Genetic Engineering

Principles and Methods

Volume 1

Edited by Jane K. Setlow

and Alexander Hollaender

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Preface

This volume is the first of a series concerning a new technology which is revolutionizing the study of biology, perhaps as profoundly as the discovery of the gene. As pointed out in the introductory chapter, we look forward to the future impact of the technology, but cannot see where it might take us. The purpose of these volumes is to follow closely the explosion of new techniques and information that is occurring as a result of the newly-acquired ability to make particular kinds of precise cuts in DNA molecules. Thus we are particularly committed to rapid publication.

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INTRODUCTION AND HISTORICAL BACKGROUND

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Not even the wisest minds can reliably predict the future course of science. One particularly striking example of utter failure is a report on future technological developments prepared in 1937 by the best talent presumed to be available in the United States (1). The report concluded, for example, that any further changes in commercial aviation would be in the direction of improved safety and comfort, although the possibility was entertained that a limited number of airplanes capable of flying at 20,000 feet and at a speed of 240 miles per hour would be built. There is no mention whatever of jet engines nor, in other spheres, of plastics or nuclear energy. It seems that the fantastic imaginations of writers of science fiction are better at prediction than are the rational minds of experts.

Modern biologists cannot expect to be better seers than were their predecessors. This chapter introduces a book that describes recent experimental approaches to the manipulation of genes in the laboratory. How many readers of the book would have predicted its scope, contents, or even its existence, six years ago? And yet, paradoxically, today the contents of the book seem neither strange nor foreign. It is in fact rather simple to trace, in retrospect, the origins of the ideas and of the technical innovations. Even the results follow logically, albeit surprisingly, from all that came before. The origins are found among observations and concepts in genetics, enzymology, molecular biology, cell biology, botany, virology, and chemistry over about the past 35 years. And the framework of the present work is defined by two already venerable paradigms (2) -- the DNA revolution and the enzyme revolution.

While the revolution in biology that crystallized around the DNA structure is only now widely perceived by interested laymen--partly as a result of the experiments described in this volume--

it is already a quarter of a century old. It is difficult to fix a precise date for its start but the initial elements surely include the proof that DNA is the genetic substance (3,4), the determination of the structure of DNA (5), and the elucidation of the genetic code (6-8). (The history of the DNA revolution has recently been described in detail (9)). Most readers of this book either internalized the revolution, bit by bit as it took place, or else, if they are young enough, were educated in the post-revolutionary period and were unaware of any cataclysm.

By itself, the DNA revolution was insufficient to permit detailed and designed manipulation of genetic systems. Given our customary time frame, it is difficult to recall that the discovery of enzymes also constituted a revolution. The enzyme revolution is as central to the successful manipulation of biological systems as is the DNA revolution. It began with the then remarkable demonstration that complex macromolecular proteins could be obtained from living organisms in the form of pure chemicals and yet still demonstrate those catalytic properties by which they contributed to the "life" of the organism. (The history of the enzyme revolution has also been described recently (10)). For the present purposes, the most fruitful development within the framework of the enzyme revolution was the discovery that other macromolecules, including polynucleotides, are substrates for specific enzymes. Chemistry itself has not yet provided techniques for the precise manipulation of biological macromolecules. Without the enzymes, the subject matter of this book would not now be attainable.

Why then, if the concepts were familiar and the paradigms of long standing, was prediction of the present results so unlikely? To a large extent, the lack of predictability stems from the diverse provenance of the antecedents of the present experiments. Furthermore, the timing of the necessary prior discoveries was not colinear with the logical development of the new methods. The two revolutions provided remarkably fruitful frameworks for the interpretation of biological observations, especially as they began to converge. The ability to move beyond interpreting observable phenomena and actually to manipulate biological systems in the fundamental manner described in this book, could not be foreseen.

With very few exceptions, the readers of this book were educated in the post-revolutionary period as far as the enzyme revolution is concerned. They will not usually concern themselves with the remarkable nature of the enzymes used to manipulate and construct precise DNA molecules. Most often those enzymes are perceived only as tools and are confronted in practice as a rather disorganized array of odd shaped tiny tubes inside a freezer. The sight inside the freezer carries no reminder that the fruits of a revolution are at hand. Nor will the investigator be reminded by the means he uses to acquire the enzymes. Some of them are now as easy to come by all over

the world as a bottle of Coca-Cola--only more expensive. Others may be obtained by persuasive begging and borrowing, and, in a tight squeeze, even stealing. But when all else fails, and it is necessary actually to prepare one of the enzymes, the impact of the revolution may be sensed. Delight and amazement inevitably accompany the emergence of a clean, exquisitely specific reagent from the equally inevitably messy beginnings. Dismay coupled with awe of successful predecessors accompany the frequent frustrating failures.

Current emphasis on the utility of reagents to manipulate DNA has also obscured the inherent and singular importance of many of the enzymes. They represent the common ground of the DNA revolution and the enzyme revolution, and in many instances their discoveries added facts of first magnitude importance to our knowledge of biology. The role of many of these enzymes in the growth and reproduction of cells is a central issue for modern biology. Study of the mechanism behind the complex yet highly specific reactions they catalyze is still a major concern of modern enzymologists. It is interesting and instructive to review the history of the discovery of these enzymes and to remind ourselves of their significance.

THE ALKALINE PHOSPHATASE OF *Escherichia coli*

The alkaline phosphatase of *E. coli* seems a very mundane reagent, devoid of any scientific glamour at all. Phosphatases catalyze a rather simple reaction, the hydrolysis of phosphomonoesters. The enzyme from *E. coli* is not even very fastidious--any phosphomonoester will do and even polyphosphates can be hydrolyzed. Besides, *E. coli* alkaline phosphatase of satisfactory purity has been available commercially for many years and it is cheap. And yet its discovery by Horiuchi, Horiuchi and Mizuno (11,12), and independently by Torriani (13), was of great interest in the late 1950s because it was made in the context of the early efforts to understand regulation of cellular processes. Earlier workers had observed that resting bacteria tend to degrade their own proteins and nucleic acids. Attempting to define this phenomenon more carefully, Torriani, as well as Horiuchi and coworkers, starved *E. coli* cells for single components. When the cells were starved for phosphate they did indeed degrade RNA but continued to make DNA and protein and to grow and multiply. A survey of the levels of a variety of enzymes under such conditions revealed a marked increase in phosphomonoesterase activity. The increased activity resulted from new synthesis of a single enzyme--the alkaline phosphatase. Under conditions of phosphate starvation this enzyme accounts for as much as 6% of the cellular protein (14). Even more dramatically, the increase in activity stopped promptly upon addition of phosphate to the cells. Thus synthesis of alkaline phosphatase is regulated

by a negative feedback system in which inorganic phosphate is the regulator. Quite separately, inorganic phosphate also interacts directly with alkaline phosphatase and inhibits its activity (14). The cell can thus respond to inorganic phosphate both in an immediate mode (inhibition of enzymatic activity) and in a long term mode (repression of enzyme synthesis).

Accompanying Torriani's paper is a report by Garen and Levinthal (14) on the purification of the phosphatase from derepressed cells. Having learned about the enzyme from their colleague Torriani, they recognized its potential for correlating changes in enzyme structure brought about by mutation, with altered susceptibility to regulation. The system still seems an attractive one, though in fact it has never been fully exploited.

Garen and Levinthal showed that alkaline phosphatase was a general, nonspecific phosphatase (14). Khorana and Viszolyi first reported that the enzyme could dephosphorylate polynucleotides (15). Fortunately, for the present purposes, Richardson (16) showed that the difficulty the enzyme has in removing phosphate from monoester ends that are either at nicks in duplex DNA, or otherwise covered by an overhanging complementary strand (first observed at the 5'-terminus of transfer RNA (17)) can be overcome by carrying out the dephosphorylation at elevated temperature.

S1 NUCLEASE

The rapid breakdown of DNA and RNA in all sorts of crude extracts is a recurrent and often vexing side effect in experiments designed for other purposes. In not a few instances during the last 25 years, purification of a nuclease became the only constructive outcome of otherwise frustrating results. Determined searches for nucleases were of course always successful. At times it seemed as though too many people and too many journal pages were devoted to the description of yet another nuclease. Nucleases were described from sources such as shark liver and mung bean and everything in between. But finally, what seemed like redundant, even trivial, investigations proved remarkably fruitful. Many of the enzymes exhibited uniquely different approaches to the degradation of polynucleotides. In recent years, these differences have been exploited to produce elegant and specific analytical procedures.

Takadiastase, a dried powder made from Aspergillus oryzae, was one of the odd sources searched for nuclease. This material is prepared in large quantity in Japan where it is commonly used to alleviate human digestive ills. It had already proven to be a convenient source of amylase, when, in 1966, Ando detected in it a nuclease now known as S1 (18). When the enzyme was purified, first by Sutton (19) and later by Vogt (20), it turned out to have a marked and very useful preference for single-stranded polynucleotides. A similar enzyme had been purified previously from

Neurospora crassa (21) but the ease with which S1 can be obtained makes it the enzyme of choice.

LAMBDA EXONUCLEASE

The availability of simple methods for the detecting of nucleases makes these enzymes ideal potential indicators of changes in the physiological state of cells. By the early 1960s, it was known that infection of E. coli with any of a number of virulent bacteriophages results in the formation of new enzymatic activities, including nucleases, and the mapping of the genes for these enzymes on the bacteriophage chromosome had begun. At the same time, some believed that lysogenic induction of cells lysogenized by those same bacteriophage did not result in new enzymatic activities. Korn and Weissbach (22,23) were interested in testing this hypothesis and chose to measure nuclease activity after induction of E. coli cells lysogenized with bacteriophage lambda. A large increase in DNase activity was readily detected and proved to be both encoded by the lambda genome and identical to the activity found in virulent infections. When the enzyme was purified to homogeneity and crystallized by Little, Lehman, and Kaiser (24), its mode of action was confirmed and it was dubbed "lambda exonuclease" (25). The products are 5'-nucleoside monophosphates (25) and, as the very first experiments suggested (22, 23), the enzyme prefers double-stranded DNA as a substrate (25,26). The lambda exonuclease turned out to be particularly interesting both mechanistically and as an analytical reagent when Little (25) determined that its exonucleolytic cleavage of DNA started at the 5'-terminus of the chain. Until that time, the only nuclease known to proceed in the 5' to 3' direction was the exonuclease of spleen, which cleaves both DNA and RNA and produces 3'-nucleoside monophosphates (27,28). The spleen enzyme was notoriously difficult to separate from relevant contaminating activities and therefore of limited analytic potential. Subsequently, the 5' to 3' exonuclease activity of DNA polymerase I was discovered (29).

The lambda exonuclease is not yet available commercially. When the enzyme is needed, the molecular biologist has an opportunity to test his mettle as enzymologist. The test is not however very rigorous. Thanks to the work of Radding and his associates (30,31), the enzyme can be prepared from induced lysogens that superproduce early lambda proteins, including lambda-exonuclease, to such an extent that only a 90-fold purification suffices to yield pure enzyme (24).

RESTRICTION ENDONUCLEASES

The power of congruence of the DNA revolution and the enzyme revolution is beautifully illustrated by the history of the

discovery of restriction endonucleases. Almost 30 years ago, the early phage workers recognized that the ability of a bacteriophage to reproduce in a particular cell type depends on the cell type in which the phage was previously grown (32). The phenomenon was referred to as host controlled variation, or host induced restriction, and appeared to be unrelated to the genetic makeup of the bacteriophage itself. To explain these odd findings, Bertani and Weigle (33) speculated in 1953 that some bacteriophage component (unspecified, but required for bacteriophage multiplication) was under the control of the host. Later, Lederberg (34) learned that a lysogenic bacteriophage could similarly restrict multiplication of a heterologous bacteriophage. Lederberg obtained the first experimental clue to the mechanism of restriction when he noted that radioactivity from the labeled DNA of an unsuccessful heterologous bacteriophage appeared in the medium. Some five years later, Arber and Dussoix (35-37) confirmed the relation between restriction and DNA degradation in the course of extensive studies on restriction. Utilizing a series of mutants, these investigators elucidated the intricate and related mechanisms behind restriction, which acts on an invading bacteriophage, and its obverse modification, which permits recognition of self. In 1965, reviewing the earlier experimental work from his laboratory, Arber (37) argued that the observed gross breakdown of the restricted DNA was probably the result of a highly specific initial cleavage followed by subsequent, nonspecific degradation. Since modification appeared to depend on methionine, Arber speculated further that the modification that inhibits restriction involves alkylation of the DNA. Though direct evidence was lacking, Arber saw it was most likely that the specificity of both processes resided in the base sequence of the DNA. He wrote (37):

> "If this last idea should be correct, one may further speculate that a restriction enzyme might provide a tool for the sequence specific cleavage of DNA. Application of enzymes of different specificity should then be useful in attempts to determine base sequences of DNA molecules."

The idea was of course correct. By then, restriction-modification systems were known to be programmed by bacterial chromosomes, by bacteriophage chromosomes, and by plasmids. So Arber may well have realized how large a catalog of enzymes of different specificities would ultimately be available (38). Nevertheless, five years went by before Arber's predictions came true. During that time, experimental work concentrated on the group of restriction endonucleases now termed class I. These enzymes are fascinating and still not completely understood, but they are not geared to fine surgery of DNA as they do not cleave at specific sites. Then, in 1970, Smith, with Wilcox (39) and with Kelly (40), described the very specific cleavage of DNA at a given nucleotide

sequence by what are now called class II enzymes. Since then we have had an avalanche of enzymes from a variety of esoteric bacteria, each cleaving its own favored base sequences. Molecular biologists have been reminded that E. coli is not, so to speak, the only fish in the sea. Any remaining doubts about the accuracy of Arber's predictions were removed by Danna and Nathan's (41) construction of a physical map of the genome of simian virus 40, using the endo R·HindII and endo R·HindIII discovered by Smith and his colleagues.

The restriction endonucleases yielded other spectacular, though unsuspected gifts. The discovery by Mertz and Davis (42) and Hedgpeth, Goodman, and Boyer (43) that the cleavage products produced by endo R·EcoRI have single-stranded, complementary, overhanging ends, and subsequent work showing that other enzymes did too, greatly improved the prospects for joining DNA fragments.

DNA LIGASE

The discovery of DNA ligase was one of the greatest "happenings" of 1967. It was another event important to the growing congruence of the DNA revolution and the enzyme revolution; and the atmosphere was enlivened because the discovery was made essentially simultaneously by several independent groups working in the laboratories of Gellert, Richardson, Lehman, Hurwitz, and Kornberg (44–48).

At the time, the most attractive proposals concerning the mechanism of genetic recombination postulated both breaking and rejoining DNA chains. Mechanisms for breakage abounded in the many demonstrable nucleases, but rejoining remained a speculative process until ligase was discovered. The importance of the enzyme was emphasized by its presence in E. coli itself, and by the synthesis of a new ligase upon infection of cells with bacteriophage T4, although the role of DNA ligase in replication itself was not yet suspected. The E. coli and T4 enzymes differ significantly in two ways. First, the E. coli ligase requires diphosphopyridine nucleotide as a cofactor while the T4 ligase requires adenosine triphosphate even though each uses its cofactor to form analogous, ligase-adenylate and DNA-adenylate intermediates (49). Second, the T4 ligase does not require overlapping complementarity on the single-stranded ends of the two chains to be joined as does the E. coli enzyme (50). Two DNA molecules with fully complementary chains can be readily joined by T4 enzyme in what is referred to as "blunt end" ligation; the only requirement is that there be a phosphomonoester end group at the 5'-terminus and a hydroxyl group at the 3'-terminal.

DNA ligase is central to the recombination of DNA in vitro. It is satisfying to realize that one of the earliest predicted results of recombinant DNA research, the construction of bacteria

that would be efficient sources of important proteins, was first realized with DNA ligase itself (51,52).

DNA POLYMERASE I

One of the most dramatic results of the juxtaposition of the DNA and enzyme revolutions was the discovery of enzymes and enzyme systems that would, outside of whole cells, copy polynucleotide templates and synthesize large, specific macromolecules. Cell-free synthesis of DNA, of RNA, and of proteins were described in rapid succession between 1958 and 1962. Discovery of the DNA polymerase I of E. coli came first, and its discovery by Kornberg and his colleagues was not accidental (53,54). Educated and accurate guesses were based on the proposed structure of DNA and its inherent implications about its own replication (5) as well as on the insight into transfer reactions involving polyphosphate esters of nucleotides that had been obtained from the earlier elucidation of the biosynthesis of nucleotides and coenzymes. Now, 20 years later, we realize that the synthesis of DNA is more complex and more diverse than might have been imagined in 1958. Still, the description of DNA polymerase I (54) and most importantly, the ability of the enzyme to copy a template faithfully (53,55), opened a new era.

Many other DNA polymerases are now known, but polymerase I remains among the most interesting, perhaps because it is the best understood. From an enzymologist's viewpoint, the interrelation between the three quite different reactions catalyzed by the two-headed protein remain intriguing. One head catalyzes phosphoryl transfer and thus synthesis, as well as hydrolysis in the 3' to 5' direction (53). The other head catalyzes hydroysis in the 5' to 3' direction (29,53), a reaction analogous to that catalyzed by lambda exonuclease. The two heads together, under the proper conditions, can start a nick in a DNA duplex, degrade the chain in the 5' to 3' direction and simultaneously rebuild the degraded chain by addition to the 3'-hydroxyl that is on the other side of the nick (53,56). The nick thus progresses down the chain--a process commonly and perhaps unfortunately referred to as nick translation, rather than nick progression. Notwithstanding the oddity of the name, the process itself is extraordinarily useful--it allows the preparation of ^{32}P-labeled DNA fragments with specific radioactivities of the order of 2×10^8 counts per minute per microgram (57).

The E. coli gene for DNA polymerase I has also been amplified in E. coli by means of recombinant DNA techniques (58).

TERMINAL NUCLEOTIDYL TRANSFERASE

The discovery of DNA polymerase I stimulated a search for similar enzymes in eukaryote sources. The result was a bonanza

and it was quickly recognized that more than one DNA polymerase coexist in cells. The polymerizing activity that was readily detected by Bollum in extracts of calf thymus was initially believed to be a DNA polymerase (59). The early work on this activity concentrated on the fact that the enzyme catalyzed the addition of deoxymononucleotide residues to a preformed polydeoxyribonucleotide primer and preferred single-stranded primers for the reaction (60). Ultimately, of course, it turned out that all the DNA polymerases require a primer. But, in contrast to the true polymerases, the major activity in calf thymus extracts does not require both a template and a primer and was eventually renamed terminal nucleotidyl transferase to reflect its mechanism of action (61-63). Calf thymus extracts contain other enzymes that can polymerize deoxynucleotide triphosphates by copying a template (63).

The preference for single-stranded primers is the basis for the need to digest DNA fragments briefly with lambda exonuclease prior to the addition of residues with terminal nucleotidyl transferase (64,65).

REVERSE TRANSCRIPTASE

Reverse transcriptase is another of the enzymatic reagents that should be appreciated as a major landmark in the process of joining the two revolutions. Data published by Temin as early as 1964 (66) suggested that such an enzyme might exist and in 1970 the predicted activity was demonstrated in preparations from RNA tumor viruses by Temin and his colleagues (67) and by Baltimore (68). Some observers saw the discovery as somehow detracting from the central position of DNA as the repository for biological information. But if the finding is viewed another way, the essential role of the enzyme in the replication of certain RNA viruses, if anything, confirms the central position of DNA in biology. Besides, now that it is recognized that the genomes of RNA viruses quite commonly reside within the genomes of cells in the form of DNA, the question of the relative importance of DNA and RNA is reduced to the old chicken and egg problem. In any case, the addition of reverse transcriptase to the catalog of known enzymes made a two-directional flow of information--DNA to RNA and RNA to DNA--feasible both in cells and in test tubes.

To carry out the RNA to DNA conversion in test tubes in a preparatively useful manner requires the availability of sufficiently purified enzyme in adequate quantities. With some variation, the large scale procedures introduced by Spiegelman in 1972 (69) still provide the bulk of the needs of investigators.

CONCLUSION

This is an introduction to a book and not meant to be an exhaustive review. Many enzymes that are being used and will be used for the manipulation of genes and chromosomes have not been mentioned. Still the histories of these few enzymes suffice to illustrate that the future impact of individual discoveries will rarely, if ever, be completely predictable. Each new discovery is likely to have pleiotropic effects and some of these effects will be apparent only later on, in the context of subsequent discoveries and theories or even new paradigms. The people who discovered the enzymes used for the manipulation of DNA did not spend time speculating on what they knew they could not yet know. Similarly, while the investigators reporting work in this book are delighted with what they have learned, neither they nor anyone else can say with certainty what may be learned in the future. The present authors will, of course, speculate; they would be neither human nor scientists if they did not. Predictions and speculations form the basis for new experiments, and some predictions, like those of Arber, may turn out to be correct.

For most scientists the frustration of not knowing the future is neither debilitating nor cause for alarm. Pessimism is rejected because it is both uninteresting and unproductive. Indeed, one might define science itself as the optimist's response to the conundrum expressed so well by the novelist and poet Robert Penn Warren (70).

> "The end of man is knowledge, but there is one thing he can't know. He can't know whether knowledge will save him or kill him. He will be killed, all right, but he can't know whether he is killed because of the knowledge which he has got or because of the knowledge which he hasn't got and which if he had it, would save him."

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