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Polyoma Virus Minichromosomes: Associated Enzyme Activities†

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Polyoma minichromosomes were isolated and fractionated on glycerol gradients as described by Gourlie et al. (J. Virol. **38**:805-814, 1981). Specific assays for DNA polymerases α, β, and γ, DNA topoisomerase I, and RNase H were carried out on each fraction. The number of units of activity in each fraction was compared with the number of total polyoma and replicative intermediate DNA molecules in each fraction determined by quantitative electron microscopy (M. R. Krauss and R. M. Benbow, J. Virol. **38**:815-825, 1981). DNA polymerase α cosedimented with polyoma replicative intermediate DNA molecules. DNA polymerase β and DNA topoisomerase I activities sedimented with mature polyoma minichromosomes. Although the bulk of RNase H activity sedimented in the minichromosome region, the peak of activity was found one fraction behind the peak of mature minichrosomes. Virtually no DNA polymerase γ activity cosedimented with polyoma minichromosomes.

All of the enzymes and proteins required for polyoma viral DNA replication are thought to be derived from the host cell, with the possible exception of T antigen, which is apparently required for the initiation of DNA replication in vivo (30). One approach to identify these enzymes is to isolate polyoma minichromosomes and proteins which are capable of carrying out DNA synthesis in vitro and to determine the enzyme and proteins which cosediment with the replicating species.

Using the soluble polyoma virus minichromosome replication system of Gourlie et al. (14), we have attempted to identify and quantitate the activity of several enzymes cosedimenting with polyoma minichromosomes which may be involved in DNA replication: DNA polymerases α, β, and γ, DNA topoisomerase I (the activity we refer to as DNA topoisomerase I does not require Mg^{2+} or ATP and is the presumptive mouse 3T6 cell type I DNA topoisomerase), and RNase H. DNA polymerase α has been suggested as the major replicative enzyme (2, 24, 34); DNA polymerase β as a repair enzyme (2, 34); and DNA polymerase γ as the mitochondrial replication enzyme (22, 34). It has been suggested that DNA topoisomerase activity plays a role in DNA replication as a "swivel" for the removal of the superhelical stress ahead of the replication fork (6). RNase H has been postulated to play a role in DNA replication either by removing the RNA primers from Okazaki fragments (25) or by functioning in initiation (17).

Using the criterion of cosedimentation with polyoma minichromosomes, we have found that DNA polymerase β, DNA topoisomerase I, and RNase H are associated with the mature viral minichromosomes and that DNA polymerase α is associated with replicative intermediate (RI) minichromosomes. In addition, calculations based on the specific activity of highly purified mouse cell DNA polymerase α (7) coupled with the quantitation of DNA molecules associated with polyoma minichromosomes (23) have allowed us to estimate the number of DNA polymerase α molecules per replicating polyoma minichromosome.

MATERIALS AND METHODS

Preparation of polyoma minichromosomes. Polyoma minichromosomes were prepared and sedimented in 5 to 20% sucrose gradients in buffer B (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], pH 7.3, 5 mM KCl, and 0.5 mM $MgCl_2$) as previously described (14). Sedimentation in 10 to 30% (wt/vol) glycerol gradients in buffer B was performed at 38,000 rpm for 90 min in a Beckman SW41 rotor with a 0.5-ml cushion of 50% sucrose at the bottom. After the gradients were collected from the bottom, equal portions of each fraction were quantified for radioactivity, using Beckman EP scintillation fluor. For most enzyme assays, the fractions were treated with 0.5 volume of buffer D (final concentration, 300 mM KCl, 20% glycerol, 2 mM dithioerythritol, and 50 mM Tris, pH 7.5) for 18 h before assay. When prela-

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beled in vivo, infected cells were incubated with 2.5 μCi of [^{14}C]thymidine (60 mCi/mmol; ICN) in 10 ml of medium for 30 or 60 min before harvest, as indicated in the figure legends.

Endogenous replication by polyoma minichromosomes from a glycerol gradient. DNA synthesis was measured in 90-μl of fractions from glycerol gradients in a total of 100 μl as described (14), using [^{3}H]dTTP (50 μCi/ml, 67.7 Ci/mmol; ICN).

DNA polymerase α assay. Assays contained 10 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 200 μg of bovine serum albumin per ml, 600 μg of activated calf thymus DNA per ml, 50 μM each dATP, dCTP, dGTP, and dTTP, and 20 μCi of [^{3}H]dTTP per ml in a total volume of 100 μl. This assay is based on conditions described by Benbow et al. (1) and measures DNA polymerase α activities (5, 7, 12). Incorporation of 1 pmol of deoxynucleotide monophosphate (dNMP) corresponds to 22 cpm.

DNA polymerase β assay. Assays contained 50 mM Tris-hydrochloride (pH 8.9), 0.4 mM $MnCl_2$, 300 μg of bovine serum albumin per ml, 120 mM KCl, polyadenylate [poly(A)]-oligodeoxythymidylate [oligo(dT_{12-18})] [50 μM poly(A) and 25 μM oligo(dT_{12-18})], 100 μM dTTP, and 75 μCi of [^{3}H]dTTP per ml in a total volume of 100 μl. This assay is based on conditions described by Joenje and Benbow (19) for DNA polymerase β. Incorporation of 1 pmol of dNMP corresponds to 100 cpm.

DNA polymerase γ assay. Assays contained 25 mM Tris-hydrochloride (pH 8.3), 50 mM K_2HPO_4 (pH 8.4), 0.5 mM $MnCl_2$, 2.5 mM dithioerythritol, 200 μg of bovine serum albumin per ml, 100 mM KCl, poly(A)-oligo(dT_{12-18}) [50 μM poly(A) and 25 μM oligo(dT_{12-18})], 50 μM dTTP, and 100 μCi of [^{3}H]dTTP per ml in a total volume of 100 μl. This assay is based on conditions described by Bertazzoni et al. (2). Incorporation of 1 pmol of dNMP corresponds to 440 cpm. The presence of 50 mM phosphate totally inhibits polymerase β activity (19, 22).

Assays were incubated at 37°C (DNA polymerase α) or 30°C (DNA polymerases β and γ) for 1 h, terminated, and precipitated with cold trichloroacetic acid; acid-insoluble [^{3}H]dNMP incorporation was determined essentially as described previously (1).

DNA topoisomerase I assay. DNA topoisomerase I activity was measured the method of Keller (20). Reaction mixtures contained, in a total volume of 50 μl: 25 mM Tris-hydrochloride (pH 7.5), 0.15 M NaCl, 0.2 mM EDTA, 0.5 μg of supercoiled ColE1 DNA (4), and 10 to 40 μl of sample. After incubation for 30 min at 30°C, the reaction was stopped by addition of sodium dodecyl sulfate, glycerol, and bromophenol blue to final concentrations of 1, 12.5, and 0.006%, respectively. The DNA was analyzed by electrophoresis at 10 V/cm in 1.0% agarose slab gels (3 by 15 by 20 mm). The running buffer and gel contained 40 mM Tris-acetate (pH 8.0), 1 mM EDTA, and 20 mM sodium acetate. After 18 h, the gels were stained with 0.5 μg of ethidium bromide per ml and photographed on a short-wavelength (302-nm) light box with Polaroid type 55 P/N film through a Kodak 23A red filter. One unit of activity is defined as the amount of enzyme that converts 1 μg of supercoiled ColE1 DNA (form I) to the relaxed form II in 30 min at 30°C.

RNase H assay. The assay of RNase H activity was based on the production of acid-soluble ^{3}H counts from [^{3}H]RNA hybridized to DNA (21). The reaction mixtures contained, in a total volume of 100 μl: 50 mM Tris-hydrochloride (pH 8.0), 9 mM $MgCl_2$, 250 μg of bovine serum albumin per ml, 1 mM β-mercaptoethanol, 17 × 10^3 cpm of poly[^{3}H](A) (5.4 Ci/mmol; New England Nuclear Corp.)·polydeoxythymidylate [poly(dT)] (P-L Biochemicals; 5:1 molar ratio of dT to A), and 50 μl of enzyme sample. After incubation for 15 min at 30°C, 0.5 ml of calf thymus DNA (5 mg/ml) was added followed by 0.5 ml of ice-cold 10% trichloroacetic acid. After 20 min at 4°C, the samples were centrifuged at 1,980 × *g* (20 min), and 0.5 ml was used to quantify radioactivity, using 4 ml of Aquasol. Less than 1% of the hybrid template was degraded by RNase A (Sigma Chemical Co.) in a range of concentrations from 25 × 10^{-6} to 25 U/ml, whereas unhybridized poly[^{3}H](A) was completely digested at all RNase A concentrations tested. Pancreatic DNase I (Sigma) at 8 ng/ml did not degrade the hybrid template.

RESULTS

Endogenous DNA synthesis by polyoma minichromosomes fractionated on a glycerol gradient. We have shown that a nuclear extract of infected 3T6 cells is capable of incorporating deoxynucleotide triphosphates in vitro (14). We now asked whether polyoma minichromosomes, further purified by gradient sedimentation, incorporated [^{3}H]dNMP in vitro in the absence of soluble factors present in the nuclear extract. Figure 1 shows fractionation of polyoma minichromosomes on a neutral 10 to 30% glycerol gradient. Endogenous DNA syn-

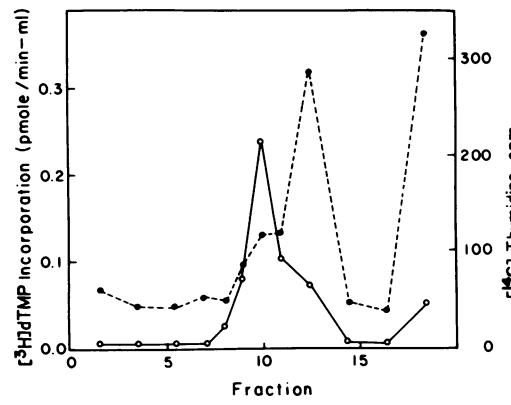

FIG. 1. *Endogenous DNA synthesis in fractions of a glycerol minichromosome gradient. Nuclear extract (0.5 ml) prepared from polyoma-infected cells at 27 h after infection was applied to a 10 to 30% glycerol gradient in buffer B. Two of the six plates of infected cells were labeled in vivo for 60 min with 0.25 μCi of [^{14}C]thymidine per ml (●), and 100-μl portions of each fraction was counted. Incorporation of [^{3}H]-dTTP in vitro (○) was determined as described in Materials and Methods.*

thesis was observed in fractions corresponding to the RI region of the gradient (fractions 8 through 11). Lower levels of synthesis were observed in fractions corresponding to sedimentation of mature minichromosomes which may have been due to elongation of early replicative intermediates (23). Approximately 50% of the activity applied to the gradient as nuclear extract was recovered.

Cosedimentation of DNA polymerase and DNA topoisomerase I activities with polyoma minichromosomes. Nuclear extract, prepared from cells labeled in vivo with [^{14}C]thymidine, was sedimented through a 5 to 20% sucrose gradient. The fractions were assayed for DNA polymerase α, β, and γ and DNA topoisomerase I activities. Polyoma DNA in each fraction was quantitated as described in the accompanying paper (23).

Figure 2A shows DNA polymerase α activity in each fraction. Of the total activity detected on the gradient, 18% sedimented with the RI and mature regions. The RI region had 1.25-fold more DNA polymerase α activity than the mature region. DNA polymerase β activity sedimented predominantly in the mature region, with a small peak in the RI region (Fig. 2B). This small peak of activity was not due to a low level of detection of DNA polymerase α activity, since less than 2% of DNA polymerase α activity is detected in the DNA polymerase β assay (12). Only very low levels of DNA polymerase γ activity were observed across the entire gradient (Fig. 2B).

DNA topoisomerase I activity, as measured by the relaxation of supercoiled ColE1 DNA (20), sedimented predominantly in the mature region of the gradient (Fig. 3). A small amount of activity was associated with the RI region. Dilutions of the peak fractions in the RI region (fraction 8) and the mature region (fraction 11) were compared for their ability to relax supercoiled ColE1 DNA. At a dilution of 1:8, fraction 11 had relaxed supercoiled ColE1 DNA to the same extent as an undiluted portion of fraction 8. It is interesting to note that most of the endogenous polyoma DNA in the mature region (fractions 11 and 12 in Fig. 3) was not relaxed. This may have been due to proteins which remained associated with the polyoma minichromosomes at the salt concentrations present in the DNA topoisomerase I assay (15, 20). These proteins were subsequently removed by treatment with sodium dodecyl sulfate before electrophoretic analysis.

Sedimentation of RNase H activity and polyoma minichromosomes. Polyoma minichromosomes were fractionated on a 10 to 30% glycerol gradient (Fig. 4). Most RNase H activity was found at the top of the gradient, but 11% of the activity sedimented with a peak of activity one fraction behind the peak of mature mini-

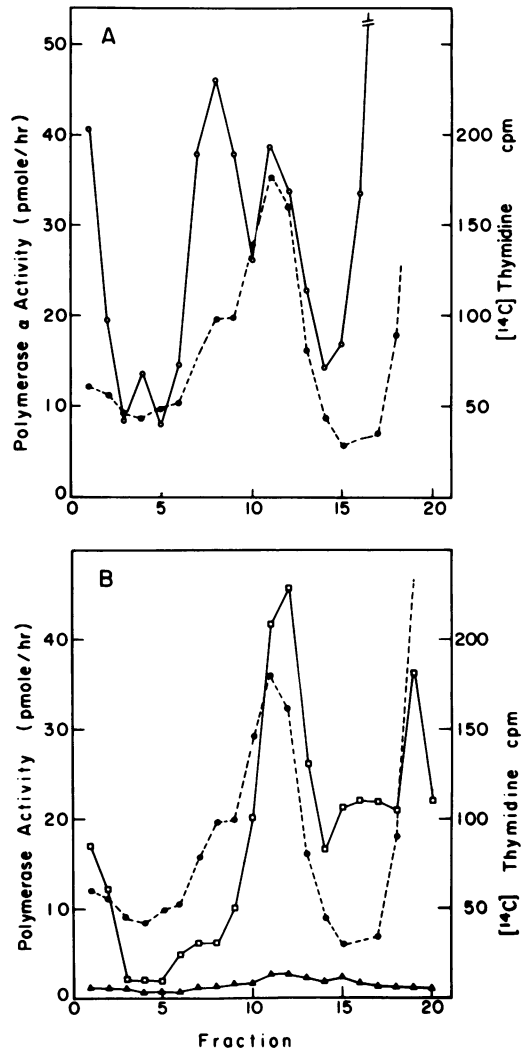

FIG. 2. *DNA polymerase activities in fractions of a sucrose minichromosome gradient. Nuclear extract (2.0 ml) prepared from polyoma-infected 3T6 cells at 27 h after infection was applied to a 5 to 25% sucrose gradient in buffer B. Sedimentation was performed at 25,000 rpm for 3 h in a Beckman SW27 rotor with a 2.0-ml cushion of 50% sucrose at the bottom. Two of the five plates of infected cells were labeled in vivo for 30 min with 0.25 μCi of [^{14}C]thymidine per ml (●), and 20-μl portions of each fraction were counted. Each fraction was adjusted to a final concentration of 0.3 M KCl, 50 mM Tris-hydrochloride (pH 7.5), 2 mM dithioerythritol, and 20% glycerol and assayed for (A) DNA polymerase α activity (○) and (B) DNA polymerase β (□) and γ (△) activities.*

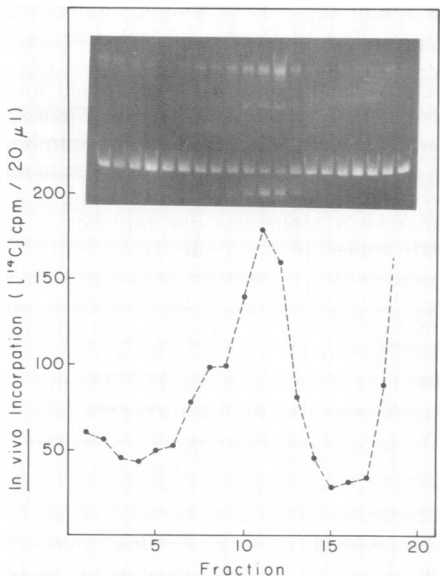

FIG. 3. *DNA topoisomerase I activity in fractions of a sucrose minichromosome gradient. Gradient fractions of 10 µl (see Fig. 2) were assayed for their ability to relax supercoiled ColE1 DNA. The peak of activity coincides with the peak of mature polyoma minichromosomes. After sodium dodecyl sulfate treatment, the polyoma DNA can be seen as bands migrating ahead of the ColE1 DNA for fractions 11, 12, and 13. The sedimentation profile for DNA labeled in vivo with [^{14}C]thymidine (●) is indicated.*

chromosomes (14). Treatment with 0.3 M KCl was necessary to detect RNase H activity cosedimenting with the minichromosomes, but not the activity present in the top of the gradient.

DISCUSSION

Polyoma minichromosomes sedimented through a glycerol gradient retained a limited ability to continue DNA synthesis in vitro (Fig. 1). These results imply cosedimentation of proteins essential for DNA replication with minichromosomes. The endogenous activity of purified minichromosomes can be increased by the addition of soluble factors (B. B. Gourlie and V. Pigiet, unpublished data; 3, 31). In this paper, we measured levels of five potential DNA replication enzyme activities cosedimenting with polyoma minichromosomes: DNA polymerases α, β, and γ, DNA topoisomerase I, and RNase H.

DNA polymerase α activity is considered the replicative polymerase activity based on a number of studies (2, 24, 34) and increases after infection with polyoma virus (35). Our results show that a significant amount of DNA polymerase α activity in the nuclear extract cosedimented with viral minichromosomes (Fig. 2A). In addition to the activity associated with RI minichromosomes (10% of the total), a significant amount of activity (8% of the total) sedimented with mature polyoma minichromosomes. The association of polymerase α with replicating minichromosomes has been observed by others for simian virus 40 (SV40) (8, 26, 31) and polyoma virus chromosomes (32). These previous studies, however, did not quantitate either the number of total and replicating viral molecules or the molecules of polymerase in each fraction.

When the amount of DNA polymerase α activity (Fig. 2A) was computed relative to the total number of polyoma DNA molecules (23; Table 1) in each gradient fraction, we found 10-fold more activity per molecule in the RI region than in the mature region (Fig. 5). This peak of activity per molecule occurred one fraction ahead of the peak of total DNA polymerase α activity (Fig. 2A) due to the smaller number of polyoma molecules and the high level of DNA polymerase α activity in fraction 7. By comparison, the activities of DNA polymerases β and γ per polyoma molecule were significantly lower and relatively constant across the gradient. The

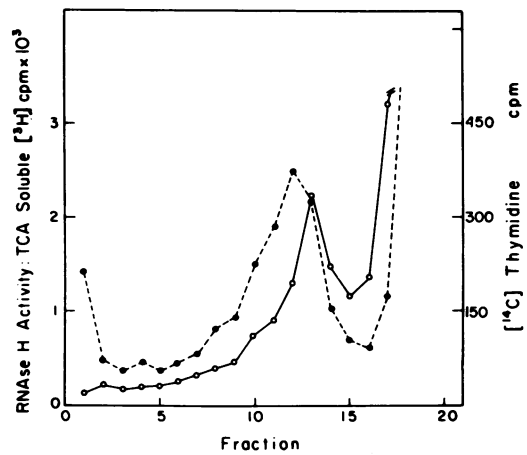

FIG. 4. *RNase H activity in fractions of a glycerol minichromosome gradient. The activity of RNase H (○) was measured in fractions of a 10 to 30% glycerol gradient (in buffer B) after centrifugation of 0.7 ml of nuclear extract. Before assay, equal portions of each gradient fraction were treated with 0.3 M KCl, 30 mM Tris-hydrochloride (pH 7.5), 2 mM dithioerythritol, and 20% glycerol. Activity was measured in 50-µl portions by release of trichloroacetic acid-soluble ^{3}H counts from the hybrid template poly[^{3}H](A)·poly(dT). One of the three plates of polyoma-infected 3T6 cells was labeled for 30 min before harvesting at 27 h after infection with 0.25 µCi of [^{14}C]thymidine per ml (●), and 30-µl portions of each fraction were counted.*

presence of DNA polymerase α in the mature region of the gradient (Fig. 2A) may have been due to its association with a class of very early replicative intermediates (23). Alternatively, DNA polymerase α may have been bound to nonreplicating form I minichromosomes.

We know the number of units of DNA polymerase α and the corresponding number of RI polyoma DNA molecules in each fraction (23; Table 1). By assuming the specific activity and molecular weight previously measured for homogeneous DNA polymerase α from mouse cells (7), we can calculate the average number of DNA polymerase α molecules cosedimenting with RI minichromosomes. The number of DNA polymerase α molecules per replicative intermediate molecule was found to be remarkably constant, with an average of 4.8 ± 0.9, even though the percentage of RI molecules varied ninefold between fractions 8 and 11 (Table 1). Some individual replicative intermediates, of course, may have no or considerably more polymerase molecules than the average. The ratio of DNA polymerase α molecules to RI molecules across the gradient is independent of the assumptions above, whereas the absolute number of polymerase molecules per replicative intermediate depends on those assumed values. One major conclusion is that the amount of DNA polymerase α in the mature region can be accounted for simply by the number of replicative intermediates found there (23). It should be pointed out that we did not distinguish between molecular species of DNA polymerase α (5, 7, 8, 12).

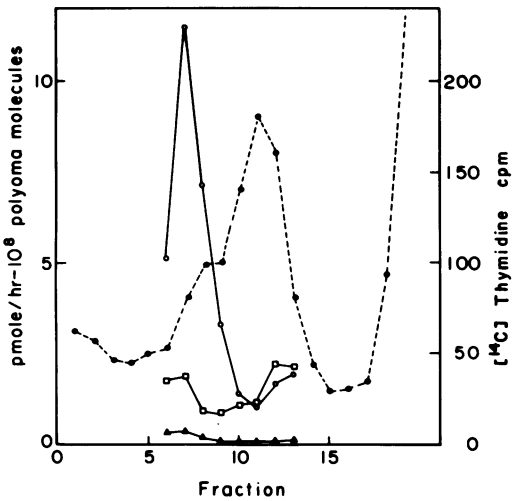

FIG. 5. *Specific activities of the DNA polymerases in fractions of a sucrose minichromosome gradient. Specific activities for DNA polymerase α (○), β (□), and γ (△) were calculated from the data in Fig. 2 and the accompanying paper (14). The sedimentation profile for DNA labeled in vivo with [¹⁴C]thymidine (●) (30-min pulse) is indicated.*

Contrary to the observations that little or no DNA polymerase β was found associated with SV40 minichromosomes (10, 26, 27), we found significant levels of DNA polymerase β activity sedimenting with mature polyoma minichro-

TABLE 1. *Molecular ratio of DNA polymerase to total and RI DNAs*[a]

Fraction	% RI[b]	Ratio					
		Pol α/total DNA[c]	Pol α/RI DNA	Pol β/total DNA[d]	Pol β/RI DNA	Pol γ/total DNA[e]	Pol γ/RI DNA
8	28.8	1.22	4.2	0.27	0.9	0.04	0.13
9	14.1	0.64	4.6	0.29	2.1	0.03	0.19
10	6.1	0.26	3.8	0.34	5.1	0.02	0.23
11	3.3	0.20	5.9	0.36	11.0	0.01	0.41
12	6.1	0.34	5.6	0.79	12.9	0.03	0.44

[a] The molecular ratios of DNA polymerase molecules per 10^8 polyoma molecules were calculated from the activity ratio in fractions of the sucrose gradient in Fig. 5. Fractions 8 through 12 were chosen because these fractions contained enough RI molecules for adequate statistical analysis.

[b] Derived from Krauss and Benbow (23).

[c] The number of DNA polymerase (Pol) α molecules was calculated from the specific activity (350 μmol/h per mg) and protomer molecular weight (1.9×10^5) observed by Chen et al. (7) for homogeneous mouse DNA polymerase α. To relate our determination to that observed by Chen et al. optimally activated calf thymus DNA was obtained from Bethesda Research Laboratories and compared with our activated calf thymus DNA. Our DNA gave 44% of the activity observed with the commercial template. The calculations presented take this correction factor into account.

[d] The number of DNA polymerase β molecules was calculated from the specific activity (380 μmol/h per mg) and molecular weight (4.5×10^4) observed by Joenje and Benbow (19) for *Xenopus laevis* oocyte DNA polymerase β.

[e] The number of DNA polymerase γ molecules was calculated from the specific activity (660 μmol/h per mg) and protomer molecular weight (4.5×10^4) observed by Yamaguchi et al. (36) for chicken embryo DNA polymerase γ.

mosomes (Fig. 2B). The constant number of DNA polymerase β molecules per total polyoma DNA molecule (Table 1) is consistent with the hypothesis that DNA polymerase β is involved in DNA repair (2, 34). In contrast to the constancy of the ratio of DNA polymerase α molecules to RI molecules (Table 1), the ratio of DNA polymerase β molecules to RI molecules varied 12-fold in fractions 8 through 12 (Table 1).

There was very little DNA polymerase γ (the presumptive mitochondrial DNA polymerase; 34) activity present in the nuclear extract, and it amounted to less than 1% of the total DNA polymerase activities found there. The small amount of activity which did cosediment with mature polyoma minichromosomes is consistent with the results of others using the SV40 system (10, 26). However, the low number of DNA polymerase γ molecules calculated per polyoma minichromosome (Table 1) and its lack of a clear role in nuclear DNA replication (3, 22) indicate that its presence may be adventitious.

Initial studies to quantitate the amount of DNA polymerase activities present in the nuclear extract relative to other fractions indicated that 18% of the total cellular DNA polymerase α activity was present in the nuclear extract, whereas 56% of DNA polymerase α activity was found in the cytoplasmic fraction remaining after preparation of nuclei for extraction. This is consistent with its tendency to leak out of nuclear preparations (11, 12). By contrast, the majority of DNA polymerase β and γ activities (58% for both) remained associated with the extracted nuclear pellet, whereas 2 and 9%, respectively, were found in the nuclear extract supernatant (data not shown).

DNA topoisomerase I activity sedimented in the mature region (fraction 11) and to a lesser extent in the RI region of the sucrose gradient (Fig. 3). Since fraction 8 contained only one-seventh the amount of polyoma DNA (23) and one-eighth the amount of DNA topoisomerase I activity, the ratio of DNA topoisomerase I activity per polyoma molecule was constant in those fractions. Although it has been reported that DNA topoisomerase I is not associated with SV40 chromatin (37), our results are consistent with other reports that DNA topoisomerase I does cosediment with SV40 minichromosomes (16, 20, 31). The function it performs in minichromosome systems is not known, but it may act as a topological swivel (29) or participate in nucleosome assembly (13).

RNase H activity (11% of the total) was found to sediment with polyoma minichromosomes (fractions 8 to 15). It is not clear whether this activity, sedimenting one fraction behind the peak of mature minichromosomes, is involved in polyoma replication. It is interesting that W. Keller (personal communication) has found that ribonucleoprotein particles sediment in this region of the gradient. However, the sedimentation of some RNase H activity in the RI and mature regions is consistent with a role for this enzyme in some stage of polyoma DNA metabolism such as excision of the RNA primers of Okazaki fragments (25) or initiation (17).

Our results are consistent with the hypothesis that enzymes involved in DNA metabolism cosediment and may be associated with polyoma minichromosomes. Of particular interest is the cosedimentation of DNA polymerase α with RI polyoma molecules. The ratio of DNA polymerase α molecules to the number of replicative intermediates in fractions of the gradient was found to be constant and, on the average, to be approximately five DNA polymerase α molecules per replicative intermediate. A simple model for these findings is the presence of one DNA polymerase α molecule on each of the leading strands of the replication forks and at least one polymerase molecule on the corresponding lagging strands.

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