Polyomavirus Minichromosomes: Associated DNA Topoisomerase II and DNA Ligase Activities

MARC R. KRAUSS, BRIAN B. GOURLIE, MARVIN L. BAYNE, AND ROBERT M. BENBOW*

Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

Received 25 May 1983/Accepted 4 October 1983

Polyomavirus minichromosomes were isolated and fractionated as described previously (B. B. Gourlie, M. R. Krauss, A. J. Buckler-White, R. M. Benbow, and V. Pigiet, *J. Virol.* **38**:805-814, 1981). Specific assays for DNA topoisomerase II and DNA ligase activity were carried out on each fraction. The enzymatic activity in each fraction was determined by quantitative electron microscopy and compared with the number of replicative intermediate and total polyomavirus DNA molecules in each fraction. DNA topoisomerase II activity cosedimented with polyomavirus replicative intermediate minichromosomes. DNA ligase activity cosedimented with mature polyomavirus minichromosomes.

The criterion of cosedimentation with polyomavirus replicative intermediate minichromosomes has been used as one line of evidence to implicate DNA polymerase α in polyomavirus DNA replication (5, 6, 9, 27, 28, 36). Using the soluble polyomavirus minichromosome system of Gourlie et al. (8), we have quantitated the activity of two additional enzymes cosedimenting with polyomavirus minichromosomes which may be involved in DNA replication: DNA topoisomerase II and DNA ligase.

DNA topoisomerase II has been implicated in DNA replication in procaryotes (reviewed in reference 7) and specifically in the initiation process in bacteriophage T4 DNA replication (20). DNA ligase has been similarly linked to DNA replication in procaryotes (reviewed by Kornberg in reference 16) and almost certainly must be involved in the joining of Okazaki fragments during polyomavirus DNA replication (22).

In this paper we develop quantitative electron microscopic assays for DNA topoisomerase II and DNA ligase activity. These assays are extensions of qualitative agarose gel electrophoresis assays for DNA topoisomerase II developed by Liu et al. (19) and for DNA ligase developed by Johnston and Nasmyth (12). Using these assays, we show that DNA topoisomerase II cosediments with replicative intermediate polyomavirus minichromosomes, whereas DNA ligase cosediments with mature polyomavirus minichromosomes.

MATERIALS AND METHODS

Preparation of polyomavirus minichromosomes. A single-plaque isolate of a large-plaque strain of polyomavirus (11, 37) was propagated at a low multiplicity of infection (10^{-5}) on primary baby mouse kidney cells. 3T6 mouse fibroblasts were seeded at 2.5×10^6 cells per 15-cm culture plate in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 5% calf serum (GIBCO) and grown at 37°C in a 5% CO₂ atmosphere. Cells were infected at a multiplicity of infection of ca. 15 on the second day after seeding (ca. 80% confluency).

At 28 h after infection, cell cultures were incubated *in vivo* with 20 μ Ci of [³H]thymidine (55 Ci/mmol; ICN Pharmaceuticals, Inc., Irvine, Calif.) in 10 ml of Dulbecco modified Eagle medium for 15 min. The cells were rinsed three times with 5 ml of hypotonic buffer A (10 mM HEPES [N-2-

hydroxyethylpiperazine-N'-2-ethanesulfonic acid], pH 7.8, 5 mM KCl, 0.5 mM MgCl₂, 0.5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride), transferred to a Dounce homogenizer, and disrupted with 10 strokes of a tight-fitting pestle.

Nuclei were collected by centrifugation ($3,000 \times g$ for 5 min) and extracted with hypotonic buffer A (0.5 ml per plate) at 0°C for 3 h (8, 34). Extracted nuclei were removed by centrifugation ($6,800 \times g$ for 15 min).

Sedimentation of polyomavirus minichromosomes. For analysis of DNA topoisomerase II (see graph, Fig. 1) and DNA ligase (see graph, Fig. 4) activities by agarose gel electrophoresis, 0.5 ml of nuclear extract (the equivalent of one culture plate) was applied to a 10-ml 5 to 20% linear sucrose gradient in buffer B (10 mM HEPES, pH 7.8, 5 mM KCl, and 0.5 mM MgCl₂) over a 0.5-ml cushion of 50% sucrose in buffer B. Sedimentation was for 90 min at 38,000 rpm ($246,000 \times g_{\max}$) in a Beckman SW41 rotor. Fractions of ca. 0.25 ml were collected from the top of the gradient.

For quantitation of DNA topoisomerase II and DNA ligase activities by electron microscopy, and for determination of the concentrations of replicative intermediate and total polyomavirus DNA molecules, 1.8 ml of nuclear extract (the equivalent of 3.6 culture plates) was applied to a 30-ml 5 to 20% linear sucrose gradient in buffer B over a 2-ml cushion of 50% sucrose in buffer B. Sedimentation was at 25,000 rpm ($111,000 \times g_{\max}$) for 3 h at 4°C in a Beckman SW27 rotor. One-ml fractions were collected as above.

DNA topoisomerase II assay. Bacteriophage P4 knotted DNA molecules (20 μ g/ml) (21) were incubated with 50 mM Tris-hydrochloride (pH 7.9)-120 mM KCl-10 mM MgCl₂-30 μ g of bovine serum albumin per ml-0.5 mM dithiothreitol-1.5 mM ATP-enzyme in a total volume of 20 μ l for 60 min (unless otherwise indicated) at 30°C (19). The reaction was terminated by adding 5 μ l of 250 mM EDTA (pH 6.8). The rate of formation of completely unknotted circular bacteriophage P4 DNA molecules (i.e., no observed intramolecular crossovers) was used to quantitate DNA topoisomerase II activity (M. R. Krauss, Ph.D. dissertation, The Johns Hopkins University, Baltimore, 1982). The unknotting of bacteriophage P4 DNA was monitored qualitatively by agarose gel electrophoresis (19). A 5- μ l sample of the reaction mixture was prepared for quantitative electron microscopy by using the aqueous conditions of Davis et al. (4) with a 1-min delay to allow for supercoil relaxation on the hypophase (17).

It should be noted that the knotted P4 DNA assay can be

* Corresponding author.

successfully used to assay for DNA topoisomerase II activity even in the presence of excess DNA topoisomerase I activity (19). This is because unknotting requires the topological passing of two crossing double-stranded DNA segments; DNA topoisomerase I activities which introduce transient single-stranded breaks cannot catalyze this reaction.

DNA ligase assay. ColE1 DNA (5.4 $\mu\text{g/ml}$) which had been singly nicked by *EcoRI* in the presence of ethidium bromide (29) was incubated with 50 mM Tris (pH 7.8)–10 mM MgCl_2 –50 μg of bovine serum albumin per ml–2 mM 2-mercaptoethanol–1 mM ATP–enzyme in a total volume of 20 μl for 30 min (unless otherwise indicated) at 30°C. The reaction was terminated by the addition of 5 μl of 250 mM EDTA (pH 6.8). DNA ligase activity was monitored qualitatively by the agarose gel assay of Johnston and Nasmyth (12). A 5- μl sample of the reaction mixture was prepared for quantitative electron microscopy in hyperphase containing 3.2 μg of ethidium bromide per ml and spread on hypophase containing 4 μg of ethidium bromide per ml. After 1 min on hypophase, the DNA was transferred to a grid and processed as in Davis et al. (4). The rate of formation of supercoiled DNA molecules (plus each supercoiled half of catenated dimers) was used to quantitate DNA ligase activity (M. R. Krauss, Ph.D. dissertation).

Quantitation of replicative intermediate and total polyomavirus DNA molecules. The number concentration (molecules per milliliter) of replicative intermediate and total polyomavirus DNA molecules was quantitated ($\pm 10\%$) relative to a standard of pXlr 11 plasmid DNA molecules as described by Krauss and Benbow (17).

RESULTS

Cosedimentation of DNA topoisomerase II activity with polyomavirus replicative intermediate minichromosomes. Polyomavirus minichromosomes were fractionated on a 5 to 20% linear sucrose gradient as described previously (8). Figure 1 illustrates the sedimentation profile of DNA topoisomerase II activity as monitored by agarose gel electrophoresis (19). Bacteriophage P4 knotted DNA incubated with fractions 16 and 18 (replicative intermediate region, see below) became fully unknotted, as did bacteriophage P4 knotted DNA incubated with fractions from the top of the gradient. Bacteriophage P4 DNA incubated with fractions 24 to 28 (mature or form I region, see below) was partially unknotted. Note that polyomavirus form I DNA was also visible as a faint band (position indicated) at the bottom of the gel in fractions 24 to 28. Control experiments showed that the smear in fractions 24 to 28 corresponded to knotted DNA and not to additional ethidium bromide staining material introduced with the polyomavirus minichromosomes. Bacteriophage P4 knotted DNA incubated with fractions 32 to 36 remained knotted. Partial unknotting was observed with fractions 6, 8, and 10, which sediment faster than polyomavirus minichromosomes.

Preliminary attempts to quantitate levels of unknotting activity in the replicative intermediate (fractions 14 and 16) and mature (fractions 22 and 24) regions by endpoint dilution as monitored by agarose gels proved inconclusive. Therefore, we developed a quantitative electron microscopic assay for unknotting of bacteriophage P4 DNA based on the formation of completely unknotted DNA molecules. Figure 2 illustrates a whole-field electron micrograph of knotted DNA (Fig. 2A) and DNA incubated with HeLa cell DNA topoisomerase II (Fig. 2B). Krauss (Ph.D. dissertation) established conditions in which the rate of formation of

completely unknotted bacteriophage P4 DNA molecules was a linear function of time of incubation and concentration of DNA topoisomerase II activity. The linear range of the assay was from 1 to 50 completely unknotted molecules per 100 molecules observed. It should be emphasized that the electron microscopic assay was linear in the range where the gel analyses were impossible to quantitate and became nonlinear above 50% complete unknotting (where the gel assay is more easily interpreted).

Figure 3A illustrates the sedimentation profile of unknotting activity in selected fractions of a polyomavirus minichromosome sucrose gradient measured by quantitative electron microscopy. Figure 3B illustrates the number concentration of replicative intermediate and total polyomavirus DNA molecules in each of these fractions, also measured by quantitative electron microscopy (17). As we showed previously (17), the replicating molecules in the mature region were predominantly early replicative intermediates. Almost all mid- and late-replicative intermediates were found in the replicative intermediate region. About 25% more unknotting activity was found in fraction 15, the peak of the replicative intermediate region, than in fraction 20 or 21, the peak of the mature (form I) region, even though the latter fractions had ninefold more total polyomavirus DNA molecules. From these data it is clear that a peak of unknotting activity cosedimented with polyomavirus replicative intermediate minichromosomes. Additional unknotting activity was found elsewhere on the gradient.

To establish that the unknotting activities in the replicative intermediate (fraction 16) and mature (fraction 21) regions were ATP-dependent, incubations were carried out for 2 h at 30°C. In the absence of ATP, medians of 0 and 0 completely unknotted molecules per 100 molecules were observed ($n = 900$) for each fraction. In the presence of 1 mM ATP, medians of 19 and 14 completely unknotted DNA molecules per 100 molecules were observed ($n = 900$) for each fraction. Agarose gel assays across the gradient gave similar results (data not shown).

To minimize the possibility that the unknotting activity was associated with RNA, the replicative intermediate (fractions 14 to 17) and mature (fractions 20 to 22) regions were pooled, dialyzed against buffer B, treated with 2 μg of heat-treated (80°C, 10 min) RNase A per ml for 15 min at 37°C, and resedimented on a 10-ml 5 to 20% linear sucrose gradient as described above. The pooled ATP-dependent unknotting activities (monitored by the quantitative electron microscopic assay) resedimented to the positions of replicative intermediate or mature polyomavirus minichromosomes, respectively. No activity (<1 completely unknotted molecule per 100 observed, $n = 500$) was found at the top of this gradient. By contrast, after incubation of minichromosomes with either 1 M KCl or pancreatic deoxyribonuclease (which disrupt minichromosome structure) no unknotting activity was found sedimenting in the positions of replicative intermediate or mature minichromosomes.

Samples of the pooled replicative intermediate and mature regions catalyzed ATP-dependent decatenation of *Crithidia fasciculata* kinetoplast DNA networks (data not shown) in the DNA topoisomerase II assay of Marini et al. (24) and exhibited an ATP-dependent increase in the rate of unwinding of supercoiled DNA when the Keller assay was used (14).

All of the above data are consistent with the hypothesis that ATP-dependent DNA topoisomerase II activity cosediments with polyomavirus replicative intermediate minichromosomes.

FIG. 1. DNA topoisomerase II activity in fractions of a polyomavirus minichromosome sucrose gradient: agarose gel analysis. The sedimentation position of mature (form I) polyomavirus minichromosomes labeled for 2 h in vivo was established by determining acid precipitable $[^3\text{H}]\text{dTMP}$ (●) in 10- μl samples of each fraction (8). The bottom of the gradient is fraction 1. Samples of 10 μl of each fraction were then assayed for DNA topoisomerase II activity as described in the text. Incubation was at 30°C for 20 h. A control experiment showed that the bacteriophage P4 DNA remained knotted and undegraded for at least 40 h at 30°C during incubation with these fractions in the absence of ATP. The right panel shows the migration of bacteriophage P4 knotted, fully unknotted, and linear DNA molecules in the agarose gels. (-) indicates input knotted P4 DNA incubated with buffer B. (+) indicates P4 DNA incubated with 20 U of HeLa cell DNA topoisomerase II.

FIG. 2. Quantitative electron microscopic analysis of DNA topoisomerase II activity. (A) Input knotted DNA molecules. Bacteriophage P4 knotted DNA molecules were spread by using the aqueous conditions of Davis et al. (4). In the field shown are 120 knotted and 1 unknotted ($\rightarrow$) DNA molecule out of a total of 121 molecules. The input DNA contained a median of 0 unknotted DNA molecules per 100 monomeric equivalents ($n = 500$ molecules). It should be emphasized that the knotted DNA molecules in these experiments are neither covalently closed nor supercoiled. (B) Bacteriophage P4 unknotted DNA molecules formed by incubating knotted bacteriophage P4 DNA with 3 U of purified HeLa cell DNA topoisomerase II (26) as described in the text. In the field shown are 11 knotted, 64 unknotted, and 2 dimeric molecules (total 79 monomeric equivalents). The product of this reaction exhibited a median value of 63 unknotted molecules per 100 monomer equivalents ($n = 500$ molecules).

FIG. 3. DNA topoisomerase II activity in fractions of a polyomavirus minichromosome sucrose gradient: electron microscopic analysis. (A) The sedimentation position of mature (form I) polyomavirus minichromosomes labeled for 2 h in vivo was established by determining acid precipitable $[^3\text{H}]\text{dTMP}$ in $10\text{-}\mu\text{l}$ samples of each fraction. The bottom of the gradient is fraction 1. Samples of $10\text{-}\mu\text{l}$ of each fraction were assayed for DNA topoisomerase II activity as described in the text. Incubation was at 30°C for 2 h. RI, Replicative intermediate. (B) The concentrations of replicative intermediate and total polyomavirus DNA molecules were determined for each fraction (17).

Cosedimentation of DNA ligase activity with mature (form I) polyomavirus minichromosomes. Polyomavirus minichromosomes were fractionated on a 5 to 20% linear sucrose gradient as described previously (8). Figure 4 illustrates the sedimentation profile of DNA ligase activity monitored by the agarose gel assay of Johnston and Nasmyth (12). DNA ligase activity was observed in the mature minichromosome region of the gradient (fractions 20, 22, and 24). Even higher levels of (presumably) free enzyme were observed at the top of the gradient (fractions 38 to 42). Much lower levels of DNA ligase activity were detected in the region of replicative intermediate minichromosomes. Preliminary attempts to quantitate DNA ligase activity in each fraction by endpoint dilution suggested that the level of DNA ligase activity in the mature (form I) region was an order of magnitude higher than in the replicative intermediate region. Accurate quantitation, however, was not possible by agarose gel assay. A quantitative electron microscopic assay for DNA ligase activity was, therefore, developed based on the rate of formation of covalently closed DNA molecules from singly nicked circular duplex DNA molecules. The ligated DNA molecules

assume the morphology of supercoiled DNA molecules when spread for electron microscopy in the presence of ethidium bromide. Figure 5 illustrates a whole-field electron micrograph of singly nicked ColE1 (Fig. 5A) and ligated ColE1 (Fig. 5B) DNA molecules spread in the presence of ethidium bromide. Extensive control experiments (M. R. Krauss, Ph.D. dissertation) established assay conditions in which the number of ligated molecules was a linear function of time of incubation and DNA ligase concentration. The linear range of the assay was from 1 to 25 ligated molecules counted.

Figure 6 illustrates the sedimentation profile of DNA ligase activity in selected fractions of a polyomavirus minichromosome sucrose gradient quantitated by electron microscopy. (The gradient is the same as that used in Fig. 3, from which the radioactivity profile was taken). Approximately eightfold more DNA ligase activity was found in the mature (form I) region than in the replicative intermediate region, in agreement with the qualitative observations made by agarose gel electrophoresis. From these data, it is clear that a peak of DNA ligase activity cosediments with mature (form I) polyomavirus minichromosomes.

To establish that the DNA ligase activity in the mature region was ATP-dependent, incubations were carried out with fraction 21 for 20 min at 30°C . In the absence of ATP, a median of 0 ligated molecules per 100 molecules was observed ($n = 900$). In the presence of 1 mM ATP, a median of 23 ligated molecules per 100 was observed ($n = 900$). Agarose gel assays across the gradient gave similar results (data not shown).

To minimize the possibility that the DNA ligase activity was associated with RNA, the mature region (fractions 20 to 23) was pooled, dialyzed against buffer B, treated with $2\text{-}\mu\text{g}$ of heat-treated RNase A per ml for 15 min, and resedimented as described above. ATP-dependent DNA ligase activity resedimented to the position of polyomavirus form I minichromosomes (a median of 8 ligated molecules per 100 molecules observed, $n = 900$, in peak fraction) with no activity found at the top of the gradient (a median of 0 ligated molecules per 100 molecules observed, $n = 900$). By contrast, after incubation of minichromosomes with either 1 M KCl or pancreatic deoxyribonuclease (which disrupts minichromosome structure), no DNA ligase activity was found sedimenting in the positions of replicative intermediate or mature minichromosomes.

Samples of the pooled mature region were also able to carry out ATP-dependent cyclization and end-to-end joining of ColE1 linear duplex DNA molecules (data not shown), two activities commonly associated with DNA ligase.

All of the data in this section are consistent with the hypothesis that ATP-dependent DNA ligase activity cosediments with mature (form I) polyomavirus minichromosomes.

DISCUSSION

Polyomavirus replicative intermediate minichromosomes sedimented through a glycerol gradient retain the ability to continue DNA synthesis in vitro (9, 38). This implies that the proteins essential for DNA replication cosediment with replicative intermediate minichromosomes. The purpose of this paper was to determine whether two potential DNA replication enzymes, DNA topoisomerase II and DNA ligase, cosediment with polyomavirus replicative intermediate minichromosomes.

DNA topoisomerase II (Fig. 1) and DNA ligase (Fig. 4) activities as measured by agarose gel electrophoresis were

FIG. 4. DNA ligase activity in fractions of a polyomavirus minichromosome sucrose gradient: agarose gel analysis. The sedimentation position of mature (form I) polyomavirus minichromosomes labeled for 2 h *in vivo* was established by determining incorporated $[^3\text{H}]\text{dTMP}$ (●) in 10- μl samples of each fraction. The bottom of the gradient is fraction 1. Samples of 10 μl of each fraction were assayed for DNA ligase activity as described in the text. Incubation was at 30°C for 10 min. Control experiments showed that no detectable ligated product was formed in the absence of ATP. The right panel shows the positions of ColE1 nicked, linear, and ligated DNA molecules. (–) indicates ColE1 singly nicked DNA incubated with buffer B. (+) indicates ColE1 singly nicked and incubated with 5 U of bacteriophage T4 DNA ligase.

observed to cosediment with polyomavirus minichromosomes. These activities also remained associated with polyomavirus minichromosomes during gel filtration on Bio-Gel A-5M and during precipitation of the minichromosomes. It was not clear from these gel assays, however, whether either activity exhibited preferential association with replicative intermediate minichromosomes. This was primarily due to the qualitative nature of the agarose gel assays.

We have previously shown that quantitative electron

microscopic assays were quite sensitive for measuring low levels of DNA replication (2) and DNA recombinase (1) activities in a complex background of competing reactions. The key to these assays was the identification by electron microscopy of unique structures which were specific to those enzyme (or multienzyme) activities. The assays for DNA topoisomerase II developed by Liu et al. (19) and for DNA ligase developed by Johnston and Nasmyth (12) also generate unique DNA structures which are in principle

FIG. 5. Quantitative electron microscopy of DNA ligase activity. (A) ColE1 singly nicked plasmid DNA molecules were spread by using the aqueous conditions of Davis et al. (4), with ethidium bromide as described in the text. In the field shown there are 0 supercoiled (form I) DNA molecules out of 54 monomeric equivalents. Seven linear (form III) DNA molecules are also visible. The input DNA contained a median value of 0 form I DNA molecules per 100 monomer equivalents ($n = 500$ molecules). (B) Ligated ColE1 plasmid DNA molecules. Singly nicked ColE1 DNA was incubated with 1.3 U of bacteriophage T4 DNA ligase for 1 h and then spread for electron microscopy in the presence of ethidium bromide as described in the text. In the field shown there are 31 supercoiled molecules and 1 catenated molecule containing a supercoiled monomer out of 41 monomeric equivalents. The ligated population exhibited a median value of 49 supercoiled DNA molecules per 100 monomer equivalents ($n = 500$ molecules).

FIG. 6. DNA ligase activity in fractions of a polyomavirus minichromosome sucrose gradient: electron microscopic analysis. Samples of 2.5 μ l of each fraction were assayed for DNA ligase activity as described in the text. Incubation was for 3 h at 30°C. The sedimentation position of mature form I minichromosomes in each fraction (taken from Fig. 3) is also shown. RI, Replicative intermediate. The bottom of the gradient is fraction 1.

amenable to analysis by quantitative electron microscopy. We opted to use this rather tedious method because preliminary attempts to quantitate these activities by agarose gel electrophoresis did not exhibit the requisite sensitivity and linear response in proportion to enzyme concentration.

Using highly purified HeLa cell DNA topoisomerase II activity and bacteriophage T4 DNA ligase activity, Krauss (Ph.D. dissertation) carried out an extensive series of control experiments to establish electron microscopic assays (Fig. 2 and 5) which were linear with respect to time and enzyme concentration. Using these assays, we have quantitated the DNA topoisomerase II (Fig. 3) and DNA ligase (Fig. 6) activities across polyomavirus minichromosome sucrose gradients. The number concentrations of replicative intermediate and total polyomavirus DNA molecules were also determined by electron microscopy as described previously (17). The ratios of enzyme activities to DNA concentration are shown in Table 1. When DNA topoisomerase II activity (arbitrary units based on the appearance of completely unknotted bacteriophage P4 DNA molecules) was computed relative to the total number of polyomavirus DNA molecules in each gradient fraction, we found 10-fold more activity per molecule in the replicative intermediate region than in the mature region. Moreover, when the activity was computed relative to the concentration of replicative intermediates, the ratio remained constant across the assayed portion of the gradient. This suggests that DNA topoisomerase II cosediments with replicative intermediate minichromosomes. By analogy with DNA polymerase α , this implicates DNA topoisomerase II in polyomavirus DNA replication. An

alternative hypothesis is that subtle factors may have contributed to a more rapid rate of topoisomerization in the replicative intermediate region, but we favor the straightforward hypothesis that there was a preferential association of DNA topoisomerase II with replicative intermediates.

When the activity of DNA ligase (ligations per hour) was computed relative to the total number of polyomavirus DNA molecules in each gradient fraction, we found slightly less activity in the replicative intermediate than in the mature region. However, when activity was computed relative to the concentration of replicative intermediates, we found 14-fold more activity in the mature than in the replicative intermediate region. This suggested that DNA ligase may be associated with (a subset of) mature minichromosomes and that the replicative DNA ligase may represent only a small fraction of the total DNA ligase. This is consistent with the results of Tseng and Goulian (35) who showed that the primary effect of adding cytosolic extracts to isolated replicating nuclei was to increase the efficiency of joining of Okazaki fragments.

A number of proteins have been shown to cosediment with partially purified papovavirus minichromosomes (reviewed in reference 25). Among these proteins three classes may potentially be distinguished: (i) enzymatic activities such as DNA polymerase α (6, 9), uracil-DNA glycosylase (18), and DNA topoisomerase II (this paper) which appear to preferentially cosediment with replicating minichromosomes; (ii) proteins and enzyme activities which appear to preferentially cosediment with mature minichromosomes (perhaps DNA polymerase β (9) and DNA ligase (this paper) fall in this class); and (iii) proteins such as cellular histones, viral proteins (VP 1 for example; reviewed in reference 25), SV40 T antigen (23, 30, 31), single-stranded DNA binding protein (28), and DNA topoisomerase I (9, 10, 15, 32, 36) which cosediment with both replicating and mature minichromosomes. The distinction between classes (ii) and (iii) is uncertain at present.

TABLE 1. Molecular ratio of DNA topoisomerase II and DNA ligase activities to total and replicative intermediate DNAs^a

Fraction	Topoisomerase II ^b Activity (Total)	Topoisomerase II Activity (RI) ^c	Ligase ^d Activity (Total)	Ligase Activity (RI)
13	1.2	4.4	0.6	2.2
14	1.7	4.2	0.7	1.7
15	2.9	5.1	0.9	1.6
16	1.3	4.4	0.7	2.3
17	0.84	3.6	0.7	3.2
18	0.75	4.0	0.8	4.4
19	0.33	3.7	0.9	9.4
20	0.09	3.7	1.0	20
21	0.26	4.4	1.2	21
22	0.27	5.3	1.1	23
23	0.40	4.0	0.9	10
24	0.75	12.5	0.8	14

^a The units in this table are arbitrary (activity divided by number of molecules). When the specific activities of homogeneous 3T3 DNA topoisomerase II and DNA ligase activities are determined these values can be converted to the number of enzyme molecules per polyomavirus DNA molecule as we did for DNA polymerase α (9).

^b DNA topoisomerase II activity is defined as the number concentration of unknotted DNA molecules created after 60 min at 37°C.

^c RI, Replicative intermediate.

^d DNA ligase activity is defined as the number concentration of supercoiled molecules created after 3 h at 30°C.

Our results are consistent with the hypothesis that enzymes involved in DNA metabolism cosediment and may be associated with polyomavirus minichromosomes. DNA topoisomerase II activity appeared to cosediment preferentially with polyomavirus replicative intermediate minichromosomes. It was perhaps surprising that DNA ligase, an enzyme which also seemingly has an important role in DNA replication, was not found associated with replicative intermediate minichromosomes. To the extent that one can extrapolate from cosedimentation studies, we would postulate that DNA polymerase α , uracil-DNA glycosylase, DNA topoisomerase II, and DNA primase (which appears to be tightly associated with DNA polymerase α [3, 13, 33, 39]) are associated (with other proteins) to form a multienzyme replication complex, whereas DNA ligase and DNA polymerase β are not.

ACKNOWLEDGMENTS

We are extraordinarily grateful to Leroy F. Liu for repeated generous gifts of bacteriophage knotted P4 DNA and HeLa cell DNA topoisomerase II, for continuing advice on DNA topoisomerases, and for his exemplary enthusiasm. We also thank Vincent Pigiet, in whose laboratory the polyomavirus minichromosomes were grown, Deborah Stowers for excellent technical assistance, and Leroy Liu, Karen Beemon, Pam Hines, Michelle Gaudette, and Alayne Senior for comments on the manuscript.

This research was supported by Public Health Service grants GM-23813, GM-25970, GM-29490, GM-00366, and CA-06304 from the National Institutes of Health.

LITERATURE CITED

- Benbow, R. M., and M. R. Krauss. 1977. Recombinant DNA formation in a cell-free system from *Xenopus laevis* eggs. *Cell* 12:191-204.
- Benbow, R. M., M. R. Krauss, and R. H. Reeder. 1978. DNA synthesis in a multienzyme system from *Xenopus laevis* eggs. *Cell* 13:307-318.
- Conaway, R. C., and I. R. Lehman. 1982. A DNA primase activity associated with DNA polymerase α from *Drosophila melanogaster* embryos. *Proc. Natl. Acad. Sci. U.S.A.* 79:2523-2527.
- Davis, R. W., M. Simon, and N. Davidson. 1971. Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. *Methods Enzymol.* 21D:413-428.
- DePamphilis, M. L., S. Anderson, R. Bar-Shavit, E. Collins, H. Edenberg, T. Herman, B. Karas, G. Kaufmann, H. Krokan, E. Shelton, R. Su, D. Tapper, and P. M. Wasserman. 1979. Replication and structure of simian virus 40 chromosomes. *Cold Spring Harbor Symp. Quant. Biol.* 43:679-692.
- Edenberg, H. J., S. Anderson, and M. L. DePamphilis. 1978. Involvement of DNA polymerase α in simian virus 40 DNA replication. *J. Biol. Chem.* 253:3273-3280.
- Gellert, M. 1981. DNA topoisomerases. *Annu. Rev. Biochem.* 50:879-910.
- Gourlie, B. B., M. R. Krauss, A. J. Buckler-White, R. M. Benbow, and V. Pigiet. 1981. Polyoma virus minichromosomes: a soluble in vitro replication system. *J. Virol.* 38:805-814.
- Gourlie, B. B., V. Pigiet, C. B. Breaux, M. R. Krauss, C. R. King, and R. M. Benbow. 1981. Polyoma virus minichromosomes: associated enzyme activities. *J. Virol.* 38:826-832.
- Hamelin, C., and M. Yaniv. 1979. Nicking-closing enzyme is associated with SV40 DNA *in vivo* as a sodium dodecyl sulfate-resistant complex. *Nucleic Acids Res.* 7:679-687.
- Israel, M. A., H. W. Chan, W. P. Rowe, and M. A. Martin. 1979. Molecular cloning of polyoma virus DNA in *Escherichia coli*: plasmid vector system. *Science* 203:883-887.
- Johnston, L. H., and K. A. Nasmyth. 1978. *Saccharomyces cerevisiae* cell cycle mutant *cdc 9* is defective in DNA ligase. *Nature (London)* 274:891-893.
- Kaufmann, G., and H. H. Falk. 1982. An oligoribonucleotide polymerase from SV40-infected cells with properties of a primase. *Nucleic Acids Res.* 10:2309-2321.
- Keller, W. 1975. Characterization of purified DNA-relaxing enzyme from human tissue culture cells. *Proc. Natl. Acad. Sci. U.S.A.* 72:2550-2554.
- Keller, W., U. Muller, I. Eicken, I. Wendel, and H. Zentgraf. 1977. Biochemical and ultrastructural analysis of SV40 chromatin. *Cold Spring Harbor Symp. Quant. Biol.* 42:227-244.
- Kornberg, A. 1980. DNA synthesis. p. 577. W. H. Freeman & Co., Publishers, San Francisco.
- Krauss, M. R., and R. M. Benbow. 1981. Polyoma virus minichromosomes: associated DNA molecules. *J. Virol.* 38:815-825.
- Krokan, H. 1981. Preferential association of uracil-DNA glycosylase activity with replicating SV40 minichromosomes. *FEBS Lett.* 133:89-91.
- Liu, L. F., J. L. Davis, and R. Calendar. 1981. Novel topologically knotted DNA from bacteriophage P4 capsids: studies with DNA topoisomerases. *Nucleic Acids Res.* 9:3979-3989.
- Liu, L. F., C. C. Liu, and B. M. Alberts. 1979. T4 DNA topoisomerase: a new ATP-dependent enzyme essential for initiation of T4 bacteriophage DNA replication. *Nature (London)* 281:456-461.
- Liu, L. F., L. Perkocha, R. Calendar, and J. C. Wang. 1981. Knotted DNA from bacteriophage capsids. *Proc. Natl. Acad. Sci. U.S.A.* 78:5498-5502.
- Magnusson, G., V. Pigiet, E. L. Winnacker, R. Abrams, and P. Reichard. 1973. RNA linked short DNA fragments during polyoma replication. *Proc. Natl. Acad. Sci. U.S.A.* 70:412-416.
- Mann, K., and T. Hunter. 1979. Association of simian virus 40 T antigen with simian virus 40 nucleoprotein complexes. *J. Virol.* 29:232-241.
- Marini, J. C., K. G. Miller, and P. T. Englund. 1980. Decatenation of kinetoplast DNA by topoisomerases. *J. Biol. Chem.* 255:4976-4979.
- Milavetz, B. I., L. D. Spotila, R. Thomas, and J. A. Huberman. 1980. Two-dimensional analysis of proteins sedimenting with simian virus 40 chromosomes. *J. Virol.* 35:854-864.
- Miller, K. G., L. F. Liu, and P. T. Englund. 1981. A homogeneous type II DNA topoisomerase from HeLa cell nuclei. *J. Biol. Chem.* 256:9334-9339.
- Otto, B., and E. Fanning. 1978. DNA polymerase- α is associated with replicating SV40 nucleoprotein complexes. *Nucleic Acids Res.* 5:1715-1728.
- Otto, B., E. Fanning, and A. Richter. 1979. DNA polymerases and a single-strand specific DNA-binding protein associated with simian virus 40 nucleoprotein complexes. *Cold Spring Harbor Symp. Quant. Biol.* 43:705-708.
- Parker, R. C., R. M. Watson, and J. Vinograd. 1977. Mapping of closed circular DNAs by cleavage with restriction endonucleases and calibration by agarose gel electrophoresis. *Proc. Natl. Acad. Sci. U.S.A.* 74:851-855.
- Reiser, J., J. Renart, L. V. Crawford, and G. R. Stark. 1980. Specific association of simian virus 40 tumor antigen with simian virus 40 chromatin. *J. Virol.* 33:78-87.
- Segawa, M., S. Sugano, and N. Yamaguchi. 1980. Association of simian virus 40 T antigen with replicating nucleoprotein complexes of simian virus 40. *J. Virol.* 35:320-330.
- Sen, A., and A. J. Levine. 1974. SV40 nucleoprotein complex activity unwinds superhelical turns in SV40 DNA. *Nature (London)* 249:343-344.
- Shioda, M., E. M. Nelson, M. L. Bayne, and R. M. Benbow. 1982. DNA primase activity associated with DNA polymerase α from *Xenopus laevis* ovaries. *Proc. Natl. Acad. Sci. U.S.A.* 79:7209-7213.
- Su, R. T., and M. L. DePamphilis. 1978. Simian virus 40 DNA replication in isolated replicating viral chromosomes. *J. Virol.* 28:53-65.
- Tseng, B. Y., and M. Goulian. 1975. DNA synthesis in human lymphocytes: intermediates in DNA synthesis. *in vitro* and *in vivo*. *J. Mol. Biol.* 99:317-337.
- Tsubota, Y., M. A. Waqar, J. F. Burke, B. I. Milavetz, M. J.

- Evans, D. Kowalski, and J. A. Huberman. 1978. Association of enzymes with replicating and nonreplicating simian virus 40 chromosomes. *Cold Spring Harbor Symp. Quant. Biol.* **43**:693–704.
37. Vogt, M., and R. Dulbecco. 1962. Studies on cells rendered neoplastic by polyoma virus: the problem of the presence of virus-related materials. *Virology* **16**:41–51.
38. Waldeck, W., U. Spaeren, G. Mastromei, R. Eliasson, and P. Reichard. 1979. Replication of polyoma DNA in nuclear extracts and nucleoprotein complexes. *J. Mol. Biol.* **135**:675–689.
39. Yagura, T., T. Koza, and T. Seno. 1982. Mouse DNA polymerase accompanied by a novel RNA polymerase activity: purification and partial characterization. *J. Biochem. (Tokyo)* **91**:607–618.