Polyoma Virus Minichromosomes: a Soluble In Vitro Replication System†

BRIAN B. GOURLIE, MARC R. KRAUSS, ALICIA J. BUCKLER-WHITE, ROBERT M. BENBOW,
AND VINCENT PIGIET*

McCollum-Pratt Institute and the Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218

Received 21 October 1980/Accepted 17 February 1981

Polyoma virus minichromosomes were isolated from infected 3T6 cells by hypotonic extraction of isolated nuclei. The kinetics of in vitro DNA synthesis in the nuclear extract was similar to that observed with intact nuclei. The majority of the products of in vitro DNA synthesis sedimented with replicative intermediate (RI) minichromosomes and migrated as two bands (RI-a and RI-b) on 1.4% agarose gels. The kinetics of deoxynucleotide monophosphate incorporation into these species was consistent with the existence of several rate-limiting steps in in vitro replication by polyoma minichromosomes. Electron microscope analysis showed that the RI-a band consisted almost entirely of RI θ structures ranging from 46 to 87% replicated, with one-half of all θ structures $67 \pm 4\%$ replicated. The RI-b material was more complex, consisting of σ and α structures with tails ranging from 7 to 114% of polyoma genome length and, less frequently, of linked and multiply linked dimeric structures.

As a foundation for the eventual reconstitution of chromatin replication in vitro, we have studied the replication of isolated polyoma minichromosomes. These small, well-defined viral genomes have a chromatin structure similar to that of the host (3, 7, 20) and provide an excellent model for eucaryotic replication (5, 22, 28, 29). With the exception of the virus-coded T antigen, which is responsible for initiating each new round of DNA replication (26), the host provides all of the machinery necessary for viral replication.

In this paper, we have characterized endogenous DNA synthesis by isolated minichromosomes and have shown the accumulation of replicative intermediates (RI-a and RI-b). In the second paper (13), the electron microscope was used to quantitate and characterize DNA species associated with mature (form I) and replicating (RI) minichromosomes. In the third paper (8), we show that several enzyme activities implicated in DNA replication cosediment with polyoma minichromosomes.

MATERIALS AND METHODS

Virus and cells. A single-plaque isolate of a large-plaque strain of polyoma virus was kindly provided by M. Martin and propagated at low multiplicity (10^{-3}) on primary baby mouse kidney cells. 3T6 mouse fibroblasts were seeded at 2.5×10^6 cells per 150-mm

culture plate (Falcon Plastics) in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 5% calf serum (GIBCO) and grown at 38°C in a 5% CO₂ atmosphere. Cells were infected on the 2nd day after seeding (approximately 80% confluency) at a multiplicity of infection of approximately 15.

Reagents. HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), dithiothreitol, phenylmethylsulfonyl fluoride, ethylene glycol-bis(B-aminoethyl ether)-N,N-tetraacetic acid, and unlabeled deoxyribonucleoside 5'-triphosphates were obtained from Sigma Chemical Co. Pyruvate kinase was obtained from Calbiochem. [³H]thymidine (55 Ci/mmol) and [α -³²P]dTTP (317 Ci/mmol) were obtained from ICN. [α -³²P]dGTP was obtained from Amersham Corp. The restriction enzyme *Bgl*I was obtained from Bethesda Research Laboratories.

Preparation of polyoma nuclear extracts (23). Twenty-seven hours after infection, cell cultures were incubated in vivo either with 20 μ Ci of [³H]thymidine (55 Ci/mmol) in 10 ml of Dulbecco modified Eagle medium for 90 min or with 100 μ Ci of [³H]thymidine in 2 ml for 5 min. The plates (now containing 4×10^7 cells and approximately 20 μ g of polyoma DNA) were rinsed three times with 5 ml of ice-cold hypotonic buffer A (10 mM HEPES, pH 7.8, 5 mM KCl, 0.5 mM MgCl₂, 0.5 mM dithiothreitol, and 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 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 with hypotonic extraction buffer (0.5 ml per plate) and extracted at 0°C for 2 h. Nuclei were

† Publication 1087 from the McCollum-Pratt Institute.

removed by centrifugation ($6,800 \times g$) for 15 min, and the supernatant solution of the nuclear extract was used for further study.

In vitro DNA synthesis (30). Viral DNA synthesis was assayed in a total volume of 100 μ l in the presence of: 40 mM NaCl, 5 mM $MgCl_2$, 5 mM ethylene glycol-bis(*B*-aminoethyl ether-*N,N'*-tetra-acetic acid, 4 mM phosphoenol pyruvate, 2 mM ATP, 3.0 IU of pyruvate kinase per ml, and 100 μ M each dATP, dCTP, and dGTP. Experiments using [3H]- and [^{32}P]dTTP were performed at 25 μ Ci/ml (18 and 300 Ci/mmol, respectively). Nuclei or nuclear extract was added in a volume of 90 μ l, and incubations were performed at 30°C. Duplicate portions of 10 or 20 μ l were removed and precipitated with 2 ml of cold 10% trichloroacetic acid containing 10 mM sodium pyrophosphate. Acid-insoluble materials were collected on glass fiber filters (Whatman GF/C) and washed four times with 2 ml of cold 5% trichloroacetic acid–5 mM sodium pyrophosphate and once with cold 95% ethanol. Radioactivity was determined on the dried filters by liquid scintillation counting in 4 ml of Beckman EP fluor.

Analysis of viral chromatin by sucrose gradient sedimentation. Nuclear extracts (0.5 ml) were sedimented through linear sucrose gradients (10 ml, 5 to 20%, wt/vol) prepared in buffer B (10 mM HEPES, pH 7.8, 5 mM KCl, and 0.5 mM $MgCl_2$) for 90 min at $255,000 \times g$ in a Beckman SW41 rotor. Fractions were collected from the bottom, and portions of each fraction were analyzed for acid-insoluble radioactivity as described above. Relative sedimentation velocity of minichromosomes was determined relative to the sedimentation of form I polyoma DNA (20S) on a parallel but separate sucrose gradient in high-salt buffer C (0.5 M NaCl, 10 mM EDTA, and 10 mM sodium acetate, pH 6.0).

Analysis of viral DNA by sedimentation on neutral and alkaline sucrose gradients. After in vitro incubation, viral DNA was prepared from viral chromatin by the method of Hirt (11). Samples (100 μ l) were sedimented through neutral sucrose gradients (3.7 ml, 5 to 20%, wt/vol) in buffer C above a 70% sucrose cushion (100 μ l) in buffer C or alkaline sucrose gradients which also contained 0.05% sodium sarcosinate and 0.3 M NaOH. Gradients were centrifuged in a Beckman SW60 rotor at $350,000 \times g$ for 110 min (neutral) or 200 min (alkaline). Fractions were collected from the bottom, and acid-precipitable radioactivity was determined as above.

Agarose gel electrophoresis of viral DNA (27). Electrophoresis was performed by using 1.4% (wt/vol) agarose slab gels (18 cm by 19 cm by 3 mm) in 40 mM Tris-hydrochloride, 5 mM sodium acetate, and 1 mM EDTA, pH 7.6. Samples of nuclear extract were treated with 0.6% sodium dodecyl sulfate, 10 mM Tris-hydrochloride, and 10 mM EDTA (pH 7.6) for 20 min at 30°C and electrophoresed for 12 to 18 h at 2.7 V/cm along with polyoma form I and II DNA markers. The gel was stained with ethidium bromide (0.5 μ g/ml, 30 min), and the fluorescent bands were photographed under UV illumination (254 nm) through a Wratten no. 25 (red) filter on Polaroid type 55 P/N film. Autoradiography was performed at 4°C on wet gels, using Kodak X-Omat R film. *Bgl*I restriction enzyme digestion of deproteinized DNA synthesized

by the nuclear extract in vitro was performed as described by the supplier.

Electron microscope analysis of viral DNA forms separated by agarose gel electrophoresis. Sections of 1.4% agarose gels corresponding to the form I, RI-a and RI-b bands were excised and dissolved in 5 volumes of saturated potassium iodide, 10 mM sodium phosphate (pH 7.0), and 1 mM sodium thiosulfate. Samples were spread for electron microscopy without further processing, using the aqueous method of Davis et al. (1a). Every DNA molecule seen was photographed at $\times 20,000$ magnification with a JEOL JEM 100B electron microscope, projected with a Nikon Shadowgraph comparator (total magnification, approximately $\times 200,000$), and traced with a Keuffel and Esser map measurer. Photographs were printed on Ilfobrom 5.1M resin-coated paper without further contrast enhancement.

RESULTS

Kinetics of in vitro DNA synthesis by the nuclear extract. Polyoma minichromosomes were extracted from virus-infected 3T6 cells at a time corresponding to maximal viral DNA synthesis (27 h postinfection in our system), using a hypotonic extraction procedure originally described for isolation of simian virus 40 (SV40) minichromosomes (22). The nuclear extract, prepared from cells labeled for either 5 or 90 min with [3H]thymidine before harvest, contained 10 to 20% of the total labeled viral DNA as determined by the Hirt procedure (11). This protocol allowed preferential labeling of either the RI (5-min pulse) or mature (90-min pulse) form I species. The efficiency of extraction was comparable to results obtained with SV40-infected cells (5, 22), but was less efficient than methods using Triton and high salt (9).

The ability of nuclear extracts to incorporate [^{32}P]dTTP was determined by using reaction conditions optimized for polyoma DNA synthesis in isolated nuclei (30). [^{32}P]dTTP incorporation into DNA was rapid during the first 5 min and reached a plateau after 10 min (Fig. 1). Maximal in vitro DNA synthesis by the nuclear extract (both initial rate and plateau) was obtained after extraction of nuclei for 2 h at 0°C. Endogenous DNA synthesis in the nuclear extract was stable for at least 8 h and decreased only 20% after storage overnight at 0°C. The rate of DNA synthesis in the nuclear extract was compared with the rate of synthesis by nuclei isolated by an isotonic procedure (20). Both reactions reached a plateau after 10 min. The initial rate of dTMP incorporation was 0.10 and 1.14 pmol/min per plate for the nuclear extract and the intact nuclear preparations, respectively. Since the nuclear extract contained 10 to 20% of the total polyoma DNA in the cell (based on the efficiency of extracting DNA labeled in

FIG. 1. Kinetics of DNA synthesis by the nuclear extract. Nuclear extract (corresponding to 3.5×10^6 cells) in 500 μ l was assayed for endogenous DNA synthesis in the presence of [α - 32 P]dTTP (158×10^3 cpm/pmol) as described in Materials and Methods. At the indicated times, 10- μ l portions were removed, and the amount of acid-precipitable [α - 32 P]dTTP was determined.

vivo), the initial rate of DNA synthesis in both preparations was roughly comparable, assuming equal extraction of a representative population of functional replicating complexes (see Discussion). The extent of de novo synthesis in vitro after 10 min can be calculated from the initial rate (see above) and the amount of replicative intermediates (0.6 pmol/plate) as approximately 120 pmol of nucleotide per mol of replicative intermediate. This calculation is an average and does not discriminate between forks that may vary in their relative efficiency. After hypotonic extraction of minichromosomes, nuclei still contained 60 to 80% of the viral DNA labeled in vivo, but less than 5% of the DNA synthetic capacity of either the nuclear extract or the intact nuclei. The cytoplasmic supernatant contained the remainder of the total polyoma DNA in the cell (10 to 20%).

Characterization of the reaction products by sedimentation analysis. The 32 P-labeled products of in vitro DNA synthesis were characterized either as viral chromatin or as deproteinized DNA. The 32 P-labeled products synthesized by the nuclear extract during a 30-min incubation sedimented between 50S and 90S, with a major peak at 84S and a shoulder at 56S relative to polyoma form I DNA (Fig. 2). For comparison, mature polyoma minichromosomes (labeled with [3 H]thymidine for 90 min in vivo) sedimented at approximately 56S, whereas minichromosomes labeled in vivo for only 5 min (predominantly RI minichromosomes) sedi-

mented at approximately 84S (Fig. 3). This sedimentation behavior was comparable to results obtained by others using different extraction conditions for polyoma virus (29) or for SV40 minichromosomes (5, 23).

The 32 P-labeled products, deproteinized by treatment with 0.6% sodium dodecyl sulfate and

FIG. 2. Characterization of the chromatin product of DNA synthesis in the nuclear extract. After incubation of the nuclear extract for 30 min with [α - 32 P]dTTP (○) as described in Fig. 1, the sample was sedimented without further treatment through a neutral sucrose gradient as described in Materials and Methods. Radioactivity was determined after acid precipitation of 10 μ l of each fraction. The viral DNA was labeled in vivo with [3 H]thymidine (●) for 30 min immediately before harvest. The arrow indicates the peak of form I polyoma DNA (20S) sedimented on a separate 5 to 20% sucrose gradient in high-salt buffer C.

FIG. 3. Sedimentation of mature (form I) and RI viral chromosomes. Infected cells (3.5×10^7 cells/plate) were labeled either with 20 μ Ci of [3 H]thymidine (55 Ci/mmol) in 10 ml for 90 min (●) or with 100 μ Ci of [3 H]thymidine in 2 ml for 5 min (○) before harvest. Portions (500 μ l) of the nuclear extracts were sedimented as described in Materials and Methods. Radioactivity was determined after acid precipitation of 10 μ l for the sample labeled for 90 min and the entire 0.5-ml fraction for the sample labeled for 5 min.

1 M NaCl (11), were characterized by sedimentation on both neutral and alkaline sucrose gradients. The *in vitro* DNA products sedimented in a neutral sucrose gradient slightly faster than polyoma form I DNA (20S) (Fig. 4), which is consistent with the sedimentation behavior of RI DNA (21). In contrast to the SV40 system (23), the majority of the *in vitro*-labeled DNA remained associated with the parental genome when analyzed either as intact chromatin (Fig. 2) or as deproteinized DNA (Fig. 4). Sedimentation under alkaline conditions of the deproteinized, ^{32}P -labeled product of a 30-min incubation *in vitro* demonstrated that the daughter strands ranged in size from 16S (full-length linear molecules) to 4S (Okazaki fragments) (Fig. 5). Significant amounts of mature form I DNA (53S) were not observed.

Characterization of the reaction products by agarose gel electrophoresis. The results of agarose gel electrophoresis of ^{32}P -labeled material synthesized *in vitro* are shown in the autoradiogram in Fig. 6. The major products of *in vitro* synthesis appeared as two bands migrating slower than relaxed circular polyoma DNA (form II). We refer to these bands as RI-a and RI-b, based on their cosedimentation with RI minichromosomes labeled *in vivo* (Fig. 3). Supercoiled Cairns-type replicative intermediates would be expected to migrate as a spectrum from form I (early replicative intermediates) to

FIG. 4. Characterization of the deproteinized product of DNA synthesis by sedimentation in neutral sucrose. After incubation of the nuclear extract for 30 min with $[\alpha\text{-}^{32}\text{P}]\text{dTTP}$ (○) as described in Fig. 1, the sample was treated according to the procedure of Hirt (11). The extracted DNA was then sedimented on a neutral sucrose gradient as described in Materials and Methods. Fractions were collected from the bottom, and acid-precipitable radioactivity was determined as described above. Viral DNA was labeled *in vivo* for 30 min immediately before harvest with $[\text{H}^3]\text{thymidine}$ (●).

FIG. 5. Characterization of the deproteinized product of DNA synthesis by sedimentation in alkaline sucrose. A portion of the same sample labeled *in vitro* and *in vivo* as described in Fig. 4 was sedimented on an alkaline sucrose gradient as described in Materials and Methods. Fractions were collected from the tube bottom and analyzed for ^{32}P *in vitro* (○) or ^3H *in vivo* (●) label.

a position slower than form II (late replicative intermediates) (16, 25). The same species in the same relative amounts were present *in vivo* as determined by pulse-labeling with $[\text{H}^3]\text{thymidine}$. The relative labelings of both RI-a and RI-b *in vitro* were also comparable when intact nuclei were used (Gourlie and Pigiet, manuscript in preparation).

Since variations in agarose concentration, electric field, and temperature differentially affect the migration of linear, circular, and supercoiled DNA of the same molecular weight (4), we investigated the effect of agarose concentrations from 0.8 to 1.4% (wt/vol). The RI-a and RI-b bands maintained the same relative mobilities to the polyoma form II marker, but became broader and more diffuse at lower gel concentrations, indicating that there may be further heterogeneity within the bands.

Labeling of the RI-a band was rapid and essentially complete after 5 min (Fig. 6). In contrast, labeling of the RI-b band increased from about 20% of the total incorporation at 5 min to about 40% of the total at 60 min. Thus, substantial labeling of RI-b continued after total incorporation had reached a plateau (10 min). Labeled form I DNA accumulated to approximately 3% of the total incorporation by 60 min, consistent with results of sucrose gradient sedimentation (Fig. 5).

FIG. 6. Characterization of the products of *in vitro* DNA synthesis by agarose gel electrophoresis. Nuclear extract was incubated as described in Fig. 1 with [α - 32 P]dTTP. At the indicated times, 50- μ l portions were removed from the incubation, treated with 0.6% sodium dodecyl sulfate, 10 mM EDTA, and 10 mM Tris-hydrochloride (pH 7.6) for 1 h at 20°C, mixed with 1/10 volume of 0.05% bromophenol blue, 25 mM EDTA, and 50% glycerol, and applied to a 1.4% agarose gel. Electrophoresis was carried out as described in Materials and Methods. Autoradiography was performed at 4°C for 14 h. Migration of form I and form II DNA is indicated.

To compare the distribution of RI-a and RI-b DNAs as a function of sedimentation of the chromatin products, agarose gel electrophoresis was performed on the DNA from individual fractions of the sucrose gradient shown in Fig. 2. The autoradiogram of this gel (Fig. 7A) showed the distribution of label incorporated into the RI-a and RI-b bands. Direct quantitation of the radioactivity in the excised bands (Fig. 7B) showed that RI-a sedimented as a single peak with an average rate of 84S (fraction 9), corresponding to the fraction which contained the maximum *in vitro* label. The RI-b DNA, in contrast, was distributed into two peaks of approximately equal magnitude at fractions 9 (84S) and 11 (68S). The ratio of label incorporated into the RI-a and RI-b bands (Fig. 7C) increased as a function of greater sedimentation rate, with RI-a being the dominant component in the sedimentation region shown to contain replicative intermediates (50 to 90S) (see Fig. 3 and reference 13).

Characterization of the reaction products by electron microscopy. To determine the composition of the RI-a and RI-b bands, we excised these regions directly from a 1.4% agarose gel run as described in Fig. 7 and prepared the DNA for electron microscopy (see Materials and Methods). Of the 22 DNA molecules observed from the RI-a band, 78% were unambig-

FIG. 7. Gel electrophoretic analysis of DNA in fractions from a minichromosome sucrose gradient. Portions (20 μ l) taken from the preparative sucrose gradient described in Fig. 3 were analyzed by agarose gel electrophoresis as described in Fig. 6. Electrophoresis was carried out for 16 h at 2.5 V/cm. Autoradiography of the gel (A) was carried out for 4°C for 12 days. Bands corresponding to RI-a ($\circ$) and RI-b ($\bullet$) were cut out of the gel and melted with 100 μ l of water for 10 min at 100°C, and radioactivity was quantified by using Beckman HP fluor (B). The dotted line represents [3 H]thymidine incorporated *in vivo* from Fig. 3. The ratios of isotope incorporated into RI-a and RI-b are plotted in C.

uous θ structures. Other structures observed included "connected" monomers (two monomers joined by a double-stranded linker of variable length) (6%) and σ structures (3%). Figure 8 shows representative micrographs of the DNA molecules found in the RI-a band. The putative replicated regions of the θ structures ranged from 46 to 87% of the total genome length. Ten of the 18 unambiguous θ structures were clustered at $67 \pm 4\%$ replicated. Of the 28 molecules observed from the RI-b band, 48% were unambiguous σ structures and 18% were α structures. Other structures observed in the RI-b band included linked and multiply linked dimers and θ structures. Figure 9 shows representative micrographs of molecules found in the RI-b band. As a control, DNA molecules extracted from the form I band were monomeric, with few if any complex structures.

Characterization of the reaction products by *Bgl*II restriction enzyme digestion. We further characterized the ^{32}P -labeled reaction products after digestion with the restriction endonuclease *Bgl*II. Cleavage of polyoma DNA occurs once near the origin of replication (10) and would convert replicative intermediates into H-like structures. Restriction digestion was performed on the DNA from a sample of nuclear extract incubated in vitro with [α - ^{32}P]dGTP for 30 min. The autoradiogram of this material after electrophoresis on a 1.2% agarose gel (Fig. 10) shows a gradation of radioactivity extending from the migration position of late Cairns species to the earliest Cairns structures migrating close to form III. A second spectrum of replicative intermediates was apparent migrating in a range intermediate between the late Cairns species and form III. This spectrum represents replicative intermediates that had replicated in a unidirectional manner and assumed a Y-type configuration after digestion with *Bgl*II (Buckler-White, Krauss, Benbow, and Pigiet, submitted for publication). Molecules comigrating with form III included all double-stranded linear molecules produced from cleavage of form I, form II, or any concatenated dimers. This pattern of isotope incorporation revealed a spectrum of replicative intermediates and was comparable to that obtained for DNA labeled in vitro with [α - ^{32}P]dGTP by nuclei prepared by an isotonic method (15) or DNA labeled in vivo with ^{32}P .

DISCUSSION

We have demonstrated that minichromosomes present in soluble nuclear extracts incorporate deoxynucleotide monophosphates into polyoma DNA in vitro. The distinctive advantage of the soluble system lies in the ability to

isolate mature and replicating minichromosomes (50 to 90S) as intact nucleoprotein complexes and to identify individual DNA molecules and enzyme activities associated with these complexes.

Interpretation of studies using isolated minichromosomes depends on understanding the nature of the materials extracted and whether they are representative of the minichromosomes present in the intact cell. The lower rate of DNA synthesis by the nuclear extract relative to isolated nuclei (about 10%) raises several questions about the synthetic capacity of the minichromosomes. The simplest interpretation is that the low incorporation of deoxynucleotide monophosphates by the nuclear extract reflects the low yield of extraction of replicating minichromosome complexes. This argument is supported by the initial rate of deoxynucleotide monophosphate incorporation, which was roughly proportional to the efficiency (10 to 20%) of extraction. The synthetic rate of nuclear extracts was comparable to the synthetic rate observed in nuclei after adjusting for this extraction efficiency. The validity of this argument rests on the assumption that the extracted minichromosomes are representative of the total population of functional complexes in nuclei.

The retention of labeled viral DNA in the nucleus, even after extensive extraction with hypotonic or hypertonic detergent buffers (1), is consistent with the finding that substantial amounts of viral DNA are tenaciously bound to the nuclear matrix. If the matrix is the site of some stages of viral replication, minichromosomes may represent a stage with no requirement for matrix attachment. Alternatively, if all stages of replication take place on the matrix (19), minichromosomes may represent species released from cells by our manipulations. It was of interest that approximately 10% of the minichromosomes were associated with amorphous material (possibly matrix components) even after high-salt extraction (13). In any event, the isolated minichromosomes are likely to prove especially valuable for identification of the proteins involved in replication even if the DNA synthesis carried out in vitro is not fully representative of events inside the nucleus.

Sedimentation analysis of the products of in vitro DNA synthesis by the nuclear extract showed that the majority of labeled DNA sedimented with the same distribution (approximately 84S) as the viral minichromosomes labeled during a short (5-min) pulse in vivo (Fig. 2 and 3). The smaller amount of in vitro label which sedimented with mature minichromosomes (Fig. 2) may correspond either to early

FIG. 8. Electron microscope analysis of DNA molecules comigrating with the RI-a band on agarose gels. The RI-a band was excised from an agarose gel, dissolved in potassium iodide, and prepared for electron microscopy as described in Materials and Methods. (A) θ Structure where the putative unreplicated region measures $0.90\ \mu\text{m}$ and the replicated arms measure 0.76 and $0.76\ \mu\text{m}$. (B) θ Structure where the putative unreplicated region measures $0.80\ \mu\text{m}$ and the replicated arms measure 0.90 and $0.85\ \mu\text{m}$. (C) θ Structure where the putative unreplicated region measures $0.60\ \mu\text{m}$ and the replicated arms measure 1.03 and $0.99\ \mu\text{m}$. (D) θ Structure where the putative unreplicated region measures $0.59\ \mu\text{m}$ and the replicated arms measure 1.10 and $1.06\ \mu\text{m}$. (E) θ Structure where the putative unreplicated region measures $0.57\ \mu\text{m}$ and the replicated arms measure 1.17 and $1.16\ \mu\text{m}$. (F) θ Structure where the putative unreplicated region measures $0.49\ \mu\text{m}$ and the replicated arms measure 1.20 and $1.19\ \mu\text{m}$. Molecules A, B, C, D, and F were printed at the same magnification; molecule E was printed at 25% higher magnification. The markers correspond to $0.5\ \mu\text{m}$.

FIG. 9. *Electron microscope analysis of DNA comigrating with the RI-b band on agarose gels. The RI-b band was excised from an agarose gel and prepared for electron microscopy as described in Fig. 8. (A) σ Structure where the circle measures $1.70\ \mu\text{m}$ and the tail measures $0.66\ \mu\text{m}$. (B) σ Structure where the circle measures $1.56\ \mu\text{m}$ and the tail measures $0.99\ \mu\text{m}$. (C) σ Structure where the circle measures $1.64\ \mu\text{m}$ and the tail measures $1.87\ \mu\text{m}$. (D) α Structure where the circle measures $1.70\ \mu\text{m}$ and the tails measure 0.51 and $0.22\ \mu\text{m}$. (E) Linked dimer structure where the two circles measure 1.74 and $1.62\ \mu\text{m}$. (F) Multiply linked dimer structure where the circles measure 1.61 and $1.59\ \mu\text{m}$. Molecules A, B, C, D, and E were printed at the same magnification; molecule F was printed at 25% higher magnification. The markers correspond to $0.5\ \mu\text{m}$.*

FIG. 10. *Bgl*I digestion of the products of *in vitro* DNA synthesis. Nuclear extract, incubated *in vitro* for 60 min with [α - 32 P]dGTP, was deproteinized and digested with *Bgl*I as described in Materials and Methods. Electrophoresis in 1.2% agarose was performed at 5 V/cm for 18 h at 4°C. Migration of form III and late Cairns (LC) structures is indicated.

replicative intermediates as detected by electron microscopy (13) or to termination products resembling mature form I DNA (14, 17, 24). Comparison of Fig. 3 and 4 shows that sedimentation of viral minichromosomes resolved the replicative intermediates from the mature forms far better than sedimentation of deproteinized DNA (Fig. 4).

Agarose gel electrophoresis of the products of DNA synthesis *in vitro* demonstrated that the majority of label appeared as two bands (RI-a and RI-b), each migrating slower than form II polyoma DNA (25), and that these forms were also present in RI DNA pulse-labeled *in vivo*. The complex contributions of molecular weight, superhelicity, and topology to the behavior of DNAs on agarose gels (4) complicate any certain identification of these bands at this time. Evidence that they are members of different conformational families (24) is supported by the increased band width observed at lower agarose concentrations and is consistent with the spectrum of intermediates seen in the *Bgl*I digest pattern (Fig. 10) and the electron microscope evidence discussed below. The kinetics of labeling of the RI-a and RI-b bands is consistent with RI-a being an initial product, rather than the

result of conversion from RI-b. In contrast to the rapid labeling of RI-a, the slower rate of labeling of RI-b is consistent with conversion of some RI-a into RI-b and/or conversion from early replicative intermediates migrating as a continuum from form I to the late Cairns position. The appearance of RI-b in two regions of the sucrose minichromosome gradient (Fig. 7) may indicate that both of these possibilities are true.

The results obtained by electron microscopy (Fig. 8 and 9) suggest that the RI-a band consisted almost entirely of unambiguous θ structures ranging from 46 to 87% replicated with a modal value of $67 \pm 4\%$ replicated. The RI-b band was more complex, consisting of σ and α structures which either could have arisen by breakage of θ structures or could have been generated by a rolling circle mechanism and branch migration. In addition, linked and multiply linked dimeric species reminiscent of the conformational families described by Sundin and Varshavsky (24) were also seen, although at lower frequencies. Although we cannot unequivocally rule out the possibility that RI-a and RI-b correspond to the 80 and 90% late Cairns species observed in SV40-infected cells (25), the kinetics of labeling and sedimentation properties of RI-a and RI-b, as well as the electron microscopy, render this possibility extremely unlikely for polyoma-infected cells. The results of this paper are consistent with, but do not prove, the hypothesis that incorporation into RI-b is due to either breakage of RI-a or maturation into topologically linked species as described by Sundin and Varshavsky (24), or both.

Mature form I polyoma DNA was not produced *in vitro* to an appreciable extent, amounting to less than 5% of the total incorporation by 60 min (Fig. 7A). This lack of conversion into form I molecules, as observed by others for intact nuclei (2, 6, 25) and soluble minichromosome systems (5, 22) may be due to the release of factors essential for termination into the cytoplasm during nuclear isolation.

ACKNOWLEDGMENTS

We acknowledge the contributions of Glen Humphrey, Carol Breaux, and Marvin Bayne for their critical evaluation of our work and manuscript. We also acknowledge the contributions of Mitchell K. Hobish, who initiated this study.

This research was supported by Public Health Service grants GM-23813 and GM-23970 and postdoctoral fellowship CA-06304 to B.B.G. from National Institutes of Health.

LITERATURE CITED

1. Buckler-White, A. J., G. N. Humphrey, and V. Pigiet. 1980. Association of polyoma T antigen and DNA with the nuclear matrix from lytically infected 3T6 cells. *Cell* 22:37-46.
- 1a. Davis, R. W., M. Simon, and N. Davidson. 1971. Elec-

- tron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. *Methods Enzymol.* **21D**:413-428.
2. DePamphilis, M. L., P. Beard, and P. Berg. 1975. Synthesis of superhelical simian virus 40 DNA in cell lysates. *J. Biol. Chem.* **250**:4340-4347.
 3. DePamphilis, M. L., and P. M. Wasserman. 1980. Replication of eukaryotic chromosomes: a close-up of the replication fork. *Annu. Rev. Biochem.* **49**:627-666.
 4. Dingman, C. W., M. P. Fisher, and T. Kakefuda. 1972. Role of molecular conformation in determining the electrophoretic properties of polynucleotides in agarose-acrylamide gels. *Biochemistry* **11**:1242-1250.
 5. Edenberg, H. J., M. J. Wagar, and J. Huberman. 1976. Subnuclear systems for synthesis of SV40 in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **73**:4392-4396.
 6. Franke, B., and T. Hunter. 1974. In vitro polyoma DNA synthesis: discontinuous chain growth. *J. Mol. Biol.* **83**:99-121.
 7. Germond, J.-E., B. Hirt, P. Oudet, M. Gross-Bellard, and P. Chambon. 1975. Folding of the DNA double helix in chromatin-like structures from simian virus 40. *Proc. Natl. Acad. Sci. U.S.A.* **72**:1843-1850.
 8. 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.
 9. Green, M. H. 1972. Biosynthetic properties of a polyoma nucleoprotein complex: evidence for replication sites. *J. Virol.* **10**:32-41.
 10. Griffith, J. D. 1975. Chromatin structure deduced from a minichromosome. *Science* **187**:1202-1204.
 11. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:365-369.
 12. Jaenisch, R., A. Mayer, and A. Levine. 1971. Replicating SV40 molecules containing closed circular template DNA strands. *Nature (London) New Biol.* **233**:72-75.
 13. Krauss, M. R., and R. M. Benbow. 1981. Polyoma virus minichromosomes: associated DNA molecules. *J. Virol.* **38**:815-825.
 14. Laipis, P. J., A. Sen, A. J. Levine, and C. Mulder. 1975. DNA replication in simian virus 40-infected cells: the structure of the 16S gap circle intermediate in simian virus 40 DNA synthesis. *J. Virol.* **68**:115-123.
 15. 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**:412-415.
 16. Martin, R. F. 1977. Analysis of polyoma virus DNA replicative intermediates by agarose gel electrophoresis. *J. Virol.* **23**:827-832.
 17. Meinke, W., and D. A. Goldstein. 1972. Studies on the structure and formation of polyoma DNA replicative intermediates. *J. Mol. Biol.* **61**:543-563.
 18. Meinke, W., M. R. Hall, and D. A. Goldstein. 1975. Proteins in intracellular simian virus nucleoprotein complexes: comparison with simian virus 40 core proteins. *J. Virol.* **15**:439-442.
 19. Pardoll, D. M., B. Vogelstein, and D. S. Coffey. 1980. A fixed site of DNA replication in eukaryotic cells. *Cell* **19**:527-536.
 20. Pigiet, V., R. Eliasson, and P. Reichard. 1974. Replication of polyoma DNA in isolated nuclei. III. The nucleotide sequence at the RNA-DNA junction of nascent strands. *J. Mol. Biol.* **84**:197-216.
 21. Seidman, M. M., C. F. Garon, and N. P. Salzman. 1978. The Relationship of SV40 replicating chromosomes to two forms of the non-replicating SV40 chromosomes. *Nucleic Acids Res.* **5**:2877-2893.
 22. 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.
 23. Su, R. T., and M. L. DePamphilis. 1978. Simian virus 40 DNA replication in isolated replicating viral chromosomes. *J. Virol.* **28**:53-65.
 24. Sundin, O., and A. Varshavsky. 1980. Terminal stages of SV40 DNA replication proceed via multiply intertwined catenated dimers. *Cell* **21**:103-114.
 25. 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.
 26. Tegtmeyer, P. 1972. Simian virus 40 DNA synthesis: the viral replicon. *J. Virol.* **10**:591-598.
 27. Tegtmeyer, P., and F. Macasaet. 1972. SV40 DNA synthesis: analysis by gel electrophoresis. *J. Virol.* **10**:599-604.
 28. Tsubota, Y., M. A. Waqar, J. F. Burke, B. I. Milavetz, M. J. Evans, D. Kowalski, and J. Huberman. 1979. Association of enzymes with replicating and non-replicating SV40 chromosomes. *Cold Spring Harbor Symp. Quant. Biol.* **43**:693-704.
 29. Waldeck, W., U. Sparen, G. Mastromei, R. Eliasson, and P. Reichard. 1979. Replication of polyoma DNA in nuclear extracts and nucleoprotein complexes. *J. Mol. Biol.* **135**:675-689.
 30. Winnacker, E.-L., G. Magnusson, and P. Reichard. 1972. Replication of polyoma DNA in isolated nuclei. I. Characterization of the system from mouse fibroblast 3T6 cells. *J. Mol. Biol.* **72**:523-537.