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He has published numerous papers on DNA polymerases from animal cells and on the molecular aspects of cellular aging, with particular attention to the machinery of DNA replication.

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Among Dr. Loeb's more than 200 publications are evidences of the breadth of and variety among his research interests. His major research interests include DNA replication in mammalian cells; molecular mechanisms for mutagenesis and carcinogenesis; the fidelity of DNA replication; and environmental carcinogenesis.

Dr. Loeb is married to Phyllis, a teacher, who facilitates his research in numerous small but important ways; they have three children.

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Chapter 1

# GENERAL INTRODUCTION

Since the first description of a DNA polymerase in an animal cell 25 years ago,[1] an immense body of information has been accumulated on eukaryotic DNA polymerases, their classification into distinct categories, prevalence, evolution, physical and catalytic properties, and roles in DNA metabolism in vivo. The substantial number of published reviews[2-16] and collected papers[17-20] which were dedicated to eukaryotic DNA polymerases bear witness to the dynamic state of research conducted on these cardinal enzymes. The rapid pace of progress in the field of DNA polymerases of animal cells has recently accelerated. The development of an arsenal of selective inhibitors of these enzymes, the detailed analysis of DNA primase, the introduction of monoclonal antibodies against DNA polymerases, and the isolation of animal cell mutants with modified DNA polymerases are of particular importance in increasing the understanding of the enzymology and biology of DNA synthesizing enzymes in eukaryotes. Furthermore, initial works show promise of the application, in the near future, of recombinant DNA techniques to the study of animal cell DNA polymerases.

The growing body of knowledge on eukaryotic DNA polymerases and the increasing interest in these enzymes appear to warrant an attempt to summarize currently available data on polymerases and to indicate possible future directions of their study. DNA replication, repair, and recombination in animal cells are highly complex processes that involve a presently inestimable number of factors. Furthermore, variations in DNA transactions appear to exist among different types of eukaryotic cells and in cells infected with various viruses. The discourse in this review is limited by necessity, however, to a narrow aspect of DNA metabolism in animal cells. First, the discussion in this book is restricted to DNA polymerases and to cofactors that appear to interact directly with them. Hence, the plethora of enzymes and factors that are involved in DNA replication and repair is not given full exposure in this work. Second, we confined our presentation to DNA polymerases of animal cells, whereas polymerases of plant cells, bacteria, viruses, and lower eukaryotes are mentioned in selected sections throughout the book merely for comparative purposes and to delineate unique aspects of animal cell polymerases. Third, in consideration of the large number of exhaustive reviews on eukaryotic DNA polymerases, which appeared between 1974 and 1984,[2-16] in this book we mainly focus on results that were obtained in the past 5 years. Regrettably, the laudable earlier efforts by pioneering authors may not be presented in full in this review. It is indeed unfortunate that, in delineating a history of concepts of biochemistry, individual findings often become a buried archeology.

In compiling the present work, we have attempted to address two distinct audiences of researchers who have interest in DNA metabolism of higher eukaryotes. First, the book is directed to investigators who are actively engaged in the study of DNA replication and repair. For these, we have undertaken to extensively summarize and synthesize recent developments in the literature and have cataloged a large number of diverse observations. A second audience, which we believe to be a rapidly growing one, is composed of advanced graduate students who have chosen DNA replication in eukaryotes as a field of future research. For these, we have analyzed current concepts and insights, made presumptive judgments, and projected future directions of study. We hope these projections will stimulate readers to challenge our interpretations and speculations.

Writing of the main body of this book was concluded in February 1985. Selected papers that were published between February and August 1985 are reviewed in special Update sections that were added in proof to some chapters.

Many colleagues have helped us in preparing this book, and it is a pleasure to acknowledge their contributions. We thank colleagues who communicated their results to us: Drs. R. A. Bambara, E. F. Baril, R. M. Benbow, U. Bertazzoni, F. J. Bollum, A. M. de Recondo, S. L. Dresler, N. B. Hecht, J. A. Huberman, U. Hübscher, J. Hurwitz, G. Kalf, D. Korn, R. Knippers, G. Krauss, M. Lieberman, A. Matsukage, M. R. Miller, K. Ono, A. B. Pardee, E. Rapaport, S. Spadari, S. H. Wilson, T. Yagura, and S. Yoshida. We are greatly indebted to Drs. R. A. Bambara, University of Rochester Medical Center, New York; K. P. Gopinathan, Indian Institute of Sciences, Bangalore, India; T. A. Kunkel, NIEHS, Research Triangle Park, N.C.; S. H. Wilson, NCI, NIH, Md.; J. Abbotts, P. K. Liu, G. M. Martin, R. J. Monnat, T. H. Norwood, M. Reyland, and E. Snow, University of Washington in Seattle for reading parts of the book and making critical comments. Inaccuracies and ambiguities in the text remain, however, ours alone. One of us (M. Fry), was on sabbatical leave from the Technion-Israel Institute of Technology at the time of the writing of this book. He wishes to express his deep gratitude to Professor George M. Martin from the University of Washington for his support and friendship. We thank Ms. Joan Hiltner, Ms. Mary Whiting, Ms. Carol Hansen, and Ms. Sharon Wes. for diligently and patiently putting to type the large number of drafts of this book.

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Chapter 2

# MULTIPLICITY OF DNA POLYMERASES IN ANIMAL CELLS

*Variety is the mother of enjoyment.*

Benjamin Disraeli
Vivian Grey

## I. BASIC ENZYMOLOGICAL CHARACTERISTICS OF DNA POLYMERASES

The enzymes responsible for the accurate synthesis of the genetic information in living cells are termed DNA-dependent DNA polymerases (DNA nucleotidyl transferases EC 2.7.7.7, herein designated DNA polymerases). These enzymes are ubiquitous in cells of all prokaryotes and eukaryotes and encoded by many of their respective viruses. The universal mechanism for catalysis by DNA polymerases was initially delineated by Kornberg et al.[1] in 1956. These enzymes are responsible for catalyzing the faithful incorporation into DNA of the four deoxyribonucleoside triphosphates, dATP, dCTP, dGTP, and dTTP. The order of incorporation is directed by a complementary DNA template strand according to the Watson-Crick base pairing rules (Figure 1).[1,2] Despite considerable variance in the structure of DNA polymerases from different sources, diverse association with auxiliary proteins, and dissimilitude in catalytic behavior and in vivo roles, generally DNA polymerases have the same basic catalytic characteristics:[1,2]

1. All DNA polymerases utilize deoxyribonucleoside triphosphates (dNTPs) for the polymerization of DNA by sequential incorporation of deoxyribonucleoside monophosphates (dNMPs) with cleavage of the $\alpha$-$\beta$ phosphodiester bond and release of pyrophosphate. The presence of a template DNA and metal ion activator are requisites for DNA polymerase-catalyzed DNA synthesis. The order of polymerization of the deoxynucleotide substrates in the product DNA is dictated by the base sequence of the template DNA. Thus, DNA polymerases and related polymerase are singular in that the specificity of catalysis is determined by another molecule.
2. All DNA polymerases require a short segment of DNA or RNA complementary to the template and having an available hydroxyl group at the third carbon position of the deoxyribose/ribose moiety. Such a nucleic acid segment acts as a primer, and polymerization of the next nucleotide residue proceeds from its 3′-hydroxyl position.
3. All DNA polymerases catalyze DNA synthesis exclusively in the 5′ → 3′ direction of the synthesized strand.

These catalytic requirements of DNA polymerase constitute the basic theoretical and experimental framework for their structural, enzymological, and functional analysis. The participation of DNA polymerases in replicative and repair synthesis of DNA in vivo must be understood in the general context of these processes. The description of general mechanisms for DNA replication and repair in animal cells is beyond the scope of this book. Knowledge of basic rules of DNA synthesis such as semiconservative mode of replication, RNA priming, leading and lagging strand-synthesis, strand displacement synthesis, excision repair, etc. are prerequisites for understanding various parts of this book. For a masterful survey of the field of DNA replication, the reader is referred to the recent book by Kornberg.[2]

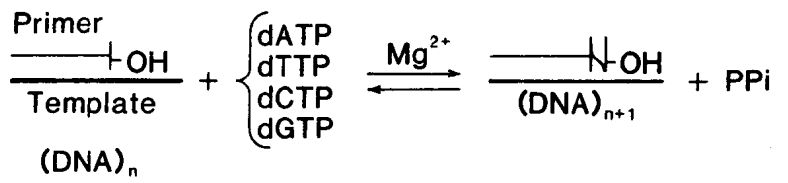


FIGURE 1. Outline of the basic mechanism of catalysis of DNA synthesis by a DNA polymerase. Details of DNA polymerase-catalyzed synthesis of DNA are described in the text. Magnesium, which is commonly the preferred metal activator of DNA polymerases, is marked in the scheme as a required component of the DNA polymerization reaction.

## II. MULTIPLICITY OF DNA POLYMERASES IN ANIMAL CELLS—A BRIEF HISTORICAL PERSPECTIVE

Almost 30 years ago, in 1956, Kornberg and associates identified in *Escherichia coli* the first known DNA polymerase, later designated *E. coli* DNA polymerase I. Shortly thereafter, Bollum and Potter[3-5] demonstrated a similar catalytic activity in a eukaryotic cell. This animal cell enzyme was later classified DNA polymerase-$\alpha$. In the ensuing decade, DNA polymerase activities were identified, partially purified and characterized in diverse organisms, tissues, and cells. In animal tissues the recovered activity of DNA polymerase paralleled the proliferative activity of the cells.[6] For this reason, initial efforts at polymerase purification were carried out using rapidly dividing cells yielding one predominant DNA polymerase activity.

The first extensive purification of an animal cell DNA polymerase was conducted by Yoneda and Bollum,[7] who isolated from calf thymus a high molecular weight enzyme that sedimented at ~12 S. The requirements for catalysis of DNA polymerization of the eukaryotic enzyme were similar to those of bacterial DNA polymerase. However, two unexpected properties of mammalian DNA polymerases were noted: the major proportion of the cellular content of that enzyme was recovered in the cytoplasm,[4] and it preferentially utilized denatured DNA as a template.[7,8] In contrast, DNA polymerase purified shortly thereafter from rapidly dividing sea urchin embryos[9] was mostly localized in the cell nucleus and utilized double-stranded DNA template more efficiently than denatured DNA.[10,11] Intracellular localization and template preference of the major animal cell DNA polymerase activity were further investigated and revised in subsequent studies (cf. Table 1). That the initially described DNA polymerase activity was associated with replication of genomic DNA was clearly gleaned from studies that showed polymerase activity increased dramatically as cell division was induced in populations of quiescent cells. Human peripheral blood lymphocytes are nonproliferative cells that can be stimulated to undergo division in culture by exposure to phytohemagglutinin, a mitogenic plant lectin. Sixteen hours after stimulation, slightly before and immediately parallel with increase in the incorporation of labeled thymidine into DNA, there is an increase in the activity of DNA polymerase [11,12] These observations are depicted in Figure 2.

For quite some time it was assumed that only a single type of DNA polymerase was present in living cells. Hence, *E. coli* DNA polymerase I was the enzyme deemed to be responsible for replication of the bacterial genomic DNA. In fact, it was even possible to demonstrate that DNA polymerase I could synthesize biologically active DNA molecules in the test tube.[13,14] Yet the catalytic properties of DNA polymerase I appeared to be inadequate for a replicative DNA polymerase.[2] A genetic approach was applied, therefore, to investigate the possibility that replication of genomic DNA is conducted in *E. coli* by an enzyme other than DNA polymerase I. Since properties of a mutant

## Table 1
## MAJOR DISTINGUISHING PROPERTIES OF ANIMAL CELL DNA POLYMERASES-$\alpha$, -$\beta$, and -$\gamma$[a]

| Property | DNA polymerase -$\alpha$ | -$\beta$ | -$\gamma$ |
|---|---|---|---|
| **Cellular** | | | |
| Relative activity (dividing cells) | 70—90% | 10—15% | 1—10% |
| Intracellular location | Nucleus | Nucleus | Mitochondria/nucleus |
| Change in activity during cell cycling | Yes | No | Minimal |
| **Physical** | | | |
| Native size (kdaltons)[b] | 200—600 Multimeric | 40 Monomeric | 180—315 Multimeric |
| Catalytic polypeptide[b] | 150—190 | 40 | 47 |
| Associated exonuclease[c] | No | No | No |
| Monospecific antibodies | Yes | Yes | Yes |
| **Catalytic** | | | |
| Preferred primer-template | Gapped DNA | Gapped DNA | Oligo(dT) · poly(rA) |
| Extension of RNA primer[d] | Yes | No | No |
| Preferred metal activator | $Mg^{2+}$ | $Mg^{2+}/Mn^{2+}$ | $Mn^{2+}$ |
| Km for dNTPs ($\mu M$)[e] | 1—15 | 5—30 | 0.2—1.0 |
| Salt (≥100 m*M*) | Inhibition | Stimulation | Stimulation |
| Processivity | Quasiprocessive | Distributive/slightly processive | Highly processive |
| Fidelity (nucleotide) | ~1/40,000 | ~1/4,000 | ~1/8,000 |
| **Major inhibitors** | | | |
| Aphidicolin (10—20 $\mu$g/m$\ell$) | Inhibition | No effect | No effect |
| *N*-Ethylmaleimide (0.5—10 m*M*) | Inhibition | No effect/slight inhibition | Inhibition |
| $d_2$TTP ($d_1$TTP/dTTP = 0.5—5.0) | No effect | Inhibition | Inhibition |
| Arylamino nucleotides | Inhibition | No effect | No effect |
| Phosphonacetate (10—100 $\mu$g/m$\ell$) | Inhibition | No effect | Inhibition |
| Arabinosyl-CTP | Inhibition | Lower inhibition | Lower inhibition |
| Inorganic phosphate (2—50 m*M*) | Limited inhibition | Inhibition | Stimulation |
| Novobiocin (0.2—0.6 mg/m$\ell$) | Inhibition | No effect | Partial inhibition |

[a] Detailed references to experimental data on the various properties of DNA polymerases-$\alpha$, -$\beta$, and -$\gamma$ are given in Chapters 3, 4, and 5, respectively.

[b] Size of native DNA polymerase-$\gamma$ and its constituent subunits were determined only for chick and mouse cells. Size of polymerases-$\alpha$ and -$\beta$ and their respective catalytic polypeptides were obtained from a large number of different cell types.

[c] In an isolated case, the association of mouse-cell polymerase-$\alpha$ with a bidirectional 3′ → 5′ and 5′ → 3′ exonuclease has been reported (Chapter 3, Section IV.A). In all other tested cases, purified DNA polymerases-$\alpha$, -$\beta$, and -$\gamma$ were devoid of detectable nuclease activity. Analysis of polymerase-$\gamma$ is at present limited to a few studies.

Table 1 (continued)
MAJOR DISTINGUISHING PROPERTIES OF ANIMAL CELL DNA POLYMERASES-$\alpha$, -$\beta$, and -$\gamma$[a]

Indicated is the ability of DNA polymerases to extend a natural RNA primer. Although DNA polymerase-$\gamma$ is able to extend synthetic ribohomopolymeric primers, it appears not to be able to utilize natural RNA primers (Chapter 5, Section I).
Km values for dNTPs vary for the various dNTPs and depend on the nature of the primer-template. Shown here is the range of Km values obtained for DNA polymerases for different sources with optimally gapped DNA and usually in experiments in which concentrations of all four dNTPs varied together.

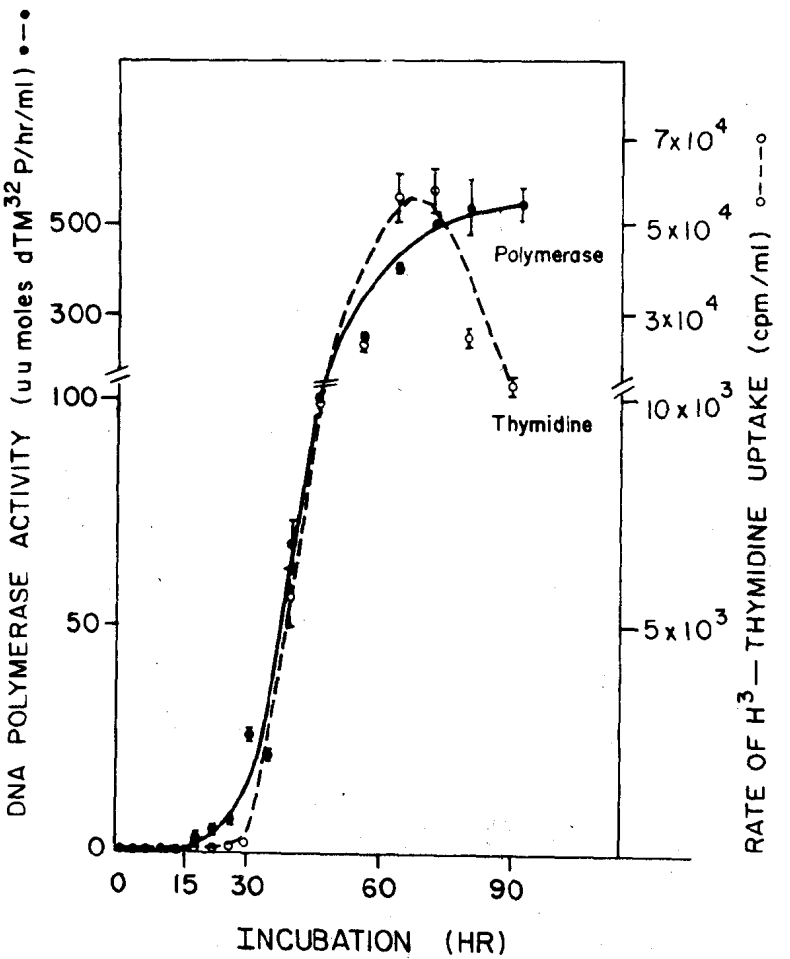


FIGURE 2. Relationship between DNA polymerase activity and rate of thymidine incorporation into DNA following phytohemagglutinin stimulation of human lymphocyte proliferation.

lacking DNA polymerase I activity could not be predicted, a large number of heavily mutagenized *E. coli* clones were quantified for cellular activity of DNA polymerase I by De Lucia and Cairns.[15] After recovering normal levels of polymerase I in 3747 clones of bacteria exposed to mutagen, the 3748th clone, designated pol A1, was found to contain 1% of the level of DNA polymerase I activity of the parental strain, yet it was viable and was able to replicate its DNA. Recovering polymerase I activity at an

expected dilution in mixtures between extracts of pol A1 and parental cell strongly suggested that the mutant did not contain an inhibitor to the polymerase.[15] These results established that *E. coli* polymerase I was not absolutely required for DNA replication and provided a source for the susequent isolation of distinct *E. coli* DNA polymerase II,[16-18] and somewhat later, DNA polymerase III.[19,20] The three types of bacterial DNA polymerase differ in size, catalytic properties determined in vitro, and associated proteins.[2,20,21] Furthermore, different in vivo roles were later assigned to the three classes of *E. coli* DNA polymerases.[2,20,21]

In contrast to prokaryotes, the study of the enzymology of DNA synthesis in animal cells did not enjoy the advantage of detailed genetics, and in particular, the establishment of conditional mutants of the synthetic process. Distinguishing among different animal cell polymerases and their associated proteins had to rely completely on biochemical approaches.

About a decade after the initial isolation of DNA polymerase-$\alpha$ from calf thymus,[5] a distinctly different polymerase, later termed DNA polymerase-$\beta$, was isolated from several cell types.[22-24] DNA polymerase-$\beta$ was distinguished from polymerase-$\alpha$ by virtue of its uniquely smaller molecular size, basic isoelectric point, and resistance to chemicals that react with the thiol group.[22,24,25] Shortly after polymerase-$\beta$ was identified, a third type of animal cell polymerase, which was later designated DNA polymerase-$\gamma$, was isolated.[26,27] Polymerase-$\gamma$ differed from $\alpha$- and $\beta$-polymerases by its synthetic template requirements, preference for $Mn^{2+}$ as a metal activator, and requirement for salt for maximum DNA synthesis in vitro, as well as its sensitivity to agents that react with protein sulfhydryl groups.[25-27]

Following their discovery, animal cell DNA polymerases of the three types were identified in a wide variety of organisms, tissues, and cells. In 1975, Weissbach et al.[25] proposed a set of basic defining criteria for the classification of known animal cell DNA polymerases under three main groups: DNA polymerases of the $\alpha$, $\beta$, and $\gamma$ types. At the time, a fourth category of a distinct mitochondrial enzyme was also included, but shortly afterwards the mitochondrial polymerase was shown to be identical with DNA polymerase-$\gamma$.[28,29] Although in the ensuing years some of the original parameters for the classification of eukaryotic DNA polymerases were modified and refined, the categorization of polymerases into the $\alpha$, $\beta$, and $\gamma$ types remains the basic working framework in this field. A property shared by all three classes of animal cell DNA polymerases is the lack of associated exonuclease activity, which is commonly found in many prokaryotic and viral DNA polymerases.[2,21,30] In 1976, an apparent fourth type of DNA polymerase that was intimately associated with 3′→5′ deoxyribonuclease activity was isolated from rabbit bone marrow.[31] That activity, denoted polymerase-$\delta$, has since been identified also in calf thymus.[32] However, it remains to be seen whether $\delta$-type polymerase is uniquely present in selected tissues or cells, or whether it is as omnipresent as are polymerases of the $\alpha$, $\beta$, and $\gamma$ classes.

Following the classification of animal cell polymerases, much progress has been made in the elucidation of their structure, association with auxiliary proteins and cofactors, catalytic activity, biology, and roles in vivo. In particular, several recent methodological developments have accelerated research on important aspects of the enzymology of DNA synthesis in animal cells. First, monoclonal and polyclonal monospecific antibodies directed against DNA polymerases have become available in recent years. Monospecific antibodies have been successfully used to study the evolution of DNA polymerases-$\alpha$ and -$\beta$.[34-36] Monoclonal antibodies are now being used for the purification of intact molecules of polymerase-$\alpha$ and for the study of their intracellular localization,[34-36] subunit structure,[37-41] and association with DNA primase, an enzyme that catalyzes the synthesis of RNA primers.[38] Moreover, monospecific antibodies are now being used in studies on the biosynthesis of animal DNA polymerases