To the Graduate Council:

I am submitting herewith a dissertation written by Sumin Mark Huang entitled "Age-associated Alteration of DNA Polymerase and in vitro DNA Synthesis of Rat Spleen." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

Wen-Kuang Yang, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

AGE-ASSOCIATED ALTERATION OF DNA POLYMERASE AND *IN VITRO* DNA SYNTHESIS OF RAT SPLEEN

A Dissertation

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Doctor of Philosophy

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ABSTRACT

Examination of the spleens of 3-month- and 25-month-old male Fischer 344 rats revealed three kinds of DNA polymerase (α, β and γ), which can be detected and characterized by their molecular weights, subcellular localizations, chromatographic migrations and template specificities.

Spleen DNA polymerases from both young and old rats are similar in their elution patterns in ion-exchange chromatography and also in their sedimentation rates in sucrose gradient centrifugation. Extracts from the cytoplasmic fraction of the young and old rat spleens contained DNA polymerase α, β and γ in approximately the same quantities. However, polymerases β and γ were found to be significantly decreased in the nuclear and large-particule fractions of the old rat spleens. Partially purified DNA polymerases from young and old rat spleens showed similar heat lability and in vitro replicational infidelity.

When included in a DNA synthesis reaction, nuclei isolated from the spleens of young rats showed a 1.2- to 1.5-fold higher dNMP incorporation than did those from old animals. Upon addition of exogenous DNA polymerase to the reaction mixture, the dNMP incorporation by the nuclei was enhanced and conversely became 3- to 4-fold higher in the old than the young. Mild acid treatment of the nuclei further increased the template activity; in this respect, the young appeared to be more responsive than the old. Kinetic analysis revealed that the relatively high template activity of the spleen nuclei from old rats represents increased substrate sites for the exogenous DNA polymerase. This large

increase in template activity also occurs in mouse spleen nuclei during the aging process, but not in the nuclei from rat liver or brain, or from mouse liver.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURE PAGE

CHAPTER 1

INTRODUCTION

Mammalian DNA Polymerase

General Statement

Until the late sixties, bacterial cells were thought to have only one DNA polymerase. Accordingly, the discovery of the multiplicity and variety of DNA polymerase in mammalian cells was not anticipated. During the past twenty years, considerable information has accumulated concerning the type and number of DNA polymerases in mammalian cells. Now it is clear that numerous mammalian DNA polymerases are distinguishable according to their distinct biochemical characteristics, the diversity of their subcellular localization, and the presumably different functions they catalyze.

Since all DNA polymerases known catalyze the polymerization of dNTPs in the presence of a suitable "template-primer," their catalytic activities are usually expressed as the quantity of nucleotide incorporated into the "template-primer" under defined reaction conditions. In early *in vitro* studies of DNA polymerase activity, denatured DNA and DNase-treated DNA were used as the template. Recently, synthetic DNA and RNA templates have been employed widely, as proper oligomer-homopolymer combination can be very effective in demonstrating DNA polymerase activity.

Early attempts to purify and characterize mammalian DNA polymerase were directed at the enzyme in calf thymus (1). Thymus possesses a particular enzyme (called terminal transferase) that polymerizes nucleotides without a template. In this article, however, we discuss only the replicative deoxynucleotidyl transferases of mammalian cells. Other eukaryotic DNA polymerases and virus-induced DNA polymerases have been the subject of several recent reviews (2, 3, 4, 5).

The Multiplicity of Mammalian DNA Polymerases

At least four different DNA polymerases are present in normal mammalian cells. Various names were used for these enzymes in different laboratories until at an international converence on DNA polymerase held in 1975, a uniform nomenclature was adopted to help organize the large number of previous studies on mammalian DNA polymerase. The four different enzymes have been named polymerase α, β, γ, and mitochondria DNA polymerase.

The first mammalian DNA polymerase to be partially purified and studied was α-DNA polymerase (6). It has been found in various mammalian cells such as human (7), murine (8), and hamster (9), among others. A characteristic property of the α-DNA polymerase is its sensitivity to inactivation by sulfhydryl group blocking reagents such as N-ethylmaleimide (10) and by salt concentrations above 25 mM (1). The molecular weight of this enzyme ranging from 70,000 to over 1,000,000 with S values of 5-12 being reported in several species. Being much larger than that of β-DNA polymerase, α-DNA polymerase is often referred

to as "high-molecular weight" DNA polymerase. Since this enzyme has a tendency to aggregate in solutions of low ionic strength (11), it is difficult to assess the significance of molecular weight variations. In addition to its replicative deoxynucleotidyl transferase activity, the calf thymus α-DNA polymerase has been reported to catalyze pyrophosphorolysis and pyrophosphate exchange (2).

In addition, the possibility that the α-DNA polymerase possesses 3'- to 5'-exonuclease activity was indicated by Chang et al. (12). However, since the α-DNA polymerase has not been purified to homogeneity, it is not clear whether the exonuclease activity is an intrinsic property of this enzyme. In contract, the β-DNA polymerase does not possess any of the above-mentioned additional activities.

Template studies have shown that α-DNA polymerase prefers "gapped" DNA such as "activated" DNA (13), and synthetic DNA templates such as poly(dA), with either oligoribo- or oligodeoxyribonucleotide as primer. RNA, on the other hand, is a poor template for the α-DNA polymerase (2).

The β-DNA polymerase, also known as low-molecular-weight DNA polymerase, is the major polymerase in the nucleus. Whereas the α-DNA polymerase of various mammalian cells occur in the cytoplasmic fraction rather than the nuclear fraction (2). The activity of the β-DNA polymerase does not vary much with proliferative states of the cell; with the use of activated calf thymus DNA, it is detected as a minor DNA polymerase activity in rapidly proliferating cells. β-DNA polymerase was first identified in HeLa cells, rat liver, and calf thymus in 1971 (10, 14, 15). The enzyme has now been purified to homogeneity from calf thymus (16) and

human KB cells (17). The purified enzyme has a molecular weight of about 45,000 and shows a sedimentation value of 3-4 S.

β-DNA polymerase is not markedly inhibited by low levels of sulfhydryl group blocking reagents such as p-chloromercuribenzoate and N-ethylmaleimide (18), which completely block the α-DNA polymerase activity. The β-DNA polymerase is, in general, an enzyme showing a high resistance to a number of chemical agents; it is not inhibited by phosphonoacetic acid (19), 5 M urea, 25% ethanol or acetone (16). Chang and Bollum have examined the template-primer specificities of the α- and β-DNA polymerase (2, 20) and showed that the β-DNA polymerase, but not the α-DNA polymerase, can copy a poly(A) template. In general, however, β-DNA polymerase is more efficient with polydeoxyribonucleotide templates than with corresponding polyribonucleotide templates.

An interesting study on the phylogeny of β-DNA polymerase by Chang (21) shows that this enzyme is ubiquitous in the animal kingdom, but is absent in bacteria, plants, and protozoa. α-DNA polymerase, however, is present in all eukaryotes that have been examined.

Another DNA polymerase that has been identified in various mammalian cells is the γ-DNA polymerase. This enzyme can be distinguished from the α- and β-DNA polymerase by its high catalytic activity in 100-300 mM KCl and 50 mM phosphate. The α-DNA polymerase is inhibited by the high salt concentration, and the activity of the β-DNA polymerase diminishes considerably in the presence of phosphate (12, 22). γ-DNA polymerase can copy, in addition to natural or synthetic DNA templates, a variety of synthetic ribohomopolymers such as poly(A) and poly(C) (22, 23).

In contrast to other DNA polymerases, the γ-DNA polymerase prefers a ribohomopolymer to deoxyribohomopolymer as template. Like the α-DNA polymerase, isolated γ-DNA polymerase appears to be heterogeneous in molecular form. For example, molecular weights from 110,000 (24) to 330,000 for the human γ-DNA polymerase (22), and multiple forms of the calf thymus γ-DNA polymerase have been reported (25). Again, the significance of the diversity of form of γ-DNA polymerase is difficult to assess due to the propensity of this enzyme to form aggregates in solution of low ionic strength (26).

DNA polymerases isolated from the mitochondria of rat liver (27), HeLa cells (28), mice (29) and other mammals have been studied. Fry and Weissbach claimed that two DNA polymerase activities can be identified in HeLa cell mitochondria (30). One of the enzymes has characteristics of γ-DNA polymerase and the other enzyme has a molecular weight of about 106,000 and is quite different from the other cellular DNA polymerases. It was later shown, however, that the cultured HeLa cells used in these studies were contaminated with mycoplasma and the 106,000-dalton enzyme is probably derived from this contaminant. Subsequently, Weissbach reinvestigated the mitochondria isolated from either mycoplasma-free HeLa cells or rat liver for DNA polymerase activity and have found, in both cases, a single DNA polymerase in the purified mitochondria. This mitochondrial associated enzyme closely resembled the γ-DNA polymerase found in the corresponding cytoplasm of these cells (31). The identity of γ-DNA polymerase and mitochondrial DNA polymerase was soon further proved in chicken embryo (32) and rat brain (33). Although early work

of Chang and Bollum (34) suggested a possible interrelationship between these DNA polymerases, recent work indicates that there is no structural relationship or polypeptide sharing between α-, β-, and γ-DNA polymerase (11, 35, 36).

Biological Role of DNA Polymerase

Biochemical processes are often compartmentalized in the cells. Since chromosomal DNA is localized in the nucleus and replication of the DNA presumably occurs in the nucleus, one may expect to find enzymes responsible for DNA synthesis localized in the nucleus. However, early works on the analysis of localization of mammalian DNA polymerases by generally acceptable procedures for subcellular fractionation usually show that the major portion of the total DNA polymerase activity was found in the soluble fraction of the cytoplasm. This has raised some questions about the possible roles of these enzymes.

Recently, techniques involving nonaqueous breakage of cells or drug-induced enucleation have indicated that 80-90% of the total DNA polymerase activity is, in fact, associated with nuclei (37, 38, 39). During the isolation of subcellular fractions by common aqueous procedures, DNA polymerases may be released from nuclei and lead to an artifactual distribution of DNA polymerase activity between nuclei and cytoplasm. However, further proof is required for excluding the possible presence of DNA polymerase activity in the cytoplasm. For example, leukemic cells appear to have a nuclear α-DNA polymerase that differing from that in the cytoplasm (40).

Since direct correlation of an enzyme reaction with mutants defective in a specific biological process is not generally possible with mammalian cells, several indirect approaches have been used to elucidate the biological roles of DNA polymerases. For example, it has been clearly demonstrated that the level of α-DNA polymerase varies according to the rate of cell proliferation and DNA synthesis. Chang and Bollum found that when L cells are stimulated to divide, there is a rise in the α-DNA polymerase activity with little change in the level of the β-DNA polymerase (12). Investigation of regenerating rat liver 30 hours after hepatectomy also gave a similar results (41). Spadari and Weissbach (42) measured the levels of DNA polymerase in the S phase of synchronized HeLa cells. They found a rise in the α-DNA polymerase activity while the cells progressed through the S phase, which continued after DNA synthesis had ceased. A concomitant increase in the γ-DNA polymerase level was noted in the early part of the S phase, whereas the β-DNA polymerase levels remained constant throughout the S phase. Perhaps the most important indication of the physiological role of the α-DNA polymerase is the ability to use a DNA template-RNA primer for in vitro DNA synthesis (2). Employing HeLa DNA that had a short RNA primer molecule hybridized to it, Spadari and Weissbach (43) found that only the α-DNA polymerase was able to synthesize DNA covalently bound to the RNA. Once the RNA-DNA molecule was formed, further extension of the DNA chain could be achieved by either α-, β-, or γ-DNA polymerase. Since short RNA molecules are generally assumed to serve as initiators during in vivo DNA replication (44,45), the results from this study, together with the evidence of increased α-DNA

polymerase activity during S phase, may imply that α-DNA polymerase is the major polymerase involved in DNA replication. Whether β- and γ-DNA polymerase are also involved in DNA replication is not clear although Mayer et al. reported that all three DNA polymerase activities increase in human lymphocyte after phytohemagglutinin induction (46). The possibility that α-DNA polymerase may form complexes with accessory proteins such as the nucleic acid helix-"unwinding" proteins from calf thymus has been raised by Herrick et al. (47). The calf thymus unwinding protein (UPI) will stimulate the activity of the α-DNA polymerase but not that of the β- or γ-DNA polymerase. This specific effect of the DNA-binding proteins on the α-DNA polymerase again provides suggestive evidence about the participation of α-DNA polymerase in <u>in vivo</u> replication.

The relatively low response of β-DNA polymerase to the stimulus of cell proliferation has led to the suggestion (41) that this enzyme is involved in a constitutive process such as DNA repair. Stenstrom et al. found that 1-β-D-arabinofuranosylcytosine triphosphate (ara-CTP) differentially inhibits replicative-type and repair-type DNA synthesis in isolated hepatocyte nuclei (48). The Ki value for the inhibition of repair-type DNA synthesis is 1000-fold higher than that of the replicative type. Since α-DNA polymerase purified from human lymphocyte is more sensitive to ara-CTP than is the β-DNA polymerase (49, 50), Stenstrom indicated that the β-DNA polymerase might be responsible for DNA repair. In addition, Bertazzoni et al., in examining the variation in DNA polymerase activities during prolonged stimulation of human lymphocyte by phytohemmagglutinin (51, 52), observed that increase of α-DNA polymerase

activity parallels the increase of DNA synthesis rate following the lectin stimulation, while the β-DNA polymerase activity reached its maximum peak at later time. In parallel studies done with ultraviolet-light-treated lymphocytes, using hydroxyurea blocking to measure repair-type DNA synthesis, Bertazzoni et al. observed that the β-DNA polymerase activity seemed to correlate with the ability of the cells to carry out repair-type DNA synthesis. It is obvious that the evidence indicating the involvement of β-DNA polymerase in repair is even more circumstantial than the evidence suggesting the involvement of the α-DNA polymerase in replicative type DNA synthesis. The actual physiological role of the β-DNA polymerase is still unknown.

The discovery of γ-DNA polymerase was prompted by the revelation of the reverse transcriptase of RNA tumor viruses (53, 54). Since both reverse transcriptase and γ-DNA polymerase use RNA templates efficiently, it was suggested that the γ-DNA polymerase may be related to the reverse transcriptase and so reflect the presence of viral particles in cell. However, γ-DNA polymerase has not been proven to be able to copy a natural RNA. Immunological studies indicate that blocking antiserum prepared against the reverse transcriptase of Rauscher leukemia virus, Woolly monkey virus, or feline virus RD-114 will not inhibit the human γ-DNA polymerase (24, 55). In addition, cellular γ-DNA polymerase and virus-induced reverse transcriptase can be clearly separated and identified in type C virus-infected cells (56, 57). Thus, the original suggestion for the involvement of γ-DNA polymerase in RNA tumor virus infection can not be supported by experimental data. The recent discovery of the identity of

γ-DNA polymerase and mitochondrial DNA polymerase leads to the suggestion that this enzyme is utilized in the synthesis of mitochondrial DNA. Since no more than 20-40% of the total γ-DNA polymerase activity is present in purified mitochondria (31, 32, 33), the γ-DNA polymerase might have functions besides mitochondrial DNA synthesis. For example, Arens et al. (58) suggests that γ-DNA polymerase is responsible for the synthesis of adenovirus.

Age-Associated Alteration of Nuclear DNA-Protein Information

Although age-related dysfunctions have attracted the attention of biologists for many years, the primary causative factors leading to senescence remain unknown. Since it has been previously proposed (59) that accumulation of errors or damage of primary genetic material might represent initiating events in cellular aging, many gerontological investigators have investigated age-associated alterations of cellular information banks, that is, of DNA and DNA-protein complexes.

In the somatic mutation theory, errors in DNA can arise in at least two ways: through miscoding in replication, or through damaged DNA left unrepaired or repaired erroneously. DNA replication is a very accurate procedure. The in vivo error rate, as measured from mutation rate data, appears to be as little as one in one billion. Since this high fidelity of DNA replication can not be explained solely by the difference in free energy between complementary and noncomplementary base pairs (60), it has been suggested that DNA polymerase might play a key role in amplifying

the base selection (61), and the fidelity of DNA polymerase could be an important factor in cellular aging (62, 63). Indeed, altered α-DNA polymerases in senescent culture chick embryo fibroblasts have been reported (64). In addition, Linn et al. have shown that the DNA polymerase isolated from the late passage of human fibroblast has decreased in vitro fidelity (65).

Direct investigation comparing DNA repair efficiency and aging has not produced consistent results. Normal fibroblasts and fibroblasts from xeroderma pigmentosum, which are defective in repair of UV-damaged DNA, have equivalent in vitro life spans (66). On the other hand, cultures of fibroblasts that have a short in vitro life-span (67) from individuals with progeria and Rothmund-Thomson syndrome (premature aging) repair DNA as well as do normal fibroblast cultures (68). Cultured skin fibroblasts from patients with Hutchinson-Guilford progeria syndrome (premature aging) are known to possess a shortened in vitro life-spam (69). Epstein et al. (70) used sedimentation in alkaline sucrose to study the rejoining of single-strand breaks in the DNA of gamma-irradiated cells from a progeriod patient and showed that the cells failed to show evidence of normal DNA rejoining. However, Regan and Setlow reinvestigated the rejoining of DNA single-strand breaks in four human diploid fibroblast cell strains and indicated that there is no inherent defect in DNA repair in progeroid cells (71). Circumstantial evidence does indicate, however, a decreased repair function in aged cells. Using exogenous DNA polymerase, Price, Modak and Makinodan found the nuclear DNA in sections of fixed brain, heart and liver tissue from aged mice possessed a higher template activity than

that from young mice. This result suggests a deficient DNA repair process, which leads to the accumulation of single stranded-region and "nicks" in DNA (72, 73). In vivo study also showed that there is an age-related increase in the incorporation of [H^3] thymidine into rat liver DNA that is independent of mitotic activity (74). In addition, Barton and Yang reported that β-DNA polymerase, presumably responsible for DNA repair, is markedly decreased in the nucleus fraction of aged mouse spleen (75).

No doubt an event such as mutation that can alter the cellular genetic information must be regarded as changes in DNA composition. However, functional deterioration at the chromatin level without mutation could also prevent or slow down the expression of cellular genetic information. For example, higher temperatures are required to dissociate the nucleoprotein complex with increasing age (76, 77, 78). Such an increase in the association of DNA with protein could potentially disturb metabolism in aged cells by interfering with the function of DNA. Indeed, a reduced RNA content of chromatin and a reduced effectiveness of nucleoprotein from old animals as template for RNA polymerase have been reported (79, 80). Another circumstantial evidence for nuclear DNA-protein complex deterioration is the decreased proliferative capacity or ability to synthesize DNA with increasing age. Such observations have been made for proliferation of mouse antibody-forming cells in vivo (81) and of human fibroblasts in vitro (82, 83). In addition, following the induction of DNA synthesis by partial hepatectomy in rat liver or isoproterenol administration in the salivary gland of mice, measurement of DNA synthesis rate show progressive lags in initiation of DNA synthesis with increasing age (79, 80).

Scope of the Present Study

The scope of the present study can be summarized as follows:

1. Identification and characterization of DNA polymerases in rat tissue. The purpose of such a study is to recognize the kinds of DNA polymerase in a rat system and to compare the polymerase properties in this system with those of other mammalian DNA polymerases. The biochemical characterization of rat DNA polymerases and the comparison of DNA polymerase activities in tissues of various mitotic index should provide an experimental foundation for subsequent age-related studies of DNA polymerase and possibly give some insight into the biological functions of these enzymes.

2. Quantitative and qualitative comparison of DNA polymerases from young and old rat tissue. Since there are age-associated alterations of DNA polymerases in some mammalian systems, it is worthwhile to examine whether such alterations occur in the aging process in the rat. A finding of alterations in DNA polymerase activity may explain the change of DNA metabolism and accumulation of mutations in aging.

3. Comparison of the structure of DNA from the tissue of young and old rats. A correlation between a quantitative or qualitative alteration of DNA polymerase and a structural alteration of DNA may suggest a cause-effect relationship between these two aging phenomena.

The primary objective of this research was to gain some insight into the understanding of the molecular genetics of aging.

CHAPTER 2

PARTIAL PURIFICATION AND CHARACTERIZATION OF THREE DNA POLYMERASES ISOLATED FROM RAT SPLEEN

Summary

We describe the partial purification and characterization of three DNA polymerases isolated from spleens of Fischer 344 rat. These three DNA polymerases can be distinguished from one another by their chromatographic migrations, template specificities, optimal pH and salt concentration for enzyme activities, divalent cation requirements, sedimentation velocities, and sensitivities to specific inhibitors. On the basis of the criteria for the nomenclature of eukaryotic DNA polymerases, two of these enzymes are clearly α- and β-DNA polymerase; the third enzyme resembles the γ-DNA polymerase in the aspects of template specificity and optimal salt concentration for enzyme activity.

Introduction

Higher eukaryotic cells contain at least three DNA polymerases which have been named DNA polymerase α, β, and γ (86). In general, these DNA polymerases can be distinguished from one another by various parameters including molecular weight, subcellular localization and enzymatic characteristics [reviewed by Bollum (2) and Weissbach (5)]. However, examination of literature often reveal variations in the

characteristic properties of the three polymerases, presumably due to different animal origins or due to different isolation procedures. In our research on DNA polymerases in relation to the aging process of mammals, three DNA polymerase activities can be detected in the spleen of Fischer 344 rats. The present study was undertaken to compare the properties of the three DNA polymerases isolated from the same Fischer 344 rat spleens and assess the applicability of criteria currently used for the identification of eukaryotic DNA polymerases.

Materials and Methods

Materials and Equipments

Whatman phosphocellulose grade P-1 was obtained from Reeve Angel, Clifton, New Jersey, and was precycled following the method of Yoneda and Bollum (87). Hydroxylapatite, Hypatite C, was purchased from Clarkson Chemical Company, Incorporated, Williamsport, Pennsylvania; poly(dT), poly(rA), and poly[d(A-T)] were obtained from Miles Laboratories, Kankakee, Illinois; $(dT)_9$ and all other synthetic template-primers were obtained from P-L Biochemicals, Incorporated, Milwaukee, Wisconsin. Native calf thymus DNA and DNase I were purchased from Worthington Biochemical Corporation (Freehold, New Jersey). Nonradioactive dNTPs (deoxynucleotide triphosphates) were products of P-L Biochemicals. Radioactively labeled dNTPs were obtained from New England Nuclear Corporation, Boston, Massachusetts. Sucrose of RNase free grade was obtained from Schwarz/Mann, Inc. (Orangeburg, New York). All centrifugation was performed in the RC-2 and RC-2B (Ivan Sorvall, Inc., Newton, Connecticut), and all

ultracentrifugation in the Model L or Model L2-65B (Beckman Instruments, Inc., Palo Alto, California).

Rats

Pathogen-free male Fischer 344 rats were raised in the Biology Division, Oak Ridge National Laboratory. Spleens which were tumor free and of normal size were removed from young adult (three months old). Tissue not immediately used was frozen in liquid nitrogen and then stored at -70°C.

Preparation of Tissue Extracts

Thirty-five grams of frozen rat spleens were thawed rapidly, minced, and then homogenized in four volumes (v/w) of 0.25 M sucrose containing 0.05 M TrisCl(pH 7.6 at 22°C), 0.025 M KCl, 1 mM dithiothreitol and 3 mM $MgCl_2$ using a teflon pestle and glass homogenizer. The homogenate was then adjusted to contain 0.5 M KCl for polymerase extraction and centrifuged at 165,000 x g for 60 minutes in a 50 Ti rotor. The supernatant fraction was used immediately for further purification by column chromatography. All steps were performed at 0-4°C.

Isolation of α, β, and γ-DNA Polymerases

The enzyme extract was adjusted to contain 0.02 M KPi (pH 7.5 at 22°C) and applied to a 1.6 cm x 14 cm Hydroxylapatite column equilibrated with 0.5 M KCl containing 0.02 M KPi, 1 mM dithiothreitol, 10% glycerol, and 3 mM $MgCl_2$. The column was washed with 60 ml of equilibrating buffer and then eluted with 500 ml of 0.02 to 0.08 M linear KPi gradient.

Fractions of 10 ml were collected with a flow rate of 1 ml/minute. Active fractions containing polymerase activity were pooled, diluted to contain 0.1 M KCl, 10% glycerol, 0.01 M KPi, 1 mM dithiothreitol, and then absorbed on a phosphocellulose column (1.6 cm x 14 cm) that was equilibrated with 0.1 M KCl, 10% glycerol, 0.01 M KPi, and 1 mM dithiothreitol. The column was washed with the starting gradient buffer and eluted with a linear KCl gradient of 0.1 to 0.6 M KCl (total volume of 500 ml). Fractions of 10 ml were collected with a flow rate of 0.5 ml/minute.

The break through from the hydroxylapatite column was diluted to contain 0.23 M KCl, 10% glycerol, 0.01 M KPi, 1 mM dithiothreitol, and then absorbed to a separate phosphocellulose column (1.6 x 18 cm) that was equilibrated with 0.23 M KCl, 10% glycerol, 0.01 M KPi, and 1 mM dithiothreitol. The column was washed with the starting gradient buffer and eluted with a linear KCl gradient of 0.23 to 0.6 M KCl (total volume of 700 ml). Active fractions containing polymerase activity were pooled, diluted to contain 0.1 M KCl with the buffer solution, and then concentrated by rechromatography in 1.5 ml size phosphocellulose column (in a disposable tuberculin syringe) using one step elution with the same buffer solution containing 0.5 M KCl (0.22 M peak) or 1.0 M KCl (0.33 M and 0.43 M peaks). The final pooled enzymes were divided into aliquots in vials. DNA polymerases were stored in 50% glycerol at -20°C. All steps were performed at 0-4°C.

Sucrose Gradient Centrifugation

Sucrose gradient centrifugation was performed by using a Spinco SW 56 Ti rotor. Partially purified DNA polymerase (60 µl) were layered on

top of 3.8 ml solution containing 10-25% sucrose linear gradient, 10 mM TrisCl(pH 8.0 at 22°C), 0.25 M KCl and 1 mM dithiothreitol in polyallomer tubes. Centrifugation was performed at 50,000 rpm for 20 hrs at 4°C. Fractions of 0.13 ml were collected from the bottom of the tube. Portion of each fraction (5 µl) was assayed for polymerase activity. Sedimentation coefficient and molecular weight of the DNA polymerases were estimated by the method of Martin and Ames (88) using the protein standard: chymotrypsinogen a (M.W. 22,000), bovine serum albumin (M.W. 67,000), and glucose oxidase (M.W. 153,000).

Determination of Polymerase Activity

DNA polymerase activity was measured by the polymerization of radioactive dNTP into acid-insoluble form in the presence of template-primer complex. Reactions were carried out in the following standard system unless otherwise indicated. Appropriate amounts of enzyme protein (within the range where protein concentration is linear-related with DNA synthesis) was added to the reaction mixture to a final volume of 50 µl which contains 20 mM TrisCl(pH 8.0 at 22°C), 5 mM dithiothreitol, 0.17 mg/ml bovine serum albumin, 25-200 mM KCl, appropriate concentration of Mg^{2+} and/or Mn^{2+}, 60 µM of appropriate dNTP including [^{3}H]dTTP or [^{3}H]dGTP at 17.5 µCi/ml, 0.02 mg/ml of synthetic template-primer complex or 0.1 mg/ml activated DNA. After incubation in a water bath at 37°C for 15 minutes, incorporation of radioactivity into acid insoluble form was measured by pipetting the reaction mixture onto a filter paper disk (Whatman 3 MM), which was subsequently washed and processed for liquid scintillation counting (89).

Activated DNA was prepared by DNase I digestion of calf thymus DNA according to the procedures of Adams et al. (90). Poly(rA)·poly(dT) was prepared by mixing an equal volume of 0.4 mg/ml poly(rA) and 0.2 mg/ml of poly(dT) in 10 mM NaCl at 45°C for ten minutes and then slowly cooling over the next three hours to 23°C. Poly(rA)·$(dT)_9$ was prepared by simply mixing the poly(rA) with $(dT)_9$ in H_2O at a base ratio of 5:1. All template-primer complexes were stored at -20°C.

Protein Determination

Protein was determined by the method of Bramhall et al. (91).

Results

Hydroxylapatite and Phosphocellulose Chromatography of DNA Polymerase

When the spleen extract was chromatographed in hydroxylapatite column using the condition described, almost all the poly(rA)·poly(dT)-dependent DNA polymerase activity in the extract passed through the hydroxylapatite column whereas most activated DNA dependent DNA polymerase was absorbed and, with the use of a KPi gradient, was eluted as a single peak at 0.035 M KPi (Figure 2.1).

The fractions containing the activated DNA-dependent activity were pooled, adjusted to contain 0.1 M KCl, and then chromatographed in a phosphocellulose column. A single peak of activity was obtained at 0.22 M region of the KCl gradient (Figure 2.2). Since this DNA polymerase can use activated DNA as template efficiently but use poly(rA) as template poorly, we assigned this DNA polymerase as α-DNA polymerase.

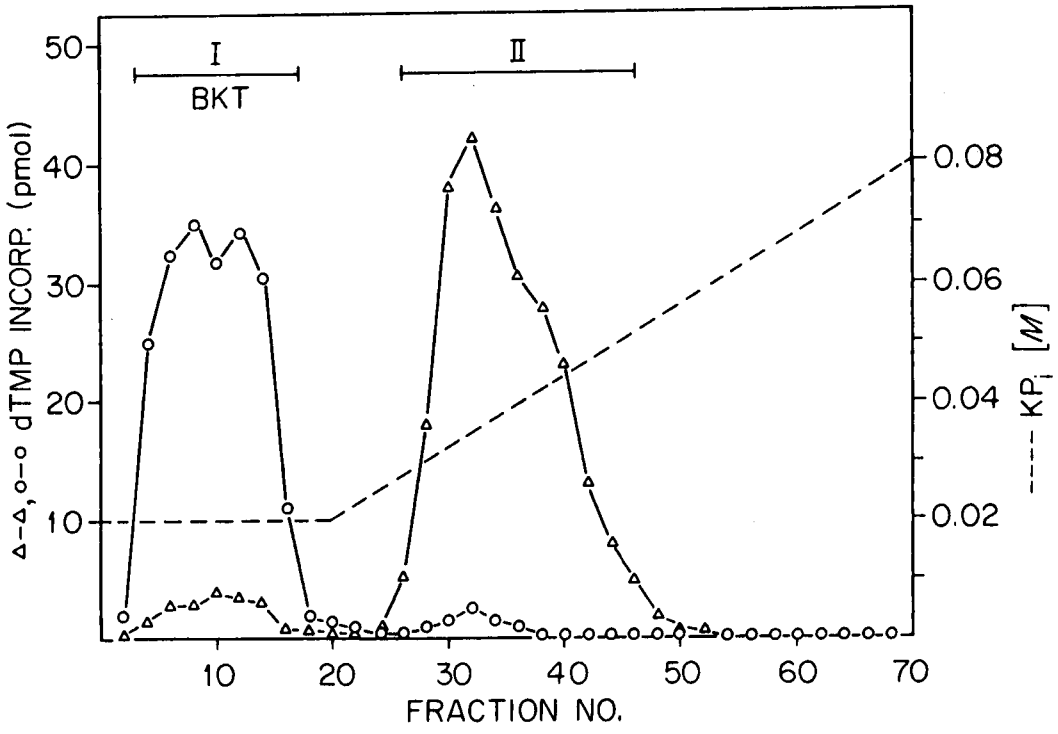

Figure 2.1. Hydroxylapatite column chromatography of rat spleen extract.

0 ——— 0 poly(rA)·poly(dT) as template

Δ ——— Δ activated DNA as template

The preparation of enzyme extract, chromatography, elution, and determination of DNA polymerase activity was performed as described under "Materials and Methods." Five µl of each fraction was used for teh assay of DNA polymerase activity. The incubation time for polymerase activity assay was 1 hr.

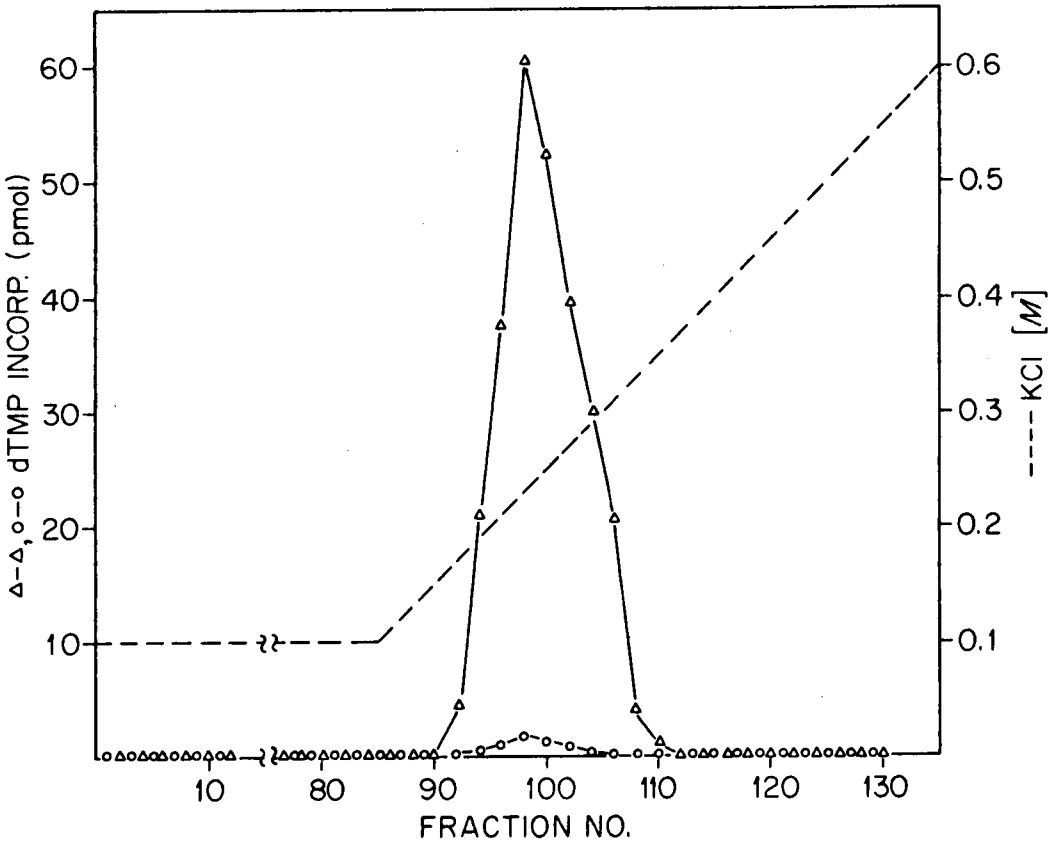

Figure 2.2. Phosphocellulose column chromatography of pool II from the hydroxylapatite column elution as shown in Figure 2.1.

Δ ——— Δ activated DNA as template

0 ——— 0 poly(rA)·poly(dT) as template

The preparation of enzyme extract, chromatography, elution, and determination of DNA polymerase activity was performed as described under "Materials and Methods." Five µl of each fraction was used for the assay of DNA polymerase activity. The incubation time for polymerase activity assay was 1 hr.

The breakthrough fractions from the hydroxylapatite column were pooled, adjusted to contain 0.23 M KCl and applied to a separate phosphocellulose column. Two peaks of activity were obtained at 0.33 M and 0.43 M regions of the KCl gradient (Figure 2.3). The polymerase of the first peak can use both poly(rA)·poly(dT) and poly(rA·$(dT)_9$ as template-primer efficiently, whereas the polymerase of the second peak can use only poly(rA)·poly(dT) as template-primer. These two peaks were apparently not the two forms of a same enzyme in equilibrium with each other, since respective rechromatography in phosphocellulose showed elution of single activities at the same KCl concentration. As we will show later, the polymerase in these two peaks are certainly distinct from each other. The polymerase in the second peak is a β-DNA polymerase, whereas the polymerase in the first peak (0.33 M KCl peak) is similar to γ-DNA polymerase in many respects.

Template Specificity

The three isolated DNA polymerases were examined with various template-primers. The reaction mixtures contained either 0.5 mM Mn^{2+} plus 1.5 mM Mg^{2+}. Under these conditions the polymerases showed no significant incorporation of deoxyribonucleotide into acid precipitable form when the template-primer complex was omitted in the reaction mixture (Tables 2.1, 2.2 and 2.3).

Activated DNA can be used by α-DNA polymerase as an efficient template. All four nucleotides were incorporated although in different rates. Synthetic DNA template-primer complex, especially poly(dA)·-

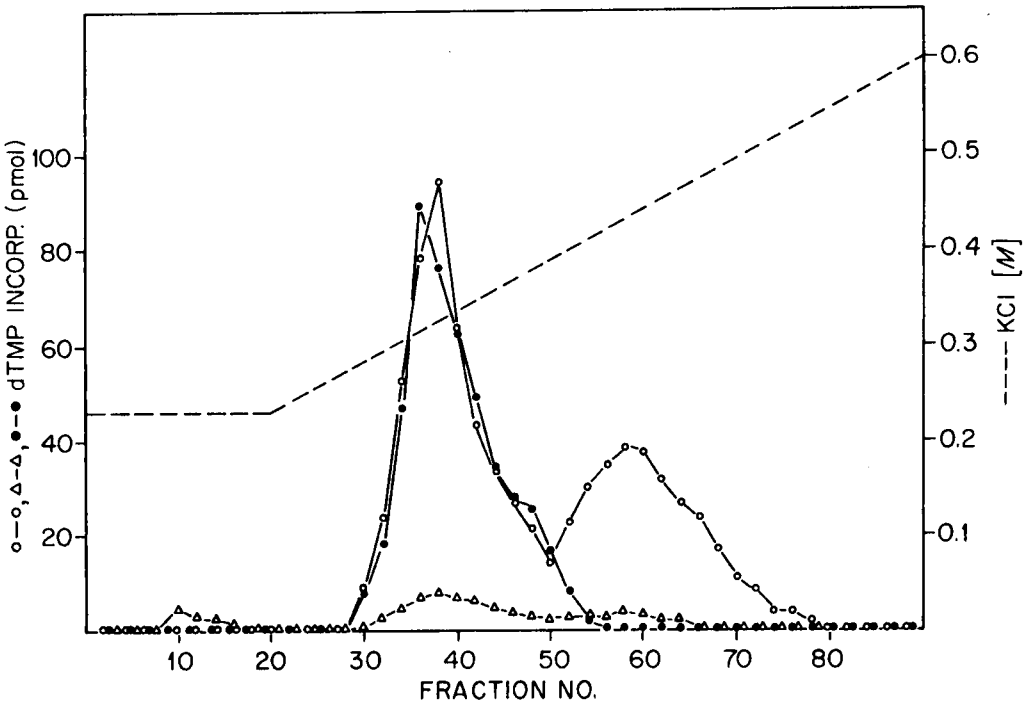

Figure 2.3. Phosphocellulose column chromatography of pool I (BKT) from the hydroxylapatite column elution as shown in Figure 2.1

Δ ——— Δ activated DNA as template

0 ——— 0 poly(rA)·poly(dT) as template

● ——— ● poly(rA)·$(dT)_9$ as template

The preparation of enxyme extract, chromatography, elution, and determination of DNA polymerase activity was performed as described under "Materials and Methods." Five µl of each fraction was used for the assay of DNA polymerase activity. The incubation time for polymerase activity assay was 1 hr.

TABLE 2. 1

TEMPLATE-PRIMER SPECIFICITY OF α-DNA POLYMERASE

Template	Primer	Deoxynucleotides		Amount of Deoxynucleotide Per Reaction[a] Incorporated (pmol)	
				Divalent Cation	
		^{3}H-Labeled	Unlabeled	Mg^{2+}(1.5 mM) Mn^{2+}(0.5 mM)	Mg^{2+}(4 mM)
None	None	dTTP	None	0	0
None	None	dGTP	None	0	0
Activated C.T.DNA	None	dTTP	dATP,dCTP,dGTP	99.20	128.75
Activated C.T.DNA	None	dATP	dTTP,dCTP,dGTP	40.97	63.20
Activated C.T.DNA	None	dGTP	dATP,dTTP,dCTP	34.68	44.23
Activated C.T.DNA	None	dCTP	dATP,dTTP,dGTP	65.90	84.35
Poly(dA)	poly(dT)	dTTP	None	12.00	3.82
Poly(dT)	poly(dA)	dATP	None	5.36	0.66
Poly(dA)	$(dT)_{12-18}$	dTTP	None	134.97	22.46
Poly(dC)	poly(dG)	dGTP	None	18.55	21.85
Poly(dC)	$(dG)_{12-18}$	dGTP	None	61.83	67.42
Poly[d(A-T)]	None	dTTP	dATP	21.14	15.39
Poly(dT)	poly(rA)	dATP	None	15.46	15.38

TABLE 2.1 (continued)

Template	Primer	Deoxynucleotides		Amount of Deoxynucleotide Per Reaction[a] Incorporated (pmol)	
				Divalent Cation	
		^{3}H-Labeled	Unlabeled	Mg^{2+}(1.5 mM) Mn^{2+}(0.5 mM)	Mg^{2+}(4 mM)
Poly(rA)	poly(dT)	dTTP	None	0.93	0.42
Poly(rA)	$(dT)_9$	dTTP	None	0.74	0.29
Poly(rC)	poly(dI)	dGTP	None	3.74	1.22
Poly(rA)	poly(rU)	dTTP	None	0.47	0.11

[a]Assays were performed under standard assay conditions as described under "Material and Methods" except that they contained the divalent cations, template primer, and deoxynucleotide as indicated. The salt concentration in reaction mixture was 25 mM, 100 mM, and 200 mM for α-, β-, and γ-DNA polymerase, respectively. Reaction mixture contained 15 μg of α-DNA polymerase protein, or 3 μg of β-DNA polymerase protein, or 10 μg of γ-DNA polymerase protein.

TABLE 2.2

TEMPLATE-PRIMER SPECIFICITY OF β-DNA POLYMERASE

Template	Primer	Deoxynucleotides		Amount of Deoxynucleotide Incorporated Per Reaction[a] (pmol)	
				Divalent Cation	
		^{3}H-Labeled	Unlabeled	Mg^{2+}(1.5 mM) Mn^{2+}(0.5 mM)	Mg^{2+}(4 mM)
None	None	dTTP	None	0	0
None	None	dGTP	None	0	0
Activated C.T.DNA	None	dTTP	dATP,dCTP,dGTP	12.62	17.40
Activated C.T.DNA	None	dATP	dTTP,dCTP,dGTP	4.99	7.25
Activated C.T.DNA	None	dCTP	dATP,dTTP,dGTP	6.61	8.88
Activated C.T.DNA	None	dGTP	dATP,dTTP,dCTP	4.30	5.81
Poly(dA)	poly(dT)	dTTP	None	224.61	46.78
Poly(dT)	poly(dA)	dATP	None	60.40	12.66
Poly(dA)	$(dT)_{12-18}$	dTTP	None	257.15	135.89
Poly(dC)	poly(dG)	dGTP	None	43.84	136.05
Poly(dC)	$(dG)_{12-18}$	dGTP	None	67.69	211.85
Poly[d(A-T)]	None	dTTP	dATP	36.09	112.93
Poly(dT)	poly(rA)	dATP	None	2.85	1.43

TABLE 2.2 (continued)

Template	Primer	Deoxynucleotides		Amount of Deoxynucleotide Per Reaction[a] Incorporated (pmol)	
				Divalent Cation	
		^{3}H-Labeled	Unlabeled	Mg^{2+}(1.5 mM) Mn^{2+}(0.5 mM)	Mg^{2+}(4 mM)
Poly(rA)	poly(dT)	dTTP	None	143.58	13.68
Poly(rA)	$(dT)_9$	dTTP	None	17.87	12.48
Poly(rC)	poly(dI)	dGTP	None	10.20	9.03
Poly(rA)	poly(rU)	dTTP	None	1.60	0.97

[a]Assays were performed under standard assay conditions as described under "Material and Methods" except that they contained the divalent cations, template primer, and deoxynucleotide as indicated. The salt concentration in reaction mixture was 25 mM, 100 mM, and 200 mM for α-, β-, and γ-DNA polymerase, respectively. Reaction mixture contained 15 μg of α-DNA polymerase protein, or 3 μg of β-DNA polymerase protein, or 10 μg of γ-DNA polymerase protein.

TABLE 2.3

TEMPLATE-PRIMER SPECIFICITY OF γ-DNA POLYMERASE

Template	Primer	^{3}H-Labeled	Unlabeled	Amount of Deoxynucleotide Per Reaction[a] Incorporated (pmol)	
				Divalent Cation	
				Mg^{2+}(1.5 mM) Mn^{2+}(0.5 mM)	Mg^{2+}(4 mM)
None	None	dTTP	None	0	0
None	None	dGTP	None	0	0
Activated C.T.DNA	None	dTTP	dATP,dCTP,dGTP	16.41	16.40
Activated C.T.DNA	None	dATP	dTTP,dCTP,dGTP	6.44	6.10
Activated C.T.DNA	None	dCTP	dATP,dTTP,dGTP	8.50	9.00
Activated C.T.DNA	None	dGTP	dCTP,dTTP,dCTP	5.87	5.88
Poly(dA)	poly(dT)	dTTP	None	37.95	3.66
Poly(dT)	poly(dA)	dATP	None	8.94	2.98
Poly(dA)	$(dT)_{12-18}$	dTTP	None	30.25	6.92
Poly(dC)	poly(dG)	dGTP	None	40.37	119.43
Poly(dC)	$(dG)_{12-18}$	dGTP	None	37.45	173.48
Poly[d(A-T)]	None	dTTP	dATP	45.12	31.12
Poly(dT)	poly(rA)	dATP	None	4.05	0.49

TABLE 2.3 (continued)

Template	Primer	Deoxynucleotides		Amount of Deoxynucleotide Per Reaction[a] Incorporated (ρmol)	
				Divadent Cation	
		^{3}H-Labeled	Unlabeled	Mg^{2+}(1.5 mM) Mn^{2+}(0.5 mM)	Mg^{2+}(4 mM)
Poly(rA)	poly(dT)	dTTP	None	151.94	11.02
Poly(rA)	$(dT)_9$	dTTP	None	195.96	92.51
Poly(rC)	poly(dI)	dGTP		15.38	32.71
Poly(rA)	poly(rU)	dTTP	None	37.46	4.01

[a]Assays were performed under standard assay conditions as described under "Material and Method" except that they contained the divalent cations, template primer, and deoxynucleotide as indicated. The salt concentration in reaction mixture was 25 mM, 100 mM, and 200 mM for α-, β-, and γ-DNA polymerase, respectively. Reaction mixture contained 15 μg of α-DNA polymerase protein, or 3μg of β-DNA polymerase protein, or 10 μg of γ-DNA polymerase protein.

$(dT)_{12-18}$, can also be used by α-DNA polymerase efficiently. The level of deoxynucleotide incorporation was higher in the presence of poly(dA)·-$(dT)_{12-18}$ and poly(dC)·$(dG)_{12-18}$ than in reactions containing poly(dA)·poly(dT), poly(dC)·poly(dG), and poly[d(A-T)]. None of the RNA templates tested can be used by α-DNA polymerase efficiently. We also tested the ability of all three DNA polymerases to use RNA initiators when DNA was used as template. The results showed that poly(rA), with poly(dT) as template, is effective initiator of polydeoxynucleotide replication catalyzed by α-DNA polymerase. Although both DNA polymerase β and DNA polymerase γ can use this template-primer system, their efficiency is much lower than that of α-DNA polymerase (Table 2.4).

β-DNA polymerase can also use activated DNA as template, however, the rate of deoxynucleotide incorporation is much lower compared with that catalyzed by α-DNA polymerase. β-DNA polymerase can use DNA templates such as poly(dA), poly(dC), and poly[d(A-T)] very efficiently, and the length of the DNA primer does not seem to change the rate of deoxynucleotide incorporations. Poly(rA)·poly(dT) is most effective among all the RNA template-DNA primer being tested by β-DNA polymerase. RNA template-RNA primer, however, is not an effective template-primer complex for this enzyme.

γ-DNA polymerase, like β-DNA polymerase, cannot use activated DNA as well as α-DNA polymerase. This enzyme shows four to five times more activity with poly(rA)·poly(dT) than with poly(dA)·poly(dT); and six times more activity with poly(rA)·$(dT)_9$ than with poly(dA)·$(dT)_{12-18}$ This preference for synthetic ribohomopolymers rather than natural or

TABLE 2.4

ACTIVITY OF DAN POLYMERASES WITH poly(dT)·poly(rA) AND poly(dT)·poly(dA) AS TEMPLATES[a]

DNA Polymerases	Activity with poly(dT)·poly(dA) ρmol of dAMP incorporated	Activity with poly(dT)·poly(rA) ρmol of dAMP incorporated	Relative Activity $\frac{\text{poly(dT)·poly(rA)}}{\text{poly(dT)·poly(dA)}}$
α	5.36	15.46	2.88
β	60.40	2.85	0.05
γ	8.94	4.05	0.45

[a]Reaction mixture contained 25 mM, 100 mM, 200 mM KCl for α-, β-, and γ-DNA polymerase, respectively. 1.5 mM Mg^{2+} and 0.5 mM Mn^{2+} were present in reaction mixture. Assays were performed under standard assay conditions as described under "Materials and Methods."

synthetic DNA templates is a characteristic property of γ-DNA polymerases, as reported for those isolated from other sources such as human tissue (23) and wheat embryos (92). Poly(dC), however, is an effective template for rat spleen γ-DNA polymerase. One of the striking template specificity of γ-DNA polymerase is its ability to use RNA template-RNA primer for deoxynucleotide incorporation, while α-, and β-DNA polymerase cannot. Wilson et al. has also reported that γ-DNA polymerase isolated from mouse myeloma can use poly(rA)·$(rU)_{10}$ as template-primer complex very efficiently (26). However, it should be cautious to imply that γ-DNA polymerase might be involved in RNA tumor virus replication since we have not been able to show that murine leukemia 70S viral RNA can be used as template by this enzyme.

Optimal pH, Optimal Salt Concentration, and Divalent Cations Requirement in Reaction Mixture

Since poly(dC)·(dG)12-18 can be used as efficient template-primer by all three DNA polymerases, we choose to use this template-primer for testing the effect of pH and salt concentration on enzyme activity of these partially purified DNA polymerases. The results showed that α-DNA polymerase has optimal activity at pH 7.8 (TrisCl buffer) whereas the optimal pH for β- and γ-DNA polymerase fall in more alkaline regions (Figure 2.4).

It has been reported that α-DNA polymerase is inhibited by salt concentrations above 25 mM (1). We are able to confirm this and show that the α-DNA polymerase enzyme activity is inhibited almost 100% when the salt concentration is raised to 150 mM. β-DNA polymerase exhibit

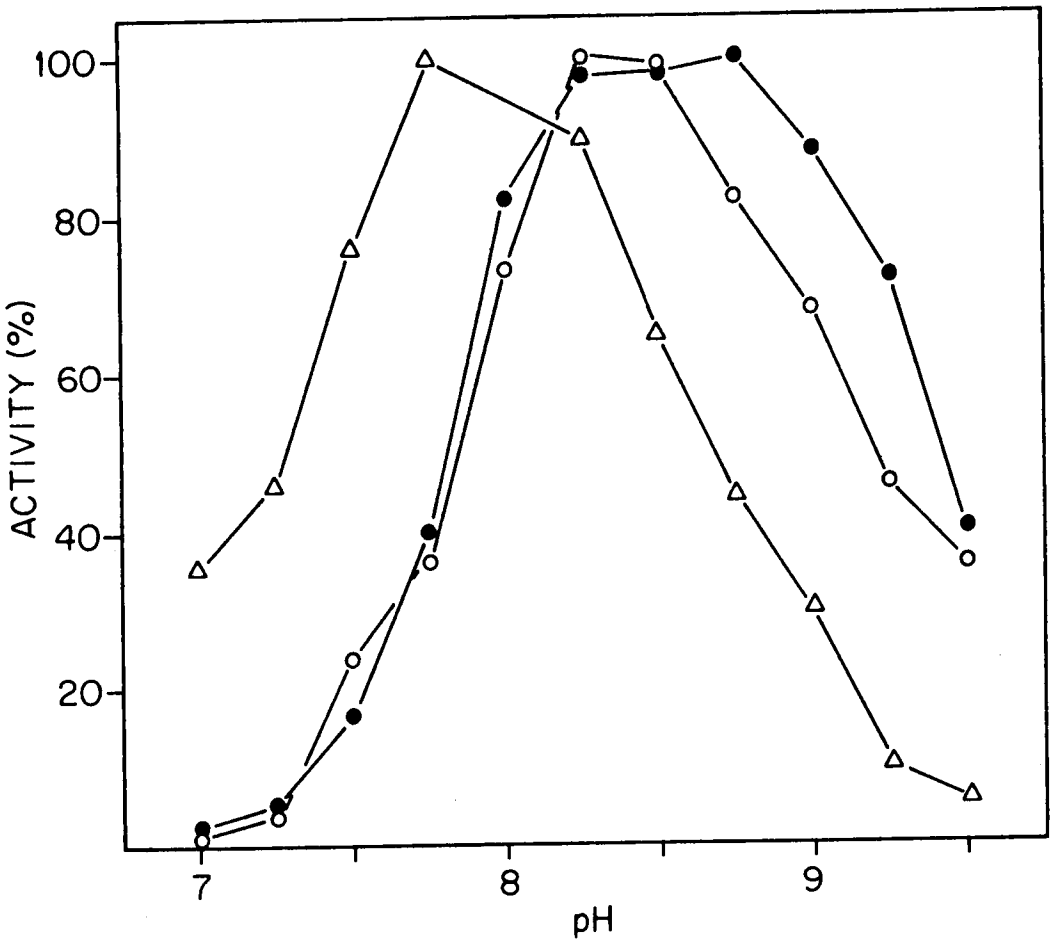

Figure 2.4. Effect of pH on rat spleen DNA polymerase activity.

Reactions were carried out in the presence of 30 mM Tris-HCl buffer at various pH, 0.02 mg/ml poly(dC)·$(dG)_{12-18}$ and 4 mM Mg^{2+} were included in each reaction mixture. The salt concentration was 25 mM, 100 mM, and 200 mM for α-, β-, and γ-DNA polymerase.

Δ ——— Δ α-DNA polymerase

0 ——— 0 β-DNA polymerase

● ——— ● γ-DNA polymerase

optimal activity at salt concentrations of 100 mM to 150 mM. The enzyme activity then decreased sharply in higher salt concentrations. The activity of γ-DNA polymerase is optimal in 200 mM KCl and still retains 75% activity when the salt concentration is as high as 500 mM. The effect of salt concentration on DNA polymerase activity is shown in Figure 2.5.

The activity of DNA polymerases is known to depend upon the presence of divalent cations in the reaction mixture. In Table 2.1 (p. 24) we have shown that when Mg^{2+} is present in reaction mixture, the addition of Mn^{2+} may increase or inhibit the DNA polymerase activity depending on the kind of template-primer utilized. Divalent cations requirement seemed to depend primarily upon template-primer used in the reaction mixture. For example, all three DNA polymerases prefer the presence of both Mg^{2+} (1.5 mM) and Mn^{2+} (0.5 mM) when poly(dA)·$(dT)_{12-18}$ is used as template-primer while the presence of Mg^{2+} alone is preferred when poly(dC)·poly(dG) is used as template-primer. However, the optimal concentration of divalent cations might also depend on the DNA polymerase used. We found that when poly(dC)·$(dG)_{12-18}$ is used as template-primer, all three DNA polymerases, especially β- and γ-DNA polymerases, require the addition of Mg^{2+} for optimal enzyme activity. Optimum Mg^{2+} concentration was 2 to 8 mM for both α- and β-DNA polymerases and 16 mM for γ-DNA polymerase. When poly-(dA)·(dT)12-18 is used as template-primer, divalent cations is also required for optimal enzyme activity. 0.5 mM Mn^{2+} enhances the enzyme activity of all three DNA polymerases, and the optimal cation concentration is 0 to 4 mM Mg^{2+} plus 0.5 mM Mn^{2+} (Figure 2.6).

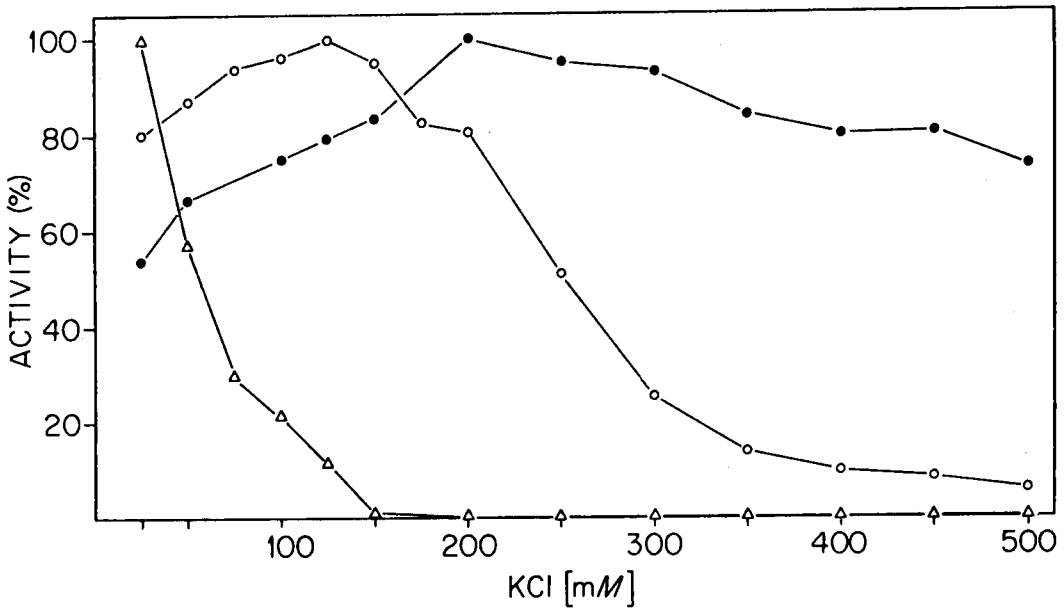

Figure 2.5. Effect of KCl concentration on DNA polymerase activity.

Assays were performed under standard assay conditions as described under "Methods" except the concentration of KCl varies as shown in the figure. The reaction mixture contains 4 mM Mg^{2+} and 0.02 mg/ml poly(dC)·$(dG)_{12-18}$.

Δ ——— Δ α-DNA polymerase

0 ——— 0 β-DNA polymerase

● ——— ● γ-DNA polymerase

Figure 2.6. Effect of divalent cations on α-, β-, and γ-DNA polymerase activity.

Assays were performed under standard assay conditions as described under "Methods" except the divalent cation concentration was varied as shown in the figure, and the KCl concentration is 25 mM, 100 mM, 200 mM for α-, β-, and γ-DNA polymerase respectively.

A: α-DNA polymerase

B: β-DNA polymerase

C: γ-DNA polymerase

● ------ ● poly(dC)·$(dG)_{12-18}$ was used as template

Δ ——— Δ poly(dA)·$(dT)_{12-18}$ was used as template with 0.5 mM Mn^{2+} in reaction mixture.

▲ ——— ▲ poly(dA)·$(dT)_{12-18}$ was used as template with 0.1 mM Mn^{2+} in reaction mixture.

0 ——— 0 poly(dA)·$(dT)_{12-18}$ was used as template

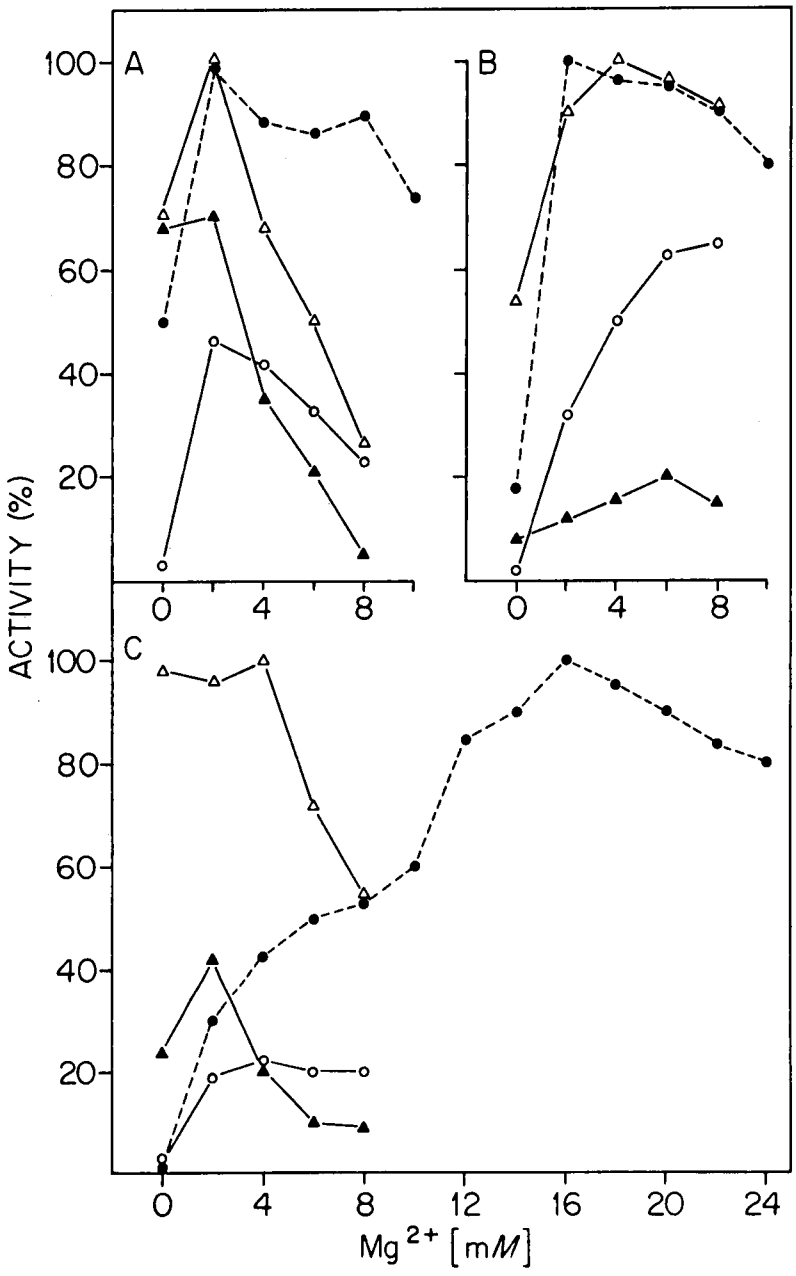

Figure 2.6

Inhibition of DNA Polymerase by Ethidium Bromide, N-ethylmaleimide, and Phosphate

Ethidium bromide is known to inhibit the activity of α-DNA polymerase (93). The sensitivity of β- and γ-DNA polymerase to ethidium bromide is not clear due to the different results reported by different investigators. We found that all three DNA polymerases are inhibited by ethidium bromide although to different extents (Figure 2.7). The γ-DNA polymerase is more resistant than α- and β-DNA polymerase.

A characteristic property of the α-DNA polymerase is its sensitivity to sulfhydryl group blocking reagent such as N-ethylmaleimide, while the β-DNA polymerase has no requirement for sulfhydryl groups and is relatively insensitive to sulfhydryl group blocking reagent (2). Here we found that α-DNA polymerase activity is completely blocked when 1 mM N-ethylmaleimide is present in reaction mixture while β- and γ-DNA polymerases are relatively insensitive to N-ethylmaleimide. Both β- and γ-DNA polymerase still retain 20% of the activity when N-ethylmaleimide is included in the reaction mixture at a concentration of 10 mM (Figure 2.8).

Knopf et al. have reported that γ-DNA polymerase is stimulated by 50 mM phosphate in the presence of KCl, whereas β-DNA polymerase activity is considerably inhibited under the same condition (12, 22). We have tested the effect of phosphate on rat spleen β- and γ-DNA polymerase activities by using poly(dC)·oligo(dG) as template-primer. The results showed that γ-DNA polymerase was stimulated only 20% in the presence of 20 mM KPO_4 and was inhibited by concentrations above 40 mM KPO_4 while β-DNA polymerase activity was inhibited 20% in 20 mM KPO_4 (Figure 2.9).

Figure 2.7. Inhibition of DNA polymerase activity by ethidium bromide.

Assays were performed under standard assay conditions as described under "Methods" except the concentration of ethidium bromide varied as shown in the figure. The template used was poly(dC)·$(dG)_{12-18}$, the concentration of Mg^{2+} was 4 mM, the salt concentration was 25 mM, 100 mM, 200 mM for α-, β-, and γ-DNA polymerase respectively.

Δ ——— Δ α-DNA polymerase

0 ——— 0 β-DNA polymerase

● ——— ● γ-DNA polymerase

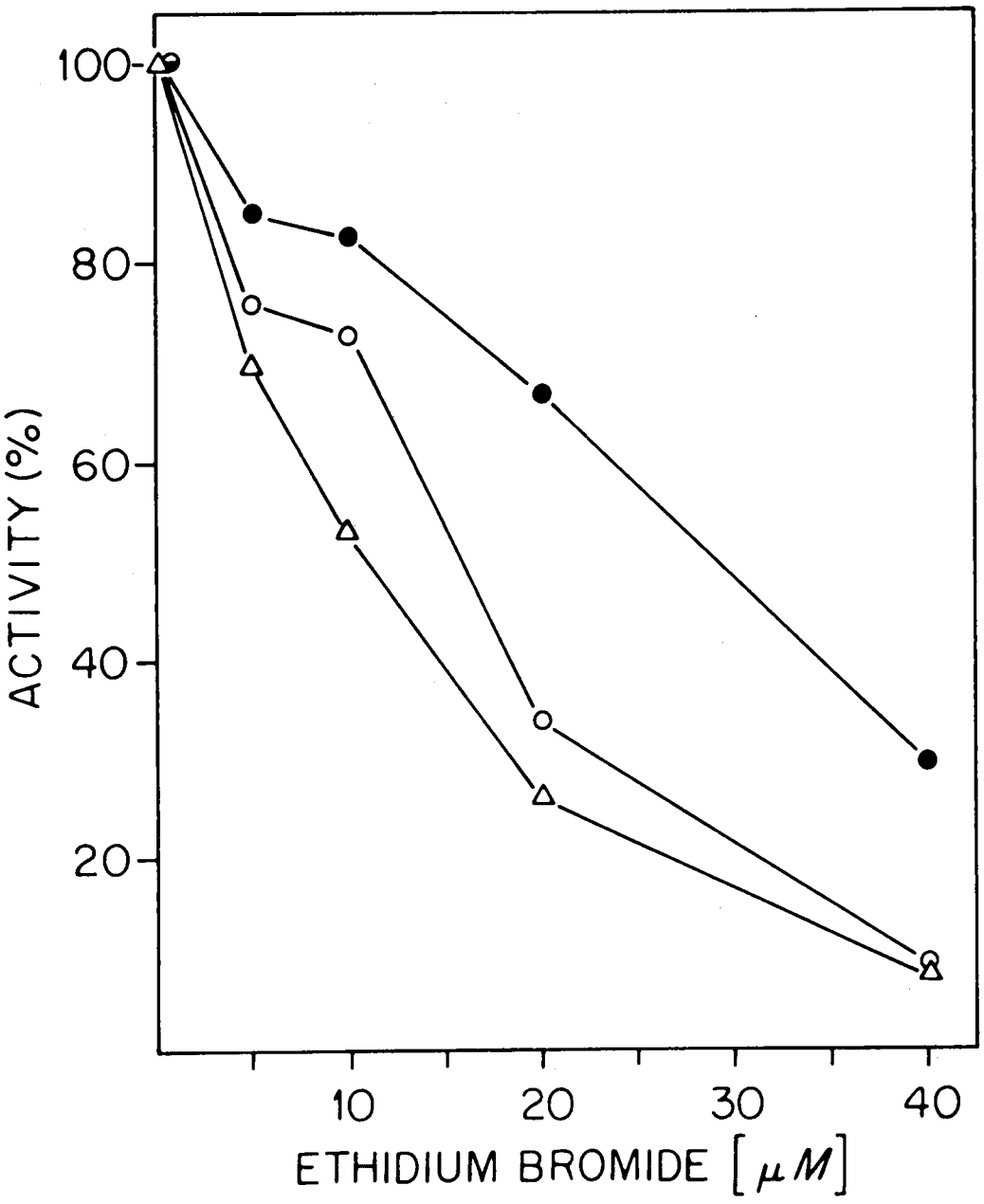

Figure 2.7

Figure 2.8. Inhibition of DNA polymerase activity by N-ethylmaleimide.

Assays were performed under standard conditions as described under "Methods" except the dithiothreitol was omitted in reaction mixture. The template used was poly(dC)·$(dG)_{12-18}$, the concentration of Mg^{2+} was 4 mM, the concentration of salt was 25 mM, 100 mM, and 200 mM for α-, β-, and γ-DNA polymerase respectively.

Δ ——— Δ α-DNA polymerase

0 ——— 0 β-DNA polymerase

● ——— ● γ-DNA polymerase

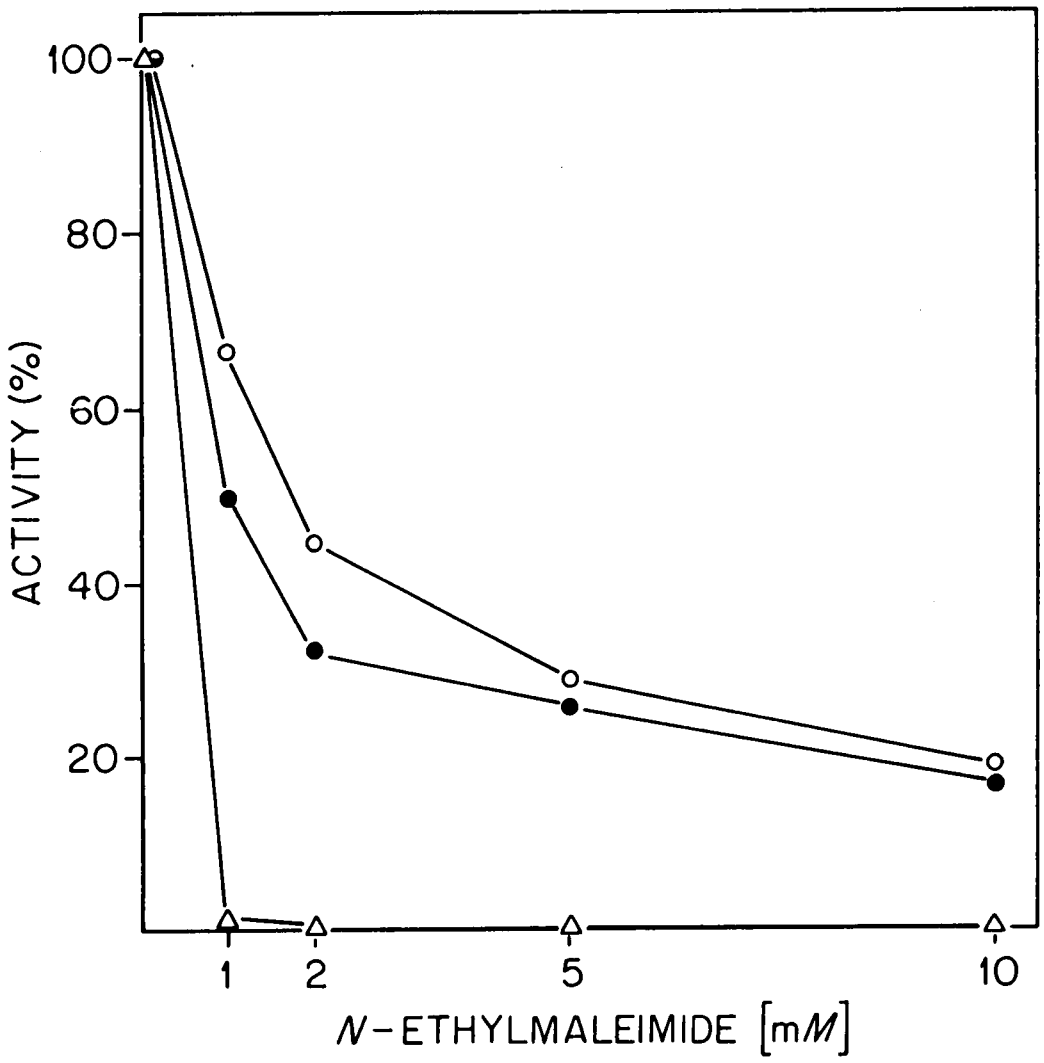

Figure 2.8

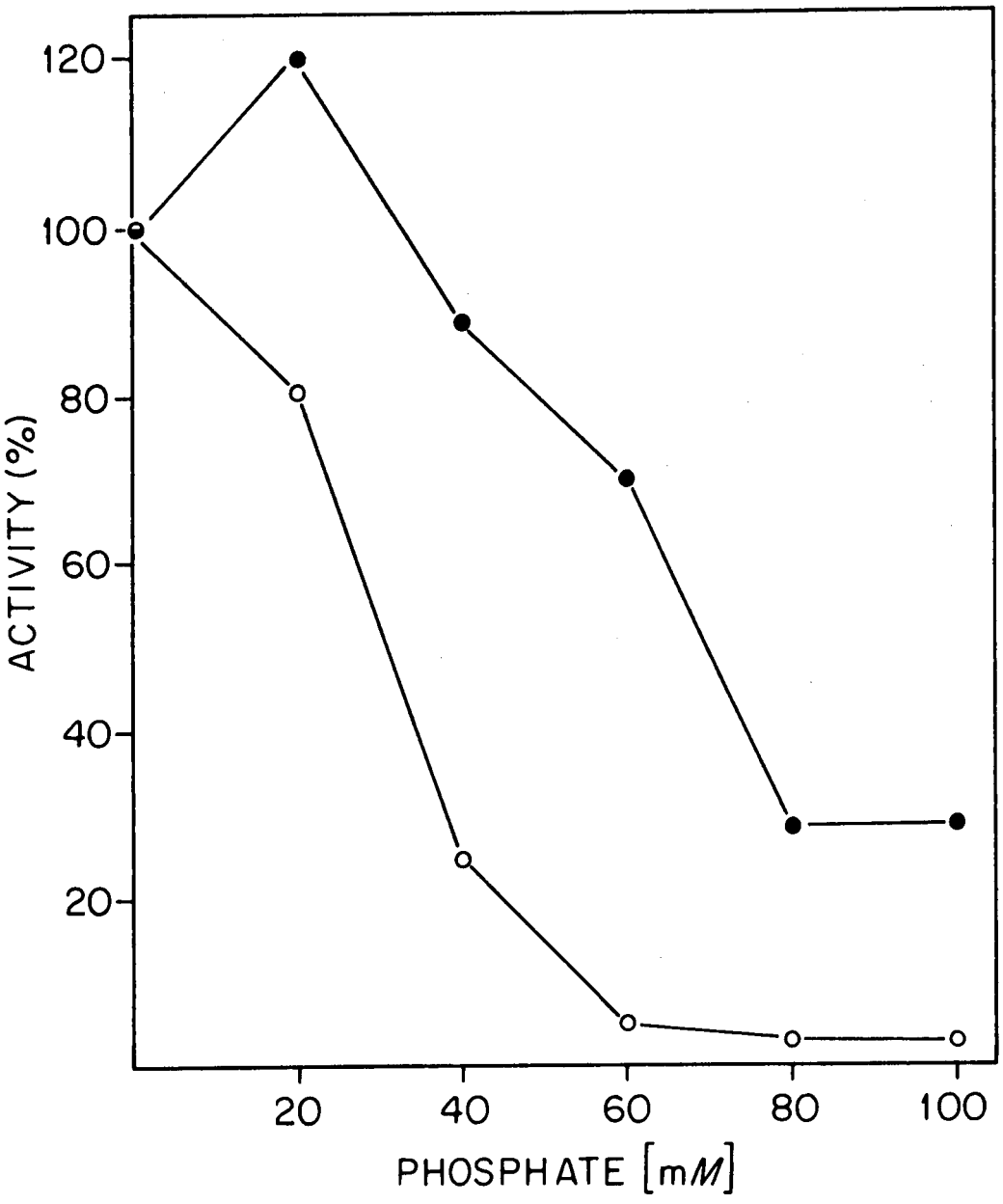

Figure 2.9. Inhibition of DNA polymerase activity by phosphate.

Poly(dC)·$(dG)_{12-18}$ was used as template, with 100 mM KCl and 4 mM Mg^{2+} in reaction mixture. Assays were performed under standard conditions as described under "Methods."

O ——— O β-DNA polymerase

● ——— ● γ-DNA polymerase

Similar results have been obtained by Weissbach et al. who test the effect of phosphate on rat liver γ-DNA polymerase (31).

Sucrose Gradient Sedimentation Analysis of DNA Polymerase

Another way of identifying DNA polymerases is the molecular weight determination. Since eukaryotic DNA polymerases are known to aggregate readily in solutions of low ionic strength (4), analysis of the partially purified polymerase was performed in high salt (0.25 m KCl) by using 10 to 25% linear sucrose gradient sedimentation. All three partially purified DNA polymerases exhibited single enzymatic peaks sedimenting respectively at 3S (β-DNA polymerase), 4S (γ-DNA polymerase), and 7S (α-DNA polymerase) (Figure 2.10). The apparent molecular weight was estimated to be 130,000 for α-DNA polymerase, 30,000 for β-DNA polymerase, and 63,000 for γ-DNA polymerase.

Discussion

The present report describes the partial purification of three DNA polymerases of rat spleen. Characterization of these enzymes indicate that they are distinct DNA polymerases.

The template utilization and preference of the cellular DNA polymerases have been used for identification purposes and examined in the hope of gaining some insight into the role of these enzymes. For example, we are able to show that the α-DNA polymerase can use DNA template-RNA primer more efficiently than β-, and γ-DNA polymerase (Table 2.4, p. 31).

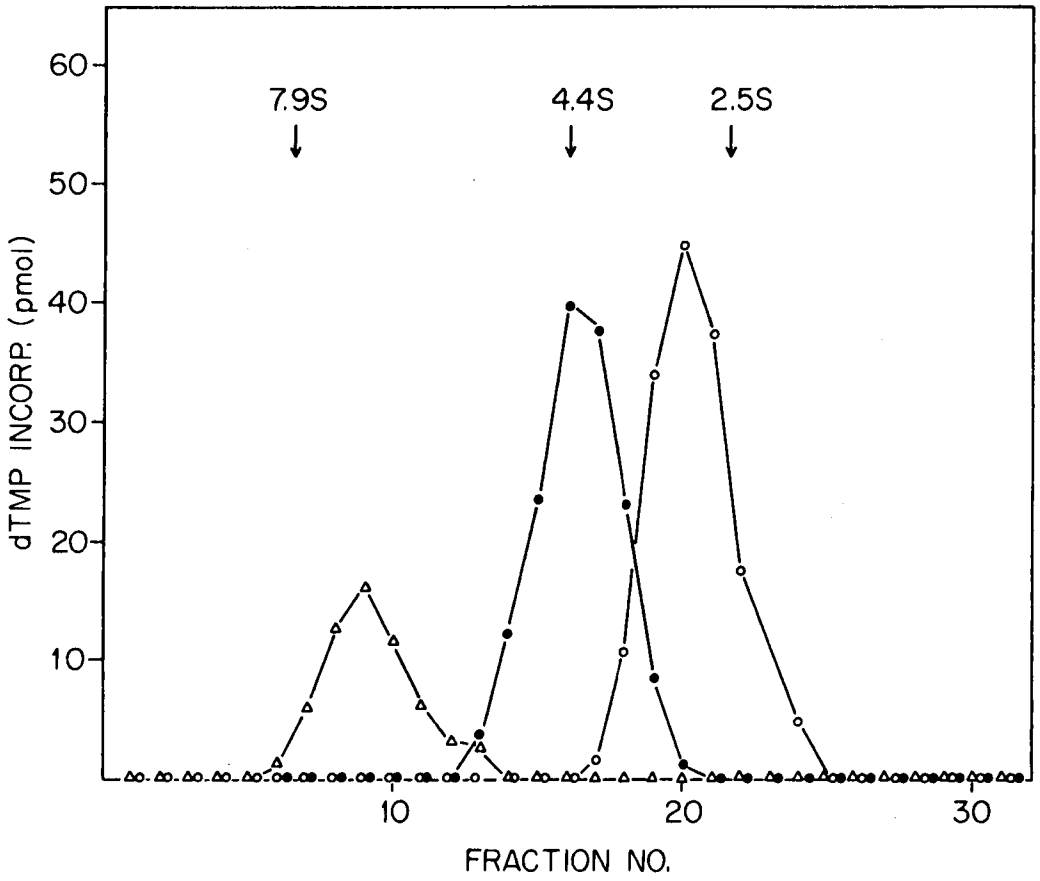

Figure 2.10. Sucrose gradient sedimentation analysis of DNA polymerase.

Sucrose gradient centrifugation and enzyme assay were performed under "Methods." Poly(dC)·$(dG)_{12-18}$ was used as template, with 4 mM Mg^{2+} in reaction mixture. The salt concentration in reaction mixture was 25 mM, 100 mM, 200 mM for α-, β-, and γ-DNA polymerase respectively.

Δ ——— Δ α-DNA polymerase

0 ——— 0 β-DNA polymerase

● ——— ● γ-DNA polymerase

Similar results had also been obtained by Bollum (2), showing that α-DNA polymerase can use oligro(rA) as primer and poly(dT) as template, while β-DNA polymerase cannot. This template-primer specificity implies that probably α-DNA polymerase, but not β-DNA polymerase, can use RNA primers provided by some RNA polymerase for new DNA synthesis in vivo. This is compatible with the finding that the level of α-DNA polymerase varies according to the rate of cell proliferation and DNA synthesis while the level of β-DNA polymerase remains relatively stable.

Parameters in reaction condition should be well defined before assaying the activity of DNA polymerases on various templates. For example, concentrations of divalent cations and salt have a dramatical effect on DNA polymerase activity upon various templates. In addition, the Km value of templates and nucleotides may differ for α-, β-, and γ-DNA polymerases.

Since none of these three DNA polymerases have been purified to homogenous form, it is conceivable that some of the characteristics we described may not be the intrinsic property of the enzyme. Nonetheless, we found most of the characteristics of rat spleen α-, β-, and γ-DNA polymerase fit in well with the criteria used for identification of eucaryotic DNA polymerases. The only exception is γ-DNA polymerase. Although the γ-DNA polymerase of rat spleen utilize poly(rA) template more efficiently than poly(dA) template, and is stimulated by 150-300 mM KCl, we cannot confirm the fact that 50 mM phosphate can stimulate the activity of γ-DNA polymerase. In addition, the sensitivity of γ-DNA polymerase to N-ethylmaleimide is similar to that of β-DNA polymerase.

Most of the γ-DNA polymerase isolated from other mammalian cells have molecular weights range from 150,000 to 300,000. The γ-DNA polymerase we isolated from rat spleen has an estimated molecular weight of only 63,000. Nonetheless, we found the rat spleen γ-DNA polymerase is similar to the rat liver γ-DNA polymerase described by Weissbach (31) who found that the mitochondria DNA polymerase is closely related to, or identical to the γ-DNA polymerase. We do not know whether the rat spleen γ-DNA polymerase we described is identical to the mitochondria DNA polymerase, but by common differential centrifugation procedures, only 10% of rat spleen γ-DNA polymerase activity can be located in mitochondrial subcellular fraction while 70% of γ-DNA polymerase activity is present in cytosol. In addition, we found higher activity of γ-DNA polymerase was present in more mitotically active tissue (94). The subcellular distribution and higher activity in mitotically active tissue of γ-DNA polymerase suggest that this enzyme may have a wider role in addition to mitochondria DNA synthesis.

The current criteria used for classification has divided the eularyotic DNA polymerase into three groups, namely α, β, and γ. However, since the DNA polymerases might diverse greatly in different eucaryotic cells, it is sometimes difficult to assign a name to certain DNA polymerase. For example, the γ-DNA polymerase of rat spleen does not fit in too well with the current criteria of nomenclature; another example is the finding of DNA polymerase located in nucleus resembles the α-DNA polymerase but possesses distinct enzymatic characteristics (95).

It should be emphasized that the number of DNA polymerase of eukaryotic cell could be greater than three. Furthermore, nomenclature based on biological function shall be more proper than on the <u>in vitro</u> enzymatic

characteristics. Further genetic and biochemical studies may help us in elucidation of the possible *in vivo* roles of these enzymes in DNA replication and repair.

CHAPTER 3

AGE-ASSOCIATED DECREASE OF RAT SPLEEN DNA POLYMERASE ACTIVITY

Summary

The DNA polymerase activity in the spleens of young adult (3 months) and old (25 months) male Fischer 344 rats have been examined. Comparisons were made by sucrose gradient sedimentation analysis of α-, β-, and γ-DNA polymerases in subcellular fractions. The α-DNA polymerase activity, demonstrated only in the cytosol fraction, was similar in the spleens of young and old rats. β-DNA polymerase activity was decreased in the spleens of old rats; the decreased activity was detected most markedly in the nuclear and mitochondrial fractions of the spleens. γ-DNA polymerase were found to be present predominantly in the cytoplasm of spleen cells, and there is a decrease of its activity in the nuclear, mitochondrial, and microsomal fractions of the spleen cells of the old rats. Stepwise extractions with increasing KCl concentrations showed consistent results without revealing difference in extractability of DNA polymerase from the particulate subcellular components of young and old rat spleen cells. DNA polymerases prepared from spleens of young and aged rats are similar in chromatographic migration, sucrose gradient sedimentation, and *in vitro* heat lability. The DNA polymerase activities in the brains and livers were also examined. Only β-DNA polymerase was detectable in rat brain; age-related decrease in DNA polymerase activity was also observed in this

tissue but to a less extent than in the spleen. None of the three DNA polymerases activities was found to decline in the liver of the old rats.

Introduction

Many studies concerning molecular mechanism of aging have been made at the DNA-chromatin level (78, 96). One phase of DNA metabolism which has been reported to be altered with aging is DNA replication. For example, the in vitro proliferative response of mouse spleen cells to phytohemagglutinin stimulation showed a marked age-related decrease (97). A delayed response of DNA synthesis and cell proliferation was also noted in the salivary glands of old rats after injection of isoproterenol (98). Another example of age-related alteration in DNA replication is the decreased fidelity of DNA polymerase activity isolated from aging human fibroblast (65).

In view of the role that DNA polymerases may play in the age-associated structural and metabolic alterations of DNA-chromatin, we initiated studies on these enzyme activities in relation to the aging process of the animal. Quantitative activity and qualitative properties of the three DNA polymerases (α-, β-, and γ-DNA polymerase) in the tissue of young and old rat were compared.

Materials and Methods

Materials and Equipments

Poly(rA) was obtained from Miles Laboratory, Kankakee, Illinois. $(dT)_9$ and poly(dA)$\cdot(dT)_{12-18}$ were purchased from P-L Biochemicals, Incorporated, Milwaukee, Wisconsin. Native calf thymus DNA and DNase I were

obtained from Worthington Biochemical Corporation, Freehold, New Jersey. Nonradioactive dNTP (deoxynucleotide triphosphates) were products of P-L Biochemicasl. Radioactively labeled dNTPs were obtained from New England Nuclear Corporation, Boston, Massachusetts. Sucrose of RNase free grade was obtained from Schwarz/Mann, Inc. (Orangebur, New York). All centrifugation was performed in the RC-2 and RC-2B (Ivan Sorvall, Inc., Newtown, Connecticut), and all untracentrifugation in the MOdel L or Model L2-65B (Bechman Instruments, Inc., Palo Alto, California).

Rats

Pathogen-free male Fischer 344 rats were raised in the Biology Division, Oak Ridge National Laboratory. Spleens and other tissues which were tumor free and of normal size were removed from young adult (3 months old) and senescent (25 months old) rats. Tissue not immediately used was frozen in liquid nitrogen and then stored at -70°C.

Preparation of Tissue Extracts

Tissues were quickly thawed, minced, and then homogenized in four volumes (v/w) of 0.25 M sucrose containing 0.05 M TrisCl(pH 7.6 at 22°C), 0.025 M KCl, 1 mM dithiothritol, and 3 mM $MgCl_2$ using a teflon pestle and glass homogenizer. The homogenate was spun at 800 x g (av.) for ten minutes and the pellet was washed once with four volumes of homogenizing medium by suspension and centrifugation to obtain the nucleus pellet. The first supernatant fraction was sequentially spun at 8000 x g (av.) for 15 minutes and 198,000 x g (av.) for 60 minutes to obtain two pellets (mitochondrial and microsomal) and a supernatant fraction. Each of the three pellets obtained by differential centrifugation was suspended in four volumes of homogenizing medium per original wet tissue weight by

Dounce homogenizers. The individual subcellular fractions were used for extraction of polymerase activity by adding KCl to final concentrations of 0.5 M for cytosol, microsomal, and mitochondrial fractions, and 0.35 M for nuclear fraction. The mixtures were then spun at 198,000 x g (av.) for 60 minutes. The supernatant fractions were then used immediately for polymerase assay by sucrose gradient centrifugation.

Sucrose Gradient Centrifugation

Sucrose gradient centrifugation was performed in a Spinco SW 56 Ti rotor. DNA polymerase extracts (same amount of protein for young and old) were layered on top of 3.8 ml solution containing 10 to 25% sucrose linear gradient, 10 mM TrisCl(pH 8.0 at 22°C), 0.25 M KCl, and 1 mM dithiothreitol in polyallomer tubes. Centrifugation was performed at 50,000 rpm for 20 hrs at 4°C. Fractions of 0.19 ml were collected from the bottom of the tube and 5 µl of each fraction was assayed for polymerase activity.

Determination of Polymerase Activity

DNA polymerase activity was measured by the polymerization of radioactive dNTP into acid insoluble form in the presence of template-primer complex. Appropriate amounts of enzyme protein (within the range where the concentration of protein is linear-related with DNA synthesis) were added to reaction mixture to a final volume of 50 µl containing 20 mM TrisCl(pH 8.0 at 22°C), 5 mM dithiothreitol, 0.17 mg/ml bovine serum albumin, 20-200 mM KCl, appropriate concentrations of Mg^{2+} and/or Mn^{2+}, 60 mM of appropraite dNTP including [^{3}H]dTTP at 17.5 µci/ml, 0.02 mg/ml of synthetic template-primer or 0.1 mg/ml activated DNA. The concentration

of divalent matal in reaction mixture depends on the template-primer being used: 8 mM Mg^{2+} for activated DNA, 1.5 mM Mg^{2+} and 0.5 mM Mn^{2+} for poly(dA)·$(dT)_{12-18}$ and poly(rA)·$(dT)_9$. After incubation in a water bath at 37°C for two hours, incorporation of radioactivity into acid insoluble form was measured by pipetting the reaction mixture onto a filter paper disk (Whatman 3 MM), which was subsequently washed and processed for liquid scintillation counting (89).

Activated DNA was prepared by DNase I digestion of calf thymus DNA according to the procedures of Adams, et al. (90). Poly(rA)·$(dT)_9$ was prepared by simple mixing the poly(rA) with $(dT)_9$ in H_2O at a base ratio of 5:1.

Heat Inactivation of DNA Polymerase

Sucrose gradient fractions with highest DNA polymerase activities were adjusted to contain 1 mg/ml bovine serum albumin and incubated at 43°C or 50°C for the indicated periods of time. Aliquots were withdrawn at various time intervals and assayed for DNA polymerase activity.

Protein Determination

Protein was determined by the method of Bramhall et al. (91).

Results

Comparison of DNA Polymerase Activities in Subcellular Fractions of Young and Aged Rat Spleens

Since our preliminary results indicated that threr is a 40% decrease of β-DNA polymerase activity and 20% decrease of γ-DNA polymerase activity

in aged rat spleen by column chromatography (data not shown), it was of interest to determine the distribution of the age-related decreased enzyme activity in subcellular fractions. Extracts of the subcellular fractions from young and old rat spleens with the same wet weight were analyzed by sucrose gradient sedimentation (Figure 3.1). For the identification and differentiation of α-, β-, and γ-DNA polymerase activities in the sucrose gradient analyses, three different template-primer complexes were used. Activated DNA with final salt concentration of 25 mM in reaction mixture was used for measurement of α-DNA polymerase activity. Poly(dA)·$(dT)_{12-18}$ with final salt concentration of 150 mM in reaction mixture was used for measurement of β-DNA polymerase activity. Since the α-DNA polymerase activity is almost completely blocked in 150 mM KCl solution, and the efficiency of γ-DNA polymerase to use poly(dA)·$(dT)_{12-18}$ is fairly low (94), the polymerase activity detected by the use of poly-(dA)·$(dT)_{12-18}$ as a template-primer should represent predominantly the β-DNA polymerase activity. Poly(rA)·$(dT)_9$ was used for measurement of γ-DNA polymerase activity since this template-primer can only be used by γ-DNA polymerase (94). Optimal salt concentration (200 mM KCl) for this enzyme was included in the reaction mixture when poly(rA)·$(dT)_9$ was used as template-primer.

The results showed that similar quantities of the α-DNA polymerase activity were detected in the spleens of young and old rats. With the procedures used for this study, this enzyme can only be detected in the cytosol fractions. β-DNA polymerase, which can be detected in all four subcellular fractions, was found to be lower in activity in the nuclear and mitochondrial fractions of the spleens of old rats than the corresponding fractions of the young. It was also found that γ-DNA polymerase

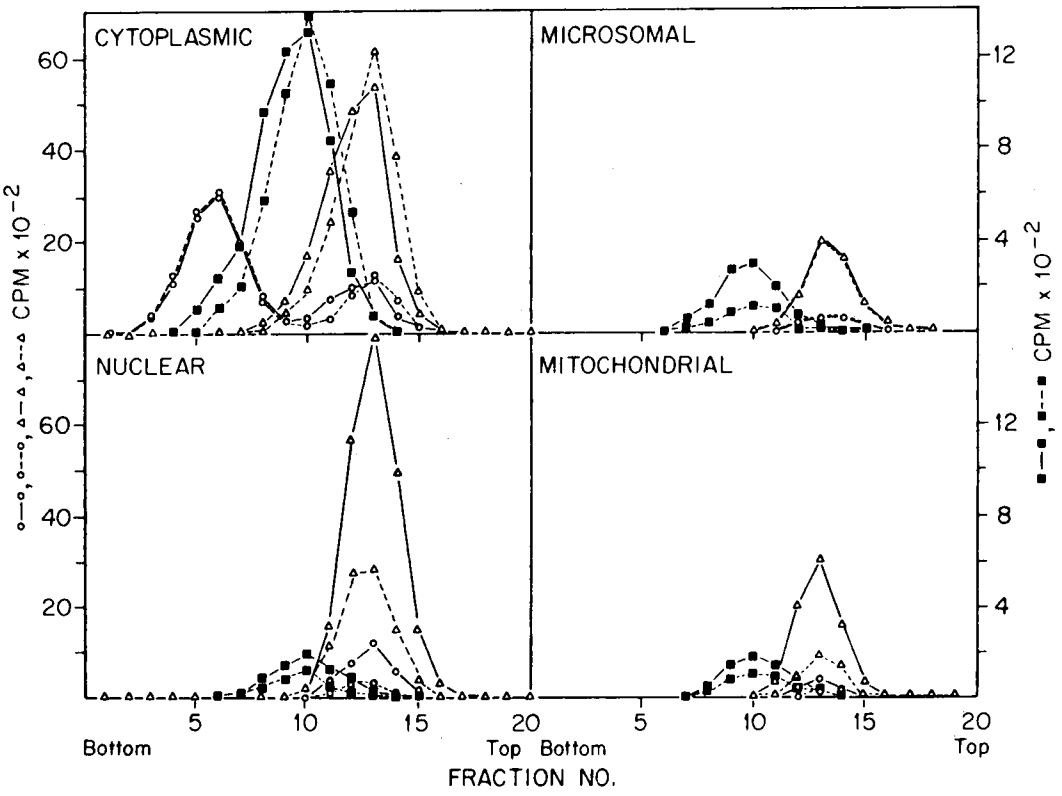

Figure 3.1. Sucrose gradient analyses of extracts of subcellular fractions from the spleens of young and aged Fischer 344 male rats.

0 ═══ 0 activated DNA

■ ═══ ■ poly(rA)·$(dT)_9$

Δ ═══ Δ poly(dA)·$(dT)_{12-18}$

Assays were performed as described under "Material and Methods." 25 mM KCl is included in the reaction mixture when activated DNA is used as template. 100 mM KCl is included in the reaction mixture when poly(dA)·$(dT)_{12-18}$ is used as template. 200 mM KCl is included in the reaction mixture when poly(rA)·$(dT)_9$ is used as template. Solid line = young, dashed line = old

showed decreased activities in the nuclear, mitochondrial, and microsomal fractions of the old. It was considered that the DNA polymerase might not be completely extracted from the aged splenic tissue; however, re-extraction with increasing salt concentration (up to 1.0 M KCl) yielded negligible additional polymerase activity. The results obtained from sucrose gradient analyses of DNA polymerase activities in the subcellular fractions of spleens pooled respectively from 35 young and 29 old rats are summarized in Table 3.1; comparison was made on the basis of wet tissue weight as well as protein contents of the extracts. These data showed that the β-DNA polymerase specific activity in the spleen nuclear fraction of aged rat is 48% of that of young adult rat; the β-DNA polymerase specific activity in the mitochondrial fraction of aged rat is only 34% of that of the young adult rat. Such decrease results in a 33% age-related decrease of β-DNA polymerase specific activity in whole cell extract. In another four separate experiments, each including 3 to 7 old rats, the spleen β-DNA polymerase activity of old rats decreased 37% to 60% of young in nuclear fraction and decreased 55% to 70% of young in the mitochondrial fraction.

The γ-DNA polymerase activity in the mitochondrial, microsomal, and nuclear fractions was also found to be decreased in the old rat spleen. However, since most of the γ-DNA polymerase activity was present in the cytosol fraction, which showed no changes between the young and the old, the total content of this polymerase in the whole cell extract was about 23% lower in the old than in the young.

TABLE 3.1

DNA POLYMERASE ACTIVITY FROM SUCROSE GRADIENT ANALYSIS OF SUBCELLULAR EXTRACTS OF SPLEEN FROM YOUNG AND AGED FISCHER 344 RATS[a]

Subcellular Fraction	α-DNA Polymerase Activity[b]		β-DNA Polymerase Activity[c]		γ-DNA Polymerase Activity[d]	
	Activity per mg of extracted protein($\times 10^{-2}$)	Activity per gram of wet tissue($\times 10^{-4}$)	Activity per mg of extracted protein($\times 10^{-3}$)	Activity per gram of wet tissue($\times 10^{-4}$)	Activity per mg of extracted protein($\times 10^{-2}$)	Activity per gram of wet tissue($\times 10^{-3}$)
young Cytosol	8.8	6.6	1.6	12	4.7	35
old Cytosol	7.9	6.6	1.5	12	4.0	34
young Microsomal	NT[e]		4.3	3.2	9.9	7.4
old Microsomal			5.6	3.2	4.8	2.7
young Mitochondrial	NT		4.4	4.8	4.0	4.3
old Mitochondrial			1.5	1.5	2.6	2.6

TABLE 3.1 (continued)

Subcellular Fraction	α-DNA Polymerase Activity[b]		β-DNA Polymerase Activity[c]		γ-DNA Polymerase Activity[d]	
	Activity per mg of extracted protein($\times 10^{-2}$)	Activity per gram of wet tissue($\times 10^{-4}$)	Activity per mg of extracted protein($\times 10^{-3}$)	Activity per gram of wet tissue($\times 10^{-4}$)	Activity per mg of extracted protein($\times 10^{-2}$)	Activity per gram of wet tissue($\times 10^{-3}$)
Nuclear young	NT		1.1	1.4	3.9	5.0
Nuclear old			5.5	5.9	2.7	2.9
Total young	6.2	6.6	3.2	34	4.9	52
Total old	6.0	6.6	2.1	23	3.8	42

[a]Activity is expressed as picomoles of dTMP incorporated per hour.

[b]Assayed with activated DNA as template, 25 mM KCl in reaction mixture.

[c]Assayed with poly(dA)·$(dT)_{12-18}$ as template, 100 mM KCl in reaction mixture.

[d]Assayed with poly(rA)·$(dT)_9$ as template, 200 mM KCl in reaction mixture.

[e]NT = Not detectable.

Examination for Presence of Inhibitors

The observed decrease of DNA polymerase activity might have resulted from the presence of inhibitors in the extracts from the aged rat spleen. One method of examining this possibility was to use the peak fractions from sucrose gradient analyses of young and aged spleen extracts and assayed separately and in a 1:1 ratio mixture. If no inhibitor was present, the enzyme activity in the mixture would be intermediate between that of the young and old spleen extracts. Results of such tests are shown in Figure 3.2. In these experiments, the polymerase activity in the mixed sample was intermediate between the individual young and old extract polymerase activity. Furthermore, no difference in the reaction time course curve was observed between the enzymes extracted from young and old rat. These results indicate that the observed decrease in polymerase activity of aged rat spleen cannot be explained as due to the presence of inhibitor in the old rat spleen extract.

Comparison of Enzyme Stability

Another possible explanation of the observed decrease in activity of enzyme from the senescent rat could be the difference in the stability of the DNA polymerase from the spleens of the two age groups. To test this possibility, peak fractions of β-DNA polymerase from sucrose gradients of young and aged spleen nuclear fractions were incubated at 43°C, the rate of loss of enzyme activity were then measured. Figure 3.3 shows that the β-DNA polymerases from young and old spleen nuclear fractions have equal stability, suggesting that both enzymes possess similar *in vitro* stability.

Figure 3.2. Assay of extracts of spleens from young and aged rats.

Peak fractions from sucrose gradient analyses were assayed alone and in a 1: 1 mixture. 25 µl of each sample (12.5 µl from young and 12.5 µl from old for 1: 1 mixture) was assayed in a 0.25 ml reaction volume at 37°C. Radioactive incorporation in a 0.05 ml was measured at different time intervals. (A) β-DNA polymerase peak fractions of nuclear fraction extracted. Poly(dA)·$(dT)_{12-18}$ was used as template. (B) β-DNA polymerase peak fractions of mitochondrial fraction extracted. Poly(dA)·$(dT)_{12-18}$ was used as template. (C) γ-DNA polymerase peak fractions of microsomal fraction extract. Poly(rA)·$(dT)_9$ was used as template.

▲ ——— ▲ Young adult

Δ ——— Δ Old

0 ——— 0 Mix.

Reaction conditions as described in "Material and Methods."

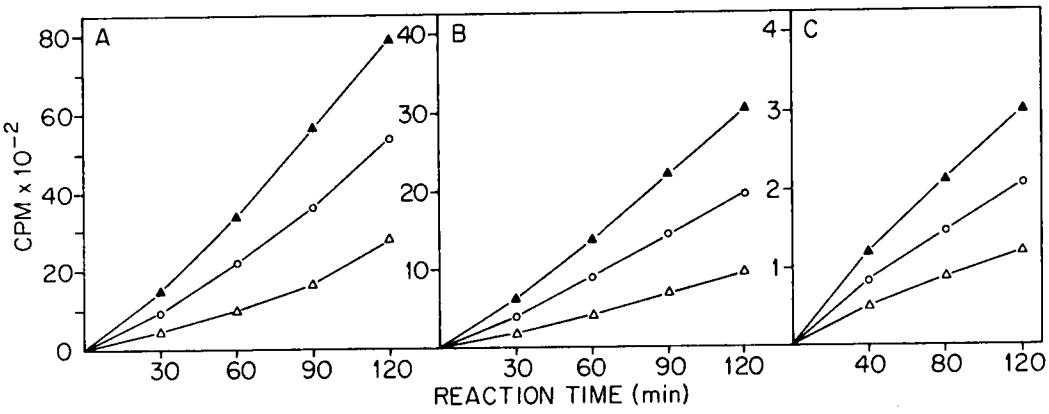
A
B
C
CPM x 10^{-2}
REACTION TIME (min)
0
20
40
60
80
10
20
30
40
1
2
3
4
30
60
90
120
40
80
120

Figure 3.2

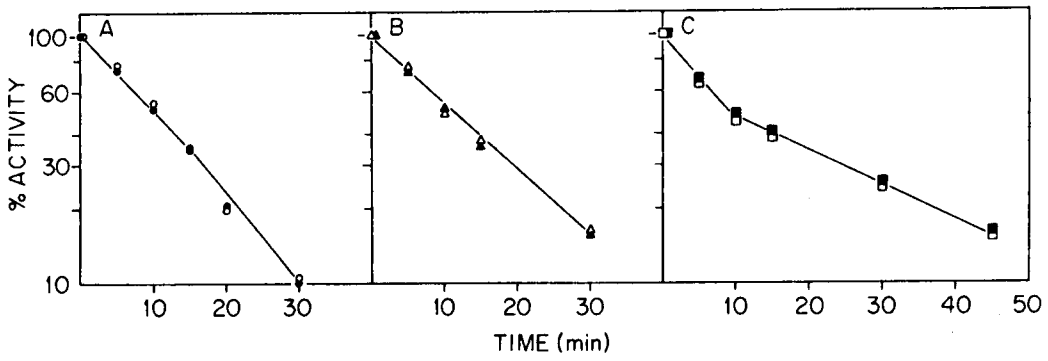

Figure 3.3. Comparison of the stability of the DNA polymerases isolated from the spleen of young and aged Fischer 344 rats.

Peak fractions from sucrose gradient analyses were used as enzyme sources. Enzymes were incubated at 43°C (β-DNA polymerase) or 50°C (α- and γ-DNA polymerase) and at different time intervals a 0.01 ml sample was removed. This sample was assayed in a 0.05 ml reaction volume at 37°C. (A) α-DNA polymerase peak fraction of cytosol extract. Activated DNA was used as template-primer. (B) β-DNA polymerase peak fraction of nuclear extract. Poly(dA)·$(dT)_{12-18}$ was used as template-primer. (C) γ-DNA polymerase peak fraction of cytosol extract. Poly-(rA)·$(dT)_9$ was used as template-primer. Closed symbols; young adult rat: open symbols: aged rat.

Since the α-DNA polymerase and γ-DNA polymerase activities were either undetectable or low in all other subcellular fractions except in the cytosal fraction, peak fractions of α- and γ-DNA polymerase from sucrose gradients of young and old spleen cytosol fractions were used to compare the enzyme stability. The results, as shown in Figure 3.3, did not reveal any significant stability difference between the enzymes from young and aged spleens.

DNA Polymerase Activities in Young and Old Rat Brains and Livers

Since age-related decreases in polymerase activities were observed in rat spleen, it was of interest to examine other tissues as well. Less mitotically active tissues such as livers and brains were examined. The homogenization, enzyme extraction, and sucrose gradient analyses were as described. The comparisons are presented quantitatively in Tables 3.2 and 3.3. In brain cells, only β-DNA polymerase can be detected, the α-DNA polymerase and γ-DNA polymerase are either absent or present in very low quantities. We found that the β-DNA polymerase activity decreased in cytosol, mitochondrial, and nuclear fractions of old rat brain, and resulted in a 32% decrease of polymerase specific activity in the whole cell extract. In rat liver cells all three DNA polymerase activities could be detected, although the α- and γ-DNA polymerase activity are much lower than that observed for the spleen. None of the three DNA polymerase activities was found to decline in livers of the old rats.

TABLE 3.2

DNA POLYMERASE ACTIVITY FROM SUCROSE GRADIENT ANALYSIS OF SUBCELLULAR EXTRACTS OF BRAIN FROM YOUNG AND AGED FISCHER 344 RATS[a]

Subcellular Fraction	α-DNA Polymerase Activity[b]		β-DNA Polymerase Activity[c]		γ-DNA Polymerase Activity[d]	
	Activity per mg of extracted protein	Activity per gram of wet tissue	Activity per mg of extracted protein($\times 10^{-2}$)	Activity per gram of wet tissue($\times 10^{-3}$)	Activity per mg of extracted protein	Activity per gram of wet tissue
young Cytosol	NT[e]		12	25	NT	
old Cytosol			8.3	15		
young Microsomal	NT		17	2.3	NT	
old Microsomal			15	2.0		
young Mitochondrial	NT		14	2.5	NT	
old Mitochondrial			9.2	1.3		

TABLE 3.2 (continued)

Subcellular Fraction	α-DNA Polymerase Activity[b]		β-DNA Polymerase Activity[c]		γ-DNA Polymerase Activity[d]	
	Activity per mg of extracted protein	Activity per gram of wet tissue	Activity per mg of extracted protein($\times 10^{-2}$)	Activity per gram of wet tissue($\times 10^{-3}$)	Activity per mg of extracted protein	Activity per gram of wet tissue
Nuclear young	NT		45	23	NT	
Nuclear old			33	14		
Total young	NT		19	53	NT	
Total old			13	33		

[a]Activity is expressed as picomoles of dTMP incorporated per hour.

[b]Assayed with activited DNA as template, 25 mM KCl in reaction mixture.

[c]Assayed with poly(dA)·$(dT)_{12-18}$ as template, 100 mM KCl in reaction mixture.

[d]Assayed with poly(rA)·$(dT)_9$ as template, 200 mM KCl in reaction mixture.

[e]NT = Not detectable.

TABLE 3.3

DNA POLYMERASE ACTIVITY FROM SUCROSE GRADIENT ANALYSIS OF SUBCELLULAR EXTRACTS OF LIVER FROM YOUNG AND AGED FISCHER 344 RATS[a]

Subcellular Fraction	α-DNA Polymerase Activity[b]		β-DNA Polymerase Activity[c]		γ-DNA Polymerase Activity[d]	
	Activity per mg of extracted protein($\times 10^{-1}$)	Activity per gram of wet tissue($\times 10^{-3}$)	Activity per mg of extracted protein($\times 10^{-3}$)	Activity per gram of wet tissue($\times 10^{-4}$)	Activity per mg of extracted protein($\times 10^{-2}$)	Activity per gram of wet tissue($\times 10^{-3}$)
young Cytosol	9.9	9.2	5.3	4.9	1.0	9.0
old Cytosol	1.0	8.2	5.6	4.4	1.0	8.0
young Microsomal	NT[e]		2.6	1.5	1.2	0.6
old Microsomal			2.6	1.0	1.6	0.6
young Mitochondrial	NT		2.8	1.2	1.2	0.5
old Mitochondrial			1.6	0.7	1.2	0.5

TABLE 3.3 (continued)

Subcellular Fraction	α-DNA Polymerase Activity[b]		β-DNA Polymerase Activity[c]		γ-DNA Polymerase Activity[d]	
	Activity per mg of extracted protein($\times 10^{-1}$)	Activity per gram of wet tissue($\times 10^{-3}$)	Activity per mg of extracted protein($\times 10^{-3}$)	Activity per gram of wet tissue($\times 10^{-4}$)	Activity per mg of extracted protein($\times 10^{-2}$)	Activity per gram of wet tissue($\times 10^{-3}$)
Nuclear young	NT		8.7	8.3	3.5	3.3
Nuclear old			10.	8.9	3.5	3.1
Total young	8.2	9.2	1.4	16.	1.2	13.
Total old	8.3	8.2	1.6	15.	1.3	12.

[a]Activity is expressed as picomoles of dTMP incorporated per hour.

[b]Assayed with activated DNA as template, 25 mM KCl in reaction mixture.

[c]Assayed with poly(dA)·$(dT)_{12-18}$ as template, 100 mM KCl in reaction mixture.

[d]Assayed with poly(rA)·$(dT)_9$ as template, 200 mM KCl in reaction mixture.

[e]NT - Not detectable.

Discussion

Our experimental results show that β-DNA polymerase is present in at least three tissues of both young and aged rat, while it has been suggested that α- and γ-DNA polymerase are directly related to *in vivo* DNA synthesis (12, 41), it is not surprising that α-DNA polymerase cannot be detected in post-mitotic cell population such as brain. Failure to detect γ-DNA polymerase in brain cells also suggests a relationship between this enzyme and DNA replication.

It is apparent from the data presented that β-DNA polymerase activity was decreased in nuclear and mitochondrial fractions of aged rat spleen. Such decrease in enzyme activity was also observed in the cytosol, nuclear, and mitochondrial fractions of aged rat brain. In both tissues, there is an approximately 33% age-related decrease of enzyme activity in whole cell extract. The γ-DNA polymerase activity also decreased markedly in mitochondrial, microsomal, and nuclear fractions of aged rat spleen. These observed decreases in DNA polymerase activities cannot be explained as the difference in extractability of the DNA polymerase from young and aged tissue since stepwise extractions with increasing salt concentration always gave consistent different results.

The mixing experiments failed to reveal any soluble inhibitor in the extract, and the comparison of reaction time course indicated that no inhibitor was reversibly bound to the polymerase. However, possibilities of the presence of inhibitors and/or activators which co-migrate with the polymerase during sucrose gradient centrifugation ca-not be ruled out because activators or inhibitors might be present in both young and aged

cell extract without any quantitative and qualitative difference and cannot be revealed by mixed-enzyme experiments. In addition, it is still possible that the protein decreased in aged rat spleen is something other than β- and γ-DNA polymerase. It could be some DNA binding protein that form complex with polymerase and facilitate the enzymatic function of the DNA polymerase.

Although it has been reported that there is an accumulation of heat-labiled enzymes in human diploid fibroblasts during the final stages of their in vitro life span (63), heat inactivation experiments did not reveal any difference in enzyme stability between the polymerases from the two age groups. Also, none of the three DNA polymerases of aged rats exhibit any aberrant behavior on sucrose gradients of column chromatography. All these results suggested that the age-related decreases in DNA polymerase activities are the result of a decreased amount of enzyme. This conclusion could be substantiated if one could obtain an antibody preparation against the DNA polymerase. by comparing the antibody inhibition of DNA polymerase in young and aged tissue extracts, one might determine whether there is an accumulation of cross-reactive, enzymatically inactive material in the extracts of aged tissue.

Since the decrease in DNA polymerase activity in aged rats is observed in certain subcellular fractions and not observed in others, this automatically indicates a change in the relative subcellular distribution of DNA polymerase activity. However, it is known that certain cell fractionation procedures may lead to an artifactual distribution of DNA polymerase activity (37, 39), it is hard to conclude whether this decrease in enzyme

activity is accompanied with redistribution of enzymes in the cell during the aging process.

Although decreased enzyme activity was observed in old rat brain (one experiment involving 10 old rats) in addition to old rat spleen, we detected no decrease in DNA polymerase activity in livers of 25-month-old rats (one experiment involving five old rats). This led us to tentatively conclude that age-related deterioration of β-DNA polymerase-associated function may occur earlier in one organ (e.g., spleen) than others (e.g., liver) in the rat. It would be interesting to study the DNA polymerase activity of rats older than 25 months; observation of further decrease in DNA polymerase activity might indicate a positive relationship between enzyme decrease and aging process. Previous studies in our laboratory have shown that β-DNA polymerase activity was decreased remarkedly in the nuclear fraction of aged BALB/c mice (75). This observation, together with the present study on Fischer 344 rats, led us to the suggestion that age-related decreases in certain DNA polymerase activity might be a general phenomenon in mammalian cells. Investigation on other mammalian species might substantiate this suggestion.

The biological significance and functional effect of decreased DNA polymerase in aged mice and rats are not clear at the present time, largely due to the fact that the precise role played by β- and γ-DNA polymerase in vivo have not been elucidated. Since β-DNA polymerase activity remains relatively constant in cells at different phases of cell cycles (42), it is reasonable to speculate that β-DNA polymerase is responsible for DNA repair or other editing functions. We are currently trying to verify this

speculation by investigating the accumulation of DNA strand breaks with increased age in rat spleens and brains.

CHAPTER 4

STUDIES ON FIDELITY OF DNA POLYMERASE FROM SPLEENS OF YOUNG AND OLD FISCHER 344 RATS

Summary

Comparison of the in vitro replicational infidelity were conducted with partially purified α-, β-, and γ-DNA polymerase from the spleens of young and old Rischer 344 male rats. No significant difference in fidelity was found between the DNA polymerases isolated from the two age groups.

Introduction

The genetic information in DNA is transmitted from parental cells to daughter cells with high fidelity. The DNA structure proposed by Watson and Crick (99) suggested that the faithful duplication of DNA is mediated primarily through deoxynucleotide base interaction by hydrogen bonding. However, analysis of the free energy involved in the interaction and the rate of spontaneous mutation indicates that a role for DNA polymerase in augmenting correct base selection appears mandatory (60). Homogeneous DNA polymerase from bacteria and bacteriaphage have an associated 3'- to 5'- exonuclease activity which has been suggested to serve in a "proof-reading" capacity (100). Such proofreading exonuclease activity does not appear to be an integral part of eukaryotic DNA polymerase (95, 101). However, purified eukaryotic DNA polymerases still copy polynucleotides

in vitro with high precision. For example, the β-DNA polymerase purified from calf thymus, which has been shown to be totally lacking in exonuclease activity, will make only one error for every 100,000 nucleotide incorporations (101). Thus, DNA polymerase must participate in base selection.

In view of the predictions made by a number of authors that alterations in the fidelity of DNA polymerase could be an important factor in cell aging (62, 63, 102, 103), we have compared the fidelities of DNA polymerases isolated from the spleens of young and aged rats.

Materials and Methods

Materials and Equipments

Synthetic polynucleotides and polydeoxynucleotides were obtained from Miles Laboratories, Kankakee, Illinois and P-L Biochemicals, Inc., Milwaukee, Wisconsin. Oligodeoxynucleotides were products of Collaborative Research, Inc., Waltham, Massachusetts and P-L Biochemicals, Inc. Nonradioactive dNTPs were products of P-L Biochemicals, Inc. and radioactively labeled dNTPs were obtained from New England Nuclear Corporation, Boston, Massachusetts. Bovine serum albumin was purchased from Worthington Biochemicals Corporation.

Rats

Pathogen free male Fischer 344 rats were raised at the Oak Ridge National Laboraotry, Oak Ridge, Tennessee. The spleens which were tumor free and normal size were removed from young (3 months old) and senescent (25 months old) rats. Tissues not immediately used were frozen in liquid nitrogen and stored at -70°C.

Tissue Homogenization and the Isolation of DNA Polymerases

Tissue homogenization and the isolation of DNA polymerases were previously described (94).

DNA Polymerase Activity

To measure the fidelity with which synthetic template is copied we measured the incorporation of correct nucleotide and incorrect nucleotide in separate assays. The two simultaneous assays were identical with respect to the following components and reaction conditions: a standard reaction mixture of 50 µl contained 5 µl DNA polymerase, 20 mM TrisCl(pH 8.0), 5 mM dithiothreitol, 0.17 mg/ml BSA, 25 mM KCl, 0.04 mg/ml synthetic template and appropriate concentration of Mg^{2+} and Mn^{2+}, 60 µM of correct doexynucleotide triphosphate and 60 µM of incorrect doexynucleotide triphosphate. Mg^{2+} (1.5 mM) and 0.5 mM Mn^{2+} were included in the reaction mixture when poly(dA)·$(dT)_{12-18}$ or poly(rA)·poly(dT) was used as template. Mg^{2+} (4mM) was included in the reaction mixture when poly[d(A-T)] or poly(dC)·$(dG)_{12-18}$ was used as template. The only difference between the two simultaneous reactions was that in one reaction the correct nucleotide was radioactively labeled (0.3 Ci/mmole) and the incorrect nucleotide was unlabeled whereas the second reaction contained the unlabeled correct nucleotide with the incorrect nucleotide being radioactively labeled (1.2 Ci/mmole). The incubations were carried out for 2 hrs at 37°C. The processing of reaction mixture for subsequent scintillation counting was described previously (89).

Results

Examination of the Infidelity of In Vitro DNA Synthesis by the DNA Polymerases of Young and Aged Rat Spleen

For comparisons of the fidelity function of *in vitro* DNA synthesis the polymerases isolated from each tissue were used at approximately the same amount of enzyme activities. With various template-primers, the reaction conditions for the polymerase were all the same except the concentrations of divalent cations in the reaction. The experimental protocol for the determination of the level of infidelity for the DNA polymerase is shown in Table 4.1. The level of infidelity is expressed as the incorporation of 1 pmol of incorrect nucleotide per X pmol of correct nucleotide. The results of such analyses on α-, β-, and γ-DNA polymerase are presented respectively in Tables 4.2, 4.3, and 4.4. As can be seen in all three tables, the polymerases from young and aged rat spleen possessed essentially the same levels of infidelity. Although a small difference in infidelity can be observed depending on template-primers being used, the difference between young and old DNA polymerase never exceeded 25% and can be explained as due to experimental error. However, there are differences in fidelity between different DNA polymerases, depending on the template-primer. For example, α-DNA polymerase showed a higher fidelity than β- and γ-DNA polymerase when poly(dA)·-$(dT)_{12-18}$ was used as template-primer. While all three DNA polymerases showed relatively similar fidelity when poly(dC)·$(dG)_{12-18}$ was used as template-primer. The significance of these differences are not clear, but it seems that the γ-DNA polymerase showed higher fidelity with

TABLE 4.1

PROTOCOL FOR DETERMINATION OF THE LEVEL OF INFIDELITY

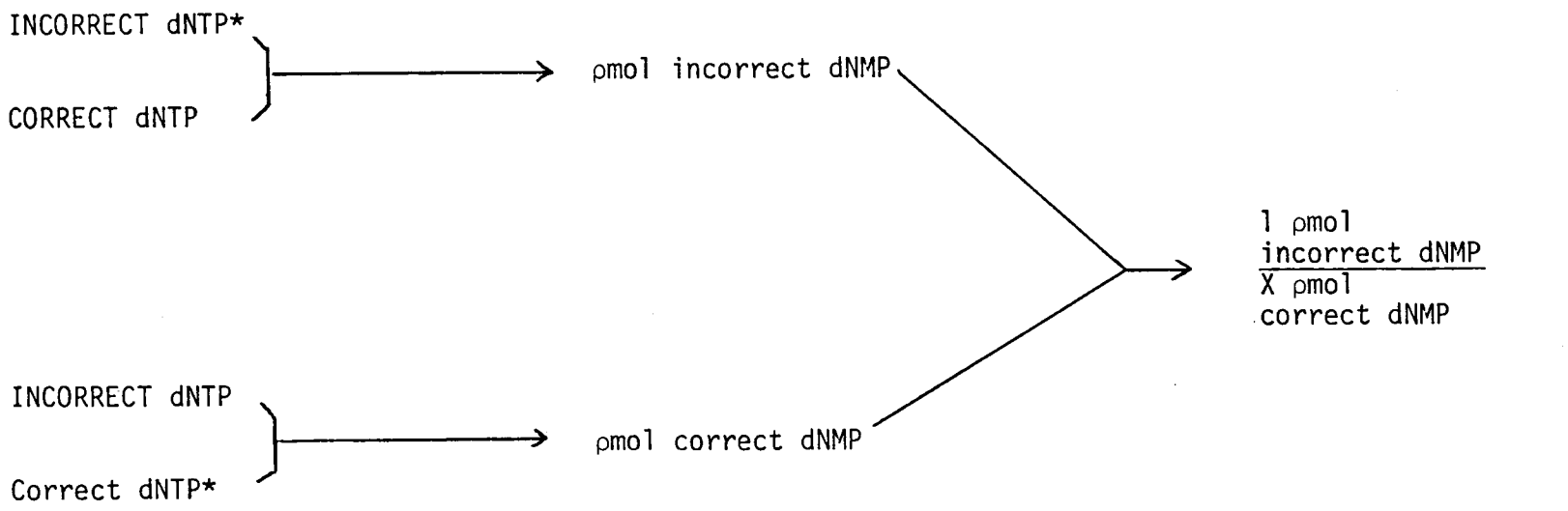

TABLE 4.2

INFIDELITY OF α-DNA POLYMERASE WITH VARIOUS TEMPLATES

Template	Enzyme Source	
	Spleen of 3-month-old rat	Spleen of 25-month-old rat
poly(dA)·$(dT)_{12-18}$	$\frac{G}{T} = \frac{1}{11597}$	$\frac{G}{T} = \frac{1}{13866}$
poly(dA)·$(dT)_{12-18}$	$\frac{C}{T} = \frac{1}{7632}$	$\frac{C}{T} = \frac{1}{7599}$
poly(dC)·$(dG)_{12-18}$	$\frac{A}{G} = \frac{1}{3358}$	$\frac{A}{G} = \frac{1}{2990}$
poly(dC)·$(dG)_{12-18}$	$\frac{T}{G} = \frac{1}{3894}$	$\frac{T}{G} = \frac{1}{3809}$

TABLE 4.3

INFIDELITY OF β-DNA POLYMERASE WITH VARIOUS TEMPLATES

Template	Enzyme Source	
	Spleen of 3-month-old rat	Spleen of 25-month-old rat
poly(dA)·$(dT)_{12-18}$	$\frac{G}{T} = \frac{1}{2443}$	$\frac{G}{T} = \frac{1}{2391}$
poly(dA)·$(dT)_{12-18}$	$\frac{C}{T} = \frac{1}{1090}$	$\frac{C}{T} = \frac{1}{1322}$
poly(rA)·poly(dT)	$\frac{G}{T} = \frac{1}{3887}$	$\frac{G}{T} = \frac{1}{3916}$
poly(rA)·poly(dT)	$\frac{C}{T} = \frac{1}{2130}$	$\frac{C}{T} = \frac{1}{2333}$
poly(dC)·$(dG)_{12-18}$	$\frac{A}{G} = \frac{1}{3926}$	$\frac{A}{G} = \frac{1}{4075}$
poly(dC)·$(dG)_{12-18}$	$\frac{T}{G} = \frac{1}{6445}$	$\frac{T}{G} = \frac{1}{7880}$

TABLE 4.4

INFIDELITY OF γ-DNA POLYMERASE WITH VARIOUS TEMPLATES

Template	Enzyme Source	
	Spleen of 3-month-old rat	Spleen of 25 month-old rat
poly(dA)·$(dT)_{12-18}$	$\frac{G}{T} = \frac{1}{1937}$	$\frac{G}{T} = \frac{1}{1879}$
poly(dA)·$(dT)_{12-18}$	$\frac{C}{T} = \frac{1}{960}$	$\frac{C}{T} = \frac{1}{1019}$
poly(rA)·poly(dT)	$\frac{G}{T} = \frac{1}{8435}$	$\frac{G}{T} = \frac{1}{7910}$
poly(rA)·poly(dT)	$\frac{C}{T} = \frac{1}{3391}$	$\frac{C}{T} = \frac{1}{3699}$
poly(dC)·$(dG)_{12-18}$	$\frac{A}{G} = \frac{1}{2893}$	$\frac{A}{G} = \frac{1}{2630}$
poly(dC)·$(dG)_{12-18}$	$\frac{T}{G} = \frac{1}{4010}$	$\frac{T}{G} = \frac{1}{4855}$

efficient template-primer such as poly(rA)·poly(dT) than the less-efficient template-primer such as poly(dA)·$(dT)_{12-18}$.

We also compared the kinetics of correct and incorrect nucleotide incorporation catalyzed by β-DNA polymerase isolated from young and old spleen. The results in Figure 4.1 and Figure 4.2 showed that when poly[d(A-T)] was used as a template-primer, no difference in the reaction time course curve was observed between the young and old enzyme preparations.

One method of amplifying the infidelity difference between DNA polymerases is to omit one of the correct nucleotide in the reaction mixture. For example, dTTP, but not dATP was included in the reaction when poly[d(A-T)] was used as template-primer. The rationale is that the reaction should stop after incorporation of a dTMP because the next nucleotide incorporation requires the presence of dATP which is omitted in the reaction mixture. However, if a dTMP mispair with the dTMP on the template due to the infidelity of the DNA polymerase, the reaction will continue on for the next nucleotide incorporation. Thus, the effect of misincorporation will be amplified by the subsequent correct incorporation.

The result of such an experiment is shown in Figure 4.3. Again, we were not able to detect any difference in the reaction time course curve between the β-DNA polymerases prepared from spleens of young and old rats.

Discussion

The production of altered DNA polymerase with faulty base-selection property could explain alterations in the genome which in turn could

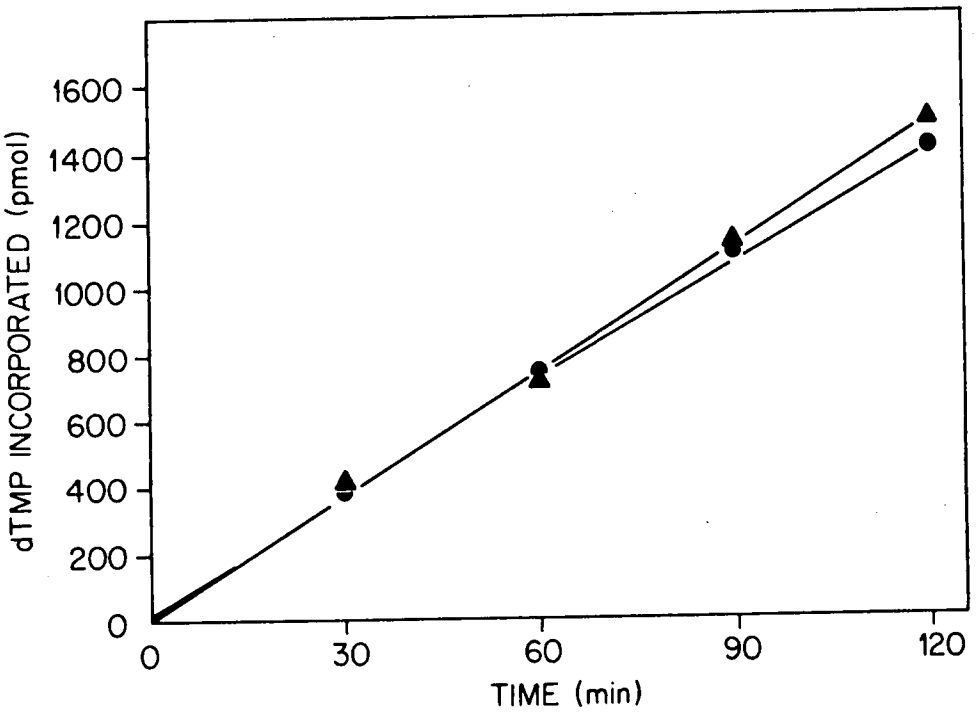

Figure 4.1. Comparison of the time course of the correct dTMP incorporation by β-DNA polymerase from young and aged rat spleen.

dATP, dTTP* (0.3 Ci/mmol), and dGTP were included in the reaction mixture. The assay procedure and reaction conditions were described in "Material and Methods." Poly[d(A-T)] was the template-primer. Some amount of enzyme protein isolated from spleens of young and old rats was used in each reaction.

● —— ● young

▲ —— ▲ old.

Figure 4.2. Comparison of the time course of the incorrect dCMP and dGMP incorporations by β-DNA polymerase from young and aged rat spleen.

dATP, dTTP, and dGTP* (0.3 Ci/mmol) or dCTP* (0.3 Ci/mmol) were included in the reaction mixture. The assay procedure and reaction conditions were as described in "Material and Methods." Poly[d(A-T)] was the template-primer. Same amount of enzyme protein isolated from spleens of young and old rats was used for each reaction.

● ——— ● dCMP incorporation by β-DNA polymerase of young spleen;

0 ------ 0 dCMP incorporation by β-DNA polymerase of old spleen;

▲ ——— ▲ dGMP incorporation by β-DNA polymerase of young spleen;

Δ ------ Δ dGMP incorporation by β-DNA polymerase of old spleen.

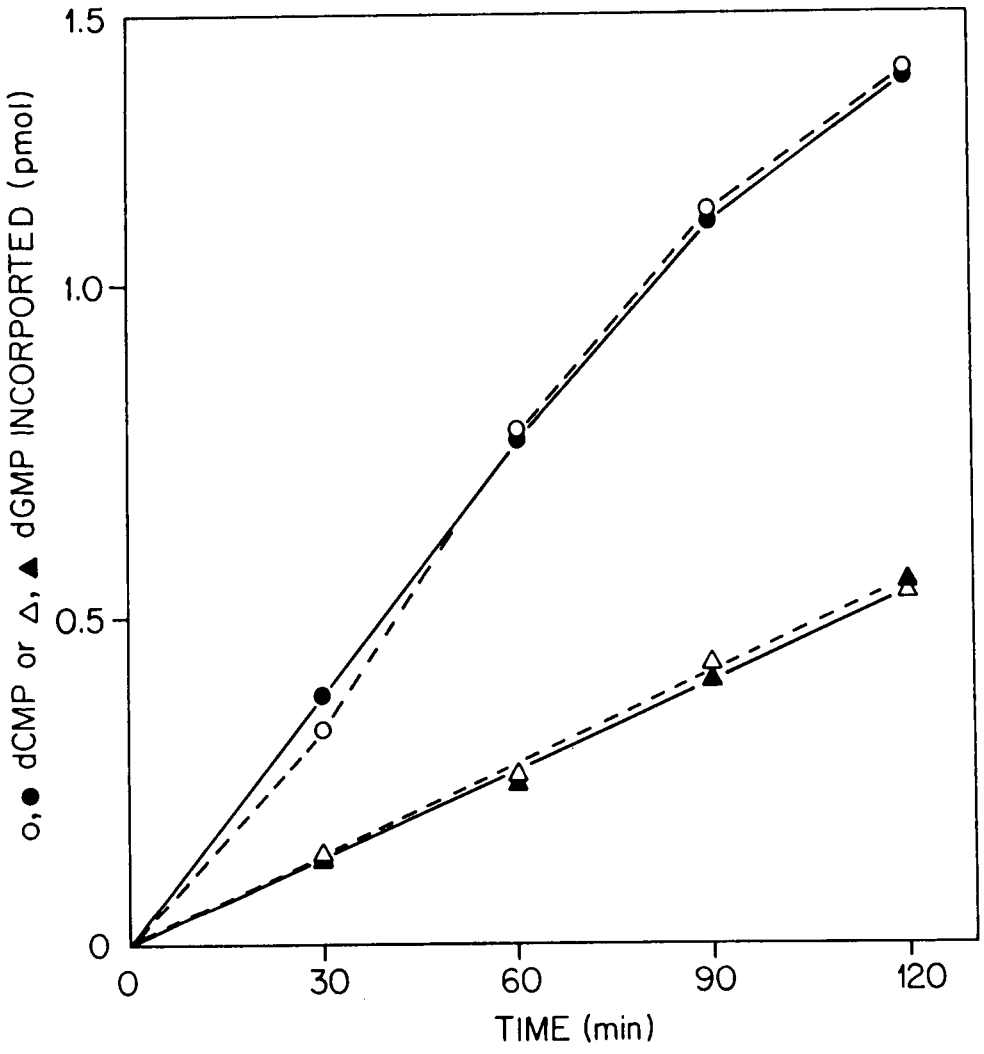
1.5
1.0
0.5
0
o, ● dCMP or Δ, ▲ dGMP INCORPORTED (pmol)
0
30
60
90
120
TIME (min)

Figure 4.2

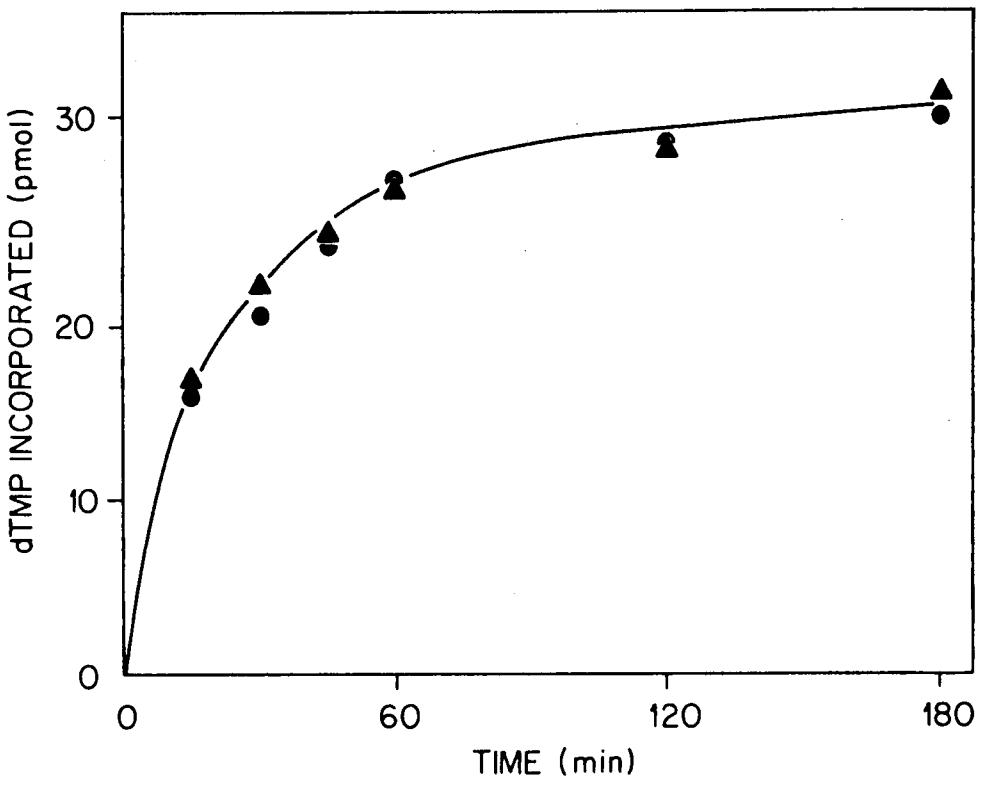

Figure 4.3. Comparison of the time course of the dTMP incorporation into poly[d(A-T)] by β-DNA polymerase isolated from young and old rat spleen when dATP is omitted from the reaction mixture.

Sixty μM dTTP* (0.3 Ci/mmol) was included in the reaction mixture. The assay procedure and reaction conditions were as described in "Material and Methods."

● ——— ● young

▲ ——— ▲ old

result in generating errors in RNA and protein synthesis during aging. Linn et al., (65) purified α-DNA polymerase from different passages of human fibroblasts and studied the fidelity of DNA synthesis. Their results suggest the presence of mutagenic DNA polymerases in senescent fibroblasts. In our analysis on the fidelity of DNA synthesis, we find no significant difference between the ability of DNA polymerases from young and old rat spleen. In addition, we cannot detect differences in the kinetics of correct and incorrect nucleotide incorporations catalyzed by the β-DNA polymerases from young and old rat spleen. The incorrect nucleotide incorporation paralleled correct nucleotide incorporation with respect to time suggests that the error incorporations were not due to terminal addition catalyzed by the contaminated terminal transferase. An interesting feature is that purine-pyrimidine mispairing (e.g., A•C) occurred at a higher frequency than purine-purine (e.g., A•G) or pyrimidine-pyrimidine (e.g., C•T) mispairing.

Although our data failed to indicate the age-related alterations in the fidelity of DNA polymerase, we cannot rule out the possibility that alterations in the fidelity of DNA polymerase could be an important factor in cell aging. The sensitivity of our assay is obviously limited. For example, the labeled incorrect nucleotide might be contaminated with correct nucleotide and the difference in DNA synthesis fidelity would be masked. Analysis of nucleotide composition of the DNA product may verify this point. The overall machinery for DNA replication is certainly far more complex than the <u>in vitro</u> system. The level of infidelity observed in our assay (10^{-3} to 10^{-4}) is much higher than the mutation rate of

mammalian cells, and it is reasonable to assume that an "editing" function associated with certain enzyme(s) must be involved in the *in vivo* DNA synthesis.

CHAPTER 5

AGE-ASSOCIATED CHANGE OF *IN VITRO* DNA SYNTHESIS IN RAT SPLEEN NUCLEI

Summary

Nuclei isolated from spleens of young adult and senescent Fischer 344 male rats were examined for DNA synthesis activity in an *in vitro* reaction mixture containing [^{3}H]dATP, dGTP, dCTP and dTTP. Low but significant dNMP incorporation into acid precipitable form was observed. This *in vitro* DNA synthesis activity was found to be 1.2- to 1.5- fold higher with nuclei preparation from the young than those from the old. Upon addition of exogenous DNA polymerase in the reaction, the dNMP incorporation into DNA of the spleen nuclei was enhanced and conversely became 3- to 4-fold higher with the old than with the young. The enhanced *in vitro* DNA synthesis by added exogenous DNA polymerase was also observed with nuclei preparation from liver and brain; however, the age-associated increase in the nuclei from the old was only slight with the brain preparations and not present with the liver preparations. Kinetic analysis revealed that the relatively high template activity of the old rat spleen nuclei represents increased substrate sites for the exogenous DNA polymerase. Mild acid treatment further increased the template activity of nuclei; in this respect, the young appeared to be more responsive than the old. This increased template activity of the nuclei was also observed in the spleen, but not in the liver, of senescent BALB/c mice.

Introduction

There appears to be correlation between the capability of DNA repair mechanism and the life span of vertebrates. Hart and Setlow (104) grew primary fibroblast cultures out of the dermis of several species and measured the DNA repair activity after exposure of these cells to ultraviolet irradiation. The most active repair activities were found in the cell cultures of human, elephant, and cow. They were nearly five times as active as the fibroblasts of rats and mice.

The results from direct investigations comparing DNA repair efficiency and aging have been conflicting among different investigations (66, 69, 70, 71). Whether or not the efficiency of the repair system changes with age is still an open question. In all repaired systems, the DNA strand which is injured is first identified and excised, then, an DNA polymerase will come to fill the gap by using information on the complementary strand to guide the repair. We have previously reported the decreased β-DNA polymerase, presumably responsible for DNA repair in mammalian cell, in the spleen of senescent Fischer 344 rat (94). In this report we will represent findings which may suggest a cause-effect relationship between the decrease of DNA polymerase activity and the alterations in the DNA of certain cell populations of old rat.

Materials and Methods

Materials and Equipments

Native calf thymus DNA were obtained from Worthington Biochemical Corporation, Freehold, New Jersey. Poly(dA)·$(dT)_{12-18}$, $(dT)_9$, non-

radioactive dNTPs (deoxyribonucleotide triphosphates), and denatured DNA cellulose were products of P-L Biochemicals. Radioactively labeled dNTPs were obtained from New England Nuclear Corporation, Boston, Massachusetts. Centrifugation was performed in the International Model PR-6 and all ultracentrifugation was performed in the Model L or Model L2-65B (Beckman Instruments, Inc., Palo Alto, California). AMV reverse transcriptase was obtained from the National Cancer Institute.

Animals

Pathogen-free male Fischer 344 rats and male BALB/c mice were raised in the Biology Division, Oak Ridge National Laboratory. Spleens and other tissue which were tumor free and of normal size were removed from young adult (3 months old) and senescent rats (25 months old) and mice (20 months old).

Isolation of Nuclei

The isolation of spleen nuclei was described previously (94). The isolation of liver and brain nuclei was done by the method of Blobel and Potter (105). The nuclei were then suspended in homogenizing buffer containing 0.2% Triton X-100. After 30 minutes at 4°C, the suspension was spun at 800 g for 10 minutes and the pellet was rewashed with homogenizing buffer twice and the pellet was suspended in 4 volumes of 10% glycerol in 0.05 M KCl, 3 mM $MgCl_2$, 0.05 M TrisCl(pH 8.0), and 1 mM dithiothreitol per original wet tissue weight. After Triton X-100 treatment, portion of the nuclei was treated with 0.01 N HCl (20 minutes, room temperature) in order to denature DNA, and rewashed twice before suspended in 4 volumes of homogenizing medium.

Purification of DNA Polymerase From Rat Spleen

The isolation and purification of DNA polymerase from rat spleen were previously described (94). The β-DNA polymerase was further purified by Sephadex G-100 gel filtration and denature DNA cellulose affinity column. Sephadex G-100, equilibrated with 0.5 M KCl, 10% glycerol, 1 mM dithiothreitol, 3 mM $MgCl_2$, and 0.02 TrisCl at pH 8.0 was used to prepare a column (5 x 68 cm) for gel filtration. The denatured DNA cellulose suspension stored at -20°C was thawed and packed into a column (0.5 x 9 cm). The column was washed with 50 ml of 1 M KCl in 10% glycerol, 1 mM dithiothreitol, 3 mM $MgCl_2$ and 0.02 TrisCl(pH 8.0) and then equilibrated with 0.15 M KCl in the buffer.

Determination of DNA Polymerase Activity

A standard reaction mixture of 50 µl contained 5 µl enzyme fraction, 20 mM TrisCl(pH 8.0), 5 mM dithiothreitol, 0.17 mg/ml BSA, 25 mM KCl, 60 µM of appropriate dNTP including [^{3}H]dTTP at 17.5 µCi/ml (0.3 Ci/mmol), 0.02 mg/ml of synthetic template-primer complex or 0.1 mg/ml activated DNA, and appropriate concentration of Mg^{2+} and Mn^{2+}. Poly(dA)·$(dT)_{12-18}$ was used as template for the assay of β-DNA polymerase activity. Poly-(rA)·$(dT)_9$ was used as template-primer for assay of γ-DNA polymerase activity and AVM reverse transcriptase activity. Mg^{2+} (1.5 mM) and 0.5 mM Mn^{2+} are included in the reaction mixture for the assay of AVM reverse transcriptase and γ-, β-DNA polymerase activity. Activated DNA (0.1 mg/ml)

and 8 mM Mg^{2+} is included in the reaction mixture for the assay of α-DNA polymerase activity. After incubation in a water bath at 37°C for a period of time, the reaction mixture was pipetted onto a filter paper disk (Whatman 3MM) for further processing and measurement of dNMPs incorporation as previously described (89).

Estimation of In Vitro DNA Synthesis

Isolated nuclei were incubated in a basal reaction mixture of 105 µl containing nuclei (5 to 150 µg DNA), 25 mM KCl, 0.17 mg/ml BSA, 8 mM $MgCl_2$, 5 mM dithiothreitol, 35 mM TrisCl(pH 8.0), and 60 µM of each of the four dNTPs including [^{3}H]dATP at 1.2 Ci/mmol. In addition to the endogenous DNA polymerase present in the nucleus, purified DNA polymerase may be included in some reactions. After incubation in a water bath at 37°C for various period of time, the reaction mixture was pipetted onto a filter paper disk (Whatman 3MM), which was subsequently washed and processed for scintillation counting (89). In all experiments, the synthesis of DNA *in vitro* have been corrected for the incorporation at zero time. The DNA synthesis is expressed as picomoles of dNMP incorporated versus amount of nuclei, expressed as µg of nuclear DNA.

DNA Determination

DNA was extracted from nuclei by the method of Ogur and Rosen (106) and then determined by the diphenylamine method (107).

Results

Further Purification of β-DNA Polymerase

β-DNA polymerase purified from phosphocellulose column (94) was dialyzed against 50% glycerol, 0.5 M KCl, 1 mM dithiothreitol, 3 mM $MgCl_2$, and 0.02 M TrisCl at pH 8.0. The concentrated β-DNA polymerase was then loaded on a Sephadex G-100 column previously equilibrated (see Materials and Methods) and eluted with the same buffer used to equilibrate the Sephadex column (Figure 5.1). The active fractions were pooled and adjusted to 0.15 M KCl in buffer and loaded on a denatured DNA cellulose column equilibrated with 0.15 M KCl in buffer. The flow rate of the column was adjusted to 0.2 ml per minute. After loading the column was washed with 100 ml of 0.15 M KCl in buffer, and the proteins were eluted with a linear KCl gradient in buffer from 0.15 M to 0.68 M. The total volume of the gradient was 100 ml. The β-DNA polymerase was eluted at about 0.57 M KCl and the elution profile is shown in Figure 5.2. The active fraction is concentrated by dialyzing against 50% glycerol, 25 mM KCl, 1 mM dithiothreitol, 3 mM $MgCl_2$, and 0.01 M phosphate buffer at pH 7.5. The dialyzed enzyme fractions were stored at -20°C before use.

In Vitro DNA Synthesis Activity of Isolated Young Rat Spleen Nuclei

In the absence of exogenous DNA polymerase, the incorporation of dNMP was proportional to the levels of spleen nuclei over at least a range of 30 to 150 μg of nuclear DNA (Figure 5.3). Since our previous experiments showed that the major DNA polymerase activity present in the nuclei of rat

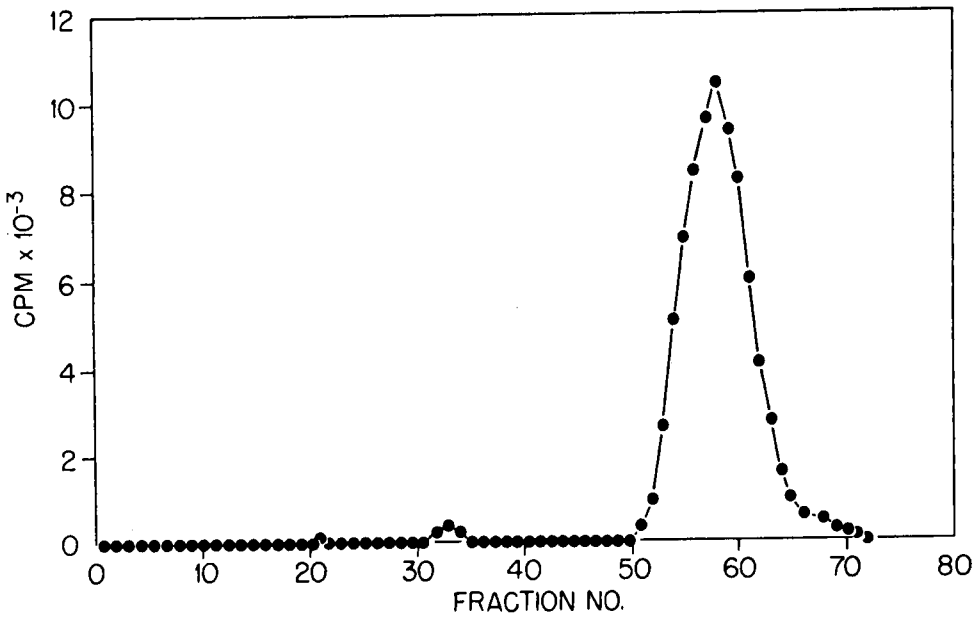

Figure 5.1. Gel filtration (Sephadex G-100) column chromatography of the β-DNA polymerase fraction of rat spleen purified by phosphocellulose chromatography.

Assay of the polymerase activity was as described in "Materials and Methods." Reaction time was 1 hr.

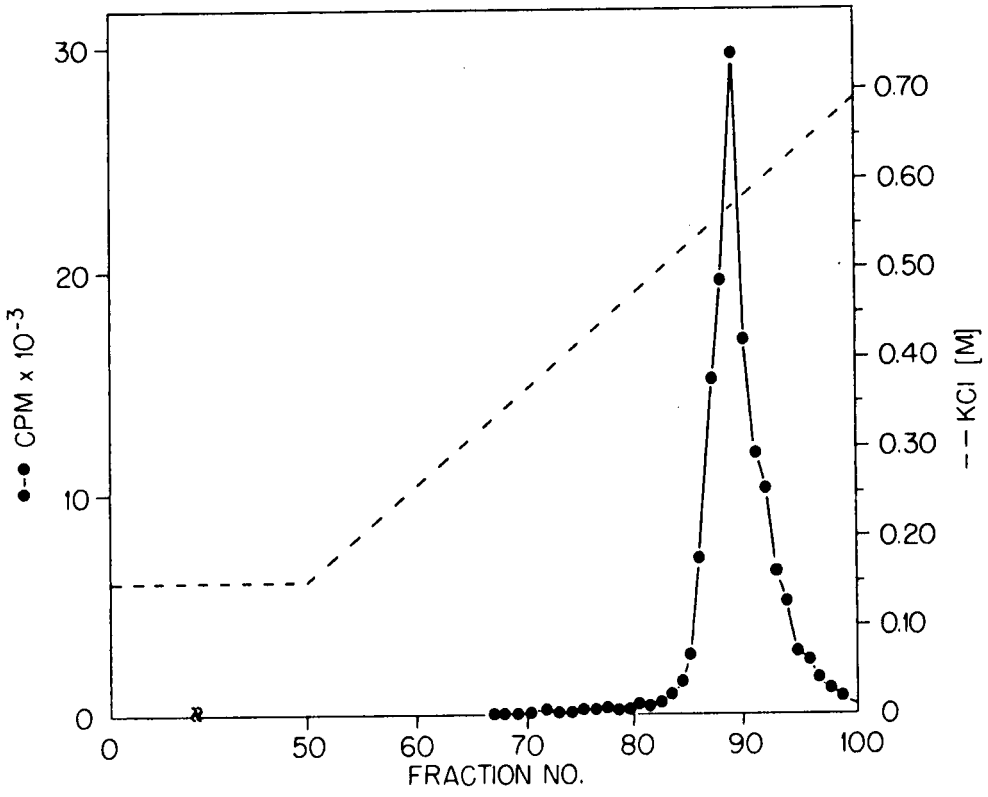

Figure 5.2. DNA-cellulose affinity chromatography of the β-DNA polymerase fraction purified by Sephadex G-100 chromatography.

Enzyme assay conditions were as described in "Materials and Methods." Incubation was carried out for 30 min.

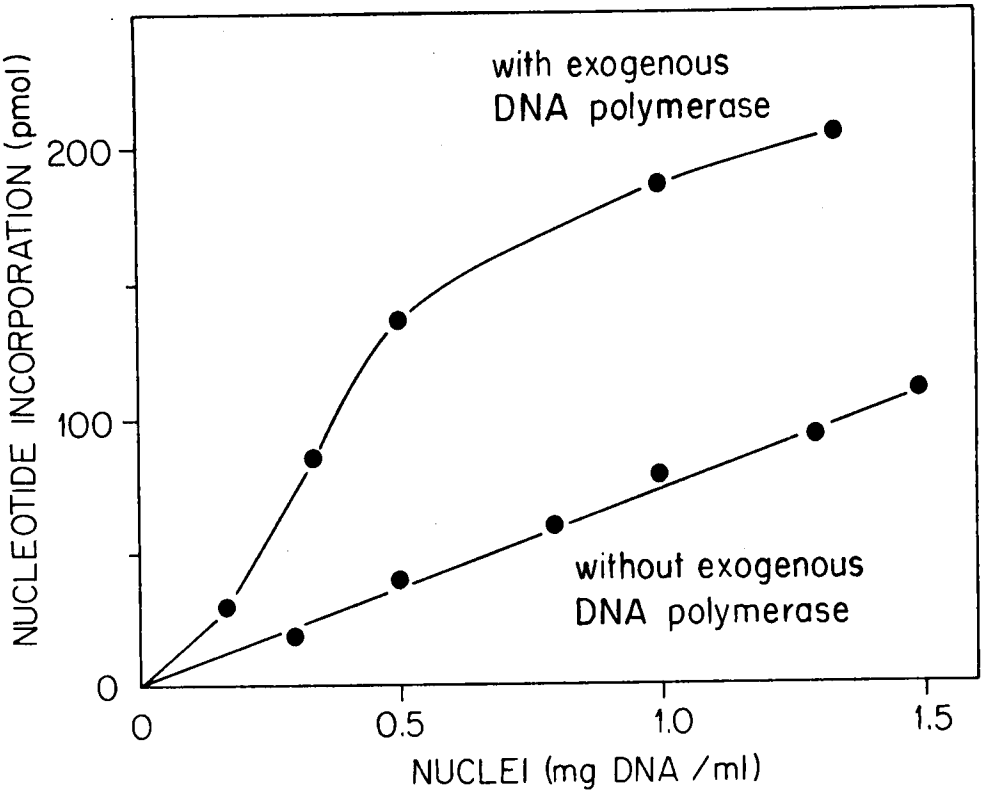

Figure 5.3. In vitro DNA synthesis activity of nuclei isolated from young rat spleen.

Reaction conditions were as described in "Materials and Methods." Incubations were carried out for 15 min. Incorporations were expressed as picomoles of dNMPs. Eighteen units of purified rat β-DNA polymerase was included in each reaction as exogenous DNA polymerase. One unit of enzyme activity is expressed as the incorporation of 300 picomoles of dNMP in 1 hr under the condition as described in "Materials and Methods."

spleen is β-DNA polymerase (94), this enzyme should be responsible for the dNMP incorporation observed. With the addition of purified rat spleen β-DNA polymerase, the dNMP incorporation increased and the rate of DNA synthesis seemed to follow the Michaelis-Menten equation although the maximum DNA synthesis rate has not been reached in the presence of 135 μg nuclear DNA included in the reaction mixture. The calculation of dNMP incorporation is based on the assumption that the amount of incorporation of all 4 dNMPs is the same. The results showed in Figure 5.3 were obtained by using [^{3}H]dATP as the labeled substrate. When [^{3}H]dTTP was used as labeled substrate, the levels of incorporation by the isolated nuclei were the same as with [^{3}H]dATP (data not shown).

In Vitro DNA Synthesis Activity of Mild Acid-Treated Nuclei of Young Rat Spleen

As shown in Figure 5.4, the incorporation of dNMP was proportional to the levels of mild acid-treated spleen nuclei in the absence of exogenous DNA polymerase. The *in vitro* DNA synthesis activity of mild acid-treated nuclei is higher than that of the untreated nuclei (compare Figure 5.3 with Figure 5.4). Obviously the β-DNA polymerase activity in the nuclei was not completely inhibited after acid treatment. It is not clear why the *in vitro* DNA synthesis activity of spleen nuclei increased after acid-treatment. It is possible, however, that after acid-treatment, more DNA polymerase and/or other nuclear protein has dissociated from non-active sites in the nuclei and then attached to the primer ends of nuclear DNA. These relocations of nuclear proteins would in turn facilitate the subsequent DNA synthesis. Another possibility is that DNA was

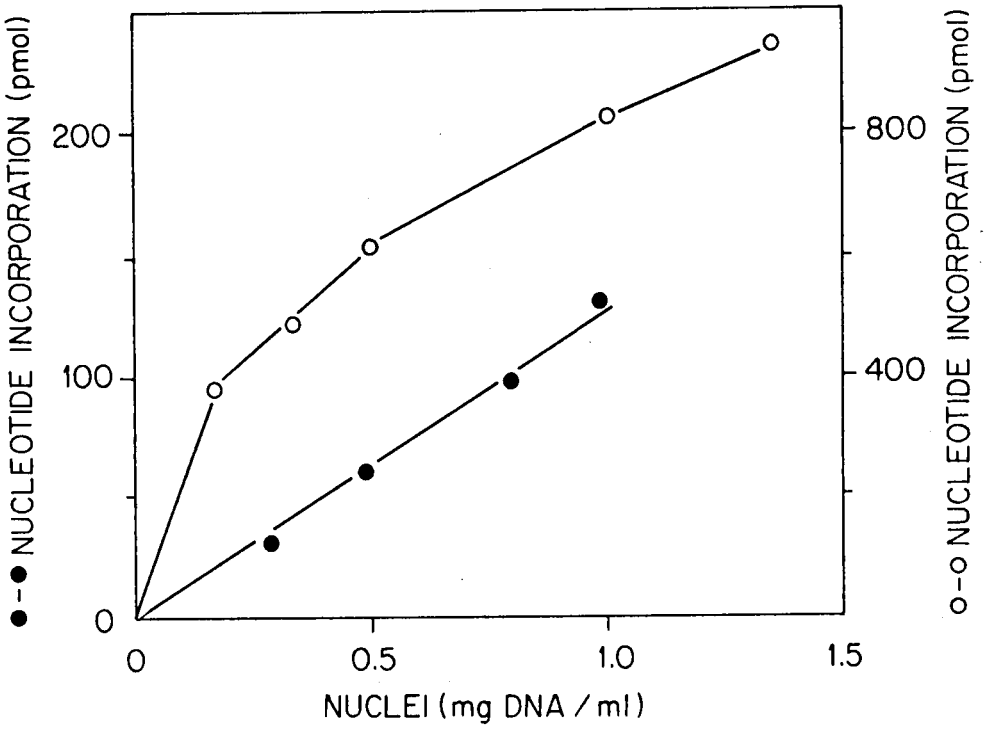

Figure 5.4. In vitro DNA synthesis activity of mild acid treated nuclei of young rat spleen.

Reaction conditions were as described in "Materials and Methods." Incubations were carried out for 15 min. The incorporations were expressed as picomoles of dNMPs. Eighteen units of purified rat β-DNA polymerase was included in each reaction as exogenous DNA polymerase. One unit of enzyme activity is expressed as the incorporation of 300 picomoles of dNMP in 1 hr under the condition as described in "Materials and Methods."

● ——— ● without exogenous DNA polymerase

o ——— o with exogenous DNA polymerase

fragmented during acid treatment and more primer ends available for DNA synthesis were produced. One should also consider, however, that acid treatment might denature the nuclear DNA which could serve as a better template for DNA synthesis.

Kinetics of dNMP Incorporation by Young Rat Spleen Nuclei in the Presence of Exogenous β-DNA Polymerase

The kinetics of DNA synthesis by spleen nuclei in the presence of exogenous β-DNA polymerase is shown in Figure 5.5. The dNMP incorporation was at nearly linear rates for the first 15 minutes, whereupon the rates fell off, especially when the untreated nuclear DNA is used as template. Again, the acid-treated nuclear DNA served as a much better template than the untreated nuclear DNA.

Effect of Addition of Various Amounts of Exogenous Rat β-DNA Polymerase on In Vitro DNA Synthesis Activity of Nuclei Isolated from Spleens of Young and Old Rats

Figure 5.6 shows the dNMP incorporation by the untreated nuclei of young and aged rat spleen in the presence of different amounts of exogenous β-DNA polymerase. In the absence of exogenous DNA polymerase, spleen nuclei of the young incorporate more dNMP than those of the old (0.80 ρmol/μg DNA versus 0.58 ρmol/μg DNA). However, as more exogenous β-DNA polymerase was included in the reaction, the DNA synthesis became conversely higher in spleen nuclei of the aged rat than those of the young.

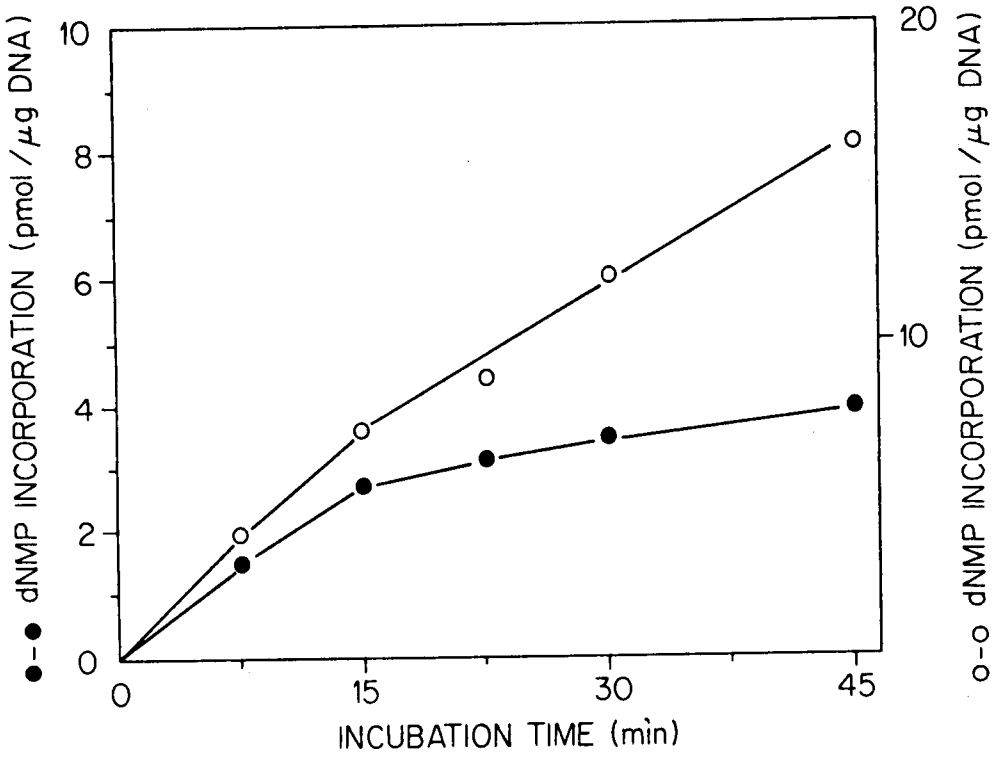

Figure 5.5. Kinetics of dNMP incorporation by young rat spleen nuclei in the presence of exogenous β-DNA polymerase.

Reaction conditions were as described in "Materials and Methods." Incorporations were expressed as picomoles of dNMP/μg nuclear DNA. Eighteen units of purified rat β-DNA polymerase was included in each reaction. One unit of enzyme activity is expressed as the incorporation of 300 picomoles of dNMP in 1 hr under the condition as described in "Materials and Methods." 30 to 50 μg DNA was included in each reaction.

● ——— ● untreated nuclei

0 ——— 0 mild acid treated nuclei.

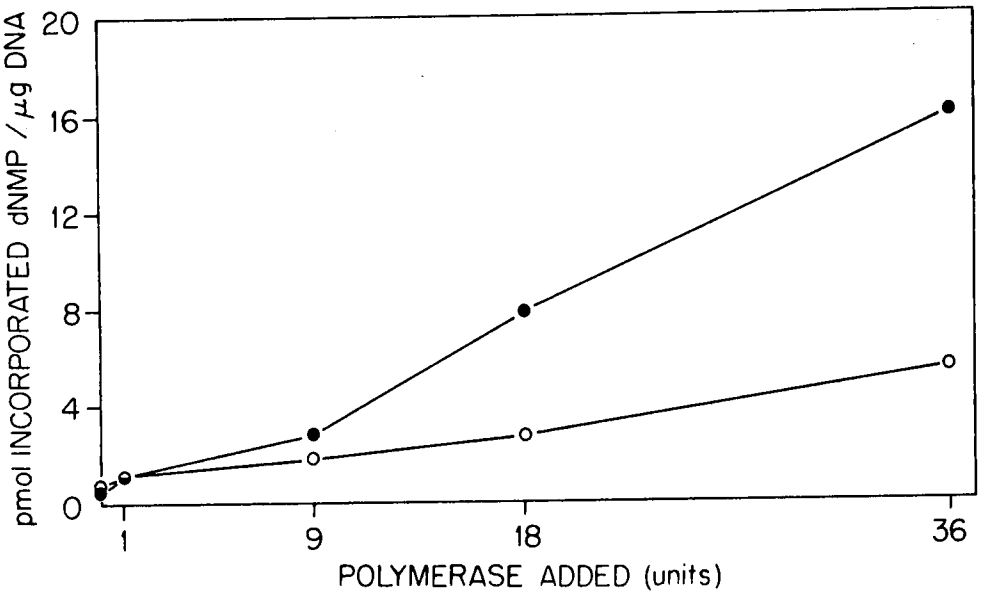

Figure 5.6. Effect of addition of various amounts of exogenous rat β-DNA polymerase on in vitro DNA synthesis activity of nuclei isolated from spleens of young and old rats.

Reaction conditions were as described in "Materials and Methods." Incubations were carried out for 15 min. Incorporations were expressed as picomoles of dNMPs/μg nuclear DNA. One unit of enzyme activity is expressed as the incorporation of 300 picomoles of dNMP in 1 hr under the conditions as described in "Materials and Methods." 30 to 50 μg DNA was included in each reaction.

● —— ● old

0 —— 0 young

Effects of Exogenous DNA Polymerase on In Vitro DNA Synthesis Activity of Nuclei Isolated from Spleen, Liver and Brain of Young and Old Rats

Tables 5.1, 5.2 and 5.3 show the dNMP incorporations by the nuclei isolated from spleen, brain, and liver of rat. The incorporations occurred either in the absence of exogenous DNA polymerase or in the presence of various DNA polymerases (purified rat α-, β-, γ-DNA polymerase and AMV reverse transcriptase). At least three points can be drawn from the results shown in Tables 5.1, 5.2 and 5.3. (1) In the absence of exogenous DNA polymerase, the dNMP incorporation is higher in the untreated young spleen nuclei than that of old (0.81 versus 0.54 pmol/μg nuclear DNA). Although such phenomenon can be observed in the liver and brain, the differences were smaller than in the spleen (0.75 versus 0.7 and 0.66 versus 0.61 pmol/μg nuclear DNA for brain and liver, respectively). In the presence of various exogenous DNA polymerases, the dNMP incorporations became conversely higher in the untreated aged spleen nuclei. Thus, the differential effects of exogenous DNA polymerase on untreated young and aged rat spleen nuclei can be demonstrated by employing β-DNA polymerase (Figure 5.6) as well as other DNA polymerases. Although the dNMP incorporation also increased in the untreated nuclei of aged brains and liver when the exogenous DNA polymerases were added (except the dNMP incorporation of liver nuclei in the presence of γ-DNA polymerase), the increase was not as remarkable (an average of 1.29-fold and 1.12-fold higher for the aged brain and liver nuclei in the presence of 4 DNA polymerases used) as that of spleen (an average of 4.05-fold higher in the aged nuclei).

TABLE 5.1

EFFECTS OF EXOGENOUS DNA POLYMERASES ON IN VITRO DNA SYNTHESIS[a] ACTIVITY OF NUCLEI ISOLATED FROM SPLEENS OF YOUNG[b] AND OLD RATS (pmol dNMP INCORPORATED PER μg NUCLEAR DNA)

Exogenous Enzyme[c]	Spleen Nuclei		Spleen Nuclei (Mild Acid Treated)	
	Young	Old	Young	Old
None	0.81 ± 0.037	0.54 ± 0.041	1.30 ± 0.085	0.87 ± 0.07
Rat α-DNA polymerase	1.42 ± 0.04	7.00 ± 0.55	11.01 ± 0.91	24.75 ± 1.80
Rat β-DNA polymerase	2.75 ± 0.07	7.94 ± 0.27	12.04 ± 1.15	20.52 ± 1.11
Rat γ-DNA polymerase	1.49 ± 0.047	5.50 ± 0.58	8.94 ± 0.53	20.36 ± 1.41
AMV reverse transcriptase	3.60 ± 0.28	16.93 ± 0.75	10.48 ± 0.88	25.06 ± 2.32

[a]Reaction conditions were as described in "Materials and Methods." Incubations were carried out for 15 min. 30 to 50 μg DNA was included in each reaction.

[b]The data in this table represent the mean value and standard error of 5 separate experiments each involved 4 to 5 young rats and 4 to 5 aged rats.

[c]The units of exogenous enzyme activities are 3.5, 18.5, 10, and 84 for rat α-, β-, γ-DNA polymerase and AMV reverse transcriptase, respectively. One unit of enzyme activity is expressed as the incorporation of 300 picomoles of dNMPs in 1 hr under the conditions described in "Materials and Methods."

TABLE 5.2

EFFECTS OF EXOGENOUS DNA POLYMERASES ON IN VITRO DNA SYNTHESIS[a] ACTIVITY OF NUCLEI ISOLATED FROM BRAINS OF YOUNG[b] AND OLD RATS (pmol dNMP INCORPORATED PER μg NUCLEAR DNA)

	Brain Nuclei		Brain Nuclei (Mild Acid Treated)	
Exogenous Enzyme	Young	Old	Young	Old
None	0.75	0.70	0.945	0.73
Rat α-DNA polymerase	0.92	1.13	1.26	1.19
Rat β-DNA polymerase	2.07	3.11	7.33	5.01
Rat γ-DNA polymerase	0.94	1.18	1.76	1.28
AMV reverse transcriptase	1.63	1.93	2.00	2.96

[a]Reaction conditions were as described in Table 5.1, except that incubations were carried out for 30 min. 10-15 μg DNA was included in each reaction.

[b]The data in this table represent the mean value of 2 separate experiments each involved 4 to 5 young rats and 4 to 5 old rats.

TABLE 5.3

EFFECTS OF EXOGENOUS DNA POLYMERASES ON IN VITRO DNA SYNTHESIS[a] ACTIVITY OF NUCLEI ISOLATED FROM LIVERS OF YOUNG AND OLD RATS
(pmol dNMP INCORPORATED PER μg NUCLEAR DNA)

	Liver Nuclei		Liver Nuclei (Mild Acid Treated)	
Exogenous enzyme	Young	Old	Young	Old
None	0.66	0.61	1.79	1.77
Rat α-DNA polymerase	0.80	0.84	3.99	3.40
Rat β-DNA polymerase	2.16	2.91	15.19	13.45
Rat γ-DNA polymerase	1.03	1.01	4.95	3.99
AMV reverse transcriptase	11.2	12.4	26.89	28.10

[a]Reaction conditions were as described in Table 5.1, except that the incubations were carried out for 30 min. 25-30 μg DNA was included in each reaction.

[b]The data in this table represent the mean value of 2 separate experiments each involved 4 to 5 young rats and 4 to 5 old rats.

(2) Acid denaturation increased the template activities of young and aged nuclear DNA in all three tissues. The nuclear DNA of young tissue was more responsive to the acid-treatment. In the presence of exogenous DNA polymerase, the aged spleen nuclear DNA still served as a better template than that of young after acid treatment, however, the average increase in template activity of aged spleen nuclei dropped from 4.05-fold to 2.16-fold after the acid treatment. Such effects can also be observed in other tissues. The aged brain and liver nuclear DNA, which served as better templates before acid treatment, were not as efficient templates as that of young brain and liver except when AMV reverse transcriptase were used as exogenous DNA polymerase.

(3) Different tissues seemed to respond differently to acid treatment in the aspect of template activation. For example, in the presence of exogenous α-DNA polymerase, the increase in template activity after acid treatment is in the order of spleen > liver > brain. In the presence of other exogenous DNA polymerases, the template activity of brain nuclear DNA did not increase as much as that of spleen and liver after acid treatment.

Comparison of In Vitro DNA Synthesis Activity of Nuclei Isolated from the Spleen and Liver of Young and Aged Mice

Nuclei isolated from tissues of 3-month and 20-month-old BALB/c mice were used to verify whether the age-associated increase of *in vitro* DNA synthesis in spleen nuclei can be identified in the mouse. The results in Table 5.4 showed that in the absence of exogenous DNA polymerase, the

TABLE 5.4

EFFECTS OF EXOGENOUS DNA POLYMERASES ON IN VITRO DNA SYNTHESIS[a] ACTIVITY OF NUCLEI ISOLATED FROM SPLEENS AND LIVERS OF YOUNG AND OLD MICE[b]
(pmol dNMP INCORPORATED PER µg NUCLEAR DNA)

	Spleen Nuclei		Liver Nuclei	
Exogenous Enzyme	Young	Old	Young	Old
None	0.94 ± 0.05	0.67 ± 0.07	NT[c]	NT
Rat α-DNA polymerase	2.91 ± 0.36	10.60 ± 0.92	2.93 ± 0.07	2.6 ± 0.07
Rat β-DNA polymerase	23.9 ± 2.80	37.60 ± 0.70	11.6 ± 1.3	10.9 ± 0.55
Rat γ-DNA polymerase	2.28 ± 0.36	7.47 ± 0.44	3.35 ± 0.02	2.93 ± 0.07
AMV reverse transcriptase	13.55 ± 0.35	26.99 ± 2.39	17.6 ± 0.20	18.6 ± 0.70

[a]Reaction conditions were as described in Table 5.1, except the incubations were carried out for 15 min (for spleen) or 30 min (for liver).

[b]The data in this table represent the mean value and standard error of 3 separate experiments each involved 2 young mice and 2 old mice.

[c]NT (not tested).

DNA synthesis of isolated mice nuclei from spleen was higher in the young than in the old. When exogenous DNA polymerases were included in the reaction, the difference became reversed. The average age-associated increase *in vitro* DNA synthesis in the presence of exogenous DNA polymerase is 2.6-fold. Nuclei isolated from livers of two groups of young and two groups of aged mice were used for the similar studies; in the presence of the four exogenous DNA polymerases, no significant difference was observed.

Kinetic Analysis of Substrate Activity of Rat Spleen Nuclear DNA

The substrate activities of rat spleen nuclear DNA under different concentrations were analyzed. Same amount of exogenous β-DNA polymerase were included in each reaction. Figure 5.7 showed that the nuclear DNA of young and aged spleen have the same Vmax value whereas the Km values, expressed by the nuclear DNA content, can be estimated to be 70 μg/ml and 330 μg/ml with the spleen nuclei of the old and young rats.

DNA Synthesis of Nuclei Isolated from Mixture of Young and Aged Rat Spleen

The observed increase of exogenous DNA polymerase-catalyzed *in vitro* DNA synthesis may have been resulted from a faster rate of release and action of deoxyribonuclease in the spleens of the aged rats than in the spleens of the young rats during the isolation. In view of this possibility, spleens from both young and aged rats were pooled together for the subsequent nuclei isolation. The isolated nuclei from mixed young and aged spleens were then assayed for *in vitro* DNA synthesis in the presence of

Figure 5.7. Kinetic analysis of substrate activity of rat spleen nuclear DNA.

Concentration of nuclei was expressed as μg DNA/ml, dNMPs incorporation was expressed as cpm (^{3}H-dATP) incorporated per min of incubation. Reaction conditions were as described in "Materials and Methods." Incubation was carried out for 15 min. Eighteen units of exogenous β-DNA polymerase was included in each reaction. The dNMPs incorporation in the absence of exogenous DNA polymerase is subtracted from the dNMPs incorporation in the presence of exogenous DNA polymerase. Nuclei preparations were isolated respectively from spleen pools of four young and four old rats.

● ——— ● old

0 ——— 0 young

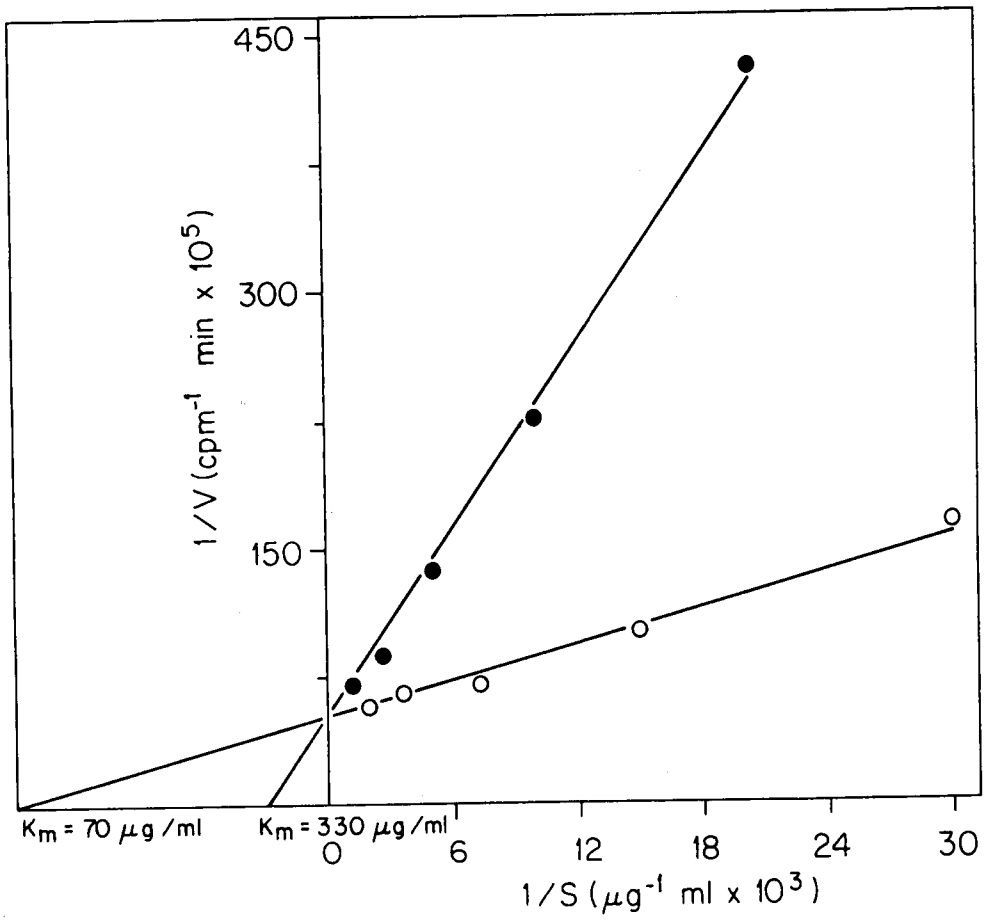
450
300
150
1/V (cpm^{-1} min x 10^5)
Km = 70 μg /ml
Km = 330 μg /ml
0
6
12
18
24
30
1/S (μg^{-1} ml x 10^3)

Figure 5.7

exogenous β-DNA polymerase. The rationale is that if deoxyribonuclease was released from the old spleen cells during isolation procedure, the enzyme should also act on the young spleen nuclear DNA. If this were true, we should expect the amount of DNA synthesized in the mixed nuclei to be similar to that in the aged spleen nuclei. Table 5.5 shows the results of an experiment which involved three young and three aged rat spleens. The *in vitro* DNA synthesis activity of mixed nuclei in the presence of exogenous β-DNA polymerase, was found to be approximately in between those individual nuclei preparations. Thus, the explanation that the increased *in vitro* DNA synthesis associated with aged rat spleen nuclei was due to faster rates of release and action of deoxyribonuclease during the isolation was not supported by the results of the mixed experiment.

Effect of ATP on the In Vitro DNA Synthesis of Rat Spleen Nuclei

Although purified DNA polymerases do not require ATP for the enzyme activities (108, 109, 110), Stenstrom et al. (111) found that ATP can stimulate *in vitro* DNA synthesis of nuclei isolated from regenerating rat liver.

In this experiment, we examined whether ATP will stimulate the *in vitro* DNA synthesis of rat spleen nuclei in the presence of exogenous β-DNA polymerase. Table 5.6 showed that in the presence of 2 mM ATP, slight stimulation of DNA synthesis can be observed (13% and 2% for young and aged, respectively). The *in vitro* DNA synthesis was considerably higher in the aged rat spleen nuclei whether ATP was present or not.

TABLE 5.5

IN VITRO DNA SYNTHESIS[a] ACTIVITY OF NUCLEI ISOLATED FROM MIXTURE[b] OF YOUNG AND AGED RAT SPLEENS

Experiment[c]	Nuclei From		
	Young	Old	Mixture
I	3.00	7.92	6.60
II	2.85	11.66	7.05
III	2.89	8.41	5.45
Average and standard error	2.91 ± 0.045	9.33 ± 1.74	6.37 ± 0.48

[a]Eighteen units of purified β-DNA polymerase of rat spleen is included in reaction mixture. Incubations were carried out for 15 min. Incorporations are represented as picomoles dNMP/μg nuclear DNA. Reaction conditions were as described in "Materials and Methods."

[b]Young spleen was cut into two pieces of equal weight, one piece is used for the assay of dNMP incorporation into the nuclear DNA of young. The other piece of young spleen, together with a piece of old spleen of the same weight, were used for the assay of dNMP incorporation into the mixed nuclear DNA. The remainder of the cut old spleen was used for teh assay of dNMP incorporation into the aged nuclear DNA.

[c]Each experiment involved one young rat and one old rat.

TABLE 5.6

EFFECT OF ATP ON THE IN VITRO DNA SYNTHESIS[a] OF RAT SPLEEN NUCLEI

Nuclei From	ATP		Nuclei From	ATP	
	Without	With[b]		Without	With[b]
Young No. 1	2.71	2.94	Old No. 1	7.94	8.01
Young No. 2	2.57	3.01	Old No. 2	6.02	6.19
Young No. 3	2.57	2.91	Old No. 3	8.99	9.42
Average and standard error	2.62 ± 0.047	2.95 ± 0.029		7.65 ± 0.87	7.87 ± 0.93

[a]Incorporations are represented as pmol dNMP/μg nuclear DNA. Eighteen units of purified β-DNA polymerase of rat spleen is included in reaction mixture. Incubations were carried out for 15 min. Reaction conditions were as described in "Materials and Methods."

[b]2 mM ATP is included in reaction mixture.

Discussion

Burnet's theory of intrinsic mutagenesis (112) argued that the rate of mutation which leads to aging is determined by the characteristics of key enzymes involved in the transmission of genetic information. Those key enzymes include replicative and repair DNA polymerases, ligase, deoxyribonuclease, and restriction enzymes. In our laboratory, we have studied the quantitative and qualitative alterations of rat DNA polymerases during aging process (94). We found that β-DNA polymerase activity, presumably responsible for DNA repair function, decreased in the aged rat tissue.

The rate of accumulation of aging injury to DNA within a cell would be a function of the rate of its occurrence and the rate of its removal. If one assumes that at any one time a cell carries a burden of potentially repairable but at that moment unrepaired aging damage, such damage would accumulate with time at a constant rate. If the repair system itself becomes less efficient during the aging process, then the mutation rate will increase in the latter part of the life span. Assuming decreased β-DNA polymerase activity does lead to a less efficient repair function, such decreases, especially in the aged spleen cells, might be critical to the aging process because accumulation of mutation in the cell system responsible for immunity could lead to the loss of autoimmunity suppression. Our attempt in this experiment was to examine whether there is a biological significance of the previously observed β-DNA polymerase decrease during aging process of aged rat. In other words, a

relationship between the decrease of DNA polymerase activity and the alteration in the DNA.

When isolated rat spleen nuclei were incubated with radioactively-labeled dNTP, the *in vitro* DNA synthesis is proportional to the nuclei included in the reaction. Upon addition of exogenous DNA polymerase, the dNMPs incorporation by the nuclei was enhanced indicating the ability of the exogenous DNA polymerase to act on the substrate sites of nuclear DNA. After acid treatment of the nuclei, the *in vitro* DNA synthesis was further increased due to either the activation of nuclear DNA and/or the nuclear proteins. The results in Figure 5.6 (p. 100) and Table 5.1 (p. 102) showed that in the absence of exogenous DNA polymerase, the dNMPs incorporation is higher in the young spleen nuclei, apparently reflecting the decreased β-DNA polymerase activity in aged rat spleen observed previously. The data also revealed that the difference between the young and the old in DNA synthesis was converted upon addition of exogenous DNA polymerase, indicating higher template activity of the spleen nuclear DNA of aged rat. Kinetic analysis of the substrate activity of the spleen nuclear DNA [Figure 5.7 (p. 108)] showed that the apparent V_{max} value was the same for both the young and the old while the Km value is much smaller for the nuclear DNA of old rat spleen. This result indicated that the higher template activity associated with aged rat spleen DNA represents increased substrate sites. Since all the DNA polymerases require a primer with free 3'-OH end in complex with the single stranded template for DNA synthesis, we suggest that such structures are present in the nuclei of young and aged rats. It is conceivable that some DNA breakage and

denaturation might be introduced during the isolation of nuclei. Another consideration is that the increased template activity of aged rat spleen may simply reflect an increased mitotic activity. To our knowledge, such phenomenon has not been observed and reported. In addition, our findings that both young and aged rat spleen possess equivalent α-DNA polymerase activity (94), which is proportional to the proliferation rate of cells, does not support this possibility.

It is possible that the increased template activity of aged rat spleen nuclei is due to an increased denaturation of DNA either during aging process or during the isolation of the nuclei. If this is the case, the levels of incorporation would be expected to be similar in both young and old nuclei after acid denaturation. However, after acid treatment, the nuclei of senescent spleen still incorporate higher amounts of dNMPs than those from young although such denaturation treatment had a greater template activation effect on the young spleen nuclei. Similar observation has been made by O'Meara et al. (79). They studied the ability of mice liver chromatin to serve as template for RNA polymerase and found that template activity increased as the protein was dissociated by the salt. At low salt concentrations, chromatin of old mice showed less template activity than the chromatin from young animals. These studies suggested that at least some nuclear proteins may be less readily removed from old chromatin.

The dNMPs incorporation also increased in the untreated nuclei of aged brain and liver upon the addition of exogenous DNA polymerase. The increase was not as remarkable (an average of 1.29-fold and 1.12-fold

higher for the aged brain and liver nuclei in the presence of four DNA polymerases used) as that of spleen (4.05-fold higher for aged nuclei). The increase in template activities seemed to correlate although not perfectly with the decrease of β-DNA polymerase activity in nucleus fraction of various tissue previously observed (60%, 39%, 0% decrease of β-DNA polymerase activity for spleen, brain, and liver, respectively).

Since it has been reported that the β-DNA polymerase activity also decreased remarkably in the aged BALB/c mice spleen, but not in liver (75), the in vitro DNA synthesis of BALB/c mice spleen and liver nuclei was also measured in this experiment. Again, upon the addition of exogenous DNA polymerase, the nuclei of senescent spleen incorporated higher amounts of dNMPs than those from young while both young and aged liver nuclei incorporated similar amounts of dNMPs (Table 5.4, p. 106).

Although it is not clear whether the single strand nick in DNA can be used as an initiation site by DNA polymerase; Bollum and Chang (113, 114) have shown that calf thymus DNA polymerase can use nicked DNA as template. In addition, Modak and Price (72) have shown that x-ray irradiation, which induce single-strand breaks but not strand separation in DNA, will cause the increase of dNMP incorporation of mice brain nuclei upon incubation with calf thymus DNA polymerase. Our results suggest that during the aging process, DNA single-strand breaks and single-strand regions accumulated in certain tissues (e.g., spleen) which render more sites become available for the dNMP incorporation catalyzed by DNA polymerase. Price, Modak and Makinodan (72, 73) have studied the template activity of DNA with calf thymus DNA polymerase and labeled dNTP

in sections of fixed brain, liver, and heart tissues from young and old mice. Following autoradiograph, grain counts were found to be higher in the nuclei of old mouse neurons, astrocytes, Kupffer cells, and heart muscle fibers. They concluded that there is an accumulation of DNA strand breaks with aging. However, their studies on lymphatic tissue showed extremely high activity that age differences could not be observed due to the high background. On the other hand, our results do suggest that there is accumulation of DNA strand breaks in the lymphatic tissue of rat during aging. We would like to further propose that DNA repair function of rat cell depends on, in addition to other factors, the function of the β-DNA polymerase. Decreased β-DNA polymerase activity may lead to the decrease of DNA repair capacity and more DNA damage were left unrepaired.

CHAPTER 6

GENERAL DISCUSSION

In summary, three major observations have been made in the present study. First, three distinctive DNA polymerase activities (α-, β-, and γ-DNA polymerase according to current nomenclature for eukaryotic cells) can be identified in the rat cells. Second, the β-DNA polymerase activity shows a considerable decrease in the spleens of the aged Fischer 344 male rat, particularly in the nuclear fraction of the cells. There is no difference in heat lability and in in vitro replicational infidelity between DNA polymerases purified from young and old rat spleens. Third, when mixed with exogenous DNA polymerases in an in vitro DNA synthesis reaction, nuclei of old rat spleen possess an increased template activity as compared with those of young. This phenomenon also occurred in aged BALB/c mouse spleen.

The DNA replication in eukaryotic cells have been studied by autoradiographic technique (115) and sedimentation analysis of newly synthesized DNA (116). The results of these investigations indicate that there are a large number of replicons in eukaryotic cells, and that DNA synthesis in the chromosome is discontinuous and may proceed in steps involving formation of relatively small discrete pieces of DNA which are eventually joined to form the completed chromosome fibers. The precise role of the cellular DNA polymerases in the DNA replication remains to be determined.

Studies with cells actively synthesizing DNA have shown that the levels of DNA polymerase α is correlated with DNA production. Chang

and Bollum (12) showed that L cells, shifting from a stationary to a dividing state, exhibit a rise in α-DNA polymerase level within 48 hours, with no change in β-DNA polymerase activity. Baril et al. (41) observed a rise in α-DNA polymerase levels in regenerating rat liver 18-30 hours post hepatectomy. Spadari and Weissbach (42) measured the levels of DNA polymerase activities in synchronized HeLa cells. They observed a rise in γ-DNA polymerase levels in the early part of the S phase, and a steady rise of α-DNA polymerase activity during the entire S phase, and no change in β-DNA polymerase levels. A common conclusion drawn from these studies is that α-DNA polymerase is one of enzymes utilized in the cellular DNA replications. In our studies of the DNA polymerase activities of rat spleen, liver and brain, both α- and γ-DNA polymerase activity appeared to correlate with cellular proliferation state of various tissued while β-DNA polymerase activity is relatively constant and can be readily detected in the spleen, liver and brain. This result further supports the previous studies on the correlation between DNA polymerase activities and DNA production and suggests that both α- and γ-DNA polymerases are important in replicative DNA synthesis.

Template-primer studies have also been used in the search for DNA polymerase function. Chang and Bollum (20) tested a series of oligoribo- and oligodeoxyribonucleotides as primers and found that oligo-adenylates are effective primers for calf thymus α-DNA polymerase but not for β-DNA polymerase. With the HeLa cell polymerase, Spadari and Weissbach (43) found that oligo-adenylate would act as a primer for all the DNA polymerases, but only α-DNA polymerase could synthesize DNA covalently bound

to a natural RNA primer. If RNA primers are necessary to initiate DNA synthesis in the nucleus (45), then one would suppose, from the above mentioned studies, that α-DNA polymerase would be required at the beginning of DNA replication. However, after the formation of the first DNA chains covalently bound to the RNA primers, further DNA synthesis could be carried out by β- or γ-DNA polymerase. In fact, Spadari and Weissbach (43) found that once a RNA-DNA primer molecule was formed by α-DNA polymerase, further extention of the DNA chain could be achieved by either α-, β-, or γ-DNA polymerase. In our studies of the template-primer specificity of rat spleen DNA polymerases, α-DNA polymerase utilizes a DNA template-RNA primer much more efficiently than β- or γ-DNA polymerase does. This result reconfirms the previous observation by other investigators and suggests that α-DNA polymerase is involved in the initial step of DNA replication.

Since DNA-repair function is required in both proliferative and post-mitotic tissue, it would be expected that the DNA polymerase responsible for repair shall be identified in all tissues. Our data showed that the β-DNA polymerase activity can be readily detected in all the tissues examined. The lack of variation of β-DNA polymerase level in various tissues might suggest that this enzyme is involved in a constitutive process such as DNA repair. Bertazzoni et al. (52) and Noy et al. (51) have examined the variation in DNA polymerase activities during prolonged stimulation of human lymphocyte by phytohemagglutinin. They observed paralleled curves of increases in the α-DNA polymerase activity and in the rate of cellular DNA synthesis; while the β-DNA polymerase activity

reached its maximum later. On the basis of parallel studies done with untraviolet light-treated lymphocytes, using hydroxyurea blocking to measure repair-type synthesis, they found that the β-DNA polymerase activity apparently correlated with the ability of cells to perform a repair-type DNA synthesis. However, it is obvious that these circumstantial evidence can not prove a definite correlation between β-DNA polymerase and DNA repair, and further experiments are required to determine the functional role of β-DNA polymerase.

In addition to α-, β-, and γ-DNA polymerases, a new species of DNA polymerase (the δ-DNA polymerase) has been isolated from the cytoplasm of rabbit erythroid hyperplastic bone marrow (117). This DNA polymerase, in contrast to all other eukaryotic DNA polymerases thus far described, is associated with a very active 3' to 5' exonuclease activity. The exonuclease activity is not separable from the DNA polymerase activity by chromatography on DEAE-Sephadex or hydroxylapatite. Upon sucrose density gradient centrifugation, both activities cosediment at 7S. In addition, both exonuclease and polymerase activities have identical rates of heat inactivation. These results suggest that the exonuclease is an integral part of δ-DNA polymerase. Although the role of the 3' to 5'-exonuclease activity associated with δ-DNA polymerase has not been established, Byrnes et al. found that this enzyme catalyzes the removal of 3'-terminal nucleotides from DNA, as well as a template-dependent conversion of deoxyribonucleoside triphosphate to monophosphate. This observation led to the suggestion that this mammalian DNA polymerase may be able to correct mistakes made during DNA polymerization and that one of the mechanisms

whereby replication fidelity is maintained in eukaryotic cells may be similar to that of prokaryotes. In addition to the rabbit bone marrow, the δ-DNA polymerase has been identified in the calf thymus (118). Whether the δ-DNA polymerase is ubiquitous in mammalian cells is not clear. In our study of the DNA polymerase activities of rat spleen, none of the three DNA polymerases identified has similar characteristics to the δ-DNA polymerase. However, since the best template for δ-DNA polymerase is poly-d(A-T), while both activated calf thymus DNA and poly(rA)·oligo(dT) are rather poor template for this enzyme (117), our use of activated calf thymus DNA, poly(rA)·poly(dT) and poly(rA)·$(dT)_9$ as templates to assay for DNA polymerase activity during purification procedures might explain why δ-DNA polymerase was not detected, if it does exist, in the rat spleen

It is obvious that understanding DNA replication requires more than mere studies on DNA polymerase. For example, several proteins specifically bind to single-stranded DNA have been isolated from calf thymus gland by Herrick and Alberts (47). These proteins will interact with DNA by ionic interaction and are able to hold long stretches of single-stranded DNA in an extended, regular, and fully hyperchromic complex. In addition, some of these DNA-binding proteins were found to stimulate the DNA polymerase activity. These unwinding proteins provide the clearest experimental demonstration of proteins factors other than DNA polymerase that might be required for DNA replication. Knowledge of the properties of accessory proteins controlling the initiation and termination of cellular DNA synthesis and removal of RNA initiators; and degradation of RNA chains of DNA-RNA hybrids, etc. would be most helpful in the understanding of the mechanism of DNA replication.

Concerning the experiments on the fidelity of DNA polymerase isolated from young and aged rat spleens, an important consideration is whether or not the experimental observation of the DNA synthesis infidelity reflects the actual in vivo situation regarding mutagenic DNA synthesis. Although the results of our experiment did not show an increased infidelity associated with aged rat spleen DNA polymerases, it does not exclude the possibility that error-making DNA polymerase molecules accumulate during aging process since the altered molecules might have been removed during purification of the enzymes. Another critical point is whether or not an editing process is active in vivo following the incorporation of an incorrect nucleotide. It is known that the high fidelity of DNA replication in prokaryotes has been ascribed, in part, to the presence of a 3' to 5' exonuclease associated with DNA polymerase. For example, this exonuclease activity is associated with DNA polymerase I, II, III of Escherichia coli and is present in all prokaryotic DNA polymerases thus far studies (119). The 3' to 5' exonuclease has a proofreading function, as it removes mismatched nucleotides incorporated at the primer terminus during DNA polymerization (100). Mutations in the gene coding for T_4 DNA polymerase can result in either an increased rate of spontaneous mutation (120) or a decreased rate (antimutator) of spontaneous mutation (121). The increased frequency of spontaneous mutation is due to a much higher ratio of DNA polymerase to exonuclease activity and, conversely, the DNA polymerase purified from the antimutator strain has a much higher nuclease to polymerase ratio (122). Thus, this 3' to 5' exonuclease

activity plays an important role in replicational fidelity and the frequency of mutation. However, the eukaryotic DNA polymerases have been reported not to be associated with any exonuclease activity (119) until the discovery of δ-DNA polymerase in rabbit bone marrow (117) and calf thymus (118). It is not clear, at the present time, whether δ-DNA polymerase is ubiquitous in the mammalian cells. In addition, it has not been established that the 3' to 5' exonuclease activity associated with δ-DNA polymerase does play an important role in maintaining the high fidelity of DNA replication in mammalian cells. Nonetheless, if such an editing process does exist, either associated with certain eukaryotic DNA polymerase or with another enzyme, then our experiment of testing the fidelity of DNA polymerase does not quite reflect what actually happens in vivo since neither α-, β-, or γ-DNA polymerase has been reported to possess a proofreading function (119). A decrease editing function in the aged cell would have even more profound effect than the mutagenic DNA polymerase on the miscoding of DNA replication. It will be interesting to compare the replicational fidelity of δ-DNA polymerase with that of other mammalian DNA polymerases which do not possess an associated exonuclease activity. Higher replicational fidelity of δ-DNA polymerase would indicate that the 3' to 5' exonuclease activity associated with the δ-DNA polymerase does have a proofreading function.

In addition to our finding of decreased β-DNA polymerase activity in aged rat spleen cells, such phenomenon has been reported in aged BALB/c mice spleen (75) and aged human granulocytes (123). It is interesting that all three instances of decreased β-DNA polymerase activity were in

tissues derived from hemopoietic stem cells. It is worthwhile to examine the polymerase activities of the various hemopoietic tissues in those animals which have an age-associated decrease of β-DNA polymerase activity in one of the hemopoietic tissues. Perhaps the decrease in β-DNA polymerase activity is a common phenomenon occurs in the hemopoietic tissue during the aging process of certain animals.

Concerning the biological effect of the decrease in β-DNA polymerase activity, it is important to consider what this phenomenon reflects alteration in DNA metabolism. It has been demonstrated that with advancing age, spleen cells of mouse (97), hamster (124), and human (125) exhibit a depressed response of DNA synthesis to phytohemagglutinin (PHA) stimulation. Hung, Perkins, and Yang (126) have further demonstrated that the reduced response of DNA synthesis to PHA stimulation could not be attributed to a decreased viability of spleen lymphocytes from old mice in <u>in vitro</u> culture condition, to different PHA dose requirements, or to longer periods of culture time for old mouse spleen cells to reach maximal activity. In addition, they found that spleen cells from old mice bind with PHA equally well, if not better, than spleen cells from young mice (127). There is neither significant alteration of binding affinity nor decreased total number of membrane receptor sites for PHA in senescent mouse spleen cells. The results of their experiment suggest that the depressed response of DNA synthesis to PHA results from, instead of marked membrane changes of aged spleen cells, defects involving various intracellular biochemical processes essential for DNA replication and initiation of cell proliferation. If the β-DNA polymerase is involved in

the response of DNA synthesis to PHA stimulation, it is expected to find a correlation between the decrease of β-DNA polymerase activity and the reduction of response to PHA stimulation. Barton and Yang (75) have observed a decrease of β-DNA polymerase activity in the spleen of aged (30 month old) BALB/c mice (medium-lived strain with a mean life span of 25 months) but not in the spleen of aged (30 months old) BC_3F_1 mice (long-lived strain with a mean life span of 31 months). Since it has been reported (128) that there is an earlier and more dramatic age-associated decrease of the response to PHA stimulation in the spleen of BALB/c mice than that in the spleen of BC_3F_1 mice, it seems that the decrease of β-DNA polymerase activity and the decrease of PHA stimulated response occur in a similar pattern during the life span of the animals.

On the other hand, if the β-DNA polymerase is involved in DNA repair process as Baril et al. have suggested (41), then decreased enzyme activity would result in a decrease in DNA repair and eventually lead to the accumulation of unrepaired DNA. In fact, the experimental results described in Chapter 5 showed that the in vitro DNA synthesis activities of the isolated nuclei from aged Fischer 344 rat and BALB/c mouse spleens are much higher than their younger counterparts when exogenous DNA polymerases were included in the reaction, indicating more gaps were left unrepaired in the nuclei of aged rat and mouse spleen. In addition, such a drastic increase in the in vitro DNA synthesis activity can not be observed in the aged mouse and rat livers in which the β-DNA polymerase activities do not decrease. It would be most enlightening to examine the in vitro template activity of nuclei isolated from spleens of animals

which do not have an age-associated decrease in β-DNA polymerase activity. Observation of correlation between these two phenomena would provide additional evidence for a cause-effect relationship between the decrease of β-DNA polymerase activity and the deteriorated DNA repair function.

The functional role of β-DNA polymerase, as well as other mammalian DNA polymerases, is still not clear at the present time largely due to the lack of pertinent mutants and adequate genetic studies. Xeroderma pigmentosum (X.P.) is a human genetic disease characterized by hypersensitivity of the skin to sunlight (129). The cells of X.P. patients are defective in the process of repair of UV radiation damage to DNA (130). Although the evidence obtained from some cell lines indicates a defect in the ability to excise UV light-induced pyrimidine dimers from their DNA (130, 131, 132), the levels of enzyme probably involved in the DNA repair process, for example, β-DNA polymerase, have not been carefully compared between normal cells and X.P. cells. Pedrini et al. have measured the DNA polymerase activity of fibroblasts obtained from normal persons and from X.P. patients and found no significant difference in the DNA polymerase level (133). While extremely interesting, their experiment was relatively crude. One would like to directly measure the individual activity of multiple species of DNA polymerases. However, Pedrini et al. have used the activated DNA, which might not be used by β- and γ-DNA polymerase efficiently (94), as template for the measurement of total DNA polymerase activity. Thus, a possible alteration of β- or γ-DNA polymerase activity in the X.P. cells might not be revealed.

Although most different forms of X.P. cells are deficient in excision-repair, a special class of X.P. patient (X.P. variants), while exhibiting the usual clinical symptoms, seem to be completely normal in excision repair of pyrimidine dimers (134). In addition, these X.P. variants have normal rates of rejoining of DNA single-strand breaks induced by ionizing radiation (135), indicating anormal level of polynucleotide ligase activity. Lehmann et al. (136) have shown that the fibroblast cultures from three X.P. variants have abnormal postreplication repair. It would be interesting to examine the level of β-DNA polymerase activity in these X.P. variants. Observation of decreased β-DNA polymerase activity would not only help elucidating the enzymatic defect associated with X.P. variant, but also indicate the possible role of β-DNA polymerase in the postreplication repair.

The decrease of specific activity of β-DNA polymerase in aged rat tissue could be due to different possibilities:

1. The accumulation of mutation in DNA. In 1961, Curtis (137) reported that exposure of ionizing radiation shortens life span. This observation naturally led many investigators to think that radiation, a known mutagen, might well be increasing the rate of aging in much the same way by which it was thought to increase the rate of mutation in germ cells. Szilard (59) has proposed that as somatic mutation accumulated in the genomes of somatic cells, genes will be affected with time in a way analogous to radiation-induced mutation. The mutant genes, for example, genes that code for DNA polymerase, would not be capable of producing a specific protein molecule in its chemically active form. In other words, such mutation would result in the synthesis of altered protein

or result in decreased activity of the protein. The cause of mutagenesis can be intrinsic as well as extrinsic. Burnet (112) has suggested that the rate of somatic mutation is determined by the characteristics of certain key enzymes involved in the transmission of genetic information. For example, the fidelity of DNA polymerases. The mutations accumulate as the DNA polymerase misincorporate error bases into the genome during DNA replication and repair. Another example of intrinsic type mutation which might occur in aging would be the conversion of a cytosine to uracil which codes for adenine rather than guanine during DNA synthesis. Estimates of the rate of spontaneous conversion of cytosine to uracil have been made by Lindahl and Nyberg (138).

2. Error in protein synthesis. In 1963 Orgel (139) proposed that the altered proteins may accumulate during aging through error in gene translation. The translational error, initially a low frequency event, could result in a gradual accumulation of altered protein molecules with age. Errors in various enzymes of the protein-synthesizing apparatus would be self-propagating and could eventually lead to an error catastrophe that would be lethal to the cell. In fact, the reduction with age of the specific activity of enzymes was found to be accompanied by an accumulation of inactive enzyme molecules in many cases; such as the aldolase (140) and isocitrate lyase (141) in Nematode, muscle aldolases of mice (142), and glutamate dehydrogenase in Neurospora crassa (143).

3. Post-translational modification. The catalytic activity of many enzymes is susceptible to physiological modulation exerted <u>in vivo</u> by both enzyme-catalyzed and nonenzyme-catalyzed covalent modification of the active functional groups of specific amino acid residues.

Recognized modification reactions include phosphorylation, methylation, deamidation, acylation, hydroxylation, adenylation, glycosylation, sulfation, peroxidation, etc. The implication of the structural and functional modification has been reviewed by Paik and Kim (144). Since a given modification reaction can virtually abolish the activity of an enzyme molecule, it is reasonable to assume that alteration of these post-translational modification reaction during aging process might lead to the decreased enzyme activity in the aged cells. Unfortunately, it seems that these post-translational modifications have received relatively insufficient emphasis in gerontological research.

The increased exogenous DNA polymerase-catalyzed in vitro DNA synthesis of the nuclei from spleens of old rats and mice indicates the absence of competent DNA-repair processes. Since β-DNA polymerase activity decreases in the spleen cells of aged rats and mice, we suggest that there is a correlation among aging, decrease in β-DNA polymerase activity, decline in DNA-repair capability, and increase in single-stranded nicks and single-stranded regions in some types of aged cell. Age-associated occurrence of unrepaired DNA lesions such as strand breakage and single-stranded regions have been reported by other investigators as well. Price, Modak, and Makinodan (73, 96) assayed the ability of isolated nuclei from brain, heart, and liver tissue of young and aged mice to provide templates for calf thymus DNA polymerase. They observed an age-related significant increase in mean autoradiographic grains in the nuclei of astrocytes, Kupffer cells, and cardiac muscle cells. Price et al. interpreted such results as an increase in single-stranded

regions of DNA in the aged cells.

Supporting evidence for increased single-stranded DNA regions in aged cells also comes from the investigation of Chetsanga et al. (145). They used single-strand-specific nuclease S_1 to detect the presence of single-stranded regions in the native DNA of mice of different ages and found an age-dependent increase of nuclease S_1 sensitivity in the DNA.

Another indication for an age-associated accumulation of unrepaired DNA lesions is the increased levels of chromosome aberrations in aged cells. Extensive investigations have observed an age-dependent direct increase in liver chromosome aberrations in mice (146), dog (147), guinea pigs (148), and Chinese hamsters (149). Such increase was also observed with human lymphocytes (150, 151). DNA cross-linking, which is another type of DNA lesions, has been found to increase with age. In one study, pregnant rats were fed with tritium, and the vital organs (brain and liver) of their offspring were examined at different ages for cross-linked products (152). With age, not only was there an increase in the frequency of DNA-protein-RNA complexes, but also their molecular size increased. In support of the formation of DNA cross-links with age, the percentage of DNA that can readily be isolated from aging liver tissue declines (153), and DNA isolated from aged organs has a higher melting temperature (154).

It is not clear how these age-associated DNA lesions might affect the basic structural organization of chromatin. The basic structural unit of chromatin consists of DNA which is periodically folded around a histone core creating a string of DNA-protein particles called

nucleosomes (155). Despite the great variety and complexity of eukaryotic chromosomes, they are at this level remarkably uniform and simple. The demonstration that the bulk of eukaryotic chromatin is organized in a repeating subunit structure raised the question as to whether all genes are packaged in a similar manner, since any model of chromatin structure should also account for the selective transcription of only a minor part of the genome. The recent evidence has revealed that there is nucleosomal heterogeneity along the DNA fiber that may be a factor in the control of differential gene activity (156, 157). In addition, DNA comprising presumptive transcribed sequences is in an extended configuration, and the packaging ratio of nucleosomes associated with these sequences is less than that observed with the bulk of the transcriptionally inert chromatin (156). The ability of bacteria RNA polymerases to transcribe genes selectively on eukaryotic chromatin templates (158) and the selective digestion of transcriptionally active genes by DNase I (159, 160) further indicate that the chromatin conformation is related to the accessibility of the chromatin to various enzymes. Thus, the observed increase in the template activity of old rat spleen nuclear DNA by exogenous DNA polymerase might imply, in addition to the accumulation of single-stranded regions, the alteration of chromatin structures in the old rat spleen cells.

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