Cell Cycle Dependent Activities of DNA Polymerases α and δ in Chinese Hamster Ovary Cells†

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ABSTRACT: The activities of DNA polymerases α and δ, in extracts from Chinese hamster ovary (CHO) cells, were assayed in order to determine whether these polymerases are regulated during the cell cycle. An exponential population of CHO cells was separated into enriched populations of G-1, S, and G-2/M phases of cell cycle by centrifugal elutriation. Total cell homogenates from each population were assayed for DNA polymerase activity by measuring labeled nucleotide incorporation into the exogenous templates oligo(dT)·poly(dA) and DNase I activated calf thymus DNA. In these experiments, specific DNA polymerase inhibitors were added to assays of the cellular extracts to allow for the independent measurement of activities of DNA polymerases α and δ. Comparisons of total DNA polymerase activity from cellular extracts, sampled from each portion of the cell cycle, demonstrated no significant change with respect to the concentration of total protein. However, results indicate that the activity of DNA polymerase δ increases with respect to that of DNA polymerase α in the G-2/M portion of the cell cycle. This difference in relative activities of DNA polymerases α and δ suggests a coordinate regulation of a specific species of DNA polymerase during the cell cycle.

DNA polymerases α and δ have been purified to near-homogeneity from several mammalian sources [reviewed by Fry and Loeb (1986)]. A functional comparison of DNA polymerases α and δ, isolated from calf thymus gland, demonstrates that these DNA polymerases have similar size and associated RNA primase activity and display a similar length of processive DNA synthesis (Crute et al., 1986). Also, DNA polymerases α and δ, isolated from several sources, have comparable sensitivity to the DNA synthesis inhibitors aphidicolin, *N*-ethylmaleimide, and ara-ATP and are resistant to dideoxythymidine triphosphate (Lee et al., 1981; Byrnes, 1984). These functional similarities between α and δ include many which have been used to target DNA polymerase α as the replicative DNA polymerase and suggest that DNA polymerase δ may also be involved in DNA replication.

A unique property of DNA polymerase δ isolated from mammalian tissues is that highly purified preparations of this enzyme have a nondissociable 3′ to 5′ exonuclease activity (Byrnes et al., 1976; Lee et al., 1980; Crute et al., 1986). Purified DNA polymerase α from higher eukaryotes either lacks an associated exonuclease [reviewed by Fry and Loeb (1986)] or copurifies with a dissociable exonuclease activity (Skarnes et al., 1986). An exception is DNA polymerase a_1 from mouse myeloma cells, which has an associated 3′ to 5′ exonuclease activity (Chen et al., 1979). However, it is possible that this enzyme is a form of DNA polymerase δ.

Typically, a polymerase-associated 3′ to 5′ exonuclease can function as a proofreader, removing mismatched nucleotides during DNA synthesis, and most prokaryotic DNA polymerases have this proofreading capacity (Kornberg, 1980). The action of 3′ to 5′ exonucleases of prokaryotic DNA polymerases is one mechanism which is responsible, in part, for the high fidelity of prokaryotic DNA replication observed in vivo [reviewed by Loeb and Kunkel (1982)]. The necessity to maintain high fidelity in mammalian DNA replication and the high error rate of purified DNA polymerase α support the suggestion that DNA polymerase α has a significant role in DNA replication in vivo.

Previous studies have indicated that DNA polymerase α activity is coordinately regulated with cellular DNA synthesis, increasing severalfold during S phase (Spadari & Weissbach, 1974; Chiu & Baril, 1975; Craig et al., 1975; Pedrali-Noy et al., 1980). In these studies, cells were synchronized by a double thymidine block, aphidicolin block, or serum deprivation. Cells synchronized by these procedures were first growth-arrested in the G-1 phase of the cell-cycle, and then the polymerase activity was quantitated after the synchronizing agent was removed. Interpretation of these results was complicated by the inability to distinguish whether the observed DNA polymerase activity increase is representative of natural entry into S phase or a result of metabolic perturbation caused by the synchronizing agent.

To assess the levels of both DNA polymerases α and δ during the cell cycle, centrifugal elutriation was employed as a method to obtain Chinese hamster ovary (CHO)[1] cells in G-1, S, and G-2/M phases of cell cycle from an exponential population (Keng, 1981). The advantage of this technique is that it does not require growth-arrested cells; therefore, results can be interpreted solely on the basis of cell cycle, not due to a release from metabolic block or a change in growth rate. In addition, the availability of monoclonal antibodies (Tanaka

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[1] Abbreviations: BuAdATP, 2-(*p*-*n*-butylanilino)-9-(2-deoxy-β-D-ribofuranosyl)adenine 5′-triphosphate; ddTTP, 2′,3′-dideoxythymidine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IgG_1, immunoglobulin G_1; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; CHO, Chinese hamster ovary; HPLC, high-performance liquid chromatography.

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et al., 1982) and butylphenylated derivatives of deoxynucleoside triphosphates, which are specific inhibitors of DNA polymerase α (Khan et al., 1984, 1985), allows for the accurate, differential quantitation of relative activities for DNA polymerase α and δ simultaneously.

Materials and Methods

Chemicals. All reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Poly(deoxyadenylic acid) with a chain length between 4000 and 5000 nucleotides [poly(dA)$_{4000-5000}$] and oligothymidylic acid with a chain length of 16 nucleotides [(dT)$_{16}$] were products of the Midland Certified Reagent Co. (Midland, TX). DTT was obtained from Boehringer Mannheim. Sephadex G-50 fine was from Pharmacia Fine Chemicals (Piscataway, NJ). [^{3}H]Thymidine 5′-triphosphate (specific activity 80 Ci mmol^{-1}) was from New England Nuclear Corp. (Boston, MA). ACS scintillation counting solution was from Amersham Corp. (Arlington Heights, IL). Deoxynucleoside 5′-triphosphates (HPLC-purified grade) were obtained from ICN Pharmaceuticals (Plainview, NY). 2-(*p*-*n*-Butylanilino)-9-(2-deoxy-β-D-ribofuranosyl)adenine 5′-triphosphate (BuAdATP) was a gift from Dr. George Wright of the Department of Pharmacology, University of Massachusetts Medical School (Shrewsbury, MA).

DNA Substrates. DNase I activated calf thymus DNA was prepared by the method of Spanos et al. (1981). Oligo(dT)$_{16}$·poly(dA)$_{4000-5000}$ was prepared as described by Crute et al. (1986) such that the interprimer distance of 165 nucleotides was obtained.

IgG Purification. A hybridoma cell line, producing a mouse monoclonal IgG antibody against human KB cell DNA polymerase α, SJK132-20 (Tanaka et al., 1982), was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's modified Eagle's medium as described by Harwell et al. (1976), except that 0.5% fetal bovine serum (J. R. Scientific) was used. IgG$_1$, SJK132-20, was purified from the cell culture supernatant solution by the method of Mishell and Shigi (1980). Control antibody, mouse myeloma protein IgG (MOPC-21), was purchased from Pharmacia (Piscataway, NJ).

Cells and Culture Conditions. CHO cells were cultured as monolayers in 150 cm^2 plastic flasks (Costar) in F-10 medium (30 mL) supplemented with sodium bicarbonate (0.15%, pH 7.5), L-glutamine (2 mM), penicillin (100 units/mL), streptomycin sulfate (100 μg/mL), and 10% fetal bovine serum (lot 9550, J. R. Scientific). Media and supplements were obtained from GIBCO (Grand Island, NY) unless otherwise noted. Cell cultures were routinely demonstrated to be *Mycoplasma* free by the *Mycotrim* assay (Du Pont, NEN). Cells (1×10^6/flask) were seeded as monolayers 2 days prior to an experiment. The cell cultures were maintained at 37 °C in a humidified incubator with an atmosphere of 95% air and 5% CO_2.

Synchronization of Cells by Centrifugal Elutriation. CHO cells were harvested with 0.25% trypsin in Hank's balanced salt solution buffer, pH 7.5 (GIBCO). After trypsinization, cells were suspended in complete F-10 medium. Cells (1×10^8) were elutriated in complete F-10 medium with 1% serum as described by Keng (1981) using the Beckman JE-6 elutriation rotor driven by a Beckman 21-C centrifuge. The cells were fractionated into subpopulations primarily by size. As cells progress through the cell cycle, the volume of a cell increases proportionally with the different stages of the cell cycle (Keng, 1981). Subpopulations which had an approximate volume ratio of 1.0:1.5:2.0 represented G-1, S, and G-2/M stages of the cell cycle, respectively. The number of cells or their size distribution was determined by a Coulter Counter and Channelyzer system (Coulter Electronics).

Flow Cytometry. Flow cytometry was used to verify the homogeneity of cell populations separated by centrifugal elutriation. From each subpopulation, cells (1.0×10^6) were fixed in 70% methanol. Cells were washed and stained with mithramycin C (100 μg/mL) and 12.5 mM $MgCl_2$ in potassium phosphate buffer. After being stained with mithramycin C overnight, the DNA content was quantitated for each cell using an EPICS V flow cytometer (Coulter Electronics). A 5-W argon ion laser operated at 457 nm and 150 mW of power was used for excitation. The percentages of G-1, S, and G-2/M cells for each population were analyzed by the cell cycle program "CCYCLE" in a Terak 8600 microcomputer (Luk et al., 1985).

Preparation of Crude Cell Extracts. The cells (2.6×10^6 cells/mL) were disrupted by freezing with liquid nitrogen in 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethanesulfonyl fluoride, 0.5 μg/mL pepstatin, 0.5 μg/mL leupeptin, 2 mM ATP, and 4 mM spermidine. Cell preparations were thawed at 4 °C after the addition of 6 mM $MgCl_2$ and 120 mM sucrose. Cellular lysis was determined by direct cell counting and by trypan blue exclusion. Crude cell extracts were used for all the DNA polymerase activity assays described in this paper.

After a cycle of freezing and thawing, the crude cell extracts (3 mL) were clarified by ultracentrifugation with a Beckman type 35 rotor at 32000 rpm and 4 °C for 1 h. The supernatant solution, pellet, and crude extract were assayed separately for DNA polymerase activity as described below. Addition of exogenous DNA template was required for assay of DNA polymerase activity. Ultracentrifugation removed 5–15% of DNA polymerase activity from the supernatant solution. On this basis, it is estimated that 85–95% of the DNA polymerase activity is solubilized by the freezing and thawing procedure.

Enzymatic Assays. Polymerase activity was determined in an assay (25 μL) containing 20 mM Tris-HCl, pH 7.5, 5.0% glycerol, 10 mM $MgCl_2$, 5.0 mM β-mercaptoethanol, dCTP, dGTP, and dATP at 40 μM each, 2 mM ATP, 250 μg/mL bovine serum albumin, and 25 μM [^{3}H]dTTP (10 Ci/mmol). Nuclease-activated calf thymus DNA (600 μM) or oligo(dT)$_{16}$·poly(dA)$_{4000-5000}$ (40 μM) was added as a substrate. Assays were carried out for a period of 30 min at 37 °C and terminated by the addition 10 μL of 0.5 M EDTA, pH 7.5. Separation of labeled, polymeric DNA from precursor monomers was achieved by sedimentation through centrifuge columns of G-50 fine resin (Penefsky, 1977). Aqueous samples from centrifuge columns were counted in ACS scintillation solution. One unit of DNA polymerase incorporates 1 nmol of nucleotide per hour at 37 °C.

Determination of DNA Polymerase Activity. In standard assays, CHO cellular extracts (4 μL) from 2.6×10^6 cells/mL had approximately 4×10^{-2} unit of DNA polymerase activity on nuclease-activated calf thymus DNA and 1×10^{-2} unit on oligo(dT)$_{16}$·poly(dA)$_{4000-5000}$. DNA polymerase inhibitors were added to the standard assays for the determination of the proportion of total DNA polymerase activity attributed to DNA polymerases α, β, γ, and δ. For all experiments, titrations of each inhibitor were performed to establish the saturating level of inhibition. A saturating concentration is defined as the minimum concentration such that a 2-fold increase will not lead to more than a 10% change in DNA synthesis rate. For comparison studies, all assays of cellular extracts were done in parallel. Extracts were thawed for assays

Table I: Effect of Inhibitors on DNA Polymerase Activity in Extracts Prepared from CHO Cells[a]

inhibitor[b]	inhibition on indicated primer-templates[c]	
	DNase I activated calf thymus DNA	homopolymeric DNA
aphidicolin	82 ± 2	75 ± 4
BuAdATP	80 ± 5	28 ± 7
SJK132-20	81 ± 4	31 ± 13
aphidicolin + BuAdATP	88 ± 2	79 ± 5
aphidicolin + SJK132-20	83 ± 2	84 ± 2
ddTTP	30 ± 11	19 ± 14
ddTTP + aphidicolin	90 ± 4	86 ± 6

[a] Extracts were prepared by a cycle of freezing and thawing from an asynchronous, exponential population of CHO cells (2.6×10^6 cells/mL). Aliquots (4 μL) of cell extract were assayed in triplicate for the incorporation of [^{3}H]dTMP into DNase I activated calf thymus DNA or oligo(dT)$_{16}$·poly(dA)$_{4000-5000}$, as the homopolymeric DNA. The extract (4 μL) had approximately 4×10^{-2} and 1×10^{-2} units of DNA polymerase activity on DNase I treated calf thymus DNA and homopolymeric DNA, respectively. [b] The assays contained the indicated inhibitors: aphidicolin (20 μg/mL); BuAdATP (1 μM); SJK132-20 antibody (50 μg/mL); ddTTP (50 μM). Appropriate control samples either were not incubated with an inhibitor or were incubated with a negative control monoclonal antibody, MOPC-21 (50 μg/mL). Control antibody did not inhibit the DNA polymerase activity significantly (maximal 5% inhibition). [c] Results are represented as the percentage of inhibition of DNA polymerase activity by inhibitors relative to control samples. The results are the average of three different experiments. The standard deviation is given.

and used immediately. Frozen extracts were stored in liquid nitrogen for not more than 15 h prior to use.

Protein Assay. The protein concentration of cellular extracts was determined by the Bradford protein assay using bovine serum albumin as a standard (Bradford, 1976).

Results

Extracts were prepared from an asynchronous, exponential population of CHO cells. A characteristic of the extracts is that 85–95% of the total DNA polymerase activity is soluble, i.e., not removable by high-speed (>100000*g*) centrifugation. Conditions for quantitation of polymerase activity are based on results of our prior studies using purified calf thymus DNA polymerases α and δ (Wahl et al., 1986) and the knowledge that template specificity and inhibitor sensitivity of these DNA polymerases are similar in all mammalian systems studied (Lee et al., 1981, 1985; Byrnes, 1984, 1985). In mammalian cell extracts, DNA polymerase β and γ activity can also contribute to the total polymerase activity. However, the addition of an appropriate concentration of ddTTP to the assay can effectively eliminate the contribution of these two DNA polymerases (Waqar et al., 1978).

The relative proportion of synthetic activity in the extracts attributable to DNA polymerases α and δ is reported in Table I. Aphidicolin is a specific inhibitor of both DNA polymerases α and δ from every mammalian tissue extract tested (Lee et al., 1981; Byrnes, 1984; Wahl, 1986). Aphidicolin at the saturating level of 20 μg/mL inhibits 75–80% of the DNA polymerase activity in CHO cellular extracts on either DNase I activated calf thymus DNA or oligo(dT)$_{16}$·poly(dA)$_{4000-5000}$, the homopolymeric primer-template. This indicates that on either primer-template, DNA polymerases α and δ account for 75–80% of synthetic activity. Dideoxy-TTP, at a saturating level of 50 μM (a 2:1 ddTTP:dTTP ratio), inhibits 20–30% of the total DNA polymerase activity on both primer-templates. This proportion of the activity is thereby attributed to DNA polymerases β and γ, since these polymerases are inhibited more than 95% at a 2:1 ddTTP:dTTP ratio, whereas DNA polymerase α is not affected by this inhibitor ratio (Waqar et al., 1978; Wahl et al., 1986). The degree of sensitivity of purified DNA polymerase δ to ddTTP depended on the purification method and conditions (Wahl et al., 1986; Lee et al., 1981). Estimations of the percent of activity represented by DNA polymerase δ in the extracts based on the fraction of activity inhibited by ddTTP and other combinations of inhibitors indicate that the level of ddTTP used does not significantly inhibit DNA polymerase δ.

Simultaneous use of aphidicolin and ddTTP inhibits approximately 90% of the total polymerase activity. Therefore, these two inhibitors essentially account for all DNA polymerase activity in cellular extracts. The observed inconsistency of 10–15% in all of the above measurements is interpreted to be the inherent variation of experiments involving use of multiple inhibitors in cell extracts.

The above methods establish that the majority of DNA polymerase activity in the extracts arises from DNA polymerases α and δ. Other inhibitors were used to distinguish the activities of these two enzymes. The monoclonal antibody SJK132-20 has been shown to be a specific inhibitor of DNA polymerases α isolated from a variety of mammalian sources (Miller et al., 1987). It is effective in neutralizing the activity of highly purified DNA polymerase α from CHO cells (Khan & Brown, 1985). However, the antibody is ineffective at neutralizing the activity of DNA polymerase δ from calf (Wahl et al., 1986), rabbit bone marrow (Byrnes, 1985), and CV-1 cells (Dr. Michael Miller, University of West Virginia, personal communication). DNA polymerase activity on DNase I activated calf thymus DNA has an identical sensitivity at saturating levels of aphidicolin and SJK132-20 antibody. This result indicates that essentially all of the aphidicolin-sensitive DNA polymerase activity on the DNase I activated calf thymus DNA substrate results from DNA polymerase α. Under comparable assay conditions, with the same cellular extract, approximately 40% of the aphidicolin-sensitive activity on the homopolymeric primer-template is sensitive to the antibody, i.e., representative of DNA polymerase α. This result suggests that approximately 60% of the aphidicolin-sensitive activity on the homopolymeric template represents DNA polymerase δ.

This conclusion is supported by the use of another specific inhibitor of DNA polymerase α, BuAdATP. In comparison studies with enzymes isolated from different organisms, purified DNA polymerase α is inhibited by BuAdATP at concentrations several hundredfold lower than purified DNA polymerase δ (Lee et al., 1985; Wahl et al., 1986).

In assays of CHO cell extracts, the percentage of inhibition in DNA synthesis observed at a saturating concentration of BuAdATP (1 μM) is comparable to the level of inhibition obtained at a saturating concentration of SJK132-20 on either primer-template.

Synchronization by Centrifugal Elutriation. CHO cells were separated by centrifugal elutriation into subpopulations enriched for G-1, S, or G-2/M DNA content. The homogeneity of each subpopulation was determined by staining a sample of the population for DNA content by mithramycin C. Cells stained with mithramycin C have a fluorescence which is proportional to DNA content and can be quantitated by flow cytometry (Luk et al., 1985). From each experiment, the percentage of cells with either G-1, S, or G-2/M DNA content in each subpopulation was determined and reported in Table II. Cell populations which are described herein as containing G-1, S, and G-2/M DNA content have an average of 92%, 85%, and 75% purity, respectively.

Table II: Evaluation of the Synchronization of Elutriated Cell Populations

cell populations[a]	% of population in each phase		
	G-1	S	G-2/M
I (G-1)	92 ± 4	6 ± 2	2 ± 2
II (S)	10 ± 4	86 ± 5	4 ± 1
III (G-2/M)	5 ± 1	20 ± 4	75 ± 6
asynchronous exponential	42 ± 9	41 ± 7	17 ± 2

[a] CHO cells were elutriated into subpopulations as described by Keng (1981). The subpopulations I, II, and III had an approximate volume ratio of 1:1.5:2 as determined by the Coulter Counter and Channelyzer (Coulter Electronics). These subpopulations were stained for cellular DNA content with mithramycin C. The DNA content was quantitated by using flow cytometry. The percentages of each subpopulation which contained G-1, S, or G-2/M DNA content were analyzed by a cell cycle program in a Terak 8600 microcomputer. The results represent the percentage of each subpopulation which contained the DNA content of G-1, S, or G-2/M cells. Asynchronous exponential cells represent the population which was not elutriated. Results are representative of three separate elutriations. The standard deviation is given.

DNA Polymerase Activity in Extracts of Synchronized Cell Populations. The total DNA polymerase activity was assayed from extracts of CHO cells enriched for G-1, S, or G-2/M portions of the cell cycle. The amount of DNA polymerase activity from 2×10^6 cells, adjusted for total cellular protein, is depicted in Figure 1. The total DNA polymerase activity was assayed at four different concentrations of extract. There is a linear relationship between the amount of extract assayed and the rate of DNA synthesis. This linear relationship implies that the primer-template is in excess, such that the rate of DNA synthesis corresponds to the total amount of active DNA polymerase that is present in each sample of extract. This relationship also implies that no concentration-dependent endogenous inhibitors or stimulators of DNA synthesis can be detected in cellular extracts from any of the phases of the cell cycle. Comparison of the rates of DNA synthesis in extracts from different phases of the cell cycle shows no significant change in the level of DNA polymerase activity on either primer-template.

Proportion of DNA Polymerase α and δ Activities in Each Synchronized Cell Population. The relative contribution of different DNA polymerase activities to the total rate of DNA synthesis obtained for each synchronized population was determined by the use of DNA polymerase inhibitors. All compared assays of cellular extracts were performed concurrently with saturating levels of aphidicolin, SJK132-20 antibody, or ddTTP present in the reactions, and expressed as the percentage of DNA synthesis in the absence of inhibitors. Figure 2, panel A, shows the DNA synthesis rate on DNase I activated calf thymus DNA, and the relative contributions of DNA polymerases α, β, and γ, as defined by sensitivity to these specific inhibitors, are indicated.

As demonstrated above, DNA polymerases α, β, and γ account for all of the synthetic activity on this template. Either aphidicolin or SJK132-20 antibody inactivates 80% of the total polymerase activity in extracts of cells from different phases of cell cycle, and this proportion, as assayed on this substrate, represents DNA polymerase α. It is evident from a comparison of panel A, Figure 1, and panel A, Figure 2, that neither the total polymerase activity nor the proportion of DNA polymerase α versus β plus γ activity varies throughout the cell cycle of an exponential cell population.

In panel B of Figure 2, cellular extracts were assayed by using the homopolymeric primer-template as a substrate. Throughout the cell cycle, the total polymerase activity that is neutralized by antibody varies, but the proportion of

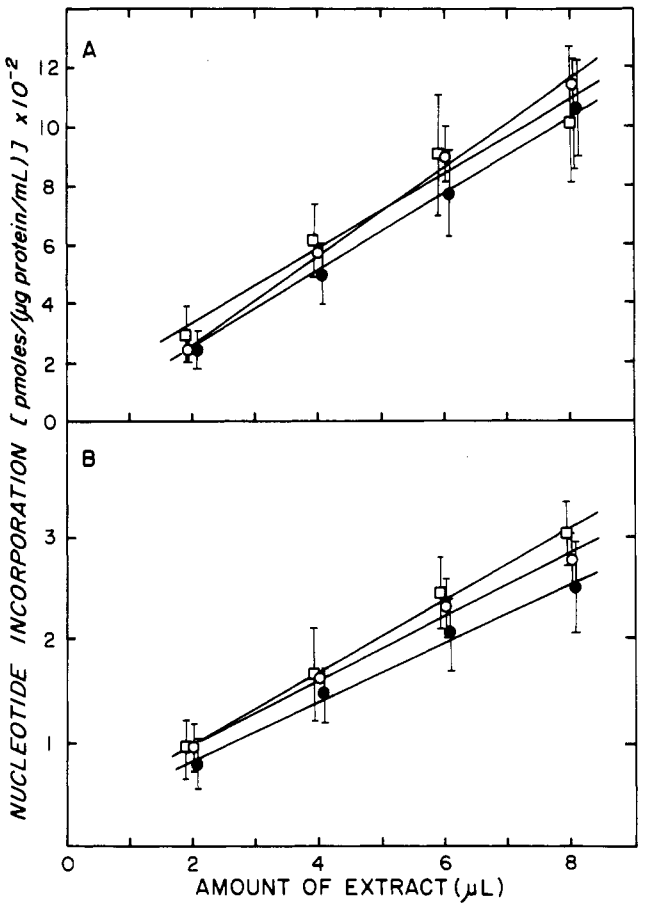

FIGURE 1: DNA polymerase activity throughout the cell cycle. CHO cells (2.6×10^6 cells/mL) from the G-1 (open circles), S (closed circles), or G-2/M (squares) portion of the cell cycle were lysed by a cycle of freezing and thawing. The protein concentration was determined (Bradford, 1976) for each cell extract. Aliquots (2–8 μL) of each cell extract were added to a standard DNA polymerase assay which contained either DNase I activated calf thymus DNA (panel A) or oligo$(dT)_{16}$·poly$(dA)_{4000-5000}$ (panel B) as a template. Each assay was done in triplicate, and the results are the average of four separate experiments. The DNA polymerase activity for each cell extract is expressed as picomoles of [^{3}H]dTMP incorporated per microgram of protein per microliter from 2.6×10^6 cells. The standard deviation is represented by the error bars.

aphidicolin-sensitive polymerase activity remains constant. On this primer-template, the difference between the percentage of total polymerase activity inhibited by the antibody compared to that inhibited by aphidicolin represents DNA polymerase δ activity. A comparison of panel B, Figure 1, and panel B, Figure 2, indicates that a major change in total polymerase activity is not observable but the proportions of those activities known to be DNA polymerases α and δ change throughout the cell cycle.

Use of BuAdATP To Verify the Changing Proportion of Activity of DNA Polymerases α and δ. Purified DNA polymerases α and δ from several mammalian sources have differential sensitivities to butylphenyl derivatives of deoxynucleotides (Byrnes, 1985; Lee et al., 1985). Specifically, purified calf DNA polymerase α is inhibited 50% at a concentration of 1 μM BuAdATP. DNA polymerase δ is much less sensitive to this inhibition by BuAdATP, demonstrating 50% inhibition at 100 μM (Wahl et al., 1986).

With DNase I activated calf thymus DNA, DNA polymerase activity in extracts from each portion of the cell cycle was inhibited approximately 80% by 1 μM BuAdATP. A titration from 1 to 370 μM BuAdATP demonstrated no significant increase in inhibition (data not shown). This result

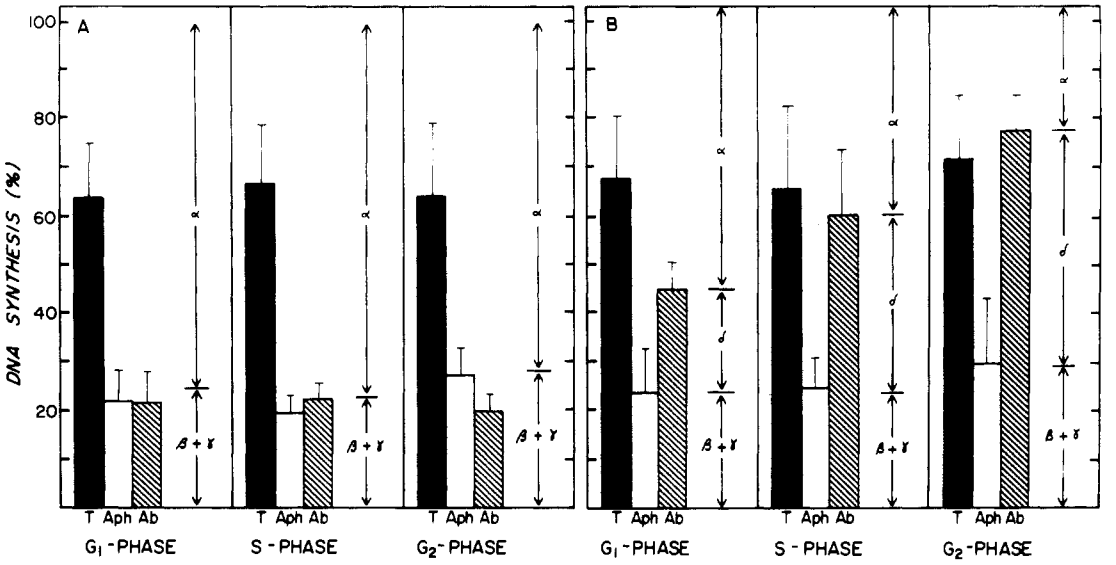

FIGURE 2: Proportion of each DNA polymerase in each cell cycle population. Extracts were prepared from CHO cells from the G-1, S, and G-2/M phase of cell cycle. Panel A depicts the incorporation of [^{3}H]dTMP into DNase I activated calf thymus DNA when DNA polymerase inhibitors were added to the extracts: aphidicolin (Aph; 20 μg/mL); antibody SJK132-20(Ab; 50 μg/mL); ddTTP (T; 50 μM). The results are expressed as the percentage of incorporation of [^{3}H]dTMP relative to a control sample without inhibitors. The control samples had approximately equal units of DNA polymerase activity and were assayed in triplicate. The proportion of activity attributed to either DNA polymerase α, β, γ, or δ is shown. Panel B shows the extracts assayed under identical conditions except that oligo(dT)$_{16}$·poly(dA)$_{4000-5000}$ was utilized as a template. The results are the average of three separate elutriations. The standard deviation is represented by the error bars. DNA polymerase α activity is obtained as 1 − mean percent inhibition by the antibody. DNA polymerase δ activity is obtained as (mean percent inhibition by aphidicolin) − (mean percent inhibition by antibody). The change in percentage of DNA polymerase activity of DNA polymerase α or δ in panel B when G-1 and G-2/M phases are compared is significant for each enzyme ($p < 0.025$). When the G-1 phase is compared to the S phase, $p < 0.15$ for either enzyme. When the S phase is compared to the G-2/M phase, $p < 0.20$ for either enzyme.

is expected since DNA polymerase α, inhibited at low concentrations of BuAdATP, represents a very large proportion of total activity on DNase I activated calf thymus DNA template. Furthermore, there is no significant change in the titration curve when extracts from populations of cells in different phases of cell cycle are compared (data not shown).

Similar titrations with BuAdATP were performed on cellular extracts from different phases of the cell cycle using oligo(dT)$_{16}$·poly(dA)$_{4000-5000}$ as the primer-template (Figure 3). Significantly less sensitivity to the inhibitor was observed in extracts from G-2/M cell populations when compared to the sensitivity in extracts from G-1 populations. This result is consistent with an increasing ratio of DNA polymerase δ to DNA polymerase α as the cell progresses through the cell cycle.

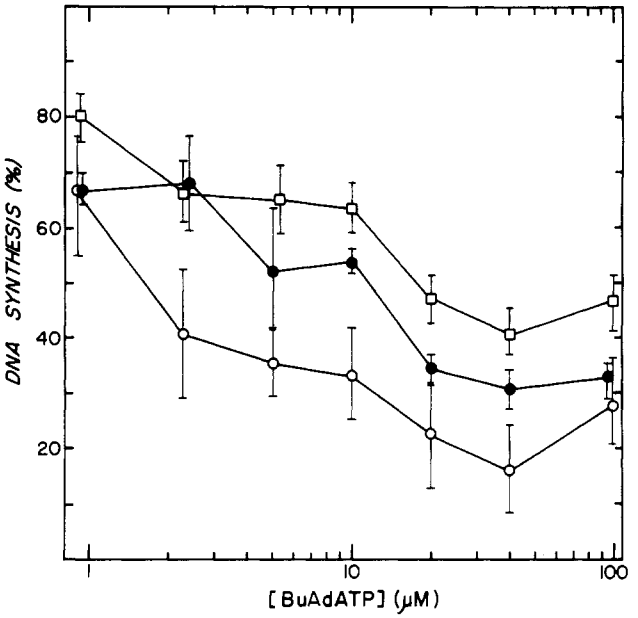

FIGURE 3: Titration of DNA polymerase activity with BuAdATP. Cell extracts from G-1 (open circles), S (closed circles), and G-2/M (squares) portions of the cell cycle were assayed in triplicate in a standard DNA polymerase assay utilizing oligo(dT)$_{16}$·poly(dA)$_{4000-5000}$ as a template. Two DNA polymerase inhibitors, ddTTP (50 μM) and BuAdATP (1–100 μM), are added to each assay. One hundred percent on the DNA synthesis axis represents [^{3}H]dTMP incorporation into the DNA template in the absence of DNA polymerase inhibitors. Each extract has approximately equal units of DNA polymerase activity in the absence of inhibitors. The results are the average of two separate elutriations. The standard deviation is represented by the error bars. When these titrations were repeated without ddTTP, the data points were shifted upward an average of 15% with no significant change in curve shape (not shown).

Discussion

A number of studies of DNA polymerase α activity in nuclei or cytoplasmic extracts from synchronized cells demonstrate at least a 2-fold increase in this activity at the onset of DNA synthesis and a rapid decline as DNA replication is completed (Spadari & Weissbach, 1974; Chiu & Baril, 1975; Craig et al., 1975; Pedrali-Noy et al., 1980). This increase in activity supports the suppositions that DNA polymerase α is the major DNA replication enzyme in mammalian cells and that its activity is coordinately regulated with the initiation of DNA synthesis. In contrast, other studies demonstrate that the majority of DNA polymerase α activity does not change with DNA synthesis or repair (Foster & Collins, 1985; Krauss & Linn, 1986). Potential causes of this discrepancy are the methods of extract preparation and, most importantly, the means by which cells are synchronized.

In this report, we have used centrifugal elutriation to separate an exponential population of CHO cells into subpopulations, representing G-1, S, and G-2/M stages of the cell cycle. This method does not require the arrest of cells in the G-1 portion of the cell cycle by serum deprivation or by metabolic inhibitors which had been used in previous experiments. Therefore, it decreases the possibility that changes in the measured levels of DNA polymerase activity are related

to alteration of the cellular metabolism induced by synchronization. Extracts from exponential CHO populations, separated by centrifugal elutriation, were then used to measure DNA polymerase activity. Our results demonstrate that the total DNA polymerase activity, corrected for total protein concentration in cellular extracts, does not change as the cell progresses through the cell cycle (Figure 1). The individual activities of DNA polymerases α and δ were then determined in these same cell extracts.

Previous results suggested four criteria by which the activities of DNA polymerases α and δ can be distinguished in CHO extracts. (1) Purified calf DNA polymerases α and δ can utilize the primer-template oligo(dT)$_{16}$·poly(dA)$_{4000-5000}$; however, only DNA polymerase α has significant activity on nuclease-activated calf thymus DNA (Crute et al., 1986; Wahl et al., 1986). (2) The antibody SJK132-20 has been shown to be an effective and specific interspecies inhibitor of DNA polymerase α (Miller et al., 1987) but is ineffective against DNA polymerase δ from calf thymus (Wahl et al., 1986) and rabbit bone marrow (Byrnes, 1985). (3) Appropriate concentrations of the inhibitor BuAdATP have been shown to inhibit completely DNA polymerase α but have no effect on DNA polymerase δ isolated from calf and human placenta (Wahl et al., 1986; Lee et al., 1985). (4) Both DNA polymerases α and δ have comparable sensitivity to aphidicolin (Lee et al., 1981; Byrnes, 1984; Wahl et al., 1986).

In CHO cell extracts, the major proportion of DNA synthesis on DNase I activated calf thymus DNA is equally sensitive to BuAdATP, antibody (SJK132-20), and aphidicolin. This pattern of sensitivity is consistent with the interpretation that most of the DNA synthesis detected by using this primer-template, approximately 80%, can be attributed to DNA polymerase α (Table I). The remainder of DNA synthetic activity must result from DNA polymerases β and γ, with virtually no contribution from DNA polymerase δ. Under similar assay conditions, the DNA polymerase α activity was determined in the extracts prepared from cells in the G-1, S, and G-2/M stages of the cell cycle. No variation of DNA polymerase α activity in proportion to total cellular protein was observed (panel A, Figure 1). These results indicate that there is a relatively constant steady-state concentration of the active form of this enzyme throughout exponential growth. Contrary to previously published results, there is no indication that the activity level of DNA polymerase α is increased to coincide with the onset of DNA synthesis, or decreased to coincide with entry into the G-2/M phase of the cell cycle.

On the homopolymeric primer-template oligo(dT)$_{16}$·poly(dA)$_{4000-5000}$, 75% of DNA synthesis, assayed in extracts of asynchronous cells, is inhibited by aphidicolin. This is comparable to the inhibition observed on nuclease-activated calf thymus DNA (Table I). BuAdATP or SJK132-20 antibody, however, inhibit only about 30% of the aphidicolin-sensitive DNA synthetic activity on the homopolymeric template (Table I). This is consistent with the conclusion that DNA polymerase δ contributes to this observed DNA synthetic activity.

Using the oligo(dT)·poly(dA) assay, we obtained evidence for a cell cycle dependent change in the level of DNA polymerase δ. In extracts from G-1, S, and G-2/M populations, the proportion of DNA synthesis that was inhibited by SJK132-20 antibody changed as the cells progressed through the cell cycle (panel B, Figure 2). A corresponding change was observed in the sensitivity of DNA synthesis to BuAdATP (Figure 3). In these same experiments, the proportion of DNA synthesis that was sensitive to ddTTP (representing DNA polymerases β and γ) or aphidicolin (representing DNA polymerases α and δ) remained relatively constant (panel B, Figure 2). Results indicate that the DNA polymerase δ activity in CHO extract increases to a maxima in the G-2/M phase of the cell cycle (Figure 2, panel B, and Figure 3). This, in turn, suggests that requirements for this enzyme change throughout the cell cycle. Although this enzyme may be required during S-phase DNA synthesis, its activity changes are inconsistent with an increased requirement at the onset of the S phase.

Although we have observed a cell cycle dependent change in the level of DNA polymerase δ, this 2-fold change is not detectable as a significant change in the total DNA polymerase activity on the homopolymeric DNA primer-template (Figure 1, panel B). The change in δ-polymerase activity, when added to the unchanging base level activities of DNA polymerases α, β, and γ (Figure 1, panel B), is evidently not measurable under the conditions of the experiment. An alternative explanation for this result is that the total polymerase activity measurement in Figure 1, panel B, is affected somewhat by an interaction among the polymerases using the homopolymeric DNA primer-template.

The similarities in structure and biochemical kinetic parameters of calf DNA polymerases α and δ suggest that one could be derived from the other. In view of these current results, one possibility is that some DNA polymerase α used in S-phase DNA synthesis, is converted to DNA polymerase δ for postreplication repair processes requiring a 3′ to 5′ exonuclease, occurring in G-2/M phase. Another possibility is that DNA polymerase δ is synthesized primarily in the G-2/M phase and converted to DNA polymerase α such that inactivated DNA polymerase α is replaced at a constant rate. Alternatively, the activities of DNA polymerases α and δ could be regulated by other proteins. Tan et al. (1986) recently reported purification of a stimulatory factor which changes the activity of DNA polymerase δ isolated from calf. This factor later was shown to be PCNA, or cyclin, a protein which stimulates simian virus 40 replication in vitro, and which itself varies in concentration with cell cycle (Prelich et al., 1987; Tan, 1982; Bravo, 1986; Celis & Celis, 1985). Experiments are now in progress to investigate the structure of purified forms of these enzymes that could distinguish these possibilities.

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The DNA Sequence of the Human β-Globin Region Is Strongly Biased in Favor of Long Strings of Contiguous Purine or Pyrimidine Residues†

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ABSTRACT: The DNA sequence of the human β-globin region, comprising over 67 kilobase pairs, has been analyzed for the occurrence of strings of contiguous purine or pyrimidine residues. Tracts of 10 or more contiguous residues are found 4 times more frequently than would be expected with a random distribution of bases, so that a long string occurs at an average of every 250 base pairs. A survey of six other human gene sequences, totaling 86 kilobase pairs, shows a remarkably similar result. No such overrepresentation of contiguous purine or pyrimidine residues is found in the bacteriophages λ or T7.

It has been known for quite some time that double-stranded synthetic polydeoxynucleotides in which one strand is exclusively purine residues have conformations that are different from DNA in which purines and pyrimidines occur on both strands. The synthetic polymers poly(dA)·poly(dT), poly(dI)·poly(dC), and poly[d(A-I)]·poly[d(T-C)] have been shown to differ from heterogeneous sequence DNA in their X-ray fiber diffraction patterns (Leslie et al., 1980). Poly(dA)·poly(dT) is known to have a helical repeat that is different from bulk DNA (Peck & Wang, 1981; Rhodes & Klug, 1981), and poly(dG)·poly(dC) is 20-fold less flexible than heterogeneous sequence DNA (Hogan et al., 1983). Neither poly(dA)·poly(dT) nor poly(dG)·poly(dC) is able to form nucleosomal structures when challenged by histones (McGhee & Felsenfeld, 1980), and a short cloned region of $(dA{\cdot}dT)_{20}$ was seen to be excluded from the central portion of nucleosomes (Kunkel & Martinson, 1981). The smallest number of contiguous purine residues that is needed for a segment of DNA in a longer strand to acquire a polypurine-like conformation is currently not known but is probably on the order of 10 base pairs or fewer.

It has been speculated that DNA sequences that have the ability to occur in conformations different from the B form,

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