Alterations in the Phosphorylation and Activity of DNA Polymerase α Correlate with the Change in Replicative DNA Synthesis as Quiescent Cells Re-enter the Cell Cycle*

(Received for publication, April 25, 1989)

Janice Cripps-Wolfman, Edgar C. Henshaw, and Robert A. Bambara
From the Departments of Biochemistry, Microbiology, and Immunology and the Cancer Center, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

The regulation of DNA polymerase α was examined in quiescent, human fibroblast cells stimulated to re-enter the cell cycle by subculturing in fresh serum-containing medium. The level of DNA polymerase α activity was measured in cell lysates and after specific immunoprecipitation. DNA polymerase α activity increased approximately 10-fold during the period of measurement. The activity increase was coincident with an approximately 60-fold increase in thymidine incorporation in the whole cells representing the first S phase. The large increase in polymerase α activity was not predominantly the result of synthesis of new polymerase, since the abundance of the enzyme changed less than 2-fold over the measured period. The quantity of [32P]phosphate incorporated into two subunits (180 and 68 kilodaltons) of DNA polymerase α increased approximately 10-fold in parallel with the increase in polymerase activity. The specific activity of the cellular ATP pool remained nearly constant over the period of measurement, indicating that the increase in labeling reflects a true increase in incorporation of phosphate. Results from other laboratories indicate that phosphorylation of DNA polymerase α increases its catalytic activity. Our results then suggest that the activity increase observed in DNA polymerase α when quiescent, human fibroblasts are stimulated to proliferate is largely caused by a phosphorylation-dependent regulatory process.

As in prokaryotes, DNA replication in eukaryotes is carried out by large multienzyme complexes as well as many accessory factors (Conaway and Lehman, 1982; Kagami et al., 1983; Huberman, 1987; Ottiger et al., 1987). An enzyme complex in eukaryotes believed to have a major role in chromosomal replication is DNA polymerase α (Bollum et al., 1975; Weissbach et al., 1979; Fry and Loeb, 1986; Yagura et al., 1987; Wahl et al., 1988). Although differences in isolation procedures have resulted in difficulty in the precise identification of the major polypeptides associated with DNA polymerase α, due to lability of the enzyme during purification (Vishwanatha et al., 1986; Wong et al., 1986; Nasheuer and Grosse, 1987), considerable progress has been made in distinguishing the major protein subunits (Wahl et al., 1984; Wang et al., 1984; Holmes et al., 1986; Wong et al., 1986; Ottiger et al., 1987; Nasheuer and Grosse, 1987; Hirose et al., 1988; Nasheuer and Grosse, 1988). There is general consensus that the enzyme consists of a polymerase α core subunit, having DNA polymerizing activity and ranging in size from 150–180 kDa, and three other subunits of 55–60, 68–73 and 45–50 kDa, some combination of which expresses DNA primase activity. The 48- and 68-kDa proteins display immunological similarity (Holmes et al., 1986). It has been suggested that the 68-kDa protein may contain regulatory activity for the DNA primase (Holmes et al., 1986).

There has been continuing interest in the regulation of DNA replication and DNA polymerase α under different conditions of cell growth and differentiation (Dell’Orco et al., 1975; Rossini et al., 1976; Adams, 1980; Sejersen et al., 1985; Katoh et al., 1985; Talbot and Jasani, 1985; Alama et al., 1986; Stein et al., 1986; Niedalski et al., 1986; Matsukage et al., 1986; Haraguchi et al., 1987; Reiter et al., 1987; Otto et al., 1988). It is known that density inhibition and serum deprivation cause human diploid fibroblasts to stop growing and enter a viable G0-phase arrested state (Rossini et al., 1976). Passage of cells, or addition of serum or growth factors, will allow the cells to re-enter the cell cycle and proliferate (Adams, 1980; Stein et al., 1986). Serum and fibroblast growth factor added to quiescent astrocytes stimulate these cells to exit their G0 state and begin to proliferate (Kniss and Burry, 1988). Treatment of growth-arrested Swiss 3T3 cells with prostaglandin F2α and insulin leads to increased DNA synthesis and elevation of DNA polymerase α activity (Otto et al., 1988). Matsukage and co-workers (1986) reported a down-regulation of DNA polymerase α and a decrease in labeling with [3H]thymidine accompanying differentiation of chick embryo lens and neural cells. These results suggest coordinated control of DNA polymerase α activity and replicative DNA synthesis with the proliferative state of cells.

Evidence from several laboratories has suggested that polymerase α may be modified by phosphorylation (Danese et al., 1981; Wong et al., 1986; Krauss et al., 1987; Donaldson and Gerner, 1987). Sylvia et al. (1986) also demonstrated that polymerase α isolated from Norman murine sarcoma tissue is a substrate for several types of protein kinases using partially purified enzymes. They showed a stimulation of DNA polymerase α synthetic activity and DNA binding capability after phosphorylation in vitro by a partially purified kinase in the presence of phosphatidylinositol 4-phosphate. Further, a 2–3-fold stimulation of polymerase α synthetic activity and enhanced fidelity has been reported to occur following incubation of the enzyme, partially purified from HeLa cells, with protein kinase C, purified from rat brain, in the presence of ATP, phosphatidyserine, diacylglycerol, and MgCl₂ (Krauss et al., 1987). Donaldson and Gerner (1987) demonstrated
phosphorylation of DNA polymerase α in specific immunoprecipitates. They found that treatment of the enzyme with alkaline phosphatase abolished its activity. However, polymerase α activity was restored after incubation with ATP but not with a non-hydrolyzable analog (Donaldson and Gerner, 1987).

Initial objectives of the present study were to determine whether DNA polymerase α activity measured in vitro and replicative DNA synthesis measured in vivo correlated in time of onset in quiescent, normal human fibroblasts stimulated to proliferate following subculturing and refeeding with fresh medium and serum. Evidence is presented that replicative DNA synthesis, DNA polymerase α activity, and phosphorylation of polymerase α are induced with a nearly identical time course following release of cells from a viable Go state. These events are not a result of a change in the overall protein level of the enzyme is also demonstrated. The ramifications of such findings with respect to causality are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**

[methyl-3H]Thymidine (80 Ci/mmol) and [methyl-3H]thymidine-5'-triphosphate (80 Ci/mmol) were purchased from Du Pont-New England Nuclear. Tran3'-label (1000 Ci/mmol) for metabolic protein labeling and [32P]orthophosphate (carrier-free) were obtained from ICN Radiochemicals and Amersham Corp., respectively. Materials for cell culturing were purchased from Flow Laboratories, VWR Scientific, and Sigma. Nucleotides, the non-inactivating anti-DNA polymerase α monoclonal antibody, SJK 237.71 (Tanaka et al., 1982), and control mouse IgG (MOPC 21) were obtained from Pharmacia LKB Biotechnology Inc. A neutralizing monoclonal antibody against DNA polymerase α, SJK 132.20 (Tanaka et al., 1982), was purchased from Pharmacia LKB Biotechnology Inc. or purified according to the method of Mihi and Shigi (1980) from the supernatant solutions of hybridoma cells (American Type Culture Collection) producing this antibody. Rabbit anti-(mouse IgG) secondary antibody was purchased from ICN or Organon-Technika (Cappel). Electrophoresis supplies were from Bethesda Research Laboratories. All other reagents were acquired from Sigma or United States Biochemicals unless otherwise indicated.

**Cells**

Human diploid, foreskin fibroblasts (HF) were a generous gift from Michael R. Miller, University of W. Virginia. They were determined to be free of mycoplasma. HF cells were routinely cultured in DMEM supplemented with 10% fetal bovine serum (J. R. Scientific), 10 units/ml penicillin (1 X) and 10 μg/ml streptomycin (1 X).

**Establishment of the Quiescent State**

HF cells were passaged to approximately 5 X 10^6 cells/75-cm² plate, and refed fresh medium containing 10% FBS every 48 h for one week. At this point the cells had just attained a confluent state and were given one final change of DMEM supplemented with 10% FBS before being left for 3-5 days. The cells were then used directly, as a quiescent population, for studies, or subcultured and refed fresh medium to examine changes occurring as the cells exit Go.

**Preparation of Cell Lysates**

Cells were harvested by washing twice with trypsin-EDTA and then incubated 1-4 min with 1 ml of trypsin-EDTA/75-cm² plate. Detached cells were pooled and rinsed once with DMEM containing 10% FBS and once with ice-cold PBS. For measurements of DNA polymerase α activity the final cell pellet was resuspended to a density of 10^7 cells/ml in a lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 1 mM each of EDTA and EGTA, 5 mM dithiothreitol, 2 mM ATP, 4 mM spermidine, 0.5 μg/ml each of leupeptin and pepstatin A, 20 μg/ml aprotinin, 5 mM p-nitrophenylphosphate and 10 mM β-glycerophosphate. The resuspended cells were immediately frozen in liquid nitrogen and stored at -80 °C. The lysate was prepared by thawing cells with addition of ice on an equal volume of 2x buffer supplemented with 240 mM sucrose and 6 mM MgCl₂. Lysates were either used directly for assay of DNA polymerase α activity or immunoprecipitated (see below) with the non-neutralizing anti-DNA polymerase α monoclonal antibody, SJK 237.71 (Tanaka et al., 1982). These immunoprecipitates were then assayed for DNA polymerase α activity.

For structural analysis of the enzyme, lysates were prepared by direct solubilization of the washed cells in lysis buffer as described above, but at pH 8.5 and containing 100 μM sodium vanadate, 10 mM phosphoserine, 100 μM sodium deoxycholate, 0.1% SDS, 125 mM sucrose, 3 mM MgCl₂, 260 mM LiCl, 10 mM p-nitrophenylphosphate, and 40 mM β-glycerophosphate (buffer S). The whole cell extract was clarified by centrifugation at 13,000 X g for 10 min and used for immunoprecipitation of DNA polymerase α using the anti-DNA polymerase α monoclonal antibody SJK 132.20 (Tanaka et al., 1982).

**Immunoprecipitation of DNA Polymerase α**

Immunoprecipitation was performed by a modification of the method of John and Firestone (1986). Clarified lysates (typically 600 μl) were preabsorbed by adding a mixture of Protein A-Sepharose (20 μl of 50%, v/v) and Sepharose CL-4B (40 μl of 50%, v/v) and 0.5 volume (200 μl) of a solution of 50 mg/ml each of cytochrome c and myoglobin such that the final concentrations of these two proteins were 12.5 mg/ml each. After a 45-60 min incubation at 4 °C with shaking, the lysates were centrifuged 3 min at 13,000 X g and the supernatant solutions retained. Each supernatant solution was divided in half, and to one aliquot was added SJK 132.20 anti-DNA polymerase α monoclonal antibody (6-15 μg). An equal amount of control mouse monoclonal IgG, was added to the other aliquot. Incubations were then continued for 1 h at 4 °C. Then the lysate-antibody solutions were mixed with Protein A-Sepharose (25 μl of 50%, v/v). The Protein A-Sepharose had been previously absorbed with rabbit anti-(mouse IgG) secondary antibody (6-10 μg/μl of primary antibody) in the presence of non-radioactive cell lysate (400 μl from 1 X 10⁷ HF cells) and 5 mg/ml each of cytochrome c and myoglobin in buffer S. Following a 30-45 min incubation with this preabsorbed Protein A-Sepharose, immunocomplexes were isolated by layering entire mixtures onto individual 500 μl of 1.0 M sucrose cushions and centrifugation in an Eppendorf microcentrifuge for 3 min. The supernatant solutions were discarded, and the cushion was overlayed with a solution of 2.0 M urea in buffer S followed by aspiration of all liquid above the pellets. The immunocomplexes were washed three times with buffer S, once with 10 mM Tris-HCl, pH 8.0, 5 mM EDTA and once with 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA (buffer T). The final immunoprecipitates were incubated for 10 min at 25 °C with 5% SDS and then boiled 10 min in 2 X Laemmli sample buffer and analyzed by SDS-PAGE (Laemmli, 1970), autoradiography, and laser densitometry.

**Immunoprecipitation of DNA polymerase α for the purpose of measurement of the activity of the enzyme was performed on lysates prepared by freeze/thawing in lysis buffer at pH 7.5. The lysates were not preabsorbed, and SJK 237.71 monoclonal antibody was used because it is non-neutralizing. The Protein A-Sepharose was preabsorbed with secondary antibody as described above except that TE buffer was used rather than buffer S, since the enzyme activity is sensitive to the presence of salt and detergents. The immunoprecipitates were washed once with TE buffer and assayed for polymerase α activity as described. After the assay the reaction mixtures were filtered onto Whatman No. 1 filters and washed with 0.5 M EDTA. Filters were washed seven times for 15-30 min each with ice-cold 5% trichloroacetic acid, twice with ice-cold ethanol, dried, and assayed for ³H incorporation in non-aqueous scintillation fluid.

**Assay for DNA Polymerase α Activity**

Aliquots (1-8 μl) of cell lysate prepared by freeze/thawing were assayed according to a standard method (Wahl et al., 1984). Briefly, incorporation of [methyl-³H]TMP into endonuclease-activated calf thymus DNA was measured in a final reaction volume of 25 μl which contained 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM MgCl₂, 250
Regulation of DNA Polymerase α

μg/ml BSA, 5 mM β-mercaptoethanol, 5% glycerol, 2 mM ATP, 0.75–1.0 mM activated calf thymus DNA prepared according to the method of Spanos et al. (1981), 40 μM each of dATP, dGTP, and dCTP, and 25 μM [methyl-3H]-dTMP at 10 Ci/mmol. The assay was started by addition of lysate, incubated for 30 min at 37 °C, and then terminated by addition of 20 μl of 0.5 M EDTA and placement of the tubes on ice. The reaction products were separated from unincorporated radioactive precursor molecules by centrifugation through 0.4-ml columns of Sephadex G-50 at 1200 rpm for 4 min (Penesky, 1977). The void volume containing the reaction products was mixed with 1.25 ml of ACS II (Amersham Corp.) aqueous scintillation fluid and counted. The incorporation of [3H]dTMP into activated calf thymus DNA was verified to be linear for the amounts of cell lysate used in these assays (data not shown).

Assay for Replicative DNA Synthesis

Replicative DNA synthesis was examined by measurement of the incorporation of [methyl-3H]-thymidine into whole cell DNA (Adams, 1980; Naito et al., 1987). Confluent (t = 0) or subcultured, re-fed HF cells in 60-mm dishes were pulse-labeled for 1 h with [methyl-3H] thymidine at 20 μCi/ml, 13 Ci/ml in DMEM supplemented with 10% FBS and 1× penicillin-streptomycin. Following the labeling period, the medium was removed and the cells washed twice with ice-cold PBS and then incubated for 12 h at 4 °C with ice-cold 5% trichloroacetic acid. This solution was then removed, and the plates were allowed to dry to completeness, and then the cells were incubated with 0.5 M NaOH at 4 °C for at least 1 h to allow for thorough solubilization of the labeled DNA. The plate was removed and immersed in methanol, dried, and autoradiographed using Kodak XAR film. Spots of [3H]ATP, established by densitometry, were quantitated by cutting and weighing. The relative specific activity of ATP in the [32P]phosphate cells was calculated from the ratio of [3H]ATP to total ATP.

Determination of the Abundance of DNA Polymerase α

Silver Staining—To determine the total amount of polymerase α protein, quiescent and subcultured HF cells were harvested and lysates prepared as described above for structural analysis. Immunoprecipitation was performed with either anti-DNA polymerase α antibody (SJK 132.20) or control mouse IgG2 (MOPC 21). The latter antibody was used to assess non-specific binding. The final immunoprecipitate was analyzed by SDS-PAGE using a 10% separating gel and a 4% stacking gel according to the method of Laemmli (1970). The resultant gel was stained with silver. Quantitation of the amounts of the 68- and 180-kDa subunits was performed using an LKB scanning laser densitometer.

Replicative DNA Polymerase α with [35S]Methionine—Quiescent or exponentially growing HF cells were labeled for 24 h with Trans-35S-label (containing approximately 70% as [35S]methionine, ICN) in minimal essential medium without methionine supplemented with 2 mM l-glutamine, 25 μM non-radioactive methionine (0.25 × 100% FBS, and 1× penicillin-streptomycin. Following the labeling period the cells were washed, harvested, and lysates prepared as described above for structural analysis. Aliquots of the clarified lysates were precipitated with 5% trichloroacetic acid and the acid-insoluble radioactivity determined.

Immuno precipitation was performed on samples containing an equal quantity of trichloroacetic acid precipitable [35S]methionine with either anti-DNA polymerase α monoclonal antibody SJK 132.20 or control mouse IgG2 to measure non-specific binding (see below). The final immunoprecipitates were analyzed by SDS-PAGE as described above. Following electrophoresis, gels were exposed at −80 °C using Kodak BBS film and a Du Pont Lightning Plus intensifying screen. The abundance of DNA polymerase α protein was quantitated by laser densitometry.

Measurement of the Phosphorylation of DNA Polymerase α

Assessment of the phosphorylation state of DNA polymerase α in whole cell lysates was made by labeling of quiescent or subcultured HF cells for 2-h periods with 150 μCi/ml carrier-free [32P]orthophosphate in phosphate-free minimum essential medium supplemented with 1 mg/ml BSA, pH 7.5, 2 mM l-glutamine, an additional 0.75% sodium bicarbonate and 1× penicillin-streptomycin. After the labeling period the medium was removed, the cells were washed twice with ice-cold PBS and then harvested as described. Immunoprecipitation was done using equal numbers of labeled cells/tube as delineated above for [35S]methionine-labeled cells. Immunoprecipitates were run on 10% SDS-polyacrylamide gels using a 4% stacking gel. Dried gels were exposed at −80 °C using preflashed Kodak XAR film and a Du Pont Lightning Plus intensifying screen. Phosphate associated with polymerase α proteins was determined by autoradiography and quantitated with an LKB laser densitometer.

Determination of the Relative Specific Activity of ATP in Quiescent and Subcultured HF Cells

Relative amounts of [32P]ATP were determined by the method of Burnette (1981). Aliquots (5 μl) from lysates prepared from [32P]phosphate-labeled cells were diluted to 100 μl and then mixed with 10 μl of a solution of 400 mM sodium tungstate, 500 mM tetrathylammonium HCl and 500 mM procaine HCl in the ratio of 5:4:1. The mixture was centrifuged at 10 μg/ml in a Beckman microfuge. 5 μl of the supernatant solutions were spotted onto polyethyleneimine thin layer chromatography (TLC) plates (J. T. Baker Chemical Co.) and the plates immersed in methanol following by drying. The TLC plates were developed in a chamber equilibrated with 0.75 M Trizma base, 0.45 M HCl, and 0.05 M LiCl. At completion, the TLC plate was removed and immersed in methanol, dried, and autoradiographed using Kodak XAR film. Spots of [32P]ATP, established by co-chromatography with an ATP standard, were scraped, and counted using non-aqueous scintillation fluid.

To determine the relative amounts of total ATP, unlabeled samples of HF cell lysates were precipitated with trichloroacetic acid and the resulting supernatant solutions extracted with Freon 113 containing 0.5 M tri-n-octylamine. Aliquots of aqueous extract (top phase) were analyzed by HPLC using a Mono Q column (Pharmacia LKB Biotechnology Inc.) and eluted with a gradient of NaCl from 100 to 450 mM. Peaks of [32P]ATP were quantitated by cutting and weighing. The relative specific activity of ATP in the [32P]phosphate cells was calculated from the ratio of [32P]ATP to total ATP.

Flow Cytometry

Flow cytometry was used to verify the homogeneity of quiescent and subcultured cell populations. Cells (1 × 10⁶) were fixed in 70% methanol, washed, and stained with thymycin C (100 μg/ml) and 12.5 mM MgCl₂ in potassium phosphate buffer. The DNA content was quantitated for each cell using an EPICS V flow cytometer ( Coulter Electronics). A 5-watt argon ion laser operated at 457 nm and 150 milliwatts of power was used for excitation.

RESULTS

Analysis of Cell Growth State

We wished to examine changes that occur in DNA polymerase α structure and activity as cells exit a quiescent Go state and re-enter the cell division cycle. To this end it was necessary to establish a viable quiescent population of cells. Cells were grown to confluence and left 3–5 days in their normal medium containing 10% fetal bovine serum. Confluent cells at 3–5 days following the last feeding were found to be greater than 99% viable (data not shown). Quiescent cells were found to consist of nearly 0% S phase cells and greater than 90% Go/G₁ phase cells (see below).

Replicative DNA Synthesis in Quiescent and Subcultured HF Cells—Measurement of the incorporation of [3H]thymidine into cellular DNA was used to assess the position of the cells within the growth cycle and to indirectly measure DNA polymerase α activity. Pulse-labeling of both quiescent (t = 0) and subcultured cells demonstrated that the HF cells could grow in synchrony for at least 24–36 h (Fig. 1). The conditions for the labeling were chosen to reduce artifactual measurements resulting from alteration in the cellular uptake or intracellular pools of thymidine nucleotides (Adams, 1980; Naito et al., 1987). There is a lengthy lag in thymidine incorporation until 12 h after subculturing. This period is presumed to represent transition from a quiescent Go state up to the beginning of S phase of the cell cycle (Naito et al., 1987). At 12 h there is a sharp transition to a high rate of thymidine incorporation representing DNA synthesis as the cell cycle progresses into S phase. The rate of synthesis increases...
The development of a procedure for preparation of cell lysates was aimed at being gentle and rapid. This consisted of freezing and thawing of cells in low ionic strength buffer followed directly by assaying for activity.

In preliminary studies lysates prepared from quiescent and non-synchronized, exponentially growing HF cells were assayed. It was found that there was at least a 10-fold difference in the polymerase α activity between these two populations (data not shown). Therefore, we measured DNA polymerase α activity as a function of time following the release of the cells from G0. DNA polymerase α activity was measured in cell lysates which were prepared at various times after HF cells were subcultured from the quiescent state and refed fresh medium and serum. As seen in Fig. 2, the activity of the enzyme was very low until approximately 12 h following passage and refeeding. Subsequent to this lag period there is a sharp transition to elevated rates of incorporation until 24 h, the latest time examined routinely in these studies. There is approximately a 10-fold increase in the polymerase α activity when comparing either quiescent and 24-h cell lysates or 12- and 24-h lysates. Other investigators have also reported large changes in the enzyme activity after release of the cells from quiescence (Spadari and Weissbach, 1974; Chiu and Baril, 1975; Reiter et al., 1987; Otto et al., 1988). It is significant that the time course for the change in DNA polymerase α activity closely parallels the change seen in replicative DNA synthesis following release of the cells from quiescence (compare Fig. 2 with Fig. 1).

That this activity determined in vitro is due solely to DNA polymerase α is very likely since the synthesis is very sensitive until approximately 20 h when it levels off until at least 24 h. Following this interval, the rate of DNA synthesis falls up to 36 h, the latest time point examined in this study. The difference in the rates of replicative DNA synthesis between 12 and 24 h is greater than 60-fold. Similar results also have been obtained using the same procedures with Chinese hamster ovary cells (data not shown).

To further assess the growth state of the quiescent and subcultured cell populations, parallel samples of cells were harvested at the same times as the labeling periods indicated in Fig. 1 and fixed in methanol for analysis of DNA content and cell size by flow cytometry as described under “Experimental Procedures.” A one-parameter analysis demonstrated that cells from t = 0 through t = 12 h had a DNA content characteristic of G1 phase cells with very few, if any, S or G2 phase cells (data not shown). After 12 h, i.e. during the time that replicative DNA synthesis increased, there was a rising percentage of cells with an S phase DNA content. By 24 h the majority of the cells were in S phase with some cells beginning to enter G2 phase (data not shown).

Evidence That Activity and Structural Modifications of DNA Polymerase α Correlate with Replication as Quiescent HF Cells Re-enter the Cell Division Cycle

DNA Polymerase α Activity in Cell Lysates—Since DNA polymerase α activity in cell lysates was found to be labile to sonication, homogenization and the presence of detergents a procedure for preparation of cell lysates was developed which would be gentle and rapid. This consisted of freezing and

3. Cripps-Wolfman and R. A. Bambara, unpublished observations.
to inhibition by aphidicolin which excludes any contribution by DNA polymerases β or γ. Furthermore, the activity is also susceptible to inhibition by the anti-DNA polymerase α antibody, SJK 132.20. Addition of this antibody to the assay causes greater than 90% inhibition of the incorporation of [3H]dTMP into activated calf thymus DNA (data not shown). It is well documented that monoclonal antibodies raised against DNA polymerase α do not interact with DNA polymerase δ (Byrnes et al., 1985; Wahl et al., 1986). To further verify these results we measured the activity of polymerase α specifically immunoprecipitated from subcultured HF cell lysates with a non-neutralizing monoclonal antibody, SJK 237.71. Again, there was at least a 10-fold change in the activity, when immunoprecipitates from 12 and 24 h were compared (Fig. 2).

**Phosphorylation of DNA Polymerase α**—Since DNA polymerase α had previously been reported to be a phosphoprotein (Wong et al., 1986), we first determined whether it was phosphorylated in the cell types used for our experiments. In the initial studies we incubated subconfluent Chinese hamster ovary cells with [32P]orthophosphate and found phosphorylation of both the 180- and 68-kDa subunits (data not shown). The same results were seen using HF cells. We then wished to explore whether this phosphorylation changed with the state of growth of the cells. Both quiescent and exponentially growing HF cells were labeled with [32P]orthophosphate as described under “Experimental Procedures,” and the subsequent immunoprecipitates of polymerase α from the labeled cells were examined by SDS-PAGE and autoradiography. There was significant phosphorylation of the 180- and 68-kDa subunits in the exponentially growing cells, but none was detected in the quiescent HF cells (data not shown).

The phosphorylation state of the 180- and 68-kDa polymerase α subunits was then examined at several times after cells were released from quiescence by subculturing and refeeding with fresh medium and serum. As seen in Fig. 3A (lane 2) phosphorylation of these two subunits is barely detectable at 12 h following subculturing and refeeding. By 18 h (Fig. 3A, lane 4) phosphate can be seen associated with both subunits. At 24 h (Fig. 3A, lane 6) an even greater amount of phosphorylation is present in both subunits. The phosphorylation is still present at 36 h, although it appears to be decreased somewhat at least in the 180-kDa band (data not shown). The degree of phosphorylation was quantitated by scanning laser densitometry, the results of which are presented in Fig. 3B. Table I summarizes the results from three independent experiments. Similar to the activity changes in DNA polymerase α, there is a 7-10-fold increase in the incorporation of phosphate into both the 180- and 68-kDa subunits between 0 or 12 and 24 h following subculturing and refeeding of the quiescent HF cells. There was very little difference in the relative specific activity of the [32P]ATP pool at 12 versus 24 h in these cells, indicating that a change in this parameter could not be responsible for the observed changes in the phosphorylation of DNA polymerase α (Table I).

**Confirmation That DNA Polymerase α Is a Stable Enzyme Complex during Changes in Cell Growth State**

**Steady State Level of DNA Polymerase α by Silver Staining**—In view of the above results, we determined whether the changes seen in polymerase activity and phosphorylation upon alteration of cell growth rate resulted from an increase in the synthesis of the enzyme itself or appeared to be part of an activation process. The total protein levels of DNA polymerase α were determined by immunoprecipitation from equal numbers of quiescent and subcultured cells and silver staining of the resultant SDS-polyacrylamide gel. A representative example from three independent experiments is shown in Fig. 4A. Lanes 1–3 are immunoprecipitations of DNA polymerase α from quiescent cells, and from cells subcultured for 12 and 24 h, respectively. Lane 4 shows a control immunoprecipitation of DNA polymerase α that was partially purified by
TABLE I

Quantitation of phosphorylation of DNA polymerase α

Values are radioactivity incorporated ± S. D. for three separate experiments except for the 180-kDa subunit at 18 h which displays the mean for two experiments. 100% in the 24-h column does not necessarily represent the same amount of phosphorylation of the two subunits.

| Time  | 0 h | 12 h | 18 h | 24 h |
|-------|-----|------|------|------|
| % 24h value |     |      |      |      |
| Subunit of polymerase α |     |      |      |      |
| 180 kDa | 0 | 15 ± 1 | 56 ± 17 | 100 |
| 68 kDa | 0 | 20 ± 12 | 29 ± 6 | 100 |
| Relative specific activity of ATP | 0.8 | 1.0 |      |      |

*Times following subculturing of quiescent HF cells. Quiescent cells are represented as time = 0.

Specific activity is expressed as the ratio of [32P]ATP to total cellular ATP.

classical methods as described by Lawton et al. (1983). Quantitation by laser densitometry was performed and the results are presented in Fig. 4B. Examination of Fig. 4 demonstrates that there are only small differences in the amounts of the 180- and 68-kDa subunits between 0 and 24 h or between 12 and 24 h. The results from three separate experiments, including that of Fig. 4, are shown in Table II. It can be seen that there is certainly less than a 2-fold difference in the abundance of either subunit of DNA polymerase α. This would seem to indicate that a change in this parameter contributes little to the increase seen in both activity and phosphorylation. These findings suggest that the observed increases in activity and phosphorylation of DNA polymerase α as quiescent cell re-enter the cell cycle are not a consequence of a significant change in the abundance of the enzyme.

Steady-state Labeling of Polymerase α with [35S]Methionine—Since the above result is central to the accurate interpretation of the phosphorylation and activity data we employed [35S]methionine labeling of cells to support the results obtained with silver staining. This method has the advantage of requiring many fewer cells than silver staining. The total protein levels of the enzyme complex were determined by labeling quiescent and exponentially growing HF cells long-term with [35S]methionine as described under “Experimental Procedures.” Immunoprecipitates of [35S]methionine-labeled DNA polymerase α from extracts of quiescent and exponential cell populations were analyzed by SDS-PAGE and autoradiography. Quantitative analysis by laser densitometry was performed and the results are shown in Fig. 5. The results demonstrate that there is only a small degree of change in the amount of labeled 180-kDa subunit in exponentially growing HF cells (Fig. 5, lane 2) compared with quiescent cells (Fig. 5, lane 3). The analysis revealed less than a 2-fold increase in the extent of labeling of the 180-kDa polymerase α subunit in exponentially growing cells versus quiescent cells. Line 1 in Fig. 5 shows the results from immunoprecipitation using MOPC 21 control antibody. Clearly, a difference of less than 2-fold in the protein level of the enzyme still could not be responsible for the observed changes in activity and is not likely to be responsible for the observed increase in phosphorylation of DNA polymerase α.

DISCUSSION

The activity of DNA polymerase α, measured in cell lysates or in specific immunoprecipitates of human fibroblasts, is controlled in parallel with replicative DNA synthesis in whole

FIG. 4. Measurement of the protein levels of DNA polymerase α subunits by protein staining. 3 × 10⁶ cells/time point were harvested as described and immunoprecipitated with approximately 0.5–1 μg of SJK 132.20 anti-DNA polymerase α monoclonal antibody. The immunoprecipitates were analyzed by SDS-PAGE and the resultant gel, stained with silver, is shown in A. Lanes 1–3 show the immunoprecipitates from quiescent cells and cells subcultured and refed fresh medium and serum for 12 and 24 h, respectively. Lane 4 shows a control immunoprecipitation with SJK 132.20 of DNA polymerase α that was purified by classical methods as described by Lawton et al. (1983). The relative amounts of the 180- and 68-kDa subunits in lanes 1–3 were quantitated by laser densitometry, the results of which are shown in B. Line numbers 1–3 refer to the lanes in A. Protein molecular weight markers (Sigma) were myosin, 200 kDa, phosphorylase b, 97.4 kDa, and BSA, 68 kDa.
TABLE II
Quantitation of DNA polymerase α protein

| Subunit of polymerase α | % 24-h value |
|------------------------|--------------|
| 180 kDa                | 124 ± 46     |
| 68 kDa                 | 122 ± 45     |

* Time following subculturing of quiescent HF cells. Quiescent cells are represented as time = 0.

![Diagram of DNA polymerase α activity](image)

FIG. 5. Steady-state protein labeling of DNA polymerase α in quiescent and exponentially growing HF cells. Quiescent and exponentially growing HF cells (4 × 10⁶) were labeled for 24 h with [³⁵S]methionine at approximately 100 μCi/ml in methionine-free medium supplemented with 10% FBS and 25 μM non-radioactive methionine as described under "Experimental Procedures." Immunoprecipitation was performed using equal amounts of acid-precipitable radioactivity for the two cell populations. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography. The amount of radioactivity in the polymerase α 180-kDa band was quantitated by laser densitometry, the results of which are shown. Lines 1 and 2 show the immunoprecipitates from exponentially growing HF cells using either control mouse IgG₁ (line 1) or anti-DNA polymerase α antibody, SJK 132.20 (line 2). Line 3 is the result of specific immunoprecipitation of DNA polymerase α from quiescent HF cells. Pre-stained protein molecular weight markers were myosin (H chain), 214 kDa and phosphorylase b, 111 kDa.

Cells. Both processes are induced with similar kinetics following release of quiescent cells from a viable G₀ state by subculturing and refeeding of fresh medium and serum. We have measured a 60-fold change in the rate of DNA synthesis and an 10-fold change in the polymerase α activity measured in the same time frame. We have also shown that the increase in activity of DNA polymerase α is coincident with phosphorylation of the 180- and 68-kDa subunits. During the same period the quantity of DNA polymerase α protein changes less than 2-fold. These results suggest that a factor other than new enzyme synthesis, possibly phosphorylation, controls the activity of DNA polymerase α. Certainly, in this system, regulatory mechanisms that govern the progression of cells from G₀ through G₁ to S phase also influence the structure and activity of an enzyme complex central to chromosomal DNA synthesis.

Our observations are consistent with earlier findings demonstrating a link between polymerase α activity and cellular proliferation. Hubscher and co-workers (1977) showed a correlation between cell growth and DNA polymerase α activity in the developing rat brain. It was also demonstrated by Haraguchi et al. (1987) that both replicative DNA synthesis, measured by incorporation of [³H]thymidine, and polymerase α activity were decreased in macrophages treated with muramyl dipeptide or lipopolysaccharide, which causes the macrophages to undergo differentiation. These results imply a coordinated regulation of polymerase α activity and DNA replication. More specifically, two independent groups of investigators have shown a stimulation of DNA synthesis and DNA polymerase α (Otto et al., 1988) or DNA polymerase α/primase (Reiter et al., 1987) activities upon treatment of quiescent mouse 3T3 cells with serum or individual growth factors. Chiu and Baril (1975) found in HeLa cells synchronized by a double thymidine block that an increase in the activity of polymerase α in nuclear extracts was temporally related to the period of S phase. Similar results were obtained by Jackson and Cook (1986a, 1986b) who demonstrated the existence of a nucleoskeletal-associated DNA polymerase α which replicated intact chromatin at 85% of the rate in vivo but was present only in S phase cells. Kozu and colleagues (1986) demonstrated an approximately 5-fold elevation in the activity of DNA polymerase α/primase complex in S phase C3H2K or Ehrlich ascites tumor cells compared with serum-starved G₁ phase or aphidicolin-arrested G₂/S phase cells. Other examples also exist demonstrating a relationship between DNA polymerase α activity and S phase DNA synthesis (Ponder and Collins, 1985; Wood and Collins, 1986; Thommes et al., 1986). In HF cells there is also a parallel and significant change in DNA polymerase α activity and replicative DNA synthesis. Therefore, our work and that of others indicates that DNA polymerase α activity is coordinately regulated with the state of cellular proliferation, that is, during progression from the G₀ to S phases of the cell cycle. Our studies show that during the first 24 h of stimulation this increased activity is primarily due to activation of pre-existing polymerase α, although a small increase in synthesis may occur.

Using the HF cell system, we have revealed a correlation between the activity of DNA polymerase α and a structural modification of the enzyme itself. There is phosphorylation of two subunits of polymerase α which occurs at the same time as the increases seen in the enzymatic activity and replicative DNA synthesis measured in the whole cells. Phosphorylation of the 180- and 68-kDa protein subunits is not detectable in quiescent HF cells and is barely detectable 12 h following passage and refeeding. The extent of labeling with [³²P]phosphate increases approximately 10-fold between 12 and 24 h, coordinately with the activity of the enzyme. A causal relationship between the two processes is suggested in the literature by measurements of elevated activity of partially purified polymerase α following treatment with protein kinases and ATP in vitro (Danse et al., 1981; Sylvia et al., 1986; Krauss et al., 1987). Most relevant are the results of Krauss and colleagues (1987). They showed a 3-4-fold increase in enzymatic activity following treatment of polymerase α with partially purified protein kinase C and ATP. They measured a decrease in the $K_m$ for binding of the enzyme to DNA, and an increase in fidelity, as measured on synthetic templates. The activation of the enzyme was inhibited when...
neutralizing antibody directed against protein kinase C was included in the reactions. There is also a growing body of evidence suggesting that at least a portion of the cellular protein kinase C is translocated to the nucleus following mitogenic stimulation of certain cell types (Cambier et al., 1987; Chen et al., 1987; Fields et al., 1988).

In addition to the above results, Doxaldson and Gerner (1987) report the presence of a phosphorylated 220-kDa protein in immunoprecipitates from 32P-labeled Rat-l(tsLA241 of their data also reveals the presence of phosphorylated bands at 180 and 68 kDa (Donaldson and Gerner, 1987). They have also suggested that phosphorylation may be a regulatory mechanism for DNA polymerase activity. They demonstrated the elimination of polymerase activity in vitro following treatment with alkaline phosphatase conjugated to agaroese. The activity was restored after incubation with either 5 or 50 μM ATP but not with ATP[γ-S]. Overall, the above results and ours are consistent with a phosphorylation-dependent activation of DNA polymerase α, coincident with the shift from a quiescent to a proliferative state of cell growth.

We interpret the increased incorporation of labeled phosphate into the polymerase as reflecting an increase in the extent of phosphorylation of the enzyme, that is, a greater proportion of molecules phosphorylated and/or more phosphate/molecule if there is more than one phosphorylation site. However, it should be noted that changes in incorporation could, in theory, be indicating simply changes in the rate of turnover of the enzyme with no change in the amount. We consider this unlikely. Consistent with the results of MacNicol et al. (1987), there were only small differences in the steady-state abundance of either the 180- or the 68-kDa subunits as cells exited Go. This result was supported by measurements of the steady-state level of the enzyme in [35S]methionine-labeled cells. The small increase measured in [35S]methionine labeling of the 180-kDa subunit in proliferating cells compared with quiescent cells may represent some new synthesis of polymerase α protein which might occur as cells exit Go and prepare for a new round of DNA synthesis and cell division. Further, Bensch et al. (1982) demonstrated that DNA polymerase α protein was present in the nucleus of all cell cycle populations using KB and BeWo cells. These data suggest that a mechanism for regulating DNA polymerase α activity exists which is not a result of changes in polymerase α protein content or nuclear translocation.

It thus appears probable that there is a significant increase in the extent of phosphorylation of the enzyme as cells emerge from quiescence. We, therefore, propose that phosphorylation is likely to be the dominant mechanism by which DNA polymerase α is activated for participation in chromosomal DNA replication when cells leave quiescence and begin to proliferate.

The next question which needs to be examined is the identity of the protein kinase that phosphorylates the enzyme in whole cells. Experiments are currently underway to explore this issue.

Acknowledgments—We thank Dr. Peter Keng for assistance with the flow cytometry studies, Lee Harwell and Dr. Edith Lord for growing the hybridoma cells, SJK 132,20, which produce the anti-DNA polymerase α monoclonal antibody, Dr. Richard Panniers for assistance with the HPLC studies, and Drs. Alan Wolfman and Richard Panniers for helpful discussions.

REFERENCES
Adams, R. L. P. (1980) Laboratory Techniques—Biochemistry and Molecular Biology (Work, T. S., and Burdon, R. H., eds) Chapt. 11, pp. 162–180, Elsevier Scientific Publishing Co., Amsterdam.
Alama, A., Nicolini, A., Conte, P. F., and Drewinko, B. (1987) Cancer Res. 47, 1902–1906.
Bensch, K. G., Tanaka, S., Hu, S.-Z., Wang, T. S.-F., and Korn, D. (1983) J. Biol. Chem. 258, 8391–8396.
Bollum, F. J. (1975) Progr. Nucleic Acids Res. Mol. Biol. 15, 109–144.
Burnecke, W. N. (1981) Anal. Biochem. 126, 195–203.
Byrnes, J. R. (1985) Biochem. Biophys. Res. Commun. 132, 628–634.
Cambier, C. J., Newell, M. K., Justement, L. B., McGuire, J. C., Leach, K. L., and Chen, Z. Z. (1987) Nature 327, 629–632.
Chen, Z. Z., McGuire, J. C., Leach, K. L., and Cambier, C. J. (1987) J. Immunol. 138, 2345–2352.
Chiu, R. W., and Brand, E. F. (1975) J. Biol. Chem. 250, 7961–7975.
Conway, R. A., and Lehman, I. R. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2523–2527.
Danse, J. M., Egly, J. M., and Kemp, J. F. (1981) FEBS Lett. 124, 84–88.
Dell'Orco, R. T., Crissman, H. A., Steinkamp, J. A., and Kraemer, P. M. (1975) Exp. Cell Res. 92, 271–274.
Donaldson, R. W., and Gerner, E. W. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 759–763.
Fields, A. P., Pettit, G. R., and May, W. S. (1988) J. Biol. Chem. 263, 8253–8260.
Foster, K. A., and Collins, J. M. (1985) J. Biol. Chem. 260, 4229–4235.
Fry, M., and Loeb, L. A. (1986) in Animal Cell DNA Polymerases, pp. 1–60, CRC, Boca Raton, FL.
Haguchi, T., Nago, S., Tanaka, A., and Nagano, H. (1987) J. Leucocyte Biol. 41, 170–176.
Hirose, F., Yamamoto, S., Yamaguchi, M., and Matsukage, A. (1988) J. Biol. Chem. 263, 2925–2933.
Holmes, A. M., Cheristlundem, E., Bollum, F. J., and Chang, L. M. S. (1986) J. Biol. Chem. 261, 11924–11930.
Huberman, J. A. (1987) Cell 48, 7–8.
Jackson, D. A., and Cook, P. R. (1986) J. Mol. Biol. 192, 65–76.
Jackson, D. A., and Cook, P. R. (1986b) J. Mol. Biol. 192, 77–86.
John, N. J., and Firestone, G. L. (1986) BioTechniques 4, 404–406.
Kaguni, L., Akiyama, K., Tanaka, M., and Mizuta, K. (1987) J. Biol. Chem. 263, 2925–2933.
Kato, Y., Kodama, K., and Ishikawa, T. (1985) Exp. Cell Res. 161, 111–116.
Kniss, D. A., and Burry, R. W. (1988) Brain Res. 439, 281–288.
Kozu, T., Sato, T., and Yagura, T. (1986) Eur. J. Biochem. 157, 251–259.
Krauss, S. W., Moehly-Rosen, D., Koschland, D. E., Jr., and Linn, S. (1987) J. Biol. Chem. 262, 3432–3435.
Laemmli, U. K. (1970) Nature 227, 680–686.
Lawton, K. G., Wierowski, J. V., Schechter, S., Hilf, R., and Bambara, R. A. (1984) Biochemistry 23, 4294–4300.
MacNicol, A. M., Banks, G. R., and Cox, R. A. (1987) FEBS Lett. 221, 48–54.
Matsukage, A., Kitani, H., Yamaguchi, M., Kusakabe, M., Morita, T., and Koshiida, Y. (1986) Dev. Biol. Chem. 117, 226–232.
Mishell, B. S., and Shig, S. M. (1980) Selected Methods in Cellular Immunology, pp. 367–370, W. H. Freeman, San Francisco.
Naito, K., Skog, S., Tribukait, B., Andersson, L., and Hisamizu, H. (1987) Cell Tissue Kinet. 20, 447–457.
Nasheuer, H.-P., and Grosse, F. (1987) Biochemistry 26, 8458–8466.
Nasheuer, H.-P., and Grosse, F. (1988) J. Biol. Chem. 263, 3981–3985.
Niedbalski, W., Zwierschowski, L., and Wasilewska, L. D. (1986) Int. J. Biochem. 18, 637–643.
Otto, H., Frei, P., Hassig, M., and Hubscher, U. (1987) Nucleic Acids Res. 15, 4769–4807.
Penelsky, H. S. (1971) J. Biol. Chem. 252, 2981–2989.
Reiter, T., Fett, R., and Knippers, R. (1987) Eur. J. Biochem. 164, 59–63.
Rossini, M., Lin, C. J., and Besrega, R. (1976) J. Cell. Physiol. 88, 1–10.
Sejersten, T., Sumegi, J., and Ringertz, N. R. (1985) J. Cell. Physiol. 125, 465–470.
Spadari, S., and Weissbach, A. (1974) J. Mol. Biol. 86, 11–20.
Stein, G. H., Atkins, L., Beeson, M., and Gordon, L. (1986) Exp. Cell Res. 163, 255–260.
Sylvia, V. L., Joe, C. O., Norman, J. O., Curtin, G. M., and Busbee, D. L. (1986) Biochem. Biophys. Res. Commun. 135, 880–885.
Regulation of DNA Polymerase α

Talbot, M. D., and Jasani, M. K. (1985) J. Immunol. Methods 84, 165–175
Tanaka, S., Hu, S.-Z., Wang, T. S.-F., and Korn, D. (1982) J. Biol. Chem. 257, 8386–8390
Thömmes, P., Reiter, T., and Knippers, R. (1986) Biochemistry 25, 1308–1314
Vishwanatha, J. K., Coughlin, S. A., Wesolowski-Owen, M., and Baril, E. F. (1986) J. Biol. Chem. 261, 6619–6628
Wahl, A. F., Kowalski, S. P., Harwell, L. W., Lord, E. M., and Bambara, R. A. (1984) Biochemistry 23, 1895–1899
Wahl, A. F., Crute, J. J., Sabatino, R. D., Bodner, J. B., Marraccino, R. L., Harwell, L. W., Lord, E. M., and Bambara, R. A. (1986) Biochemistry 25, 7821–7827
Wahl, A. F., Geis, A. M., Spain, B. H., Wong, S. W., Korn, D., and Wang, T. S.-F. (1988) Mol. Cell. Biol. 8, 5016–5025
Wang, T. S.-F., Hu, S.-Z., and Korn, D. (1984) J. Biol. Chem. 259, 1854–1865
Weisbach, A. (1979) Arch. Biochem. Biophys. 198, 386–396
Wong, S. W., Paborsky, L. R., Fisher, P. A., Wang, T. S.-F., and Korn, D. (1986) J. Biol. Chem. 261, 7958–7968
Wood, S. H., and Collins, J. M. (1986) J. Biol. Chem. 261, 7119–7122
Yagura, T., Kozu, T., Seno, T., and Tanaka, S. (1987) Biochemistry 26, 7749–7754