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Chromatin-Like Structures in Polyoma Virus and Simian Virus 40 Lytic Cycle

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Nucleoprotein complexes containing viral DNA and cellular histones were extracted from nuclei of permissive cells infected with polyoma virus or simian virus 40 (SV40) and examined by electron microscopy. Polyoma and SV40 nucleoprotein complexes are almost identical. They appear as relaxed circular molecules consisting of 20 to 21 globular particles interconnected by thin filaments. Their contour length in 0.02 M salt is 2.7 times shorter than that of viral DNA form I obtained after dissociation of the proteins in 1 M NaCl. The nucleosomes have an average diameter of 12.5 nm. Each nucleosome contains 175 to 205 DNA base pairs condensed fivefold in length. The nucleosomes are regularly spaced on the circular molecule. The internucleosomal filaments are made of naked DNA, and each filament contains about 55 base pairs. The partial sensitivity of the nucleoprotein complex to cleavage by *Eco*R1 endonuclease suggests that the nucleosomes are not formed at specific sites on the viral genome. Faster sedimenting nucleoprotein complexes containing replicative intermediates were studied. Isopycnic centrifugation in metrizamide gradients in the absence of aldehyde fixation showed that these molecules conserved the same DNA-to-protein ratio as the form I DNA-containing complexes.

Infection of permissive cells with polyoma virus (Py) or simian virus 40 (SV40) leads to the production of progeny virus and to the destruction of the host cells. The synthesis of viral DNA takes place in the nucleus and is accompanied by a simultaneous stimulation of viral-induced cellular DNA and histone synthesis (14, 31). Nucleoprotein complexes extracted from infected nuclei contain viral DNA associated with cellular histones (18-20, 27). It has been shown (9, 27) that during replication viral DNA is also associated with proteins.

Cellular chromatin, recently studied by electron microscopy, appears as a series of spherical particles, which have been named ν bodies (23) or nucleosomes (24) and are interconnected by naked DNA filaments. The nucleosomes contain the cellular histones except H1 and approximately 200 base pairs (BP) of DNA (24). Recent models were proposed for the association between DNA and histones in these structures (15, 28).

We analyzed and compared the structure of polyoma and SV40 nucleoprotein complexes isolated from infected cells and investigated whether such complexes are conserved during replication. We also tried to determine if any DNA specificity is required for protein association in the nucleosomes. Our electron microscopic results are in agreement with recent results on SV40 nucleoprotein complexes isolated from infected cells (12) or associated in vitro (8).

MATERIALS AND METHODS

Cell cultures and infection conditions. Mouse fibroblasts (3T6 cell line obtained from M. Fried) and an African green monkey kidney cell line, HP8, a subclone of CV1, were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (Grand Island Biological Co.) in plastic petri dishes (Falcon, 88 mm in diameter).

A plaque-purified, large-plaque strain of Py virus (originally obtained from N. Acheson) was propagated on primary mouse kidney cells (30). Digestion of the viral DNA with HpaII endonuclease gave a fragment pattern identical to that described by Griffin et al. (11). A plaque-purified, wild-type strain of SV40 was a gift of R. Cassingena. The virus was propagated at low multiplicity in HP8 cells.

For the preparation of nucleoprotein complexes, 3T6 cells were infected in exponential phase with Py virus at a multiplicity of 50 to 100 PFU/cell. HP8 cells were infected with SV40 at the same multiplicity 24 h after reaching confluency. The virus was allowed to absorb for 90 min at 37 C in a CO_2 incubator. Thereafter, the cultures were covered

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with 10 ml of Dulbecco modified Eagle medium containing 1% serum.

Purification of nucleoprotein complexes. Infected cultures were labeled with 10 μCi of [^{14}C]-thymidine (C.E.A., 55 mCi/mmol) per plate containing 3 ml of medium from 25 to 30 h postinfection. During the last 10 min, 500 μCi of [^{3}H]thymidine (C.E.A., 20 Ci/mmol) was added per plate. After the pulse labeling, the monolayers were washed twice with cold Tris-buffered saline, and the nuclei were isolated by the procedure of Burgoyne et al. (4). Nucleoprotein complexes were extracted with the technique described by Green et al. (10): the isolated nuclei were incubated in a solution containing 0.25% Triton X-100, 0.01 M Tris-hydrochloride, pH 7.9, and 0.001 M EDTA (0.18 ml/petri dish) with occasional gentle mixing for 30 min at room temperature, and then 0.02 ml of 2 M NaCl was added. The supernatant obtained after centrifugation at 800 × *g* contained the nucleoprotein complex and could be stored for several days at 4 C.

The nucleoprotein complexes were partially purified by sedimentation on linear 5 to 20% (wt/wt) sucrose gradients in 0.01 M Tris-hydrochloride, pH 7.9, 0.001 M EDTA, 0.2 M NaCl for 2 h at 36,000 rpm in an SW50.1 rotor at 4 C.

Metrizamide density gradients. Metrizamide (Nyegaard & Co., Oslo) is an iodinated glucose derivative [2-(3-acetamido-5-*N*-methylacetamido-2, 4,6-triodobenzamido)-2-deoxy-D-glucose] recently described by Birnie et al. (3). Metrizamide solutions were prepared in a buffer containing 0.01 M *N*-2-hydroxyethyl-piperazine-*N'*-2'-ethanesulfonic acid (Calbiochem), pH 7.9, 0.001 M EDTA, and 0.2 M NaCl. Nucleoprotein complex samples (0.5 ml) isolated by sedimentation on sucrose gradients were layered onto discontinuous gradients made up of 1.1-ml cushions of metrizamide solutions at densities of 1.31, 1.24, and 1.16 g/cm^3. Gradients were centrifuged at 50,000 rpm for 3 h at 4 C in an SW56 rotor. The density of the gradient fractions was calculated from the refraction index (*c*) measured with a Zeiss refractometer according to the formula: $\rho = 3.350\,c - 3.462$ (3).

Restriction endonuclease digestion. Endonuclease *Eco*R1 was purified from *Escherichia coli* RY13 (obtained from H. Boyer) according to the procedure of R. Yoshimori (Ph.D. thesis, Univ. of California, San Francisco, 1971) to the hydroxyapatite column step. No exo- or endonuclease contaminations could be detected in this preparation. Viral DNA or nucleoprotein complexes from sucrose gradients were incubated with excess *Eco*R1 for 1 h at 37 C in 0.01 M Tris-hydrochloride, pH 7.4, 0.01 M $MgCl_2$. Under these conditions, total conversion of deproteinized circular DNA to linear molecules was observed.

Electron microscopy. Nucleoprotein complexes were prepared for electron microscopy using the method of Dubochet et al. (6). Samples were diluted 20-fold with a buffer containing 0.01 M Tris-hydrochloride, pH 7.9, and 0.001 M EDTA. A drop of the solution was applied to a carbon-coated grid (400 mesh), which was previously activated by alternating current glow discharge in amylamine vapor. After 2 min, the excess liquid was removed from the grid, and the adsorbed material was stained for 1 min with a 2% aqueous solution of uranyl acetate. The grids were dried on filter paper and rotary shadowed with platinum-palladium (80:20) at an angle of 8°. Samples were observed with a Siemens Elmiskop 101 electron microscope, and pictures were taken at a magnification of 16,000. Measurements were made on 4× to 10× enlargements with the help of a laboratory-made coordinatometer connected to a POP-8 digital computer, or with a lens with 0.1-mm graduations. Calibration was based on examination of a carbon-grating replica (E. F. Fullamn, Inc.; 54,800 lines/inch). φX174 replicative form DNA (a gift of N. Truffault) was analyzed under the same conditions. A value of 5,500 ± 100 BP (F. Sanger, personal communication) was taken for this DNA. This value was obtained by comparison of the mobilities of restriction enzyme fragments of φX DNA with DNA fragments of known sequence.

RESULTS

Isolation and electron microscopic analysis of nucleoprotein complexes. Cells were infected with a multiplicity of 50 to 100 PFU/cell and labeled from 25 to 30 h after infection with [^{14}C]thymidine. During the last 10 min, [^{3}H]thymidine was added to preferentially label the replicative intermediates. The nucleoprotein complexes were extracted from isolated nuclei with 0.25% Triton X-100 and analyzed on neutral sucrose gradients as described in Materials and Methods. In agreement with previous results on Py and SV40 nucleoprotein complexes (9, 13, 27), the ^{14}C-labeled complexes containing form I DNA (Fig. 1) sedimented at about 55*S* on neutral sucrose gradients. To acquire more information on the molecular structure of the viral nucleoprotein complexes, we utilized the electron microscopic technique recently developed by Dubochet for the observation of DNA-protein complexes (6). With samples originating from the ^{14}C peak of the sucrose gradient shown in Fig. 1, we observed (Fig. 2a, b, d) relaxed circular structures composed of ν bodies (nucleosomes) with interconnecting filaments of DNA. The structure of nucleoprotein complexes from SV40- or Py-infected cells is very similar (Fig. 2a, b). Photographic enlargements of these complexes (Fig. 2d) permitted the measurement of the filament length and nucleosome diameter. These data are summarized in Table 1. On the average, 20 to 21 nucleosomes about 12.5 nm in diameter are observed per molecule. They are connected by filaments of variable length. The length measurement of these filaments shows a unimodal distribution (Fig. 3). The mode was 11.6 nm for Py and 13.3 nm for SV40 complexes,

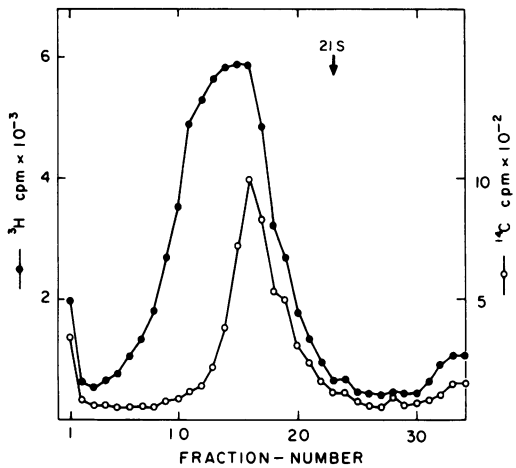

FIG. 1. *Sucrose gradient sedimentation of nucleoprotein complexes. SV40-infected HP8 monkey cells were labeled for 5 h with [^{14}C]thymidine and pulse labeled for 10 min with [^{3}H]thymidine before extraction of the nucleoprotein complexes as described in Materials and Methods. The nuclear extract was layered on to a 5.0-ml 5 to 20% sucrose gradient and centrifuged for 2 h at 35,000 rpm at 4 C in an SW50.1 Spinco rotor. Fractions were collected and assayed for trichloroacetic acid-insoluble radioactivity by liquid scintillation counting. The position of the 21S DNA marker was taken from a separate gradient run in parallel. Symbols: (●) [^{3}H]thymidine; (○) [^{14}C]thymidine. Similar results were obtained for Py nucleoprotein complexes.*

whereas the means were about 17.7 nm in both cases (Table 1). The same average filament length was obtained by subtracting the sum of the diameters of the nucleosomes in a molecule from the nucleoprotein complex length and dividing it by the number of filaments.

The precise number of BP in the nucleosomes and internucleosomal filaments depends on a precise estimate of the DNA BP content of Py or SV40 molecules. A number of 4,800 to 5,100 BP is generally accepted for SV40. When we calibrated polyacrylamide gels with HpaII or HindII,III fragments of λ DNA (1, 2), or HindII,III fragments of SV40 DNA (4,880 BP; 2), a value of 5,000 ± 300 BP was obtained for Py DNA. A more precise determination should await comparison of the electrophoretic mobilities of Py and SV40 DNA fragments with fragments of known molecular weight. The number of BP estimated by sequence studies for φX174 DNA is 5,500 ± 100 (F. Sanger, personal communication). When replicative form DNA of φX174 was spread on electron microscope grids and measured, the distance between the planes of neighboring BPs was calculated to be 0.332 nm. Dividing our length measurements of SV40 and Py DNA (Table 1) by this value, the number of BPs obtained for SV40 and Py DNA is 4,840 and 5,190, respectively.

The length of DNA contained in the nucleosomes was calculated by deducting the sum of the internucleosomal filament lengths from the contour length of deproteinized DNA molecules. A nucleosome contained approximately 175 to 205 BP. The internucleosomal filaments had the same width as deproteinized DNA when analyzed under the same experimental conditions and contained approximately 55 BP (Table 1). The DNA in the nucleosome is condensed fivefold as compared with deproteinized DNA. The nucleoprotein complex itself is altogether about 2.7 times more compact than naked DNA (Fig. 2a, c; Table 1). Previous physical studies showed that the SV40 nucleoprotein complex sediments faster than the Py complex (13). However, the differences we observed between both complexes (Table 1) are within the error limits of the measurements, and a more accurate study is needed to propose a correlation of our results with the differences in sedimentation rates.

Increase in the ionic strength affects the structure of the complex (10). When the Py or SV40 nucleoprotein complex was treated with 0.5 M NaCl (30 min at 20 C, followed by dilution to low ionic strength), the number of nucleosomes observed per molecule decreased, whereas the contour length of the molecules increased. After this salt treatment, the nucleoprotein complex sedimented at about 35*S* (data not shown). Treatment of the nucleoprotein complex with 1 M NaCl removed the bound histones, and naked DNA molecules were observed by electron microscopy (Fig. 2c). The majority of these molecules was superhelical, whereas a minority was constituted by relaxed circles. Length measurements were mostly done on relaxed circular DNA. The bulk of 1 M NaCl-treated material sedimented as purified form I DNA on neutral sucrose gradients.

*Eco*R1 restriction endonuclease cleaves SV40 DNA at a unique site (21). The SV40 nucleoprotein complex was treated with *Eco*R1, and the samples were scored by electron microscopy for the presence of linear complexes. Only a fraction of the complex molecules, 15 to 27% in two different experiments (Table 2), became linear in the presence of excess enzyme. The linear complexes contained an average of 21 nucleosomes that were indistinguishable from those in the circular complexes. Under the same experimental conditions, naked SV40 DNA added to the complex was totally cleaved

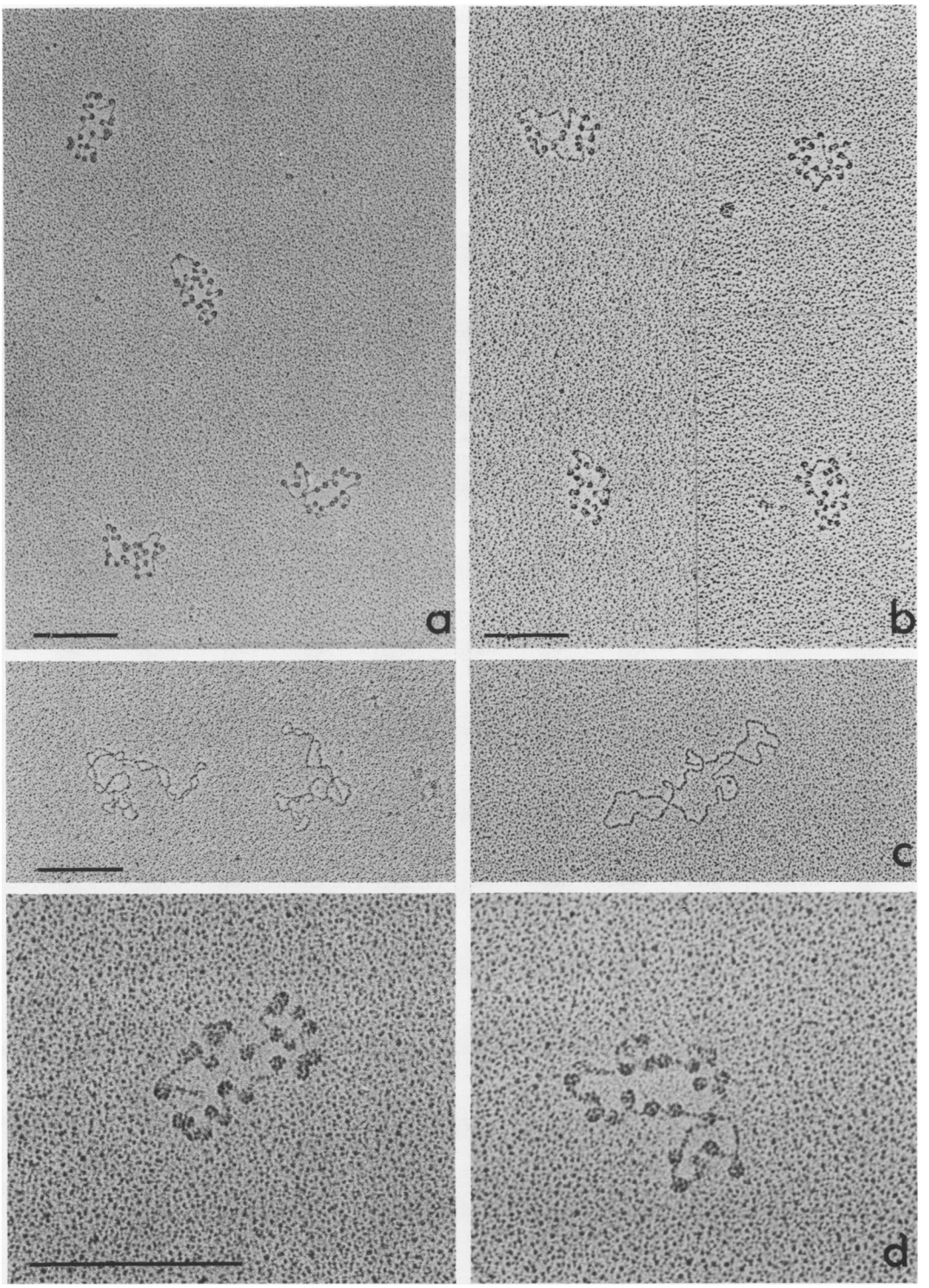

FIG. 2. *Electron micrographs of Py and SV40 nucleoprotein complexes. (a) and (d) Complexes extracted from SV40-infected cells; (b) nucleoprotein complexes extracted from Py-infected cells; (c) deproteinized DNA, obtained by treatment of SV40 nucleoprotein complexes with 1 M NaCl. Samples were prepared, stained, and shadowed as described in Materials and Methods. Bar, 200 nm.*

TABLE 1. *Py virus and SV40 nucleoprotein complex dimensions*[a]

Determination	Py virus	SV40
Nucleoprotein complex length (nm)	631 ± 81 (21)	605 ± 123 (33)
Nucleosomes/molecule	20 ± 1.5 (60)	21 ± 1.5 (69)
Nucleosome diameter (nm)	13.1 ± 1.1 (59)	11.8 ± 1.8 (43)
BP/nucleosome	205	175
Internucleosomal filament length (nm)	17.7 ± 10.1 (287)	17.6 ± 11.8 (236)
BP/filament	55	55
Deproteinized DNA length (nm)	1,670 ± 120 (39)	1,560 ± 89 (95)
Nucleosome packing ratio		
Nucleosome DNA length/nucleosome diameter	5.0	4.8
Condensation ratio		
Deproteinized DNA length/complex length	2.7	2.6

[a] The number of observations is given in parentheses. Diameters and widths have been corrected for the fractional increase by deposited platinum-palladium. The average number of BPs was estimated by dividing the length of DNA by 0.322, taken as the distance in nanometers between the planes of neighboring BPs (see Results). The length of the DNA packed in a nucleosome was calculated by subtracting from the Py or SV40 deproteinized DNA length the sum of the internucleosomal segment lengths, and by dividing the result by the number of nucleosomes per molecule. Length measurements of Py or SV40 *Eco*R1 linears with the replicative form of ϕX174 DNA as internal standard gave values identical to those given for the circular DNA.

to full-length linear duplexes. No exchange of histones was observed between the complex and the naked DNA during the *Eco*R1 digestion. About 20% of the DNA in the complex is situated in the internucleosomal filaments free of histones (Table 1). The frequency of cleavage with *Eco*R1 indicates that the distribution of the nucleosomes is probably random along the SV40 genome, and that no movements of the nucleosomes occur during the incubation.

Replicating intermediate complexes. Trying to understand whether such nucleoprotein complexes are conserved during replication, we have attempted to elucidate the structure of replicating complexes by direct observation with the electron microscope and by density determinations. The material labeled for 10 min with [^{3}H]thymidine sediments faster than 55*S*, in accordance with previous reports (9, 13, 27) (Fig. 1).

Several iodinated derivatives were recently used for density gradient studies of nucleoprotein complexes in the absence of aldehyde fixation (5). Metrizamide, a nonionic iodinated benzamido derivative of glucose, was used for the study of cellular chromatin (26) or polyoma nucleoprotein complexes (18). Fractions from neutral sucrose gradients containing both form I and replicative intermediate nucleoprotein complexes were pooled and applied to a discontinuous metrizamide density gradient without previous aldehyde fixation. Both replicative intermediates and form I DNA-containing complexes band in the same position, at a density of 1.164 g/cm^3 (Fig. 4). No separation between "mature" and "replicative" complexes was obtained after longer runs that generated less steep density gradients. Under the same conditions, purified DNA bands at a density of 1.118 g/cm^3. It was shown that the density of DNA protein complexes depends on the relative ratio of protein to DNA (3). In reconstruction experiments, Birnie et al. (3) have obtained a density of 1.168 g/cm^3 in metrizamide gradients for DNA-protein complexes with a ratio of one. Our results thus indicate that, in the nucleoprotein complexes, the DNA-to-protein ratio does not change during replication and that it is probably equal to one. These results are in agreement with the buoyant density determinations reported for glutaraldehyde-fixed SV40 and Py nucleoprotein complexes (9, 27). The nucleoprotein complexes observed by electron microscopy after metrizamide gradients were not fundamentally modified, and the typical nucleosome structure was conserved. However, when compared to the nucleoprotein complex measurements given in Table 1, the molecules were about 40% longer and had generally lost two nucleosomes each, whereas the internucleosomal filament length was very variable and usually doubled. The centrifugation of the complex in metrizamide decreased the number of BPs in the nucleosomes (150 BP), possibly by unwrapping a fraction of the DNA (P. F. Pignatti, C. Crémisi, O. Croissant, and M. Yaniv, FEBS Lett., in press).

DISCUSSION

Recent electron microscopic studies of eukaryotic chromatin showed the presence of a series of globular structures called ν bodies (23) or nucleosomes (24) that constitute the chroma-

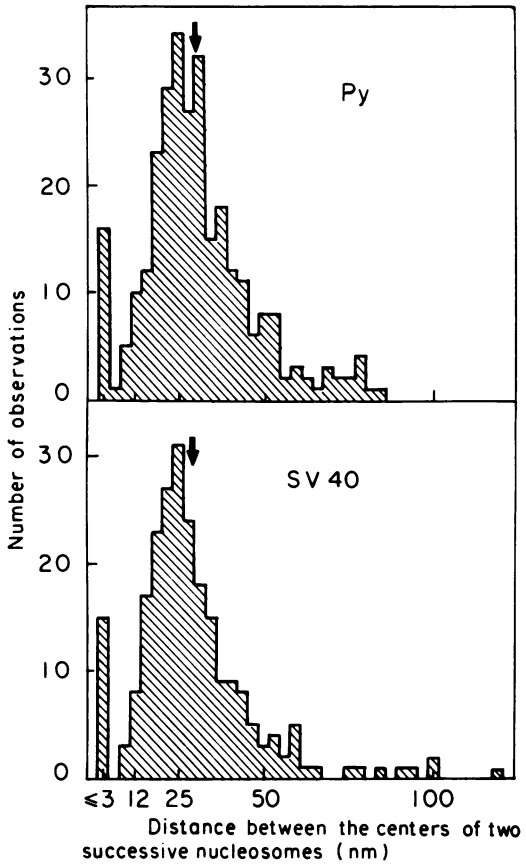

FIG. 3. *Distance between the centers of two neighboring nucleosomes. The distance between the centers of adjacent nucleosomes was measured on photographic enlargements of nucleoprotein complex molecules isolated from SV40- or Py-infected cells (see Materials and Methods). Measures ≤12 nm in the two histograms represent the number of observations in which nucleosomes were in contact with each other, or superimposed, and could represent nucleosome multimers. The arrow indicates the position of the mean. The internucleosomal filament length was calculated from these data by subtraction of the nucleosome diameter. The total number of determinations was 287 for polyoma and 236 for SV40 nucleoprotein complexes.*

tin fibers. The four histones (H2a, H2b, H3, and H4) are associated with the cellular DNA in this structure (16, 24). Previous biochemical studies showed that both SV40 and Py viral DNA are associated with four cellular histones in the virion cores or in the nuclei (18–20, 27). Griffith has shown that the SV40 nucleoprotein complex extracted from infected cells is circular and contains 21 nucleosomes (12). Experiments on in vitro association of SV40 DNA and cellular histones showed that the formation of 21 nucleosomes on the viral DNA explain the presence of 21 superhelical turns on the deproteinized DNA (8). Our images of both SV40 and Py nucleoprotein complexes are similar to those described by Griffith for SV40 minichromosome spread in 0.015 M salt (12). According to our measurements, the nucleosome of the viral chromatin contains about 190 BP and the internucleosomal connecting filament contains about 55 BP. Our values for the SV40 nucleoprotein complex are slightly higher than the values of 170 and 40 BP given by Griffith (12). The repetitive structure of the cellular and viral chromatin was also deduced from the production of discrete DNA segments by nuclease digestion. Louie (18) showed that endonuclease digestion of Py nucleoprotein complexes generated DNA fragments of 200 or 400 BP. Using micrococcal nuclease digestion of chromatin, Noll (22) found 205 BP per nucleosome monomer, 405 for dimers and 605 for trimers. These dimers and trimers originate from a minor fraction of the nucleosomes (see Fig. 3 for the possible presence of nucleosome multimers). The internucleosomal DNA, judging from its width, does not seem to be associated with proteins. This is in agreement with the sensitivity of the internucleosomal DNA to nuclease digestion (18). The association between the viral DNA and the cellular histones allows a 2.7-fold condensation of the DNA relative to the deproteinized DNA molecule. These nucleoprotein complexes have the possibility of becoming even more

TABLE 2. *Nucleoprotein complex cleavage by EcoR1 restriction endonuclease*[a]

Determination	Expt 1		Expt 2	
	*Eco*R1	Control	*Eco*R1	Control
No. of molecules counted	230	205	163	120
Linear molecules (%)	20	5	35	8
Molecules (%) cleaved by *Eco*R1	15		27	

[a] SV40 nucleoprotein complexes from the sucrose gradient peak fractions were incubated in the presence or absence of *Eco*R1 in 0.01 M Tris-hydrochloride, pH 7.4, 0.01 M $MgCl_2$ for 1 h at 37 C. The reaction mixture was then chilled in ice, samples were prepared for electron microscopy as described in Materials and Methods, and the complex molecules were scored for linears and circulars. When naked circular SV40 DNA was used for similar experiments together with nucleoprotein complexes, it was totally cleaved to full-length linear molecules.

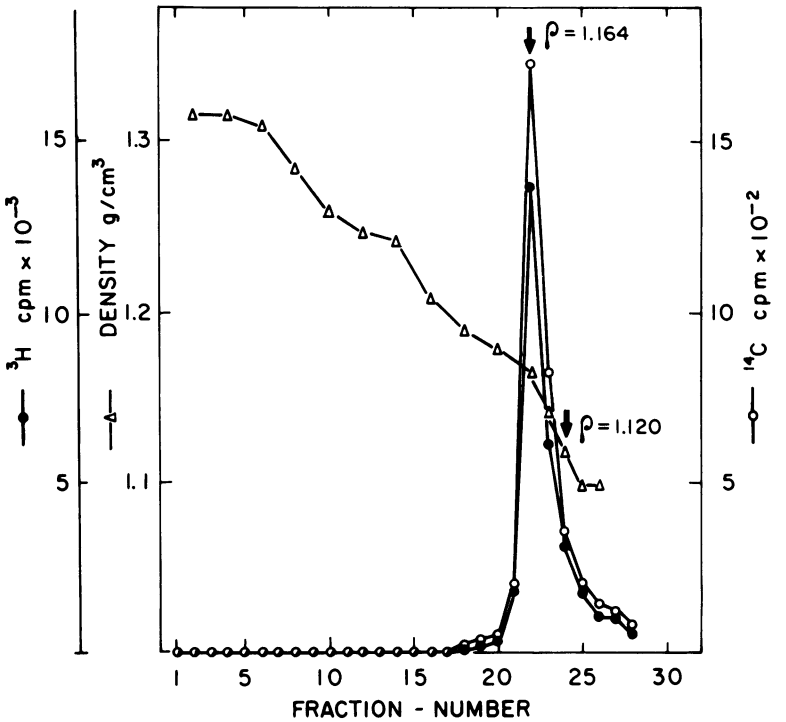

FIG. 4. *Metrizamide density gradient analysis of nucleoprotein complexes. Aliquots of fractions 9 to 19 of the gradient shown in Fig. 1 containing partially purified SV40 nucleoprotein complexes were pooled and centrifuged in a discontinuous metrizamide gradient, as described in Materials and Methods. Fractions were collected, their refractive indexes were measured, and the trichloroacetic acid-insoluble radioactivity was counted by liquid scintillation. The position of free DNA marker was taken from a separate gradient run in parallel. Symbols:* (●) *[^{3}H]thymidine;* (○) *[^{14}C]thymidine;* (△) *density. Similar results were obtained for Py nucleoprotein complexes.*

compact for encapsidation, as suggested by the flexibility of the internucleosomal DNA shown in the electron microscopic images (Fig. 2). Griffith utilized a different technique for looking at SV40 nucleoprotein complexes (12). When spread in 0.15 M NaCl, he observed a more condensed form of the molecules, which he called "native minichromosome." This structure was condensed sevenfold relative to the deproteinized DNA.

To study the role of histones during DNA replication, we compared the density of mature DNA and replicative DNA protein complexes. In the absence of glutaraldehyde fixation, both complexes had identical densities in metrizamide density gradients. Electron microscopy observation showed that the association of the DNA with the histones was conserved during isopycnic centrifugation. These results suggest that the DNA-histone association is conserved during replication. It was shown that Py and SV40 DNA replication is discontinuous: 4S segments (150 to 200 nucleotides) are synthesized in less than 15 s on the two strands at the level of the replicating fork (7, 25). No specific sequence was found to initiate fragment synthesis (25). These facts suggest that discontinuous DNA synthesis might be related to the presence of the nucleosomes along the DNA molecules. The histones could leave the DNA during replication for a very short time, and the newly naked DNA (about 200 nucleotides) could be replicated and immediately reassociated with the cellular histones. A similar explanation for discontinuous DNA synthesis was proposed by Kornberg (15) from observations on *Drosophila* DNA replication (17). Studies are in progress in an attempt to obtain unambiguous images of replicative DNA-histone complexes after cleavage with restriction endonucleases.

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