamydial[56] outer membrane proteins may also contribute to protective immunity.

The second group of candidates for engineered antigens to serve as antibacterial vaccines are virulence factors and toxins. Pathogenic *Neisseria* and *H influenzae* produce an IgA protease whose role in disease has been unclear. The genes for these proteases have been cloned and expressed in *E coli* and genetic systems for their reintroduction to the species of origin have been established (S. Falkow, PhD, verbal communication, 1982). Genetic manipulation of IgA protease genes should facilitate an understanding of their role in the pathogenesis of disease.

Bacterial exotoxins represent another area where recombinant DNA technology has had an impact both in the understanding of basic pathogenic mechanism and by offering vaccine potential. The gene for the heat-labile toxin of enterotoxic *E coli* has been cloned and sequenced.[57] The genes specifying attachment and toxic activity have been defined, permitting construction of a vaccine comprised only of the binding subunit.

It should be possible to clone several medically important toxins, such as those elaborated by *Clostridium difficile, Pseudomonas,* the erythrogenic toxins of streptococcal and staphylococcal strains and other toxins associated with food poisoning. With a cloned gene, structure-function studies can be done that could separate toxic from immunogenic portions of the molecule, leading eventually to "toxoid" vaccines in which the toxic moieties have been genetically deleted.

The final vaccine candidates I will describe are the capsular polysaccharides. *E coli* capsular antigen K1, which is immunochemically identical to the capsule of group B meningococcus, is associated with many *E coli* strains causing meningitis. Using cosmid cloning, Silver and associates[58] transferred the gene(s) specifying K1 biosynthesis to unencapsulated *E coli* K12 laboratory strains. This represents the first use of recombinant DNA to study capsular polysaccharides.[58]

In my laboratory we have cloned a 2.1-kilobase (kb) segment of *Treponema pallidum* DNA that directs the synthesis of a protease-resistant periodate-sensitive molecule. We have isolated an identical molecule from *T pallidum*. The cloned antigen reacts specifically with serum from patients who have had syphilis. It is highly immunogenic for rabbits and induces the formation of antibody that immobilizes virulent *T pallidum* in vitro (T. Fehniger, A. Walfield, PhD, and M. A. Lovett, MD, PhD, 1983, unpublished data). Vaccination experiments are now in progress for experimental syphilis.

Synthesizing cell surface polysaccharide from pathogenic bacterial species may be important for several reasons. Stable and abundant production of antigen can be achieved without the difficulty or the potential bio-hazard inherent in cultivating large volumes of a fastidious or noncultivatable pathogen. There is also the potential to engineer genetically the enzymes involved in polysaccharide synthesis so that polymers of altered composition and antigenicity can be created.

Recombinant DNA in Diagnosing Infectious Disease

Using cloned genes for *E coli* heat-labile enterotoxin, Moseley and co-workers[59] have shown that DNA hybridization is a powerful tool to detect the presence of a pathogen directly in clinical specimens. "DNA probes" have been constructed that detect *Chlamydia trachomatis, N gonorrhoeae* and a variety of enteric pathogens (S. Falkow, PhD, verbal communication, 1982). DNA hybridization has been used to detect cytomegalovirus DNA in urine.[60]

Because certain genes encoding antimicrobial resistance can be readily cloned, DNA probes will probably identify a pathogen and will also show its antimicrobial resistance. DNA probes could also be used as a rapid diagnostic adjunct to tissue culture or conventional bacterial culture techniques. Before DNA probes can be routinely used in clinical microbiology laboratories, however, methods of labeling the DNA must be developed to avoid the use of radioisotopes. The hybridization itself can be done in less than three hours.

Recombinant DNA technology can provide large quantities of pure microbial antigens for accurate and reproducible serotesting. The cloned antigens may be used directly in the serotests; they could also be used in monoclonal antibody-based tests. A *T pallidum* antigen synthesized in *E coli* has been a better serodiagnostic antigen for diagnosing syphilis than conventional and treponemal tests (M. A. Lovett, MD, PhD, 1983, unpublished data).

Applications of Cloned Genes

LARRY J. SHAPIRO, MD:* Almost immediately after the discovery that recombinant DNA techniques could be applied to eukaryotic and mammalian systems, intense interest developed in the possible application of these methods to several vexing problems in clinical medicine.[61-63] The fact that these capabilities have come into being as the fields of medical genetics and prenatal diagnosis are moving into their adolescence has assured an enthusiastic reception for the attendant new ideas and concepts in this sector of medical practice. At present, although applications of recombinant DNA technology to clinical genetics are relatively limited in a technical sense, there are few workers in this area who have not been gripped by the intense excitement and sense of optimism that has come about as a result

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of the first few forays into this new arena. In the hope of sharing some of that excitement, I will review briefly some recent developments and strategies involving the application of this technology to genetic counseling, prenatal diagnosis and eventually to the treatment of inherited diseases.

Prenatal diagnosis is now a well-established discipline that has developed around techniques of fetal visualization (such as ultrasound, fetoscopy and radiography), the biopsy of fetal tissues and the analysis of amniotic fluid and its cellular constituents.[64] Cultured amniotic fluid cells have been widely used for prenatal detection of chromosome defects and inborn errors of metabolism. Biochemical diagnosis depends, however, on a relatively precise knowledge of the nature of the genetic defect in question, on the normal expression of the function in question in relatively undifferentiated amniotic fluid cells and on normal levels of expression of this function to an extent that the analysis of the number of cells obtained after a few weeks of growth in culture can be carried out relatively rapidly in the second trimester of pregnancy.

The use of suitable cloned probes for diagnosing genetic defects by direct analysis of fetal cellular DNA promises several advances in this process. First and most important, the primary genetic abnormality can be examined directly. Expression of a detectable cellular phenotype is not required and therefore one is not limited to the repertoire of inherent responses of amniotic fluid cells. The examination of globin genes or genes related to liver-specific proteins becomes possible even though the cells to be studied do not produce globin or liver proteins. Second, the possibility of detecting prenatally conditions in which the primary genetic defect is unknown or uncertain becomes tenable through genetic linkage analysis. This strategy is not completely novel. Initial results using recombinant DNA-generated markers suggest that the high degree of variability or polymorphism, which will be detectable throughout the human genome using suitable molecular probes, will logarithmically enhance our capabilities to deal with new diseases both by genetic counseling and by prenatal diagnosis. Finally, the technical nature of the procedures involved should make possible more rapid laboratory diagnosis using materials other than just growing and dividing amniotic fluid cells. Direct analysis of the DNA of uncultured cells is possible. In addition, attention has been directed recently toward the use of transcervical chorionic villus biopsy to obtain tissue suitable for fetal DNA analysis during the first trimester of pregnancy. Thus, the duration of maternal anxiety, the medical risk of pregnancy termination and psychosocial concerns related to mid-trimester abortion can all be abated.[65,66]

The requisites for detecting any given disease at a genomic level are the availability of a suitable cloned probe for the gene in question and a detailed knowledge of the molecular nature of the defect for which the fetus is at risk. The only clinical experiences reported to date involve disorders of the human α- and β-globin loci, which are due to the early availability of probes for these regions of the genome and the high frequency with which clinically significant disorders involving these genes are found in human populations. Experience with these conditions will serve as a prototype for other diseases.

Many patients with α-thalassemia and some patients with β-thalassemia or $\delta\beta$-thalassemia seem to have actual deletions of genetic material involving all or part of the relevant chromosomal regions. Such defects can be detected by molecular hybridization with suitable probes either in solution or on Southern blots.[67,68] The interpretation of such experiments is quite straightforward as homozygous affected subjects would be missing an entire band corresponding to the deleted gene sequence.

Although many heritable diseases may be the result of gross deletions, several other conditions have resulted from smaller alterations of the genome. Single-base changes may result in amino acid substitutions in the structure of a protein affecting either the function, stability or location of the gene product. In addition, small deletions, mutations producing early termination of protein elongation and frame-shift mutations leading to complete alteration of the gene product have all been described in mammalian systems. Furthermore, the relatively recent recognition of intervening sequences (introns) described by Dr Cederbaum suggests new possibilities for the genesis of hereditary disorders. The complexity of the pathway of maturation of messenger RNA, including splicing out of the introns from primary transcripts, polyadenylation at the 3′ end of messenger molecules and the addition of a translation site at the 5′ end before transport from nucleus to cytoplasm, suggests a host of other possibilities for mutations to interfere with the production of normal amounts of protein; these gene products are of normal structure and function though quantitatively reduced. Indeed, the study of the numerous thalassemia mutations in humans has, by their disruption of globin synthesis, provided the most detailed information on these processes.

To provide potentially useful diagnostic information in instances of this type, detailed information is required about the molecular lesion in the specific defect for which the person in question is at risk. In patients with well-documented sickle cell disease, a single A to T base substitution in the middle position of codon 6 changes the specified amino acid from glutamic acid to valine. This defect may be present in most sickle hemoglobin genes. However, as discussed previously, the molecular defects in the thalassemias are very heterogeneous and are the result of a wide spectrum of genetic alterations. It is therefore necessary to be able to study the relevant mutant gene in either a heterozygous or homozygous state before attempting prenatal diagnosis. Certain disorders, like sickle cell disease in blacks or Tay-Sachs disease in Ashkenazi Jews, may turn out to be less heterogeneous, but many

other defects, even those with very similar phenotypes, will show a great deal of molecular variability.

There are presently two strategies that should be widely applicable for the detection of discrete genetic defects by molecular hybridization techniques. These involve the use of synthetic oligonucleotide probes and the use of site-specific restriction endonucleases to identify alterations in cellular DNA. A recent example of the use of synthetic oligonucleotide probes has been provided by workers at the City of Hope Medical Center (Duarte, California).[69] They have chemically synthesized two probes that correspond to 19 bases of the β-globin sequence. The probes were identical except at a single position corresponding to the alteration in the β^S-globin gene. By controlling conditions of hybridization of such radioactively labeled probes with great care, specific hybridization of each probe with its homologous gene was obtained. The considerable technical advances in synthetic oligonucleotide chemistry make it plausible to generate such probes where any specific single nucleotide substitution in a mutant gene has been identified. However, this specific knowledge will be needed and exquisite care in the control of the temperature and conditions of hybridization may limit the widespread applicability of such methods. Finally, the specific nature of the nucleotide substitution examined will alter the relative stabilities of properly matched and mismatched hybridization between probe and cellular DNA.

An approach to prenatal or postnatal diagnosis by analysis of genomic DNA that may be more general makes use of the site-specific restriction nucleases described earlier in this conference. About 200 restriction enzymes with various specificities have been isolated and characterized.[70] With the knowledge of a specific nucleotide sequence alteration in a given genetic disorder, it should be possible to search through the known restriction enzymes and find one such reagent whose recognition site would be destroyed or an alternate site created by the mutation in question. After digesting a patient's DNA with the relevant enzyme and locating by electrophoresis specific fragments on Southern blots with labeled probes, size and hence electrophoretic differences between mutant and normal genes should be uncovered. Several technical requisites are necessary, however, before such a strategy may be used. These include the use of appropriate probes, the identification of enzymes that generate restriction fragments, which differ sufficiently in length in mutant and normal genes, and careful control of reaction conditions to ensure complete digestion of the DNA. A final problem is the possible pitfall of restriction site polymorphism unrelated to the mutation in question, which could confound the Southern blot analysis. There is substantial, benign variation in nucleotide sequence from one person to another; some of this variation might occur close to the site being studied, causing erroneous interpretation of results. Effective restriction enzyme tests for sickle cell disease have now been reported based on the use of Dde I or Mst II, with the latter enzyme being more generally useful.[71,72] Mst II cleaves at the sequence CCTNAGG, where N is any nucleotide. This sequence is included in normal β-globin genes (CCTGAGGAG) but not in a β^S-globin gene (CCTGTGGAG). A sickle gene, therefore, will not be cut at this position and a longer gene fragment results.

Much attention has been given recently to the use of benign restriction endonuclease site polymorphism as genetic markers to provide counseling and diagnostic information to families at risk for genetic disorders of as-yet-uncertain causes. This approach is predicated on the use of family linkage data and gene mapping technology reviewed recently by Sparkes and colleagues.[73] The use of DNA analysis in such endeavors is not fundamentally different from the use of any other genetic markers; the DNA polymorphism, however, should be more readily detectable, giving rise to more recognizable variability at more locations throughout the genome.[74]

At least three significant limitations are inherent in this strategy. First, because one is using a linked polymorphism as a genetic marker, by definition that marker lies at some measurable distance from the mutant gene whose transmission is to be studied in a pedigree. There is, therefore, a finite probability that the polymorphisms and the gene of concern will be separated from one another by recombinational events. The second limitation is based on the fact that no matter how frequently one observes variability at a given polymorphic region, some subjects in pedigrees will not be informative about transmission of presumably linked genes. This problem obviously diminishes with increasing numbers of polymorphisms detectable in the same region. For example, a single variant at a genetic locus present in less than 10% of the population will not be terribly useful for linkage studies. The same variant, if present in 50% of the genes in a population, will be much more useful but many people (half) will be homozygous for one or the other variant and thus uninformative about transmission of genes to their offspring. A single genetic locus or region with many different alternative alleles present in the population at relatively high frequency, as best typified by the human leukocyte antigen region, provides the best situation for linkage analysis. Third, the main deterrent to the use of linkage analysis is the need to do relatively complete family studies for each diagnostic procedure. This is necessary to establish the phase relationship of the mutant gene and the marker in each pedigree. Despite these limitations, linkage analysis is increasingly being done as the only reasonable means presently available to provide diagnostic information to families at risk for disorders such as pseudohypertrophic (Duchenne) muscular dystrophy, Huntington's chorea or cystic fibrosis where few clues to the primary genetic defects are available.

Two types of probes have been used in attempts to identify usefully polymorphic linkage markers: (1) probes for genes already cloned and of known func-

tion, and (2) quasi-random cloned fragments of DNA of unknown function but whose location in the genome can be determined and whose genes show polymorphism as an "anonymous sequence." A substantial amount of effort has gone into identifying polymorphic restriction sites near the β-globin locus, which has proved useful for the study of mutations leading to hemoglobinopathies and thalassemias. One of the first such polymorphisms identified was the absence (or presence) of an Hpa I restriction site in the 3′ flanking DNA of the β-globin gene.[75] This polymorphism results in Hpa I-digested DNA that contains the β-globin gene in a 7,600 base-pair fragment (7.6-kb) or in a 13,000 base-pair (13.0-kb) fragment depending on whether the recognition site is present or absent. Kan and Dozy[75] found that most, but not all, β^S-genes were associated with a 13.0-kb fragment and most β^A-genes were associated with a 7.6-kb fragment. If family studies were done to verify the phase relation of the restriction fragment polymorphism and the β-gene, the former marker could be used for the prenatal diagnosis of sickle cell disease. Subsequent work by several groups has identified numerous other linked polymorphisms in the β-globin region, which should be increasingly useful for the diagnosis of defects in this portion of the genome, especially in relatively inbred populations.[76-78]

By analogy, random cloned fragments of DNA from other regions of the genome should also be useful if they show significant polymorphisms.[79] Such probes need not be derived from any functional gene but must represent unique sequences within the genome. Some DNA sequences are repeated several or even thousands of times in the genome. Segments of DNA to be used to search for polymorphism must represent discrete genetic loci. One of the several strategies used to identify such probes is chromosome isolation, either physically[10] or in somatic cell hybrids.[11] Such chromosome-specific DNAs can be regionally mapped in the standard gene-mapping fashion using hybrid cell panels derived from patients with translocations. Once such probes are located, polymorphism can be sought using a reasonable number of test subjects and a variety of restriction endonucleases. Finally, linkage with a specific disease gene can be sought by studying multigenerational families, segregating both the DNA polymorphism and the disorder in question. An example of such application is the work of Davies and associates[80] in identifying a probe from the short arm of the X chromosome, which has a polymorphic restriction site nearby and appears linked to the locus for pseudohypertrophic muscular dystrophy. Although these results are preliminary and the genetic distance involved is too great to be of clinical use, studies such as these point the way toward developing a high-resolution map of the human genome based on these polymorphisms. This map would permit genetic counseling and prenatal diagnosis even for disorders whose cause is unknown.

In addition to these diagnostic implications, molecular technology will surely improve the therapeutic approach to genetic diseases. The use of recombinant DNA methods to generate protein products is being investigated. Some of these products, however, could be enzymes, or growth factors, that various patients might be missing on a genetic basis. For example, numerous clinical trials have been reported of enzyme replacement therapy for lysosomal storage diseases. However, most of these experiments have been impeded by the lack of a ready and available supply of human enzyme. Such a source of enzyme might eventually be found in bacterial production systems.

Of more long-range applicability is the direct insertion of genes into mammalian cells and even into whole organisms. There are two conceptually disparate approaches. The first involves introducing functional genes into individual somatic cells of a patient, probably in vitro, and then returning these engineered cells to the patient. The second approach involves introducing new genetic information to all of a person's cells, including germ cells, which would thus involve a true genetic change that could be transmitted to progeny. These two approaches differ from each other in technical difficulty and certainly in moral and ethical content. Although the technical capacity to introduce genes either into cultured cells or into fertilized mouse zygotes is clearly at hand, procedures to ensure site-specific integration into the genome and appropriately regulated expression are not yet available. Nonetheless, Cline and co-workers[81] have reported transferring dihydrofolate reductase genes to murine bone marrow cells and selecting methotrexate-resistant recipient cells in intact animals. Several laboratories have introduced cloned genes by microinjection into fertilized mouse eggs and detected the integration, transmission to progeny and expression of these genes.[82-88] Perhaps the most dramatic experiment reported to date involves the introduction of a cloned rat growth hormone gene (linked to a metallothionein promoter) into such mice.[26] This resulted in animals that had as much as 800 times normal levels of circulating growth hormone and that achieved twice the size of their normal littermates.

Clearly, the advent of recombinant DNA techniques and their application to biology and medicine has ushered in one of the most exciting eras of human intellectual endeavor. Although many technical and conceptual barriers remain, there is clear promise to improve the outcome for families affected by genetic diseases through better diagnosis, counseling and treatment.

Summary

DR CEDERBAUM: *We have reviewed some of the basic principles of recombinant DNA technology and some areas of extant or imminent impact on clinical medicine. We believe that the true potential of this technology has just begun to be exploited and that it is reasonable to anticipate major strides in the next few years. These advances will be felt in the production of new and more useful biologics and drugs; in the de-*

velopment of probes providing a deeper understanding of normal function, including that of development of the immune system; in the understanding of gene expression in embryologic development and maturation, and in the existence, nature and control of "oncogenes" that may play a role in cancer. Ultimately cloned genes, or their complementary DNA, will be introduced and expressed in foreign cells in a predictable and reproducible way and serve as the basis for gene therapy. We are confident that involvement of recombinant DNA technology will increase at the forefront of medicine.

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