APPLIED GENETIC ENGINEERING

Future Trends and Problems

by

Morris A. Levin

George H. Kidd

Robert H. Zaugg

Jeffrey R. Swarz

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Morris A. Levin
Environmental Protection Agency
Washington, DC

George H. Kidd
International Plant Research Institute
San Carlos, CA

Robert H. Zaugg
AgroBiotics Inc.
Baltimore, MD

Jeffrey R. Swarz
AgroBiotics Inc.
Baltimore, MD

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Preface

The book is divided into six parts. Chapters 1 and 2 provide, respectively, historical background for and a description of the techniques utilized by the applied genetics industry. Chapter 3 contains an overview of the biotechnology industry looking back from the year 1990, and analyzes potential hazards summarizing our general understanding of the sources and magnitudes of the potential adverse environmental and health effects of applied genetics. Questions requiring further study are identified, as are possible areas of future concern. As will be apparent, all of the trends or possibilities identified in later chapters will not develop into significant industrial applications. Chapter 3 examines the probabilities of successes based on the interaction of economies, regulating forces and technological input.

The information in Chapter 4 is organized according to industrial sector. The following industries are examined:

- Pharmaceuticals
- Industrial chemicals
- Energy
- Mining
- Pollution and waste management
- Electronics

Chapter 5 contains a description of agricultural applications and trends relevant to biotechnology. Some of the more specialized organisms and techniques are discussed in terms of application, potential and possible hazards. Chapter 6 documents the involvement of and relationship among academic, government, and commercial concerns that have a

stake in the applied genetics industry. Both foreign and domestic concerns are included.

The material in this book is based on a series of reports prepared for the Office of Exploratory Research of the United States Environmental Protection Agency. The authors were involved either in preparing or organizing one or more of them. Although the initial reports were reviewed by the Agency, the opinions herein are those of the authors, and do not necessarily reflect the views and policies of the United States Environmental Protection Agency.

Washington, DC
September, 1982

Morris A. Levin

ABOUT THE AUTHORS

Morris A. Levin, Ph.D., is presently a member of the interdisciplinary staff of the Office of Strategic Assessment and Special Studies of the U.S. Environmental Protection Agency, Washington, DC.

George H. Kidd, Ph.D., is the Director of Marketing for the International Plant Research Institute in San Carlos, California. He is an editorial adviser for *Genetic Technology News* and has advised several major corporations on their genetic engineering programs.

Robert H. Zaugg, Ph.D., is presently with Sandoz Incorporated in East Hanover, New Jersey. He was previously Vice-President of AgroBiotics Incorporated, Baltimore, Maryland.

Jeffrey R. Swarz, Ph.D., is presently with Pall Corporation of Glen Cove, New York. He was previously President of AgroBiotics, Baltimore, Maryland.

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1

Introduction

BRIEF HISTORY

The purposeful manipulation of hereditary information in plants and animals by humans, as well as the exploitation of microbial processes, has occurred since the time mankind formed societies. An understanding of the biological nature of these processes has been acquired only recently (i.e., during the past 100 years), and their chemical basis has been unravelled even more recently (in the past 35 years). A variety of terms is now employed to encompass this field of knowledge, including applied genetics, biotechnology, bioengineering, and genetic engineering. While recognizing that these general terms connote subtle differences in scope, we will use them interchangeably in this book. However, certain bioengineering procedures, such as recombinant DNA technology, entail specific activities that require more careful definition. These techniques are described in detail in Chapter 2.

Examples of genetic practices and microbial processes that have ancient origin include the following: alcohol fermentation, cheese production, food crop and domestic animal breeding, crop rotation, and the use of human and animal wastes as fertilizers. The utility of animal and plant breeding and selection was long ago recognized as a controlled method of generating improved strains of vital food crops and hardier domesticated animals. This ancient realization likely arose from the observation that children tended to possess various features characteristic of each of the parents, although the reasons for these similarities were unknown. Alcohol and cheese fermentations were undertaken long before the microbial basis for these processes was recognized. Likewise, occasional planting of fields with leguminous crops, such as soybeans,

peas and alfalfa, proved to be a helpful, often crucial, means of replenishing spent soil before it became known that bacteria were responsible for this outcome by virtue of their ability to convert atmospheric nitrogen into usable, chemically reduced forms, such as ammonia. This is the process of nitrogen fixation. And, lastly, ignorance of the role of soil bacteria in recycling human and animal solid wastes did not prevent ancient cultures from employing this rich source of nutrients to improve crop production.

The biological basis of these various processes was recognized beginning in the latter half of the nineteenth century. Two separate findings were essential to the genesis of this understanding. First, during the years 1856 to 1868, an Austrian monk named Gregor Mendel demonstrated in his experiments with peas that numerous observable traits, such as flower and seed colors, are passed from parent to offspring in the form of discrete units of heredity and that each parent supplied independent traits. These revolutionary findings, which were ignored by the scientific community until early in the twentieth century, provide the basis for the gene theory of inheritance, which states that the multitude of traits that constitute an individual organism are expressions of discrete hereditary units, called genes. In higher organisms, these genes are located on chromosomes within the nucleus of each cell. In lower forms of life, such as bacteria, which lack a defined nucleus, the chromosomes nevertheless consist of genes. In all life forms, genes provide the information that determines the make-up of the organism itself, as well as the means whereby traits are extended to the next generation.

The second fundamental discovery that led to an understanding of the biological nature of ancient endeavors in the realm of applied genetics was that of Louis Pasteur. In 1860, he demonstrated that alcohol production from fermentable substrates depended on the presence of viable microorganisms called yeasts. This finding provided the initial example of a living microbe performing a commercially useful process. Today's genetic engineering industry holds the promise that many thousands of commercially useful products and processes will result from applications of recent discoveries in biology that owe their heritage in part to the findings of Mendel and Pasteur.

The chemical basis of genetics was uncovered only recently. Although DNA (deoxyribonucleic acid) was located in cell nuclei in 1869, its role as the bearer of genetic information was not revealed until 1944 by Oswald Avery and co-workers. They demonstrated that pure DNA isolated from virulent pneumococci bacteria was absorbed by a nonvirulent pneumonia strain which was thereupon transfored to the virulent form. Further substantiation of the genetic function of DNA was provided in 1952 by A.D. Hershey and M. Chase, who radioactively labeled both protein and DNA constituents of bacteriophage viruses. (Bacteriophage

are simple viruses that infect bacteria; they consist solely of a protein coat surrounding a DNA core.) Infection of susceptible bacteria by these radiolabeled viruses resulted in the finding that viral DNA is necessary and sufficient to mediate the infection. Viral protein is not required.

The above-mentioned studies confirmed the role of DNA as the bearer of genetic information in living systems. It is now well-established that DNA alone serves this purpose in all forms of life, both plants and animals, both primitive and advanced. The information contained within the chemical structure of DNA determines to the full extent the biological nature of the organism (i.e., its appearance and its life functions). (The only exception to the universality of DNA as the genetic material is certain viruses, called retroviruses, that employ ribonucleic acid, or RNA, in this role. Although they constitute an exceedingly small proportion of the total biota on the planet, these viruses are important because they induce malignant tumors in mammals including, probably, humans. Even so, retrovirus RNA is copied into DNA during the process of infection.)

Knowledge of the chemical means whereby DNA maintains and replicates the cell's store of genetic information evolved during the 1950s and 1960s. Many scientific investigators contributed during this time to this advance in understanding, but several steps in particular bear mentioning. In 1953, James D. Watson and Francis Crick proposed a double-helical structure for DNA. This model readily suggested a mechanism whereby DNA could be faithfully reproduced. During the mid-1960s, Arthur Kornberg and associates worked out many of the biochemical details of this replication process. Meanwhile, the genetic code was being broken, most notably by Marshall Nirenberg and colleagues. This code determines how the sequence of chemical constituents in DNA is translated into a specific sequence of amino acids (via a nucleic acid intermediate called messenger RNA or mRNA). Amino acids are the chemical building blocks of proteins which, in turn, provide structural integrity and mediate metabolic activities within every cell of every organism. The steps in the pathway from DNA to protein are diagrammed in Figure 1.1.

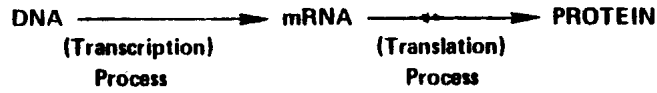

Figure 1.1: The expression of genetic information in the cell. (Modified from Harsanyi and Karney, Office of Technology Assessment, Report on Biotechnology, OTA HR 132.)

This basic research in molecular biology and genetics paved the way for developments during the 1970s that have given rise to the technology of recombinant DNA. These later achievements and procedures will be detailed in Chapter 2. It must be recognized that the modern field of applied genetics, with all its promise for future benefits to mankind (and its potential dangers), could not exist today but for the numerous accomplishments in basic research in biology and biochemistry over the past several decades, only a few of which are mentioned above.

2

Technology of Applied Genetics

Applied genetics as practiced by ancient societies involved a minimum of human intervention and consisted of little more than allowing nature to take its course. Thus, alcohol and cheese fermentation and the recycling of wastes, processes that we now know to be mediated by microorganisms, were undertaken merely by exposing the appropriate raw materials to the environment, whereupon a transformation of the substrates took place. Controlled animal and plant breeding was implemented by placing prospective parents in proximity to one another. Ancient bioengineering technology, therefore, succeeded by virtue of man's ability to manipulate crudely the biology of his environment.

By contrast, the emergence of modern biotechnology as a scientific discipline that holds enormous potential for benefiting mankind stems from our recently acquired ability to comprehend and manipulate the chemistry of living systems. Thus, the currently popular notion that modern society is embarking on the "Age of Biology" could be slightly rephrased to become the "Age of Biochemistry."

The modern technology of applied genetics encompasses a variety of procedures and processes. Each of these will be dealt with separately in the remainder of this chapter.

RECOMBINANT DNA TECHNIQUES

Recombinant DNA technology refers to the ability to isolate fragments of DNA from separate sources and to splice them together chemically into a functional unit. The DNA fragments can derive from the same organisms or from different organisms in the same species (tech-

niques that have considerable future potential for gene therapy application in humans), but the currently most promising technique involves the joining of DNA segments from disparate species of organisms, such as bacteria and humans. This latter approach has been utilized, for example, in recent efforts to mass-produce human interferon, a drug that may combat viral diseases and cancer.

A review of the recent developments in molecular biology that have led to the emergence of recombinant DNA technology can best be presented by considering those specific laboratory procedures necessary to carry out such experiments. There exist six distinct phases in the process.

(1) Isolation and Purification of DNA–Since DNA exists naturally as a long, fragile, chain-like structure, techniques for gently isolating extended sequences containing intact genes were needed. Such procedures, which include high-speed centrifugation and electrophoresis, were developed during the early 1960s, largely by Julius Marmur and colleagues.

(2) Fragmentation of DNA into Reassociable Segments–This crucial step is mediated by a class of bacterial enzymes, called restriction endonucleases, that introduce widely spaced breaks at specific sites in the DNA chain. The nature of the cuts is such that the separated ends (so-called "sticky ends") can readily reassociate with one another, thereby regenerating the original cleavage site. The rejoining can involve two DNA segments that each derive from different sources, so long as the DNA from each source was clipped into fragments by the same restriction endonuclease. Discovery of these enzymes and elucidation of their physiological role are largely credited to Werner Arber in Switzerland and to Dan Nathans and Hamilton Smith at Johns Hopkins.

(3) Sealing DNA Fragments Together–The rejoining of DNA fragments by way of their sticky ends requires a further step for the full stabilization of the recombined unit. Another enzyme, called polynucleotide ligase or simply ligase, performs this function. The ligase enzymes were discovered independently by a number of investigators, including Malcolm Gefter at MIT and Arthur Kornberg at Stanford.

(4) Replication and Maintenance of Recombinant DNA Molecules–Once DNA fragments have been cut-and-spliced together *in vitro*, a suitable host organism must be found into which the recombinant DNA can be stably incorporated and reproduced. The enteric bacterium, *Escherichia coli*, (or *E. coli*), was the obvious first choice as a host since more is known about the genetics and molecular biology of this microbe than of any other organism. The replication of a DNA segment by *E. coli* requires that the segment contain a specific short sequence of DNA that serves as a signal to the enzymatic machinery inside the cell. This signal, sometimes called the origin of replication, can be found on certain

small, self-replicating loops of DNA, called plasmids, that are commonly found inside bacterial cells. (Plasmids reproduce themselves independently of the major chromosome in bacteria and they are readily transferred between different bacterial strains. In addition to other functions, plasmids are responsible for the resistance to numerous antibiotics that has become a major medical problem in recent years.) Thus, incorporation *in vitro* of the recombinant DNA molecule into a bacterial plasmid, followed by reintroduction of the hybrid plasmid into the bacterial cell, will permit stable replication of the recombinant DNA.

Alternatively, if the recombinant DNA could be incorporated into the major chromosome of the host bacterium, then it would be replicated as part of the chromosome. This is possible through the use of a particular bacteriophage, called lambda, that infects *E. coli.* Upon infection, lambda DNA becomes incorporated into the bacterial chromosome where it replicates along with the host chromosome. Thus, attachment of the recombinant DNA molecule to lambda DNA prior to the infection of *E. coli.* will similarly allow replication of the recombinant DNA.

Both plasmids and lambda bacteriophage are termed vectors owing to this ability to transfer recombinant DNA into suitable hosts for replication. A number of scientists pioneered the effort to demonstrate the usefulness of vectors in gaining expression of exogenous or foreign DNA in *E. coli.,* including Stanley Cohen and Paul Berg at Stanford, and Herb Boyer at the University of California, San Francisco.

There exists a direct method of putting foreign DNA into host bacteria without the need for intact viruses or plasmids. Pure, naked DNA can be absorbed by bacterial cells in a process called transformation. This is the procedure used by Avery and co-workers in 1944 to "transform" nonvirulent pneumococcus strains into virulent bacteria. Some bacterial strains, including *E. coli,* must undergo a simple chemical pretreatment with calcium salts in order to make them amenable to DNA uptake.

(5) Selection of Cells Containing Recombinant DNA–Since only a small percentage of potential host bacteria do in fact acquire recombinant DNA by way of these procedures, it is necessary to perform a selection step. Depending on the type of vector used, it is possible to screen for antibiotic resistance (when the vector is a plasmid containing an antibiotic resistance gene) or to screen for the presence of viable bacteriophage viruses (when lambda is used as the vector). These selection methods give rise to clones of bacterial hosts containing recombinant DNA; that is, each bacterium in the clone is derived from a single progenitor cell that multiplied repeatedly, with exact copies of the cell's DNA having been distributed into each daughter cell. The segment of recombinant DNA contained therein is also replicated; that is, it has been cloned.

(6) Expression of Recombinant DNA into Gene Products (Proteins)– The recently acquired ability to incorporate exogenous DNA into bacteria, and to have that DNA replicated as though part of the bacterial genetic complement, is of considerable scientific interest. But commercial applications of this new technology demand that foreign genes implanted into bacteria be expressed into the proteins encoded by that DNA. For example, in order to convert *E. coli* into "factories" capable of producing human insulin, it is necessary both that the gene for insulin is stably maintained in the bacteria *and* that the human DNA segment is transcribed into messenger RNA, then translated into insulin (see Figures 1.1 and 4.1). As mentioned above, gene replication (maintenance) is assured by the presence of certain genetic signals. Similarly, the processes of transcription and translation rely on signals that inform the cell's enzymatic machinery where to start and where to terminate each of these processes. All of these various signals must be present at the appropriate locations in the DNA in order for gene expression by recombinant DNA methodology to be successful.

Once a bacterial cell has been "tricked" into manufacturing a human or other foreign protein, additional problems arise. The bacterium may recognize insulin as a "foreign" protein and may degrade it before it can be recovered. If stable, the foreign protein may simply accumulate inside the bacterial cell, necessitating its recovery by breaking open the cells–a tedious and inefficient process. Ideally, the foreign protein will be excreted out of the host cell into the growth medium from which it can be readily purified. Clever techniques are now available to bring this about, and improvements are being made continuously.

One additional roadblock bears mentioning. Many human proteins possess attachments that consist of sugar molecules. These glycoproteins are especially common in blood serum; e.g., interferon as a glycoprotein, although insulin is not. Bacteria do not possess the machinery to synthesize or attach sugars to proteins. Although the precise function of the sugars is unclear, it is probable that they serve a useful, perhaps crucial, role in maintaining the physiological activity of the protein. Thus, considerable effort is underway to develop microbial host organisms that *can* attach sugars to proteins. Common brewer's yeast, or *Saccharomyces cerevisiae,* is likely to be the preferred host cell for this purpose. Although it is a single-celled microbe, yeasts belong to the general class of higher organisms that include humans, namely eukaryotes. Eukaryotic organisms are classified on the basis of their having a nuclear membrane surrounding the genetic material within each cell. Bacteria and certain algae, on the other hand, compose the class of organisms called prokaryotes (i.e., those lacking a defined nuclear membrane). Although researchers in recombinant DNA have predominately utilized *E. coli* as the host organism, there is no doubt that the future com-