SIXTH EDITION

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Principles and Applications of Recombinant DNA

Bernard R. Glick and Cheryl L. Patten

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Bernard R. Glick
Department of Biology, University of Waterloo
Waterloo, Ontario, Canada

Cheryl L. Patten
Biology Department, University of New Brunswick
Fredericton, New Brunswick, Canada

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B. R. Glick
C. L. Patten

About the Companion Website

This book is accompanied by a companion website for instructors.

www.wiley.com/go/glick/molbiotech6

This website includes:

- Powerpoints of Figures and Tables from the book

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Preface to the Sixth Edition

When the first edition of *Molecular Biotechnology: Principles and Applications of Recombinant DNA* was published in 1994, nearly all of the transgenic organisms that were produced included only a single foreign gene or cDNA. Now, nearly 30 years later it is common for scientific researchers to genetically engineer organisms by modifying both the activity and regulation of one or more existing genes or by introducing entire new pathways. Genetic engineering is no longer limited to only a small number of common bacterial strains using recombinant DNA technology. Scientists now routinely modify many different microorganisms, animals, and plants using the techniques of PCR, chemical DNA synthesis, directed mutagenesis, and genome editing. This progress has been greatly facilitated by advances in DNA and RNA sequencing, monoclonal antibody production, genomics, proteomics, and metabolomics. Scientists worldwide can understand and purposefully manipulate the biological world as never before.

In 1994, only a very few products produced by recombinant DNA technology had been commercialized. Today, as a consequence of molecular biotechnology hundreds of new therapeutic agents, diagnostics tests, and vaccines are available in the marketplace with many more in the pipeline. At the time of this writing, RNA vaccines have been produced in record time and approved for the first time by regulatory authorities worldwide to control the devastating COVID-19 global pandemic, which has also spurred advances in molecular diagnostics. Scientists have also genetically modified hundreds of different plants to improve crop yields and traits, with dozens of these transgenic plants already commercialized, and many more in the works. DNA technologies have become a cornerstone of modern forensics, paternity testing, and ancestry determination. The list goes on and on. Molecular biotechnology has clearly lived up to its promise and all of the original hype that has existed since the late 1970s. Worldwide there are several thousand biotechnology companies and research institutes, operating in virtually every corner of the globe, employing hundreds of thousands of highly skilled scientists. In addition to all of the scientific studies with commercial potential that are being conducted, there is also an enormous amount of exciting and innovative

fundamental biological research, using the many techniques of molecular biotechnology, being done at universities and government labs around the world. Never before in recorded history has the world witnessed such a vast amount of discovery and fundamental change in the biological sciences, and there is every indication that there is still much more to come. This sixth edition of *Molecular Biotechnology* builds upon the fundamentals that were established in the previous five editions, and endeavors to provide readers with a window on many of the major developments in this ever growing and ever important field. Given the enormity of the field of molecular biotechnology, we have had to be highly selective in choosing the material that we have included in this edition. Moreover, the window that we are looking through is continually moving. With the many changes in this field that have occurred since the first edition of *Molecular Biotechnology*, we both expect and look forward to a considerable amount of additional change in the coming years, including the implementation and commercialization of many of the discoveries that are discussed here.

We have throughout endeavored to make the text as reader-friendly as possible by minimizing the use of technical jargon and unnecessary abbreviations. When an important term appears for the first time in the text, it is generally followed in parentheses with a synonym or brief explanation. The figures and tables are not just restatements of the data from the original literature; rather, we have made an effort to conceptualize the experimenters' thinking and rationale. We have endeavored to be as up-to-date as possible, expanding on some previous discussions and providing large numbers of practical examples.

Each chapter opens with an outline of topics and concludes with a summary and list of review questions to sharpen students' critical thinking skills. All of the key ideas in the book are illustrated by the more than 585 full-color figures and elaborated in nearly 100 tables. After introducing molecular biotechnology as a scientific and economic venture in Chapter 1, the next two chapters explain the detailed methodologies of molecular biotechnology. These chapters provide a solid scientific base for the remainder of the book. Chapters 4 to 8 present examples of applications for microbial molecular biotechnology covering such topics as diagnostic techniques, both protein and nucleic acid therapeutic agents, vaccines, bioremediation of pollutants, the production of metabolites, and biomass utilization by industry. Chapter 9 describes some of the key components of large-scale fermentation processes using recombinant microorganisms. Chapters 10 to 12 describe the molecular manipulation of plants and animals addressing both fundamental approaches and a wide range of applications, with a particular emphasis on agricultural improvements. The book concludes in Chapter 13 with a discussion of the interaction of molecular biotechnology with society including some discussion of controversies that have occurred as a consequence of this technology, coverage of the regulation of molecular biotechnology, and patents.

Throughout the text we have relied extensively upon the recent published work of many researchers. In all cases, although not cited directly in the body of a chapter, the original published articles are cited in the references section of the appropriate chapter. In some cases, we have taken "pedagogic license" and either extracted or reformulated data from the original publications. Clearly, we are responsible for any distortions or

misrepresentations from these simplifications, although we hope that none have occurred. The references section also contains other sources that we used in a general way, which might, if consulted, bring the readers closer to a particular subject.

Bernard R. Glick
Cheryl L. Patten

1 The Development of Molecular Biotechnology

Emergence of Molecular Biotechnology

Long before we knew that microorganisms existed or that genes were the units of inheritance, humans looked to the natural world to develop methods to increase food production, preserve food, and heal the sick. Our ancestors discovered that grains could be preserved through fermentation into beer, that storing horse saddles in a warm, damp corner of the stable resulted in the growth of a saddle mold that could heal infected saddle sores, that intentional exposure to a "contagion" could somehow provide protection from an infectious disease on subsequent exposures, and that plants and animals with enhanced production traits could be developed through crossbreeding. Following the discovery of the microscopic world in the 17th century, microorganisms have been employed in the development of numerous useful processes and products. Many of these are found in our households and backyards. Lactic acid bacteria are used to prepare yogurts and probiotics, insecticide-producing bacteria are sprayed on many of the plants from which the vegetables in our refrigerator are harvested, nitrogen-fixing bacteria are added in the soil used for cultivation of legumes, the enzymatic stain removers in laundry detergent come from a microorganism, and antibiotics that are derived from common soil microbes are used to treat infectious diseases. These are just a few examples of traditional biotechnologies that have improved our lives. Up to the early 1970s, however, biotechnology was not a well-recognized scientific discipline, and research in this area was centered in departments of chemical engineering and occasionally in specialized microbiology programs.

Molecular Biotechnology: Principles and Applications of Recombinant DNA, Sixth Edition. Bernard R. Glick and Cheryl L. Patten.

In a broad sense, biotechnology is concerned with the manipulation of organisms to develop and manufacture useful products. The term "biotechnology" was first used in 1917 by a Hungarian engineer, Karl Ereky, to describe an integrated process for the large-scale production of pigs by using sugar beets as the source of food. According to Ereky, biotechnology was "all lines of work by which products are produced from raw materials with the aid of living things." This fairly precise definition was more or less ignored. For a number of years, biotechnology was used to describe two very different engineering disciplines. On one hand, it referred to industrial fermentation. On the other, it was used for the study of efficiency in the workplace—what is now called ergonomics. This ambiguity ended in 1961 when the Swedish microbiologist Carl Göran Hedén recommended that the title of a scientific journal dedicated to publishing research in the fields of applied microbiology and industrial fermentation be changed from the *Journal of Microbiological and Biochemical Engineering and Technology* to *Biotechnology and Bioengineering*. From that time on, biotechnology has been defined as the application of scientific and engineering principles to the processing of material by biological agents to provide goods and services. It is grounded on expertise in microbiology, genetics, biochemistry, immunology, cell biology, and chemical engineering.

Large-scale production of commodities from natural organisms is often considerably less than optimal. Initial efforts to enhance yields of microbial products focused on creating variants (mutants) using chemical mutagens or radiation to induce changes in the genetic constitution of existing strains. The level of improvement that could be achieved in this way was usually limited biologically. If, for example, a bacterium was mutated to produce high levels of a compound, other metabolic functions often were impaired, thereby causing the bacterium's growth during large-scale fermentation to be less than desired. Despite this constraint, the traditional "induced mutagenesis and selection" strategies of strain improvement were extremely successful for a number of processes, such as the production of increased levels of antibiotics.

The traditional genetic improvement regimens were tedious, time-consuming, and costly because of the large numbers of microbial cells that had to be screened and tested. The best result that could be expected with this approach was the improvement of an existing inherited property of a microorganism rather than the expansion of its genetic capabilities. Despite these limitations, by the late 1970s, effective processes for the mass production of a wide range of commercial products from microorganisms had been perfected.

Today we have acquired sufficient knowledge of the biochemistry, genetics, and molecular biology of microbes and other organisms to significantly accelerate the development of useful and improved biological products and processes and to create new products that would not otherwise occur. Distinct from traditional biotechnology, the modern methods require knowledge of and manipulation of genes, the functional units of inheritance, and the discipline that is concerned with the manipulation of genes for the purpose of producing useful goods and services using living organisms is known as molecular biotechnology. The pivotal developments that enabled this technology were the establishment of techniques to isolate genes and to transfer them from one organism to another. The joining of DNA molecules from different sources was first demonstrated in 1971 by biochemist Paul Berg at Stanford University, who inserted genes from the bacterial

virus (bacteriophage) lambda into simian virus 40 DNA. This technology is known as recombinant DNA technology, and it was further developed by two scientists working in different fields who met at a scientific conference in 1972. In his laboratory at Stanford University in California, Stanley Cohen had been developing methods to transfer plasmids, small circular DNA molecules that replicate independently of chromosomal DNA, into bacterial cells. Meanwhile, Herbert Boyer at the University of California at San Francisco was working with enzymes that cut DNA at specific nucleotide sequences. Over lunch at a scientific meeting in Hawaii, they reasoned that Boyer's enzyme could be used to splice a specific segment of DNA into a plasmid and then the modified (recombinant) plasmid could be introduced into a host bacterium using Cohen's method.

Recombinant DNA Technology

It was clear to Cohen and Boyer, and others, that recombinant DNA technology had far-reaching possibilities. As Cohen noted at the time, "It may be possible to introduce in *E. coli*, genes specifying metabolic or synthetic functions such as photosynthesis or antibiotic production indigenous to other biological classes." The first commercial product produced using recombinant DNA technology was human insulin, which is used in the treatment of diabetes. The DNA sequence that encodes human insulin was synthesized, a remarkable feat in itself at the time, and was inserted into a plasmid that could be maintained in a nonpathogenic strain of the bacterium *Escherichia coli*. The bacterial host cells acted as biological factories for the production of the two peptide chains of human insulin that could be purified and combined and used to treat diabetics who were allergic to the commercially available porcine (pig) insulin. Today, this type of genetic engineering is commonplace.

The nature of biotechnology was changed forever by the development of recombinant DNA technology. Genetic engineering provided the means to create, rather than merely isolate, highly productive microbes and other organisms. Not long after the production of the first commercial preparation of recombinant human insulin in 1982, bacteria and then eukaryotic cells were used for the production of other therapeutic proteins, such as interferon, growth hormone, and viral antigens. Recombinant DNA technology also facilitated the biological production of large amounts of useful low-molecular-weight compounds and macromolecules that occur naturally in minuscule quantities. Plants and animals became natural bioreactors for producing new or altered gene products that could never have been created either by mutagenesis and selection or by crossbreeding. From its modest beginnings, around 50 years ago, molecular biotechnology has become the standard method for developing living systems with novel functions and capabilities for the synthesis of thousands of important commercial products.

Most new scientific disciplines do not arise solely on their own. They are often formed by the synthesis of knowledge from different areas of research. For molecular biotechnology, the biotechnology component was perfected by industrial microbiologists and chemical engineers, whereas the recombinant DNA technology portion owes much to discoveries in molecular biology, bacterial genetics, and nucleic acid enzymology (Table 1.1). In a broad sense, molecular biotechnology draws on knowledge from a diverse set of fundamental scientific disciplines to create products that are useful in a wide range of applications (Fig. 1.1).

Table 1.1 Selected developments in the history of molecular biotechnology[a]

Date	Event
1917	Hungarian agricultural engineer Karl Ereky coins the term "biotechnology"
1940	Danish microbiologist A. Jost coins the term "genetic engineering" in a lecture on sexual reproduction in yeast
1943	Penicillin is produced on an industrial scale
1944	Avery, MacLeod, and McCarty demonstrate that DNA is the genetic material
1953	Watson and Crick determine the structure of DNA
1961–1966	Entire genetic code deciphered
1970	First restriction endonuclease isolated
1972	Khorana and coworkers synthesize an entire tRNA gene
1973	Boyer and Cohen establish recombinant DNA technology
1975	Kohler and Milstein describe the production of monoclonal antibodies
1976	First guidelines for the conduct of recombinant DNA research are issued
1976	Techniques are developed to determine the sequence of DNA
1978	Genentech produces human insulin in *E. coli*
1980	U.S. Supreme Court rules in the case of Diamond vs Chakrabarty that genetically manipulated microorganisms can be patented
1981	First commercial, automated DNA synthesizers are sold
1981	First monoclonal antibody-based diagnostic kit is approved for use in the United States
1982	First animal vaccine produced by recombinant DNA methodologies is approved for use in Europe
1983	Engineered Ti plasmids are used to transform plants
1988	U.S. patent is granted for a genetically engineered mouse susceptible to cancer
1988	PCR method is published
1990	Approval is granted in the United States for a trial of human somatic cell gene therapy
1990	Recombinant chymosin is used for cheese making in the United States
1994–1995	Detailed genetic and physical maps of human chromosomes are published
1994	FDA announces that genetically engineered tomatoes are as safe as conventionally bred tomatoes
1995	First genome of a cellular organism, the bacterium *Haemophilus influenzae*, is sequenced
1996	Recombinant protein erythropoietin exceeds $1 billion in annual sales
1996	Complete DNA sequence of all the chromosomes of a eukaryotic organism, the yeast *Saccharomyces cerevisiae*, is determined
1996	Commercial planting of genetically modified crops
1997	A sheep is cloned by somatic cell nuclear transfer
1998	FDA approves first antisense drug
1999	FDA approves recombinant fusion protein (diphtheria toxin–interleukin-2) to treat cutaneous T-cell lymphoma
2000	*Arabidopsis* genome is sequenced
2000	Monoclonal antibodies exceed $2 billion in annual sales
2000	Development of "Golden Rice" (provitamin-A-producing rice) is announced
2001	Human genome sequence is published
2002	Complete human gene microarrays are commercially available
2002	FDA approves first nucleic acid test to screen whole blood from donors for HIV and HCV
2004	Large-scale sequencing of the Sargasso Sea metagenome
2005	NCBI announces 100 gigabases of nucleotides in GenBank sequence database
2006	Recombinant cancer vaccine (Gardasil) is available to protect against cervical cancer
2009	FDA approves first drug produced in a genetically engineered animal (goat)

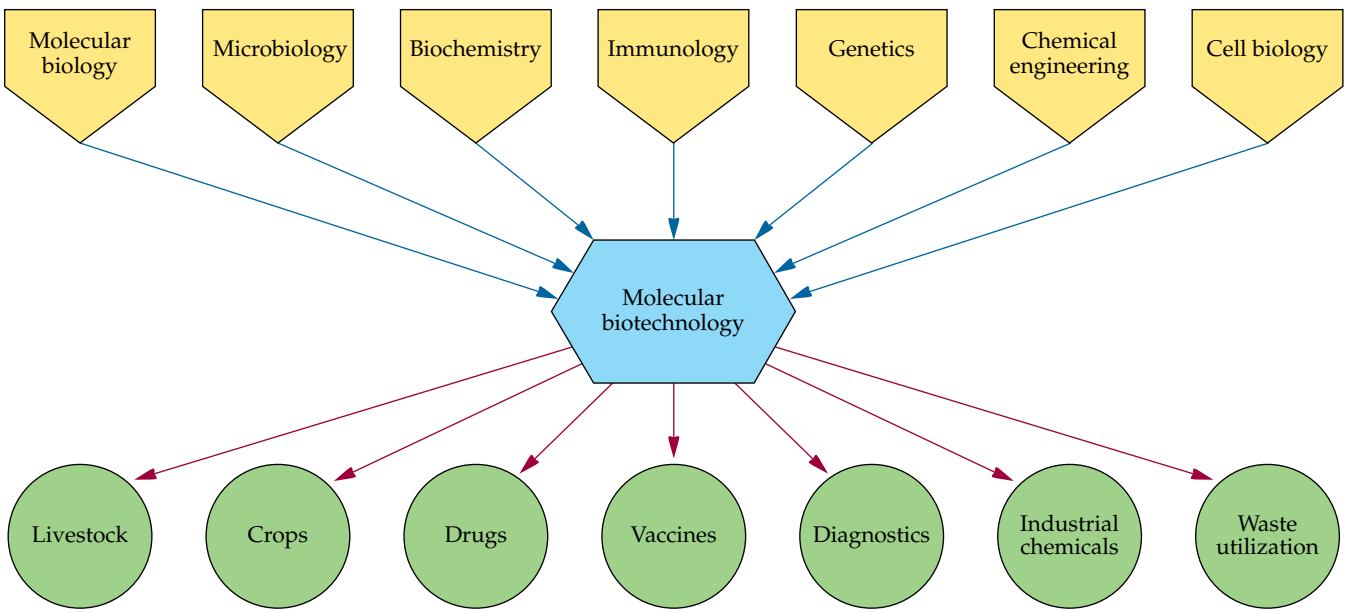
(continued)

Table 1.1 *(continued)*

Date	Event
2009	First clinical trial using embryonic stem cells
2010	Researchers create the first synthetic cell
2012	Doudna and Charpentier demonstrate targeted cleavage of DNA by CRISPR-Cas system
2013	U.S. Supreme Court rules that isolated genes are not eligible for patenting
2014	Patent granted for CRISPR-Cas systems and methods for altering expression of gene products
2015	FDA approves first transgenic animal (salmon) for human consumption
2016	National Bioengineered Food Disclosure Law is passed by the U.S. Congress requiring labeling of genetically engineered food
2017	FDA approves *in vivo* gene therapy for treatment of an inherited form of retinal dystrophy
2018	First RNA interference therapy (to treat hereditary transthyretin amyloidosis) approved in the United States and Europe
2018	Birth of genome-edited babies in China triggers widespread criticism
2019	Genetically engineered crops are grown in 29 countries on 190 million hectares
2019	Clinical trials begin to test the safety and efficacy of CRISPR-Cas genome editing to treat two blood disorders, β-thalassemia and sickle cell anemia
2019	Philippines is first Asian country to receive safety approval for genetically modified Golden Rice
2020	Results from the first CRISPR clinical trial indicate that the technique is safe
2020	First report of genome-edited mitochondria
2020	COVID-19 pandemic impels development of innovative molecular diagnostic tests, treatments, and vaccines
2021	Trial release of genetically modified mosquitoes in the United States

[a]FDA, Food and Drug Administration; HCV, hepatitis C virus; HIV, human immunodeficiency virus; NCBI, National Center for Biotechnology Information; NIH, National Institutes of Health; PCR, polymerase chain reaction; tRNA, transfer ribonucleic acid; CRISPR, clustered regularly interspaced short palindromic repeats.

Figure 1.1 Many scientific disciplines contribute to molecular biotechnology, which generates a wide range of commercial products.



Construction of Biologically Functional Bacterial Plasmids *In Vitro*

S. N. Cohen, A. C. Y. Chang, H. W. Boyer, and R. B. Helling
Proc. Natl. Acad. Sci. USA **70:**3240–3244, 1973

milestone

The landmark study of Cohen et al. established the scientific foundation for recombinant DNA technology by showing how genetic information from different sources could be joined to create a novel, replicable genetic structure. In this case, the new genetic entities were derived from bacterial autonomously replicating extrachromosomal DNA structures called plasmids. In a previous study, Cohen and Chang (*Proc. Natl. Acad. Sci. USA* **70:**1293–1297, 1973) produced a small plasmid from a large naturally occurring plasmid by shearing the larger plasmid into smaller random pieces and introducing the mixture of pieces into a host cell, the bacterium *E. coli*. One of the fragments that was about 1/10 the size of the original plasmid was perpetuated as a functional plasmid. To overcome the randomness of this approach and to make the genetic manipulation of plasmids more manageable, Cohen and his coworkers used an enzyme (restriction endonuclease) that cuts a DNA molecule at a specific nucleotide sequence and produces a short single-stranded extension at each end. The extensions of the cut ends of a restriction endonuclease-treated DNA molecule can combine (due to complementarity of the nitrogenous bases) with the extensions of another DNA molecule that has been cleaved with the same restriction endonuclease. Consequently, when DNA molecules from different sources are treated with the same restriction endonuclease and mixed together, new DNA combinations (recombinant DNA) that never existed before can be formed. In this way, Cohen et al. not only introduced a gene from one plasmid into another plasmid but also demonstrated that the introduced gene was biologically active. To their credit, these authors fully appreciated that their strategy was "potentially useful for insertion of specific sequences from prokaryotic or eukaryotic chromosomes or extrachromosomal DNA into independently replicating bacterial plasmids." In other words, any gene from any organism could theoretically be cloned into a plasmid which, after introduction into a host cell, would be maintained indefinitely and, perhaps, produce the protein encoded by the cloned gene. By demonstrating the feasibility of gene cloning, Cohen et al. provided the experimental basis for recombinant DNA technology and established that plasmids could act as vehicles (vectors) for maintaining cloned genes. This motivated others to pursue research in this area that rapidly led to the development of more sophisticated vectors and gene cloning strategies. It also engendered concerns about the safety and ethics of this kind of research that, in turn, were responsible for the establishment of official guidelines and governmental agencies for conducting and regulating recombinant DNA research, respectively, and contributed to the formation of the molecular biotechnology industry.

The Cohen and Boyer strategy for gene cloning was an experiment "heard round the world" (see Milestone). Once their concept was made public, many other researchers immediately appreciated the power of its potential. Consequently, scientists created a large variety of experimental protocols that made identifying, isolating, characterizing, and utilizing genes more efficient and relatively easy. These technological developments have had an enormous impact on generating new knowledge in practically all biological disciplines. Indeed, the emergence of the field of genomics was dependent on the ability to clone large fragments of DNA into plasmids in preparation for sequence determination.

Commercialization of Molecular Biotechnology

The potential of recombinant DNA technology reached the public with a frenzy of excitement, and many people became rich on its promise. Indeed, within 20 minutes of the start of trading on the New York Stock Exchange on 14 October 1980, the price of shares in Genentech, the company founded

by Boyer with chemist and entrepreneur Robert Swanson that produced recombinant human insulin, went from $35 to $89. This was the fastest increase in the value of any initial public offering in the history of the market up to that time. It was predicted that some genetically engineered microorganisms would replace chemical fertilizers and others would eat up oil spills; plants with inherited resistance to a variety of pests and exceptional nutritional content would be created; and livestock would have faster growing times, more efficient feed utilization, and meat with low fat content. Many were convinced that as long as a biological characteristic was genetically determined by one or a few genes, organisms with novel genetic constitutions could be readily created. Today, in many cases, the promise of recombinant DNA technology has become a reality.

Since the commercial production of recombinant human insulin, hundreds of drugs produced by recombinant DNA technology have been developed to treat diseases such as cancer, multiple sclerosis, rheumatoid arthritis, cystic fibrosis, and strokes, and to provide protection against numerous infectious diseases. The majority of these are therapeutic monoclonal antibodies, hormones, and growth factors, many of which are more effective and have fewer side effects than other therapies. Very recently, a small number of nucleic acid therapies have been approved to target diseases caused by specific mutations, including gene therapies to replace defective genes that cause blindness and spinal muscular atrophy, and RNA therapies to treat an inherited form of amyloidosis and Duchenne muscular dystrophy. Hundreds more new biological drugs and therapies are in the process of being tested in human clinical trials, most to treat various cancers and also genetic, neurological, autoimmune, and infectious diseases. Beyond medical applications, many molecular biotechnology products are available to enhance crop and livestock yields, decrease pesticide use, and improve industrial processes such as the manufacture of pulp and paper, food, energy, and textiles.

The impact on agriculture has been tremendous. While the global population is expanding rapidly, yield increases of all major crops have decreased due to poor agricultural management practices, decreased acreage of arable land, and increased reliance on fertilizers and pesticides that diminish soil quality. To produce more food on less land, in 2019, 17 million farmers in 29 countries planted genetically engineered crops on 190 million hectares of land. These crops are predominantly soybeans, corn, cotton, and canola that are resistant to herbicides and insects, although many others such as drought-resistant sugarcane and nonbrowning potatoes and apples are produced. The global market value of genetically modified crops is currently around $20 billion. Small resource-poor farmers benefit substantially from agricultural biotechnology. In a comparative study of small cotton farms in South Africa, it was found, over three seasons, that the yield of cotton from plants that were genetically engineered to produce a bacterial insecticide was on average about 70% greater than those from non-genetically modified plants. Higher yields and reduced pesticide and labor costs translated into doubled revenues despite the slightly higher costs of the transgenic seeds. In a recent 2-year study in Bangladesh, the net returns per hectare for insect-resistant eggplant were six times higher than that for conventional eggplant.

The ultimate objective of all biotechnology research is the development of commercial products. Consequently, molecular biotechnology is driven

to a great extent by the prospect of financial gain. By nightfall on 14 October 1980, the principal shareholders of Genentech stock were worth millions of dollars. The unprecedented enthusiastic public response to Genentech encouraged others to follow. Between 1980 and 1983, about 200 small biotechnology companies were founded in the United States with the help of tax incentives and funding from both stock market speculation and private investment. Like Herbert Boyer, who was first a research scientist at the University of California at San Francisco and then a vice president of Genentech, university professors started many of the early companies.

Today, there are about 8,000 biotechnology companies worldwide, most in the United States and Europe, with annual earnings in the hundreds of billions of dollars. The biotechnology industry in these regions employs more than 200,000 people. Large multinational chemical and pharmaceutical companies, such as Bayer, DuPont, Pfizer, GlaxoSmithKline, Merck, Novartis, Hoffmann-LaRoche, Gilead Sciences, and Amgen, to name but a few, have made significant research commitments to molecular biotechnology. The roster of biotechnology companies is extensive and includes those focused on vaccines, protein and nucleic acid therapeutics, drug delivery, molecular diagnostics, genomics, industrial processing, and agricultural biotechnology.

Concerns and Consequences

While many people appreciate the potential of molecular biotechnology to solve important problems in agriculture, medicine, and industry, they recognize the need to be cautious about its widespread application. Indeed, one of the first scientific responses to recombinant DNA technology was a voluntary moratorium on certain experiments that were thought to be potentially hazardous. This research ban was self-imposed by a group of molecular biologists, including Cohen and Boyer. They were concerned that combining genes from two different organisms might unintentionally create a novel organism with undesirable or even dangerous properties. Within a few years, however, these apprehensions were allayed as scientists gained laboratory experience with the technology and safety guidelines were formulated for recombinant DNA research. The temporary cessation of some recombinant DNA research projects did not dampen the enthusiasm for genetic engineering. In fact, the new technology continued to receive unprecedented attention from both the public and the scientific community.

Molecular biotechnology can benefit humanity by

- Providing opportunities to accurately diagnose, prevent, treat, or cure a wide range of infectious and genetic diseases
- Increasing crop yields by creating plants that are resistant to insect predation, fungal and viral diseases, and environmental stresses such as short-term drought and excessive heat and at the same time reducing applications of hazardous agrichemicals
- Creating microorganisms that will produce metabolites (products of metabolism), polymers, amino acids, enzymes, and additives that are important for food production and other industries