

Properties of Nucleoprotein Complexes Containing Replicating Polyoma DNA

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Short-lived nucleoprotein complexes (r-py complex) containing replicating polyoma DNA were isolated from infected cells after lysis with Triton X-100. The Triton lysing procedure of Green, Miller, and Hendler (1971) releases most complexes containing supercoiled viral DNA (py complex) from nuclei, but liberates only a portion of r-py complexes. r-py Complexes are associated more strongly with nuclear sites but can be extracted by prolonged incubation of nuclei in lysing solution. Complexes containing replicating polyoma DNA appear to be precursors to stable complexes containing supercoiled DNA. Sedimentation and buoyant density studies indicate that protein is bound to both r-py complexes and py complexes at a ratio of protein to DNA of about 1 to 2/1. Both types of complexes sediment as if the viral DNA is more compact than free DNA and both undergo major reversible configurational changes with increased salt concentration. Changes resulting from enzymatic and chemical treatment indicate that there may be two or more protein components in both r-py complex and py complex. One component is digested by Pronase and trypsin while another is resistant to the enzymes but released by deoxycholate. The abundance and similarity in chemical and physical properties of protein bound to all forms of polyoma DNA suggest that part of the protein molecules may serve in a structural capacity.

A polyoma DNA-protein complex (py complex) which sediments at 55S can be isolated from infected mouse cells by lysis with the nonionic detergent Triton X-100 (14). Similarly, a 44S DNA protein complex is found by Triton lysis of simian virus 40 (SV40)-infected BSC-1 cells (28). The DNA released from these complexes by high salt, Pronase, or sodium dodecyl sulfate (SDS) appears to be supercoiled viral DNA. The origin of the protein is not known.

When infected cells are pulsed with ^3H -thymidine for only 5 min prior to lysis, some of the labeled DNA in the complex is not supercoiled and may represent viral DNA in the process of replication (14, 28). Our interest was to isolate and characterize the polyoma DNA-protein complex found after short pulses and to determine what relationship it had to the complex found after long pulses.

In this study, we show that a short-lived nucleoprotein complex containing replicating polyoma DNA (r-py complex) can be isolated after short pulses of ^3H -thymidine. This complex is bound to the nuclear fraction more

strongly than the complex containing supercoiled viral DNA (py complex). The r-py complex has a protein-to-DNA ratio identical to the py complex, and the majority of the proteins react in a similar manner to chemical and enzymatic treatments. Both complexes may contain two or more types of protein. Although the function of the complex in DNA replication has not been determined, the amount and similarity of the protein in both r-py complex and py complex suggests a role in the stability and maturation of viral DNA.

MATERIALS AND METHODS

Virus and cells. The small-plaque variant of polyoma virus used throughout this study was produced on monolayers of primary baby mouse kidney cells in plastic petri dishes (AS Nunc, 9 cm). Origin of the virus, preparation of cells, and methods used in their culture have been previously described (20).

Polyoma DNA from virions. The methods used to purify polyoma virus and extract labeled viral DNA have been reported (20).

SDS extraction of polyoma DNA from infected cells. Viral DNA was extracted from infected mouse

kidney monolayers by the selective extraction procedure of Hirt (17).

Triton lysis of infected cells. Cells were lysed with Triton and polyoma DNA-protein complexes extracted by a modification of the procedures described by Green et al. (14). After labeling, cells were washed twice with ice-cold Tris-buffered saline containing magnesium and calcium (TBS) (29). Each petri plate was then treated with 0.9 ml of a hypotonic lysing fluid designated TTE containing 0.25% Triton X-100, 0.01 M Tris-hydrochloride (pH 7.9), and 0.01 M EDTA. The cells were allowed to swell for 10 min at room temperature, and 0.1 ml of 2.0 M NaCl was added. Addition of NaCl caused the cells to lyse; however, nuclei remained morphologically intact and clumped to form large aggregates. The lysate and nuclear aggregates were decanted from the plate or gently scraped with a rubber policeman and poured into an ice-cold, 12-ml centrifuge tube. Nuclear aggregates and large cellular debris were pelleted by centrifugation at 2,000 rpm for 5 min at 4 C in the HL-4 swinging bucket rotor of a Sorvall GLC centrifuge. The Triton supernatant fluid was decanted and stored at 4 C. The polyoma DNA-protein complex in this supernatant fraction was designated TSC. Pellets were resuspended in various extraction buffers and treated as described below.

SDS extraction of polyoma DNA from pellets of Triton cell lysates. The pellet obtained from the low-speed centrifugation of the Triton cell lysate was resuspended in 0.01 M EDTA (pH 7.5) (0.9 ml per petri dish). SDS was added to a final concentration of 1%, and the viscous nuclear lysate was mixed gently to avoid shearing nuclear DNA. The solution was incubated at 50 C for 20 min and cooled to room temperature, and polyoma DNA was extracted by the Hirt selective extraction procedure (17).

Triton extraction of polyoma DNA-protein complexes from pellets of Triton cell lysates. The pellet obtained from the low-speed centrifugation of the Triton cell lysate was resuspended in ice-cold TTE (0.9 ml per petri dish). One-tenth volume of 2.0 M NaCl was added, and the solution was incubated for 3 h on ice. Nuclei remained morphologically intact and were pelleted as previously described. The supernatant fluid containing nuclear polyoma DNA-protein complex, designated nuclear extraction complex (NEC), was decanted and stored at 4 C. Polyoma DNA remaining in the nuclear pellet was extracted by the modified Hirt selective extraction procedure described above.

Sucrose density gradients. Linear sucrose gradients were prepared in TTE buffer containing 0.2 M NaCl (STTE) and chilled to 4 C. For preparative runs, 0.4- to 0.5-ml samples were layered on 10.0-ml gradients (10-30%, wt/wt), covered with mineral oil, and centrifuged at 35,000 rpm for 165 min at 4 C in a Spinco SW41 rotor. For analytical runs, 0.1- to 0.2-ml samples were layered onto 4.8 ml (5-20%, wt/wt) gradients and centrifuged at 36,000 rpm for 120 min at 4 C in a Spinco SW50.1 rotor (14).

Tubes were punctured at the bottom, and fractions were collected through a needle either into the wells of microtiter plates or directly onto filter disks

(Schleicher and Schuell, 740-E). Filters were dried and assayed for acid-precipitable radioactivity by techniques previously described (20).

Buoyant density centrifugation of glutaraldehyde-fixed polyoma DNA-protein complexes. Linear CsCl gradients (1.3 g/cm³ to 1.6 g/cm³) were prepared in 0.01 M Tris-hydrochloride, 0.01 M EDTA, 0.1% Triton X-100 (pH 8.0). Samples (0.2 ml) were layered onto 4.8-ml gradients and centrifuged at 35,000 rpm for 5 to 7 h at 4 C in a Spinco SW50.1 rotor. For density determinations fractions were collected under mineral oil in microtiter plates, and the index of refraction was measured (Zeiss Refractometer).

In some cases CsCl crystals were added to samples dialyzed into TTE buffer and the density was adjusted to 1.45 g/cm³ in a volume of 3.0 ml. The solutions were covered with mineral oil and centrifuged at 33,000 rpm at 4 C in the Spinco SW50.1 rotor for 48 h or longer.

CsCl band centrifugation. Analysis of DNA by CsCl velocity centrifugation was performed as previously described (20).

Dye-buoyant density centrifugation. Supercoiled and replicating polyoma DNA molecules were separated from open circular and linear forms by equilibrium centrifugation in CsCl solutions containing propidium diiodide (8). Samples were prepared by mixing 1.9 ml of the DNA sample in 0.02 M Tris-hydrochloride (pH 8.0), 0.001 M EDTA with 0.5 ml of a 3 mg/ml propidium diiodide solution and 2.1 g of CsCl. Solutions were adjusted to a density of 1.515 to 1.517 g/cm³ and centrifuged at 35,000 rpm for 40 to 60 h at 25 C in a Spinco SW50.1 rotor.

Alkaline digestion and trichloroacetic acid precipitation of subcellular fractions. To determine the total alkali-resistant trichloroacetic acid-precipitable radioactivity, duplicate 50- μ liter samples were diluted to 0.8 ml total volume in 0.02 M Tris-hydrochloride, 0.001 M EDTA (pH 8.0) and made to 1 N NaOH. Samples were incubated at 50 C for 20 min and cooled on ice, and 1.33 volumes of 50% trichloroacetic acid was added. Yeast transfer RNA was then added as carrier (50 μ g/ml), the mixture was placed on ice for 40 min, and acid-precipitable material was collected by filtering through Whatman GFC filters. Filters were rinsed twice with 5% trichloroacetic acid, twice with 95% ethanol, dried, and counted in toluene scintillation fluid by use of a Nuclear Chicago Unilux II scintillation counter.

Glutaraldehyde and formaldehyde fixation. Peak fractions of polyoma DNA-protein complexes from sucrose gradients were pooled and fixed for 2 to 24 h at 0 C in 5% neutralized glutaraldehyde by the procedure of Baltimore and Huang (2). In some instances duplicate samples were also fixed in 5% neutralized formaldehyde under similar conditions. Fixed samples were layered on linear CsCl gradients or dialyzed against TTE buffer and centrifuged to equilibrium in CsCl solutions as described above.

Chemicals and enzymes. Chemicals and enzymes were obtained from the following sources: thymidine-*methyl*-³H (56-50 Ci/mmol) and thymidine-2-¹⁴C (56.5 mCi/mmol), New England Nuclear Corp. (Boston, Mass.); RNase (bovine pancreas), neuraminidase

(*C. perfringens*), and Triton X-100, Sigma Chemical Co. (St. Louis, Mo.); Eagle minimal essential medium, Grand Island Biological Co. (Grand Island, N.Y.); Pronase and propidium diiodide, Calbiochem (San Diego, Calif.); trypsin, Worthington Biochemicals Corp. (Freehold, N.J.); Nonidet P-40 (NP-40), Shell Chemical Co. (New York, N.Y.); glutaraldehyde, Aldrich Chemical Co. (Milwaukee, Wis.); formaldehyde, Matheson, Coleman and Bell (Norwood, Ohio); CsCl (optical grade), Harshaw Chemical Co. (Solon, Ohio); and sucrose (RNase-free), Schwarz-Mann (New York).

RESULTS

Isolation of polyoma nucleoprotein complexes. At 29 h after infection ^3H -thymidine was added to two sets of cultures. After 10 min, one set was lysed and the other set was chased for 30 min in unlabeled medium and then lysed. Half of the plates in each set were lysed with SDS and the rest with Triton. Polyoma DNA was prepared from the SDS lysates by the Hirt selective extraction procedure (17). These latter samples were designated Hirt extracts.

After lysis of cells with Triton, nuclei were pelleted by low-speed centrifugation. The supernatant fluids were designated as the Triton supernatant fractions. Nuclear pellets were resuspended in EDTA buffer, and nuclei were reextracted by a modification of the Hirt SDS extraction procedure (see Materials and Methods). Supernatant fluids prepared by modified Hirt extraction of nuclear pellets were designated Triton pellet fractions.

Triton supernatant fractions were analyzed in neutral sucrose gradients (Fig. 1). A broad peak of ^3H -labeled material sedimenting at about 50S with a distinct leading shoulder is observed in the Triton supernatant fluid prepared from cells pulsed for 10 min (Fig. 1, top). A peak of radioactivity also occurs at the top of the gradient. In the Triton supernatant fluid prepared after a chase, ^3H -labeled material sediments as a more homogeneous component at a position slightly higher in the gradient (Fig. 1, bottom). Similar results have been reported for the sedimentation of nucleoproteins extracted from SV40-infected cells (28). These complexes do not appear to result from nonspecific binding of protein to viral DNA since labeled polyoma DNA added to cells at the time of lysis sedimented in sucrose gradients to a position characteristic of free viral DNA.

The Triton supernatant fluids were treated with SDS to remove protein from the complexes, and the DNA was analyzed by velocity sedimentation in CsCl gradients. Triton pellet fractions and Hirt extracts were also sedi-

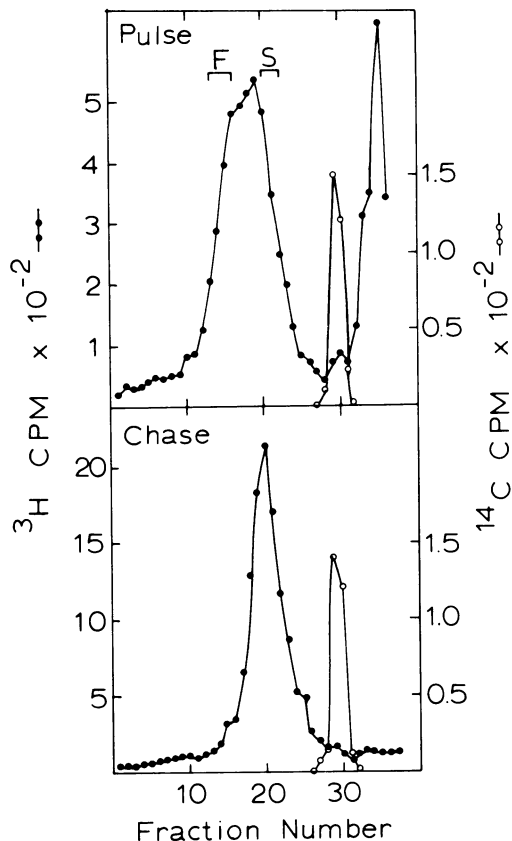


FIG. 1. Velocity sedimentation in 10 to 30% neutral sucrose gradients of polyoma nucleoprotein complexes. At 29 h after infection, cell cultures were pulse labeled with ^3H -thymidine (70 $\mu\text{Ci/ml}$) for 10 min. One-half of the plates were lysed and half chased for 30 min in medium containing 100 μg of thymidine per ml and 10 μg of deoxycytidine per ml (20). The supernatant fractions prepared from Triton lysates were mixed with ^{14}C -labeled polyoma DNA and centrifuged as described in Materials and Methods. Sedimentation is from right to left. Brackets indicate fractions which were pooled and later analyzed (see Fig. 3).

mented in CsCl gradients. The sedimentation patterns are shown in Fig. 2. After a 10-min pulse, both the Hirt extracts and the Triton supernatant fluids show two peaks of DNA, a 25S and a 20S peak (Fig. 2, top). The 25S DNA has previously been shown to be polyoma specific and has properties characteristic of a replicative intermediate (RI) (4, 5, 19, 20, 23). However, the Triton supernatant fluid had only about half the amount of 25S DNA as the Hirt extract. The 25S DNA not found in the Triton supernatant fraction appeared to be located in the pellet fraction (Fig. 2, open circles). With

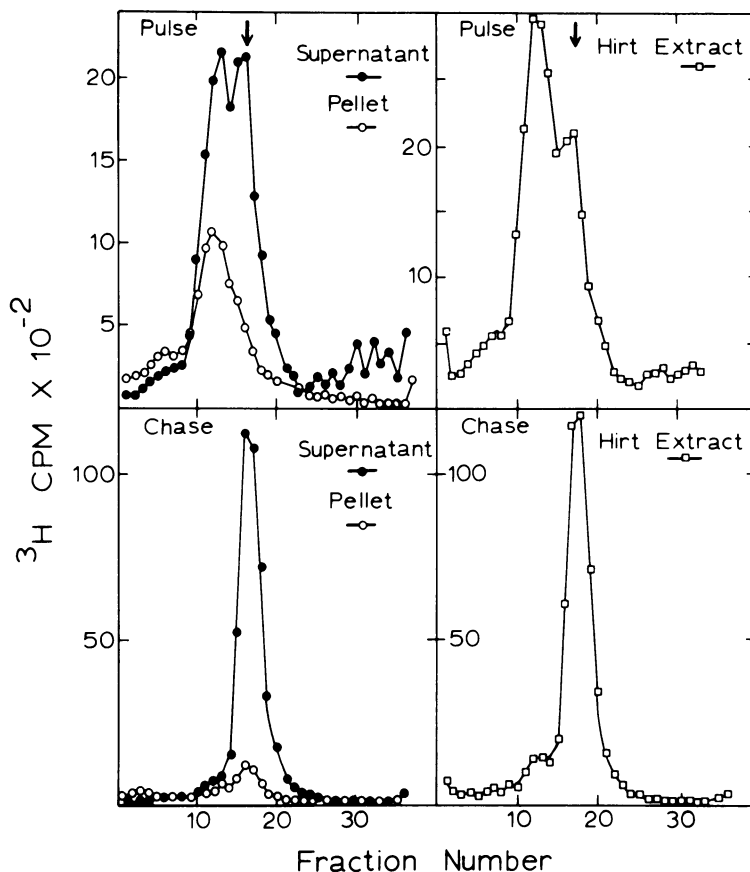


FIG. 2. Velocity sedimentation in CsCl gradients of DNA from Triton and Hirt extracts. Part of the cell cultures described in Fig. 1 were lysed with Triton and the rest by the Hirt procedure. The Triton lysates were separated into supernatant fluid and pellet fractions. Polyoma DNA was extracted from the Triton pellet fractions by an SDS extraction procedure (see Materials and Methods). Samples from SDS-treated Triton pellet fractions, SDS-treated Triton supernatant fractions, and Hirt extracts were sedimented in CsCl (density 1.5 g/cm^3) gradients (20). The arrows indicate the positions of a 20S ^{14}C -labeled polyoma DNA marker included in each tube. All samples were run in separate tubes, but the Triton pellet and supernatant fractions are plotted together for purposes of comparison.

even shorter pulses (3–8 min), very little of the total 25S DNA was found in the Triton supernatant fluid, but it could be extracted from the pellet fraction. In several experiments, the sum of the radioactivity in the 25S peak from the Triton pellet fraction added to that from the Triton supernatant fraction was about equal to the total 25S radioactivity in the Hirt extracts.

After a short pulse, velocity gradients of the Triton supernatant fluid also show a small quantity of radioactivity near the top of the gradient (Fig. 2, top left). This material is apparently derived from radioactive DNA released from the very slow sedimenting material previously shown at the top of sucrose gradients (Fig. 1, top) and is not found in material extracted after a chase (Fig. 2, bottom). In the present study no attempt was made to deter-

mine the origin of this slowly sedimenting DNA.

After a chase, the sedimentation pattern of the DNA from the Triton supernatant fluid was similar to that of the Hirt extract (Fig. 2, bottom). In each extract the DNA sedimented as a 20S component. Almost all of the 20S material was supercoiled viral DNA as determined by equilibrium centrifugation in CsCl -propidium diiodide (CsCl -PI) gradients (8). The DNA remaining in the Triton pellet was only a small fraction of the total polyoma DNA (Fig. 2, bottom).

These experiments show that at least two polyoma DNA-protein complexes can be extracted by the Triton procedure. One of the radioactive complexes is extracted quantitatively into the Triton supernatant fluid from cells either pulsed with ^3H -thymidine or pulsed

and then chased before lysis. This complex (py complex) contains 20S supercoiled polyoma DNA. The other complex (r-py complex), apparently short-lived, is detected only after a short pulse. It appears to be more strongly bound to the nuclear fraction and is only partially released from nuclei by Triton. This complex contains polyoma 25S RI DNA which can be extracted from nuclei by SDS.

With regard to the polyoma DNA extracted by SDS from the Triton pellet, it was important to determine whether the procedure for its extraction also liberated some labeled cellular DNA. Therefore, extracts from Triton pellets were centrifuged in velocity gradients for short periods (60–90 min) in order to detect any possible high-molecular-weight cellular DNA contamination. Radioactivity was never found sedimenting in these gradients ahead of polyoma 25S DNA.

As described above, in sucrose gradients the nucleoprotein complex extracted from pulse-

labeled cells is more heterogeneous than the nucleoprotein complex extracted after a chase. White and Eason (28) reported that after a 5-min pulse the heterogeneous leading portion of the nucleoprotein peak contains SV40 RI DNA, while the trailing portion contains supercoiled DNA. Since a heterogeneous peak is also Triton extractable from polyoma-infected cells after a short pulse, specific fractions were pooled from the "fast" (69S to 60S) and "slow" (51S to 42S) portions of the peak (Fig. 1, top). Protein was removed by SDS treatment, and fractions were analyzed by velocity sedimentation in CsCl and by equilibrium sedimentation in CsCl-PI gradients. The data shown in Fig. 3 are in agreement with the results of White and Eason. DNA released from the leading portion of the nucleoprotein peak sediments ahead of the polyoma 20S DNA marker in velocity gradients. In CsCl-PI gradients, most of the fast sedimenting DNA has a buoyant density lighter than the supercoiled DNA marker. Part of this

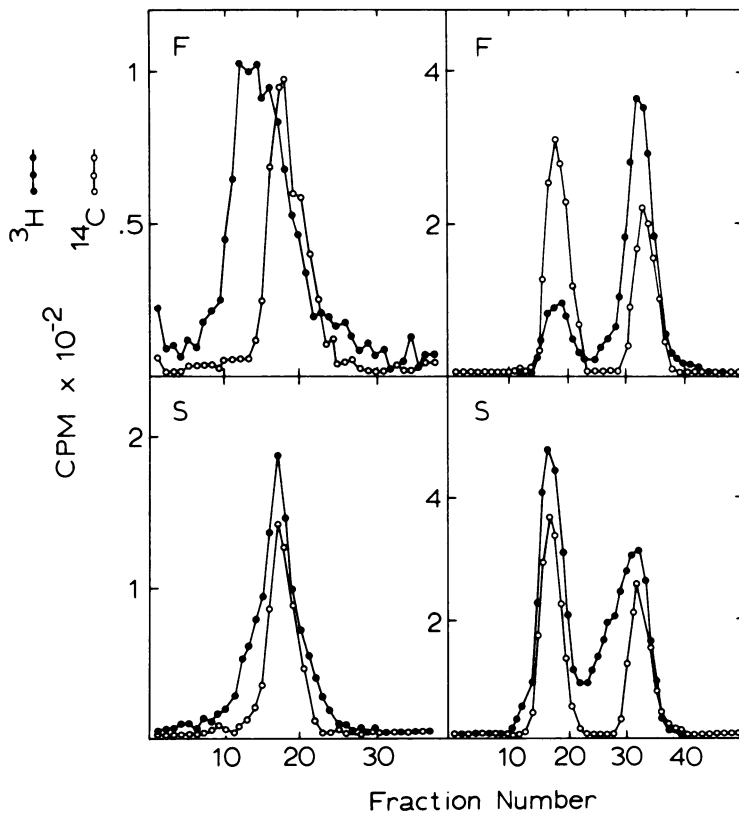


FIG. 3. Velocity sedimentation and CsCl-PI equilibrium centrifugation of polyoma DNA released from fast and slow sedimenting nucleoprotein complexes. Fractions were pooled from the leading (F) and trailing (S) portions of the polyoma nucleoprotein peak as indicated by the brackets shown in Fig. 1. Samples were treated with SDS to remove protein, and ^{14}C -labeled polyoma DNA was added as a marker. Part of each sample was analyzed by velocity sedimentation in CsCl (left) and part by equilibrium centrifugation in CsCl-PI density gradients (right).

light DNA also bands in a position intermediate between supercoiled and open circular DNA. The DNA banding in the intermediate and light regions of the gradients is typical of RI (4, 19, 23).

Most of the DNA released from the slower sedimenting region of the DNA-protein complex cosediments with the polyoma 20S DNA marker (Fig. 3, bottom). However, CsCl-PI gradients show that only about half of this 20S material is supercoiled. The remainder bands at an intermediate position or with open circular polyoma DNA marker characteristic of replicating DNA. Thus, after a short pulse, slow sedimenting complexes contain some slow sedimenting RI DNA in addition to 20S supercoiled viral DNA. The correlation between fast and slow sedimenting complexes and fast and slow sedimenting DNA suggests specific binding of protein to DNA regardless of the form of the DNA.

Extraction of polyoma DNA-protein complexes from nuclei. We next attempted to determine what factors caused only some RI DNA to be Triton extractable as a DNA-protein complex while the rest remained tightly bound to the nucleus. In practice, the amount of RI DNA extracted into the supernatant fraction as a DNA-protein complex was found to be variable, and preliminary experiments indicated that the amount extracted was strongly dependent on the precise conditions of lysis and subsequent treatment. Conditions for the extraction of RI were analyzed in several experiments. In one, six sets of cultures were pulse labeled with ^3H -thymidine for 8 min at 28 h after infection. Petri plates were washed with ice-cold TBS buffer, one set was lysed with SDS by the Hirt procedure, and the rest was covered with Triton lysing solution and then incubated at room temperature for 10 min. NaCl was added to a final concentration of 0.2 M, lysates were scraped from plates, and the nuclei were pelleted by low-speed centrifugation. Triton supernatant fluids were removed, and the pellet from one set was extracted by the modified Hirt procedure (see Materials and Methods). Pellets from the remaining four sets were suspended in TTE and made 0.2 M in NaCl, and the solutions were incubated for 30 min as follows: (i) 0 C, (ii) room temperature, (iii) 0 C with 0.01% deoxycholate (DOC), and (iv) 0 C with NaCl at a final concentration of 0.4 M. After incubation the extraction solutions containing nuclei were centrifuged, the supernatant fluids were removed, and the final pellets were extracted by the modified Hirt procedure described above.

Incubation of nuclei for 30 min at 0 C in

STTE buffer containing 0.01% DOC extracted about twice the amount of radioactivity that could be extracted by Triton lysis of cells (Table 1). Treatment of the pellet fraction with STTE buffer or STTE made 0.4 M in NaCl also released radioactivity from the nuclei (Table 1). The buffer containing DOC extracted the greatest quantity of protein-DNA complex.

In similar experiments, nuclei were extracted with other chemicals and enzymes. Extraction with phospholipase C, neuraminidase, or 0.8 M NaCl did not increase the yield of complex relative to extraction with STTE. Less complex was released when nuclei were incubated with NP-40, 1% Triton, or 0.25% Triton in reticulocyte-standard buffer (RSB) (20).

The complexes obtained from nuclei were examined in sucrose gradients. Figure 4 (top) shows a comparison of the polyoma DNA-protein complex from the Triton supernatant fraction (TSC) to the polyoma DNA-protein complex obtained by extraction of nuclei with STTE buffer (NEC). The TSC as well as the NEC cosedimented as heterogeneous peaks. Similar sedimentation patterns were obtained with material extracted at room temperature in STTE buffer and at 0 C in STTE made 0.4 M NaCl. However, the DOC-extracted complex partially degraded upon storage.

The DNA released from NEC by SDS sedimented mainly as a 25S component, whereas DNA from TSC sedimented as two almost equal peaks, one at 25S and the other at 20S (Fig. 4, bottom). The fast sedimenting and slow sedimenting portions of the DNA peaks (Fig. 4, bottom) were analyzed in CsCl-PI equilibrium density gradients. Most fast (25S-21S) as well as slow (21-16S) DNA from NEC banded as polyoma RI with densities in the intermediate and light regions of CsCl-PI gradients (Fig. 5, open circles, top and bottom). Fast sedimenting DNA from TSC also banded mainly in the intermediate and light regions of the gradient, indicating that fast sedimenting DNA in TSC is RI (see also Fig. 3, top). However, slow sedimenting DNA from TSC banded as about an equal mixture of supercoiled and open circular forms (Fig. 5, bottom). These results are consistent with the previous analysis of TSC shown in Fig. 3, which indicated that fast sedimenting DNA-protein complexes contain fast sedimenting RI DNA, whereas slower sedimenting complexes contain a mixture of supercoiled DNA and slow sedimenting RI.

Stability and chemical properties of RI DNA-protein complex. Since polyoma RI DNA-protein complexes (r-py complex) could

TABLE 1. *Extraction of polyoma DNA-protein complexes from nuclei*^a

Method of extraction	Fraction (counts/min)			Total counts/min
	Triton supernatant	Triton pellet	SDS pellet	
1. Hirt extract				25,866
2. Triton lysis	7,107		19,986 ^b	27,093
3. Triton lysis and extraction of nuclei in STTE buffer				
0 C	7,929	5,385	8,517 ^c	21,831
22 C	7,788	12,045	3,729 ^c	23,562
0 C + 0.01% DOC	7,863	12,552	4,764 ^c	25,179
0 C + 0.4 M NaCl	8,565	8,019	4,260 ^c	20,844

^a Hirt extract and Triton lysates prepared from cells after an 8-min pulse with ³H-thymidine and treated as described in the text.

^b Nuclei obtained by Triton lysis extracted directly by the modified Hirt procedure.

^c Nuclei extracted in STTE buffer or STTE containing either DOC or 0.4 M NaCl were recentrifuged and extracted by the modified Hirt procedure.

be extracted from nuclei, it was of interest to compare its properties to that of the complex containing supercoiled polyoma DNA which is Triton extractable by cell lysis (py complex). First, it was necessary to decide on a suitable extraction procedure which would yield r-py complex in an undegraded state. Extraction and storage in 0.4 M NaCl or in low concentrations of DOC resulted in a gradual breakdown of the complex to forms with *S* values between 30 and 50S. Nuclear complexes were stable in STTE, and, although extraction of nuclei with this buffer did not release all of the complex, about 75% could be extracted by a 3-h incubation period of nuclei at 0 C. This was adopted as a standard extraction procedure (see Materials and Methods).

The chemical properties of ³H-labeled NEC extracted after a 20-min pulse were compared to ¹⁴C-labeled TSC containing mostly supercoiled polyoma DNA. This latter complex was prepared by incubating cells for 6 h (24–30 h after infection) in the presence of ¹⁴C-thymidine and preparing a Triton supernatant fraction. Over 90% of the polyoma DNA in this ¹⁴C-labeled fraction was supercoiled.

³H-labeled NEC (20-min pulse) and ¹⁴C-labeled TSC (6-h pulse) were mixed and incubated for 1 or 2 h with several chemicals and enzymes. After incubation, samples were analyzed in sucrose density gradients. A typical experiment is shown in Fig. 6. After a 20-min pulse, NEC sediments as two peaks (Fig. 6,

control). The leading peak is r-py complex whereas the trailing peak is a mixture of complexes containing slow sedimenting RI DNA and a small amount of 20S supercoiled DNA. In this respect 20-min NEC resembles 10-min TSC. Treatments with Pronase, trypsin, or DOC reduce the sedimentation coefficients of both NEC and TSC to values intermediate between that of the intact complexes and 20S polyoma DNA. With these treatments the peaks of NEC and TSC sharpen and the complexes co-sediment. One-hour treatments with phospholipase C, neuraminidase, low concentrations of DOC (0.1%), or NaCl (0.4 M) do not alter the sedimentation coefficients of the complexes.

Treatment with 1 μ g of RNase per ml increased the sedimentation coefficients as shown in Fig. 6; however, treatment with 10 or 100 μ g/ml caused both complexes to pellet when centrifuged in our gradients. These results are in agreement with previous reports that indicated binding of RNase to the complexes. RNase binding appears to require the presence of the protein moiety since the sedimentation coefficient of polyoma marker DNA is not altered by incubation with 10 μ g of RNase per ml. The results of these experiments are summarized in Table 2.

Buoyant densities of glutaraldehyde- and formaldehyde-fixed polyoma nucleoprotein-complexes in CsCl. To obtain an estimate of the amount of protein bound to polyoma DNA, peak fractions were pooled from sucrose gradi-

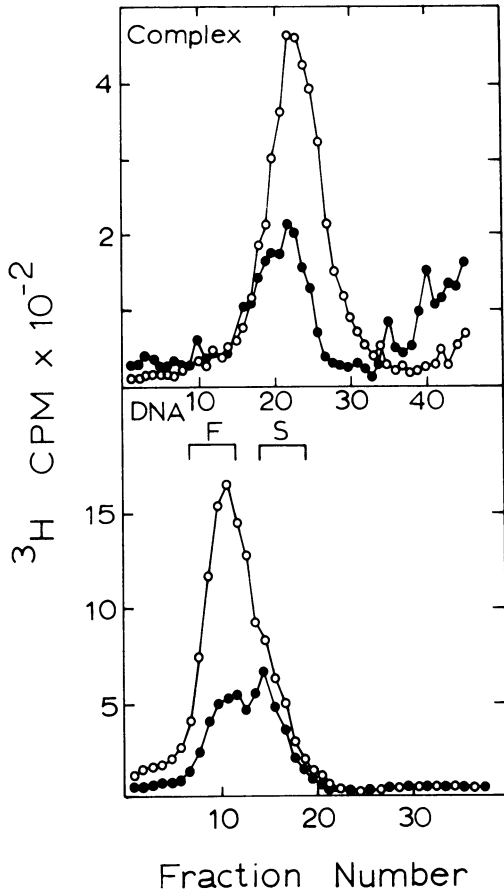


FIG. 4. Velocity sedimentation of polyoma nucleoprotein complexes and DNA from Triton supernatant fractions and from extracts of Triton pellet fractions. Cells were pulse-labeled with ^3H -thymidine for 8 min at 28 h after infection. Cells were lysed with Triton, and nuclei were pelleted by centrifugation for 5 min at $800 \times g$. The TSC was removed and stored at 4 C. The nuclear pellet was resuspended in 0.9 ml TTE, 0.1 ml of 2 M NaCl was added, the mixture was incubated for 30 min at 0 C, and the nuclei were pelleted as above. The supernatant fluid resulting from extraction of nuclei (NEC) was removed and stored at 4 C. Top: Sedimentation in a 10 to 30% sucrose gradient of polyoma DNA-protein complex from the TSC (\bullet) and from the NEC (\circ). Bottom: Velocity sedimentation in a CsCl gradient of TSC (\bullet) and NEC (\circ) after treatment with SDS.

ents, fixed in glutaraldehyde, and centrifuged to equilibrium in preformed CsCl density gradients (2). As shown in Fig. 7, NEC and TSC band at an identical position in these gradients. The average buoyant density from 16 determinations of several different preparations is $1.437 \pm 0.008 \text{ g/cm}^3$. Peak fractions, pooled and dialyzed to remove CsCl, sedimented in sucrose velocity gradients with values characteristic of unfixed complexes.

Densities were also determined after fixation with formaldehyde. This was done by centrifuging formaldehyde-fixed complexes with the glutaraldehyde-fixed complex as a marker either in the same tube or in separate tubes. The average density for formaldehyde-fixed complexes from four determinations was $1.476 \pm 0.010 \text{ g/cm}^3$. The same density was obtained if the formaldehyde-fixed complex was exposed to glutaraldehyde after fixation, so the difference between glutaraldehyde and formaldehyde fixation does

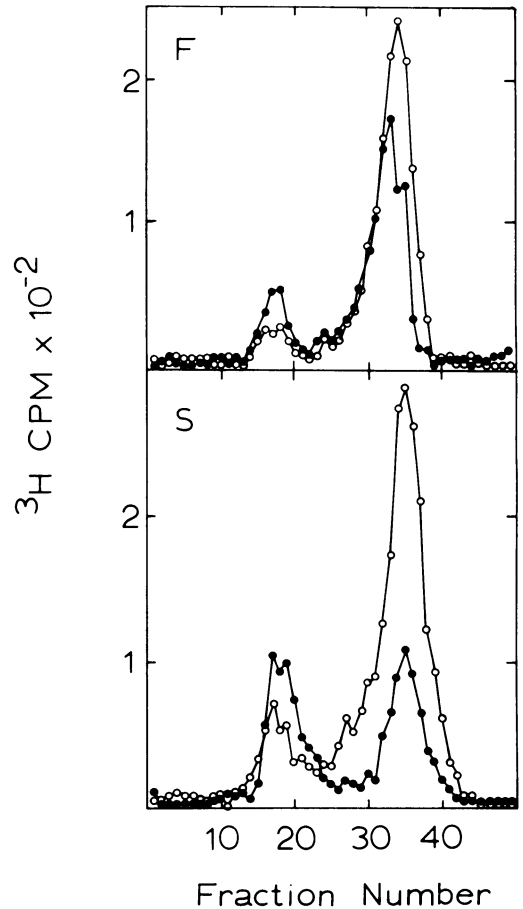


FIG. 5. Equilibrium centrifugation of polyoma DNA prepared from TSC and NEC. Fractions were pooled from the fast (F) and slow (S) sedimenting portions of the DNA peaks indicated by brackets in the bottom segment of Fig. 4. ^{14}C -labeled polyoma DNA marker, CsCl, and propidium diiodide were added, and each solution was adjusted to a final density of 1.515 g/cm^3 before centrifugation. The number of fractions separating ^{14}C -labeled polyoma supercoiled DNA from open circular DNA was identical in each of the four gradients, and the F and S portions are plotted together for purposes of comparison. Symbols: \circ , DNA from the NEC; \bullet , DNA from the TSC.

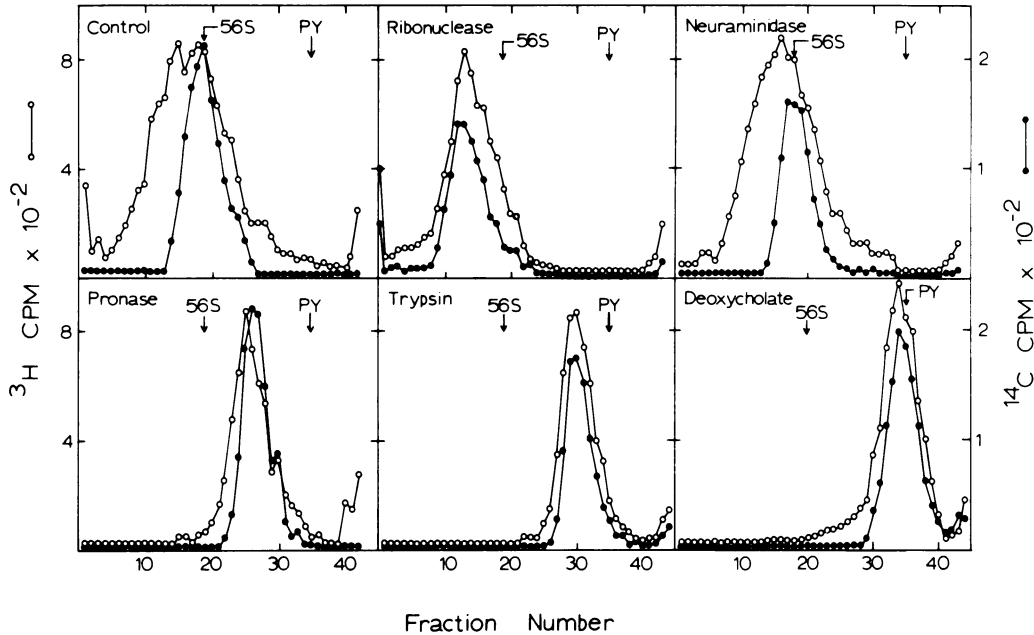


FIG. 6. Velocity sedimentation of TSC and NEC treated with selected chemicals and enzymes. TSC was prepared from cells pulse labeled with ^{14}C -thymidine from 24 to 30 h after infection. NEC was prepared from cells pulse labeled for 20 min with ^3H -thymidine at 29 h after infection. Samples of ^{14}C -labeled TSC and ^3H -thymidine-labeled NEC were mixed, treated as described below, and sedimented in 5 to 20% sucrose gradients. Samples were treated under the following conditions: control, 60 min at 0 C; RNase (heat treated) (1 $\mu\text{g}/\text{ml}$), 60 min at 22 C; neuraminidase (1 unit/ml), 60 min at 22 C; Pronase (auto-digested) (100 $\mu\text{g}/\text{ml}$), 60 min at 22 C; trypsin (50 $\mu\text{g}/\text{ml}$), 120 min at 28 C; deoxycholate (1%), 60 min at 0 C. Symbols: O, NEC; ●, TSC.

not seem to be a consequence of one chemical fixing more protein to the DNA than the other. Higher densities have also been reported for formaldehyde-fixed ribosomes as compared to glutaraldehyde-fixed ribosomes (2, 16, 26).

Fixation with glutaraldehyde was rapid, and complexes were stable for long periods. Low values of density were obtained for formaldehyde-fixed complexes if exposure was for short periods (1 to 2 h). Because of the reproducibility and stability of the glutaraldehyde-fixed complexes, the glutaraldehyde technique was adopted for all subsequent studies.

To obtain an estimate of the molecular weights of the complexes from their buoyant density in CsCl , it is assumed that the density contributions of the separate components are additive. This assumption appears to be valid for polyoma virions. For example, from the chemical composition (10, 21) and the density of the DNA (27) and protein (21) in CsCl , a value of 1.334 g/cm^3 can be calculated for the density of intact virions. Experimentally determined values are 1.327 (21), 1.32 (6, 7), and 1.339 (29).

Accordingly, taking the density of polyoma protein in CsCl as an estimate of the density of the protein moiety of the complex, namely 1.281

g/cm^3 (21), and the density of polyoma DNA as 1.709 g/cm^3 (27), a ratio of protein to DNA of 1.744 can be calculated from the density of the glutaraldehyde-fixed complex. This represents a composition of about 64% protein and 36% DNA and a molecular weight of about 8 million. Similar calculations using the density of the complex fixed with formaldehyde give a composition of about 55% protein and 45% DNA and a molecular weight of 6.6 million. Allowing for the uncertainty in the density of the protein moiety, the molecular weight of the py complex could range anywhere from about 6 to 9 million.

The fact that the buoyant densities of r-py complex and py complex are identical suggest that protein is bound stoichiometrically to all forms of polyoma DNA. This is consistent with the sedimentation properties as described above. Molecular weights of r-py complexes would therefore be expected to range from that of the py complex, representing early replicating DNA protein complexes, to values about twice the molecular weight of py complex (late replicating DNA-protein complexes).

Sedimentation and buoyant density of complexes exposed to different concentrations of NaCl. Treatment with concentrations of NaCl exceeding 0.2 M causes a decrease in

TABLE 2. Effect of enzymes and deoxycholate on polyoma DNA-protein complexes^a

Treatment	Time (min)	Concn	Temp (C)	Sedimentation coefficient	
				TSC	NEC
Control	60		0	56 ^b	67, 58 ^b
	60		22	56	67, 58
DOC	60	0.10%	0	56	67, 58
	60	0.25%	0	56	67, 58
	60	1.00%	0	23	23
Neuraminidase	60	1 unit/ml	22	56	67, 58
Pronase	60	100 μg/ml	22	38	41
Phospholipase C	60	200 μg/ml	22	56	67, 58
RNase	60	100 μg/ml	22	Pellet	Pellet
	60	10 μg/ml	22	Pellet	Pellet
	60	1 μg/ml	22	71	69
Trypsin	30	100 μg/ml	22	38	
	120	50 μg/ml	28	32	32

^a ³H-labeled NEC (20-min pulse) and ¹⁴C-labeled TSC (6-h pulse) were mixed and incubated as described. After incubation, samples were analyzed in 5 to 20% sucrose gradients.

^b S values were based on the position of the large ribosomal subunit in these gradients (15, 24, 25).

^c Samples treated with low concentrations of DOC showed a marked reduction in sedimentation rate upon storage for 24 h or longer.

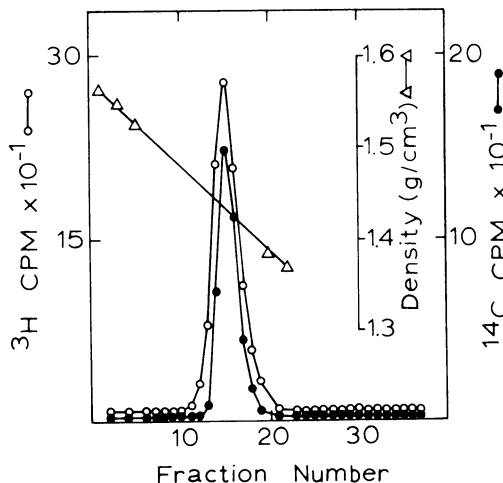


FIG. 7. Equilibrium centrifugation of glutaraldehyde-fixed nucleoprotein complexes from Triton supernatant and nuclear fractions. ³H-labeled NEC and ¹⁴C-labeled TSC prepared as described in the legend to Fig. 6 were sedimented in separate 5 to 20% sucrose gradients. Peak fractions from each gradient were pooled and treated with 5% glutaraldehyde for 2 h at 0°C. Fixed samples were combined, layered onto preformed CsCl gradients, and centrifuged to equilibrium (2).

the sedimentation coefficients of both complexes. Density measurements indicate, however, that much less protein is removed than would be predicted from the changes in sedimentation. For example, exposure to 0.5 M NaCl causes at least a 30% decrease in the rate of sedimentation while density measurements indicate little change in total mass (Table 3). Actually, part or all of the observed increase in density (0.018 g/cm³) could be caused by a change in hydration and/or binding of CsCl due to a configurational change. Thus it appears that 0.5 M NaCl strongly affects the configuration of the complexes resulting in increased frictional coefficients. The configurational change in going from 0.2 to 0.5 M NaCl is completely reversible (Table 3). r-py complex and py complex behave similarly under these conditions.

Exposure of the complexes to 1.0 M NaCl results in about a 50% decrease in sedimentation coefficient, whereas density measurements indicate a loss of only about 25% in mass. The 1 M NaCl-treated sample (density 1.487 g/cm³) has about the same density as a 38S trypsin-treated complex (density 1.492 g/cm³), and yet the trypsin-treated complex sediments about 25% faster. The configurational change caused by the 1 M NaCl is partially reversible upon dilution to 0.2 M NaCl (Table 3). That protein can bind to free polyoma DNA at 1 M NaCl was demonstrated by incubating ¹⁴C-labeled polyoma DNA marker in ³H-labeled Triton lysates at 1 M NaCl. As shown in Fig. 8, about 20% of the polyoma marker cosediments with ³H-labeled complex when about 0.1 μg of DNA was added to 0.2 ml of a Triton extract.

DISCUSSION

When polyoma-infected cells are pulsed for short periods with ³H-thymidine and lysed by the Triton procedure, most of the newly replicated supercoiled viral DNA is released from the nuclear fraction as nucleoprotein complexes. However, this procedure releases only a portion of the polyoma replicating DNA. The shorter the pulse, the greater is the amount of total replicating DNA which remains in the nuclear fraction. Polyoma replicating DNA can be reextracted from the nuclear fractions as a nucleoprotein complex (r-py complex) by re-swelling the nuclei in hypotonic buffer containing Triton, adding salt, and incubating the nuclei for several hours on ice. Reextraction with NP-40, neuraminidase, or phospholipase does not increase the amount of r-py complex released from nuclei.

Recently, it was reported that lysis of SV40-

TABLE 3. *Effect of NaCl on py complex^a*

Treatment for 1 h (0°C) at:	S	Buoyant density (g/cm ³) ^c	Computed S ^d	Mol wt ($\times 10^{-6}$) ^e	σ protein
1. Standard conditions (0.2 M NaCl), sedimented in 0.2 M NaCl, 5–20% sucrose gradient	56 ^f	1.437		8.23	63.6
2. 0.5 M, sedimented in 0.5 M NaCl, 5–20% sucrose gradient	40 ^f	1.455	52	7.38	59.4
3. 0.5 M, diluted to 0.2 M NaCl (20 min), sedimented in 0.2 M NaCl, 5–20% sucrose gradient	56	1.437	56	8.23	63.6
4. 1.0 M, sedimented in 1.0 M NaCl, 5–20% sucrose gradient	29 ^f	1.487	46	6.23	51.9
5. 1.0 M, diluted to 0.2 M NaCl (20 min) sedimented in 0.2 M NaCl 5–20% sucrose gradient	38	1.477	48	6.55	54.2

^a ³H-labeled NEC and ¹⁴C-labeled TSC were prepared as described in Fig. 6. Both complexes reacted similarly to the treatments. All values are for ¹⁴C-labeled TSC only.

^b Buoyant densities in CsCl of fractions pooled from sucrose gradients and fixed in glutaraldehyde. Buoyant densities of formaldehyde-fixed complexes are greater by 0.04 g/cm³.

^c S at standard conditions. Computed from change in mass and density of particle but no change in configuration. The value of partial specific volume used in this calculation is estimated from reciprocal of density.

^d Molecular weights computed using protein to DNA ratio estimated from densities of glutaraldehyde-fixed complexes. It is assumed that each complex molecule consists of only polyoma DNA (3×10^6 daltons) and protein.

^e Measured as described in Table 2.

^f Determined from the position of the 56S complex and corrected for viscosity and density to "standard conditions".

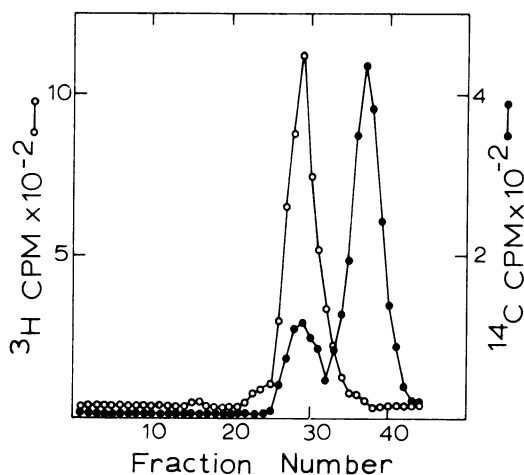


FIG. 8. Velocity sedimentation of ³H-py complex and ¹⁴C-polyoma DNA in a 5 to 20% sucrose gradient containing 1 M NaCl. A portion of a Triton supernatant fraction prepared after a 15-min pulse with ³H-thymidine (75 μ Ci/ml) was mixed with ¹⁴C-labeled polyoma DNA (14,000 counts per min per μ g) extracted from virus. The mixture was made to 1 M in NaCl and incubated for 60 min at 0°C. A 0.2-ml sample was layered onto a linear sucrose gradient made by mixing 5 and 20% solutions of sucrose in TTE containing 1 M NaCl and centrifuged as previously described.

infected CV-1 cells releases the majority of virions from nuclei irrespective of the ionic strength of the buffer or the presence of detergent and divalent cation (12). Green and co-workers found that py complex could be extracted from nuclei without detergent (14). In our studies, py complex was extracted by a variety of procedures, but the Triton procedure of Green and co-workers (14) worked equally well or better than most. For example, cell lysis at 0°C, substitution of NP-40 for Triton, lower concentrations of EDTA, or inclusion of magnesium and calcium in the lysing buffers resulted in lower yields of py complex (data not presented above). Although the concentration of Triton was not found to be critical, swelling of nuclei in relatively high concentrations of EDTA was apparently necessary to render nuclei sufficiently leaky to release all the py complex.

The fact that many r-py complexes are not released during the initial cell lysis indicates that these complexes exist in a different physical state in the nuclei, presumably associated with replicative sites. Previously, we presented evidence for a fixed number of replicative sites from kinetic data on the formation of replicative intermediates (20). Whether or not these sites

are associated with membranes is difficult to conclude from our data since enzymes which attack membranes such as neuraminidase and phospholipase did not appear to be more effective in extracting r-py complexes from nuclei.

Triton lysis after a short pulse always releases some r-py complex into the Triton supernatant fluid. Whether this represents preferential release of r-py complexes from replicative sites at some specific stage in replication was determined by comparing the DNA of nuclear r-py complex to the DNA of r-py complex from the Triton supernatant fluid in CsCl-PI equilibrium gradients. "Young" replicating DNA can be distinguished from "mature" replicating DNA in these gradients since young replicating molecules are found closer to the supercoiled peak, whereas mature replicating molecules have densities more like open circular DNA (4, 19, 23). In CsCl-PI density gradients, the distribution of densities of RI extracted from the nuclear fraction was found to be identical to the distribution of RI in the Triton supernatant fluid so it does not appear that Triton lysis causes preferential release of r-py complexes containing either young or mature forms of replicating polyoma DNA. Since r-py complex is released slowly from nuclei over a period of hours by the same buffer used to lyse cells, it is likely that the amount of r-py complex released during the Triton lysis represents a nonspecific fraction liberated during the initial lysis. Nevertheless, this does not rule out the possibility that more than one type of replication site exists in the cell with each having a different susceptibility to Triton.

A similarity exists between the sedimentation profiles of nucleoprotein complexes and the sedimentation profiles of DNA released from them. For example, 69 to 60S nucleoprotein complexes contain polyoma DNA which sediments at 25 to 21S, whereas 51 to 42S complexes contain DNA which sediments at about 20S (both supercoiled and RI). If binding of the protein alters the configuration and flexibility of the different forms of DNA to about the same extent, the sedimentation coefficients of the complexes should be approximately proportional to their mass. Should this be the case, then the sedimentation coefficients of the complexes suggest an almost stoichiometric binding of protein to all forms of polyoma DNA. Buoyant density measurements of the glutaraldehyde-fixed complexes appear to support this. The average buoyant densities of all the glutaraldehyde-fixed polyoma complexes differ by less than 0.01 g/cm³.

A crucial question is the origin of the protein moiety. The proteins in the r-py complex and the py complex appear to react similarly to enzymatic and chemical treatment. Neither Pronase nor trypsin treatment at room temperature reduce the sedimentation rate of the complexes to *S* values characteristic of free DNA. From the buoyant densities of the trypsin-treated, glutaraldehyde-fixed complexes in CsCl, it was estimated that only about 40% of the protein is removed by this treatment. Green reported that complexes with sedimentation coefficients of 35S resulted if cells were exposed to cycloheximide prior to pulse labeling with ³H-thymidine (13). We have found that both r-py complex and py complex sediment between 37 and 30S depending on the time of pretreatment with cycloheximide (Goldstein et al., unpublished data). These results taken together suggest that both complexes may have a major protein component sensitive to both trypsin and Pronase which requires de novo protein synthesis for association with newly replicated polyoma DNA.

A second component which is resistant to Pronase and trypsin can be removed by treatment with high concentrations of DOC. A small amount of material appears to remain associated with DNA even after DOC treatment, and this may represent a third component. It is not certain that either the DOC-sensitive or -resistant material is composed entirely of protein. The fact that complexes were resistant to neuraminidase and phospholipase does not eliminate the possibility that they may contain glycoproteins or lipoproteins since these components might be sequestered in the intact complex. It is also difficult to rule out an RNA component since RNase binds strongly to the complex.

r-py complex and py complex sediment in 0.2 M NaCl more rapidly than would be expected for complexes containing DNA in the same configuration as free DNA. For example, taking into account the difference in densities between py complex and free supercoiled DNA, it can be calculated that py complex with a mass between 6 and 9 million daltons would sediment in the range of about 28 to 42S, instead of 56S. Pettijohn and Kamiya (22) have actually formed complexes with these molecular weights and *S* values by mixing polyoma supercoiled DNA with purified protein (*Escherichia coli* RNA polymerase).

A reversible configurational change resulting in a reduced sedimentation rate occurs when the NaCl concentration is made 0.5 M. This change is accompanied by a slight increase in

buoyant density (Table 3). A further decrease in *S* value and an apparent loss of protein takes place when the NaCl concentration is made 1.0 M. Nonetheless, density measurements indicate that about 50% of the protein is still bound to polyoma complexes at this salt concentration.

Green et al. (14) and White and Eason (28) observed that treatment with 1 M NaCl caused complexes to cosediment with viral DNA marker. It should be noted, however, that our conditions of treatment and sedimentation were slightly different from theirs. In our experiments, sucrose gradients were made in the Triton lysing buffer (STTE), whereas Triton was not included in their gradients. We found that polyoma DNA marker sediments faster in sucrose gradients if Triton is omitted. Additionally, as noted in Results, protein binds to polyoma DNA in 1 M NaCl, and a small excess of protein would be sufficient to bind all viral DNA marker of high specific activity. The fact that no free viral DNA is found after Triton lysis suggests that an excess of protein does in fact exist in infected cells.

Protein comprises over half the mass of both r-py complex and py complex. For example, this amount of protein could represent the binding of roughly 100 protein molecules of 50,000 molecular weight per polyoma DNA molecule (3×10^6 mol wt). Since the r-py complex and the py complex have the same buoyant density, it is reasonable to conclude that protein is attached to replicated DNA as well as unreplicated DNA in the r-py complex. This protein is most likely added to newly replicated regions immediately after the base sequences are copied. This suggests that the nucleoprotein complexes may contain structural, "repressor," or maturation proteins. Subviral deoxynucleoprotein complexes with sedimentation coefficients between 46 and 25S have been isolated by mild alkali treatment of SV40 and polyoma virions (1, 3, 9, 11, 18). A comparison of the protein in py complex with subviral protein might provide considerable insight into the function of the subcellular polyoma complexes.

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ADDENDUM IN PROOF

We recently received a manuscript submitted for publication by T. Seebeck and R. Weil in which they describe the isolation of polyoma nucleoprotein complexes from host cell chromatin. Fast-sedimenting complexes containing replicating polyoma DNA and slow sedimenting complexes containing supercoiled DNA were fixed with formaldehyde and found to have identical buoyant densities in CsCl, namely, 1.470 g/cm³.

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