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Synthesis of Multimeric Polyoma Virus DNA in Mouse L-Cells: Role of the *ts*A1S9 Gene Product

PETER R. GANZ[1] AND ROSE SHEININ[2]*

Department of Microbiology, University of Toronto, Toronto, Ontario, Canada M5S 1A1,[2] and Blood Transfusion Service, Canadian Red Cross, Ottawa, Ontario, Canada K1S 3E2[1]

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Several different forms of progeny viral DNA can be identified in polyoma virus (Py)-infected mouse L-cells. The majority comprise mature form I superhelical DNA and the circular, double-stranded "theta" replicating intermediates in which the progeny DNA strands never exceed the unit genome length of the template. There is formed, in addition, a minority fraction of multimeric, linear, double-stranded Py DNA molecules that sediment heterogeneously at 28 to 35S and >35S. Restriction enzyme analysis of these large Py DNA molecules reveals them to be tandem arrays of multiple unit genome lengths, covalently linked head to tail. It is estimated that the 28 to 35S multimeric DNA has an average size of about 20 megadaltons, made up of 6 to 20 Py genome units. The >35S Py DNA is, of course, larger. Kinetic analysis indicates that formation of the monomeric progeny viral DNA and the 28 to 35S multimeric Py DNA reaches a peak at about 35 to 36 h postinfection. Synthesis of the very large linear molecules of >35S is first detected after this interval and continues thereafter. The de novo synthesis of all of these progeny Py DNA molecules proceeds apparently normally in Py-infected *ts*A1S9 mouse L-cells incubated at 38.5°C under conditions which restrict normal cellular DNA replication. These findings suggest that the cellular DNA topoisomerase II activity, encoded in the *ts*A1S9 locus (R. W. Colwill and R. Sheinin, submitted for publication), is not required for de novo formation of any form of Py DNA. However, the total amount made and the rate of synthesis of the large molecular weight Py DNA are affected very late in temperature-inactivated *ts*A1S9 cells.

Most studies of the replication of polyoma virus (Py) DNA have yielded information compatible with the model that this double-stranded, covalently closed genome is synthesized semiconservatively (20) from a replicative intermediate beginning at an unique origin (11, 12) and proceeding bidirectionally around the unit genome template (7, 10–12, 28). This so-called "theta" mechanism of replication would normally yield progeny replication intermediates of increasing size, never exceeding the unit genome length of the monomeric template and the final mature form I DNA of 3.4×10^6 daltons (11, 19).

Relatively recently Bjrsell (5) identified circular Py DNA molecules carrying "tailed" linear segments of four to five times the unit size of Py genome in mouse cells treated with an inhibitor of initiation. Similar molecules were detected in isolated nuclei still synthesizing Py DNA, but in an aberrant manner (6). These tailed molecules were interpreted as being intermediates of replication generated by the "rolling circle" mechanism initially suggested by Gilbert and Dressler (18) to explain certain aspects of bacteriophage DNA replication.

We have reexamined this problem using the Py–mouse L-cell system. This particular study was prompted by an earlier finding (33) that the temperature-sensitive *ts*A1S9 mouse L-cell supports apparently normal multiplication of Py and of viral DNA by the theta mechanism, under conditions in which cellular DNA synthesis is severely inhibited by temperature inactivation of the *ts*A1S9 gene product. Our most recent studies suggest that the *ts*A1S9 genetic locus encodes information for DNA topoisomerase II activity (R. W. Colwill and R. Sheinin, submitted for publication). It supports our earlier conclusion (32–36) that temperature-inactivated *ts*A1S9 cells carry normal enzymes of polydeoxyribonucleotide chain synthesis.

As indicated above, possible rolling circle replication of Py DNA had been observed under conditions in which cellular DNA synthesis had been interrupted by chemical or physical manipulation. The experiments set out herein were performed to test the postulate that such rolling

circle replication might be enhanced, or rendered more easily detectable, in Py-infected *ts*A1S9 mouse L-cells in which DNA replication was inactivated at some level other than that of polydeoxyribonucleotide chain synthesis.

MATERIALS AND METHODS

Cells. Wild-type (WT-4) and *ts*A1S9 mouse L-cells, whose properties have been described elsewhere (31–36), were grown in suspension culture containing α-minimal essential medium (40) supplemented with 7.5% (vol/vol) fetal calf serum. In all cases cells were grown to early logarithmic phase (1×10^5 to 2×10^5 cells per ml) and then mock infected or infected with virus as noted below. The permissive temperature was 34°C, the nonpermissive temperature 38.5°C.

Polyoma infection. The Toronto small plaque strain (TSP1) of Py (39) was used. A high-titer stock (6.8×10^{10} PFU/ml) was prepared as described earlier (33). Logarithmically growing cells were collected by centrifugation at approximately $800 \times g$ and suspended in 3.5 ml of virus suspension to give a multiplicity of infection of 1,000. (This high multiplicity of infection is required to ensure synchronous infection and optimal virus production in 100% of mouse cells, irrespective of strain origin [33, 37].) Mock-infected cells were taken up in an equivalent volume of suspending medium. The virus was allowed to absorb at 34 or 38.5°C for 1.5 h, after which the infected cells were diluted to $\simeq 2 \times 10^5$/ml and incubated further as required.

Radiolabeling and analyses of DNA. Unless otherwise noted, cultures were incubated at 34 or 38.5°C for 16 to 24 h, a period adequate to permit full expression of the *ts*A1S9 lesion (32). They were then infected or mock infected, cultured further for an interval noted in specific figure legends, and incubated in the presence of [*methyl*-^{3}H]thymidine (approximate specific activity, 20 mCi/mmol; final level, 1 μCi/ml) for 6 h to label the newly made DNA. The cells were harvested by centrifugation at $800 \times g$ and washed twice with phosphate-buffered saline (16). The DNA was fractionated and partially purified by the procedure of Hirt (21). The final supernatant carried small-molecular-weight DNA. The large-molecular-weight DNA in the sodium dodecyl sulfate (SDS)-NaCl precipitate was suspended in 2 ml of 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.8]) (24) and digested with RNase (50 μg/ml) at 25°C for 1 h and then with proteinase K (50 μg/ml) at 37°C for 12 h. The resulting viscous solution was extracted twice with an equal volume of TE (10 mM Tris-hydrochloride [pH 7.6], 1 mM EDTA)-saturated phenol. The aqueous phase was clarified by chloroform extraction and dialyzed against TE buffer overnight.

The Hirt supernatant DNA and the purified Hirt precipitate DNA were analyzed by sedimentation velocity in 15 to 30% neutral sucrose gradients containing 10 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA–1 M NaCl (8). Centrifugation was carried out for 16 h at 22,000 rpm in the L5-65 Beckman ultracentrifuge. Mouse L-cell 18S and 28S rRNA and ^{32}P-labeled bacteriophage lambda DNA (35S) were used as molecular weight markers. Fractions were assayed for acid-insoluble radioactivity and counted in toluene-based scintillation fluid as described previously (16). Where noted DNA was further purified from pooled fractions in the 20 to 22S, 22 to 28S, 28 to 35S, and >35S regions of the resulting gradients. These pooled DNA lots were resedimented, and those fractions recovered at 20 to 22S, 22 to 28S, 28 to 35S, and >35S, respectively, were pooled, dialyzed against TE buffer, and stored at −70°C pending further analysis. Py form I and II DNA, utilized as standards in some experiments, was extracted from virus-infected secondary mouse embryo cells (37) by the procedure of Hirt (21) and purified by the method of Radloff et al. (27).

Restriction enzyme digestion and agarose gel electrophoresis of DNA. Digestion of purified DNA by *Hin*dIII, *Eco*RI, and *Bgl*II restriction enzymes was carried out according to the specifications of the manufacturer (Bethesda Research Laboratories). Agarose (Seakem) slab gels (0.4 to 1%) were prepared in TEA buffer (10 mM Tris-acetate, 1 mM EDTA, 20 mM sodium acetate [pH 8.3]). The DNA was subjected to electrophoresis at 2 V/cm for 12 to 14 h. Lambda DNA fragments (Miles Laboratories, Inc.) obtained by digestion with *Hin*dIII endonuclease served as molecular weight markers. The electrophoretically separated DNA molecules were stained with ethidium bromide (10 μg/ml) and photographed over a UV transilluminator with Polaroid 55 P/N film (25).

Analysis of DNA by molecular hybridization. PDNA sequences were identified by hybridization as follows. DNA electrophoretically separated in agarose gels was denatured with alkali and transferred by the blotting method of Southern (38) to nitrocellulose filters, which were then baked under vacuum at 80°C for 12 h to fix the DNA. Before hybridization, the filter-bound DNA was incubated overnight in 6× SSC plus 0.02% each of Ficoll 400, polyvinylpyrrolidone, and bovine serum albumin at 65°C as described by Denhardt (14).

Hybridization was carried out in sealed plastic bags for 34 h at 65°C in 6× SSC containing 200 μg of heat-denatured and sonically sheared calf thymus DNA per ml plus 3×10^{-2} μg of ^{32}P-labeled Py DNA probe (1.4×10^8 cpm/μg), prepared by nick-translation with *Escherichia coli* DNA polymerase I (Boehringer Mannheim Corp.) as described by Rigby et al. (30). When hybridization was complete, the nitrocellulose filters were washed at 65°C in four changes of 2× SSC and rinsed for 1 h in 1.5 mM sodium pyrophosphate–25 mM potassium phosphate (pH 7.0)–0.1% SDS. To detect the hybridized complementary [^{32}P]DNA on the filters, these were dried and radioautographed by exposure to Kodak XR-1 X-ray film at −70°C.

The number of Py DNA equivalents in any DNA preparation was determined by measuring the rate of reassociation of ^{32}P-labeled Py DNA in the presence of the test DNA (17). Test DNA (250 μg/ml) premixed with ^{32}P-labeled Py DNA (2×10^{-4} to 3.4×10^{-3} μg/ml; 4.3×10^7 cpm/μg) was fragmented by ultrasound (Raytheon sonic oscillator model DF 101; 1 A for 30 s) and then denatured for 3 min at 100°C in 10 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA. The DNA was allowed to cool at ambient temperature for 1 min, and NaCl was added to 0.6 M. Annealing of the DNA was carried out at 68°C. At various intervals, 0.5-ml samples of the reaction mixture were withdrawn and frozen in 2.5 ml of 30 mM sodium acetate (pH 4.55)–0.5 mM $ZnCl_2$ pending further analysis. Thawed samples were digested with *Aspergillus oryzae* S1 nuclease (Miles Laboratories, 800 U/ml) at 37°C

for 3 h to degrade nonhybridized, single-stranded DNA. The residual ^{32}P-labeled, double-stranded DNA was counted by liquid scintillation spectrometry. The data obtained were used to calculate the equivalents of viral DNA per diploid quantity of cellular DNA as described by Gelb et al. (17).

Electron microscopy of DNA. DNA was spread for electron microscopy under aqueous conditions by using the Davis et al. (14) modification of the Kleinschmidt procedure. Grids were rotary shadowed with platinum-palladium and examined in a Phillips EM 300 electron microscope. Purified PM2 DNA (6.3 megadaltons) mixed in excess with test DNA served as a molecular weight marker.

RESULTS

Synthesis of Py DNA in mouse L-cells. As a prelude to analysis of the state of Py DNA in infected mouse L-cells, we established the kinetics of viral DNA synthesis as follows. Cells were incubated at 34 or 38.5°C for 16 h to permit temperature inactivation of the *ts*A1S9 gene product and then infected with Py as described above. At 5 h before the times noted in Table 1, cultures received [^{3}H]thymidine to label newly made DNA. The cultures were processed for separation by the Hirt procedure (21) of a supernatant fraction containing replicative intermediate, form II Py DNA and mature form I DNA. Incorporation of [^{3}H]thymidine into 20S form I DNA (subsequently purified further by velocity sedimentation in neutral sucrose density gradients, as noted above; see also legend to Fig. 1) was assessed.

As indicated in Table 1, the amount of ^{3}H-labeled Py DNA form I, barely detectable before 20 h postinfection (PI) in virus-infected *ts*A1S9 cells incubated at 34 or 38.5°C, subsequently increased throughout infection at both temperatures and reached a maximum between 35 and 40 h PI.

As expected, no DNA with the properties of Py DNA was recovered from mock-infected cells. The ^{3}H-labeled Py DNA form I yielded the two expected Py-specific DNA fragments (molecular weights, 2.0×10^6 and 1.4×10^6) after digestion with the restriction endonuclease *Hin*dIII (19) (data not shown). These results are in agreement with earlier studies which indicate that Py grows equally well in *ts*A1S9 cells under permissive and restrictive conditions (33).

Velocity sedimentation analysis of high-molecular-weight DNA newly made in Py-infected mouse L-cells. Having confirmed that unit size Py DNA genome is synthesized in *ts*A1S9 and in WT-4 cells incubated at 34 or 38.5°C (33), we sought to determine whether viral DNA is present as larger-molecular-weight material. Cells were infected with Py, incubated at these temperatures, and labeled with [^{3}H]thymidine between 20 and 30 h PI. The cells were then extracted by the Hirt procedure as described above to obtain material precipitated with NaCl and SDS and known to contain large-molecular-weight DNA (21). The precipitated DNA was analyzed by velocity sedimentation in neutral sucrose density gradients as described in the legend of Fig. 1.

TABLE 1. Synthesis of Py DNA in *ts*A1S9 mouse L-cells at 34 and 38.5°C

h PI	34°C	38.5°C
20	0.4	0.4
25	2.0	2.9
30	4.2	5.1
35	8.3	9.6
40	7.8	8.5
45	5.3	6.7
50	5.9	5.4

[a] Fractions of 18 to 21S cut from neutral sucrose gradients (run as noted in Fig. 1 legend) of Hirt supernatant material cosedimenting with marker Py DNA derived from virion particles and further identified by molecular hybridization with the Py DNA probe.

Figure 1B presents data obtained with DNA from mock-infected WT-4 cells incubated at 34 and 38.5°C. It shows that the majority of the NaCl–SDS–precipitated DNA is collected as large-molecular-weight material onto a 70% sucrose cushion (fraction cut D). This DNA has a sedimentation constant in excess of 35S relative to an added external marker. DNA sedimenting at 28 to 35S (fraction cut C) was also detected.

Similar results were obtained with mock-infected *ts*A1S9 cells incubated at 34°C (Fig. 1A). In the case of *ts*A1S9 cells incubated (for a total of 50 h) at the nonpermissive temperature, the total amount of NaCl-SDS–precipitable material was reduced. In addition, the size distribution was somewhat lower than that observed for control cell DNA. These observations, which are in accord with previous findings (33), will not be commented upon further here. Most important was the observation that little or no material sedimenting as monomeric Py DNA (see below) was detected in any mock infection, with specific molecular hybridization as a criterion.

The sedimentation profiles in Fig. 1C and D were obtained with DNA extracted from Py-infected *ts*A1S9 and WT-4 cells, respectively. They show essentially the same qualitative pattern in all cases. Four separate DNA size ranges were identified by comparison with marker molecules: A, 18 to 21S; B, 22 to 28S; C, 28 to 35S; and D, >35S. The major quantitative difference is seen in the fact that Py-infected *ts*A1S9 cells incubated at 38.5°C produce comparatively little >35S DNA (fraction cut D). This was expected on the basis of previous findings (33).

Detection of newly made Py DNA in the Hirt

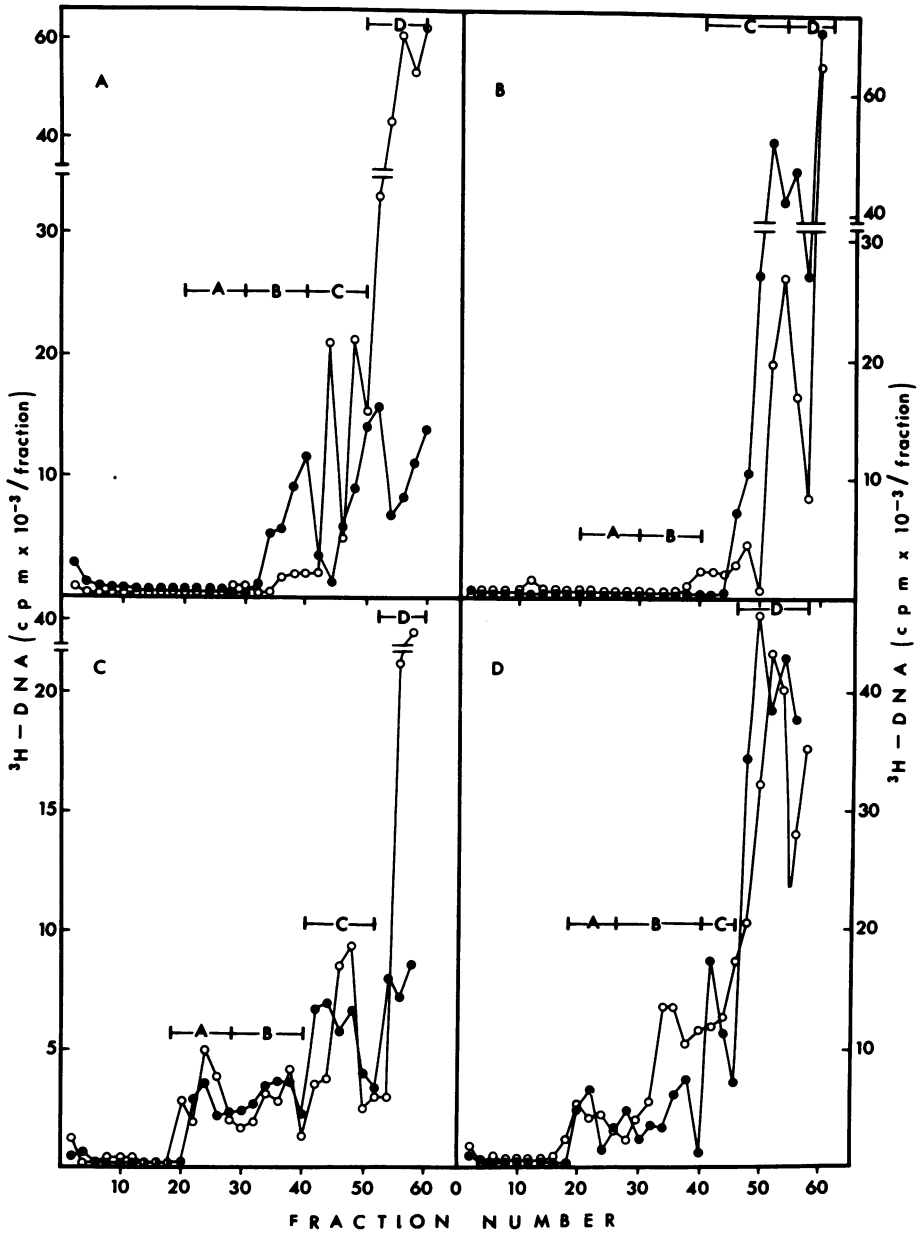

FIG. 1. Velocity sedimentation analysis of the Hirt precipitate DNA newly made in Py-infected or mock-infected mouse L-cells. The DNA newly made between 24 and 30 h PI in Py-infected or mock-infected WT-4 or *ts*A1S9 mouse cells incubated at 34°C (○) or at 38.5°C (●) was labeled with [^{3}H]thymidine as described in the text. The cells were processed by the Hirt procedure to precipitate large-molecular-weight DNA with NaCl-SDS. This was further purified and analyzed by velocity sedimentation in neutral sucrose density gradients. (A and B) Mock-infected *ts*A1S9 and WT-4 cells, respectively. (C and D) Py-infected *ts*A1S9 and WT-4 cells, respectively. Fraction cuts A, B, C, and D indicate material sedimenting at 18 to 21S, 22 to 28S, 28 to 35S, and >35S, respectively. These size estimates are based on the positions of sedimentation of the following markers: 3.4 megadalton 18 to 21S Py DNA, forms II and I, 22 to 28S monomeric replicative intermediate Py DNA, 35S bacteriophage λ DNA, and 18S and 28S rRNA.

precipitate derived from Py-infected mouse L-cells. It was of special interest here to determine whether the large-molecular-weight DNA made in Py-infected mouse L-cells carried viral DNA. To do this we first purified the DNA through an initial velocity sedimentation step as described above (see also Fig. 1). DNA fraction cuts, A, B, C, and D were made; the DNA was purified through a second cycle of velocity sedimentation and phenol extraction as described above and then analyzed further.

It was established that fraction cut A of 18 to 21S DNA contained newly made Py DNA primarily of the form I conformation. This was assessed by the following criteria: molecular hybridization (to be discussed below), electrophoresis in agarose gels (25), and susceptibility to cutting by *Eco*RI endonuclease thereby generating unit genome-length, double-stranded linear DNA of 3.4 megadaltons (19) (data not shown). We presume that this monomeric, newly replicated progeny Py DNA was present in the SDS-NaCl Hirt precipitate as a contaminant from the majority component of the Hirt supernatant. A similar analysis was made of the 22 to 28S fraction B DNA cuts from Py-infected cells (data not shown). By analogy with previous work (7, 8, 33), we concluded that this material, which runs in 1% agarose gels (25) very close to the exclusion limit, contains monomeric replicative intermediate Py DNA.

Of special interest in the present context are the large-molecular-weight DNA fractions, sedimenting heterogeneously at 28 to 35S and >35S. We therefore focused on the fraction C and D cuts noted in Fig. 1C and D. The DNA was extensively purified as noted above and then examined by electron microscopy to assess the possible presence of double-stranded, circular DNA molecules. None was detected on any of more than 50 grids examined. There was, however, much linear, double-stranded DNA. We probed quantitatively for Py DNA-containing molecules, by DNA-DNA reassociation kinetic analysis, as described above. We determined that Py-infected Wt-4 and *ts*A1S9 cells incubated at 34°C for 24 h PI contained ≈130 and 147 (respectively) equivalents of Py DNA sediment-

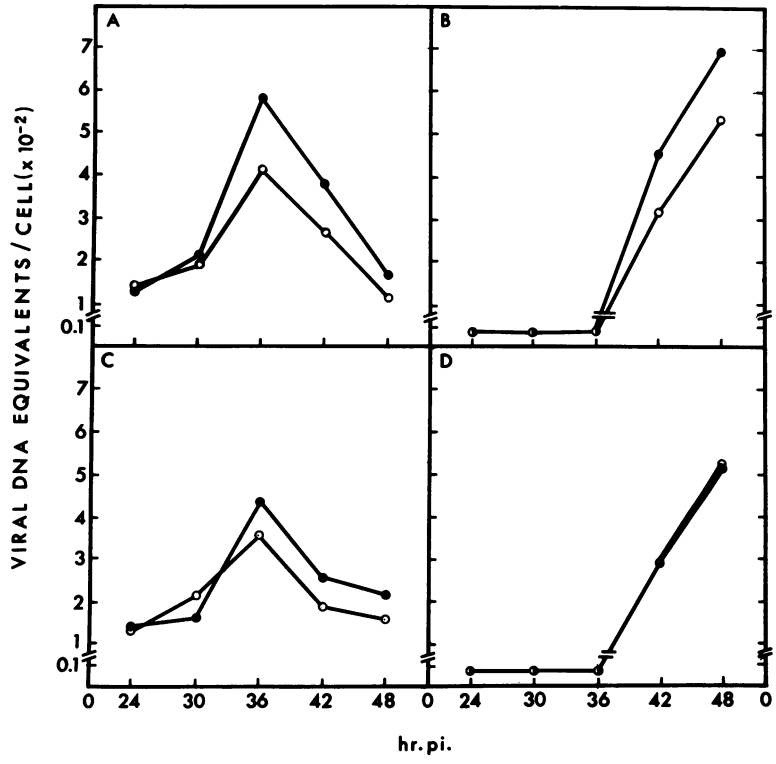

FIG. 2. Time course of synthesis of Py DNA forms in mouse L-cells. Cultures of Py-infected WT-4 or *ts*A1S9 cells were incubated at 34°C (○) or 38.5°C (●). After various intervals the 28 to 35S (A and C) and >35S (B and D) DNA fractions were cut from neutral sucrose gradients as noted in the legend to Fig. 1, purified further extensively, and analyzed by molecular hybridization for the presence of Py DNA, using DNA-DNA renaturation kinetics as described in the text.

ing at 28 to 35S per cell. The analogous results for cells incubated at 38.5°C were 144 and 132 copies of viral DNA per cell. For the >35S material, the values were less than one genome equivalent of viral DNA per cell in all instances.

Time course of synthesis of high-molecular-weight Py DNA. The foregoing studies revealed that high-molecular-weight forms of Py DNA can be recovered from virus-infected mouse L-cells incubated at 34 or 38.5°C. We therefore examined the kinetics of formation of the 28 to 35S and >35S Py DNA components throughout the infectious cycle (Fig. 2).

The data presented in Fig. 2A and C reveal similar patterns of formation of 28 to 35S Py DNA in WT-4 and *ts*A1S9 cells at both 34 and 38.5°C. Thus, such synthesis was first detected ≈24 h PI; it rose rapidly to reach a peak 36 h PI, after which it declined. These results are similar to those obtained with mature Py DNA (Table 1). They also show that the synthesis of the 28 to 35S Py DNA proceeds, in temperature-inactivated *ts*A1S9 cells, to very near the same level as that which occurs in wild-type cells and in *ts*A1S9 cells cultivated at the permissive temperature.

Figures 2B and D present the kinetics of formation of >35S viral DNA. In Py-infected WT-4 and *ts*A1S9 cultures incubated at 34 or 38.5°C, little or no >35S viral DNA was detected before ≈36 h PI. Thereafter, its formation increased steadily throughout the latter part of the infectious cycle. It should be noted, however, that the rate of accumulation of >35S Py DNA was ≈1.3 times higher in WT-4 cells cultured at 38.5°C as compared with the rate in cells incubated at 34°C. No such augmentation was observed in the case of Py-infected *ts*A1S9 cells incubated at the nonpermissive temperature. There may also be some impact of expression of the *ts*A1S9 mutation on the total amount of 28 to 35S Py DNA made (e.g., at 36 h PI).

Organization of high-molecular-weight Py DNA in mouse L-cells. The foregoing studies suggest that Py DNA can be recovered as material of larger than unit genome size (>3.4 megadaltons) in productively infected mouse L-cells. Restriction enzyme analyses were performed (Fig. 3) to examine the organization of such Py DNA and to assess its possible association with cellular DNA.

Attention was first focused on the 28 to 35S DNA isolated from Py-infected *ts*A1S9 and WT-4 cells 36 h PI, i.e., at the time of maximal accumulation. As is evident in the Fig. 3A electropherograms obtained with ethidium bromide-stained DNA, 28 to 35S material from *ts*A1S9 and WT-4 cells run in slots 1 and 3, respectively, was recovered at the exclusion limit of a 1% agarose gel. When such DNA was reacted with ^{32}P-labeled Py DNA probe, the radioautograms of slots 1 and 3 in segment B of Fig. 3 were obtained. They reveal the presence of viral DNA in a form which is much larger than unit Py genome. When such experiments were repeated with agarose gels of 0.4 or 0.7%, this 28 to 35S DNA migrated heterogeneously (data not shown) as material of 20 to 30 megadaltons as indicated by marker DNA.

The observation that the 28 to 35S DNA component carries Py DNA of very large molecular weight cannot be attributed to spurious, nonspecific trapping of unit-length Py genome with much larger cellular DNA, since the 28 to 35S DNA appeared to be totally free of detectable amounts of form I and II Py DNA as revealed by electron microscopy. This conclusion was confirmed by the following reconstruction experiment. Py DNA extracted from purified virions (7) was predigested with *Eco*RI endonuclease and then mixed with the 28 to 35S DNA of mock-infected WT-4 cells and subjected to electrophoresis and Southern blot analysis (Fig. 3). All of the Py DNA was recovered as unit genome length DNA comigrating with marker (Fig. 3B, slot 5), well away from the cellular DNA excluded from the gel and detected by ethidium bromide staining in Fig. 3A, slot 5. Similar results were obtained in analogous mixing experiments performed with the 18 to 21S Py DNA isolated from virus-infected cells (data not shown). These various studies suggest that the Py DNA in the 28 to 35S DNA component of virus-infected mouse L-cells is present as molecules of very large molecular weight or that it is covalently associated with large-molecular-weight cellular DNA.

The DNA patterns shown in slots 2 and 4 of Fig. 3 derive from *Eco*RI digestion of 28 to 35S DNA purified from *ts*A1S9 and WT-4 cells, respectively. The ethidium bromide-stained material (Fig. 3A) reveals a diffuse pattern of both large- and small-molecular-weight digestion products. Only one band was identified as Py DNA by molecular hybridization and by its comigration with marker Py DNA (Fig. 3B, slot 5). It should be noted that, whereas all of the undigested, Py-specific DNA in the 28 to 35S DNA fractions remains at the exclusion limit of the gels (Fig. 3, slots 1 and 3) all of the digested material is recovered as unit-length Py DNA (Fig. 3, slots 2 and 4). Such a result would be obtained if the Py DNA is present as a large-molecular-weight multimer, free or integrated into cellular DNA, at or near the *Eco*RI restriction site on the viral DNA.

If Py DNA were covalently joined to cellular DNA, digestion with the restriction enzyme *Bgl*II (which does not cleave Py DNA [4]) should produce DNA fragments larger than unit-

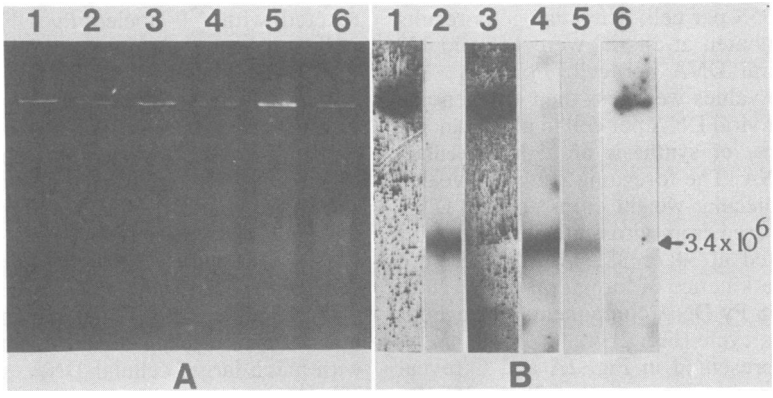

FIG. 3. Restriction enzyme analysis of the 28 to 35S Py DNA made in mouse L-cells. The 28 to 35S Py DNA synthesized in WT-4 or *ts*A1S9 cells incubated at 38.5°C was purified, treated with *Eco*RI or *Bgl*II, and analyzed by agarose gel electrophoresis as described in the text. Segment A presents ethidium bromide-stained DNA gel patterns of DNA preparations (1 to 2 μg each). Segment B gives the analogous Southern blots, using ^{32}P-labeled Py DNA as a probe for viral DNA. Slots: 1 and 2, 28 to 35S DNA from Py-infected *ts*A1S9 cells without and with *Eco*RI digestion; 3 and 4, 28 to 35S DNA from Py-infected WT-4 cells before and after *Eco*RI digestion; 5, *Eco*RI-treated Py DNA mixed with DNA from mock-infected WT-4 cells; 6, 28 to 35S DNA from *ts*A1S9 cells treated with *Bgl*II.

length Py DNA. The size of these molecules would be a function of the proximity of cellular *Bgl*II sites to Py DNA.

The results of *Bgl*II endonuclease treatment of material derived from Py-infected *ts*A1S9 cells incubated at 38.5°C for 36 h is shown in Fig. 3, slots 6. All of the Py-specific DNA remained at the exclusion limit of the gel (Fig. 3B, slot 6). No Py-specific DNA was detected migrating between the DNA at this exclusion limit, with a putative molecular weight of 20×10^6 and unit-length Py DNA of 3.4 megadaltons (Fig. 3B, slot 5). The migration of heterogenous Py-specific DNA of ≥20 megadaltons in agarose gels of lower porosity (e.g., 0.4 and 0.7%) was similarly not affected by *Bgl*II enzyme digestion (data not shown). Analogous results were obtained with the 28 to 35S DNA purified from virus-infected *ts*A1S9 cells incubated for 36 h at 34°C and from Py-infected WT-4 cells incubated at 34 or 38.5°C for 36 h. The foregoing findings suggest that the 28 to 35S Py DNA may not be covalently bound to cellular DNA, but may exist as free, large-molecular-weight, viral DNA multimers of 20 to 30 megadaltons in size (calculated on the basis of sedimentation value).

Organization of Py genomes within the high-molecular-weight viral DNA. Information about the molecular organization of the Py genome units just described was sought by using sensitivity to *Eco*RI endonuclease as a probe. The possibilities are that the viral DNA is arranged in a head-to-head or in a head-to-tail configuration. Only single Py DNA molecules were released by digestion of the 28 to 35S Py DNA with *Eco*RI endonuclease (Fig. 3, slots 2 and 4). These were of unit genome size (i.e., 3.4 megadaltons), as expected if the viral DNA is linked head-to-tail in a multimeric structure.

Electron microscopy examination of large-molecular-weight Py DNA. Purified large-molecular-weight Py DNA was examined by electron microscopy to determine whether the DNA has a circular or a linear configuration after digestion with the *Bgl*II endonuclease. This enzyme would be expected to degrade the cellular DNA while leaving Py DNA intact.

Fractions of 28 to 35S and >35S DNA were cut from gradients (as described in the legend of Fig. 1) run to partially purify large-molecular-weight material from Py- amd mock-infected mouse L-cells. This DNA was digested to completion with *Bgl*II endonuclease as recommended by the manufacturers, subjected to a second cycle of velocity sedimentation, and then processed for electron microscopic examination.

Electron microscope grids prepared after final processing of mock-infected cellular material yielded little or nothing identifiable as DNA. In contrast, a majority of molecules examined in the purified 28 to 35S DNA and all of the >35S DNA molecules derived from Py-infected cells were similar to those shown in Fig. 4.

Over 99% of the DNA molecules recovered as *Bgl*II-resistant material from Py-infected cells were found to be linear and double stranded (Fig. 4). Fewer than 1% were circular, and these carried short linear segments of less than genome length. Most of the molecules in the 28 to 35S DNA fractions ranged in size from 10 to 17 μm. This corresponds to 20 to 30 megadaltons or about 6 to 10 times the unit size of the Py

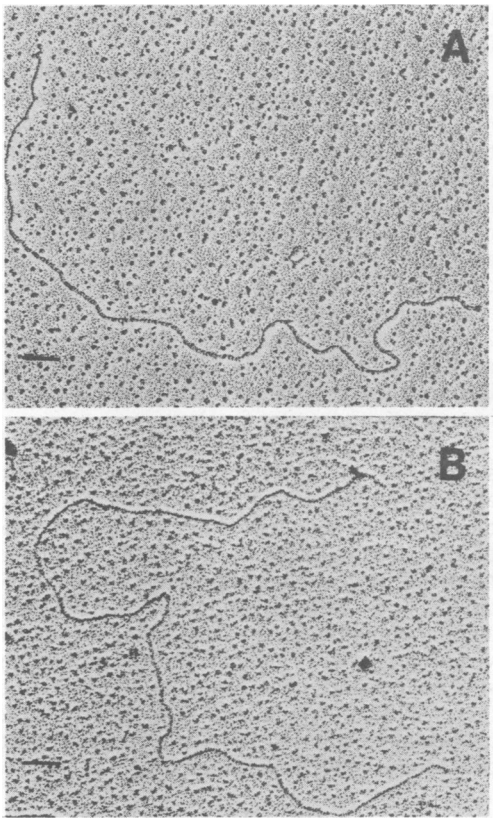

FIG. 4. Electron micrographs of DNA molecules in the large-molecular-weight, viral DNA-containing fractions of Py-infected mouse L-cells. The 28 to 35S and >35S DNA from Py-infected *ts*A1S9 cells incubated at 38.5°C for 36 h was collected by velocity sedimentation as described in the legend of Fig. 1, digested with *Bgl*II, purified further extensively, and examined by electron microscopy as described in the text. Bacteriophage PM2 DNA (~6.3 megadaltons) served as the internal size marker from which molecular weights of molecules were determined.

genome. These various observations can be interpreted to suggest that the 28 to 35S and >35S Py DNA produced in virus-infected mouse L-cells occurs in the form of long, linear sequences.

DISCUSSION

In previous studies (33) it was demonstrated that temperature-inactivated *ts*A1S9 cells are able to replicate Py virus at the nonpermissive temperature and to synthesize de novo monomeric forms I (20S) and II (18S) from the 22 to 28S monomeric replicative intermediate. Using somewhat different experimental conditions and highly radiolabelled probes, we have here confirmed this finding. In addition, we have shown that *ts*A1S9 cells also make large-molecular-weight forms of Py DNA under conditions which restrict formation of normal, large-molecular-weight cellular DNA. Molecules of two size classes have been examined. These, which sediment, respectively, at 28 to 35S and at >35S, are also synthesized by wild-type WT-4 cells incubated at 34 or 38.5°C, and by *ts*A1S9 cells maintained at the permissive temperature.

The large-molecular-weight Py DNA (i.e., >28S) constitutes at most 0.07% of the total DNA newly made in virus-infected L-cells. It is therefore perhaps not surprising that it was undetected in an early study (33), which used less sensitive molecular probes than herein. It is clearly associated with productive infection because, as already demonstrated (33), all L-cells give rise to infectious centers under the conditions of infection used herein.

The significance of the large-molecular-weight Py DNA remains unknown. Its synthesis does not likely reflect host cell specificity because this was observed by Björsell using Py-infected 3T6 mouse fibroblasts (5). Nor is the phenomenon restricted to Py virus. Rigby and Berg (29) reported the formation of large-molecular-weight forms of simian virus 40 DNA, with kinetics of synthesis similar to those observed in the present study, by using virus-infected CV-1 cells. This raises the possibility that all of the B-type papovaviruses may exhibit the same physiology of viral DNA synthesis throughout the multiplication cycle.

There are a number of possible explanations for the finding of large-molecular-weight Py (and simian virus 40) DNA in productively infected cells. One invokes the so-called rolling circle mechanism of DNA replication, initially formulated by Gilbert and Dressler (18), to interpret specific aspects of procaryotic DNA synthesis. It implies a replicative intermediate which can be visualized by electron microscopy as a double-stranded circle of unit genome size, carrying a linear, double-stranded segment of varied length which can greatly exceed the genome molecular weight. The rolling circle mechanism has been suggested to function as a minority component in the replication of Py in 3T6 cells (5) and of simian virus 40 in CV-1 cells (26). In the former study, few putative replicative intermediates with very long linear segments were observed. Only short-tailed molecules were noted in the latter investigation, in which monomeric molecular weight viral DNA intermediates were examined in cells infected, under restrictive conditions, with *ts* virus.

In the present study less than 1% of the large-molecular-weight Py DNA molecules examined by electron microscopy were found to be circular, and these carried linear segments with vari-

able-length tails all much less than one genome unit in length. Although these could be indicative of replication by a rolling circle mechanism, it should be recalled that unit-length Py DNA circles carrying short linear segments of up to one genome unit length may derive from the denaturation of molecules replicating by the usual semiconservative mechanism when this is interrupted, for example, by inhibition of de novo protein synthesis (7). In a study of simian virus 40-infected CV-1 cells which revealed linear multimeric viral DNA (29), circular molecules with the properties of putative rolling circle intermediates were not identified. Such observations do not entirely exclude some rolling circle replication.

It is, however, possible to envisage another mechanism for the formation of small amounts of large-molecular-weight Py DNA as seen herein, i.e., through recombination. Such recombination could occur as an aberrant event associated with the formation of double-stranded linear form III Py DNA (10) or pseudovirion DNA (42) known to occur very late during infection (10, 31). Recombination could also result from integration (43) and excision events (23).

As already implied above, electron microscopic analysis of the 28 to 35S and >35S DNA fractions of Py-infected cells revealed that a majority were linear and double stranded. Based on their contour lengths and the sedimentation rates, the 28 to 35S and >35S Py DNA molecules are calculated to be 20 to 30 and >35 megadaltons, respectively, i.e., 6 to 10 and more than 10 Py genome units in length. Digestion of the large-molecular-weight Py DNA with *Eco*RI endonuclease released only unit-length viral DNA segments. This finding suggests that the concatemeric Py DNA comprises only viral genomes linked in head-to-tail arrangement. The absence of significantly large sequences of cellular DNA in these structures is indicated by the fact that treatment with the *Bgl*II restriction enzyme does not change their mobility in agarose gels nor their size as revealed by electron microscopy. However, extremely small residual terminal cell DNA sequences would not have been detected.

The detection of large-molecular-weight Py DNA is in itself an interesting observation. It becomes even more so because of the kinetics of its formation. Thus, the pattern of synthesis of 28 to 35S Py DNA closely parallels that for mature form I DNA, being readily detectable 24 h PI increasing steadily to reach a peak at 36 h PI, and declining thereafter. In contrast, formation of >35S Py DNA was first measurable ≈36 h PI and proceeded at least throughout the next 12 h. Thus, synthesis of the >35S Py DNA occurred very late during infection, beginning at the time when formation of the other Py DNA forms began to decline.

In this context it is of interest to recall that polymeric bacteriophage lambda DNA is formed by a rolling circle mechanism in association with the late processes of packaging and virion maturation (1, 41). Recent electron microscopic studies with simian virus 40 (9) suggest that oligomeric viral DNA may participate in virion assembly. Synthesis of large-molecular-weight concatemers of pseudorabies virus DNA has also been detected late during infection (2, 3, 22). It is suggested that these also participate in packaging of DNA into capsids. Perhaps the large-molecular-weight Py DNA described herein may serve a similar function.

The studies set out here and elsewhere (33) suggest that the DNA topoisomerase II activity affected by expression of the *ts*A1S9 mutation of mouse L-cells is not essential for the replication of the several forms of Py DNA. It should, however, be noted that expression of the *ts*A1S9 defect does ultimately impact on the total amount of multimeric Py DNA made very late in virus-infected cells incubated at the nonpermissive temperature. The possible contribution of the *ts*A1S9 protein in the formation of large-molecular-weight Py DNA under these latter conditions is therefore under study.

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