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CHAPTER 3

Molecular Biology Is Finally Being Exploited—Let Us Count Some Ways

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Molecular biology is now being utilized in industry. DNA sequencing, recombinant DNA, cell fusion, and other techniques have made this possible. Applications in the medical-pharmaceutical field are discovery of new antibiotics and yield improvement, production of mammalian proteins by microorganisms, and monoclonal antibodies. There is a large potential for production of proteins such as human insulin, and interferon(s) may represent the most provocative medical opportunity for the new biology. In nonmedical fields, microbial competition for big chemical markets such as substances derived from ethylene—ethanol, glycols, epoxides—should result from molecular biology advances. Reverse genetics to produce desired phenotypes, and "biophore" industrial microorganisms "tailored" to specific objectives are exciting conceptional approaches; examples are discussed.

INTRODUCTION

Molecular biology, belatedly perhaps, is now being energetically practiced in industry, and a variety of tangible, commercial applications are being pursued. It has been said: "Anything a chemist can do a living organism can do better." And now, with recombinant DNA, cell fusion, monoclonal antibodies, and more, biology can in fact accomplish things chemistry could not presume to address.

It is fascinating to reflect on how life processes have been shown to employ the cleverest aspects of engineering design seen in computers and other nonbiological developments. Nature, it turns out, uses blueprints, working drawings, switches, molecules to operate the switches, feedback, and codes. These are not metaphors but physical realities. It is satisfying to recall the predictions of the triplet nature of codons, of the existence of adapter (transfer) RNA and of repressors, and of the race to find them (Gilbert and Müller-Hill 1970; Ptashne 1971). Yet, about 10 yr ago, Stent (1969) proclaimed that the excitement had largely gone from molecular biology—all that was left was a mopping up operation on which a respectable scientist should carefully consider whether or not to squander his only career.

Five years ago, Pontecorvo (1976) scolded a meeting of industrial microbial geneticists on their failure to exploit meaningfully the treasure trove of insights available to them. Although there were some admirable examples of "elegant" experimental design in industry (Lago et al. 1972; Ball and Azevedo 1976), by and large industry viewed molecular biology as esoteric and useless; or perhaps industrialists believed Stent.

Then why has there been a virtual explosion of activity in the past few years? More than for any other reason it is because new tools have been developed. When Stent

made his pessimistic pronouncement, the most astonishing techniques now facilitating breakthrough after breakthrough were neither available nor foreseen. The practice of genetics in industry was a pragmatic and laborious "mutate and hunt" affair, where one looked for new therapeutic moieties and higher yields of ones already discovered. "Genetics with the eyes closed," it might be called. Now consider the techniques available today: DNA sequencing (Maxam and Gilbert 1977; Sanger et al. 1977) so precise that, combined with knowledge of the genetic code, nucleotide sequence information has been used to *correct* older amino acid sequence information in proteins (Martial et al.1979); hybridization (Southern 1975) and electron microscopy (Tilghman et al. 1978) are techniques which similarly elucidate both structure and function at the level of the individual codon; even a new generation of chromatographic elegance (O'Farrell 1975) which provides unbelievable detail in inspecting the spectrum of proteins in a cell; and last, merely because they're becoming household words, the techniques mentioned earlier, i.e., recombinant DNA, cell fusion, and monoclonal antibodies. It all adds up to "genetics with the eyes open." It provides scientists in industry with a far more plausible story to tell their managements in asking for support in this area. Perhaps the most effective word to be used in selling these new technologies to managements is "speed." As Calam et al. (1976) stressed in defending the behavior of industrial microbiologists against Pontecorvo's charges, executives are under great time pressures and often cannot wait for several years for positive feedback from research decisions. This creates an atmosphere antithetical to fundamental, theoretical research. Today's techniques, mostly developments of the last 5 yr, not only amplify our ability to do exciting things, they also speed it up.

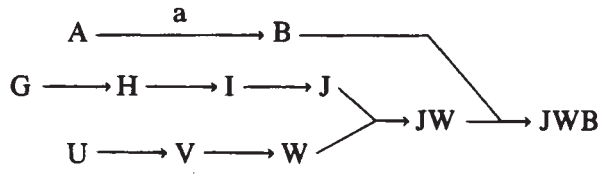
Discussion

It is now of interest to describe some of the areas in which molecular biology currently is being applied commercially.

Medical Applications

Antibiotic production. In the antibiotic field there are two important problems which can now be addressed in new ways. One is the discovery of new antibiotics, the other is yield improvement. First, consider the discovery of new antibiotics. Traditional new antibiotic searches using blind empiricism are running out of steam. Hopwood et al. (1977) and Baltz (1978) have demonstrated successful protoplast fusion in streptomycetes. Others (Godfrey et al. 1978) pursue this technique within a species as well as extending it to interspecies fusions to combine biosynthetic pathways.

Ten years ago what is now proposed would have been considered ridiculous. A couple of background observations render these new approaches more plausible. A number of authors (Shier et al. 1969; Nagakoa and Demain 1975) have demonstrated a phenomenon variously described as mutasynthesis or mutational biosynthesis. The idea is to block mutationally a biosynthetic pathway which contributes to the ultimate synthesis of the antibiotic and to feed the fermentation with an analog of the precursor whose synthesis the mutational block prevents. For example, if the antibiotic JWB is made as follows:



and the synthesis of B is blocked by deleting or otherwise removing the activity of the gene for coding enzyme a, then the new compound JWC can be made by feeding C. The hybrimycins and mutamycins were made with this strategy. It shows that some flexibility in utilization of intermediates exists.

The other background observation concerns expectations regarding the kind of molecules likely to be synthesized by particular organisms. Ten years ago, one might have generalized that β-lactams were to be sought principally, if not exclusively, in fungi. Now we know of cephamycins and thienamycin from streptomycetes.

The proposal to use the new technique of cell fusion to cram together in one cell certain selected biosynthetic pathways never before known to exist together in the hopes of thereby facilitating the synthesis of entirely new antibiotics is not at all far fetched. In fact, Fleck (1979) claims to have done just this, creating a variant of the macrolide violamycin by fusing a mutant of its producer with a mutant of a turimycin producer. He calls the new compound iremycin (*i*nterspecific *re*combinant).

The other important problem in the antibiotic industry is yield improvement. We are all familiar with the blind mutation, cloning, and hopefully intelligent selection of mutant phenotypes which have, used together, characterized this work. There has been very little knowledge or, for that matter, concern about the genotypes responsible for strain improvement. Now, again using fusion of chosen strains, we see reports of significant yield improvement. Hamlyn and Ball (1979) fused fungal protoplasts to get a recombinant with 40% better cephalosporin C yield than the higher yielding parent.

There are many other avenues to higher yields. No longer are we constrained to consider commercial production only in the strain, species, or even genus in which a compound is discovered. Techniques of plasmid manipulation resulting in massive increases in gene dosage make it possible to so marshal the cell's resources that fully 70% of its protein synthesizing productivity can be devoted to a particular protein of choice (O'Farrell et al. 1978).

Protein production. The next major application is also from the pharmaceutical industry. It is the manufacture, in bacteria, of mammalian and, most interestingly, of human proteins. In contrast to the antibiotic problems where perhaps one could envisage alternative solutions, one cannot imagine any practical route to human growth hormone or to interferon save through various techniques of molecular biology. So much that is wonderful has been accomplished in this field in the past 3 yr—and the newspaper announcements have sometimes caught the excitement but confused the details—that it is worthwhile to recapitulate the sequence of announcements.

First a word about jargon. When we talk about cloning, we are usually referring to the splicing of a DNA sequence (usually a gene or more) from a heterologous source (from another strain in the simplest case or another totally unrelated genus in the

extreme case) into a host cell's DNA, usually on a plasmid or a temperate virus, where the foreign DNA replicates whenever the host DNA replicates. Thus the splice is perpetuated in the progeny. When we talk about expression, we mean that the foreign DNA has caused the formation of protein for which its codon sequence codes the amino acid sequence. But expression does not necessarily insure biological function. For example, someone with great dexterity in genetic engineering might succeed in splicing the DNA for nitrogen fixation, the nif genes from *Rhizobium* or *Klebsiella,* into the DNA of a corn cell. Perhaps one might even succeed in getting expression, the actual formation in the corn cell of the complex protein nitrogenase. But considering the complexities of nitrogen fixation (Brill 1979), it will be appreciated that even this is far from accomplishing the fixation of atmospheric nitrogen by a corn plant. Therefore beyond expression is function.

With that background, here is a sequence of recent announcements:

May 1976.	First breaking of the "barrier," or better, demonstration of the absence of a barrier to the expression of a eukaryotic (yeast) gene in a prokaryote (bacterium) (Struhl et al. 1976).
1976.	Cloning of a mammalian gene (rabbit globin) in bacteria (Maniatis et al. 1976; Rabbits 1976; Higuchi et al. 1976).
March 1977.	Expression in bacteria of rat growth hormone (Seeburg et al. 1977).
May 1977.	Cloning of gene for rat insulin. Expression moot (Ullrich et al. 1977).
November 1977.	Synthesis and cloning of a gene to code for human somatostatin and expression of immunologically active material (Itakura et al. 1977). Interestingly, the synthetic gene may not correspond exactly to the sequence of nucleotides used as codons in the human genome; the degeneracy of the genetic code permits the chemist to make choices among alternative nucleotide sequences which code for the same amino acids. For several important technical reasons, this protein was expressed as a fusion product with a bacterial protein, using a technique developed earlier called "read-through expression" (Polisky et al. 1976).
December 1977.	Cloning of a natural human gene for chorionic somatomammotropin (placental lactogen) (Shine et al. 1977).
August 1978.	Cloning of rat insulin and its read-through expression as a fusion product with a bacterial protein (β-lactamase) intended for "export." The fused protein ended up in the periplasmic space—not yet secretion, a necessity for immobilized cell continuous fermentation, but a step in the right direction (Villa-Komaroff et al. 1978).

September 1978.	Synthesis and cloning of genes coding for human A and B insulin chains and expression of those chains, which on mixing produce immunologically active material (Crea et al. 1978).
October 1978.	First demonstration of *function* (as opposed to mere expression) of a mammalian gene in *E. coli*—in this case, the gene coding for mouse dihydrofolate reductase (Chang et al. 1978).
December 1978.	Expression and delivery to periplasmic space of chicken ovalbumin (Fraser and Bruce 1978).
May 1979.	Two separate groups cloned and expressed virus antigens (for influenza and hepatitis) in programs intended to lead to vaccines based on such antigens alone, thus obviating the need in vaccines for any viruses, dead or alive (*New York Times* May 23, 1979; Burrell et al. 1979).
July 1979.	Almost simultaneously, two separate groups announced the expression of human growth hormone in *E. coli* (Martial et al. 1979; Goeddel and Seeburg 1979).

An interesting comparison: Gilbert's group originally reported production of rat insulin at the level of about 100 molecules per cell. Both groups involved in the human growth hormone announcements indicate production of more than 200,000 molecules per cell.

The preceding chronology is intended to be merely illustrative of the pace of progress. As the announcements blend into each other, like the news reports of successive moon landings, the actual accomplishments tend to merge in the memories of all but the most involved workers. But the take home lesson is clear: the level of activity is very high; the accomplishments are very exciting. We can expect the stream of announcements and "breakthroughs" to continue as, for the first time, more human proteins become available in quantity. Human insulin will undoubtedly become available commercially and the new product will certainly represent some improvement over insulin derived from the cow or the pig. But whether it will command a premium price, whether it will expand the market, or merely partially replace existing insulin sources remains to be seen. In any event, the total annual world market for insulin, while considerable, is not enormous, being estimated at $200-300 million. Of interest here is the recent agreement by Eli Lilly to license, free of royalties, any technology (including genetic engineering technology) relating to insulin. Thus, insulin is not a huge, profitable market, nor will it likely remain captive to a few suppliers.

Interferon(s) may represent the most commercially provocative medical opportunity for the new biology. Highly promising against viruses and cancer, the price of human interferon has been quoted at $22 *billion* per pound. This indicates that virtually none is available. Clearly, there is no way to provide large quantities other than by genetic engineering. There are several strategies available to clone and express functional interferon and we, along with others, are pursuing these various courses. They include the use of polynucleotide "probes" to obtain messenger RNA for interferon, the production of complementary DNA, the development of cell lines with high levels of messenger RNA for interferon, the development of procedures for enriching for interferon messenger, the improvement of assays to detect appropriate clones, developing

ways to use known control elements to enhance expression, and so forth. An intriguing extension of the example mentioned earlier where nucleotide sequence was used to corroborate or correct previously reported amino acid sequences is the use we and others are making of amino acid sequences to guide our synthesis of polynucleotide probes.

Before leaving examples of industrial opportunities in the health field, worth mentioning is the use of hybridomas to make monoclonal antibodies. This remarkable capability has diagnostic, research, and therapeutic possibilities rivaling those attributable to recombinant DNA and many industrial research groups have this new capability. New markets, only dimly perceived now, will materialize; new companies have already been formed; and products, impossible to imagine just a few years ago, are being promoted.

Non-Medical Applications

Despite the overwhelming stress in the publicity surrounding the so-called medical applications of genetic engineering, I am convinced that comparably exciting and profitable opportunities exist in other fields. Reflecting this, our company has extensive nonpharmaceutical programs; well over half its resources are devoted to nonmedical goals. The most romantic medical opportunities seem to *require* genetic engineering. But while they are romantic, there are always questions like, "Will there *really* be a multi-billion dollar market for interferon?" Much less romantic are opportunities in the energy, chemical, food, minerals, and other industries. In these fields, biology enters as an *alternative* way to an existing objective, an alternative way to supply products for huge, existing markets. Here the question is not "does the market exist?", but rather, "can biology compete?" A few familiar and unfamiliar examples are cited below.

First the familiar—fermentation of ethanol is as old as history (at least). To a purist, the term fermentation should, in fact, be restricted largely to alcohol production, a nuance long since ignored. The technology of biologically produced alcohol for nonbeverage uses lost out to petroleum (ethylene)-based organic chemistry in the cheap oil economy we used to enjoy. Even today with the gasohol proposal, little, if any, "new biology" is reflected in the present fermentation "state of the art." Our company is well into a major effort to improve the technology for ethanol production, and clearly the impetus to do so is largely a function of the skyrocketing price of ethylene!

We also have developed a biological way to make glycols and epoxides from alkenes. Here again, competing chemical technology is well entrenched, but the traditional chemical methods may be vulnerable. In general, biological processes are less energy-demanding and less polluting than chemical processes. For these reasons, and also because enzymatic conversions permit the use of less expensive substrate streams, biology may come in at lower cost in many big chemical markets. Our process targets products such as ethylene and propylene oxides and glycols, with a present combined annual U.S. market in the $2.5 billion range.

Traditionalists in industry have two principal concerns about using fermentation, directly or indirectly (i.e., using enzymes produced by fermentation), for large volume, low-to-moderate priced products. First, intuitively they doubt that biological processes can proceed quickly enough to keep capital costs within reason. Our data indicate that this should never be prejudged; each case should be studied for its own specifics.

Tentatively, we are competitive. The second objection to using biology in large-scale production situations arises from the obvious unsuitability of batch processes to large volumes. When making a product worth $22 billion per pound, maybe a bathtub with a propeller in it will do. Ethanol, however, should cost about one twenty-two billionth of that, so we have to look to engineering, both conventional and genetic. Moreover, chemical and genetic engineers must hold hands, as it were, from early in the undertaking. Dramatic new engineering developments include massive continuous airlift fermenters, such as the one recently installed by ICI at Teesside in England. Also, the need for continuous processes (such as our ethanol process) speak to the need for cell recycle and/or the use of enzymes or immobilized cells. This latter is still a developing art.

Conceptual Approaches

Reverse genetics. What do we mean by reverse genetics? Let me contrast it with the conventional genetics of the past, herewith by implication, dubbed "forward genetics." In the past, phenotypes have been observed first and genotypes elucidated subsequently. First one produced mutants and then tried to figure out why they were mutants: how did the genetic lesions map; were they point mutations, deletions, or translocations; could they be complemented; and most recently and elegantly, what were the base sequences that characterize the locus and all its various alleles. At the same time, again at the molecular level, what were the pathways; what were the intermediates; what were the changes in the proteins caused by the mutations; and how, in the best cases, did all these phenomena add up to what we call phenotype?

Reverse genetics is the use of all we know about genetics and biochemistry to *predict* a desired phenotype and to try to make certain changes in DNA to cause the desired phenotype to come about. This description is a pretty good answer to the question, "What is genetic engineering?" Some background may help here. Weissmann et al. (1979), studying the β-globin gene of the rabbit, cloned the gene and its neighboring regions, determined the appropriate nucleotide sequences, made specific changes ("site-directed mutagenesis") in these sequences, and then studied expression: what changes are seen in the nature of the protein and its control (i.e., the phenotype, at the molecular level)? Extending this approach, using different methods, we can clone nonhuman proinsulin DNA and change it to pseudo-human proinsulin DNA (i.e., sequences that, like the somatostatin example described above, may not mirror the base sequence in human DNA, but which will nonetheless code precisely for the amino acid sequence of human proinsulin). Also, in a nonmedical application, we can "import" a foreign gene with the prediction that it will lower the ATP requirement for a particular individual process, and thus lower the cost of the product. These are not daydreams; they are now the subject of industrial projects.

Biophores. The "biophore," is a term coined by Joshua Lederberg to describe the ideal industrial microorganism into which one will "stitch" new genetic material. Actually, we envision a collection of such organisms, including cell cultures from animal and plant sources as well. Each biophore would be "tailored" to specific objectives, depending on the nature of the product and the process, but safety, economics, and genetic considerations would dictate what was done in each instance. Two issues (there

are many more) will illustrate the intellectual excitement and give you an idea of the challenges that go hand-in-hand with the opportunities. To me, it is particularly thrilling that these industrial considerations deal with scientific issues at the very frontier of the underlying molecular biology. For once, the scientific content of the industrial problem is at the same level as that demanding the obsessive attention of professors and students around the world.

The first challenge concerns an issue referred to above: industry prefers continuous rather than batch processes. Indeed, it goes beyond "preference"; in many cases, large-scale, continuous processes are the only practical way to go in producing moderate price products. This immediately brings to mind a conceptual picture of the reactor as a column or tube with substrate being continuously fed in one end and product stream coming out the other. Two biologically based ingredients can be imagined in the tube: immobilized enzymes or whole cells. We will focus on the latter. If the process is to be efficient, we look for active "delivery" of the product out of the cells and into the effluent stream. We know quite a bit now about how cells give directions to the proteins they synthesize; there are many examples of "address signals" incorporated into leader sequences (e.g., preproinsulin—Chan et al. 1976). There is much more to be learned. An alternative, which in one project has already served well, involves the apparent delivery to the outside of the cell, but still attached to it, of a necessary enzyme. In this case, the immobilized cell acts as an immobilized enzyme, and we are spared separating the enzyme from the cell in a separate, expensive step.

The second challenge concerns the fact that *E. coli* is not omnipotent. There are many things bacteria do not ordinarily do. Some things they can be taught to do—this is the charm of recombinant DNA. The so-called "barrier" between eukaryotic and prokaryotic DNA has been shown to be largely a figment of pessimistic imagination. It is the absence of any real barrier and the universality of the genetic code that in fact permits genetic engineering to direct *E. coli* to make insulin.

We must be careful in this moment of triumph. We already know that control elements (e.g., ribosome binding sites or promoters) may differ between species. One of the most intriguing developments of the past several years has been the discovery and study of intervening sequences (introns) between the structural expressed sequences (exons) of the DNA of many animal cells and viruses. Perhaps this is true of plants too. It used to be (as recently as 1 or 2 yr ago) a truism that higher organism genes would best be expressed in eukaryotic biophores. But there are at least two reasons to wonder about this glib oversimplification. It seems that messenger RNA is transcribed in the nucleus of eukaryotic cells, including the introns, which are then removed and the exons are spliced together to form mature messenger RNA. This is called post-transcriptional processing. In the past 2 yr, there has been much talk of yeast, being relatively simple yet understood and safe, as being an ideal eukaryotic biophore. The exon/intron phenomenon has been shown to exist in yeast tRNA (Van Ommen et al. 1979). It is therefore disappointing to learn (van den Berg et al. 1979) that yeast does *not* process the messenger RNA for rabbit β-globin. Maybe further study will indicate why. The failure of cloned rabbit β-globin genes to be transcribed and spliced properly in *S. cerevisae* does not preclude the possibility that other genes, including those without intervening sequences, may be processed correctly. Indeed, it is not known how many eukaryotic genes of interest actually contain intervening sequences and require mRNA processing.

In the meantime, because it is so-called cDNA (complementary DNA, made from messenger RNA using reverse transcriptase), not genomic DNA with its introns and exons which is being frequently cloned in current projects, this problem is not a big roadblock. If cDNA is being used, it may be that a prokaryote, which doesn't "expect" introns and exons, is the biophore of choice for the time being in *all* projects. But there are prokaryotes and prokaryotes. *E. coli* has the advantage stemming from the wealth of available genetic information available in that system. *B. subtilis*, on the other hand, has its advantages too (easier to cause it to excrete, generally regarded as safe (GRAS), and now the availability of highly efficient transformation techniques, Chang and Cohen 1979).

All of which notwithstanding, there are reasons to develop eukaryotic biophores. For example, interferon is not a simple protein. It is glycosylated with a specificity and to a purpose not yet understood. We believe that nonglycosylated interferon will "work." It may even be better. We would like to have the opportunity to compare before making a choice, and it is not clear how or whether a prokaryotic biophore can be made to glycosylate a protein. Yeast has, on the other hand, been known to produce and excrete glycosylated enzymes. Therefore, it is attractive to continue to study this and a wide variety of other biophores; we and others are doing so.

In conclusion, there is no longer a paucity, indeed there is a plethora, of activity, and what is more, of advances in the commercial applications of molecular biology. They cover a wide range of industrial applications and involve scientific activity at the very frontiers.

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