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Polyoma Viral DNA Replicated as a Nucleoprotein Complex in Close Association with the Host Cell Chromatin

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Polyoma viral DNA is shown to be replicated in close association with the mouse cell chromatin. Two virus-specific nucleoprotein complexes, designated complex A and B, can be dissociated from the isolated chromatin by gentle homogenization in 0.5 M NaCl. Complex A contains only replicating polyoma (Py) DNA whereas complex B contains only mature Py DNA I. The results show, furthermore, that complex A, containing viral DNA in different stages of replication, and complex B are both nucleoproteins with the same buoyant density. The data presently available suggest that newly synthesized stretches of Py DNA are immediately complexed with mouse cell histones and that complex B becomes the "core" of progeny Py virions. These results suggested that Py-induced replication of the mouse cell chromatin may be necessary to provide replicating Py DNA with histones.

Infection of primary confluent mouse kidney cell cultures with polyoma virus (Py) leads to the production of progeny virus and to the destruction of the host cells. The synthesis of the viral DNA takes place in the nucleus; it is accompanied by the replication of the host cell chromatin, which may be a necessary condition for the replication of the viral DNA (10, 23). The close temporal and functional relationship between the synthesis of mouse chromosomal and Py viral DNA points to the possibility that this small viral DNA (molecular weight 3×10^6) might use the chromosome replication machinery of the host cell for its own replication.

In the present paper we report the isolation of two Py-specific DNA-protein complexes, designated complex A and B, from the chromatin of Py-infected mouse kidney cell cultures. We show that complex A contains only Py DNA in statu nascendi whereas complex B contains only mature, superhelical Py DNA I. (This paper was submitted to partially fulfill the requirements for a Ph.D. thesis to the Univ. of Geneva by T.S.)

MATERIAL AND METHODS

Cell cultures and virus. Primary mouse kidney cell cultures were prepared by the method of Winocour (24) from 10-day-old CR-1 mice (obtained from the Swiss Institute of Experimental Cancer Research in Lausanne, Switzerland). Cells were plated in large plastic petri dishes (88 mm in diameter), using reinforced Eagle medium supplemented with 10% calf serum (Microbiological Associates Inc.). Under the conditions used, the cultures reached confluence 3 days after plating and contained then approximately 10^5 cells/cm^2. They were infected with 0.4 ml of crude Py viral lysates (containing approximately 10^8 PFU/ml) 1 or 2 days after reaching confluence. The virus was adsorbed at 37 C in a CO_2 incubator for 2 h; thereafter the cultures were covered with 10 ml of reinforced Eagle medium containing no serum per culture.

Labeling of DNA with [^{3}H]dT: (i) Pulse labeling. At the times indicated in Results, the medium was thoroughly removed and the cultures were covered with 1 ml of serum-free medium, warmed to 37 C, containing 100 μCi of [^{3}H]thymidine (dT) per ml (New England Nuclear, 20 to 25 Ci/mmol). The labeling was stopped after 3 or 5 min by putting the dishes onto ice water. After removal of the radioactive medium, the cultures were washed twice with 5 ml of ice-cold isotonic buffer (0.13 M NaCl-0.005 M KCl-0.001 M Na_2HPO_4-0.025 M Tris, pH 7.5 at 2 C) and then immediately processed as described below.

(ii) Pulse-chase. The cultures were labeled with [^{3}H]dT for 5 min under the conditions described above. To end the pulse, the radioactive medium was removed and the cultures were washed twice with 5 ml of medium and then covered with 10 ml of medium, warmed to 37 C and containing 20 μg of dT per ml (Calbiochem). Thereafter they were incubated at 37 C ("chase") for the lengths of time indicated in Results and then processed as described below.

Preparation of the nucleoprotein complexes. Unless indicated otherwise, four cultures were used for

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each experimental point. The whole isolation procedure was carried out at 2 to 4 C.

Collection of the cells. The ice-cold isotonic buffer was thoroughly removed from the cultures. Thereafter 2 ml of hypotonic buffer (0.01 M Tris-hydrochloride-0.01 M KCl-0.005 M $CaCl_2$; pH 7.4 at 2 C) was added to the first dish. The cells were scraped off with a rubber policeman and the cell suspension was transferred to the second dish; the same procedure was repeated for the third and the fourth dish. The final cell suspension (containing approximately 3×10^7 cells in 2 ml) was transferred into a Blaessig glass homogenizer tube.

Isolation of the nuclei. The cells were kept in the ice-cold hypotonic buffer for about 10 min and then disrupted by passing 10 times slowly through a 21-gauge needle. This procedure broke more than 90% of the cells as judged by phase-contrast microscopy. The suspension was then centrifuged in the homogenizer tube in a Sorvall HB 4 rotor at 2,000 rpm ($600 \times g$) for 5 min at 2 C. The supernatant ("cytoplasm") was discarded. The nuclear pellet was then processed as described below.

Isolation of the chromatin. The procedure used for the isolation of the chromatin was essentially that described by Hancock (9). The nuclear pellet was resuspended in 2 ml of "chromatin buffer" (0.08 M NaCl-0.02 M EDTA, pH 7.2 at 2 C) and homogenized with 20 strokes in the Blaessig homogenizer. The homogenate was centrifuged in the homogenizer tube in a Sorvall HB 4 rotor at 4,000 rpm ($2,500 \times g$) for 15 min at 2 C. The pellet was resuspended in 2 ml of chromatin buffer and subjected to four additional cycles of homogenization and centrifugation. All supernatants were pooled and designated as "nucleoplasm." From the final pellet ("chromatin"), the viral complex A and B were isolated as described below.

Isolation of the viral nucleoprotein complexes A and B. The chromatin was rinsed once with 1 ml of NEN buffer (0.5 M NaCl-0.01 M EDTA-0.01 M sodium acetate, pH 6.0 at 2 C), which was discarded. Thereafter, the chromatin was homogenized (20 strokes) in 1 ml of NEN buffer, incubated at 2 C for 60 min, and then transferred into a polypropylene tube and centrifuged in a Sorvall HB 4 rotor at 8,000 rpm ($10,000 \times g$) for 10 min at 2 C. The resulting supernatant ("NEN supernatant"), containing the two viral complexes, was layered on top of a linear sucrose density gradient (5 to 20% [wt/vol] in NEN buffer) and was centrifuged in a Spinco SW40 rotor at 40,000 rpm ($113,000 \times g$) for 180 min at 2 C. Fractions containing 10 drops each were collected from the bottom of the tube.

Extraction of the DNA from complex A and B. The fractions containing complex A or B (see Results) were pooled and the DNA was extracted by one of the following three methods which gave essentially the same results.

Method (i) ("Hirt procedure"; 11). The pooled fractions were mixed with 1/10 volume of 20% sodium dodecyl sulfate (SDS) (Serva Heidelberg, 20% [wt/vol] in double-distilled water) and incubated at 37 C for 15 min. Thereafter, 1/5 volume of 5 M NaCl was added, and the solution was mixed on a Vortex mixer for 15 s and then incubated at 2 C for about 12 h. The SDS-protein precipitate was removed by centrifugation in a Sorvall HB 4 rotor at 12,000 rpm ($23,000 \times g$) for 20 min at 2 C. The supernatant contained more than 90% of the radioactive DNA originally present in the pooled fractions.

Method (ii) ("Sarkosyl extraction"). The pooled fractions were mixed with 1/10 volume of Sarkosyl N-30 (Geigy AG, 10% [vol/vol] in double-distilled water) and incubated at 37 C for 15 min. The DNA was then directly used for analysis. This method gives 90 to 100% recovery of acid-precipitable radioactivity.

Method (iii) ("SDS-Pronase treatment"). The pooled fractions were mixed with 1/20 volume of 1% SDS and with 1/20 volume of Pronase (Calbiochem A grade, 1 mg/ml in double-distilled water, self-digested at 37 C for 2 h) and incubated at 37 C for 16 h. Solid CsCl was added to a density of 1.70 g/cm^3, and the solution was centrifuged to equilibrium in a Spinco SW65 rotor at 35,000 rpm for 24 h at 20 C. Fractions containing 8 drops each were collected from the bottom of the tube. The fractions containing the radioactive DNA were pooled, dialyzed twice against 100 volumes of 0.1 × SSC (1 × SSC: 0.15 M NaCl-0.015 M sodium citrate, pH 7.2), and then used for analysis. DNA to be used for DNA-DNA hybridization experiments was always purified by this method which resulted in a 70 to 80% recovery.

Extraction of mouse cell DNA. Uninfected confluent mouse kidney cell cultures were lysed with 1% SDS (0.4 ml/dish). The lysate was incubated at 60 C for 15 min, mixed with 1/5 volume of 5 M NaCl, and then extracted four times with 1 volume of $CHCl_3$-isoamyl alcohol (23:1, vol/vol). DNA was precipitated from the water phase with 3 volumes of ethanol, collected with a glass rod, and washed three times with ethanol and once with ether. It was then dissolved in 0.1 × SSC (200 to 400 μg of DNA/ml), digested with RNase (Calbiochem A grade, 50 μg/ml, 2 h at 37 C), and then with Pronase (50 μg/ml, 2 h at 37 C), adjusted to 1 × SSC, and extracted twice with 1 volume of $CHCl_3$-isoamyl alcohol (no more interphase visible). The water phase was then extracted four times with ether, dialyzed twice against 100 volumes of 0.1 × SSC, and adjusted with solid CsCl to a density of 1.70 g/cm^3, and DNA was centrifuged to equilibrium in a Spinco SW65 rotor at 35,000 rpm for 30 h at 20 C. Fractions containing 8 drops each were collected from the bottom of the tube.

DNA-DNA hybridization: isolation of Py DNA I from purified virus. Crude viral lysates were incubated with DNase and RNase (14) and then purified by differential and CsCl equilibrium density gradient centrifugation (24). The fractions of the CsCl gradient containing the virus band (14) were pooled and dialyzed twice against 100 volumes of 0.001 M phosphate buffer (pH 7.2). The dialyzed preparation was mixed with 1/10 volume of 10% SDS. After incubation at 37 C for 15 min, the DNA was extracted three times with 1 volume of $CHCl_3$-isoamyl alcohol. The viral DNA was then subjected to ethidium bromide (EBr) CsCl equilibrium density gradient centrifugation (15). Fractions containing 6 drops each were collected from the bottom of the tube and those containing Py DNA I

(heavy band) were pooled, extracted twice with 1 volume of isopropanol to remove the dye, and then dialyzed three times against 100 volumes of 0.1 × SSC.

Preparation of Py DNA-containing filters. Py DNA I (10 μg/ml) was converted to single strands by boiling in 0.1 × SSC for 30 min, diluted into 100 volumes of ice-cold 6 × SSC, and fixed (2 μg of DNA/filter) by slow filtration onto 25-mm membrane filters (Schleicher & Schüll, B 6). Each filter was washed twice by filtration of 25 ml of 6 × SSC, dried over $CaCl_2$ at room temperature for about 12 h, and then heated to 80 C for 2 h. Blank filters without DNA were prepared in the same way.

Hybridization. Hybridization was carried out essentially by the urea method described by Kourilsky et al. (13). A portion (10 μliters, 1,000 counts/min, 0.1 μg) of ^{14}C-labeled Py DNA I was added to each sample of ^{3}H-labeled DNA to be analyzed (50 to 100 μliters; less than 0.1 μg in 0.1 × SSC). The solution was then mixed with 1/10 volume of 5 N NaOH, boiled for 7 min (18), and then quenched in ice water and neutralized with 1/5 volume of 5 M NaH_2PO_4. It was adjusted to 550 μliters with double-distilled water and then mixed with an equal volume of 8 M urea (Mann)-4 × SSC-0.2% SDS. Duplicate portions (500 μliters) were then incubated each with one filter in a screw-cap glass vial (Packard) at 40 C for 20 to 24 h. Studies of the hybridization kinetics have shown that this period results in maximum hybridization, corresponding to approximately 50% of the radioactive input viral DNA (unpublished results).

After hybridization, the filters were washed in the incubation vials at room temperature four times for 15 min with 5 ml of 4 M urea-2 × SSC and then twice for 5 min with 5 ml of 2 × SSC. They were dried and radioactivity was determined in 2.5 ml of toluene-based scintillation fluid in a Nuclear Chicago Mark II scintillation counter. All results were corrected for the counting background (10 counts/min for ^{3}H and 5 counts/min for ^{14}C) and for the overlap of ^{14}C into the ^{3}H channel (25%). The overlap of ^{3}H into the ^{14}C channel (less than 0.1%) was neglected.

RESULTS

As determined by autoradiography, the onset of Py-induced DNA synthesis is asynchronous. It begins in a small number of cells about 12 h postinfection (p.i.); the number of DNA-synthesizing cells increases rapidly and reaches a maximum around 28 to 30 h p.i. when 70 to 80% of the cells are synthesizing DNA (20). The experiments reported here were therefore performed around 28 h p.i.

Replication of Py viral DNA in close association with the mouse cell chromatin. In three experiments, Py-infected mouse kidney cell cultures were labeled with [^{3}H]dT (10 μCi/ml, 20 Ci/mmol) at 30 h p.i. for 5, 10, and 30 min, respectively; at the end of the pulse, the chromatin was isolated as described in Material and Methods. The results (not shown) demonstrated that in these experiments 80 to 90% of the acid-precipitable radioactivity present in the nuclei was recovered in the chromatin while the remainder was present in the nucleoplasm.

In other experiments the relative amounts of ^{3}H-labeled, mature, superhelical Py DNA I and of replicating Py DNA molecules were determined in (i) total cells, (ii) chromatin, and (iii) nucleoplasm. Four Py-infected cultures were labeled with [^{3}H]dT (10 μCi/ml) at 30 h p.i. for 3 min. At the end of the pulse, viral DNA was selectively extracted from two cultures (11); from the two remaining cultures chromatin and nucleoplasm were isolated and viral DNA was selectively extracted from each preparation. The DNA preparations were then subjected to EBr-CsCl equilibrium density gradient centrifugation (Fig. 1A-C). In this gradient, superhelical DNA bands at a higher density than relaxed circular or linear DNA whereas replicating circular DNA bands at intermediate densities (1, 12, 15, 17). The results in Fig. 1A-C show that essentially the same amounts and the same species of radioactive DNA are present in extracts from isolated chromatin as are found in extracts from total cells; only a small fraction of radioactive Py DNA (about 10%) can be detected in the nucleoplasm.

Isolation of two virus-specific DNA-protein complexes. The results presented in this and the following sections show that most or all of the free, i.e., non-encapsidated, nuclear Py DNA is present in the form of two nucleoprotein complexes. These complexes are associated with the host cell chromatin but can be dissociated from it by gentle homogenization in a buffer containing 0.5 M NaCl. The faster sedimenting complex A contains replicating Py DNA molecules whereas the more slowly sedimenting complex B contains only mature Py DNA I molecules.

Complex A. Py-infected cultures were labeled with 100 μCi of [^{3}H]dT per ml at 28 h p.i. for 3 min. At the end of the pulse the chromatin was isolated and then homogenized in NEN buffer as described in Material and Methods. The NEN supernatant was analyzed on a linear sucrose density gradient (5 to 20%). Figure 2 shows that most of the radioactive DNA recovered from the gradient is present as a single broad peak which cosedimented with bacteriophage R17 added as a sedimentation marker (80*S* in 1 × SSC; 6). This band always contained 30 to 40% of the total radioactivity present in the isolated chromatin; the remain-

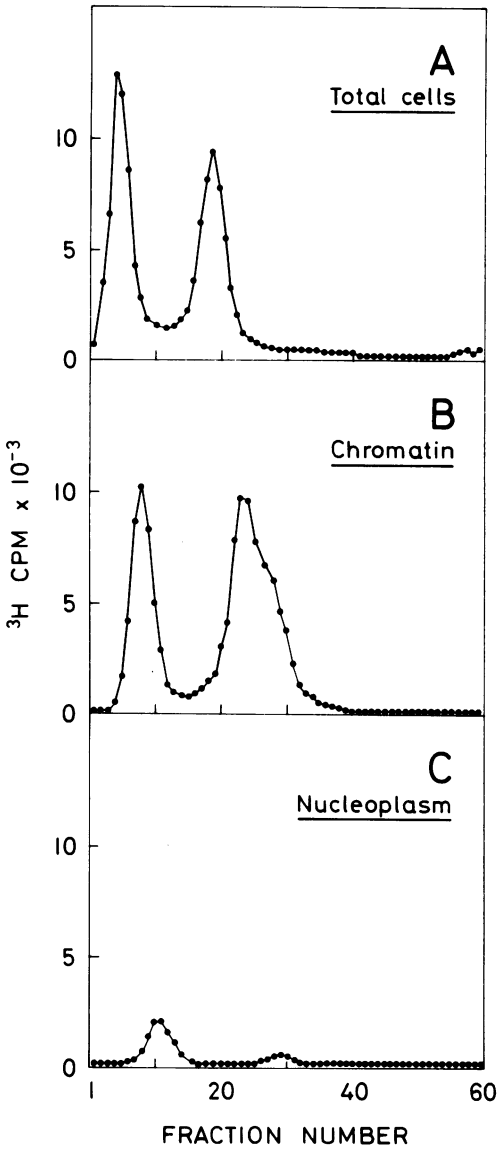

FIG. 1. *Evidence that Py DNA replicates in close association with the mouse cell chromatin. Py-infected cultures were labeled with 10 μCi of [^{3}H]dT per ml 28 h p.i. for 30 min. Py DNA was selectively extracted from (A) total cells, (B) isolated chromatin, and (C) nucleoplasm. The DNA samples were adjusted to 3.15 ml with 1 mM phosphate buffer (pH 7.2), mixed with 0.35 ml of EBr (1 mg/ml in water) and 3.14 g of solid CsCl, and then centrifuged to equilibrium in a Spinco Ti50 rotor at 40,000 rpm for 60 h at 20 C. Fractions of 3 drops were collected from the bottom of the tube onto filter paper disks. These were washed with 10% trichloroacetic acid, then 5% trichloroacetic acid, then 70% ethanol, dried, and counted for radioactivity in 2.5 ml of toluene-based scintillation fluid.*

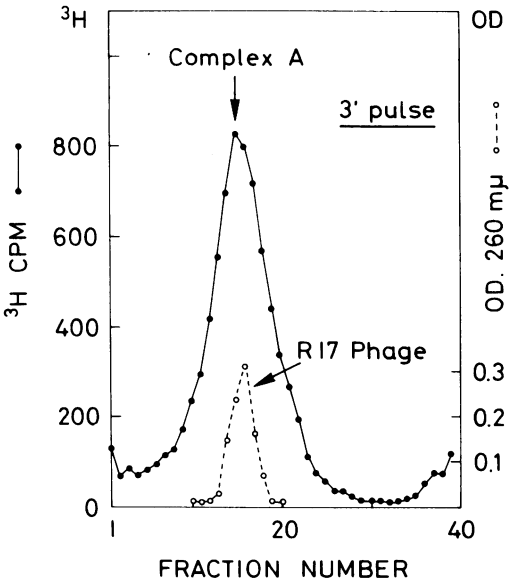

FIG. 2. *Isolation of complex A. Three Py-infected cultures were labeled with 100 μCi of [^{3}H]dT per ml 28 h p.i. for 3 min. Immediately after the pulse the chromatin was isolated and homogenized in NEN buffer. The NEN supernatant was mixed with unlabeled bacteriophage R17 (60 μg) and sedimented through a linear sucrose density gradient (5 to 20% [wt/vol] in NEN buffer, 13 ml) in a Spinco SW40 rotor at 40,000 rpm for 180 min at 2 C. Fractions of 10 drops were collected from the bottom of the tube and 20 μliters of each fraction were spotted onto filter paper disks which were dried and then counted for radioactivity. The position of R17 bacteriophage (arrow) was determined by measuring the absorbance at 260 nm. It should be noted that the sedimentation pattern of complex A is not influenced by the presence of the bacteriophage (unpublished observation).*

der was recovered in the NEN pellet and exhibited in CsCl density equilibrium gradients the density distribution of mouse cell DNA.

Complex B. After labeling with [^{3}H]dT for longer than 5 to 10 min, a second, more slowly sedimenting radioactive peak appeared. The amount of label in this second peak increases with the length of the pulse, and the peak becomes predominant by about 20 min (not shown). The same labeling and sedimentation pattern is observed in experiments where a 5-min pulse with [^{3}H]dT was followed by a chase in nonradioactive medium (Fig. 3). These results suggest the existence of a precursor-product relationship between the radioactive DNA molecules contained in complex A and B.

Absence of comparable DNA-protein complexes in uninfected mouse kidney cells. In several experiments, uninfected mouse kidney

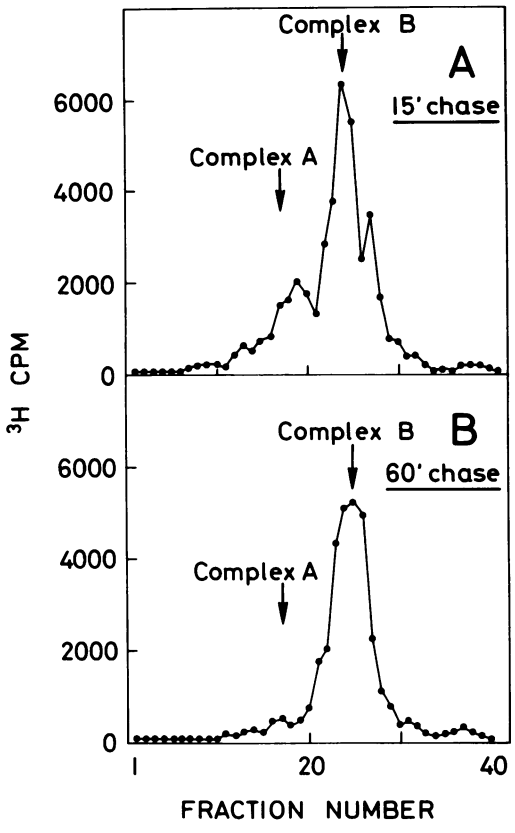

FIG. 3. *Isolation of complex B. Py-infected cultures were labeled with 100 μCi of [^{3}H]dT per ml 28 h p.i. for 5 min. The cultures were washed and incubated in nonradioactive medium containing 20 μg of dT per ml for 15 min or 60 min, respectively (chase) (seven cultures for each point). Chromatin was isolated and homogenized in NEN buffer, and the NEN supernatant was sedimented as described in Fig. 2. A 50-μliter amount of each fraction was spotted onto filter paper disks and then counted for radioactivity. A, 15-min chase; B, 60-min chase.*

cell cultures, both growing and confluent, were labeled with 100 μCi of [^{3}H]dT per ml for 5 min. The chromatin was isolated and extracted with NEN buffer, and the NEN supernatant was analyzed in sucrose density gradients as described above. In these experiments only 1 to 5% of the acid-precipitable radioactivity originally present in the isolated chromatin could be recovered on top of the gradients (in the last 4 or 5 of 40 fractions); no radioactivity could be detected elsewhere in the gradient (data not shown).

Reconstruction experiments. (i) Since DNA tends to form aggregates with basic proteins or polylysine, the possibility was considered that complex A and B might be the result of a nonspecific association of free Py DNA with cellular proteins during the isolation procedure. To test this possibility, ^{3}H-labeled Py DNA I (2 μg per 20,000 counts per min) was added either to a cell suspension from infected cultures immediately prior to the isolation of the nuclei or, alternatively, to isolated nuclei immediately prior to the isolation of the chromatin. In these experiments, less than 10% of the radioactive Py DNA was found associated with the isolated chromatin; virtually all of this chromatin-associated radioactivity was released into the NEN supernatant and, in sucrose density gradients, exhibited the sedimentation pattern of Py DNA I (not shown).

(ii) To examine the possibility that the association of complex A and B with the chromatin is an artifact of extraction, preparations of ^{3}H-labeled complex A or B were mixed with isolated nuclei from infected cells. Chromatin and nucleoplasm were then isolated from these nuclei; 80 to 90% of the radioactive complexes were recovered in the nucleoplasm. These results showed that purified complex A or B does not aggregate with chromatin during the isolation procedure.

Characterization of the DNA present in complex A. Py-infected cultures were labeled with 100 μCi of [^{3}H]dT per ml at 29 h p.i. for 5 min; complex A was then isolated from a sucrose density gradient as described above. Total DNA was extracted from complex A (see Material and Methods) and analyzed by several methods. One portion was sedimented through a neutral CsCl solution (pH 7.2) (19) (Fig. 4A). The results show that the bulk of the DNA forms a broad peak at 25*S*. Sedimentation through neutral sucrose density gradients (5 to 20%) resulted in a similar pattern (data not shown). Another portion of the DNA was sedimented through an alkaline sucrose density gradient (15 to 30%) (Fig. 4B). The results show that the bulk of the radioactive DNA has sedimentation coefficients of 16*S* or less; thus it exhibits the sedimentation pattern of linear, single-stranded DNA molecules of the size of one complete strand of Py DNA or less. Only about 5% of the radioactivity is found at the position expected for double-stranded cyclic coils (53*S*; 21). Another portion of the DNA was subjected to EBr-CsCl equilibrium density gradient centrifugation (Fig. 4C). The results show that the bulk of the radioactive DNA bands at densities expected for replicating Py DNA molecules (1, 12, 17) whereas only a small fraction bands at the density of Py DNA I. The results shown in Fig. 4A-C lead to the conclusion that complex A contains Py DNA molecules in different stages of replication.

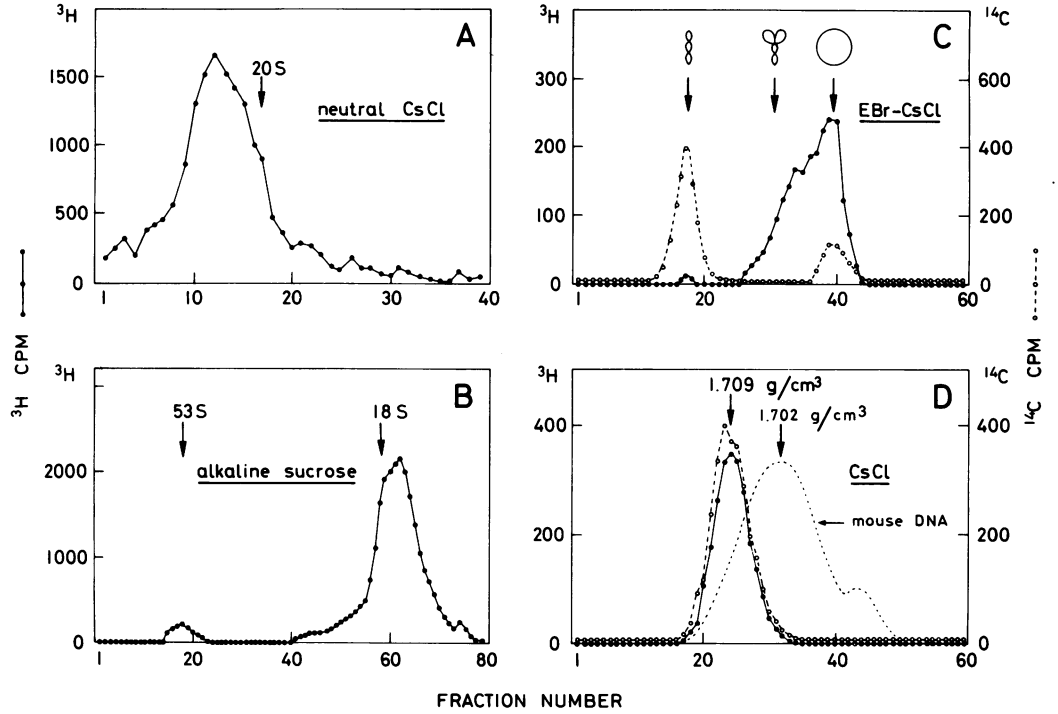

FIG. 4. *Characterization of the DNA from complex A. Py-infected cultures were labeled with 100 μCi of [³H]dT per ml 29 h p.i. for 5 min. Complex A was isolated as described in Fig. 2, and DNA was quantitatively extracted with Sarkosyl. ³H-labeled DNA samples to be analyzed were mixed with 2,000 counts/min of ¹⁴C-labeled Py DNA (about 80% form I and 20% form II) added as a marker, and then analyzed as follows. A, Sedimentation through a neutral CsCl solution: a sample (0.2 ml) was layered on top of 3.5 ml of CsCl solution (density 1.5 g/cm³ in 1 mM phosphate buffer, pH 7.2) and centrifuged in a Spinco SW65 rotor at 45,000 rpm for 3 h at 20 C. Fractions of 3 drops were collected from the bottom of the tube on filter paper disks which were then processed as described in Fig. 1. The arrow indicates the position of the marker Py DNA I. B, Sedimentation through an alkaline sucrose density gradient: a sample (0.2 ml) was mixed with 20 μliters of 3 M NaOH and then sedimented through a linear alkaline sucrose density gradient (15 to 30% [wt/vol] in 0.3 M NaOH-0.001 M EDTA-0.1% SDS) in an SW65 rotor at 45,000 rpm for 4.25 h at 20 C. Fractions of 4 drops were collected from the bottom of the tube and were processed as in Fig. 1. The arrows indicate the position of double-stranded cyclic coils (53S) and of single-stranded circular and linear molecules (16 to 18S) derived from the ¹⁴C-labeled marker Py DNA. C, EBr-CsCl equilibrium density gradient centrifugation: a sample was centrifuged to equilibrium and collected as described in Fig. 1. The profile of the ¹⁴C-labeled marker Py DNA is shown in the figure (form I—heavy band, density 1.592 g/cm³; form II—light band, density 1.556 g/cm³). D, CsCl equilibrium density gradient centrifugation: a sample was adjusted to 3.0 ml with 1 mM phosphate buffer (pH 7.2); solid CsCl (3.84 g) was added to yield a density of 1.702 g/cm³. The sample was then centrifuged to equilibrium in a Ti50 rotor at 33,000 rpm for 72 h at 20 C. Fractions of 3 drops were collected from the bottom of the tube and processed as described in Fig. 1. The dotted line indicates the density distribution of DNA from uninfected mouse cells determined in a parallel gradient (buoyant density 1.702 g/cm³).*

DNA from complex A was furthermore hybridized with unlabeled Py DNA. The results presented in Table 1 show that the DNA from complex A hybridizes to the same extent to Py DNA as does Py DNA I extracted from Py virions. This leads to the conclusion that complex A contains mainly Py DNA, a notion which is also supported by the perfect coincidence of the bands formed by DNA from complex A and by Py DNA I when analyzed together in shallow CsCl equilibrium density gradients (3) (Fig. 4D).

Characterization of the DNA present in complex B. Py-infected cultures were labeled with 100 μCi of [³H]dT per ml at 28 h p.i. for 5 min and then incubated for 3 h (chase) in nonradioactive medium containing 20 μg of dT per ml; complex B was then isolated from a sucrose density gradient as described above. DNA was extracted and analyzed by the same methods as in the preceding section. Figure 5A shows that the DNA from complex B cosediments in neutral CsCl solutions with ¹⁴C-labeled Py DNA I added as sedimentation

marker. Sedimentation through neutral sucrose density gradients (5 to 20%) gives essentially the same pattern (not shown). Figure 5B shows that, in alkaline sucrose density gradients (15 to 30%), this DNA exhibits the sedimentation pattern expected for double-stranded cyclic coils (53S). In EBr-CsCl equilibrium density gradients (Fig. 5C), most of the DNA forms a band which coincides with the band of ^{14}C-labeled Py DNA I; only about 2% or less of the DNA from complex B bands with the ^{14}C-labeled Py DNA II. These results suggest that the DNA from complex B consists essentially of mature Py DNA I molecules, an interpretation which is supported by the results from hybridization with Py DNA I (Table 1) and from CsCl equilibrium density gradient centrifugation (Fig. 5D).

TABLE 1. *Hybridization of DNA extracted from complex A and B*

Source of DNA	Input hybridized[a] (%)
Complex A	50.5[b]
Complex B	49.9[b]
Py DNA I	50.0[b]
Mouse cell DNA	0.8[c]

[a] Filters containing 2 μg of Py DNA were used throughout; each incubation mix contained 1,000 to 2,000 counts/min of ^{3}H-labeled DNA.
[b] Average of five experiments.
[c] Average of three experiments.

Complex A and B may be nucleoprotein complexes. Preparations of ^{3}H-labeled complex A or B were mixed with an equal volume of 12% formaldehyde (Merck 37%, diluted with 2 volumes of NEN buffer, pH 6.0) and incubated for about 20 h at 2 C; this procedure cross-links proteins and nucleic acids (2, 5, 8). The samples were dialyzed four times against 100 volumes of 0.001 M phosphate buffer (pH 7.2) and then centrifuged to equilibrium in a preformed neutral CsCl density gradient. Figure 6 shows that both complex A and B form uniform bands at a density of 1.470 ± 0.005 g/cm^{3}. These results are compatible with the hypothesis that complex A and B are DNA-protein complexes. In the

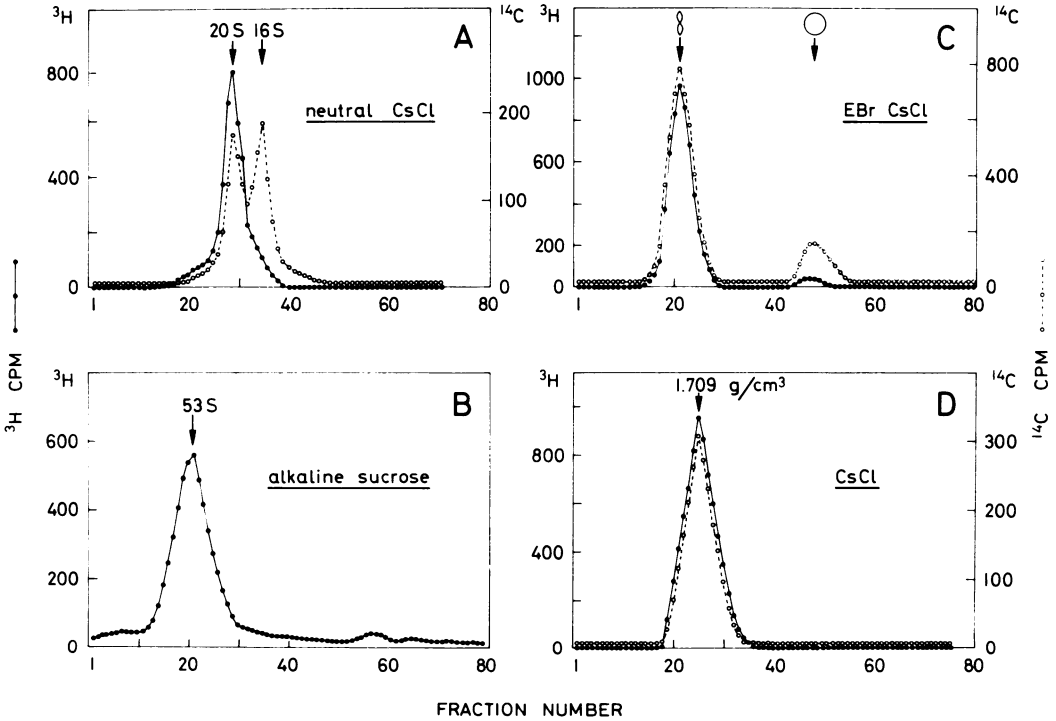

FIG. 5. *Characterization of the DNA from complex B. Py-infected cultures were labeled with 100 μCi of [^{3}H]dT per ml 28 h p.i. for 5 min. The cultures were then washed and incubated for 3 h (chase) in nonradioactive medium containing 20 μg of dT per ml. Complex B was isolated as described in Fig. 3, and DNA was extracted with Sarkosyl. ^{3}H-labeled DNA samples to be analyzed were mixed with ^{14}C-labeled marker Py DNA and analyzed as described in Fig. 4. A, Sedimentation through a neutral CsCl solution; B, sedimentation through an alkaline sucrose density gradient; C, EBr-CsCl equilibrium density gradient centrifugation; D, CsCl equilibrium density gradient centrifugation.*

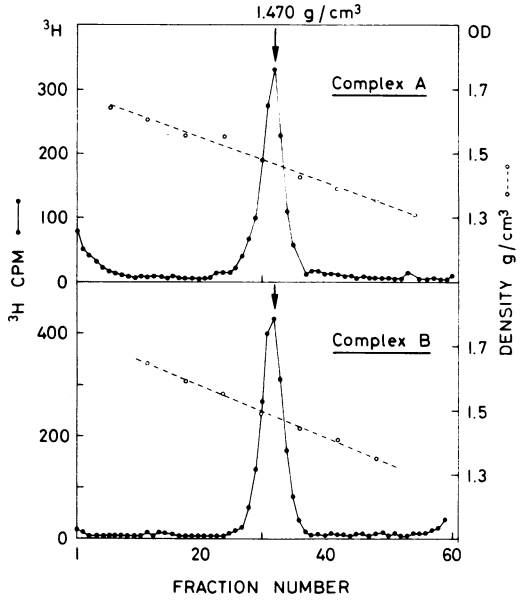

FIG. 6. *Buoyant density of formaldehyde-treated complex A and B in CsCl density gradients. Complex A and B were isolated as described in Fig. 2 and 3, and were then treated with formaldehyde (see text for details). Samples were layered on a preformed linear CsCl density gradient (1.20 to 1.60 g/cm³, 4.5 ml) and centrifuged to equilibrium in an SW65 rotor at 35,000 rpm for 24 h at 20 C. Fractions of 3 drops were collected from the bottom of the tubes on filter paper disks; one out of six fractions was collected directly into tubes containing paraffin oil and the refractive index was immediately measured in a Zeiss-Abbé refractometer at 19 to 21 C.*

native complexes the protein appears to be bound noncovalently to the DNA since it is removed from the DNA if the complexes are treated with detergents or incubated in 7.5 M CsCl.

DNA-to-protein ratio appears to remain unchanged during Py DNA replication. In sucrose density gradients, complex A sediments as a relatively broad peak. The results of this section show that faster sedimenting particles of complex A contain Py DNA in advanced stages of replication whereas more slowly sedimenting particles contain Py DNA in earlier stages of replication and all of them appear to have the same DNA-to-protein ratio. Infected cultures were labeled with 100 μCi of [³H]dT per ml at 28 h p.i. for 5 min. Complex A was isolated from a sucrose density gradient as described above. Fractions from (i) the front, (ii) the center, and (iii) the back of the peak formed by complex A were pooled separately. From a portion of each of the three samples, DNA was extracted and then analyzed in an EBr-CsCl equilibrium density gradient. The results in Fig. 7 show that the DNA molecules from sample (i) (front) exhibit the density distribution of Py DNA in late stages of replication, that a large fraction of the DNA from sample (iii) (back) shows properties of Py DNA in early stages of replication, and that DNA from sample (ii) (center) shows a profile which is intermediate between those of

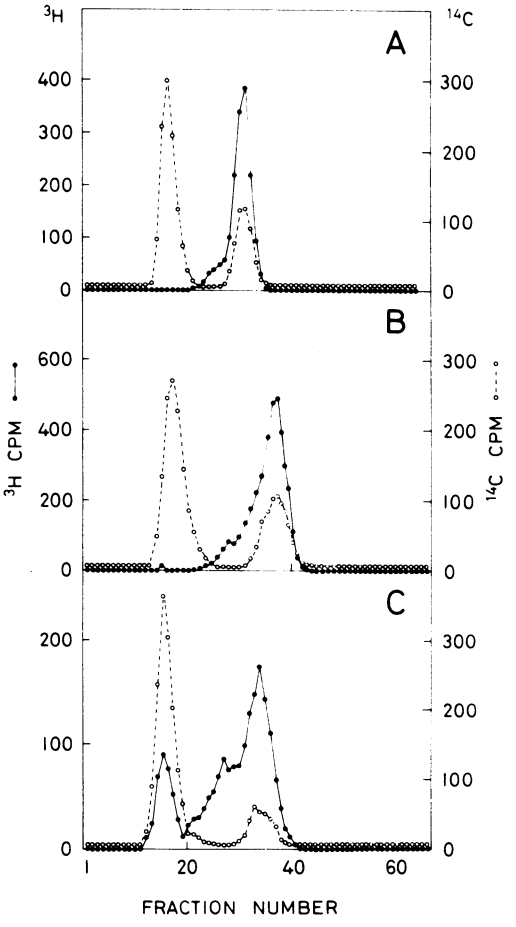

FIG. 7. *Evidence that complex A contains Py DNA in different stages of replication. Py-infected cultures were labeled with 100 μCi of [³H]dT per ml 28 h p.i. for 5 min. Complex A was isolated and sedimented as described in Fig. 2. Fractions containing (A) the front, (B) the center, and (C) the back of the peak formed by complex A were pooled separately (corresponding to fractions 8 to 13, 14 to 17, and 18 to 22 in Fig. 2, respectively). From a sample of each of the three pools DNA was extracted with Sarkosyl, mixed with ¹⁴C-labeled marker Py DNA and then subjected to EBr-CsCl equilibrium density gradient centrifugation as described in Fig. 1 and 4C. A, DNA from the front; B, DNA from the center; C, DNA from the back. The profile of the ¹⁴C-labeled marker Py DNA is shown in the figure.*

samples (i) and (iii). The presence of Py DNA I molecules in the slower sedimenting fractions of complex A suggests a "contamination" with complex B.

Other samples of samples (i), (ii) and (iii) were fixed with formaldehyde and then subjected to CsCl equilibrium density gradient centrifugation (Fig. 8). The results show that the particles from the three samples exhibit the same buoyant density (1.470 ± 0.005 g/cm^3) despite the fact that they contain markedly different amounts of DNA. These results suggest that the DNA-to-protein ratio remains virtually unchanged throughout the entire replication of a Py DNA molecule, thus indicating that newly replicated stretches of Py DNA are immediately complexed with proteins.

DISCUSSION

Two Py-specific nucleoprotein complexes, designated as complex A and B, can be isolated from mouse kidney cell cultures undergoing a lytic infection with Py virus. Although both complexes remain physically associated with the host cell chromatin during the isolation procedure used, they can be dissociated from the isolated chromatin by gentle homogenization in a buffer containing 0.5 M NaCl. The results presented above make it appear unlikely that the two complexes and their association with the chromatin are experimental artifacts; they do not, however, rigorously rule out this possibility.

The DNA from complex A and B has been characterized by velocity sedimentation under neutral and alkaline conditions, by CsCl equilibrium density gradient centrifugation in the presence or absence of ethidium bromide, and by hybridization with Py DNA I. The results show (i) that both complexes contain exclusively Py DNA, (ii) that complex A contains Py DNA molecules in different stages of replication, and (iii) that complex B contains only mature Py DNA I molecules. Complex B may correspond to the viral nucleoprotein complex, previously isolated from Py-infected mouse cell cultures by Green et al. (7, 8). Their complex consists of mature Py DNA I molecules which are complexed to proteins. A probably analogous complex has also been isolated from simian virus 40-infected monkey cells (22).

Isolated complex A and B were treated with formaldehyde under conditions which cross-link DNA and proteins and were then subjected to equilibrium density gradient centrifugation in CsCl density gradients. The results suggest that complex A and complex B are both nucleoproteins with the same buoyant density (1.470 g/cm^3). In turn, this suggests that newly synthesized stretches of Py DNA are immediately complexed with proteins and that the DNA-to-protein ratio of the complex remains unchanged throughout replication. In this context it may be recalled that a specific DNA-to-protein ratio is also maintained during the replication of the mouse cell chromatin.

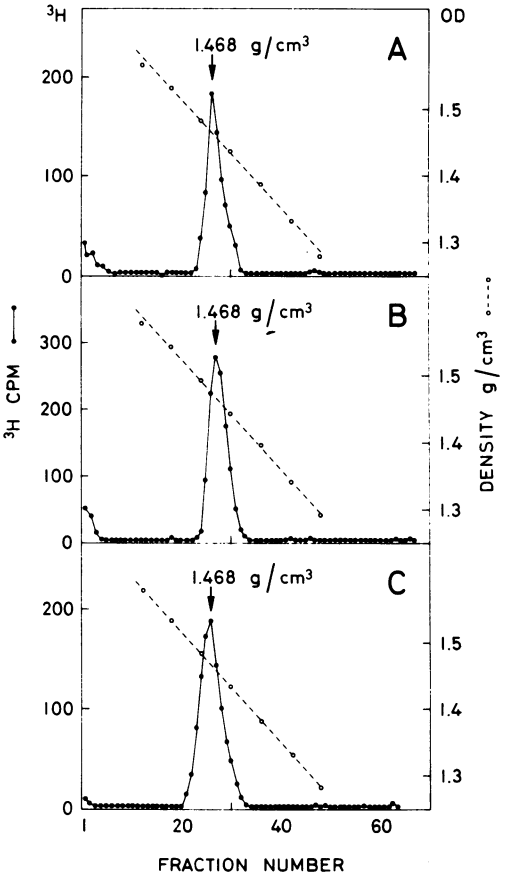

FIG. 8. *Evidence that the DNA-protein ratio remains virtually constant during replication of Py DNA. Samples from the three pools described in Fig. 7 were treated with formaldehyde and centrifuged to equilibrium in a CsCl density gradient as described in Fig. 6. A, Front; B, center; C, back.*

The proteins in complex A and B (manuscript in preparation) exhibit in acrylamide gels essentially the same pattern as the small proteins (PV IV-VII) present in purified Py virions (4, 16; Consigli et al. and B. Hirt, manuscripts in preparation). These proteins are host cell histones (B. Hirt, manuscript in preparation) synthesized during Py-induced chromatin replication (Seehafer and R. Weil, Virology, in press). We are therefore led to the tentative

conclusion that replicating Py DNA is immediately complexed with mouse cell histones and that complex B may become the "core" of progeny Py virions. Our results are compatible with the hypothesis that Py DNA uses the chromosome replication machinery for its own synthesis and that, among others, Py-induced replication of the host cell chromatin is necessary to provide the replicating Py DNA with histones.

On the basis of the experimental evidence presently available we consider Py DNA to be a very simplified chromosomal replication unit and expect that studies on the replication of Py DNA may provide information on the role of histones in the replication of the chromatin in mammalian cells.

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