Contribution of DNA polymerase δ to DNA replication in permeable CHO cells synchronized in S phase

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ABSTRACT

To evaluate the relative contributions of DNA polymerase α and DNA polymerase δ in chromosome replication during the S phase of the cell cycle, we have used the permeable cell system for replication as a functional assay. We carried out the analysis of DNA polymerases both in quiescent cells stimulated to proliferate and progress through the cell cycle (monolayers) and in actively growing cells separated into progressive stages of the cell cycle by centrifugal elutriation (suspension cultures). DNA polymerase α was measured by using the inhibitor butylphenyl dGTP at low concentrations. Using several inhibitors such as aphidicolin, ddTTP and butylphenyl dGTP, we found that DNA polymerase α and δ activity were coordinately increased during S phase and declined at the end. However, DNA polymerase δ was performing about 80% of the total replication and DNA polymerase α performed only 20%. This high ratio of DNA polymerase δ to DNA polymerase α replication activity was maintained throughout S phase in two entirely different experimental approaches.

INTRODUCTION

The replication of nuclear DNA in eukaryotic cells is limited to the S phase of the cell cycle. It has long been known that DNA polymerase α plays a major role in DNA replication (1). However, the recent discovery of DNA polymerase δ has raised an important but unsettled question about the relationship of these two polymerases and their roles in DNA replication (2). Recent studies have indicated that both polymerases may be involved in the replication of mammalian chromosomes (3-6), but there has been some dispute over their relative contributions. Several studies indicated that DNA polymerase α activity is coordinately regulated with cellular DNA synthesis, increasing several-fold during S phase (7-10). However other studies showed that DNA polymerase α activity does not change with DNA synthesis or repair (11,12). Furthermore, studies with CHO cells using enriched populations in G1, S, and G2/M phases of the cell cycle showed no significant change in total DNA polymerase activity but an increasing ratio of DNA polymerase δ to DNA polymerase α as the cells progress through the cell cycle (4). These discrepancies may in part be due to the manner in which DNA polymerase is measured. DNA polymerase assays are often done in cell extracts, using

synthetic templates such as oligo(dT)-poly(dA) and may not necessarily measure the level of *in vivo* functional enzyme. Accordingly, we undertook the study of DNA polymerases α and δ during the S phase of the cell cycle using chromosome replication as a functional assay. We have used a permeable cell system for the replication of chromosomes with the expectation that the high degree of structural and physiological integrity of permeabilized cells would allow a better understanding of DNA synthesis and its regulation than studies in a disrupted cell system.

Another possible cause for the discrepancies concerning the relative roles of DNA polymerase α and δ is that the regulatory mechanisms in actively cycling cells may be different from those operating in quiescent cells that are stimulated to proliferate. Hence, we carried out our studies both in quiescent cells stimulated to progress through the cell cycle and in actively growing cells separated into progressive stages of the cell cycle by centrifugal elutriation. Our results show that in both experimental systems DNA polymerase δ is the dominant activity throughout S phase. DNA polymerase α activity is present throughout the cell cycle but amounts to only about 20% of the total replication activity.

MATERIALS AND METHODS

Chemicals

(*p*-*n*-Butylphenyl) deoxyguanosine triphosphate (BuPdGTP) and (*p*-*n*-butylanilino) deoxyadenosine triphosphate (BuAdATP) were kindly provided by Dr. G. Wright. [^3H]Thymidine (66 Ci/mmol) and [^3H]thymidine 5'-triphosphate (54 Ci/mmol) were from ICN. All other reagents were from Sigma. Growth media and serum were obtained from GIBCO.

Cell Cultures

Chinese hamster ovary cells (CHOK1, ATCC *CCL61) were grown either in suspension culture in spinner flasks using minimal Eagle's medium (MEM) plus 0.3 mM L-proline or in plastic 75 cm² flasks using F12 Ham's medium both supplemented with 10% fetal bovine serum (FBS).

Fractionation of Cells by Centrifugal Elutriation

CHOK1 cells were grown for 24 h for a final concentration of 4×10^5 cells/ml (exponential population). Cells were harvested by centrifugation at 650xg for 10 min at room temperature and suspended in MEM with 1% FBS. The cell suspension was passed through an elutriation rotor (Beckman JE6B) maintained at 2250 rpm in J2-21 (Beckman) centrifuge at a temperature of 22°C (14). Fractions elutriated at increasing flow rates (16-50 ml/min) in MEM with 1% FBS were collected by centrifugation and used for *in vitro* DNA replication and for [^3H]thymidine incorporation assays. Cells (2×10^8) were separated into 4 elutriation fractions at flow rates of 21 to 27, 28 to 33, 34 to 40, and 41 to 48 ml/min. The first few fractions containing primarily G₁ cells and the last fractions containing cell aggregates were discarded.

Cell Synchronization by Serum Deprivation

Cells were plated at 6700 cells/cm² in plastic 75 cm² flasks and were grown in 10% CO₂. After 24 h cells were washed and grown in F12 medium without serum for 66 hrs to arrest them in G₀. Cells were allowed to recover in F12 medium with 10% FBS for 4 h, followed by the addition of hydroxyurea to 1.5 mM. Fourteen hours later, cells were washed 3 times with Ca/Mg-free Dulbecco's phosphate buffered saline (PBS) and supplied by fresh F12 medium with 10% FBS but without hydroxyurea (15). DNA replication activity was measured by incubating the cells in the presence of 0.66 μ Ci/ml [³H]thymidine for 1 h before trypsinization. For suspending the cells from the surface of flasks, 0.05% trypsin in PBS treatment was used. Trypsinization was stopped by the addition of 10 volumes of F12 medium with 10% FBS. Cells were collected by centrifugation at room temperature and made permeable with lysolecithin treatment for *in vitro* DNA synthesis.

Flow Cytometry

Flow cytometry was used to analyze the quality of cell synchronization after centrifugal elutriation or serum deprivation. Cells were stained by suspension in 50 μ g/ml propidium iodide in 0.1 M ammonium citrate for 15 min at 0°C, followed by the addition of an equal volume of 70% ethanol. Cells were analyzed in a Becton-Dickinson fluorescence-activated cell sorter. All experiments were repeated at least three times with similar results. The nuclear DNA content, expressed in C values (a C value of 1 corresponds to a haploid DNA content per cell) increases from 2C to 4C as the cell progresses through S phase. Thus, the C value provides a quantitative measure of a cell's progress through S phase. Since the flow cytometry profile gives the distribution of DNA content in a cell population, it can be used to calculate the average DNA content (C value) of a sample. For convenience, the cell population of each elutriated fraction was divided into 12 classes based on DNA content and the fraction of the cell population corresponding to each class determined by the appropriate area under the flow cytometry profile. These were then averaged to yield the mean DNA content (or C value) of the elutriated fraction.

DNA Synthesis in Lysolecithin-treated Permeable Cells

Cells (0.2-1.0 x 10⁶) from each fraction were washed with buffer A (150 mM sucrose, 80 mM KCl, 35 mM Hepes pH 7.4, 5 mM KPO₄ pH 7.4, 5 mM MgCl₂, and 0.5 mM CaCl₂), resuspended in the same buffer, and permeabilized by treatment with 250 μ g/ml lysolecithin for 1 min at 0°C (13). Permeabilization was monitored by trypan blue uptake. The cells were diluted in a DNA synthesis mixture which contained 0.25 mM ATP, 0.1 mM each of dATP, dGTP, and dCTP, 0.03 mM [³H]dTTP (0.6-1.0 mCi/mmol), 1 mM dithiothreitol, 10 mM phosphoenolpyruvate and 5 μ g/ml pyruvate kinase. After 30 min incubation at 37°C, for studies with inhibitors an equal volume of 10% trichloroacetic acid was added to stop the reaction. The mixture was incubated for 1 h at 0°C. Precipitates were washed on glass filters and counted for insoluble

Figure 1 Flow cytometric analysis of CHO cells synchronized by (A) centrifugal elutriation and (B) serum deprivation. DNA content is indicated on the abscissa and cell number on the ordinate. The fractions analyzed are indicated in Fig. 2.

radioactivity. DNA synthesis was measured in a linear range with respect to incubation time and cell concentration.

RESULTS

Synchronization by Centrifugal Elutriation and Chemical Means

To obtain cells at different stages of S phase, exponentially growing CHOK1 cells in suspension culture were subjected to centrifugal elutriation. The elutriated cell population was divided into four subpopulations. The amount of cells in each fraction after elutriation was 8.2×10^6 , 25.0×10^6 , 37.5×10^6 , and 15.0×10^6 . The rate of [^3H]thymidine incorporation into the cells in different elutriated fractions showed that these represented subpopulations of the entire S phase, with a ten-times higher rate of incorporation in the middle fractions (Fig. 2A).

Whereas the above experiment was done using suspension cultures in which the cells are continuously traversing the cell cycle, we were also interested in examining cells that were stimulated to enter the cell cycle from the quiescent

Figure 2 Incorporation of [³H]thymidine into CHO cells during S phase. Cells were synchronized by (A) centrifugal elutriation and by (B) serum deprivation with hydroxyurea block. Cell synchronized by elutriation were washed with buffer A and incubated in the presence of 0.66 μ Ci/ml [³H]thymidine for 1 h at 37°C. Monolayer cells synchronized by serum deprivation were grown in the presence of [³H]thymidine 1 h before washing with buffer A. Fractions E₁ to E₄ and R₁ to R₄ indicate cell samples taken for the flow cytometric analysis shown in Fig. 1.

state. Monolayers of CHOK1 cells were deprived of serum to accumulate in G₀ phase. After the cells were induced to proliferate by addition of complete medium, followed by a brief hydroxyurea block to synchronize entry into S phase, they were harvested at various times in S phase. [³H]Thymidine incorporation into growing cells showed a well-synchronized population with

Figure 3 Inhibition of [³H]dTTP incorporation into permeable cells from exponentially growing populations in the presence of BuPdGTP (●—●), BuAdATP (○—○), ddTTP (X—X), aphidicolin (■—■), and araCTP (□—□).

TABLE 1
Inhibition of DNA polymerase activities in permeable CHO cells
synchronized by centrifugal elutriation

Elutriate fraction	DNA polymerase activity		Inhibition (%)			
			ddTTP 50 μ M	Aphidicolin 20 μ g/ml	BuPdGTP	
	Total synthesized pmoles	pmol [3 H]dTTP per 10^6 cells in 30 min			100 μ M	5 μ M
I	7.4	0.9	28	60	64	12
II	167.5	6.7	20	78	84	13
III	438.8	11.1	18	81	81	15
IV	91.5	6.1	17	81	82	11

the peak of DNA synthesis at 5 h and a duration of S-phase of about 8 h as shown in Fig. 2B. The flow cytometry profiles showed that the selected cell populations had an average of 86%, 92%, 98%, and 90% S-phase purity (Fig. 1B).

DNA Replication in Permeable Cells and its Response to Inhibitors

To determine which enzymes are responsible for DNA synthesis by permeable CHO cells under our conditions of assay, a variety of inhibitors specific for different eukaryotic DNA polymerases were used. Fig. 3A shows that the replication of permeable CHO cells from exponentially growing cultures are inhibited by aphidicolin and araCTP in a dose-dependent manner. Aphidicolin and araCTP have been shown to inhibit both DNA polymerases α and δ isolated from a variety of sources (16). Our data therefore suggests that DNA polymerases α and/or δ are involved in replication of CHO cells. We have also used two recently discovered inhibitors, BuPdGTP and BuAdATP, which inhibit DNA polymerase α at low concentrations (17,18). Figure 3B shows that 5 μ M

TABLE 2
Inhibition of DNA polymerase activities in permeable CHO cells
synchronized by serum deprivation

Time points	DNA polymerase activity		Inhibition (%)			
			ddTTP 50 μ M	Aphidicolin 20 μ g/ml	BuPdGTP	
	Total synthesized pmoles	pmol [3 H]dTTP per 10^6 cells in 30 min			100 μ M	5 μ M
I	191	8.9	27	73	72	15
II	342	15.9	21	79	77	13
III	237	11.0	12	76	82	16
IV	148	6.9	14	73	79	18

TABLE 3
DNA polymerase α and DNA polymerase δ activity during
replication at various stages of S phase

Type of cells	Fraction	Stage in S phase (C value)	% of total DNA synthesis ^a		pmol [³ H]dTTP per 10 ⁶ cells in 30 min ^a		DNA pol δ / DNA pol α
			DNA pol α	DNA pol δ	DNA pol α	DNA pol δ	
Actively cycling cells	E ₁	2.00	12	52	0.11	0.47	4.33
	E ₂	2.09	13	71	0.87	4.76	5.46
	E ₃	2.83	13	68	1.44	7.55	5.23
	E ₄	3.48	11	71	0.67	4.33	6.45
Quiescent cells stimulated to proliferate	R ₁	2.51	15	57	1.34	5.07	3.80
	R ₂	3.22	13	64	2.07	10.18	4.92
	R ₃	3.75	16	66	1.76	7.26	4.13
	R ₄	3.85	18	61	1.24	4.21	3.38

^aDNA polymerase α and DNA polymerase δ percentages were calculated from Table 1 and Table 2 from inhibition data as described in the text. These values were used to calculate pmol [³H]dTTP per 10⁶ cells in 30 min by DNA polymerase α and polymerase δ .

BuPdGTP, which is known to inhibit DNA polymerase α more than 98% (17), inhibited replication in permeable CHO cells by only 20%. However at high concentrations (100 μ M) of BuPdGTP, about 80% of the total activity is was abolished. A similar pattern of inhibition was observed with BuAdATP but DNA synthesis was more sensitive than BuPdGTP, 50% inhibition being achieved at about 12 μ M and 30 μ M, respectively (Fig. 3B). DNA polymerase β and γ activity during replication in permeable cells was assessed by the use of dideoxy-TTP. At a level of 50 μ M, this nucleotide analog completely inhibits these activities without effect on DNA polymerases α and δ (19). We observed about 20% inhibition by 50 μ M dideoxy-TTP, suggesting that most DNA synthesis in permeable cells derived from the exponential stage of growth does not involve DNA polymerases β and γ (Fig. 3B).

DNA Polymerase α and δ Activities at Different Stages of S Phase

The contribution of DNA polymerases α and δ to DNA synthesis was determined in four fractions containing cells at different stages of S phase, obtained from either suspension cultures (Table 1) or monolayers (Table 2) as described above. DNA replication was measured in permeable cells with and without various inhibitors at appropriate concentrations for distinguishing between the activity due to DNA polymerase α and δ . The rate of DNA synthesis as measured by [³H]dTTP incorporation at various stages of S phase paralleled the incorporation of [³H]thymidine by the growing cells (compare the data in Figure 2 and in Tables 1 and 2). Since aphidicolin inhibits specifically both DNA polymerase α and δ , the inhibition achieved at high concentrations of aphidicolin (20 μ g/ml) measures the total contribution of DNA polymerases α

and δ . At a low concentration (5 μ M), BuPdGTP is a specific DNA polymerase α inhibitor (3,5,17,18) whereas at a very high concentration (100 μ M), BuPdGTP inhibits both polymerase α and δ , similar to aphidicolin. Accordingly, BuPdGTP can be used to calculate the activity of DNA polymerase α as well as the total activity due to both α and δ (3,5,17,18). Using this rationale, we have determined the activities of DNA polymerases α and δ at different stages of S phase (Table 3). In view of the fact that 5 μ M BuPdGTP may slightly inhibit DNA polymerase δ (3), the values of the ratio of δ/α polymerase calculated by this procedure are minimum values. Cycling cells traversing S phase and quiescent cells stimulated to enter S phase yielded the following strikingly similar results: (i) Total DNA synthesis reached similar maximum levels in mid-S phase; (ii) the activity due to polymerase β and γ , based on inhibition of DNA synthesis by ddTTP, was about 28% at the beginning of S phase and fell to 15-20% in mid- and late-S phase; (iii) inhibition by aphidicolin (20 μ g/ml) or BuPdGTP (100 μ M) was 60-70% at the beginning and 80-85% in mid-S phase; (iv) contribution due to DNA polymerase α , estimated from the inhibition by 5 μ M BuPdGTP, was 12-18% of the total activity and did not change significantly throughout S phase, indicating that most replication activity during S phase was due to DNA polymerase δ ranging from 55% at the onset to 65-70% in mid-S phase (Table 3); and (v) virtually all DNA polymerase activity can be accounted for by inhibition achieved by ddTTP plus aphidicolin.

DISCUSSION

In this study, we have addressed the following questions: which of the DNA polymerases are functionally active in chromosomal replication during the S phase of the cell cycle, and what are their relative contribution to this process? These questions cannot be answered by measuring the relative amounts of the DNA polymerases at different stages of the cell cycle but requires an assay for DNA replicative activity attributable to each of the DNA polymerases. Our replication assay used lysolecithin-treated permeable CHO cells, which are known to perform semiconservative replication in a cell cycle dependent manner (13). That DNA replication in lysolecithin-permeabilized cells reflects DNA replication is also supported by our recent observation that the replication of various genes throughout the S phase in this system occurs in a reproducible temporal order which agrees with the replication order observed *in vivo* (21). Additional evidence for the integrity of DNA replication in lysolecithin-permeabilized cells is the continuous initiation of replication at the origin of SV40 genome observed in permeable infected CV-1 cells (22). Our system of permeable CHO cells synthesizes DNA *in vitro* in the range which other laboratories observed for CHO cells (13), CV-1 cells (3) and human fibroblasts (5). At the peak of S phase, we observed 85 pmole of DNA per 10^6 cells/h which is comparable to the value of 73 pmoles of DNA/ 10^6 cells/h for other cell types (3,5). In this system, about 20% of the cells are actively synthesizing DNA; using this value, our rate of *in vitro* DNA synthesis is about 23% of the *in vivo* rate of DNA replication and about 3.0% of DNA is synthesized per cell per hour.

Using this replication assay, our studies show that DNA polymerase δ contributes 3-6 times more to DNA synthesis than DNA polymerase α throughout S phase, both in actively cycling cells synchronized by elutriation and in quiescent cells that are stimulated to proliferate. The functional activity of these enzymes could, of course, not be measured directly but had to be deduced from the effect of specific inhibitors. The availability of an inhibitor specific for DNA polymerase α was therefore of critical importance. This inhibitor, BuPdGTP has been shown to block DNA polymerase α preferentially (95% or more) at a concentration of 5 μ M, without appreciable effect on DNA polymerase δ (3-6,17,18,20,23,24). However, care must be used in interpreting results based on the use of BuPdGTP, which at 10 μ M can inhibit purified DNA polymerase δ 30-50%; as a result, the use of higher concentration of BuPdGTP will cause an overestimation of the contribution of DNA polymerase α (3). An alternative to using nucleotide analogs in order to discriminate between two different DNA polymerases would be the use of antibodies against DNA polymerase α . However, the use of such antibodies is questionable since the inhibition of DNA replication in permeable cells requires several orders of magnitude higher concentration of antibodies than those which inhibit isolated DNA polymerase α (25). Moreover, monoclonal antibodies to DNA polymerase α have been shown to interact with subfractions of DNA polymerase δ at higher concentrations (26,27).

The relative contributions of DNA polymerase α and DNA polymerase δ to DNA replication do not change significantly at various stages of S phase, even though the replicative activities of the both enzymes rise strikingly at the onset of S phase and decline near its end, basically following the pattern of [3 H]thymidine incorporation (Table 3 and Fig. 2). The cyclic change in the replicative activity of both DNA polymerases was seen in cell fractions obtained by centrifuge elutriation of exponentially growing suspension cultures and in monolayer cells at various times after entry into S-phase after serum stimulation of quiescent cultures (Table 3). The smaller apparent spread of polymerase activities in stimulated quiescent cells is due to the narrower spread of C values in the fractions examined (Table 3). This behavior differs strikingly from the cell cycle-dependent changes in the *in vitro* activity of DNA polymerase α (4,11,12) and DNA polymerase δ (4). For example, Bambara and coworkers (4) measured total activity of DNA polymerases α and δ in cell extracts, using synthetic DNA templates, and found no major changes throughout the cell cycle and an activity ratio of about one. Although our results cannot be directly compared, since we measured replication function and not the enzyme content, we do not observe any difference between the continuously cycling cells and quiescent cells stimulated to proliferate with respect to replication activity due to DNA polymerase α (Table 3), whereas Korn and coworkers (28) find a constant level of DNA polymerase α activity at all stages in cycling cells but substantial increase from a low level during the activation of quiescent cells (28).

Our results are consistent with the experiments of Dresler and Frattini that DNA polymerase δ participates in DNA replication in human fibroblasts

(5,29). We are also in agreement with studies implicating both DNA polymerase α and δ in DNA replication of CV-1 cells, although we differ in the quantitative interpretation of the participation of the two DNA polymerases (3). *In vitro* replication of SV40 chromatin and plasmid DNA containing the SV40 replication origin has been shown to involve DNA polymerase δ (24,30). Injected plasmid replication in *Xenopus laevis* eggs and egg chromosome replication revealed the participation of DNA polymerase δ to the extent of 70% and 30% of total DNA synthesis, respectively (31). On the other hand, a major portion of DNA repair synthesis induced by UV irradiation in human fibroblasts is due to DNA polymerase δ (5,29,32). Thus, a wide variety of biological systems seem to involve DNA polymerase δ .

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