Genetic Engineering

H.W. Boyer and S. Nicosia
Editors

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and

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PREFACE

This year marks the 25th anniversary of Watson and Crick's publication of the structure of DNA which stands as one of the most significant events in the history of science. Since that time thousands of scientists have been investigating numerous aspects of the structure and function of DNA in order to understand how genetic information is contained within this molecule and how it is processed in such an incredibly ordered manner. The ultimate goals of these investigative efforts include the very basic human desire to understand the molecular processes of life and to determine the basis of the many genetic and viral diseases of man as the first step in their eradication. As a result of these efforts we now have a solid foundation of information in molecular genetics for future investigations. The development of recombinant DNA technology over the past five years has excited the imaginations of scientists because it offers a powerful methodology for unravelling the many genetic complexities of higher organisms and for the genetic engineering of microorganisms designed to produce useful products for the biomedical sciences and industry.

The international symposium on Genetic Engineering hosted by the Giovanni Lorenzini Foundation in March 1978 and sponsored by this Foundation and the World Health Organisation, Geneva, focused on some of the latest and most exciting research in this field. These topics ranged from basic and applied genetics to discussions of the establishment and comparison of guidelines thought to be necessary for conducting recombinant DNA research adopted by the various countries of the world engaged in this type of research. The presentations of that symposium are compiled in this volume. If the rate of progress toward our goals continues to accelerate as in the past few years, the next twenty-five years of genetic research should be gratifying to the investigative scientist and the society that supports these efforts.

H. Boyer
S. Nicosia

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REPLACING NATURE AS AN INNOVATOR

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Technological developments show a consistent pattern of continuous acceleration in the rate of progress. It is enough to think of the speed of the fastest means of transportation available. For many thousands of years the horse offered the only improvement over our own legs for terrestrial transportation. But recently, the internal combustion engine and more recently still, jet engines have increased the speed of transportation by orders of magnitude. In the biological field we have witnessed some remarkable improvements in mortality rates, especially those of the young. But some of the most recent, most important and prestigious conquests, like that of chemotherapeutics and antibiotics are already being threatened by the adaptive capacities of microorganisms. In antiviral or anticancer therapy advances witnessed so far have been less impressive. Even more striking is the hiatus between the discoveries of molecular biology at the theoretical level and the lack of applications. But recent events have proved that in the next years the situation is likely to change.

The common name for the new development is "genetic engineering". But in my view, there is no real discontinuity between the first attempts at forcing living organisms to improve their services to us, and the present attempts that go under the name of genetic engineering. Basically, they can be mostly described as some form of hybridization. It may be objected that in genetic engineering there is a real attempt at replacing nature as an innovator, for instance using "unnatural" means of hybridization. But not even this need be true. Restriction enzymes are very "natural" and their role in nature may be, at least in part, similar to the one which is asked of them in the test tube. It is also perfectly possible that DNA recombination has played an evolutionary role. If I were to look for examples, I would try where there are more chances of it happening, for instance in intracellular symbioses. We cannot today exclude that mitochondria were the result of gene splicing, from an intracellular microorganism and a eukaryote; part of the spliced DNA may have been extruded as a ring in the cytoplasm at the beginning or at some later stage. For other intracellular symbioses there may have been exchange

of parts of DNA between host and guest. When the exchange is not too old and subsequent evolution not too pronounced there may be better chances of proving it.

It is perhaps correct to say that genetic engineering does not replace nature as an innovator, but just uses ways and means which are not so commonly found in nature. This is no diminution of genetic engineering. As a parallel example one certainly does not diminish the importance and interest of B. Mintz's artificial chimeras in mice if we remember that a certain fraction of twins in man are natural chimeras. The title of this talk is correct if we interpret the word "innovation" somewhat loosely. Strictly speaking, of course, the word innovation is reminiscent more of mutation than of hybridization. The possibility of influencing more and more specifically the mutational process is of course an approach of great interest but presently success is expected from the manufacture of recombinants which would not ordinarily be formed, e.g. between highly unrelated species, or between a natural and a synthetic DNA. Directing recombination between organisms, even when they have been separated by billions of years of divergent evolution, seems to be a much easier task than that of directing mutation, and this is the preferential though not exclusive approach of genetic engineering today.

There already have been, in this century some breakthroughs in plant breeding: hybrid corn and the green revolution are the outstanding examples. There have been improvements in the quality of animal stocks. There have been substantial decreases in the price of antibiotics made possible, in part at least, by the selection of appropriate mutants. All this however seems far less striking than the advances in other technologies, from plastic to computers, communication, energy production. Are possibilities of application in genetics likely to be as important as those of other branches? It is too early to say, but one can have some confidence that the answer will be positive. There is an old experience to draw upon, so old that it dates back before written history. Unknown probably even to many fellow geneticists, the very basis of modern civilization is a consequence of genetic technology. Man started some 10,000 years ago to domesticate all cultivated plants and all animals that are bred today. The transition from food collection to food production that thus took place was the major determinant of social change. It allowed the increase of population size by three orders of magnitude, it forced people to become sedentary, it generated the multitude of villages, towns and cities, which exists today, and thus created the necessity of civil administration and government. Only with the higher potential density of people permitted by food production did modern society become possible and indeed necessary.

These developments took place independently in various parts of the world, but mostly where some kind of cereal was available as a wild plant and could be successfully domesticated. The modern wheats which we still use to make bread and spaghetti were developed mostly as polyploids of wild types in the Near East. Specific mutants were selected for, e.g. strong rachides which do not break when harvested with sickles. Ordinary wild type wheats have a brittle rachis and the seeds scatter if harvested with sickles; they have to be plucked by hand, a less efficient procedure.

At least at the beginning, much of this development need not have been very deliberate. But the existence of a constant effort to improve yields is well documented by archeological evidence. Corn used in the early process of domestication was much smaller than that with which we are familiar today. Cobs were at the beginning not much larger than a penny. They have increased steadily in size, at a rate which can be measured on the basis of the archeological record to be about one inch every 1500 years. The constancy of the growth rate probably reflects a constant effort in selecting the best grain for sowing. The source of genetic variation was most probably mutation, perhaps gene duplication, and must have been of very low frequency. The observed tempo of change gives an idea of what the genetic engineers of the time could do. This conference should reassure us that present day genetic engineers count on faster results.

In addition to telling us about the state of the art, we expect this conference to give us thoughts on cost - benefit analysis. We all know that this is still a major issue. There has been, among biologists, a tendency to split into three major categories: those who thought genetic engineering - and in particular the dominant technique, DNA recombination - could do no harm, so that every precaution taken is a complete waste of time and money. Others made prediction of doom and gloom, while an intermediate category of people suggested taking reasonable precautions against foreseeable risk. This last opinion has prevailed so far, and thus the NIH Guidelines were born. It is a first in technological development, that elaborate precautions were taken before any accident; the custom is to wait until something happens. Personally, I am relieved that fellow biologists have taken this cautious attitude. I wish there existed NIH Guidelines also for violence on TV, or that guidelines had been developed for environmental pollution before many big cities came close to being the disaster areas which they are today. The same could be said for car safety.

We have found in the process that there are serious dangers inherent in warning against danger. Caution may scare especially when it involves, as in the present case, fear of the unknown. We have witnessed an epidemic of fear spreading even among scientists. With exceptions, the epidemic has perhaps hit more severely, on average, those who had more limited contact with the disciplines of medical microbiology or pathology. The epidemic has of course spread widely outside the circle of scientists. Few people were alert to this danger. If this is one of the costs of being cautious, one must, however, accept it, even if objectively, the chances of danger seem low. If nothing else, the evolutionary consideration that remote crossings are very unlikely to happen spontaneously should be reassuring. Living organisms are often protected against them, when possible, because the misfits thus generated are very unlikely to reproduce efficiently enough. Virulence of microorganisms is very difficult to keep in strains grown in the laboratory. Adaptation to life outside the guest's environment is difficult to many parasites and usually incompatible with the maintenance of virulence. Even the deliberate creation of super dangerous organisms by super sophisticated DNA technology does not seem at all an easy task. Other technologies provide much better chances to the warmonger or to the terrorist. But even so, we have had so much negative experiences from the careless use of new technologies that we should discipline ourselves to be cautious.

We all desire to learn about the benefits. The most immediate ones are perhaps more likely to be at the industrial level. For the branch in which I have a special interest, human genetics, there are a few possibilities which are not too remote. One important difficulty is that most genetic diseases are rare. Unfortunately, for those few diseases that are less rare the chances of using present genetic engineering techniques are more remote. The most frequent genetic abnormality among Caucasians, Down's syndrome (1/700) is a trisomy and I see no simple application of DNA recombination for treating it successfully. But there is fortunately efficient prophylaxis. An apparently good recessive is cystic fibrosis, with an incidence of 1/2000 among Caucasians. Here the knowledge of the primary defect is practically zero, making it difficult to think of any simple genetic engineering technique. Even more frequent among most Blacks and a few South Eastern European populations is sickle cell anemia, for which we know almost everything except successful treatment. Replacing a given nucleotide (the one which affects change of glutamic acid to valine in the 6th Aminoacid of the β chain of hemoglo-

bin) would be relatively simple in microorganisms, but is still a very ambitious project indeed in Man.

Attempts at replacing erroneous DNA affecting synthesis in human cells have been reported and will have to be seriously reconsidered in the future. It is not impossible to think of ways of making, by genetic engineering a normal human enzyme and administering it to the patient unable to produce it, in a form favouring long survival or slow administration. Although phenylketonuria can be successfully treated with prophylactic diet, the possibility of administering the missing enzyme, phenylalanine hydroxylase, made in vitro, may be considered as a useful alternative. Other inborn errors of metabolism could be similarly treated, if the human enzyme could be made in vitro at a reasonable cost.

Chances of successful use would be even higher for proteins for which replacement therapy is already working. The classical case is hemophilia. Here, in the A form, it is AHG, antihemophilic globulin, which is not produced. Administration of the human globulin allows normal life. But the lifelong administration of an expensive product obtained from fractionation of human plasma is extremely expensive, and carries a substantial danger of hepatitis. Unfortunately, AHG is a big molecule, and making it by DNA cloning is no mean project.

Applications in human genetics are limited more than anything else by the rarity of the relevant diseases. Hemophilia is one of the most frequent ones and yet the incidence is of order one in 10,000. It will increase in the future, given that the balance between mutation and selection which kept its incidence low has been broken. Today, in developed countries the life expectancy of the hemophiliac is normal. The incidence can be expected to double in about a century. In most other cases the increase of incidence due to therapy will not be so dramatic. Medicine can be dysgenic to some extent when it cures successfully a genetic disease, but except for dominant diseases the increase of incidence due to selection relaxation is extremely slow. Sex linked recessives, like hemophilia are closer to dominants in this respect. With dominants that are strongly selected against, successful therapy may cause a doubling of incidence every generation. This fear is only theoretical because we are still unable to treat successfully dominant diseases.

There is one area, bordering on genetics, where DNA recombination of some sort may prove even more useful than in bona fide genetic disease. This is immunology. The specific site of antibodies is only some 100 aminoacids long. The manufacture of specific antibodies could thus be within reach of present techniques.

The number of antibiotic resistant infections increases daily. The chances of antiviral and anticancer therapy with antibodies may be relatively high. The number of different antibodies which our body manufactures is, however, staggering. Important help may come from new recent developments using hybrid clones. By cloning cells which make a specific antibody, say against diphteria or tetanus toxin, enough chemically homogeneous antibody could be produced for sequencing the variable region of this antibody. Perhaps the mRNA from such cells could be used directly, or an artificial DNA made once the aminoacid sequence is known. Perhaps even faster techniques can be developed. Making antitoxins against diphtheria or tetanus, especially if of human origin, would have the advantage of improving considerably already existing, very useful prophylactic or therapeutic products.

I have insisted on medical applications because I am more familiar with them. Maybe the real future of genetic engineering is elsewhere, in food production or in pollution control. When our ancestors started developing domesticated plants and animals they could hardly imagine that they would eventually multiply their own number by a factor of 1000 or 10,000. We are now reaching the end of the human "logarithmic" growth phase. Food shortage is perhaps as much of a problem, or even more so than it was at the beginning of agriculture. We are reaching the limits of growth, however, not only because we are exhausting means of food production and other limited resources but also because of the accumulation of toxic products. In a way, all these problems were created by the genetic engineers of some 10,000 years ago, who started the population growth spurt of which we are the latest products. It would, of course, be too much to expect that genetic engineering will solve all our present problems. But it can hopefully contribute to relieve some of the pressures which afflict us today.

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THE ISOLATION OF AN E. COLI PLASMID DETERMINANT THAT ENCODES FOR THE PRODUCTION OF A HEAT-LABILE ENTEROTOXIN

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ABSTRACT

A segment of plasmid DNA has been identified that carries the genes for the biosynthesis of E. coli heat labile enterotoxin, LT. Three polypeptides associated with LT have been identified in E. coli minicell extracts. Two of these polypeptides are related to cholera toxin. It seems likely that the LT genes can be modified suitably for use in a vaccine.

INTRODUCTION

About ten years ago clinical investigators in several different countries demonstrated that human diarrheal disease could be caused by toxigenic Escherichia coli. The study of toxigenic E. coli has been the subject of a series of studies beginning in the field of veterinary medicine, touching upon basic findings in cholera research, and, subsequently, encompassing microbial genetics and molecular biology. The relatively short but extraordinary history of the role of toxigenic E. coli in animal and human diarrhea has been the subject of two recent reviews[1,2]. Several aspects of this work that has been of particular interest to us have been the findings that there were two general classes of E. coli enterotoxins[1] and that the genes specifying these toxins were, more often than not, plasmid-mediated[2,3,4].

The two general classes of enterotoxins that have been described are found in cell-free supernatant fluid following the growth of cells in broth; that is they are exotoxins. One class of enterotoxins, called ST, is heat stable (to boiling for 30 min)[1]. The other enterotoxin, called LT, is heat labile (at 60C for 15 min). Enterotoxigenic E. coli may produce one or both of these enterotoxins[1,5]. Moreover, plasmids (designated Ent) encoding for one or both of these enterotoxins have been described[3,4].

ST is a non-antigenic polypeptide of low molecular weight (about 4,000 daltons)[1]. The response of sensitive small bowel epithelial cells to ST is characterized by an immediate secretion of H_2O and electrolytes[6], and is likely mediated by stimulation of cyclic GMP production within cells (M. Gill, private communication). The duration of ST action is short-lived.

LT appears to be distinctly different from ST[1]. LT is an immunogenic protein which has been variously estimated to be from 24,000 daltons to over 100,000 daltons

in mass[1,7]. The E. coli LT toxin shares partial antigenic identity with the well-characterized enterotoxin of Vibrio cholerae, and there are indications that LT's from diverse origins are immunologically homogeneous[8,9]. Cholera toxin has been shown to exert its effect on small bowel epithelial cells through the activation of adenyl cyclase and it is now well established that the effects of LT are also mediated by this mechanism[10,11].

About one year ago we reported the successful use of recombinant DNA technology to isolate a fragment of plasmid DNA containing the gene(s) for ST synthesis[12]. M. So and F. Heffron (in preparation) have determined the DNA sequence for the portion of this fragment encoding for ST and have further shown that the ST gene(s) are flanked by IS1 sequences and constitute a transposon. In this paper, we report the preliminary results we have obtained for the isolation of the structural gene(s) of LT biosynthesis.

MATERIAL AND METHODS

Bacterial Strains and Plasmids. The E. coli K-12 sublines employed in this study as well as the Ent P307 plasmid have been described in detail previously[12,13]. The cloning vehicles RSF2124[14] and pBR313 have been described.

Recombinant DNA Methods. The methods used to cleave plasmid DNA with restriction endonucleases and the ligation of these fragments have been described by So et.al.[12]. The experiments described here were performed under P2-EK1 conditions as specified in the NIH Guidelines for Recombinant DNA Research.

Protein Synthesis in E. coli Minicells. E. coli minicells were isolated and labelled from plasmid-containing E. coli DS410 strains as described by Dougan and Sherratt[16]. Labelled minicell extracts were analyzed by the electrophoresis method of Studier[17].

RESULTS

Molecular Cloning of an LT Fragment From P307.

The plasmid P307 was isolated from an enteropathogenic E. coli strain which caused an outbreak of diarrheal disease in piglets[4,5]. P307 has been characterized as a 60 x 10^6 dalton conjugative plasmid of the FI compatibility group[13,18]. The Ent plasmid was transferred to E. coli K-12 by conjugation and E. coli K-12 strains carrying this plasmid elaborate an enterotoxin that can be readily assayed in tissue cultures of Y-1 adrenal cells[10] or Chinese Hamster Ovary (CHO) cells[11].

P307 plasmid DNA was cleaved with the EcoRI restriction endonuclease and the fragment ligated to similarly cleaved DNA of the ColE1 cloning vehicle RSF2124. The ligated DNA was used to transform E. coli C600 cells with selection for ampicillin resistance (Ap^r). Ap^r transformant clones which were colicin sensitive (i.e. contained DNA inserted into the ColE1 structural gene) were further tested in the CHO cells assay system. No cells that produced functional LT were isolated.