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DNA Polymerase Activities Induced by Polyoma Virus Infection of 3T3 Mouse Fibroblasts

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Resting mouse 3T3 fibroblasts were stimulated to synthesize DNA either by infection with polyoma virus or by injection of fresh serum. Changes in the levels of DNA polymerase (α-, β-, and γ-enzymes) were measured in the cytoplasm and the cell nucleus. Both types of stimulation gave very similar increases for all three enzyme activities. In the cell nucleus, both α- and γ-polymerases increased almost tenfold, whereas the β-enzyme only was stimulated twofold. In the cytoplasm α- and γ-polymerases increased two- to fourfold. Only insignificant amounts of the β-enzyme were found in the cytoplasm.

Three DNA polymerase activities (α, β, and γ) have been identified in mammalian cells (2, 4, 6, 7, 17, 18, 20). α-Polymerase (*S* value, 6 to 8) is predominantly present in the cytoplasm. β-Polymerase (*S* value, 3.4) is localized in the nucleus, and γ-polymerase is present both in the cytoplasm and in the nucleus. The activity of the γ-enzyme is very low in most cells. It was identified by its ability to copy polyribonucleotide templates using oligodeoxynucleotides as primers. Recent studies have, however, shown that also the β-polymerase utilizes such templates (5).

The function of the three polymerases in DNA replication is unknown. Both α- and γ-polymerase activities increase during DNA replication, whereas the β-polymerase activity has been reported to be relatively constant during all phases of the cell cycle (2, 6, 7, 10, 19).

Infection of mouse cells with polyoma virus increases the activity of cellular DNA polymerases. Earlier studies were done before the recognition of more than one DNA polymerase (9). To gain further insight into the involvement of different DNA polymerases in the synthesis of polyoma DNA, we now have studied the effect of virus infection of resting 3T3 cells on the activity of all three enzymes. We also investigated the effects of serum stimulation.

MATERIALS AND METHODS

Sources of most materials were described earlier (15, 21). Poly[r(A)]homopolymer and oligo[d(T)] (12-18) were obtained from Collaborative Research, Inc., and poly[d(AT)] was obtained from Miles Laboratories, Inc., Elkhart, Ind. Activated calf thymus DNA was prepared by treating commercial DNA with pancreatic DNase to an acid solubility of 20 to 25% (1). Membrane filters were purchased from Schleicher & Schüll.

Cell cultures. Swiss 3T3 mouse fibroblasts, obtained from H. Green, Massachusetts Institute of Technology, Cambridge, Mass., were grown at 37 C in 9-cm petri dishes in Earle minimal essential medium containing 10% fetal calf serum. The medium was changed every second day. The cells were routinely passaged at 1.6×10^4 cells/cm^2.

On the third day after seeding, the cells had reached a density of 5×10^4 to 6×10^4 cells/cm^2. These cells were infected with polyoma virus at a multiplicity of about 100 PFU/cell for 60 min at 37 C. The original medium was replaced after infection. Mock infection of cells was done similarly but in the absence of virus.

Serum stimulation of resting cells was done by adding fresh medium containing 20% fetal calf serum to cells grown for six days (6×10^4 cells/cm^2) with a change of medium every second day.

The rate of DNA synthesis was measured by radioactive thymidine incorporation. [^{3}H]thymidine (6.7 Ci/mmol) was added to the culture medium at a final concentration of 1 μM. After 60 min at 37 C, the cells were harvested. Viral DNA was separated from high-molecular-weight cellular DNA by the method of Hirt (11). Cellular DNA was dissolved in 0.3 M NaOH, and the radioactivity incorporated into viral and cellular DNA was determined after acid precipitation. The radioactivity was then normalized to the DNA content (3) of each culture. The number of cells incorporating [^{3}H]thymidine was determined by autoradiography of parallel cultures.

Enzyme preparation. Nuclei were prepared from 15 to 30 petri dishes as described previously (21). After homogenization of cells, nuclei were sedimented by low-speed centrifugation. The supernatant solution containing 15 to 30 mg of protein was used for determinations of cytoplasmic enzyme activities. The sedimented nuclei were resuspended in an equal volume of isotonic *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (21), and enzymes were extracted for 20 min at 0 C by addition of 10 volumes of 1 M NaCl in isotonic HEPES. These extracts contained about 2 to 4 mg of protein. The cytoplasmic extract was clarified by centrifuga-

tion for 60 min at 4 C at 100,000 × g (5 to 10 mg of protein).

DNA was removed from crude nuclear extracts by batchwise addition of DEAE-cellulose equilibrated with 1 M NaCl in isotonic HEPES. A 0.3-mg portion of packed DEAE-cellulose was added per unit of optical density at 260 nm (OD_{260}). The cytoplasmic extract was adjusted to 1 M NaCl and treated in the same way. DEAE-cellulose was removed by centrifugation at 100,000 × g for 60 min at 4 C. The supernatants were dialyzed against 50 mM potassium phosphate buffer (pH 7.5)-20% glycerol-5 mM dithiothreitol. The dialyzed material was then absorbed onto a DEAE-cellulose column (0.4 to 1.2 ml) at a ratio of 3 to 5 mg of protein per ml of DEAE-cellulose bed volume. The column had previously been equilibrated with the buffer used during dialysis. The enzymes were eluted with a linear gradient from 50 to 400 mM potassium phosphate buffer, pH 7.5, in 20% glycerol-5 mM dithiothreitol (8 column volumes) as described by Weissbach et al. (20).

Assay of DNA polymerase activities. DNA polymerase was measured by the incorporation of [^{3}H]dTTP (300 to 600 counts/min per pmol) into an acid-insoluble product, using one of three different templates for the assay. Activated calf thymus DNA was used for the assay of the α- and β-polymerases (7), poly[d(AT)] for the β-polymerase (5), and poly[r(A)]-oligo[d(T)] for the β- and γ-polymerases (5, 18). All incubations were carried out for 15 or 30 min in a final volume of 50 μl. The incorporation of labeled nucleotide into acid-insoluble material was linear for at least 30 min.

With activated calf thymus DNA (200 μg/ml) as template incubations were done at 37 C with: 50 mM Tris-hydrochloride (pH 8.0); 6 mM $MgCl_2$; 5 mM DTT; 0.6 mg of bovine serum albumin per ml; and dATP, dGTP, dCTP, and [^{3}H]dTTP, each at 100 μM. Incubations with poly[d(AT)] (50 μg/ml) were made at 37 C with 50 mM Tris-hydrochloride (pH 8.5), 6 mM $MgCl_2$, 5 mM DTT, 0.6 mg of bovine serum albumin per ml, 100 mM KCl, 100 μM dATP, and 40 μM [^{3}H]dTTP. Incubations with poly[r(A)]-oligo[d(T)] {400 μg/ml; 10 poly[r(A)]:1 oligo[d(T)]} were done at 30 C with 50 mM Tris-hydrochloride (pH 7.6), 1 mM $MnCl_2$, 2 mM DTT, 0.2 mg of bovine serum albumin per ml, 100 mM KCl, and 100 μM [^{3}H]dTTP. Reactions were stopped by addition of Sarkosyl to a final concentration of 0.15%.

Acid-insoluble material was precipitated with 1 ml of ice-cold 10% trichloroacetic acid-0.1 M sodium pyrophosphate and collected onto membrane filters. The filters were washed with 40 ml of cold acid and dried. Radioactivity was determined in a toluene-based liquid scintillation fluid.

One enzyme unit was defined as the incorporation of 1 nmol of [^{3}H]dTTP in 30 min at 37 or 30 C.

Protein was precipitated with 5% trichloroacetic acid and then determined by the method of Lowry et al. (12).

RESULTS

Our experiments were aimed at a comparison of the activity of the different DNA-polymerases induced in confluent 3T3 cells by polyoma infection and by serum stimulation.

Confluent 3T3 cultures for virus infection were prepared by growing cells until they had reached a density of 5×10^4 to 6×10^4 cells/cm^2. In such cultures 10 to 15% of the cells synthesized DNA as measured by autoradiography after a 60-min pulse of [^{3}H]thymidine. Even though this value could be decreased further, the DNA synthesis of cultures grown for a longer time period could no longer be efficiently stimulated by polyoma infection. Virus infection of cultures prepared as described resulted in an increase of both cellular and viral DNA synthesis starting at about 20 h after infection. The maximal rate of cellular and viral DNA synthesis was reached after 30 h. At this time 40 to 50% of the cells synthesized DNA as measured by autoradiography. In parallel mock-infected cultures only 2% of the cells incorporated [^{3}H]thymidine.

DNA polymerase activities were determined in nuclear and cytoplasmic extracts from five sets of cultures: (i) uninfected cells harvested at the time of infection; (ii) mock-infected cells harvested 30 h after infection; and (iii) infected cells harvested at 23, 30, and 37 h, respectively, after infection. The time course of the total induced DNA polymerase activity, as determined with activated calf thymus DNA as template, as well as incorporation of [^{3}H]thymidine into total cellular and viral DNA is shown in Fig. 1. Enzyme induction clearly occurred in parallel with induction of DNA synthesis and was maximal 30 h after infection. At that time DNA polymerase activity was increased fourfold over the activity in mock-infected cultures.

DNA polymerases were then separated by DEAE-cellulose chromatography of nuclear and cytoplasmic extracts, respectively. The three different enzyme activities were defined by their positions on the chromatogram and by their template specificities. Figure 2 gives the results with the nuclear enzymes from mock-infected (Fig. 2A) and virus-infected (Fig. 2B) cultures. In both chromatograms the first sharp peak of enzyme activity was obtained with all three templates and represents the β-enzyme. Virus infection increased this activity about twofold. The second major peak, which was much broader, was obtained only with activated calf thymus DNA and corresponds to the α-enzyme. This activity was low in mock-infected cells and increased almost tenfold after virus infection. Finally, a third minor peak was identified at the leading edge of the α-polymerase. This activity, which was observed exclusively with the synthetic polymer poly[r(A)]-oligo[d(T)], corresponded to the γ-enzyme. This

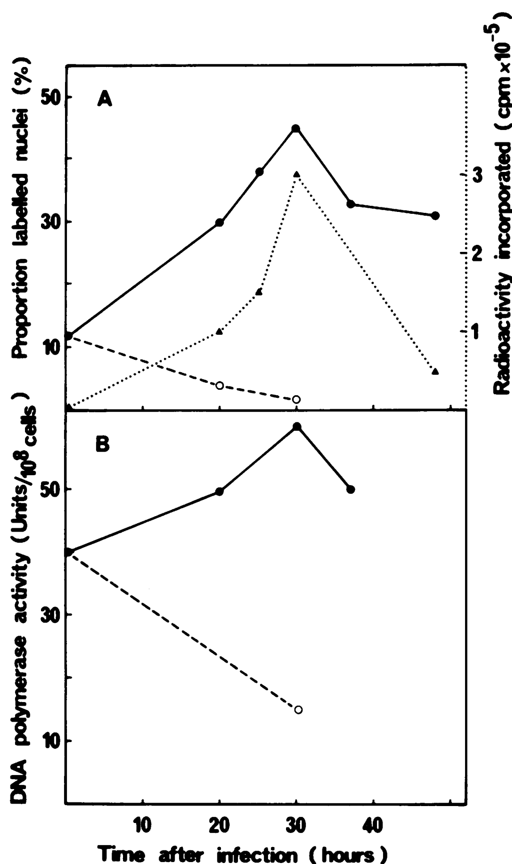

FIG. 1. *Effect of polyoma virus infection on DNA synthesis and DNA polymerase activity in 3T3 cells. The rate of DNA synthesis after virus infection of resting 3T3 cultures was measured by labeling the cells with [3H]thymidine for 60 min. (A) Proportion of nuclei incorporating radioactivity as measured by autoradiography (●) and the amount of acid-precipitable radioactivity in selectively extracted viral DNA (▲). (B) Content of DNA polymerase activity extracted from parallel cultures. Enzyme activity was measured using activated calf thymus DNA as a template. Open symbols represent results from mock-infected cultures.*

peak was badly visible in nuclear extracts from mock-infected cells.

Similar chromatograms of cytoplasmic extracts are shown in Fig. 3. Here the α-polymerase dominates both mock- and virus-infected extracts. The α-polymerase as well as the γ-activity increased about threefold after infection. The β-polymerase was almost completely absent from the cytoplasmic extracts.

A more detailed analysis of the changes in the activities of different DNA polymerases in the cell nucleus and cytoplasm at different times after polyoma infection is given in Table 1. The table also includes results from a second experiment in which DNA synthesis of resting 3T3 cells was stimulated by a change of medium containing 20% fetal calf serum. In the latter experiment about 1% of the cells was synthesizing DNA, as judged from autoradiography at the time of medium change. This value increased to 35% 20 h later. DNA polymerases were measured at both time points.

The values recorded in Table 1 were calculated from chromatograms similar to those shown in Fig. 2 and 3. Each total polymerase activity was obtained by addition of the enzyme activities of the separated chromatographic fractions, and the values were normalized for 10^8 cells. The α-enzyme was calculated from values obtained with activated calf thymus DNA as template, the β-enzyme from values with poly[d(AT)], and the γ-enzyme from values with poly[r(A)]-oligo[d(T)]. The overall recovery was always better than 70% in each chromatogram.

Similar to many earlier observations in other

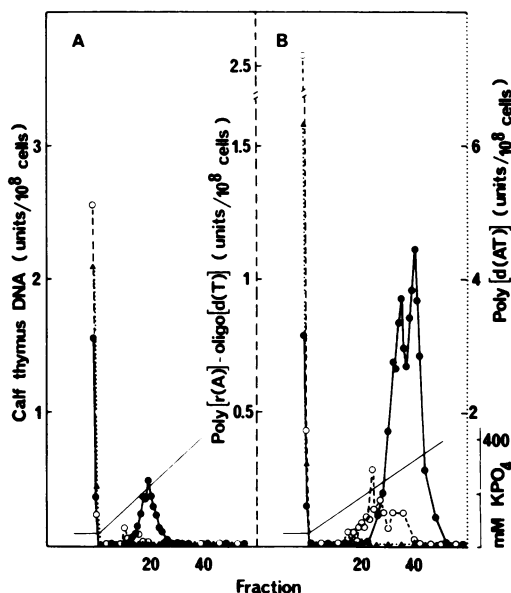

FIG. 2. *Separation of nuclear DNA polymerases by DEAE-cellulose chromatography. Extracts were prepared from nuclei of mock-infected cells (A) and 30 h after infection of cells (B) and chromatographed as described in Materials and Methods. The three DNA polymerase activities were identified by their preferential activities with different template-primers. The β-polymerase preferentially copied poly[d(AT)], the γ-polymerase preferred poly[r(A)]/oligo[d(T)] as a template, and the α-enzyme had the highest activity with activated calf thymus DNA. Symbols: ▲, poly[d(AT)]; ○, poly[r(A)]/oligo[d(T)]; ●, calf thymus DNA.*

systems, resting or mock-infected 3T3 cells contain essentially all the β-activity in the cell nucleus, whereas the α-enzyme is preponderant in the cytoplasm (2, 6, 7, 10, 19). Also most of the γ-enzyme is recovered in the cytoplasm, but the low activity of this polymerase puts a considerable uncertainty on the absolute values.

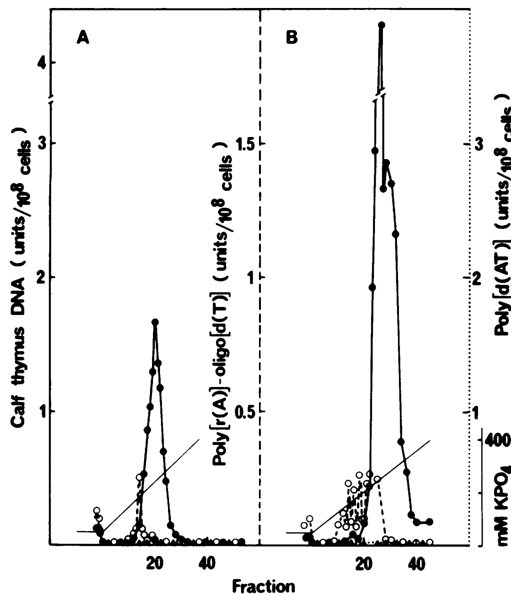

FIG. 3. *Separation of cytoplasmic DNA polymerase activities by DEAE-cellulose chromatography. Extracts were prepared from cytoplasmic fractions of mock-infected cells (A) and 30 h after infection of cells (B). Assay of DNA polymerase activity was done as described in legend to Fig. 2.* ▲, *Poly[d(AT)];* ○, *poly[r(A)]/oligo[d(T)]; and* ●, *calf thymus DNA.*

Infection with polyoma or stimulation of DNA synthesis by serum gave almost identical results: there is a large increase of the α-enzyme in the cell nucleus and a somewhat smaller increase in the cytoplasm. Similar changes also occur with the γ-enzyme. Also the β-polymerase of the nucleus increased after stimulation of DNA synthesis, but the increase was much smaller than that of the two other enzymes. The peak activities of all three enzymes in the nucleus occurred in parallel with the peak of DNA synthesis (cf. Table 1 and Fig. 1) after polyoma infection.

DISCUSSION

Earlier studies from this laboratory on the mechanism of polyoma DNA replication in isolated cell nuclei from mouse 3T6 cells led to the suggestion that DNA chain elongation is a discontinuous process that involves at least two different DNA polymerases (13, 14, 16). It was proposed that one of the enzymes might be involved in the elongation of short DNA fragments that had been initiated by a polyribonucleotide about 10 nucleotides in length (initiator RNA). The second DNA polymerase would then close the gaps created between the DNA fragments after removal of initiator RNA.

The present study was undertaken in order to investigate the relative amounts of the different DNA polymerases present in nuclei from normal and polyoma-infected mouse fibroblasts. Earlier work by others had shown that: (i) the activities of the α- and γ-enzymes were much higher in cells engaged in DNA synthesis than in resting cells (2, 6, 7, 10, 19) and that the increase of the α-enzyme to a large extent oc-

TABLE 1. *DNA polymerase activities in mouse fibroblast cells*[a]

Determination	Nuclear extract (DNA polymerase)			Cytoplasmic extract (DNA polymerase)			Calculated DNA polymerase activities in whole cells (DNA polymerase)		
	α	β	γ	α	β	γ	α	β	γ
Polyoma virus infection of 3T3 cells:									
Mock-infected cells 30 h postinfection	3.4	5.0	0.16	10.1	0.43	0.37	13.5	5.43	0.53
Infected cells									
23 h postinfection	15.2	7.3	0.87						
30 h postinfection	26.0	10.6	1.20	30.5	0.46	1.30	56.6	11.1	2.50
37 h postinfection	17.9	7.0	0.85						
Serum stimulation of 3T3 cells:									
Nongrowing cells	1.6	5.5	0.14	9.0	0.44	1.26	10.6	5.94	1.40
Serum-stimulated cells	17.6	12.0	1.20	32.0	0.34	2.00	49.6	12.3	3.20

[a] The enzyme activities are given as total units per 10^8 cells. The separated α-, β-, and γ-polymerase activities were determined with activated calf thymus DNA, poly[d(AT)], and poly[r(A)] oligo[d(T)] as a template-primer, respectively, as described in Materials and Methods.

curred in the cell nucleus (2, 10, 19) and (ii) polyoma infection resulted in a large increase of the total DNA polymerase activity of cells (9).

The method used in the present study relies on the earlier described separation of the three enzymes on DEAE-cellulose (19, 20) as well as on their different template specificities (5, 7, 18). We did not obtain a clearcut separation of the γ- and α-enzymes (Fig. 1 and 2). Nevertheless, by using poly[r(A)]-oligo[d(T)] as a template the γ-enzyme could be determined with reasonable accuracy, since the α-enzyme was inactive under the conditions of assay.

We found that polyoma infection resulted in an increase of all three enzyme activities in the cell nucleus and that this increase occurred in parallel with the stimulation of DNA synthesis. The increase was largest for the α- and γ-enzymes (five- to tenfold) but was also quite apparent for the β-enzyme (twofold). In the cytoplasm only insignificant amounts of the β-enzyme were found in all cases. There the α- and γ-enzymes also increased after infection, but to a smaller extent than in the cell nucleus. Nevertheless, the total amount of α- and γ-activities was always larger in the cytoplasm than in the cell nucleus.

The significance of the presence of DNA polymerase in the cytoplasm is not evident. It may be an artifact caused by leakage of enzymes from the nucleus during cell fractionation in aqueous media. In this case the relative increase of enzyme activity in the nucleus during DNA synthesis may be the result of a tight binding of polymerases to an active replication complex in the nucleus, causing less leakage. On the other hand it also seems possible that both α- and γ-polymerase are present in the cytoplasm in cells not engaged in DNA synthesis and are transported to the nucleus during S phase.

Also serum stimulation of DNA synthesis gave an increase of all three polymerases, mostly localized to the nucleus, and thus showed effects very similar to polyoma infection. From this we conclude that the influence of polyoma is not specific for the virus infection but rather is a result of the more general effect on DNA synthesis. Our results leave open the question raised in the beginning of the discussion concerning the involvement of a given polymerase in specific steps of chain elongation.

Wintersberger and Wintersberger (22) recently independently investigated the effect of polyoma infection on the levels of α- and β-polymerases in resting mouse kidney cells. They reported a large increase in the α-enzyme, in particular in the cell nucleus, but found no change in the β-enzyme. However, their method of analysis may not have been sensitive enough to find minor changes in this enzyme activity.

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