Polyoma Virus Minichromosomes: Characterization of the Products of In Vitro DNA Synthesis†

BRIAN B. GOURLIE AND VINCENT P. PIGIET*

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

Received 28 July 1982/Accepted 12 October 1982

A two-dimensional (neutral-alkali) agarose gel electrophoretic system was used to separate three families of replicative intermediate (RI) polyoma virus DNA molecules (form I, form II, and form III RIs). Two of these families, form II and III RIs, are the result of artifactual nicking of one of the parental strands of supercoiled RIs (form I RIs) during in vitro replication of soluble minichromosomes. Kinetic studies in vitro showed that the nicked RIs serve as templates for limited DNA synthesis. The nicked species are not converted into normal products, however. The nicking reaction, which appears to be specific for the parental strands, is dependent on magnesium ions and occurs concurrently with the in vitro synthesis of DNA.

Papovavirus minichromosomes have served as an excellent model system to study the structure and replication of eucaryotic chromatin. The technical advantages of this system derive from the small well-defined genome (7), the dependence on the replication machinery of the host (2, 3, 16), and the ability to carry out limited DNA synthesis in a completely soluble system (5, 13, 16). The utility of such an in vitro system, however, depends on its ability to faithfully mimic the in vivo processes. In an earlier study we characterized an in vitro replication system, using soluble polyoma virus minichromosomes (5), and identified the major labeled products as RIa and RIb, which migrated on a 1.4% agarose gel slower than open circular (form II) DNA. Electron microscopy suggested that these species were nicked forms of the normally supercoiled replicative intermediates (form I RIs).

By two-dimensional (neutral-alkali) agarose gel electrophoresis (14), we have now identified these major products as two families of nicked RIs. One family consists of relaxed or nonsupercoiled RIs (form II), which arise due to a single-stranded break in either the replicated or the unreplicated region. The other family consists of RIs containing a circle with a linear, double-stranded tail (form III RI) and is thought to arise due to a single-stranded break in a parental strand at the replication fork. The kinetics of appearance in vitro and the electrophoretic migration properties of these species indicate that the form III RIs can be derived from form II RIs by fork elongation to a nick in the unreplicated region. With extended incubation in vitro the

broken parental strand can be ligated to the daughter strand, resulting in a rolling-circle-type molecule.

Analysis of experimental conditions for isolation and incubation of the minichromosomes has led us to conclude that nicking of the parent strands results from a magnesium-dependent reaction, possibly an aberrant topoisomerase reaction.

MATERIALS AND METHODS

Preparation of polyoma minichromosomes. Mouse 3T6 cells were infected as described previously (5) with a large-plaque strain of polyoma virus grown from a single-plaque isolate at low multiplicity (10^{-3}) on baby mouse kidney cells. Infected cells were harvested at 26 h postinfection. Where applicable, viral DNA was labeled in vivo with [3 H]thymidine (100 μ Ci in 1 ml of medium; 55 Ci/mmol; Amersham Corp.) for 5 min (37°C) immediately before cell harvest. Nuclear extracts containing soluble minichromosomes were prepared from nuclei as follows. Infected-cell monolayers were rinsed three times with ice-cold hypotonic buffer A (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], pH 7.8, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and drained. The cells were removed with a rubber scraper, transferred to an ice-cold Dounce homogenizer, and disrupted with 10 strokes of a tight-fitting pestle. Nuclei were pelleted at $3,000 \times g$ in a Sorvall type SS-34 rotor for 5 min. The supernatant was removed, and the nuclear pellet was suspended in buffer A (0.5 ml per 15-cm plate) and extracted at 0°C for 2 h. Nuclei were removed by centrifugation ($6,800 \times g$) for 15 min, and the supernatant solution containing soluble polyoma minichromosomes was used directly for further study. Where noted, $MgCl_2$ was omitted from buffer A.

In vitro DNA synthesis. In vitro viral DNA synthesis was performed as previously described (5) in a total

† Contribution no. 1185 from the McCollum Pratt Institute and the Department of Biology.

volume of 100 μ l, including 4 μ mol of NaCl, 0.5 μ mol of $MgCl_2$, 0.5 μ mol of EDTA, 0.4 μ mol of phosphoenolpyruvate, 0.2 μ mol of ATP, 3 IU of pyruvate kinase per ml (Calbiochem), 100 μ mol each of dATP, dCTP, and dTTP, and 50 μ Ci of [α - ^{32}P]dGTP (300 Ci/mmol; Amersham). Acid-insoluble radioactivity was determined as previously described (5).

Two-dimensional agarose gel electrophoresis of viral DNA. Viral DNA was prepared either directly from infected cells by the method of Hirt (8) or from samples of nuclear extract treated with 0.6% sodium dodecyl sulfate–10 mM Tris-hydrochloride–10 mM EDTA (pH 7.6) for 20 min at 30°C, followed by extraction once with phenol and twice with chloroform-isoamyl alcohol (20:1).

Electrophoresis in the first (neutral) dimension was performed submerged, using 0.8% (wt/vol) agarose (Bethesda Research Laboratories) slab gels (18 by 10 by 0.3 cm) in 40 mM Tris-hydrochloride–5 mM sodium acetate–1 mM EDTA (pH 7.6) for 10 h at 1.75 V/cm and 8°C.

For ^{32}P -labeled samples, autoradiography was performed on wet gels at 4°C, using Kodak X-Omat R film. For 3H -labeled samples, duplicate lanes were prepared for fluorography as described below.

Electrophoresis in the second (alkaline) dimension was performed as described by Sundin and Varshavsky (14). Strips of the first-dimension gel were soaked for 2 h with four changes of alkaline buffer [30 mM NaOH, 2 mM EDTA, 1 mM ethylene glycol-bis(β -aminoethyl ether)- N,N -tetraacetic acid] containing 5 μ g of bromophenol blue per ml. The first-dimension strips (0.5 cm wide) were then placed horizontally into slots in the second-dimension alkaline gel (1.5% agarose; 18 by 10 by 0.3 cm) and immobilized with 0.4% (wt/vol) agarose in alkaline buffer.

Electrophoresis was performed submerged for 20 h at 2.5 V/cm. Gels containing ^{32}P -labeled material were dried onto filter paper before autoradiography. Gels containing 3H -labeled material were treated with En 3 Hance (Beckman Instruments, Inc.) and washed as described by the manufacturer before fluorography, using Kodak X-Omat R film.

Re-electrophoresis of RIa and RIb. Sections of 1.4% agarose gels corresponding to the migration position of RIa and RIb bands (detected by wet autoradiography) were excised and dissolved in 5 volumes of saturated potassium iodide–10 mM sodium phosphate–1 mM sodium thiosulfate (pH 7.0). DNA was purified by the glass-fiber filter-binding method (17) and re-electrophoresed on 1.4% agarose.

RESULTS

Kinetics of labeling RIa and RIb in vitro.

Earlier in vitro studies (5) showed that soluble polyoma minichromosomes incorporated α - ^{32}P -labeled deoxynucleotide triphosphates predominantly into species migrating as bands designated RIa and RIb, separable by electrophoresis on 1.4% agarose gels. Detailed analysis presented here demonstrates the kinetic relationship between the species migrating in these bands. Figure 1A illustrates that a spectrum of products migrated between the positions corresponding to superhelical form I molecules at the bottom and

the RIs, approaching completion (i.e., late Cairns structures) at the top of the gel. In addition, there are the two prominent bands corresponding to RIa and RIb. The percentage of the total label incorporated into the species migrating with the individual bands shows a rapid, initial labeling of RIa, followed by a progressive increase in the formation of RIb (Fig. 1B). Extended incubation (3 h) resulted in a limited amount of labeling of supercoiled form I molecules (<10%). These kinetics are consistent with RIa as a precursor of RIb, but the data could not exclude the independent generation of RIb from the spectrum of RIs migrating between the positions of form I and the late Cairns structures. Pulse-chase kinetic experiments (data not shown) supported the first interpretation. Direct demonstration of the derivation of RIb from RIa was obtained by extraction of each band from the agarose gel followed by re-electrophoresis. Figure 2 shows that whereas re-electrophoresis of the extracted RIa band generated a significant amount of RIb (~22%), re-electrophoresis did not affect migration of the extracted RIb. This indicated that RIa was converted to RIb by handling and that RIb was a product of RIa degradation in the system.

In vivo labeled RIs are converted into RIa and RIb in vitro. Nuclear extract was prepared from polyoma virus-infected cells and incubated in vivo with [3H]thymidine for 5 min to selectively label the RI DNA (13). The nuclear extract containing the minichromosomes was then incubated in vitro with [α - ^{32}P]dGTP. Figure 3 (top) shows the kinetics of incorporation of [^{32}P]dGMP into acid-precipitable material in vitro. The amount of [3H]thymidine label remained unchanged throughout the incubation, indicating no further incorporation after preparation of the minichromosomes and no degradative loss of the in vivo labeled DNA.

Electrophoretic analysis showed that before in vitro incubation the in vivo [3H]thymidine label was predominantly in the RIa region (0 min; Fig. 3, bottom). This result is consistent with the accumulation of late Cairns structures, as noted previously for both polyoma virus and simian virus 40 (13, 19). There was also considerable 3H label distributed between the form I position and the migration position of RIa. After incubation for 1 min in vitro, ^{32}P label appeared predominantly in the region of RIa, with some label distributed in the migration positions corresponding to RIb and form II. After incubation for 15 min, ^{32}P label appeared in the region of form I and in the same relative distribution as the 3H label in the other regions of the gel (15 min). Prolonged incubation (60 min) resulted in a progressively greater proportion of both 3H and ^{32}P labels migrating in the position of RIb.

FIG. 1. Kinetics of labeling of RIa and RIb. Nuclear extract was incubated with [α - 32 P]dGTP as described in the text. At the indicated times, 50- μ l aliquots were treated with 0.6% sodium dodecyl sulfate–10 mM EDTA–Tris-hydrochloride (pH 7.6) and then phenol extracted. Electrophoresis on 1.4% agarose was performed at 5 V/cm for 18 h. The autoradiograph of the dried gel is shown in (A). Integration of the autoradiographic intensities was performed with a Helena Quick Scan R and D to determine the relative amount of label corresponding to RIa (●), RIb (■), and form II (▲) (B). The length of exposure of the 2-min time point was increased three times to enhance the autoradiographic image in (A).

Furthermore, as the incubation time progressed, relatively less label appeared in the migration region between RIa and RIb, and the band widths of RIa and RIb became progressively narrower. This result is consistent with the maturation and conversion of the spectrum of supercoiled RIs into RI species migrating predominantly in the RIb position.

Two-dimensional agarose gel electrophoresis of in vitro products. Upon decreasing the agarose concentration from 1.4 to 0.8%, the bands corresponding to the RIa and RIb species became broader and more diffuse. Furthermore, when electrophoresis was carried out in the second dimension in alkali, the RIa and RIb species (distinguished as relatively discrete bands on 1.4% agarose gels) now appeared as a spectrum (or family) of daughter strands which varied in length from nearly full-length molecules (5,200 bases) to oligonucleotides (~500 bases) (Fig. 4D).

The products of in vitro DNA synthesis in the nuclear extract were examined by two-dimensional (neutral-alkali) agarose gel electrophoresis after various lengths of incubation time in vitro (Fig. 4). At the earliest incubation time (5 min) three families of RIs were clearly apparent. One family extended from the position of the late Cairns structures (upper left, Fig. 4D) to the position of form I (lower right, Fig. 4D); the second family extended from the position of the

late Cairns structures to the position of form II; and a third family extended from somewhat ahead of the position of the late Cairns structures to the position of form II. We refer to these families as the form I RI, II RI, and III RI families, respectively (see Fig. 4D). With longer incubation times in vitro, the relative amount of isotope in the form I RI family decreased, with a concomitant increase in intensity of the form II and III RI families. Throughout the time course, isotope labeling of the form I and II species also increased. Supercoiled molecules of increasing superhelical density (i.e., Keller bands; 10) were also apparent (particularly at longer incubation times) and extended from the respective positions of form II to form I. In addition, a family of daughter strands of greater than unit length extended from the position above the longest form III RI position and intercepted the position of the linear single strands (labeled ss1) which derive from form II molecules. This latter family may represent form III RI molecules, one of whose daughter strands was ligated to the broken parental strand. This family would be identical to the products of replication by a rolling-circle mechanism (see Discussion).

Two-dimensional analysis of in vivo products. Two-dimensional (neutral-alkali) agarose gel electrophoresis of papovavirus DNA RIs were originally described by Sundin and Varshavsky (14), using simian virus 40 DNA pulse-labeled in

FIG. 2. Electrophoresis of extracted RIa and RIb. DNA from agarose gel slices containing RIa or RIb was eluted by the glass-fiber filter-binding method (17), ethanol precipitated, and re-electrophoresed on 1.4% agarose along with total nuclear extract (NE) as described in the legend to Fig. 1.

vivo with [^3H]thymidine. Their results showed only one continuous family of labeled daughter strands extending from the position of the late Cairns structures to the position of form I molecules, consistent with our description of the form I RI family. We therefore asked whether our *in vitro* incubation conditions were producing the form II and III RI families.

Polyoma virus-infected cells were labeled briefly *in vivo* with [^3H]thymidine for 5 min to label only RIs. Viral DNA was isolated from cells either with detergent, using the method of Hirt (8), or as a nuclear extract yielding minichromosomes (5). The nuclear extract was further incubated for 30 min *in vitro* in the complete replication assay mix containing unlabeled deoxynucleotide triphosphates. When analyzed by fluorography after two-dimensional gel electrophoresis, the form I RI family was the predominant *in vivo* labeled product in both the Hirt extract and the unincubated nuclear extract (Fig. 5A and B). This indicated that preparation

of minichromosomes was not responsible for the loss or conversion of the form I RI family. However, after 30 min of incubation in the *in vitro* assay mix, the form I RI family disappeared and form II and III RI families now appeared as the prominent species (Fig. 5C). Also, two additional families appeared with

FIG. 3. Fate of *in vivo* [^3H]thymidine pulse after *in vitro* incubation with [$\alpha\text{-}^{32}\text{P}$]dGTP. Nuclear extract was prepared from polyoma virus-infected cells which had been incubated with [^3H]thymidine (100 $\mu\text{Ci}/\text{ml}$) for 5 min before extraction ($\bullet$). The nuclear extract was then incubated *in vitro* with [$\alpha\text{-}^{32}\text{P}$]dGTP at 30°C ($\circ$). At the indicated times, aliquots were acid precipitated to determine isotope incorporation (top) or prepared for electrophoresis on 1.4% agarose as described in the legend to Fig. 1. After electrophoresis, the gel was cut into 1-mm slices, and the ^{32}P (---) and ^3H (—) labels were quantified in Beckman HP fluor (bottom).

FIG. 4. Two-dimensional (neutral-alkali) agarose gel electrophoresis of in vitro labeled products. DNA from nuclear extract, incubated in vitro with $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$ at 30°C for 5 (A), 15 (B), and 60 (C) min, was subjected to electrophoresis (4 h at 5 V/cm) in 0.8% agarose in the first dimension. Autoradiography was performed on the wet gel, and the individual lanes were cut out, soaked in alkali for 2 h, and then cast into an alkaline agarose (1.5%) slab gel. After electrophoresis at 1.75 V/cm the gel was dried and subjected to autoradiography. The exposure of the first-dimension gel is placed above the second dimension for reference. The diagram (D) indicates the positions in the first (neutral) dimension of the late Cairns (LC) species, forms I, II, and III. In the second (alkali) dimension, the positions of single-strand circles (ssc), single-strand linear molecules (ssl), the B and C catenated dimer families (B, C) (14), the supercoiled intermediate or Keller bands (KB), the rolling-circle family of linear molecules (RC), and the form I, II, and III RI families (I-RI, II-RI, III-RI) are shown.

daughter strands greater than unit length (see Fig. 4 and 6; see below). Nuclear extract incubated at 30°C for 30 min in the absence of assay mix showed no appreciable breakdown of the form I RI family (data not shown).

Components of the assay mix responsible for breakdown of form I RIs. To determine the

components of the assay mix which were responsible for the breakdown or conversion of the form I RI family, nuclear extract was incubated in vitro with $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$ for 30 min under different assay conditions (Table 1). Omission of MgCl_2 in the assay mix produced the greatest relative amount of synthesis of the form I RI

FIG. 5. Two-dimensional (neutral-alkali) agarose gel electrophoresis of in vivo labeled products. Polyoma virus-infected cells were labeled in vivo with [^3H]thymidine (100 $\mu\text{Ci/ml}$) for 5 min before extraction. DNA was prepared by the method of Hirt (8): (A) directly from the infected cell monolayer; (B) directly from the nuclear extract; or (C) from the nuclear extract after incubation for 30 min in assay mix containing only unlabeled deoxynucleotide triphosphates. After two-dimensional agarose gel electrophoresis, the gel was soaked in En^3Hance (Beckman) for 2 h, dried, and subjected to fluorography. The exposure of the first-dimension gel is placed above the second dimension for reference.

family (Fig. 7B). Since net synthesis was only 16% of that obtained with the complete assay mix, it was not clear whether the absence of labeled form II and III RIs was due to decreased de novo synthesis of form II and III RI families or to blocking the breakdown of the form I RI family under conditions restrictive for DNA synthesis. Omission of other components of the reaction mix still resulted in significant breakdown of the form I RI family in vitro.

Since extraction of minichromosomes from infected cells usually involves the use of MgCl_2 (0.5 mM) at a level one-tenth the amount present in the assay mix (5 mM), nuclear extract was also prepared without MgCl_2 in the extraction buffer. In this latter case, recovery of in vivo label and incorporation in vitro were both 85% of that obtained with minichromosomes extracted in buffer containing 0.5 mM MgCl_2 , indicating no loss of DNA synthetic capacity. We estimate the endogenous magnesium concentration in the nuclear extract to be approximately one-half of the amount of the total magnesium concentration in the extract prepared in the presence of 0.5 mM MgCl_2 . Furthermore, the minichromosomes prepared without MgCl_2 in the extraction buffer showed the same distribution of form I, II, and III RIs as found with minichromosomes extracted in the presence of 0.5 mM MgCl_2 when various components of the assay mix were omitted (not shown).

Addition of ribonucleoside triphosphates (100

μM) or calf thymus DNA (75 $\mu\text{g/ml}$) to the complete reaction mix did not prevent form I RI breakdown (data not shown).

DISCUSSION

We have presented evidence that in vitro replication of polyoma virus minichromosomes results in the appearance of two families of nicked RIs (forms II and III). These two families (originally referred to as RIa and RIb, respectively [5] are distinguishable from the unnicked supercoiled RIs (form I) by the migration of their labeled daughter strands in two-dimensional (neutral-alkali) agarose gels (14). Second-dimension electrophoresis in alkali is a powerful tool for analyzing families of RIs since the daughter strands form a continuous spectrum or arc extending from the latest (>90% replicated) to the earliest (<10% replicated) RIs.

The earliest RIs (i.e., RIs replicated to only a small degree) migrate in the first (neutral) dimension slightly slower than their unreplicated counterparts (11, 15). Their short daughter strands migrate furthest in the second (alkali) dimension, and their spectrum is asymptotic to the position of the unreplicated species. For example, in the second (alkali) dimension the daughter strands of the form I RI family are asymptotic with the position of the supercoiled form I position, and the daughter strands of the form II and III RI families are asymptotic with the

FIG. 6. Proposed mechanism for the origin of nicked RIs. A single-strand nick in the parent strand of a supercoiled RI (I-RI) in either the unreplicated region (not shown) or the replicated region (1) would result in a relaxed RI (II-RI). Elongation of the replication fork to the site of the nick would result in a linearized RI (III-RI). This III-RI structure would also result from a nick at the replication fork (2). Ligation of the daughter strand to the nicked parent strand after elongation by either the leading daughter strand or gap filling to the lagging daughter strand (not shown) would result in a rolling-circle molecule.

position of relaxed circular form II (see Fig. 4D).

The latest or most mature RIs of the form I and II RI families both migrate in the first (neutral) dimension with the late Cairns structures since the most mature form I RI molecules would possess little or no superhelicity (5). In the second (alkali) dimension the long daughter strands, derived from these molecules, migrate with full-length linear molecules (see ssl position, Fig. 4D). The latest (or most mature) RIs of the form III RI family do not migrate in the first dimension with the latest Cairns structures probably due to the higher mobility contribution by the linear tail (4).

In the first dimension, undenatured form III RI molecules would be indistinguishable from RIs with a linear tail generated by a rolling-circle mechanism (see Fig. 6). However, after denaturation one of the daughter strands of the rolling-circle molecule would be covalently attached to a parental strand and would migrate in the second dimension much slower than a full-length linear molecule (see Fig. 4D, RC). These longer daughter strands are seen after incubation for 30 min in vitro in Fig. 4, 5, and 7. These results confirm earlier electron microscopic analysis of RIa and RIb bands excised from 1.4% agarose

gels which showed these bands to be composed predominantly of θ and σ structures, respectively (5). In addition, the small percentage of form III RI molecules present after in vivo labeling of

TABLE 1. Effect of in vitro incubation conditions

Reaction mix	Relative synthesis in vitro (%) ^a	
	Nuclear extract + MgCl ₂ ^b	Nuclear extract - MgCl ₂ ^c
Complete	100	100
- MgCl ₂	16	6
- ATP	135	117
- Phosphoenolpyruvate	85	81
- Pyruvate kinase	122	115

^a Data are presented as in vitro incorporation relative to complete reaction mix.

^b Nuclear extract prepared by the standard technique with magnesium-containing buffer (0.5 mM). 100% represents a net dGMP incorporation of 3.5×10^6 cpm per plate equivalent.

^c Nuclear extract prepared in the absence of magnesium chloride in the extraction buffer. 100% = 3.0×10^6 cpm per plate equivalent. Recovery of in vivo [³H]thymidine (labeled in vivo for 60 min) was 85% that obtained with extraction buffer containing MgCl₂.

FIG. 7. Effect of in vitro incubation on the breakdown of form I RIs. Autoradiogram of ^{32}P -labeled DNA from nuclear extract incubated for 30 min in (A) complete reaction mix, (B) reaction mix minus MgCl_2 , (C) reaction mix minus ATP, (D) reaction mix minus phosphoenolpyruvate, after two-dimensional (neutral-alkali) agarose gel electrophoresis. Materials were prepared as described in Table 1. Equivalent amounts of radioactivity were loaded onto each gel, and autoradiography was carried out on dried gels for 48 h at -70°C .

RIs (Fig. 5A and B) is consistent with the amount of rolling-circle molecules observed by electron microscopy (1).

It may be important that we see little evidence for nicking of daughter strands, since nicking would result in considerable heterogeneity of chain lengths of daughter strands in any one member of an RI family. Our results, however, are consistent with the occurrence of a single-strand nick in the parental strand of a supercoiled RI (form I RI; see Fig. 6). If the nick occurred in the unreplicated region, a relaxed RI (form II RI) would result (pathway 1, Fig. 6). If the nick occurred in the single-stranded region of a parental strand at the replication fork, then a linearized RI (form III RI) would result (pathway 2, Fig. 6). This form III RI structure also would result from a nick somewhat ahead of the replication fork and subsequent fork elongation to the nick, resulting in the release of one of the arms of the fork. This is consistent with the kinetic evidence showing an increase in form III RIs with longer incubation time in vitro (Fig. 5). A single-strand nick in the parental strand in the replicated region would result in a form II RI with no further conversion to form III RI. If the released arm of a form III RI had been on the lagging side of the replication fork, then elongation of the leading strand, followed by a simple

ligation event, would produce a rolling-circle molecule (see Fig. 6). On the other hand, if the released arm had been the leading side of the replication fork, a simple gap filling of the lagging strand gap followed by ligation would produce a similar rolling-circle molecule (not shown on Fig. 6).

We have not observed significant amounts of double-strand breakage of RIs (12, 18). A double-stranded break in the unreplicated region would result in linear molecules with a replication bubble which would migrate as a family of linear molecules asymptotic with the form III position, which we do not see. Furthermore, a double-stranded break in the replicated region would produce broken daughter strands much shorter than we observed.

Analysis of our in vitro replication conditions indicated that magnesium ion may play a role in the single-strand nicking activity (Table 1; Fig. 7), since its omission or 10-fold reduction results in less relative production of form II and III RIs. Furthermore, attempts to reduce this nicking activity by extensive washing of the nuclei before extraction and Bio-Gel A5M chromatography of the minichromosomes still resulted in extensive nicking during in vitro replication. This may indicate that the nicking activity is associated with minichromosomes themselves,

perhaps as an aberrant topoisomerase activity located ahead of the replication fork. Topoisomerase activity has been found associated with polyoma and simian virus 40 minichromosomes (6, 9, 16).

This analysis points out the obvious high resolving power of the two-dimensional gel method and the possible shortcomings of gel analysis of RIs by simple one-dimensional methods. Although we have identified a source of nicking that compromises maximal rates of synthesis *in vitro*, we hope that further studies will permit its suppression or elimination.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant GM-23813 from the National Institutes of Health and postdoctoral fellowship A-06304 to B.B.G.

LITERATURE CITED

1. Bjursell, G. 1978. Effects of 2'-deoxy-2'-azidocytidine on polyoma virus DNA replication: evidence for rolling circle-type mechanism. *J. Virol.* **26**:136-142.
2. DePamphilis, M. L., S. Anderson, R. Bar-Shavit, E. Collins, H. Edenberg, T. Herman, B. Karar, G. Kaufmann, H. Krokan, E. Shelton, R. Su, D. Tepper, and P. M. Wassarman. 1979. Replication and structure of simian virus 40 chromosomes. *Cold Spring Harbor Symp. Quant. Biol.* **43**:679-692.
3. DePamphilis, M. L., and P. M. Wassarman. 1980. Replication of eukaryotic chromosomes: a close-up of the replication fork. *Annu. Rev. Biochem.* **49**:627-666.
4. Fisher, M. P., and C. W. Dingman. 1971. Role of molecular conformation in determining the electrophoretic properties of polynucleotides in agarose-acrylamide composite gels. *Biochemistry* **10**:1895-1902.
5. 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.
6. 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.
7. Griffin, B. E. 1977. A fine structure map of polyoma virus DNA. *J. Mol. Biol.* **117**:447-471.
8. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:364-369.
9. Keller, W. 1975. Characterization of purified DNA-relaxing enzyme from human tissue culture cells. *Proc. Natl. Acad. Sci. U.S.A.* **72**:2550-2554.
10. Keller, W. 1975. Determination of the number of superhelical turns in simian virus 40 DNA by gel electrophoresis. *Proc. Natl. Acad. Sci. U.S.A.* **72**:4876-4880.
11. Martin, R. F. 1977. Analysis of polyoma virus DNA replicative intermediates by agarose gel electrophoresis. *J. Virol.* **23**:827-832.
12. Scott, W. A., and D. J. Wigmore. 1978. Sites in simian virus 40 chromatin which are preferentially cleaved by endonucleases. *Cell* **15**:1511-1518.
13. Su, R. T., and M. L. DePamphilis. 1976. *In vitro* replication of SV40 DNA in a nucleoprotein complex. *Proc. Natl. Acad. Sci. U.S.A.* **73**:3466-3470.
14. Sundin, O., and A. Varshavsky. 1980. Terminal stages of SV40 DNA replication proceed via multiply intertwined catenated dimers. *Cell* **21**:103-114.
15. Tapper, D. P., and M. L. DePamphilis. 1978. Discontinuous DNA replication: accumulation of SV40 DNA at specific stages in its replication. *J. Mol. Biol.* **120**:401-422.
16. Tsubota, Y., M. A. Waqar, J. F. Burke, B. I. Milavetz, M. J. Evans, D. Kowalski, and J. A. Huberman. 1979. Association of enzymes with replicating and non-replicating SV40 chromosomes. *Cold Spring Harbor Symp. Quant. Biol.* **43**:693-704.
17. Yang, R. C.-A., J. Lis, and R. Wu. 1979. Elution of DNA from agarose gels after electrophoresis. *Methods Enzymol.* **68**:176-182.
18. Waldeck, W., B. Fohring, K. Chowdury, P. Gruss, and G. Sauer. 1978. Origin of DNA replication in papova virus chromatin is recognized by endogenous endonuclease. *Proc. Natl. Acad. Sci. U.S.A.* **75**:5964-5968.
19. 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.