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Intracellular Forms of Simian Virus 40 Nucleoprotein Complexes

I. Methods of Isolation and Characterization in CV-1 Cells

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A new method was developed for isolation of intracellular forms of simian virus 40 (SV40) nucleoprotein complexes from SV40-infected CV-1 cells late in the infectious cycle. In contrast to the Triton extraction method, which yields only a 60-70S complex, this new procedure yielded three forms of SV40 nucleoprotein complexes: complex I, complex II, and the mature virion (V). The three nucleoprotein complexes differed in physical as well as biochemical properties. Complex I, which is only a small portion of the total SV40 nucleoprotein complexes late during infection, was active in synthesizing both SV40-specific DNA and RNA. Pulse-labeling experiments suggest the following metabolic pathway: I → II → V. Conversion of complex I to II occurred shortly after the completion of SV40 DNA replication and resulted in the inactivation of the biosynthetic activities of I.

Simian virus 40 (SV40) chromatin isolated from lytically infected cells exhibits many properties similar to those of eucaryotic cell chromatin. It is composed mainly of a histone-DNA complex arranged in the nucleosome structure (8) and is associated with both DNA (19) and RNA (6) polymerase activities.

We are interested in using SV40 chromatin as a model system for studying the relation between the structure and function of eucaryotic chromatin. During our initial study of SV40 chromatin isolated by the Triton extraction procedure (7), we were puzzled by the fact that no mature virions were ever recovered in the extract even when mature virions could be isolated by banding infected cell lysate in a CsCl gradient. Further investigation showed that virions as well as many intracellular nucleoprotein (NP) complexes were disrupted by Triton treatment to yield a single species sedimenting at about 60S. This observation prompted us to develop a new isolation procedure which preserves the intracellular forms of the SV40 NP complexes. With the new procedure three forms of SV40 NP complexes were isolated. A minor component, complex I, sedimenting at about 70S, was found to be actively synthesizing SV40 RNA and DNA. In addition, a more condensed form of SV40 chromatin (complex II), which is derived from complex I shortly after the completion of DNA replication, was isolated as well as the mature virion.

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In the present communication we describe the method for isolating these intracellular forms of SV40 NP complexes and the initial characterization of their physical and biochemical properties.

MATERIALS AND METHODS

Cell and virus. The SVS strain of SV40 was used to infect CV-1 cells. The description of virus, the growth of cells and virus, and the infection procedure have been reported (10).

Extraction of SV40 complexes. Two alternative methods were used for extraction of SV40 complexes. (i) For total cell extract, 48 h after infection, cells were washed once with TD buffer (25 mM Tris-hydrochloride, pH 7.4, 0.136 M NaCl, 7 mM KCl, 0.7 mM Na_2HPO_4) and were scraped off the plate into 2 ml of cold hypotonic buffer (25 mM Tris, pH 7.9, 1 mM $MgCl_2$, 0.4 mM $CaCl_2$, 0.5 mM dithiothreitol). The cells were broken by homogenization (30 strokes) in a tightly fitting glass Dounce homogenizer. The nuclei were spun down by centrifugation in an IEC PR-6000 centrifuge at 2,000 rpm for 5 min. The supernatant was then layered on a sucrose gradient for isolation of SV40 NP complexes. (ii) For nuclear extract, cells were washed once with TD buffer and were scraped into TD buffer at 10^7 cells per ml. The cells were spun down (1,500 rpm, 3 min) and resuspended in TD buffer at 10^7 cells per ml, and the detergent Nonidet P-40 (Shell) was added to 0.5%. After brief mixing, nuclei were spun down (2,000 rpm, 5 min) and resuspended in 2 ml of cold TD buffer, and the nuclei were homogenized by Dounce homogenization (30 strokes). Nuclei were spun down as before, and the nuclear extract was analyzed by using a sucrose gradient.

Sedimentation velocity analysis of SV40 NP complexes. A 5 to 20% sucrose gradient was used to

analyze the properties of SV40 complexes. In later experiments we used a 5 to 40% sucrose gradient, which gives better resolution of different forms of SV40 complexes. The buffer used is indicated in the figure legends. The conditions for centrifugation were: SW40 rotor, 37,000 rpm, 70 min, 4°C.

Glutaraldehyde fixation of NP complexes and determination of buoyant density. Fractions from a sucrose gradient of SV40 complex were fixed with glutaraldehyde according to the procedure of Baltimore and Huang (1). Fixed samples were layered onto a preformed CsCl gradient (1.2 to 1.6 g/ml in 0.01 M Tris-hydrochloride, pH 7.9, 0.01 M EDTA, 0.1% Triton X-100) and centrifuged in an SW65 rotor at 32,000 rpm for 12 h at 20°C. Fractions, 100 μl, were collected into microtiter plates. A drop of mineral oil was then added to each fraction to prevent evaporation. The density of fractions was determined by the refractive index.

Unfixed samples were analyzed in the same gradient except that a 1-ml cushion of a 1.7-g/ml CsCl solution was included. Unfixed samples were also analyzed in a 44% (wt/vol) metrizamide (Nyegaard) gradient in 50 mM Tris-hydrochloride, pH 7.4. The gradients were spun at 32,000 rpm for 60 h at 20°C in an SW65 rotor. Gradients were fractionated as in the CsCl gradient, and the density (ρ) of metrizamide solution was determined from the refractive index (n), using the formula: ρ (5°C) = 3.453 n (20°C) − 3.601.

Gel electrophoresis. Low-salt agarose gel electrophoresis for analyzing the mobility of SV40 complex was performed according to Varshavsky et al. (20). SV40 complexes were electrophoresed in a 0.4, 1.0, or 2% agarose tube gel (12 by 0.7 cm) containing 10 mM Tris, pH 7.4, at 50 V for 9 h. After electrophoresis the gels were either stained with 0.5 μg of ethidium bromide per ml or 0.2% Coomassie brilliant blue or the gels were sliced into 2-mm slices for analyzing the radioactivity. Agarose gel electrophoresis for analyzing supercoiled DNA was performed in a 2% agarose slab gel containing 40 mM Tris-30 mM NaH_2PO_4-1 mM EDTA, pH 7.8, according to the procedure of Shure and Vinograd (17). SV40 DNA or DNA fragments obtained after micrococcal nuclease digestion were analyzed in either 1.4 or 2.5% agarose under the conditions described previously (10).

Protein samples were analyzed by electrophoresis on a 14% polyacrylamide-sodium dodecyl sulfate slab gel as described by Laemmli (13).

DNA, RNA, and protein labeling, extraction, and RNA-DNA hybridization. SV40 DNA was isolated from cells infected at low multiplicity as described previously (10). The DNA was labeled with 200 μCi of [^{3}H]thymidine (New England Nuclear, 20 Ci/mmol) per plate, and the radioactivity was chased by washing the cells with fresh medium and incubating them in the medium containing 100 mM thymidine (Calbiochem) for 1 h. For double-labeling experiments, SV40 DNA was labeled with 20 μCi of [^{14}C]thymidine (New England Nuclear, 40 to 60 mCi/mmol) per plate.

To prepare pulse-labeled RNA, cells were incubated with 1 mCi of [^{3}H]uridine (New England Nuclear, >25 Ci/mmol) per ml for 1 min and immediately quenched on ice with the simultaneous addition of 10 ml of ice-cold TD buffer. The RNA was isolated from SV40 NP complexes after sucrose gradient fractionation by extraction with phenol (saturated with buffer containing 0.1 M NaCl, 0.01 M Tris, pH 7.4, 0.01 M EDTA, 0.1% sodium dodecyl sulfate) and chloroform-isoamyl alcohol (24:1). Conditions for the hybridization of labeled RNA to the SV40 DNA filter are described in reference 12.

Labeled protein was obtained from cells labeled with [^{3}H]lysine (New England Nuclear, 60 to 80 Ci/mmol), 50 μCi/ml, for 24 to 48 h postinfection. To isolate CV-1 cell histone proteins, monolayer cell cultures were washed twice with 10 ml of TD buffer, and the cells were scraped off the plates into TD buffer and collected by low-speed centrifugation. The histone protein was extracted from the cell pellet with 0.25 N H_2SO_4 at 4°C followed by further extraction with 0.4 N H_2SO_4. The two extracts were combined, 10 volumes of cold acetone was added, and the protein was precipitated at −20°C. (Calf thymus histones were a gift of G. Vidali.)

In vitro assay of RNA polymerase activities. Endogenous RNA polymerase activities associated with SV40 complexes were assayed according to the conditions of Green and Brooks (6). In vitro transcription of SV40 complexes, with *Escherichia coli* RNA polymerase (purified according to the procedure of Burgess and Jendrisak [3]), was performed in 40 mM Tris (pH 7.9), 10 mM $MgCl_2$, 150 mM KCl, 0.4 mM K_2HPO_4, 0.1 mM dithiothreitol, 0.15 mM each ATP, GTP, and CTP, and 10 μCi of [^{3}H]UTP (New England Nuclear, 35 to 50 Ci/mmol) per ml at 37°C for 30 min.

Micrococcal nuclease digestion. SV40 complexes isolated after sucrose gradient fractionation were digested with 500 U of micrococcal nuclease (Worthington) per ml for 10 min in buffer containing 25 mM Tris (pH 7.5)-50 mM KCl-10 mM NaCl-10 mM mercaptoethanol-10 mM $MgCl_2$-1 mM $CaCl_2$-0.15 mM spermine-0.5 mM spermidine. The reaction was stopped by the addition of sodium dodecyl sulfate to 0.5% and EDTA to 25 mM, and the DNA was extracted with phenol-chloroform-isoamyl alcohol (24:1) and precipitated with 2 volumes of ethanol.

Electron microscopy. A drop of solution containing SV40 complexes in TD buffer or in 0.1 M Tris (pH 7.4) was placed on a sheet of Parafilm. Immediately after the drop was placed on Parafilm, grids coated with pallodion were touched to the drop for 5 s and washed in distilled water for 5 s. Alternatively, a drop of solution containing complexes was placed on a grid for 5 s, and the excess liquid was removed with filter paper. Grids were shadowed with platinum-palladium (80:20) with or without prior staining in 5×10^{-5} M uranyl acetate in 90% ethanol. The method for spreading of SV40 DNA in 50% formamide or the SV40 complexes in 50 to 70% formamide has been described (11).

RESULTS

Isolation of SV40 DNA-protein complexes. Previously, the isolation of intracellular SV40 NP complexes has been achieved by using Triton extraction method (7). This extraction method yields a single SV40 DNA protein complex sedimenting at about 60-70S in the sucrose

gradient (Fig. 1). However, repeated efforts with this method to recover mature virions from cells late after infection failed, even when a large amount of virion could be isolated from similar cultures after CsCl gradient fractionation of extracts obtained from cells lysed by repeated freeze-thawing. The absence of any mature virion in the Triton extract strongly suggests that the intracellular forms of SV40 NP complexes are disrupted during isolation. Further investigation showed that 40 to 50% of [^{3}H]thymidine-labeled virions purified on a CsCl gradient are converted into a species sedimenting at 60S when coextracted with unlabeled SV40-infected cells by the Triton method. The disruption is dependent on the presence of Triton-treated cellular material because no disruption was observed when purified virus alone was extracted with Triton. These observations indicate that the 60-70S complex extracted by the Triton method could be derived from many different forms of NP complexes, including mature virions. To avoid the disruption of intracellular NP complexes, a new isolation procedure was developed. Briefly, SV40-infected cells in hypotonic buffer or nuclei in isotonic buffer obtained by Nonidet P-40 lysis of SV40-infected cells were homogenized in a tightly fitting Dounce homogenizer (see Materials and Methods). SV40 complexes selectively leaked out from the nuclei and could be separated from cellular chromatin by low-speed centrifugation. SV40 complexes extracted by this new procedure exhibit very different profiles in the sucrose gradient from those obtained by the Triton method (Fig. 1 and 2). When analyzed in a 5 to 20% sucrose gradient, major components were observed sedimenting at 70S (NP-I) and 180-200S (Fig. 1). The heavier species can be further resolved into two components when the complexes are pulse-labeled and analyzed in 5 to 40% sucrose gradients (Fig. 2). The material sedimenting at about 200S is mostly SV40 virion as defined by the criteria of electron microscopic morphology and CsCl gradient banding (virion bands at a density of 1.35 g/ml). The heterogeneous material sedimenting between NP-I and SV40 virions (V) was operationally designated NP-II.

The possibility that SV40 complexes obtained by the new procedure are due to artifacts generated during the isolation procedure was examined in a series of control experiments. ^{3}H-labeled NP-I, NP-II, or virions were added to unlabeled SV40-infected cells and extracted by using the new procedure. If NP-I or NP-II was derived from virions during homogenization, then coextraction of labeled virion with unlabeled infected cells should yield labeled material sedimenting at NP-I and NP-II positions. Such results were not obtained in two trials. Similarly, NP-II could be derived from NP-I by aggregation with itself or with other cellular components during extraction. Coextraction of labeled NP-I with unlabeled infected cells demonstrated that

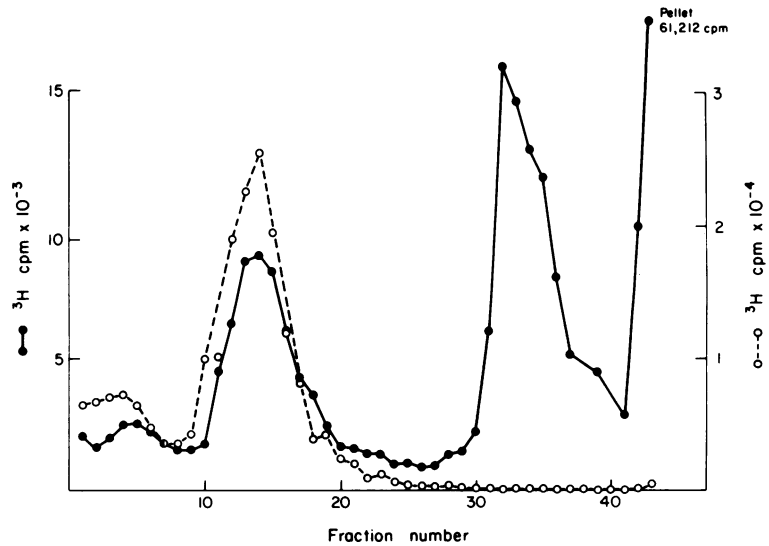

FIG. 1. *Sucrose gradient analysis of SV40 chromatin isolated by the Triton extraction method* (○) *and by the new method as described in the text* (●). *Cells infected with SV40 were labeled with 200 μCi of [^{3}H]-thymidine per 100-mm dish of cells from 36 to 48 h postinfection. SV40 chromatin was extracted and analyzed in a 5 to 20% sucrose gradient containing 50 mM Tris, pH 7.9, in an SW40 rotor (37,000 rpm, 60 min, 4°C).*

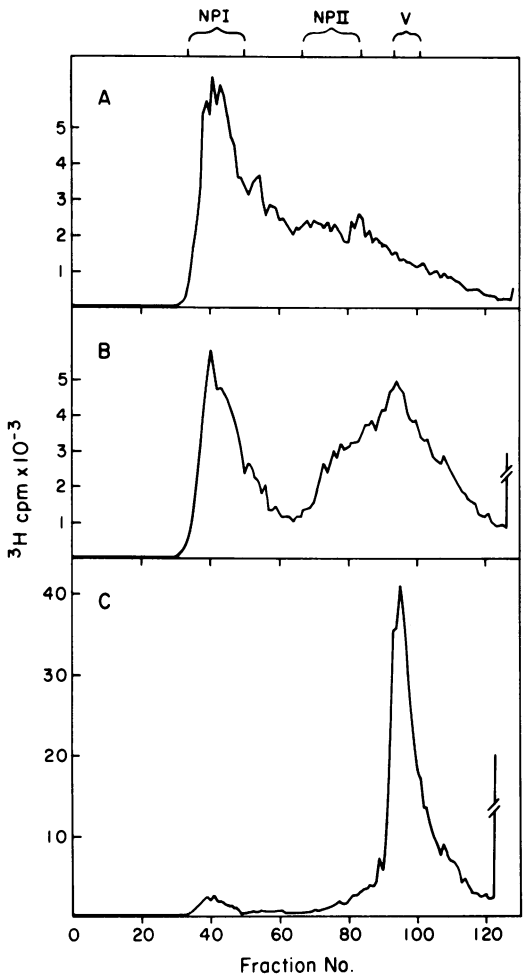

FIG. 2. *Five to 40% sucrose gradient analysis of SV40 DNA protein complexes extracted by the new method (see text). SV40-infected cells were pulse-labeled with [^{3}H]thymidine for (a) 30 min, (b) 90 min, or (c) 24 h, with the end point of labeling at 48 h postinfection. SV40 complexes were extracted from infected cells by Dounce homogenization in hypotonic solution and analyzed in a 5 to 40% sucrose gradient containing 2 mM Tris, pH 7.4, in an SW40 rotor (37,000 rpm, 70 min, 4°C). Gradients were fractionated from the top into 100-μl fractions. Similar profiles were obtained when the buffer in the sucrose gradient was isotonic TD buffer. In TD buffer NP-I sediments slightly faster, whereas virions sediment slightly slower.*

this is not the case. Similar arguments indicate that NP-I could not be derived from NP-II during extraction. These experiments demonstrate that the isolated NP complexes retain their characteristic sedimentation properties upon reextraction and are not artifacts of the extraction procedure; therefore, they probably represent genuine intracellular forms of SV40 NPs.

The efficiency of extracting SV40 complexes by the new procedure was estimated as follows. SV40-infected cells were labeled for 24 h (24 to 48 h postinfection) with [^{3}H]thymidine. The radioactive label present in the three classes of SV40 complexes was compared with the label present in the Hirt extract of the same nuclei remaining after prior extraction of complexes. The ratio of label present in the isolated complexes to that in the Hirt extraction is 1.1×10^6 cpm/1.0×10^6 cpm or about 1, indicating that the extraction efficiency is about 50%. Analysis of the material present in the two extracts showed that more than 98% of the material present in the three classes of complexes is SV40 DNA, whereas, at most, 50% of the material in the Hirt extract is SV40 circles. This suggests that the efficiency of extraction must be at least 50% and perhaps as much as 70%. In another experiment, nuclei collected after Dounce homogenization were reextracted in Triton extraction buffer, and the extract was analyzed on a sucrose gradient. No 70S complex was observed in the sucrose gradient, suggesting that extraction with the new procedure is at least as efficient as Triton extraction.

The extraction of [^{3}H]thymidine-labeled NP complexes from total cells and from nuclei prepared from Nonidet P-40-lysed cells yields the same profile in the sucrose gradient. However, in the whole-cell extract there is extensive contamination of NP-I by cytoplasmic material, especially ribosomes. These contaminations were mostly eliminated by extracting SV40 complexes from the isolated nuclei obtained by lysing infected cells with Nonidet P-40. Figure 3 shows the sucrose gradient profile of a nuclear extract labeled with [^{3}H]thymidine and [^{3}H]lysine from 24 to 48 h postinfection and with ^{32}P from 36 to 48 h postinfection. Host protein contamination was greatly reduced (Fig. 3). Complexes obtained from nuclear extract were therefore used for analyzing its protein compositions.

Properties of SV40 NP complexes. The physical as well as biochemical properties of SV40 complexes are summarized in Table 1. They are discussed below.

(i) Physical properties. The following physical properties of SV40 complexes isolated in sucrose gradient were analyzed.

(a) Morphology of SV40 complexes was analyzed by electron microscopy. Different fractions in the sucrose gradient containing isotonic buffer corresponding to NP-I, NP-II, and V were diluted in either isotonic TD buffer or 0.1 M Tris, pH 7.4, and deposited on the electron microscope grids as described in Materials and Methods. When samples were prepared in TD buffer and extensive surface tension during sample

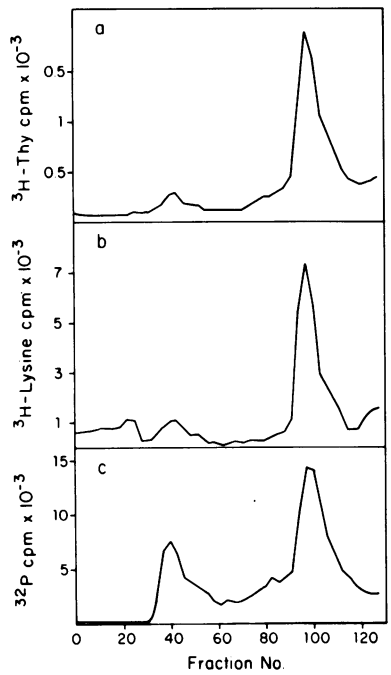

FIG. 3. *Profile of SV40 complexes in a 5 to 40% sucrose gradient labeled with (a) [^{3}H]thymidine and (b) [^{3}H]lysine from 24 to 48 h postinfection and (c) with ^{32}P from 36 to 48 h postinfection. SV40 complexes were extracted from the nuclei of infected cells as described in the text. Compare the profile of 12-h ^{32}P labeling with that of 12-h [^{3}H]thymidine labeling, as shown in Fig. 13e.*

preparation was avoided, NP-I was seen as a "beaded" nucleosomal structure (Fig. 4B). In the same method of sample preparation, NP-II appeared as a more condensed structure (Fig. 4C). When the samples were prepared in 0.1 M Tris (pH 7.4), NP-I was reproducibly observed to consist of thin DNA-protein fiber about 80 Å thick. Most of the NP-I complex spread under this condition showed a supercoiled structure (Fig. 4A, dashed arrows) similar to that of the free SV40 DNA. Occasionally relaxed open circles can be observed (Fig. 4A, solid arrow). The contour length of these open circles is about 1.5 μm, similar to that of free DNA. In contrast, NP-II, when spread under the same conditions, showed a reproducibly more compact structure (Fig. 4D). Occasionally, thin fiber similar to that observed in NP-I samples could be seen as part of the NP-II molecule. These observations demonstrate that NP-I and NP-II have a different conformation, which can be easily differentiated in the electron microscope.

(b) Buoyant density of SV40 complexes was analyzed in CsCl gradients after glutaraldehyde fixation or in a metrizamide gradient without fixation. NP-I has a density of 1.45 g/ml in the CsCl gradient, whereas NP-II and the virion have densities of 1.35 g/ml (Fig. 5). Although fixed NP-II and the virion have the same density in CsCl, unfixed NP-II is disrupted into free protein and DNA components in a CsCl gradient whereas the mature virion is not. In metrizamide gradient analysis, NP-I and NP-II have the same density, 1.18 to 1.20 g/ml, whereas the mature virion has a density of 1.26 g/ml (Fig. 5). The second component of density 1.2 g/ml in Fig. 5C is due to the presence of NP-II material in a virus peak in the sucrose gradient. The presence of NP-II material in the virus peak is also observed by electron microscopy as described above and by analyzing unfixed material in the virus peak in the CsCl gradient.

(c) Electrophoresis of SV40 complexes in a 1.0% agarose gel shows that NP-I, NP-II, and virion have different mobilities (Fig. 6). NP-I and NP-II have broad distribution in the gel. However, the ratio of [^{3}H]lysine to [^{14}C]thymidine label remains approximately constant throughout the band, indicating that the breadth of the band is not the result of dissociation of proteins during electrophoresis, but rather due to the inherent heterogeneity of the samples.

(ii) Biochemical characteristics. The DNA of SV40 NP complexes is mainly supercoiled DNA (≥90%), as determined by electron microscopy and agarose gel electrophoresis. The number of superhelical turns in the DNA of all three complexes appears to be the same as when analyzed by agarose gel electrophoresis (14). The DNA is arranged in a nucleosome structure in

TABLE 1. *Physical and biochemical properties of SV40 complexes*

Property	NP-I	NP-II	Virion
Sedimentation coefficient	70S	180S	210S
Density (CsCl; μg/ml)	1.45	1.35	1.35
Density (metrizamide; μg/ml)	1.18	1.18	1.26
Morphology (electron microscopy)	Open	Condensed	Virion
Replication activity in vivo	Yes	No	No
Transcription activity in vivo	Yes	No	No
Replication activity in vitro	Yes	No	No
Transcription activity in vitro	Yes	Variable	No
Supercoil density (DNA)	Same	Same	Same
Micrococcal nuclease digestion	Nucleosome	Nucleosome	
Histone H1	Yes	Yes	No

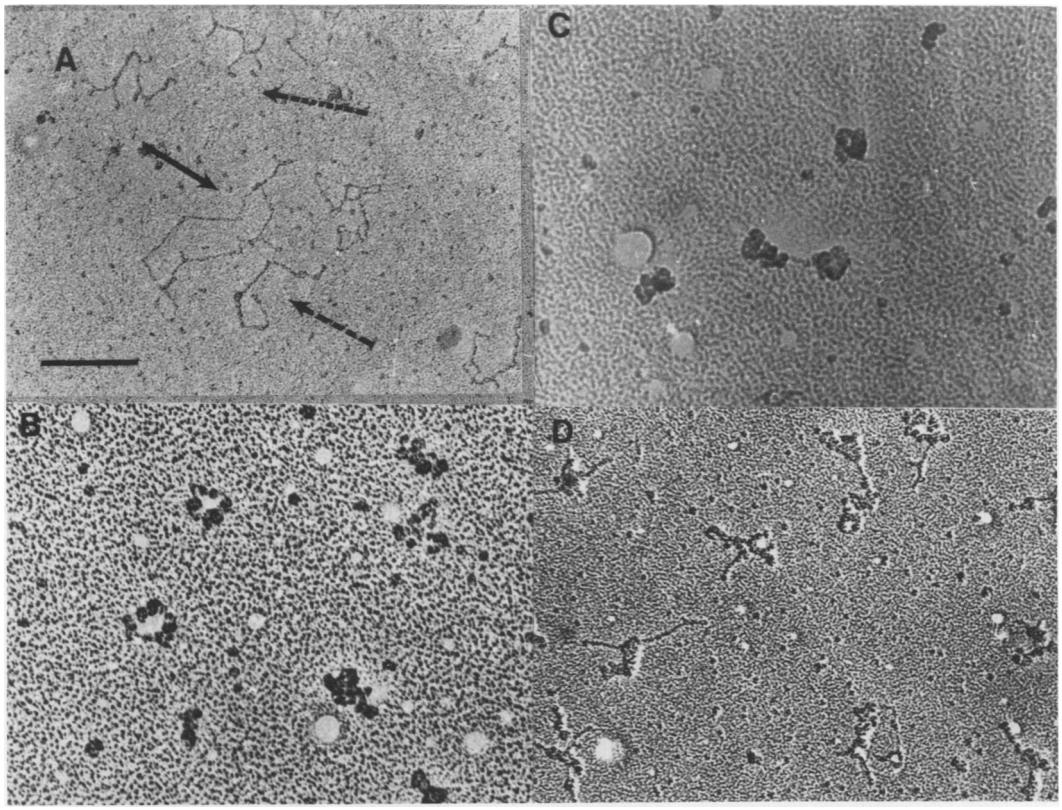

FIG. 4. *Electron micrographs of SV40 NP complexes. SV40 NP complexes obtained from a sucrose gradient in TD buffer were diluted into TD buffer (B, C) or 0.1 M Tris, pH 7.4 (A, D). Pallodion-coated grids were touched to a drop of complex solution for 5 s. After excess solution was removed, the grids were shadowed with platinum-palladium (80:20). (A, B) NP-I; (C, D) NP-II.*

NP-I and NP-II as manifested by micrococcus nuclease digestion (Fig. 7). A resistant core of about 150 nucleotides in length was observed. This result is similar to that obtained for polyoma virus by Ponder et al. (16).

(a) Preliminary analysis of the protein components of SV40 complexes is given in Fig. 8. NP-I is composed mainly of the five groups of histones and material migrating in the same position as VP-1 protein of SV40 virus in a sodium dodecyl sulfate-polyacrylamide gel. The major proteins in NP-II, however, are the viral capsid proteins VP-1, VP-2, and VP-3. Histone proteins are also present but in lower amounts. Histones, except for H1, are present in the mature virion. The presence of H1 in NP-I and NP-II but not in the mature virion has also been confirmed by the acid-urea gel electrophoresis technique (15; M. Coca-Prados, G. Vidali, and M.-T. Hsu, manuscript in preparation). Further characterization of the protein components of the SV40 NP complexes and their modification will be reported elsewhere.

(b) Endogenous RNA and DNA polymerase activities associated with SV40 complexes were analyzed by in vivo and in vitro incorporation of RNA or DNA precursors. To analyze the in vivo transcription activities, SV40-infected cells were pulse-labeled with [^{3}H]uridine for 1 min at 37°C. (A separate biochemical analysis of 1-min pulse-labeled RNA showed that they represent growing RNA chains [5].) SV40 complexes were then extracted from total cells and separated in a 5 to 20% sucrose gradient. Labeled RNAs were extracted from each fraction of the gradient and assayed for SV40-specific RNA by hybridizing to an SV40 DNA filter. Figure 9 shows that 1-min labeled SV40 RNA is associated with NP-I and not with NP-II or virion. The endogenous RNA polymerase activities were also assayed in vitro for the incorporation of [^{3}H]UTP or α-[^{32}P]UTP into acid-precipitable material. NP-I can be reproducibly found to be active in transcription (Fig. 10). NP-II, on the other hand, gave low but variable activities. At present we do not know the cause of such variability in the activity of NP-II. The specificity of the in vitro transcription of NP-I was assayed by hybridizing

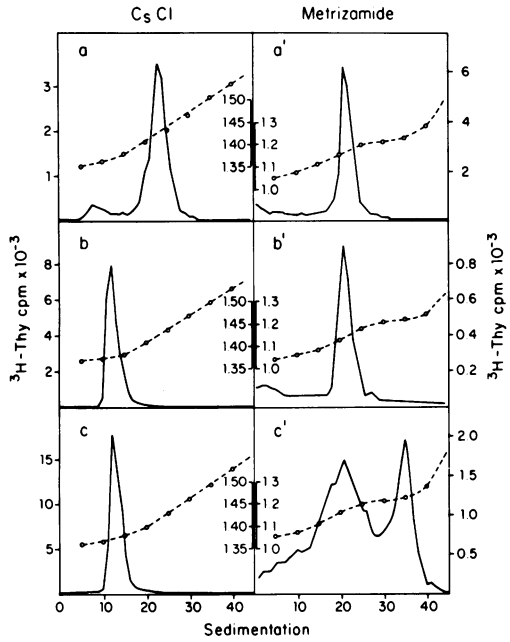

FIG. 5. *Analysis of density of SV40 complexes. SV40 complexes were labeled with [^{3}H]thymidine from 24 to 48 h postinfection, as in Fig. 2c. Fractions corresponding to complexes I and II and virion (see Fig. 2c) were analyzed in a 44% (wt/vol) metrizamide gradient in 50 mM Tris, pH 7.4 (a', b', c') or in a preformed CsCl gradient after fixation of SV40 complexes with glutaraldehyde (a, b, c). Solid curves represent radioactivity. Dashed curves represent densities. (a, a') NP-I; (b, b') NP-II; (c, c') virion.*

labeled product to SV40 DNA filters. About 50% of the label became hybridized to SV40 DNA filters after 24 h of incubation at 65°C and treatment of the filters extensively with RNases. Under the same conditions, labeled complementary RNA transcribed from SV40 supercoiled DNA by *Escherichia coli* polymerase hybridized to the extent of 60%. Thus, most, if not all, of the RNA transcribed in vitro represents viral sequences. Since almost no host DNA was observed in the DNA extracted from NP-I complex when analyzed in the electron microscope, we believe that the lower efficiency of hybridization of RNA transcribed from NP-I as compared to complementary RNA is probably not due to transcription of host DNA. However, the nature of in vitro transcripts as to its sizes and locations on the SV40 genome has not been characterized.

SV40 transcription intermediates in SV40-infected cells could be observed in the electron microscope. When nuclei of SV40-infected cells were added to a solution containing either 70% (Fig. 11A, B, and C) or 50% (Fig. 11D) formamide and immediately spread onto a hypophase containing 40 or 20% formamide, elongating RNA could be seen associated with SV40 DNA. The frequency of observing such structures is about 1% of the total SV40 DNA scored on the grid. This frequency remained approximately the same even when the sample was diluted to the extent that no more than two SV40 DNAs could be seen in the same grid area in a 300-mesh grid. This suggests that the structures observed are unlikely to be the result of the superimposition of free RNA and DNA on the grid. Treatment of nuclei with RNase before spreading eliminated these SV40 DNA-associated chains, demonstrating that these chains are RNA in nature. The extended RNA chain in 70% formamide spreading (Fig. 11A, B, and C) could be seen to collapse into secondary structures in 50% formamide (Fig. 11D, arrow), a typical behavior of single-stranded RNA. An RNA chain almost as long as the SV40 genome length could be observed (Fig. 11B). Rarely, replicating molecules with RNA chain could also be observed (Fig. 11C).

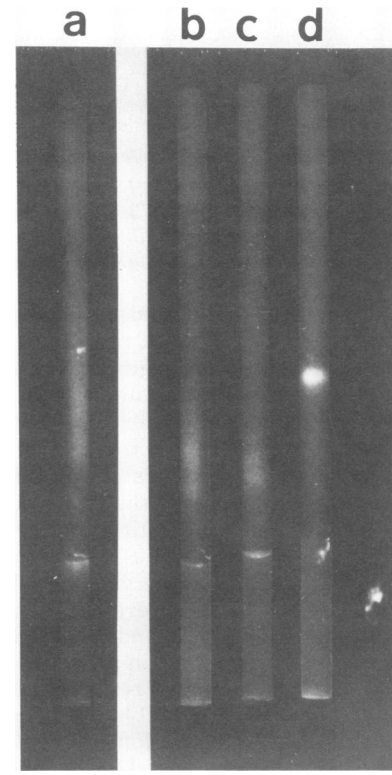

FIG. 6. *Low-salt agarose gel electrophoresis of SV40 complexes. SV40 complexes corresponding to fractions 42 (a), 61 (b), 78 (c), and 96 (d) in Fig. 2c were electrophoresed in 0.4% agarose in 10 mM Tris (pH 7.4) at 50 V for 9 h. The gel is stained with 0.5 μg of ethidium bromide per ml.*

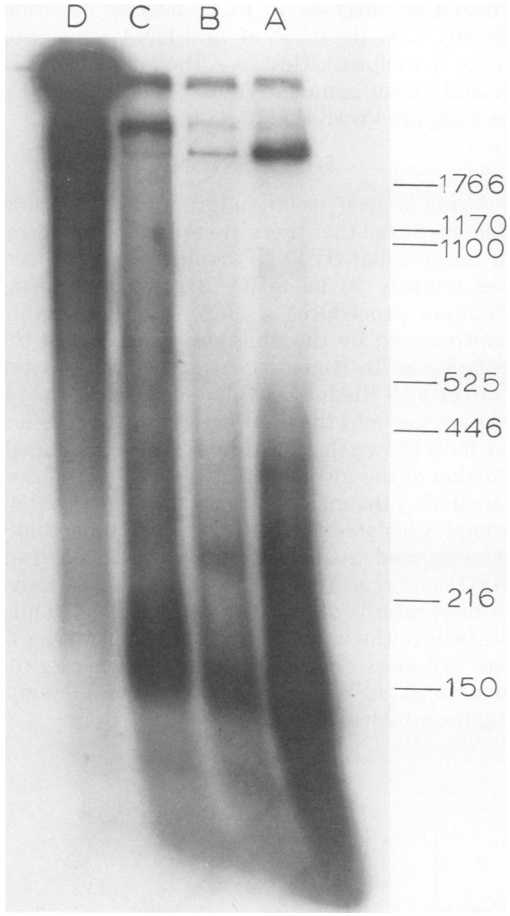

FIG. 7. *Micrococcal nuclease digestion of SV40 complexes. ^{32}P-labeled SV40 complexes corresponding to those in Fig. 6 (approximately 1 to 5 μg) were digested with 500 U of micrococcal nuclease per ml for 10 min at 37°C. The reaction was stopped by adding EDTA to 0.1 M and sodium dodecyl sulfate to 0.5%. DNA was extracted by phenol and chloroform and analyzed in a 2.5% agarose gel. (A) Complex I; (B, C) complex II; (D) virion.*

DNA-synthesizing activities of SV40 complexes were also analyzed. Pulse-labeling in vivo for 10 min with [^{3}H]thymidine demonstrated that the majority of DNA-synthesizing activity is associated with NP-I (Fig. 12). A similar conclusion was obtained by assaying in vitro incorporation of [^{3}H]TTP (data not shown).

Relationship among SV40 NP complexes. To determine whether there might be a precursor-product relationship among the three forms of SV40 complexes, we performed a pulse-chase experiment (see Fig. 13). As described previously, short pulses with [^{3}H]thymidine only label NP-I. A chase for 1 h with 100 mM unlabeled thymidine resulted in the appearance of label in the NP-II region. This result suggests that NP-II is derived from NP-I. Since NP-II and virions are not very well separated in the 5 to 20% sucrose gradient, we further characterized the complex in the NP-II-virion region by a CsCl gradient. Mature virions band at a density of 1.34 g/ml, whereas SV40 complexes are dissociated into DNA and protein components, which band at densities of 1.7 and 1.2 g/ml, respectively. Such an analysis was carried out using SV40 complexes pulse-labeled with [^{3}H]thymidine for different lengths of time, with the end point of labeling always at 48 h postinfection. The complexes extracted were first separated in

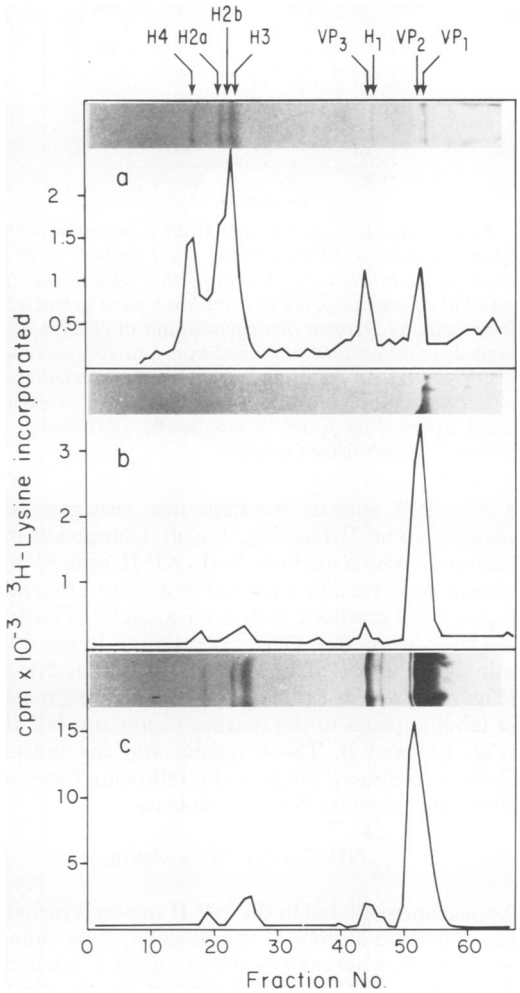

FIG. 8. *Sodium dodecyl sulfate-polyacrylamide gel (14%) electrophoresis analysis of proteins in SV40 chromatin. (a) Complex I; (b) complex II; (c) virion. Proteins were labeled with [^{3}H]lysine from 24 to 48 h postinfection. Gel was stained with 0.2% Coomassie brilliant blue (inserts), sliced into 1-mm slices, and counted (solid curves).*

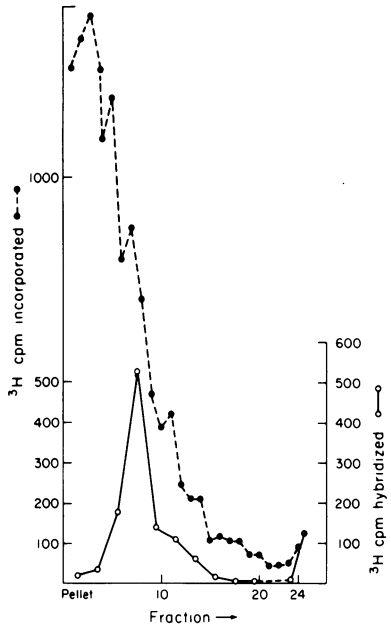

FIG. 9. *Analysis of nascent RNA associated with SV40 complexes. SV40-infected CV-1 cells (5 × 10^7) were pulse-labeled for 1 min with [^{3}H]uridine (1 mCi/ml of medium). SV40 complexes were extracted from cells by Dounce homogenization of cells in hypotonic solution and analyzed in a 5 to 20% sucrose gradient. RNAs from each fraction were extracted with phenol and chloroform and hybridized to SV40 DNA filters (1 μg/filter). Symbols: (●) total incorporation; (○) hybridized counts.*

a 5 to 20% sucrose gradient into two general regions, I and II (see Fig. 13a–e). Complexes in region II which include both NP-II and SV40 virions were further analyzed in a 1.2 to 1.6-g/ml linear CsCl gradient with a 1.7-g/ml CsCl cushion to trap the free DNA (Fig. 13f–g). In the 30-min label, most of the label appears in NP-I (Fig. 13a), whereas during the 12-h labeling most of label appears in the mature virion and NP-II (Fig. 13e and j). These results and the pulse-chase experiment suggest the following biosynthetic pathway for SV40 complexes:

$$\text{NP-I} \rightarrow \text{NP-II} \rightarrow \text{virion}.$$

Appearance of label in the NP-II region is rather rapid (within 30 min of the pulse-labeling time, see Fig. 2), whereas the SV40 virion is labeled only after 1 h of pulse-labeling. Since the time required for one round of SV40 DNA replication is about 10 to 15 min, this result implies that shortly after the completion of DNA replication NP-I is converted into NP-II.

Additional experiments have shown that a similar kinetic relationship could be demonstrated as early as 16 to 17 h after infection, shortly after the onset of viral DNA replication (data not shown). However, the amount of NP-II and virion is much reduced relative to NP-I as compared with that at 48 h postinfection.

DISCUSSION

Using a new isolation procedure, we have demonstrated that there are at least three forms of intracellular SV40 NP complexes. That they are unlikely to be artifacts generated during isolation procedures is shown by the various controls and by the differential labeling of the complexes. In contrast, extraction with Triton converts all the intracellular complexes, including virions, into the 60S-70S form (NP-I). Since we have shown that NP-I represents only a small portion of the intracellular SV40 NP complexes late during the infection cycle, the 60S-70S SV40 complex isolated by the Triton method actually is composed mainly of material derived from NP-II and mature virions, which are not active in biosynthesis of SV40 RNA and DNA. Thus, we believe the isolation procedure is superior to the Triton extraction method in preserving the structures of SV40 NP complexes and for studying their biological functions.

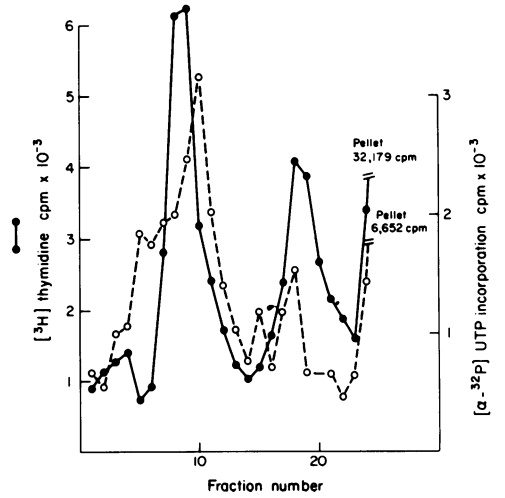

FIG. 10. *In vitro incorporation of α-[^{32}P]UTP into acid-precipitable RNA by SV40 complexes. SV40-infected cells were labeled with [^{3}H]thymidine from 44 to 48 h after infection. SV40 complexes extracted from total cells by Dounce homogenization of cells in hypotonic buffer were separated in a 5 to 20% sucrose gradient containing 50 mM Tris (pH 7.9), 50 mM KCl, and 0.5 mM dithiothreitol. Fractions collected from the sucrose gradient were assayed for the activity of incorporation of α-[^{32}P]UTP into acid-precipitable RNA. Symbols: (○) α-[^{32}P]UTP incorporation; (●) [^{3}H]thymidine incorporation. Pellet represents contaminating nuclei.*

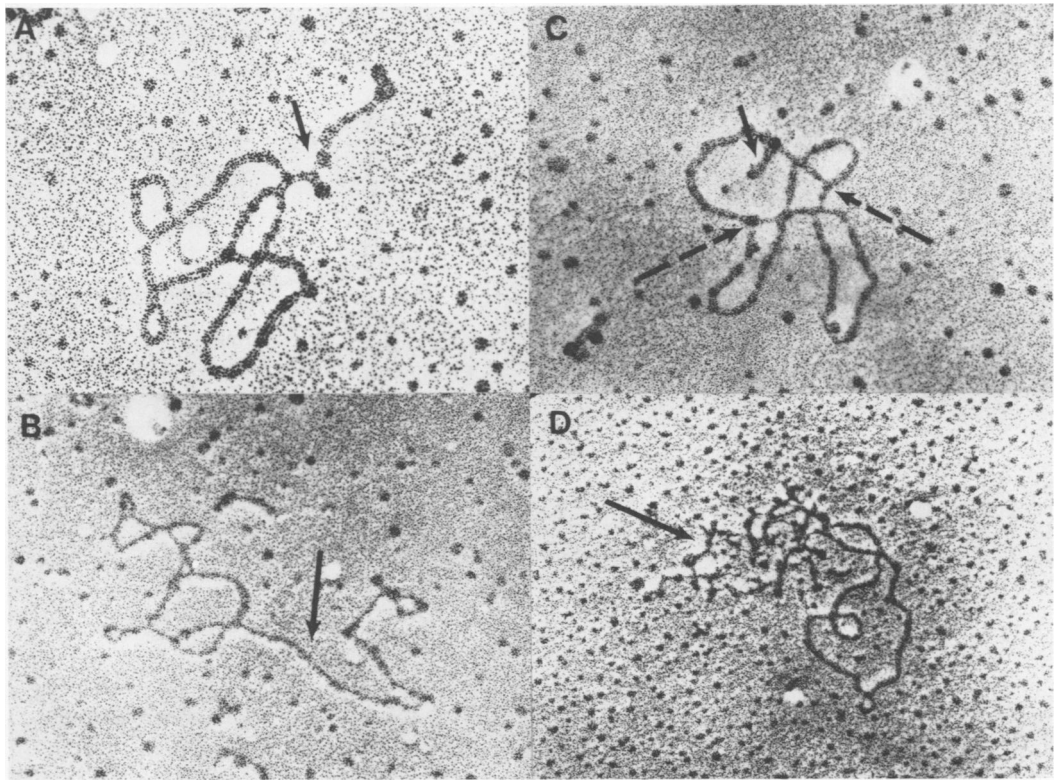

FIG. 11. *Electron micrographs of SV40 transcription complexes. Nuclei from SV40-infected cells were spread in 70% formamide (A, B, C) or 50% formamide (D) in 0.1 M Tris (pH 9.5)-0.01 M EDTA. Solid arrows indicate the nascent RNA chains. In 70% formamide these RNA chains are in extended conformation, whereas in 50% formamide the chains collapse into "bushes" due to secondary structures (D). At the junction between DNA template and RNA chain, a dark sphere is always present. It may represent RNA polymerase. (C) is a replicating molecule. The two replicating forks are indicated by broken arrows.*

The three forms of SV40 NP complexes differ in both physical and biochemical properties. NP-I is active in the synthesis of SV40 DNA and RNA. The DNA in NP-I is more sensitive to DNase I digestion than NP-II (E. Derman and M.-T. Hsu, unpublished observations). Analysis of both its physical and biochemical properties suggests that NP-I is the active chromatin of SV40. Because it can be isolated uncontaminated by the other forms of SV40 NP complexes and by the cellular chromatin and because of its simple genetic complexity, we believe that NP-I represents an excellent model system for studying the structures and functions of active chromatin in eucaryotic cells.

In contrast to reports by other workers, SV40 NP-I complex was observed as a uniform 8-mm DNA-protein fiber when spread in 0.1 M Tris, pH 7.4. This structure may be derived from the nucleosomal structure by unfolding the nucleosomes as a result of charge repulsion between DNA-protein fibers in a low-salt environment. Variability of chromatin conformation observed in the electron microscope as a result of the difference in methods and buffers used for preparing samples is well known (17). In our experience, not only are salt concentration and pH important factors in spreading SV40 complexes, but surface tension of the sample is a major factor contributing to the variability of structure observed in the electron microscope. This variability notwithstanding, the SV40 NP-I and NP-II complexes, when spread under identical conditions, showed reproducibly recognizable differences in morphology under the electron microscope.

The major protein components present in complex I are composed of histones and a protein that comigrates with VP1. At present we do not know the function of VP1 in NP-I. The possibility that it is aggregated with NP-I during isolation cannot be excluded. The presence of the histone H1 fraction in SV40 NP complex extracted by the Triton method has been reported by Varshavsky et al. (20) but not by others (2, 14). Our results show that H1 is pres-

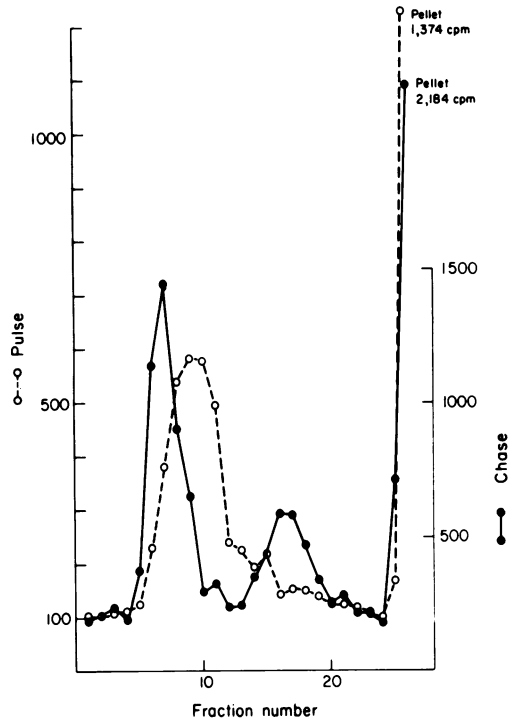

FIG. 12. *Pulse-chase of SV40 complexes with [^{3}H]thymidine. A total of 10^7 SV40-infected cells were pulse-labeled with [^{3}H]thymidine (1 mCi/5 ml of medium) for 10 min (○) and chased with 100 mM thymidine for 1 h (●). SV40 complexes in total cell extract were analyzed in a 5 to 20% sucrose gradient containing 50 mM Tris, pH 7.9. Pellet represents contaminating cell nuclei as analyzed with the electron microscope.*

ent in NP-I and NP-II but not in virion. This implies that during SV40 infection H1 first becomes associated with the uncoated virus and is later removed when SV40 virus is assembled. Further analysis is necessary to understand the functional role of H1 in the virus infection process.

The histones present in NP-I complex are highly modified by phosphorylation and acetylation. In addition, highly phosphorylated and acetylated non-histone proteins were also observed (Coca-Prados et al., manuscript in preparation).

The role of NP-II in the SV40 infection cycle is still unknown. It is derived from NP-I within 20 min after completion of DNA replication of NP-I. At present we do not know what is the control process that determines the conversion of NP-I to NP-II with the concomitant change of conformation and inactivation of the biosynthetic activities of SV40 chromatin. Perhaps it is triggered by the addition of viral coat proteins

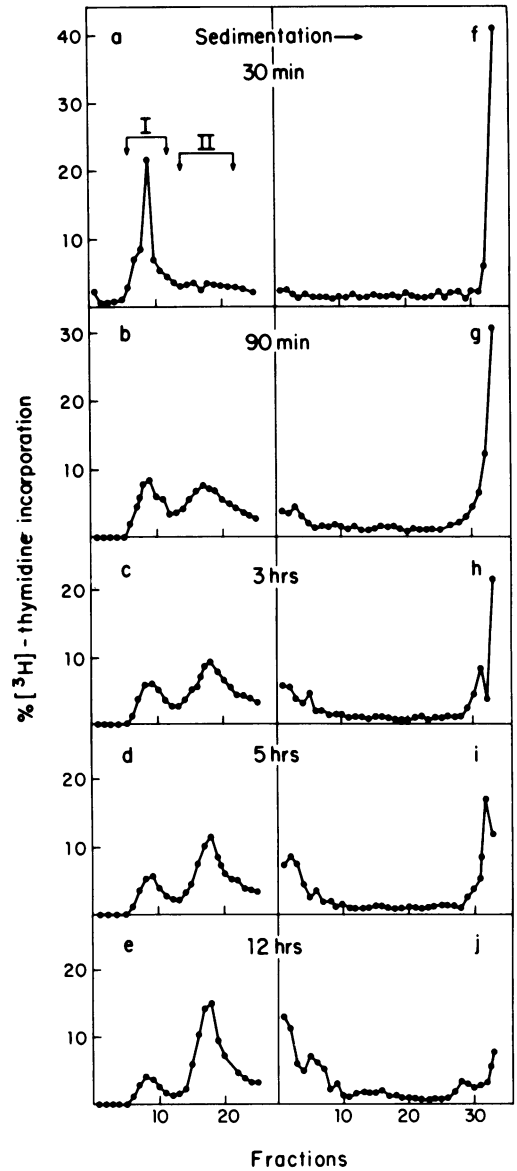

FIG. 13. *Labeling kinetics of SV40 DNA present in SV40 complexes. SV40-infected cells were pulse-labeled with [^{3}H]thymidine for 30 min (a, f), 90 min (b, g), 3 h (c, h), 5 h (d, i), or 12 h (e, j). The end point of labeling periods was always at 48 h after infection. SV40 complexes obtained in total cell extracts were analyzed first in a 5 to 20% sucrose gradient containing 50 mM Tris, pH 7.4. Portions of the gradient labeled "II" were pooled and layered on a 1.42-g/ml CsCl gradient with a 1-ml cushion of 1.7 g of CsCl per ml. The gradient was spun at 33,000 rpm for 20 h in a type 65 rotor. Sucrose gradients (a–c); CsCl gradient (f–j). Sedimentation is from left to right. In the CsCl gradient profile, the virion bands at the top of the gradient (f–j, left), whereas free DNA molecules sediment to the bottom of the gradient (f–j, right).*

(e.g., VP3 which replaces H1 histones) which are present in large quantity in NP-II. These added proteins must be in a relatively loose structure since NP-II has the same density as NP-I in a metrizamide gradient and its DNA is accessible to micrococcus nuclease digestion. Study of the mechanism of NP-I to NP-II conversion should yield information as to how the gene activity of chromatin is regulated at the level of the structure of chromatin.

The buoyant density of NP-II is similar to that of NP-I chromatin in the metrizamide gradient whereas in the CsCl gradient it is similar to that of SV40 virions. The protein-to-DNA ratio of NP-II calculated from its protein composition predicts that its buoyant density should be similar to that of SV40 virions. The lower than expected density of NP-II in a nonionic metrizamide gradient is probably due to extensive hydration of the loosely structured NP-II complex. SV40 chromatin in NP-II is as accessible to micrococcal nuclease digestion as the NP-I complex, suggesting that the capsid proteins in NP-II are probably only loosely attached to SV40 chromatin. The "porous" structure of NP-II allows water molecules to freely penetrate inside the complex, whereas the interior of the mature virion is less accessible. This may explain why NP-II bands at a lower density than the virion even though they have a similar protein-to-DNA ratios.

The population of NP-II is rather heterogeneous. It probably contains different intermediates between the active chromatin (NP-I) and the assembled virus. Further analysis of NP-II, using the electron microscope technique to study the process of virus assembly, will be reported (manuscript in preparation).

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