Synthesis of DNA Polymerase α Analyzed by Immunoprecipitation from Synchronously Proliferating Cells†

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ABSTRACT: Synchronously proliferating TC7 monkey and 3T3 mouse cells were pulse labeled with [^{35}S]-methionine. Radioactively labeled DNA polymerase α was immunoprecipitated with polymerase-specific monoclonal antibodies. The precipitated polypeptides were identified by gel electrophoresis and fluorography. The increase in DNA polymerase α activity during S phase was accompanied by an increased synthesis of the enzyme. Some DNA polymerase α was synthesized in growth-arrested TC7 cells whereas the synthesis of the large polymerase subunit in 3T3 cells was strictly coupled to the replicative phase of the cell cycle. We also found that DNA polymerase α was more prone to proteolysis in TC7 cells than in 3T3 cells. In 3T3 cells, a polymerase subunit with an apparent molecular weight of 186 000 was observed; this subunit was most probably associated with two smaller subunits of M_r 74 000 and 52 000. Synthesis of these three polymerase-associated polypeptides appeared to be regulated differently.

Replicative DNA synthesis in eukaryotic cells is most likely catalyzed by DNA polymerase α. The evidence for this proposition is circumstantial, but nevertheless compelling [reviewed by Fry (1983) and Hübscher (1983)]. Many authors (Table I) have reported that the activity of DNA polymerase α increases during the DNA replication phase (S phase) of the cell cycle while the activities of DNA polymerases β and γ do not change significantly.

It has not been clear whether the observed increase in activity is due to modification of the enzyme or whether new enzyme is synthesized. Previous work with cultured HeLa cells and regenerating rat liver cells has shown that the increase in DNA polymerase α activity at the beginning of the S phase was largely suppressed by cycloheximide, an inhibitor of protein synthesis, and by actinomycin D, an inhibitor of transcription (Chiu & Baril, 1975; Davis et al., 1976). These data suggest, but do not necessarily prove, that the increase in enzymatic activity is due to de novo synthesis of DNA polymerase α. Alternative explanations, such as the synthesis of an activator, are equally likely. A reinvestigation of this problem was clearly necessary. The availability of monoclonal antibodies against DNA polymerase α (Tanaka et al., 1982; Masaki et al., 1982; Karawya et al., 1984) suggested an approach involving the immunoprecipitation of pulse-labeled DNA polymerase α polypeptide chains. This technique provides information about the rate of enzyme synthesis and the structure of the enzyme at various stages during the prereplicative and replicative phases of the cell cycle.

In this paper, we report studies with two mammalian cell lines, the African green monkey kidney cell line TC7 and mouse fibroblast 3T3 cells. Synchronized cells were pulse labeled with [^{35}S]methionine; the proteins were extracted and immunoprecipitated with the polymerase-specific monoclonal antibodies of Tanaka et al. (1982). We found that the increase in DNA polymerase α activity during S phase (Table I) was accompanied by an increased synthesis of the enzyme.

Materials and Methods

Synchronization and Labeling of Cells. The African green monkey kidney cell line TC7 (Gluzman et al., 1977; Hiscott & Defendi, 1979) and the mouse fibroblast line 3T3 (Aaronson & Todaro, 1968) were grown as monolayers in Dulbecco's modified Eagle's (DME) medium containing 10% newborn calf serum.

For synchronization, 2×10^5 3T3 cells were plated per 90-mm plate and grown under standard conditions for 4 days at 37 °C. DME medium (plus serum) was then replaced by serum-free medium. After 18–24 h, 10% calf serum was added. Progression through the cell cycle was monitored by [^{3}H]thymidine incorporation (see below and Figure 1A). With TC7, 10^6 cells were plated per 90-mm dish and incubated at 37 °C for about 10 days without changing the medium. Cells became confluent after 3–4 days, and DNA synthesis ceased after 6–7 days. After addition of fresh medium (plus serum), the cells went through at least one S phase (Figure 1B).

The rate of DNA synthesis was determined by labeling with 1 μCi/mL [^{3}H]thymidine (46 Ci/mmol; Amersham) for 30 min. Incorporation was stopped by removal of medium and washing with ice-cold phosphate-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4). The cells were scraped off the plate, washed once in ice-cold PBS, and lysed in 10 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), pH 7.5, containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1% sodium dodecyl sulfate. Nucleic acids were precipitated in 5% trichloroacetic acid, collected, washed on glass fiber filters, and counted.

For [^{35}S]methionine labeling, DME medium was replaced by 4 mL of methionine-free medium containing 2% dialyzed newborn calf serum and 20 μCi/mL [^{35}S]methionine (800 Ci/nmol; Amersham). After 2 h, the cells were washed with ice-cold PBS and used to prepare cell extracts.

Preparation of Cell Extracts. About 3×10^7 cells were resuspended in 1 mL of buffer A [300 mM KCl, 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 7.8, 1 mM dithioerythritol, 0.1 mM EDTA, 10 mM $Na_2S_2O_5$, 2 μg/mL pepstatin A, 2 μg/mL aprotinin, 0.5 mM phenylmethanesulfonyl fluoride, 0.5% Triton X-100, and 20% glycerol]. In some experiments, buffer A contained 100 mM Tris-HCl (pH 9) instead of Hepes (see legends to Figures 4–6). After 30 min on ice, insoluble material was removed by centrifugation at 10000*g* for 15 min. The pellet was essentially free of DNA polymerase activity. The supernatants

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Table I: Increase of DNA Polymerase α Activity during S Phase of Synchronously Proliferating Cells

cell type	method of synchronization	DNA polymerase α act. increase in S-phase cells	ref
permanently cultured cells			
HeLa	double thymidine block	2–3-fold; 7–10-fold	Spadari & Weissbach (1974); Baril et al. (1975)
	aphidicolin block	2-fold	Pedrali-Noy et al. (1980)
BHK/21-C3	serum depletion	4-fold	Craig et al. (1975)
CV1	serum depletion	2–4-fold	Riva et al. (1978); Tanabe et al. (1984)
mouse L cells	dilution of suspension culture	5–10-fold	Chang et al. (1973)
primary cultures of lymphocytes	stimulation with Con A or with phytohemagglutinin	5–20-fold	Coleman et al. (1974); Bertazzoni et al. (1976); Sons et al. (1976); Spadari et al. (1978)
liver cells in vivo	partial hepatectomy	6–10-fold	Chang & Bollum (1972); Baril et al. (1973); Davis et al. (1976)

were either used directly or stored at −70 °C before use.

DNA polymerase α activity was determined in 1–5-μL aliquots as described by König et al. (1983) using DNase I treated ("activated") calf thymus DNA as primer-template and [α-^{32}P]dATP as the labeled substrate. One enzyme unit corresponds to an activity incorporating 1 nmol of deoxyribonucleotides in 1 h at 37 °C.

Protein concentrations were determined according to Schaffner and Weissmann (1973).

Antibodies and Immunoprecipitation. The hybridoma cell lines STK1, SJK 132–20, SJK 237–71, and SJK 287–38, which produce antibodies against DNA polymerase α from human KB cells (Tanaka et al., 1982), were obtained through the American Type Culture Collection. Antibodies were purified from culture supernatants by protein A–Sepharose chromatography as suggested by the supplier (Pharmacia). The final immunoglobulin concentrations were greater than 1 mg/mL. The monoclonal antibodies SJK 237–71 and STK-1 (Tanaka et al., 1982), which bind to DNA polymerase α without disturbing its polymerizing activity, were used to assay activity in the immunopellet. In all other experiments, we used the strongly binding, inhibiting antibodies SJK 132–20 and SJK 287–38, either as separate components or in combination.

Since our antibody preparations also contained immunoglobulins which were present in the fetal calf serum required for the cultivation of hybridoma cells, we routinely used immunoglobulins prepared from fetal calf serum as controls in the experiments reported below. Other controls were done with the monoclonal antibody pAB 122 against the transformation-specific protein p53 (Gurney et al., 1980) and gave essentially the same results.

The monoclonal antibodies against human DNA polymerase α reacted well with TC7 and 3T3 polymerase α. For example, 5 μg of SJK 237–71 antibody precipitated all of the active polymerase α in an extract from 10^6 TC7 cells. The STK-1 antibody was less effective under these conditions, precipitating only about 80% of the active TC7 enzyme. About 90% of the polymerase activity in an immunoprecipitate could be inhibited by 20 μg/mL aphidicolin, a specific inhibitor of DNA polymerase α (see Figure 1).

The inhibiting antibodies SJK 132–20 and 237–38 were tested by adding increasing amounts of antibody to TC7, 3T3, and (human) HeLa cell extracts. These antibodies inhibited DNA polymerase α activity in extracts from all three cell lines. For example, 0.2 μg of SJK 132–20 inhibited 60–65% of the activity from 5×10^4 HeLa or TC7 cells and about 50% of the activity from 5×10^4 3T3 cells. Similar data were obtained for SJK 237–38 antibody.

Forty micrograms of a mixture of inhibiting antibodies was used to immunoprecipitate DNA polymerase α from extracts of 5×10^6 [^{35}S]methionine-labeled cells. After 60 min on ice, we added 60 μL of a 10% suspension of *Staphylococcus aureus* (strain Cowan I; Kessler, 1975) that had been fixed with formaldehyde and then thoroughly washed. [Since these monoclonal antibodies belong to the $IgG_{\gamma 1}$ subclass (Tanaka et al., 1982), which binds poorly to protein A at pH values below 8.0, we originally used rabbit anti-mouse immunoglobulins, bound to *Staphylococcus aureus*, as an absorbant for the immunocomplexes. Later, we prepared the protein extracts and performed the immunoprecipitations at pH 9, omitting the second antibody.] The aggregates were precipitated and washed 3 times with NET buffer (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 0.05% Nonidet P-40, and 0.1% gelatine) (Smith et al., 1978) containing 0.5 M LiCl and then washed 3 times with NET buffer without LiCl. Precipitates were resuspended in 30 μL of sample buffer and electrophoresed on a 7.5% polyacrylamide slab gel according to Laemmli (1970). The gels were stained with Coomassie Blue, treated for 30 min in Amplify (Amersham), dried, and exposed to SB2 Kodak film for 3–20 days at −70 °C. Molecular weight markers were myosin (M_r 205 000), *Escherichia coli* RNA polymerase (M_r 160 000, 150 000, 70 000, and 35 000), β-galactosidase (M_r 116 000), phosphorylase B (M_r 94 000), and bovine serum albumin (M_r 67 000). In some experiments, radioactive polypeptides were cut out of the gel, dissolved, and counted in Lipoluma-Lumasolve (Baker).

To test enzyme activity, the immunoprecipitated DNA polymerase α was washed 3 times with polymerase assay buffer (without deoxyribonucleoside triphosphates) and finally resuspended in the reaction buffer of König et al. (1983).

Results

DNA Polymerase α Activity in Serum-Stimulated Cells. Growth-arrested TC7 monkey cells and 3T3 mouse fibroblasts were induced for DNA synthesis by addition of fresh medium containing newborn calf serum. DNA synthesis, monitored by [^{3}H]thymidine incorporation, was first detected 8–12 h after serum addition. Maximal rates of DNA synthesis were reached after 16–17 h in TC7 cells and after 24 h in 3T3 cells. The rate of DNA synthesis then declined rapidly in TC7 cells while 3T3 cells continued to incorporate [^{3}H]thymidine, albeit at a reduced rate (Figure 1).

In both cell lines, we observed that DNA polymerase α activity increased at the beginning of S phase and reached a maximum at times of highest [^{3}H]thymidine incorporation. The polymerase activity remained high even when thymidine incorporation returned to low levels. In several independent experiments, DNA polymerase α activity increased by a factor of 2–3.

The DNA polymerase assay used does not unambiguously differentiate between the three different DNA polymerase species of the cell. However, two observations strongly suggest that we were measuring the increase in polymerase α activity.

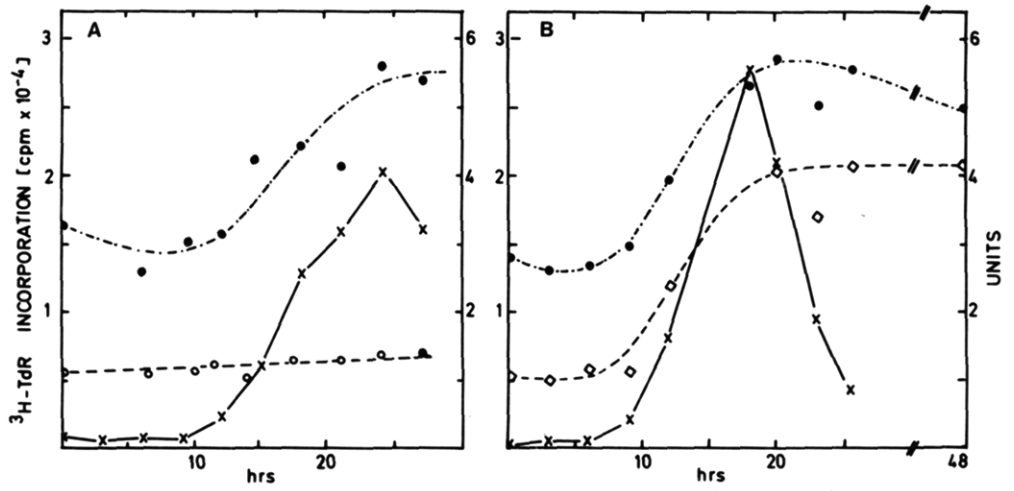

FIGURE 1: DNA polymerase α activity in serum-activated cells. Growth-arrested cells were activated by addition of fresh serum as described under Materials and Methods. At the times indicated, DNA synthesis was measured by pulse labeling with [^{3}H]thymidine. The results are given in ^{3}H cpm incorporated per 10^7 cells (×). DNA polymerase α activity was measured in protein extracts (König et al., 1983). The results are expressed in enzyme units per milligram of protein (●). In panel A, the enzyme activity in the presence of aphidicolin (20 μg/mL) is shown (○). In panel B, the DNA polymerase α activity tests in immunoprecipitates with the noninhibiting monoclonal antibodies SJK 237-71 and STK 1 are shown (◇). (A) 3T3 cells, (B) TC7 cells.

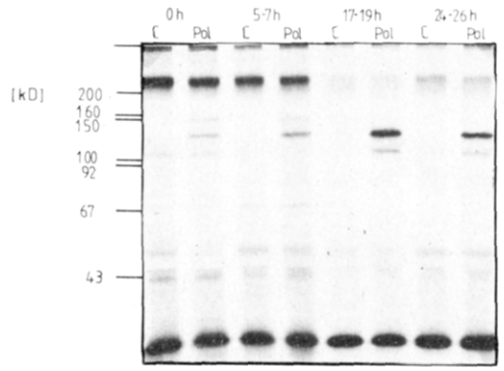

FIGURE 2: Fluorograms of the electrophoresed immunoprecipitates from TC7 cells. Cells were pulse labeled for 2 h with [^{35}S]methionine. The labeling intervals are indicated at the top of the figure. The lanes marked 0 h contain samples labeled for 2 h before addition of serum. C, lanes contain immunoprecipitates with unspecific antibodies; Pol, lanes contain immunoprecipitates from a mixture of the polymerase-specific monoclonal antibodies SJK 132-20 and SJK 287-38.

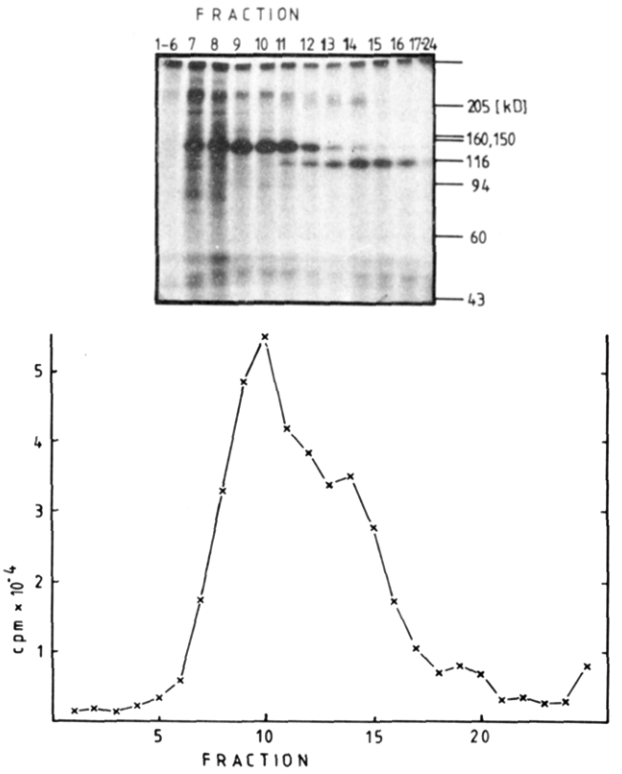

FIGURE 3: Zone velocity sedimentation of TC7 DNA polymerase α. A protein extract (0.2 mL) from 10^7 exponentially growing, [^{35}S]-methionine-labeled TC7 cells was sedimented at 0 °C for 35 h through a 15–35% glycerol gradient (in buffer A, pH 7.8) using the Beckman SW55 rotor at 45 000 rpm. The activity of DNA polymerase α was determined in 5-μL aliquots as described (König et al., 1983). The remainder of each fraction was immunoprecipitated by using a mixture of the monoclonal antibodies SJK 287-38 and SJK 132-20. The sedimentation coefficients of the polymerase peaks in fractions 10 and 14 were determined to be 8.8 and 7.7 S, respectively, using catalase (11.2 S), immunoglobulin G (7.2 S), and hemoglobin (4.3 S) as sedimentation markers (centrifuged under identical conditions in a parallel tube).

First, aphidicolin, a highly specific inhibitor of DNA polymerase α [reviewed by Huberman (1981)], completely blocked that fraction of total polymerase activity which increased in S-phase cells (Figure 1A). Second, the polymerase activity precipitated with noninhibiting monoclonal antibodies SKJ 237-71 and STK-1 increased as the cell cycle progressed (Figure 1B).

Biosynthesis of DNA Polymerase α in TC7 Cells. TC7 cells were labeled with [^{35}S]methionine for 2 h at various times after addition of serum to growth-arrested cells. We detected some labeled polypeptides which were specifically precipitated by the monoclonal antibodies (Figure 2). At early times after addition of serum, we detected labeled proteins of apparent molecular weight values of approximately 150000 and 125000. At later times, the M_r 150000 polypeptide disappeared. Instead, a M_r 105000 polypeptide appeared. The M_r 125000 polypeptide remained the most prominent band on the gel. When extracts, containing labeled proteins, were centrifuged through glycerol gradients, we found that the immunoprecipitable proteins cosedimented with DNA polymerase α activities (Figure 3). This observation strongly supports our conclusion that the immunoprecipitable polypeptides are related to DNA polymerase α. [The biphasic distribution of polymerase α activity during zone sedimentation has been described and discussed by König et al. (1983). Further explanations for this observation will be given below.]

To determine the relative rate of polymerase α synthesis, we cut out the specific gel bands (Figure 2) and determined their radioactivity by scintillation counting. In several inde-

Table II: Incorporation of [^{35}S]Methionine into Immunoprecipitable DNA Polymerase α (TC7 Cells)

expt	time (h) after addn of serum to resting TC7 cells[a]	total radioact. (cpm × 10^{-7})[b]	radioact. in DNA polymerase α (cpm)[c]	rel biosynthesis rates[d]
1	−2 to 0	1.36	440	1.00
	5 to 7	1.48	563	1.17
	17 to 19	1.84	1038	1.74
	24 to 26	2.38	725	0.93
2	−3 to 0	1.75	280	1.0
	8 to 11	2.53	624	1.54
	16 to 19	2.43	875	2.25

[a] Experiment 1 is described in Figure 2. Experiment 2 was performed as experiment 1, except that the labeling times were 3 h. [b] Before immunoprecipitation, 10-μL aliquots were removed from the protein extracts to determine the total [^{35}S]methionine incorporation into acid-precipitable proteins. [c] The bands of specifically precipitated proteins were cut out of the gel, dissolved in Lumasolve (Baker), and counted. The sum of the radioactivities is given when more than one specific protein was detected by fluorography (see Figure 2 and text). [d] The ratios of the radioactivity in immunoprecipitated polymerase bands over total radioactivity were determined. That ratio before serum additon is given as 1.00. Ratios obtained for samples taken at later times are divided by the ratio obtained at zero time.

pendent experiments, we found that the total radioactivity in the polymerase bands increased by a factor of 2.4 after stimulation of quiescent cells with serum. During the same time, the overall rate of [^{35}S]methionine incorporation increased by a factor of 1.6, demonstrating that the increase in polymerase α synthesis exceeded the average increase for protein synthesis in serum-activated TC7 cells (Table II). We therefore propose that the higher biosynthetic rates account for the increase in enzymatic activities described here (Figure 1) and by others (Table I).

The polymerase bands seen in the immunoprecipitates from TC7 cells remained essentially unchanged when the 2-h [^{35}S]methionine pulse was followed by a 6-h chase with an excess of unlabeled methionine. After a 24-h chase, the immunoprecipitated polypeptides still had two-thirds of the radioactivity incorporated during the pulse (data not shown), suggesting that the half-life of the enzyme in TC7 cells must be longer than 24 h. This result explains why polymerase activity remained high even after completion of the S phase (Figure 1), while the synthesis of polymerase decreased (Table II).

The disappearance of the M_r 150 000 polypeptide may be due to a higher proteolytic activity at later stages of the cell cycle that would lead to the degradation of the large polypeptide during its preparation. Schwyzer et al. (1980) have previously observed that proteolytic cleavage of the protein coded for by gene A of simian virus 40 can be largely suppressed when TC7 cells are extracted at pH 9 instead of pH 7. Accordingly, we prepared protein extracts from exponentially growing TC7 cells at pH 7.8 and 9 and compared the immunoprecipitates. Only one polypeptide of M_r 150 000 was detected when the preparation was carried out at pH 9, whereas two polypeptides of M_r 125 000 and 105 000 were seen when the experiment was performed at pH 7.8 (Figure 4). This result indicates that the M_r 125 000 and 105 000 polypeptides are probably products of proteolytic degradation. When the experiment of Figure 2 was repeated at pH 9, we found only the polypeptide of M_r 150 000 (not shown). The radioactivity in this band increased with time after serum addition essentially as shown in Figure 2 and Table II.

Purified preparations of DNA polymerase α from various sources contain at least one large subunit of molecular weight values greater than 100 000 and two or three smaller subunits

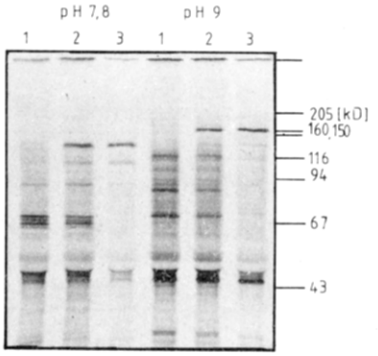

FIGURE 4: Uncontrolled proteolysis of DNA polymerase α in extracts from TC7 cells. Exponentially growing TC7 cells were labeled for 2 h with [^{35}S]methionine, harvested, and divided into two parts. Proteins were extracted from one part in buffer A, pH 7.8, and from the second part in buffer A, adjusted to pH 9.0. Immunoprecipitations were performed at the respective pH values. Lanes 1, controls using immunoglobulins from fetal calf serum. Lanes 2, immunoprecipitates with polymerase-specific monoclonal antibodies SJK 287-38 and SJK 132-20, washed with NET buffer without LiCl. Lanes 3, immunoprecipitates with polymerase-specific monoclonal antibodies, washed under standard conditions (see Materials and Methods).

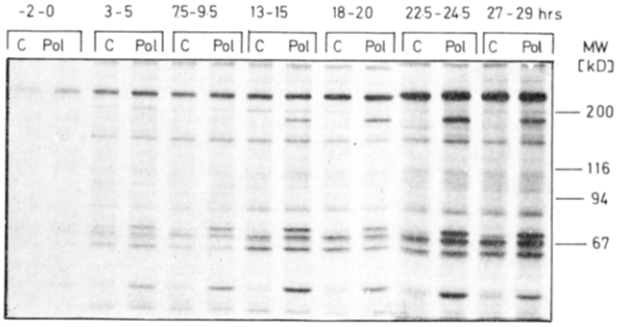

FIGURE 5: Biosynthesis of DNA polymerase α in 3T3 cells. The labeling periods are given at the top of the figure. C, control immunoprecipitations, using antibodies from fetal calf serum; Pol, immunoprecipitations using the polymerase-specific monoclonal antibodies SJK 287-38 and SJK 132-20. Molecular weight markers (see Materials and Methods) are indicated at the right-hand margin.

[summarized by Hübscher (1983)]. However, in all our experiments with TC7 cells, we only observed proteins with apparent molecular weight values of 150 000, 125 000, and 105 000 and never detected the smaller polymerase subunits. Although the epitopes recognized by the antibodies used in our experiments are most likely located on the large polymerase subunit (Tanaka et al., 1982; Wang et al., 1984), we reasoned that the small subunits should be present in immunocomplexes since the polymerase quaternary structure appears to be stable during the long biochemical procedure required for purification. However, it could not be excluded that the rather stringent washing procedure used to obtain immunoprecipitates may cause a dissociation of the quaternary structure. For this reason, we performed an experiment omitting LiCl from the NET wash buffer. As shown in Figure 4, the immunoprecipitates thus obtained contained more nonspecific radioactive proteins but no additional specific bands. Similar results were obtained when the detergent Nonidet P-40 was also omitted from the washing buffer (not shown).

Biosynthesis of DNA Polymerase α in Serum-Stimulated 3T3 Cells. Serum-stimulated 3T3 cells were pulse labeled with [^{35}S]methionine, and protein extracts were immunoprecipitated with the SJK 132–20 and SJK 287–38 antibodies. An analysis of the immunoprecipitates is shown in Figure 5.

From extracts prepared more than 15 h after serum addition, we specifically immunoprecipitated *three* polypeptides

Table III: Synthesis of Polypeptides Specifically Immunoprecipitated from Extracts of [^{35}S]Methionine-Labeled 3T3 Cells

time (h) after serum addition to growth-arrested 3T3 cells	total radioact. (cpm × 10^{-7})[a]	radioact. recovered in immunoprecipitated polypeptides (cpm)[b]		
		M_r 186 000	M_r 74 000	M_r 52 000
−2 to 0	0.29	nd[c]	nd	40
3–5	0.94	nd	112	129
7.5–9.5	1.14	15	137	145
13–15	1.17	96	235	325
18–20	1.67	220	187	212
22.5–24.5	2.05	470	375	406
27–29	2.81	415	362	313

[a] Acid-precipitable radioactivity was determined in aliquots from the protein extracts used for immunoprecipitation. The values given in the table are the total radioactivity in these extracts. [b] Specifically immunoprecipitated proteins, identified as bands in the fluorogram of Figure 5, were excised from the gel, dissolved, and counted. Backgrounds were subtracted. [c] nd, not detectable.

of apparent M_r 186 000, 74 000, and 52 000. The radioactivity in these bands increased with time after serum addition (Table III), indicating that the increase in enzymatic activity (Figure 1) was largely due to a higher rate of biosynthesis.

The protein extracts were prepared at pH 9 to minimize proteolysis (Figure 4). With extraction buffers of pH 7.8, drastically different results were obtained: only one large polypeptide with an apparent molecular weight value of approximately 160 000 appeared; the smaller polypeptides (M_r 74 000 and 52 000) were not detected (data not shown). This observation suggests that the M_r 160 000 subunit is generated by proteolysis of the M_r 186 000 subunit and, furthermore, that during immunoprecipitation the small subunits remain stably associated with the M_r 186 000 protein but not with the M_r 160 000 protein.

The fluorogram of Figure 5 was quantitated by densitometry (Figure 6). We found that the radioactivity in the large subunit (M_r 186 000) increased by a factor of more than 10 during the course of the experiment. Increases of the biosynthesis of the M_r 74 000 and 52 000 proteins were less pronounced. A comparison of the relative biosynthesis rates of the three subunits (Table III; Figure 6) suggests that the synthesis of the large polymerase subunit is more tightly coupled to the S phase of the cell cycle than is the synthesis of the two smaller subunits.

Discussion

According to previous observations (Table I), the activity of DNA polymerase α increases when animal cells enter the replication phase of the cell cycle. The results presented in this paper suggest that this increase is due to an elevated rate of polymerase synthesis, which is highest when DNA synthesis reaches its maximum, and which declines again in postreplicative phases. However, the *activity* of DNA polymerase α remains high in postreplicative cells, suggesting that polymerase, once formed, is relatively stable at least in the two cell lines studied. Pulse–chase experiments indicate that it has a half-life of more than 24 h.

Following nuclear breakdown during mitosis, DNA polymerase α is probably distributed to the two daughter cells (Bensch et al., 1982). De novo enzyme biosynthesis may then be required to provide these cells with the full complement of enzymatic activity necessary for genome replication. This may explain why the increase in enzymatic activity never exceeds a factor of 2–3.

The mechanism that regulates the synthesis of DNA polymerase α is unknown. It does not depend on the level of *active* polymerase, since aphidicolin in concentrations sufficient to block most ongoing DNA synthesis in vivo has no effect on the rate of [^{35}S]methionine incorporation into polymerase α (unpublished observation). Enzyme synthesis is probably

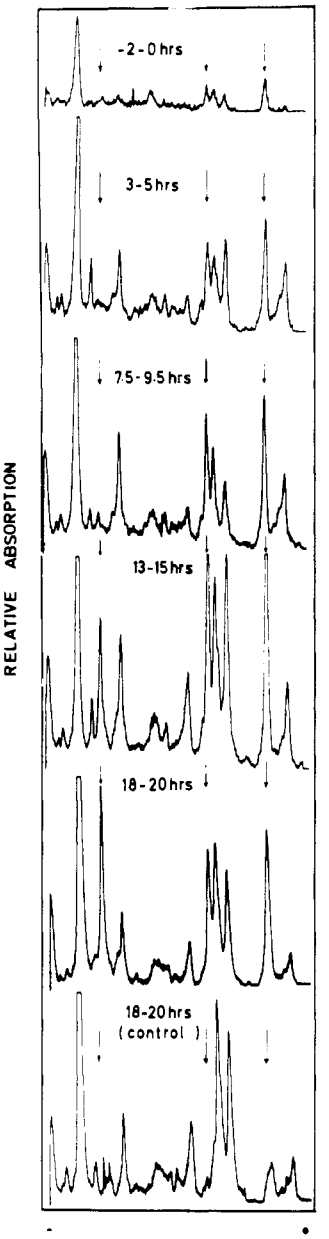

FIGURE 6: Synthesis of immunoprecipitated polypeptides. The fluorogram of Figure 5 was scanned by laser densitometry. We show the results obtained with immunoprecipitated samples prepared up to 20 h after stimulation of growth-arrested 3T3 cells with serum. The control panel (bottom) gives the results obtained with antibodies from fetal calf serum. The arrows indicate the three specifically immunoprecipitated polypeptides. Note that the peak of the M_r 186 000 protein (left arrow) became detectable at the beginning of S phase (13–15 h; Figure 1A) whereas the M_r 74 000 protein (middle arrow) and the M_r 52 000 protein (right arrow) already appeared in prereplicative phases of the cell cycle.

also not dependent on active DNA replication, since hydroxyurea, a potent inhibitor of in vivo DNA synthesis, does not block the increase in polymerase activity during the S phase (Chiu & Baril, 1975).

Growth-arrested TC7 cells continue to synthesize DNA polymerase α at low rates (Figure 2), whereas [^{35}S]methionine incorporation into the large polymerase α subunit was not detectable in resting 3T3 cells (Figure 5). Regulation of DNA polymerase α expression may be related to the transformed phenotype of the cell. No DNA polymerase α was detected by immunocytochemical means in resting normal diploid human fibroblasts (Bensch et al., 1982).

Our data also contribute to the ongoing debate on the subunit structure of DNA polymerase α. In pH 9 buffers, in which proteolysis is reduced, we specifically immunoprecipitated a polypeptide of M_r 150000 from TC7 cells. At pH 7.8, this polypeptide was apparently degraded into products with molecular weight values of 125000 and 105000 (Figure 4).

In extracts from 3T3 cells, we detected a M_r 160000 protein at pH 7.8. At pH 9, however, a large protein with an apparent molecular weight value of 186000 could be immunoprecipitated. In addition, at pH 9, two smaller subunits with molecular weight values around 74000 and 52000 were seen (Figure 5). This observation suggests that the M_r 186000 protein can be cleaved to give a fragment of M_r 160000 and that the smaller polymerase subunits remain stably associated only with the undegraded large subunit. Previous work with DNA polymerase α from *Drosophila melanogaster* (Sauer & Lehman, 1982; Kaguni et al., 1983a) and calf thymus (Masaki et al., 1984) has also shown that a subunit with an approximate molecular weight value of 180000 was converted into a smaller polypeptide. If TC7 extracts possess an intrinsically high protease activity, our inability to detect the M_r 180000 subunit in these cells is understandable.

The monoclonal antibodies SJK 132-20 and SJK 287-38 recognize epitopes on the large polymerase subunit (Tanaka et al., 1982; Wang et al., 1984). Coprecipitation of two additional proteins indicates a close association of these proteins in a large quaternary complex. Why are these complexes preserved only when the large subunit has an apparent molecular weight value of 186000? It is possible that proteolytic cleavage of the large polypeptide removes or destroys the contact regions between the three subunits. It is also possible that proteolysis destabilizes protein–protein interactions, resulting in the dissociation of the enzyme's quaternary structure upon immunocomplex formation. It has been shown (Stahl et al., 1985) that monoclonal antibodies of defined specificity occasionally cause a disruption of tight protein–protein contacts.

In 3T3 cells, all three detectable polymerase subunits are synthesized at elevated rates during the replicative phase of the cell cycle. However, whereas significant synthesis of the large subunit occurs only during the replication phase, incorporation of [^{35}S]methionine into the M_r 74000 and 52000 subunits could be observed relatively soon after addition of serum to arrested cells. Further work will be necessary to elucidate the function of these small subunits. They may be required for primer strand synthesis (Kaguni et al., 1983b) or for the processivity of the enzyme (Wierowski et al., 1983; Riedel et al., 1984).

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Forms of the Chemotactic Adenosine 3′,5′-Cyclic Phosphate Receptor in Isolated *Dictyostelium discoideum* Membranes and Interconversions Induced by Guanine Nucleotides

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ABSTRACT: Aggregating *Dictyostelium discoideum* cells possess receptors for the chemoattractant cAMP on their cell surface. Membranes enriched in these receptors were isolated. Kinetic studies indicated the same receptor heterogeneity in membranes as found for intact cells [van Haastert, P. J. M., & De Wit, R. J. W. (1984) *J. Biol. Chem. 259*, 13321–13328]. Dissociation kinetics revealed at least three receptor forms: one form, called SS, with $k_{-1} = 0.9 \times 10^{-3}$ s^{-1} and $K_D = 6.5$ nM; one form, called S, with $k_{-1} = 1.3 \times 10^{-2}$ s^{-1} and $K_D = \sim 6$ nM; and one or more forms, called F, with $k_{-1} > 0.1$ s^{-1}. The contribution of the SS form to the dissociation process was lower in the presence of millimolar concentrations of cAMP compared to dissociation induced by dilution only. Guanosine di- and triphosphates decreased the affinity of membranes for cAMP by increasing the dissociation rate of the cAMP–receptor complex. This was shown to result from a reduction in the number of sites of the slowly dissociating, high-affinity receptor form SS and probably also the high-affinity form S. Because the total number of cAMP binding sites was not changed by guanine nucleotides, it is inferred that the SS and S receptor forms are converted to other more rapidly dissociating receptor forms with lower affinities than SS and S. We propose that cAMP receptors in *Dictyostelium* membranes interact with G protein which binds guanosine di- and triphosphates. The different complexes between receptor and occupied or unoccupied G protein explain the different receptor forms and their interconversions.

In the course of a developmental program, induced by starvation, cells of the cellular slime mould *Dictyostelium discoideum* become sensitive to extracellular cAMP. Extracellular cAMP is used as a chemoattractant which guides the solitary cells to aggregation sites (Devreotes, 1982). Here, a fruiting body is formed. Furthermore, extracellular cAMP also stimulates cell differentiation (Sussman, 1982; Schaap & van Driel, 1985). Extracellular cAMP is produced by cells during cell aggregation, by means of a signal relay mechanism in which cell-surface receptors specific for cAMP are functionally coupled to adenylate cyclase. Receptor occupation results in a rapid, 10-fold activation of adenylate cyclase. Subsequently, within a few minutes, the enzyme is inactivated by an adaptation process (Devreotes, 1982).

The cAMP receptor–adenylate cyclase system in *Dictyostelium discoideum* is used as a model system for signal transduction in lower eukaryotes. Receptor binding studies with intact cells have demonstrated the occurrence of site heterogeneity (multiple receptor sites with different kinetic constants), ligand-dependent interconversion of binding sites (cooperativity) (van Haastert & De Wit, 1984; van Haastert, 1985), and ligand-induced decrease of the number of binding sites (down-regulation) (Klein & Juliani, 1977). Two fast dissociating types of cAMP binding sites (called H and L; K_d's of 60 and 450 nM, respectively) and one slowly dissociating site (called S, $K_D = 12.5$ nM) have been identified (van Haastert & De Wit, 1984). Studies with intact cells are limited, however, by the fact that the cAMP that is produced

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