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DNA polymerase α is associated with replicating SV40 nucleoprotein complexes*

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ABSTRACT

Simian virus 40 (SV40) nucleoprotein complexes were extracted from nuclei of infected monkey cells and fractionated on neutral sucrose density gradients. Complexes which contained replicating SV40 DNA (95S) separated well from those containing closed circular supercoiled viral DNA (75S). DNA polymerase activity was associated with the replicating nucleoprotein complexes but not with the slower sedimenting complexes. This DNA polymerase activity coprecipitated with the nucleoprotein complexes in the presence of $MgCl_2$ and remained associated with the 95S complexes. This DNA polymerase activity has been identified as primarily DNA polymerase α on the basis of its sedimentation behavior, optimum salt concentration, and sensitivity to N-ethylmaleimide. DNA polymerase γ activity was also detected in the complexes, but DNA polymerase ß was not associated with the complexes.

INTRODUCTION

Recently, subnuclear systems for SV40 DNA replication in vitro have been developed (1,2,3,4). Hypotonic extracts of nuclei from SV40-infected monkey cells contain nucleoprotein complexes composed in part of replicating SV40 DNA which can be elongated and completed in vitro (2,4). Since the replication of SV40 DNA is dependent on cellular replication enzymes, except for a viral gene product involved in initiation of replication (5,6), this system offers the possibility of defining the proteins and enzyme activities which carry out the various steps in mammalian DNA replication. The best studied enzyme activities involved in mammalian cell DNA replication are the DNA polymerases α, ß and γ (reviewed in reference 7). The evidence available suggests that the α-polymerase is responsible for cell DNA replication, whereas the ß-activity may be involved in repair or recombination (8). The role of the

γ -polymerase in cell DNA replication is not clear (7,9,10). If DNA polymerase α is responsible for cell DNA replication, one might expect to find it associated preferentially with replicating chromatin. Since replicating SV40 chromatin can be simply separated from nonreplicating SV40 complexes by zone velocity sedimentation (2), the SV40 system lends itself to an investigation of this question.

This communication reports the association of DNA polymerase α activity with replicating SV40 nucleoprotein complexes. Little or no DNA polymerase activity was found associated with nonreplicating complexes. DNA polymerase γ , but not ß, activity was detected in SV40 nucleoprotein complexes.

MATERIAL AND METHODS

Cell culture. CV-1 cells were a gift from G.Sauer, Heidelberg, and the TC-7 clone of CV-1 cells (11) was donated by K.Huebner, Philadelphia. The cells were grown in monolayer cultures in plastic dishes (C.A.Greiner, Nürtingen) in Dulbecco-modified Eagle medium (GIBCO) supplemented with 10% calf serum (Flow Labs) at 37° in a 5% CO_2 atmosphere.

Virus. Simian virus 40 (SV40), strain Rh911 (12) was originally obtained from G.Sauer. SV40 was propagated in confluent CV-1 or TC-7 cells infected with three times plaque-purified virus at 0.01 PFU per cell, harvested as described earlier (13), and titered by plaque formation on CV-1 cells (14).

Reagents. N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, dithiothreitol (DTT), dithioerythritol, bovine serum albumin (BSA) and calf thymus DNA were purchased from Sigma. Unlabeled ribo- and deoxyribonucleotide triphosphates and poly(A)·$(dT)_{12-18}$ were obtained from Boehringer, Mannheim. Phenylmethylsulfonyl fluoride and N-ethylmaleimide were supplied by Serva, and all other chemicals, by Merck, Darmstadt. Radioisotopes were supplied by Amersham, Braunschweig.

Preparation of SV40 nucleoprotein complexes was carried out essentially as described (2). CV-1 cells (4-5 x 10^6 cells per 15 cm diameter dish) were infected with 1-5 PFU per cell of SV40. In some experiments the cells were labeled from 24 to 36 h postinfection with 1-2 μCi/ml of (^{3}H)thymidine (22-24 Ci/mmol) or 0.1 μCi/ml of (^{14}C)thymidine (56 mCi/mmol). At 36-38 h

postinfection the complexes were either pulse-labeled for 5-30 min with 50-100 uCi/ml of (^{3}H)thymidine or harvested directly. The cells were scraped from each plate in 2 ml of 10mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.8, 5mM KCl, 0.5mM $MgCl_2$, 1mM DTT (Buffer A) and disrupted with 6-8 strokes of a Dounce homogenizer tightly fitted with a Teflon pestle. The nuclei were pelleted at 3000 x g, and the cytoplasmic fraction was removed. The nuclei were extracted with Buffer A (4-5 x 10^7 nuclei/ml) at 0° for 1 h, and then pelleted twice at 8000 x g. The supernatant containing the complexes was free of nuclei. With this procedure, 25-50% of the SV40 DNA in the nuclei was recovered as complexes.

Precipitation of SV40 complexes with $MgCl_2$. The supernatant containing the complexes was made 20mM in $MgCl_2$ and incubated for 1 h at 0° (15). The complexes were pelleted at 12,000 x g for 10 min, washed with Buffer A containing 20mM $MgCl_2$ and resuspended in Buffer A.

Zone velocity sedimentation of SV40 complexes was performed essentially as described (2). Sucrose density gradients (5-30%, w/v) were made up in Buffer A containing 1mM phenylmethylsulfonyl fluoride and assembled on top of a 50% sucrose cushion. The conditions of sedimentation are stated in the figure legends. ^{3}H-labeled bacteriophage T7 DNA (34S), obtained from J.Bauer, Konstanz, was run in a parallel gradient as a sedimentation marker.

Zone velocity sedimentation in alkaline sucrose density gradients was carried out as reported earlier (16).

Zone velocity sedimentation of enzyme activities. $MgCl_2$-precipitated nucleoprotein complexes were made 0.25 M in $(NH_4)_2SO_4$ and layered on a 0-15% linear sucrose density gradient made up in 50mM Tris-HCl, pH 8.0, 1mM EDTA, 5mM DTT, 250mM $(NH_4)_2SO_4$, 20% glycerol and 500 ug/ml of BSA. The gradients were centrifuged at 4° in the SW50.1 rotor for 20 h at 50,000 rpm or in the SW41 rotor for 48 h at 35,000 rpm. Bovine hemoglobin included as a sedimentation marker (4.4.S) was detected by its optical absorption at 425 nm.

Standard DNA polymerase assay. Each reaction mixture (0.1 ml) contained 50mM Tris-HCl, pH 7.5, 5mM $MgCl_2$, 5mM DTT, 0.5 mg/ml

of BSA, 100/ug/ml of activated calf thymus DNA (17), 100/uM each dCTP, dGTP, and dTTP and 20/uM (α -^{32}P)dATP. After 60 min at 37°, the reaction was terminated by adding 50/ul of 0.1 M EDTA. Acid-precipitable radioactivity was collected on glass fiber filters, dried and counted in a toluene-based scintillator. Background was subtracted before the data were plotted.

Selective assays for DNA polymerases α, ß and γ were performed according to Hübscher et al. (18) with minor modifications. The α -polymerase assay contained 100/ug/ml of activated calf thymus DNA, 50/uM dCTP, dATP and dGTP, 20/uM (^{3}H)dTTP, 20mM K-phosphate, pH 7.2, 5mM $MgCl_2$, 5mM DTT and 400/ug/ml of BSA. The ß-polymerase assay contained 100/ug/ml of activated DNA, 50/uM dCTP, dATP, dGTP, 20/uM (^{3}H)dTTP, 50mM Tris-HCl, pH 8.3, 5mM $MgCl_2$, 100mM NaCl, and 400/ug/ml of BSA. The aliquots to be assayed were preincubated in 5mM N-ethylmaleimide at 0° for 30 min. Both α-and ß-polymerase assays were incubated at 37° for 60 min. Acid-precipitable radioactivity was determined as in the standard assay. The γ -polymerase assay contained 50/ug/ml of poly(A)·$(dT)_{12-18}$, 5/uM (^{3}H)dTTP, 25mM Tris-HCl, pH 8.3, 50mM K-phosphate, pH 8.4, 0.5mM $MnCl_2$, 100mM KCl, 2mM DTT and 400/ug/ml of BSA. Assays for γ -polymerase were incubated at 30° for 60 min. Acid-precipitable radioactivity was collected on nitrocellulose filters, dried and counted as above.

RESULTS

DNA polymerase activity in replicating SV40 nucleoprotein complexes. SV40-infected CV-1 cells were labeled with (^{14}C) thymidine for 12 h and pulse-labeled with (^{3}H)thymidine. SV40 nucleoprotein complexes were extracted and fractionated by zone velocity sedimentation. Replicating SV40 complexes, labeled with (^{3}H)thymidine for 5 min (Fig. 1A), could be easily distinguished from slower sedimenting complexes which appeared after longer labeling periods (Fig. 1A,B). The complexes sedimented at approximately 95S and 75S, respectively, based on T7 DNA. Zone velocity sedimentation in alkaline sucrose density gradients demonstrated that DNA labeled for 12 h was mostly closed circular supercoiled SV40 DNA (53S). DNA labeled for 5 min included unit length nicked viral DNA (16-18S), as

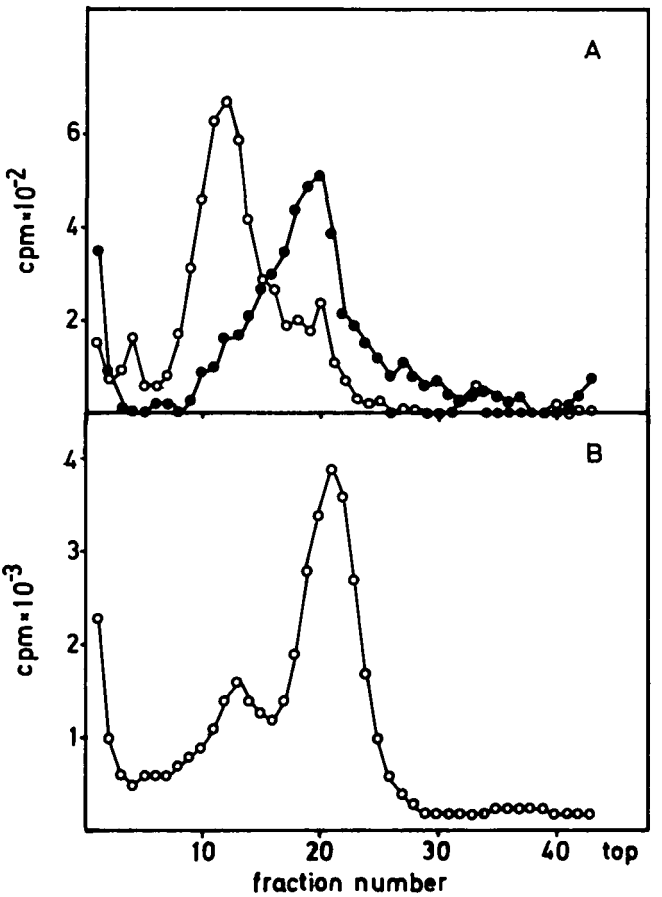

Figure 1. Separation of replicating SV40 nucleoprotein complexes from nonreplicating complexes. SV40 nucleoprotein complexes were extracted from SV40-infected cells double-labeled with (^{14}C)thymidine for 12 h and (^{3}H)thymidine for 5 min (A) or labeled with (^{3}H)thymidine for 30 min (B). The complexes were fractionated in 5-30% sucrose density gradients in the Spinco SW41 rotor at 0° and 40,000 rpm for 1.5 h. Fractions were collected from the bottom and 100-200 µl aliquots were acid-precipitated, collected on glass fiber filters, dried and counted. o-o, ^{3}H; •-•, ^{14}C.

well as smaller fragments of DNA, in agreement with published reports (reviewed in references 19 and 20) (data not shown).

Replicating and complete SV40 nucleoprotein complexes labeled in vivo for 15 min were fractionated on neutral sucrose gradients. DNA polymerase activity was detected in the replicating complexes, as well as free at the top of the gradient (Fig. 2).

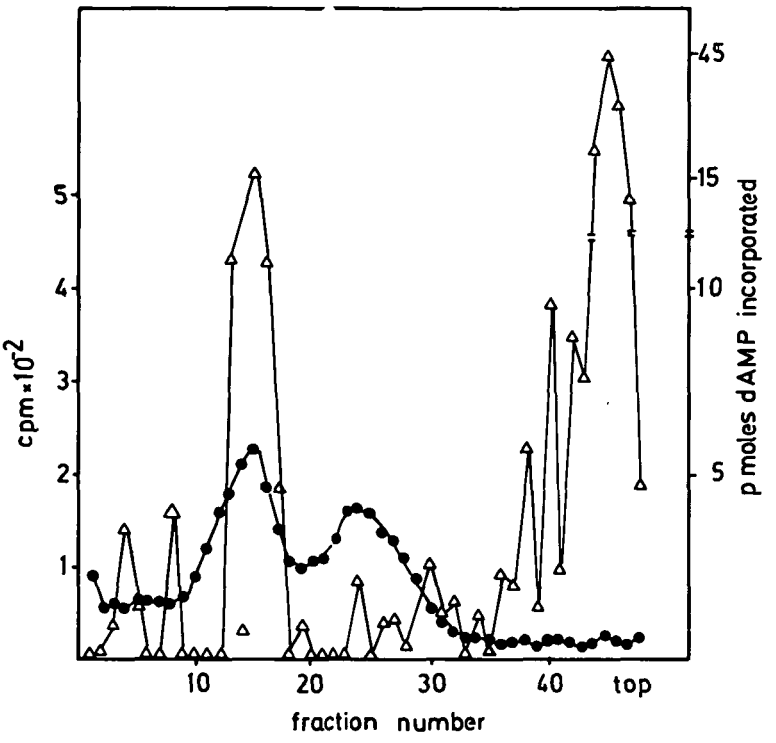

Figure 2. Association of DNA polymerase activity with replicating SV40 nucleoprotein complexes. SV40 nucleoprotein complexes prepared from infected cells labeled with (^{3}H)thymidine for 15 min were fractionated as in Fig. 1, except that the Spinco SW50.1 rotor was used (45,000 rpm for 1 h). Acid-precipitable radioactivity and DNA polymerase activity, using the standard assay (400 cpm/pmol), were determined in 20 µl aliquots of each fraction. ●-●, ^{3}H, ▲-▲, ^{32}P.

The fraction of DNA polymerase activity associated with the complexes varied but generally comprised about 10-20% of the total activity detected on the gradient. In Fig. 2, for example, about 34 pmol of dAMP were incorporated by the polymerase activity bound to replicating complexes, and about 140 pmol by the soluble activity at the top of the gradient. Little or no polymerase activity was observed in the 75S complexes, although activated calf thymus was included in the assay mix so that polymerase activity could have been detected in the absence of SV40 DNA primer-template. These data suggest that the polymerase activity may be specifically associated with replicating complexes.

DNA polymerase activity in Mg^{++}-precipitated nucleoprotein complexes. Like cellular chromatin (15), SV40 nucleoprotein complexes may be precipitated in the presence of Mg^{++}. A preparation of SV40 complexes labeled in vivo for 12 h with (^{14}C) thymidine and pulse-labeled for 5 min with (^{3}H)thymidine was divided into two parts. One part was treated with 20mM $MgCl_2$, and then both parts were analyzed on neutral sucrose density gradients (Fig. 3). Replicating and complete nucleoprotein com-

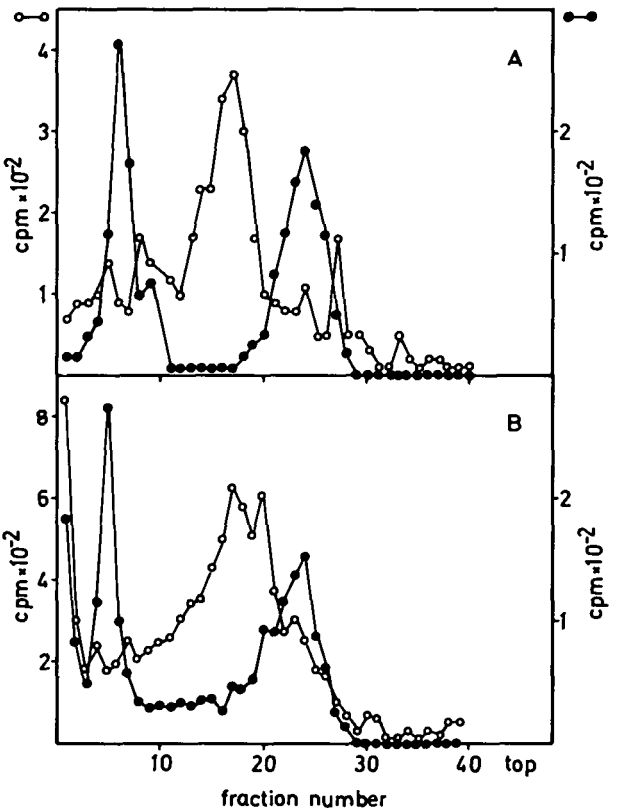

Figure 3. Mg^{++} precipitation of replicating and complete SV40 nucleoprotein complexes. An extract containing SV40 nucleoprotein complexes from infected cells labeled with (^{14}C)thymidine for 12 h and (^{3}H)thymidine for 5 min was divided into two parts. One part was fractionated directly, as in Fig. 1, and acid-precipitable radioactivity in 100 µl aliquots was determined (A). The complexes in the other half of the extract were precipitated in 20mM $MgCl_2$, resuspended, and fractionated by zone velocity sedimentation. Acid-precipitable radioactivity in 200 µl aliquots was determined (B). o-o, ^{3}H; •-•, ^{14}C.

plexes precipitated in the presence of Mg^{++} to approximately the same extent. Generally 70-85% of the labeled complexes in the nuclear extract were recovered in the precipitate. Aggregation of SV40 complexes to fast sedimenting forms which also precipitated in the presence of $MgCl_2$ was occasionally observed.

DNA polymerase activity in 5 and 10 µl aliquots of a crude nuclear extract (4×10^7 nuclei) containing SV40 complexes was measured using the standard assay with (^{3}H)dTTP, both before treatment with $MgCl_2$ and in the resuspended precipitate. The total incorporation of dTMP before $MgCl_2$ precipitation was 26, 200 pmol and in the resuspended pellet, 2720 pmol. In other words, about 10% of the polymerase activity present in nuclear extracts was precipitated in the presence of $MgCl_2$, a value in good agreement with the results presented in Fig. 2.

In order to determine whether the DNA polymerase activity which precipitated in $MgCl_2$ was bound to SV40 chromatin or whether unbound polymerase activity was precipitated, SV40 nucleoprotein complexes labeled in vivo for 25 min were precipitated in 20mM $MgCl_2$, washed, resuspended and analyzed by zone velocity sedimentation. DNA polymerase activity was detected primarily in the replicating complexes (Fig. 4). Little free DNA polymerase activity was observed in Mg^{++}-precipitated material. Thus, polymerase activity in the nucleoprotein complexes coprecipitated with them, whereas free polymerase activity was not precipitated by $MgCl_2$. Moreover, the polymerase activity remained associated only with the replicating complexes.

Identification of the DNA polymerase activity in SV40 nucleoprotein complexes. In nuclear extracts containing SV40 nucleoprotein complexes, DNA polymerase activity measured without exogenous template primer in the assay mix was negligible (Table 1), although it is possible to measure endogenous DNA synthesis in these extracts using a conventional assay for DNA replication in vitro (2,3,4, authors' unpublished results). When exogenous template was included in the assay, DNA polymerase α, β and γ activities in nuclear extracts containing SV40 nucleoprotein complexes could be determined using selective assays (18) (Table 1). Nearly 90% of the polymerase activity in nuclear extracts appeared to be DNA polymerase α, but small

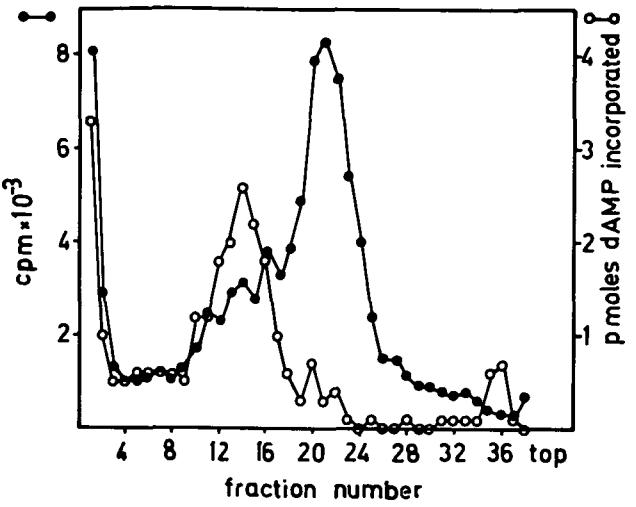

Figure 4. DNA polymerase activity coprecipitates with replicating SV40 nucleoprotein complexes. Mg^{++}-precipitated complexes prepared from SV40-infected cells which had been labeled with (^{3}H)thymidine for 25 min were analyzed on sucrose gradients as in Fig. 1. Acid-precipitable radioactivity in 50 µl aliquots of each fraction was determined. DNA polymerase activity in 20 µl aliquots of each fraction was tested using the standard assay (1000 cpm/pmol). ●-●, ^{3}H, o-o, ^{32}P.

amounts of the ß and γ activities were also eluted from the nuclei and could be optimally detected if the nuclear extracts were treated with 0.5 M NaCl prior to the enzyme assays, suggesting that these enzymes might be bound to template or otherwise unable to use the exogenous template-primer provided in the assay mix. Salt treatment of the nuclear extract prior to the α-polymerase assay, however, had no effect on the activity measured.

Since the α-polymerase is the main DNA polymerase activity in nuclei of growing cells (21,22), and since only about 10% of the polymerase activity in the nuclear extract is bound to SV40 complexes (Fig.2,4), the prominence of DNA polymerase α activity in the nuclear extract need not mean that it is associated with the complexes.

Thus, the polymerase activity associated with the replicating complexes was separated from free activity in the nuclear extract by Mg^{++} precipitation of the complexes. DNA polymerase activity in the Mg^{++}-precipitate was dissociated from the com-

Table 1. DNA polymerase activities in a nuclear extract containing SV40 nucleoprotein complexes [a]

Assay [b]	Incorporation of dTMP 5 µl of extract		10 µl of extract	
	cpm [c]	pmol	cpm [c]	pmol
DNA polymerase α	11425	8.8	22055	17.0
DNA polymerase β	474	0.4	1200	0.9
DNA polymerase γ	900	0.7	1196	0.9
Endogenous DNA polymerase [d]	24	0.002	34	0.003

[a] Crude SV40 nucleoprotein complexes were extracted in 0.3 ml of Buffer A from 10^7 nuclei, as described in Material and Methods.

[b] Selective assays for α, β and γ polymerase were as described in Materials and Methods, except that in the β and γ polymerase assays, nuclear extract was treated with 0.5 M NaCl prior to the assay and then diluted into the assay mixture.

[c] Average cpm of duplicate determinations after subtraction of background.

[d] Polymerase activity was determined using the standard assay, except that (^{3}H)dTTP (1300 cpm/pmol) was used and activated calf thymus DNA was omitted.

plexes and partially purified by zone velocity sedimentation (Fig. 5A). DNA polymerase activity extracted from CV-1 nuclei was analyzed in a parallel gradient (Fig. 5B). CV-1 nuclei contained DNA polymerases α (8-9S), β (3-4S) and γ (7S), but the SV40 nucleoprotein complexes contained only the α and γ activities. The proportion of γ-polymerase relative to total polymerase activity is somewhat higher in the partially purified enzymes from Mg^{++}-precipitated complexes (Fig. 5) than in the nuclear extracts containing SV40 complexes (Table 1). At least two explanations of this discrepancy are possible. First, only about 10% of the polymerase activity in the nuclear extract is associated with nucleoprotein complexes, and second, the activities of either enzyme may be different in nuclear extracts than after Mg^{++}-precipitation and sedimentation.

Since the SV40 DNA was dissociated from the DNA polymerase activity but not removed from the sample before centrifugation, it sedimented to the bottom of the tube. Occasionally, however, some polymerase activity failed to dissociate from the DNA, resulting in a fast-sedimenting peak of polymerase activity

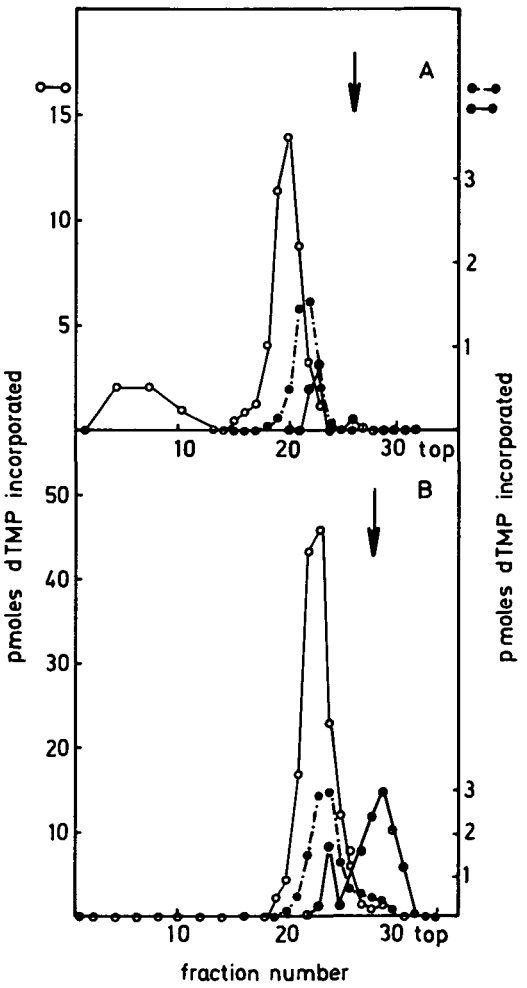

Figure 5. Zone velocity sedimentation of DNA polymerase activity from SV40 nucleoprotein complexes and CV-1 nuclei. Mg^{++}-precipitated nucleoprotein complexes were resuspended in Buffer A and made 0.25 M in $(NH_4)_2SO_4$ to dissociate DNA polymerase activity from the complexes. This material (A), or material extracted from CV-1 nuclei in Buffer A containing 0.25 M $(NH_4)_2SO_4$ (B), was layered on 0-15% linear sucrose density gradients and centrifuged in the SW41 rotor at 4° and 35,000 rpm for 48 h. Bovine hemoglobin (4.4S) was included as a sedimentation marker (arrow). Aliquots of each fraction were assayed for DNA polymerase α (10/ul), ß (10/ul) and γ (20/ul activity using (^{3}H)dTTP (1300 cpm/pmol) (18). o-o, α-polymerase assay; •-•, ß-polymerase assay; •.-.•, γ-polymerase assay.

(Fig. 5A). A small peak of polymerase activity (7S) detected with the ß-polymerase assay is most likely due to incomplete inhibition of γ-polymerase by the N-ethylmaleimide present in the assay (9,10,18). When the polymerase activity from the complexes was tested with the standard assay, however, DNA polymerase activity was completely inhibited by 2mM N-ethylmaleimide, confirming the absence of ß-polymerase, which is not sensitive to this inhibitor (23). The optimum salt concentration for the pooled fractions from the complexes was 20-25mM NaCl when activated calf thymus DNA was used as template-primer and 150-175mM NaCl when poly(A)·$(dT)_{12-18}$ was used. These data are consistent with the presence of two different DNA polymerase activities, α and γ, in SV 40 nucleoprotein complexes.

DISCUSSION

The hypothesis that DNA polymerase α is the major polymerase activity responsible for eucaryotic DNA replication is based primarily on the correlation of the amount of α-polymerase activity in cells with their growth state or phase in the cell cycle (reviewed in reference 7). The level of DNA polymerase α activity has also been found to increase in growing monkey cells infected with SV40 (24) and resting mouse cells infected with polyoma virus (25,26). This quantitative approach, while suggestive, cannot provide conclusive evidence that α-polymerase is the major enzyme involved in papova virus or cell DNA replication.

Recently, however, chromatin from stimulated bovine lymphocytes was shown to contain α-polymerase activity, while that from resting lymphocytes contained little or no α activity (E.J.Schlaeger, H.J.van Telgen, K.H.Klempnauer and R.Knippers, Eur.J.Biochem., in press). Using the SV40 system, in which replicating nucleoprotein complexes can be separated from nonreplicating complexes, we have demonstrated that DNA polymerase α is associated with replicating SV40 chromatin and is not associated with nonreplicating chromatin. These results provide additional evidence for the participation of the α-polymerase in SV40 DNA replication.

DNA polymerase γ is also associated with SV40 nucleoprotein complexes, but assigning it to either replicating or complete

complexes has proved difficult. Preliminary results, however, indicate that the level of γ activity is highest in the material sedimenting between the replicating and complete complexes. The different distribution of α- and γ-polymerases within the complexes may indicate that they fulfill different functions in replication (26). DNA polymerase γ activity has been found in adenovirus DNA replication complexes (27,28), but its role in DNA replication in that system is also unclear.

DNA polymerase ß was not detected in SV40 nucleoprotein complexes which had been separated from soluble enzyme activities by Mg^{++}-precipitation (Fig. 5A). DNA polymerase ß activity was demonstrated, however, in extracts of crude SV40 complexes (Table 1), in nuclei from infected host cells (Fig. 5B) and in the material which remains soluble at the top of the sucrose gradients used to separate replicating and complete SV40 complexes (authors' unpublished data). These results suggest that DNA polymerase ß probably does not function in SV40 DNA replication. Moreover, the fact that no detectable DNA polymerase ß was present in the complexes supports the notion that the association of DNA polymerase α with the replicating complexes is a specific one.

Though the presence of DNA polymerases α and γ in SV40 complexes suggests that these activities may carry out SV40 DNA replication, further evidence is necessary to show that they are both in fact required for replication.

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