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Late Modifications of Simian Virus 40 Chromatin During the Lytic Cycle Occur in an Immature Form of Virion

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Two main modifications of the simian virus 40 chromatin were found to occur during the lytic cycle. One was the progressive increase in the acetylation level in the four non-H1 histones as the 75S deoxynucleoprotein complexes (minichromosomes) became assembled into heavier structures. The other was the final elimination from viral chromatin of histone H1. An important stage in the course of these changes was represented by an intracellular simian virus 40 particle, in which the virus-coded proteins were already assembled, but properties distinct from those of mature virions were still present. This particle resembled the mature virions in morphology, sedimentation rate, and buoyant density. It was distinguished by the instability, the presence of histone H1, the uptake of radioactive acetate, and the lower infectivity. Its significance appears to be that of an immature virion on the basis of these characters and of the consistent kinetic behavior during the lytic cycle.

The histones contained as internal proteins in simian virus 40 (SV40) and polyoma virions are considerably more acetylated than those associated with the chromatin of uninfected host cells (23). In a previous study (16) we found that during the lytic cycle of SV40, the level of histone acetylation in the host chromatin does not rise significantly above that of the uninfected cells and that the intracellular viral chromatin of recent synthesis (the native 75S deoxynucleoproteins complexes, often referred to as minichromosomes) contains histones which are somewhat more acetylated than the host histones but definitely less acetylated than the virion histones. These results indicated that the 75S minichromosomes could not be the immediate precursor of the mature virion, for lack of the proper degree of histone acetylation. In the same study it was also found that the acetyl turnover of minichromosome histones differed from that of cell histones because of a lower rate of acetate removal, as though the viral chromatin were partially shielded from deacetylase action. It was thus suggested that the final high level of histone acetylation typical of virion chromatin might be attained through intermediate complexes (between the 75S minichromosomes and mature virions), still allowing the transfer of acetyl groups to histones and thus producing a gradual net acetylation increase.

The present work shows the presence, within permissive cells infected with SV40, of a population of fully assembled particles with properties partly similar to, and partly different from, those of mature virions. The sedimentation rate, the density in CsCl gradients, and the general morphology are not distinguishable from those of mature virions. The histone pattern which includes H1, the stability in certain saline environments, the infectivity, and the capacity to incorporate acetate into histones are distinct. These particles predominate before cell lysis begins, but at the end of the lytic cycle, only the stabler virion forms are detected, with little loss of viral DNA. The particle characters and the consistent [^{3}H]thymidine labeling patterns indicate that they represent immature forms of SV40 virions. In them, the viral chromatin completes the acetylation of histones.

MATERIALS AND METHODS

Cell media and chemicals. The synthetic media and serum for cell growth were from Eurobio, Paris, France; reagents for the preparation of polyacrylamide gels were from Serva, Heidelberg, West Germany; radioisotopes were from New England Nuclear Corp., Munich, West Germany.

Cell culture and virus. CV-1 cells were grown in 10-cm plastic dishes in Dulbecco medium supplemented with nonessential amino acids, 10% tryptose phosphate broth, and 10% calf serum. Confluent cultures were infected with a large-plaque clone (18) of SV40 at a multiplicity of 5 to 10. When infected cells were allowed to lyse completely (5 days after infection), the virus was recovered from the 10,000 × g supernatant of the lysate by centrifugation at 33,000 rpm for 3 h in a Spinco 35 rotor. Viral pellets were suspended in 100 mM NaCl-10 mM Tris and centrifuged either in sucrose gradients similar to those used for cell extracts (see below) or in CsCl gradients (6).

Cell fractionation and isolation of SV40 complexes. Cells collected by scraping were washed in Earle saline and swollen for 5 min in a hypotonic buffer (10 mM Tris [pH 7.4], 10 mM NaCl, 0.1 mM

EDTA, 0.3 mg of phenylmethylsulfonyl fluoride per ml). Cells were then disrupted by 25 quick strokes of a tight-fitting pestle in a Dounce homogenizer and centrifuged at 10,000 × *g* for 5 min in a Sorvall refrigerated centrifuge. The supernatant was made 1 mM in EDTA immediately before layering onto a 15 to 30% sucrose gradient in 10 mM Tris (pH 7.4)–100 mM NaCl and then centrifuged for 110 min at 40,000 rpm in a Spinco SW41 rotor. Fractions were collected through an automatic-recording Gilford spectrophotometer. The following are critical details concerning this extraction procedure. (i) The exposure to EDTA above certain limits of time and concentration disrupts some viral complexes (10; this paper). On the other hand, the careful use of EDTA, particularly when dealing with concentrated samples, lowers the risk of unspecific associations and dissociates the polyribosomes; this in turn prevents entrapment effects and allows the monitoring of particle absorbance after sucrose gradient centrifugation (see Fig. 1). The preservation of viral complexes exposed to EDTA under the above conditions was ascertained with appropriate parallel experiments using hypotonic buffers with and without EDTA (reticulocyte standard buffer, etc.). Depending, however, on the dilution of samples, the time required for multisample processing, the necessity of absorbance profiles, etc., it might sometimes be preferable to exclude the EDTA either from the hypotonic buffer or even totally. No difference could be observed by substituting Tris with PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid] (10) in our protocol. (ii) During a reasonably rapid extraction, the use of inhibitors (3, 24, 27) to prevent histone deacetylation in vitro does not appear to be necessary, since identical results were obtained when we tested in the above procedure the effect of the presence and the absence of 10 mM butyrate. As a precaution, however, this compound was added in experiments requiring longer manipulations. (iii) The effectiveness of extraction (hence, the number of strokes to be used with a homogenizer of a given tightness) was monitored by applying the Hirt procedure (14) to the 10,000 × *g* pellet. Centrifugation in sodium dodecyl sulfate (SDS)-sucrose gradients of the Hirt extracts (18) and of suitable samples of nucleoprotein complexes indicated that our procedure extracted at least 90% of the complexes, as measured by the 21S DNA mass. For complexes labeled with radioactive thymidine in 30- to 60-min pulses, we obtained a comparable, albeit more variable (80 to 90%), effectiveness by adding a second extraction of the 10,000 × *g* pellet. The same was done for complexes formed at early times after infection.

Histone extraction and analysis. Cellular histones were extracted from the 10,000 × *g* pellet with 0.4 N H_2SO_4 for 1 h under magnetic stirring in an ice bath. The same procedure was applied to the SV40 complexes isolated by sucrose gradient centrifugation, after precipitating the corresponding fractions with 2 volumes of ethanol. The acid extracts were precipitated with 10 volumes of acetone. Histones were electrophoresed in 15-cm (6-mm inside diameter) acetic acid-polyacrylamide gels containing 2.5 M urea, prepared by a modification of the Panyim and Chalkley procedure (21) (17% acrylamide, 0.085% ethylene diacrylate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, 0.16% ammonium persulfate). Samples were dissolved in 5 M urea–4 mM dithiothreitol–0.9 N acetic acid and heated to 90°C for 1 min. After an overnight prerun, electrophoresis was carried out for 24 h at 6 V/cm. Bands were stained with amido black, and densitometric tracings were obtained with either a Gilford 2410 or an E-C 910 densitometer.

Sucrose (15 to 30%) in SDS buffer (10 mM Tris [pH 7.4], 100 mM NaCl, 1 mM EDTA, 0.5% SDS) was used to quantitate and separate viral DNA from the proteins to be analyzed in SDS-polyacrylamide gels. SV40 complexes precipitated from sucrose gradients as indicated above were redissolved in SDS buffer–2 mM dithiothreitol and centrifuged for 14 h at 30,000 rpm (25°C) in the SW41 Spinco rotor; proteins were precipitated from the top fractions with 5 volumes of ethanol.

Histone labeling. [^{3}H]acetate (6.4 Ci/mmol) was dried by vacuum and dissolved in normal culture medium before administration to cells. Labeling with [^{14}C]lysine and [^{14}C]arginine (300 mCi/mmol) was carried out in a medium that differed from the growth medium in that it contained dialyzed serum and Joklik synthetic components. After electrophoresis, gel slices were hydrolyzed and assayed for radioactivity.

RESULTS

Distribution of SV40 DNA among the main species of nucleoprotein particles during the lytic cycle. Inside infected cells, SV40 DNA is found associated with proteins in multiple forms of complexes (7, 8, 10, 16, 25, 26). Some of these have short lifetimes and are relatively minor components in mass terms, such as replication and transcription complexes and possible partially assembled structures. The exposure of nucleoprotein particles to an improper environment can also increase their heterogeneity because of variable disruption of these structures (10). In total extracts of infected cells under conditions that do not degrade viral structures (see above and below), the mass of SV40 DNA was detected within two major populations of intracellular particles. The first appeared with the onset of viral DNA synthesis, sedimented in sucrose gradients at about 75S, and corresponded to the structures also called minichromosomes. The other became evident during the lytic cycle several hours after the first and cosedimented in sucrose gradients with mature virions at about 180S. The sedimentation rates indicated in the present experiments are based on linear extrapolation from the positions of the two ribosomal subunits of the host cell, taken as reference points with the conventional values of 60 and 40S in 15 to 30% sucrose gradients. The sedimentation rate usually assigned to SV40 is higher (240S, as determined in 1 M NaCl); in our gradients, however, the SV40 virions sedimented slightly ahead of the ribosomal tetramer of animal polysomes, i.e., at about 180S (20). The same coefficient has been reported

when the sedimentation rate of SV40 has been compared with that of poliovirus (11). The sedimentation rate of minichromosomes reported recently is 70 to 75S (6, 8, 10, 16, 26), but it should be considered, as already noted by Varshavsky et al. (26), that a coefficient obtained with ribosomal subunits as markers is lower than that obtained with the 21S viral DNA as the marker. Figure 1A illustrates the fractionation by sucrose gradient centrifugation of an infected-cell extract, prepared at a time near the middle of a 5-day lytic cycle. The absorbance profile shows the separation of two main species of deoxynucleoprotein particles: the heavier one sedimented in the 180S position, and the lighter one emerged as a shoulder on the left side of the large ribosomal subunit peak, derived from the dissociation of total cell ribosomes. The label distribution shows that after a 70-min exposure of cells to radioactive thymidine, most radioactivity was associated with the 75S particles and very little was associated with the 180S particles. In this experiment the two peaks of particles were eventually precipitated with ethanol, dissolved with 1% SDS, and analyzed in sucrose-SDS gradients; the same was done with a parallel sample which had been labeled for 22 h. The results of these analyses (not shown here) indicated that: (i) as measured from optical density tracings, the 180 and 75S particles contained nearly equal amounts of 21S DNA (29 and 26 μg/dish of about 7×10^6 cells, respectively); (ii) the 180S peak contained no other nucleic acid except 21S DNA; and (iii) the viral DNA accounted for all of the radioactivity incorporated in the two particle peaks during both the short and long labeling periods.

The large specific activity difference in the DNA of the two particles, observed at a stage when comparable masses of both are present inside the cells, suggests that newly synthesized viral DNA enters first the 75S and then the 180S population. Figure 1B shows the results of an experiment in which the radioactive thymidine incorporated in a 45-min pulse was chased for 4 h. The radioactivity in the 75S peak decreased during the chase, and that in the 180S peak increased (although in a nonlinear fashion, presumably due to particle release); this result supports the suggestion that viral DNA moves from

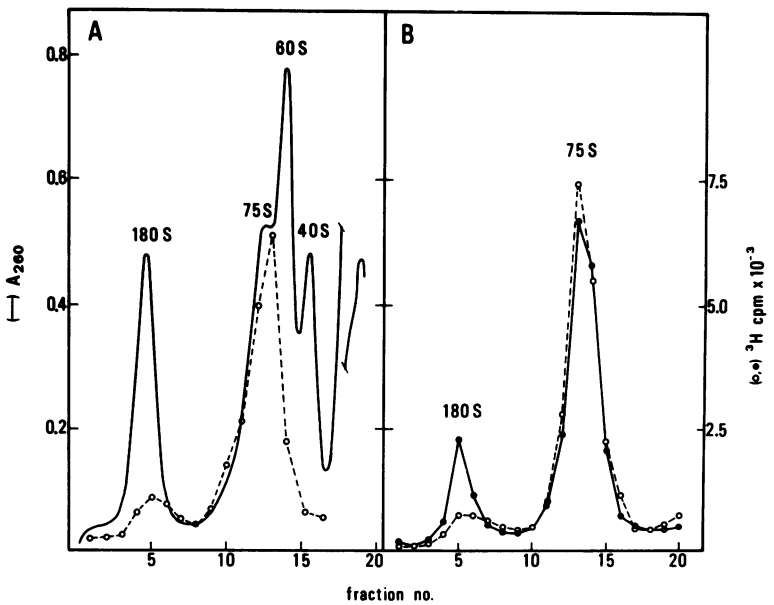

FIG. 1. *Sucrose gradient fractionation of infected-cell extracts. Extracts from CV-1 cultures infected with SV40 were prepared as described in the text and centrifuged in 15 to 30% sucrose gradients for 110 min in the SW41 Spinco rotor. The gradients were collected through an automatic-recording Gilford spectrophotometer. Suitable samples of the gradient fractions were precipitated with trichloroacetic acid and assayed for radioactivity. Fractions 3 to 6 and 11 to 15 were eventually precipitated with ethanol and analyzed in SDS-sucrose gradients for viral DNA content (see text). A total of (A) 7×10^6 cells in one 100-mm dish were labeled at 62 h postinfection for 70 min with [^{3}H]thymidine (10 μCi/ml; specific activity, 6.7 Ci/mmol). (B) Two 60-mm dishes containing 2.5×10^6 cells received [^{3}H]thymidine as described above at 60 h postinfection for 45 min. One culture was extracted at the end of this period (○); the other was extracted after another 4-h incubation in new medium containing 5×10^{-4} M unlabeled thymidine (●). A_{260}, Absorbance at 260 nm.*

the first to the second particle.

Figure 2 shows the mass distribution of the total 21S viral DNA produced during the lytic cycle among the 75S particles, the intracellular 180S particles, and the 180S particles spontaneously released in the growth medium. The infected cells were also labeled for 60 min soon after the start of viral DNA synthesis to follow the fate of 21S molecules synthesized at the beginning of the cycle. From the DNA mass diagram (Fig. 2A), it appears that the 21S DNA contained in the 75S particles increased slowly for more than half the cycle and then declined, whereas the 21S DNA associated with the 180S particles was detectable several hours later, rapidly reached its maximum toward the middle of the cycle, and then slowly decreased. After their appearance inside the cell, the 180S particles were also found in the medium and there continuously increased as the culture progressed toward lysis. The 180S particles released in the medium were separately quantitated to present a full picture of viral DNA distribution among the particles constituting the major stations of viral chromatin in the lytic cycle. Figure 2B shows the percent distribution of the 21S radioactivity incorporated during a [^{3}H]thymidine pulse administered at 26 h postinfection among the particles described above. The DNA molecules synthesized early in the lytic cycle emerged successively in the 75S and the intracellular and extracellular 180S particles. Conclusions concerning the rate of this passage, however, are only approximate because the notable increase in the total 21S radioactivity in the course of the cycle (see the legend to Fig. 2) for that part which was not due to an imperfect initial extraction of labeled molecules resulted in a proportional increase in the apparent transit time from the 75S to the 180S particles. This also prevented an unambiguous answer to the question of whether the label left the 75S particles linearly or exponentially (i.e., whether these particles were processed in their order of synthesis or randomly). This kinetics helps in the understanding of the correspondence between the biological and operational identities of the particles isolated during viral growth. It should be noted that the viral DNA weight detected in the 180S particles (intracellular plus extracellular) at between 60 and 70 h postinfection was more than 80% recovered in the sum of the particles at the end of the lytic period. The overall diagram also confirms that SV40 DNA is efficiently encapsidated (10).

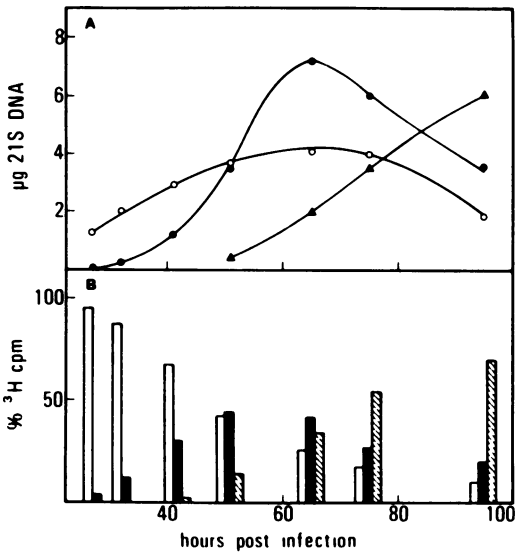

FIG. 2. *Distribution of 21S SV40 DNA among 75S particles, intracellular 180S particles, and 180S particles released in the medium. A series of 60-mm dishes containing confluent cultures was labeled at 26 h after infection with [^{3}H]thymidine (20 μCi/ml) for 60 min. At the end of this period, the cultures received new medium containing 5 × 10^{-4} M unlabeled thymidine. At the times indicated, cell extracts were prepared and analyzed in sucrose gradients as shown in Fig. 1. The 180 and 75S fractions were precipitated with ethanol, dissolved with 1% SDS, and centrifuged in SDS-sucrose gradients (see text). The released particles were concentrated by centrifuging a 10,000 × g supernatant from the culture medium at 38,000 rpm for 3 h in a Spinco 50 Ti rotor. (A) In the spectrophotometric tracings obtained from SDS-sucrose gradients, the area of the 21S peaks was quantitated by a graphic method; the amount of viral DNA was determined by applying the equivalence 1 optical density unit at 260 nm = 50 μg of DNA per ml (14, 18). Symbols: ○, ●, and ▲: 21S DNA from 75S, intracellular 180S, and released 180S particles, respectively. (B) Radioactivity associated with the 21S DNA of the viral particles illustrated above. (The 100% value increased with time from about 110,000 cpm initially to about 190,000 cpm at 75 h postinfection; this was probably due to more than one concurring error, such as an incomplete chase, imperfect initial extraction of highly radioactive molecules, and recycling of incorporated thymidine.) Symbols: □, ■, and ▨: 21S radioactivity from 75S, intracellular 180S, and released 180S particles, respectively. During the fastest rise of 180S, the hourly increase of its 21S DNA content was between 3 and 6% of the total 21S (A); during the exponential-like phase of the 75S radioactivity decrease (B), the apparent decay rate was approximately 5%/h.*

Early 180S particles are similar, but not identical, to mature virions. The experiments to detect an SV40 intermediate complex on which the final acetylation of viral chromatin could take place resulted in the finding of a likely candidate: a discrete peak of particles heavier than 75S and capable of rapidly incor-

porating radioactive acetate into histones. Because of abnormal sedimentation properties, however, we also realized that these particles were unstable in some ionic environments, which still left the mature virions unaltered, and that under different saline conditions these particles cosedimented with mature virions. Figure 3 shows the results of an experiment in which 180S particles labeled with [^{3}H]thymidine and isolated at 60 h after infection were mixed with virions labeled with [^{14}C]thymidine and collected at the end of the lytic period (each isotope had been added to one half of the same infected culture at 36 h after infection). Both species of particles had been first isolated separately in sucrose density gradients. This mixture was partly made 0.015 M NaCl-0.01 M EDTA-0.01 M Tris (pH 7.4) and partly left in 0.1 M NaCl-0.01 M Tris (pH 7.4), incubated for 15 h at 20°C, and finally centrifuged in sucrose density gradients. The profiles show that the two types of particles cosedimented when maintained in 0.1 M NaCl-0.01 M Tris (pH 7.4), but sedimented separately when incubated with EDTA at a low-salt concentration; the ^{14}C-labeled particles did not change their sedimentation rate, whereas the ^{3}H-labeled particles sedimented more slowly. Similar experiments (not shown here) showed that: (i) EDTA and low salt, each alone, had little effect; (ii) the transition kinetics from the fast- to the slow-sedimenting form of the unstable particles occurred via a shorter-lived intermediate stage; (iii) with different preparations, the result shown in Fig. 3 was also obtained at 5°C (or at 20°C, in a shorter time); and (iv) the transition from one discrete form of unstable particles to the other was initially over 50% reversible, but eventually a decay into a heterogeneous set of smaller complexes took place. The 180S particles behaved in an analogous manner when centrifuged to equilibrium in CsCl gradients (Fig. 4). A mixture of ^{14}C-labeled and ^{3}H-labeled particles similar to that described in the experiment of Fig. 3 was first incubated with EDTA at a low-salt concentration, then suspended (without fixation) in a CsCl solution with a final density of 1.36 g/cm^3, and centrifuged for 26 h at 38,000 rpm. The distribution of the two isotopes at the end of the run formed two peaks essentially superimposed at a density of 1.345 g/cm^3 in the unincubated control (Fig. 4A). The EDTA-treated sample (Fig. 4B) showed the presence of the ^{14}C peak in the same position of the control, but the ^{3}H radioactivity was distributed rather heterogeneously in the denser region of the gradient. This suggests that the ^{3}H-labeled particles that had been treated with EDTA progressively lost proteins when centrifuged in a CsCl gradient. Such a process of dissociation seemed to be rather slow for it was not apparent after a 90-min centrifugation in a CsCl gradient carried out by the method of Christiansen et al.

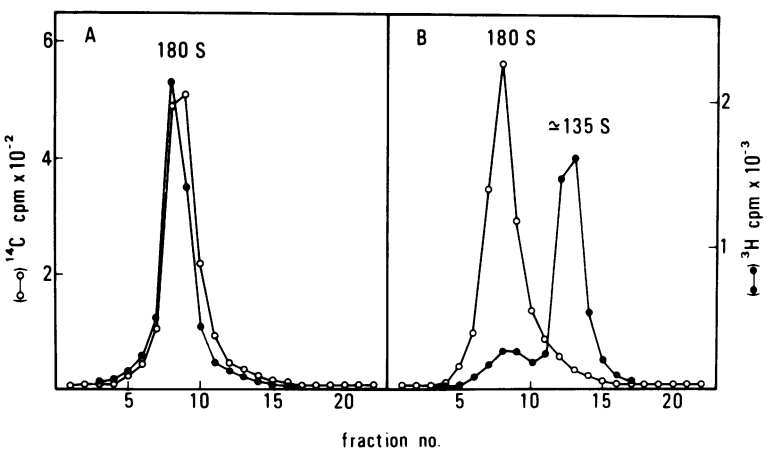

FIG. 3. *Effect of EDTA at a low ionic strength on the sedimentation rate of mature virions and intracellular 180S particles. Two infected cultures were labeled at 36 h postinfection, one with [^{3}H]thymidine (5 μCi/ml) and the other with [^{14}C]thymidine (0.12 μCi/ml; specific activity, 47 mCi/mmol). At 60 h postinfection, the ^{3}H-labeled culture was extracted and analyzed as shown in Fig. 1. The ^{14}C-labeled culture was allowed to lyse completely; the lysate (collected at 120 h postinfection) was rapidly frozen and thawed five times and centrifuged for 10 min at 10,000 × g; the supernatant was then sedimented at 35,000 rpm for 3 h in a Spinco 42.1 rotor. The pellet was suspended and centrifuged in a sucrose gradient as with the ^{3}H-labeled extract; the top fractions of the ^{14}C and ^{3}H 180S peaks thus obtained were mixed. Part of this mixture was left in 0.1 M NaCl-0.1 M Tris (pH 7.4) (A); part was diluted to 0.015 M NaCl and made 0.01 M in EDTA (B). The two samples were incubated for 15 h at 20°C and then centrifuged in sucrose gradients as in Fig. 1 for 90 min.*

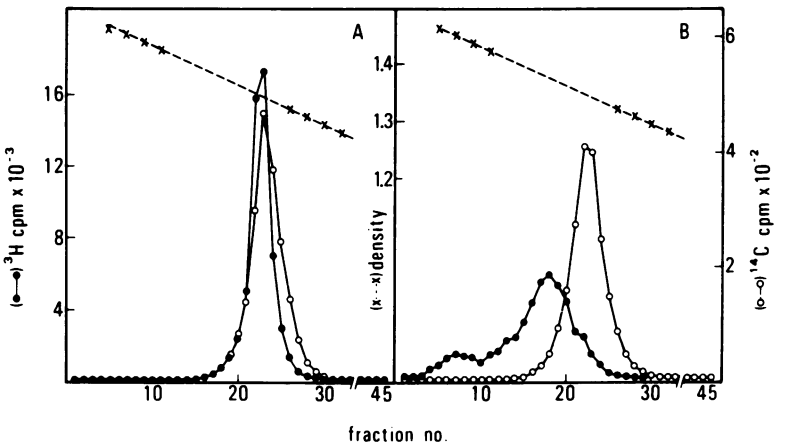

FIG. 4. *CsCl gradient analysis of mature virions and intracellular 180S particles incubated with EDTA at a low ionic strength. A mixture of ^{14}C-labeled mature virions and ^{3}H-labeled 180S particles was prepared and incubated with EDTA at a low-salt concentration as described in the legend to Fig. 3. The samples were then brought up to 3 ml with a CsCl solution (final density, approximately 1.36 g/cm^3) and centrifuged for 26 h at 38,000 rpm and 10°C in the SW50.1 Spinco rotor. (A) Unincubated control; (B) sample incubated with 0.01 M EDTA-0.015 M NaCl.*

(6), but it became evident when the same centrifugation was prolonged for 24 h (data not shown).

Electron microscopic pictures of the 180S particles extracted from cells at 60 h postinfection did not reveal any remarkable difference between their morphology and that of mature virus collected at the end of the lytic cycle (Fig. 5).

The structural proteins contained in these particles were analyzed by polyacrylamide gel electrophoresis in the presence of either SDS or acetic acid-urea (21). The patterns obtained with SDS-polyacrylamide gels are shown in Fig. 6A. VP1, VP2, and VP3, as well as histones, were present in the intracellular 180S particles (gel c). In one detail, the pattern of these particles differed qualitatively from that of more mature virions (gels a and b): the VP3 band of the former was closely followed by a small band absent in the latter. This difference was not very evident here because in this type of gel, histone H1 was split into two bands, the lower one of which comigrated with VP3 (see the uninfected-cell histones of gel d). This difference, however, became quite clear in the acetic acid-urea gels, which were more suitable for resolving histones (Fig. 6B). These gels show that histone H1 was present in the intracellular 180S particles, whereas it was absent from the late virion particles (Fig. 6B, gels c and d). For the remaining aspects, the two histone patterns were very similar; in particular, the acetylation level observed in both was higher than that in the 75S histones (gel b), which in turn was slightly higher than that in cellular histones (gel a).

A histone analysis was also performed on a mixture of intracellular 180S and late virions, whose proteins had been labeled with different isotopes. This was done to rule out hypothetical artifacts (such as selective losses and changes in dye affinity) that might have resulted in the disappearance of H1 from late virions. An infected culture was labeled at 24 hours after infection, half with [^{3}H]lysine and half with [^{14}C]lysine. The incubation with [^{3}H]lysine was stopped at 60 h after infection and that with [^{14}C]lysine was stopped at 130 h after infection, when the lysis was completed. Particles sedimenting at 180S were extracted from the homogenized cellular structures and from the growth media and centrifuged in sucrose gradients. The histones extracted from the mixture of the two particles are shown in Fig. 7A. The radioactivity distribution confirms the strong reduction of the histone H1 peak in virion particles (labeled with ^{14}C) collected at the end of the lytic cycle.

When infected CV-1 cells were allowed to lyse completely, the bulk of virions was spontaneously released into the culture medium, and the yield could be increased further by the widely used procedure of freezing and thawing the lysate. A residual fraction of virions, however, usually less than 15% of the total, could only be recovered by mechanical disruption of cells and cell debris in a homogenizer. These virions, which somehow conserved a tighter association with cell structures until the end, were also isolated and analyzed for the histone content. In their pattern, only a very faint band of histone

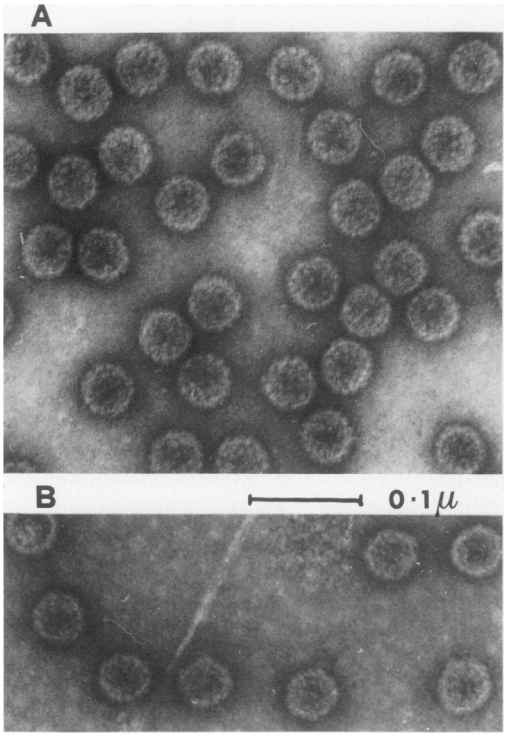

FIG. 5. *Electron micrographs of intracellular 180S particles (at 60 h postinfection) (A) and mature virions (B). 180S particles and mature virions were prepared and isolated in sucrose gradients as described in the legend to Fig. 3. Five microliters from the gradient peaks was absorbed for 3 min onto grids covered with a collodion film and carbon coated. The material was stained without fixation for 30 s with 2% uranyl acetate.*

H1 was present: this can be observed better in densitometric profiles (Fig. 7B, curve c). In a large-scale experiment, it was found conversely that the 180S particles released early in the medium (at 60 h after infection) contained slightly less histone H1 than did the intracellular 180S particles (curves a and b).

The possibility of a subtler contamination artifact affecting the intracellular 180S particles was examined carefully, especially because a change in their histone pattern was observed after centrifugation in CsCl. 180S particles, labeled with radioactive thymidine, were first isolated in standard sucrose gradients. The peak fractions were then diluted and centrifuged over a 34% CsCl solution by the method of Christiansen et al. (6). At the end, 94% of the particles were recovered in a sharp band (halfway into the CsCl gradient, as expected), but histone H1 was then absent, or nearly so, from their histone pattern. The extraction of the CsCl-sucrose boundary, followed by an analysis in acetic acid-urea gels, showed the presence of a single gel band corresponding to histone H1 in an amount roughly complementing the histone pattern from particles (Fig. 8, gels a and b). The first explanation considered was that an artificial H1 absorption to the capsid of truly H1-less viral particles might have occurred during cell homogenization, followed by H1 release upon exposure to a high CsCl concentration. The alternative possibility was obviously that of a physiological association of H1 with the particles, with the salt extracting H1 prematurely. We then carried out several reconstruction experiments in which concentrated mature virions were homogenized with uninfected cells and then isolated with the usual procedure in sucrose gradients. In all cases, no trace of histone H1 was found associated with virions (Fig. 8, gel c). From these results (together with the consistently regular aspect of the five-histone pattern in sucrose-isolated particles and the observation that the host histones were fairly well conserved also at the end of cell lysis), we concluded that the CsCl centrifugation resulted in the production, rather than in the elimination, of an artifact.

The specific infectivity of viral particles was measured in experiments in which the preparative procedures were kept as parallel as possible (Table 1). The virus particles collected at the end of the lytic cycle consistently presented a higher specific infectivity than did the intracellular 180S particles isolated at 60 h after infection: the lowest value observed in the former was still three times larger than the highest value in the latter. Table 1 also shows the specific infectivity of the 75S particles, but these data should be considered as indicating only the minimum difference from virions, since separation by sucrose gradient centrifugation, which is adequate for biochemical purposes, is presumably inadequate for biological assays.

Final stages of viral chromatin acetylation: uptake and retention of radioactive acetate. The maturation and assembly of viral deoxynucleoprotein complexes into the definitive virion products appears to take place through a series of intermediates (8, 10; unpublished observations). The short-lived intermediates are only small components in terms of chromatin mass, but can be detected by labeling the viral DNA with short radioactive pulses. With this approach and suitable chase experiments, Garber et al. identified four intracellular complexes which the viral DNA enters in succession: (1) a DNA replicative intermediate (sedimenting slightly faster than the 75S complex); (2) a 75S complex, which is a product of the replicative

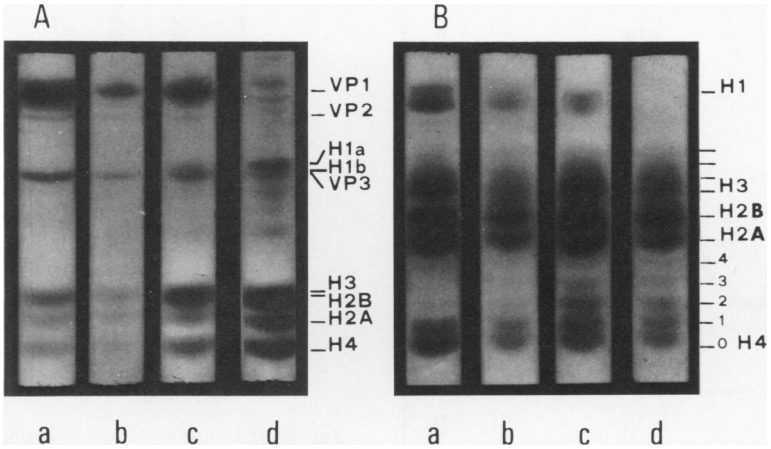

FIG. 6. *Electrophoretic analysis of proteins extracted from mature virions and intracellular 180S particles. The two types of particles were prepared as described in the legend to Fig. 3. (A) SDS-polyacrylamide gels. a and b, Proteins from mature virions, 1 dose and 0.5 dose, respectively; c, proteins from 180S particles; d, histones from uninfected CV-1 cells. Three milliamperes per gel was applied for 16 h to 10-cm , 15% polyacrylamide gels, cross-linked with ethylene diacrylate. (B) Acetic acid-urea gels. a, b, c, and d, Histones extracted from infected-cell nuclei, 75S complexes, 180S particles (at 60 h postinfection), and mature virions, respectively. For other technical details, see the text.*

form; (3) a precursor of fully assembled particles (sedimenting slightly slower than assembled particles); and (4) a fully assembled particle. Complexes 2 and 4 correspond to the two major particles that we have been considering, whereas complexes 1 and 3 in our experiments so far were partly left out and were partly minor contaminants of the particles obtained from the 75 and 180S peaks. In a scaled-up experiment similar to that shown in Fig. 1, we analyzed the histones of the particles sedimenting in the regions of complexes 1 and 3, that is, ahead of the 75S peak and behind the 180S peak. These histones are shown in Fig. 9. The fraction enriched in replicative forms (gel b) contained histones with a degree of acetylation very similar to that of the 75S complexes (gel a). In histone H4, in which the acetylation level could be observed more easily, the two lower bands (corresponding to molecules with 0 or 1 acetyllysine) were clearly predominant over the upper bands. The fractions enriched in the precursor of the 180S particles showed a higher degree of histone acetylation (gel c), very near that of the 180S particles (gel d). Here the relative distribution of histone H4 among the five bands had changed in favor of the upper bands. These patterns were consistent with the idea that viral chromatin is progressively modified by an increase in histone acetylation during successive stages of virion maturation.

Conceivably, at the stage represented by the intracellular 180S particles, the histone acetylation processes are either completed or nearly completed. Any activity still occurring in these particles should be revealed by the analysis of their histones after a short exposure of cells to radioactive acetate. The following two experiments were carried out first to measure the total uptake and retention of [^{3}H]acetate in the histones from 180S particles and then to determine whether the chromatin carried into the 180S particles by precursor particles could account for all of the label associated with these histones or whether a direct histone acetylation might also occur.

In experiment 1, histones were labeled for 20 min with [^{3}H]acetate, and the incorporation was measured after 20 and 90 min of chase (the 20-min chase substituted for the simple pulse because the chase was not completely effective and the incorporation continued to increase for the first 20 min). The results are shown in Fig. 10, which also shows, for a comparison, the host cell and the 75S histone patterns. The cellular histones and the 75S histones incorporated [^{3}H]-acetate as expected; at the end of the chase, the cell histones had lost about 40% of the radioactive acetyl groups present 70 min earlier (Fig. 10c and c'). The corresponding loss in histones associated with the 75S viral chromatin was only about 5% (Fig. 10b, and b'). There was no loss and actually a minor increase in acetate radioactivity after the chase in the histone extracted from the intracellular 180S peak (Fig. 10a and a'). This experiment confirmed that histone acetylation in the viral chromatin has a different turnover from that in the host chromatin (16).

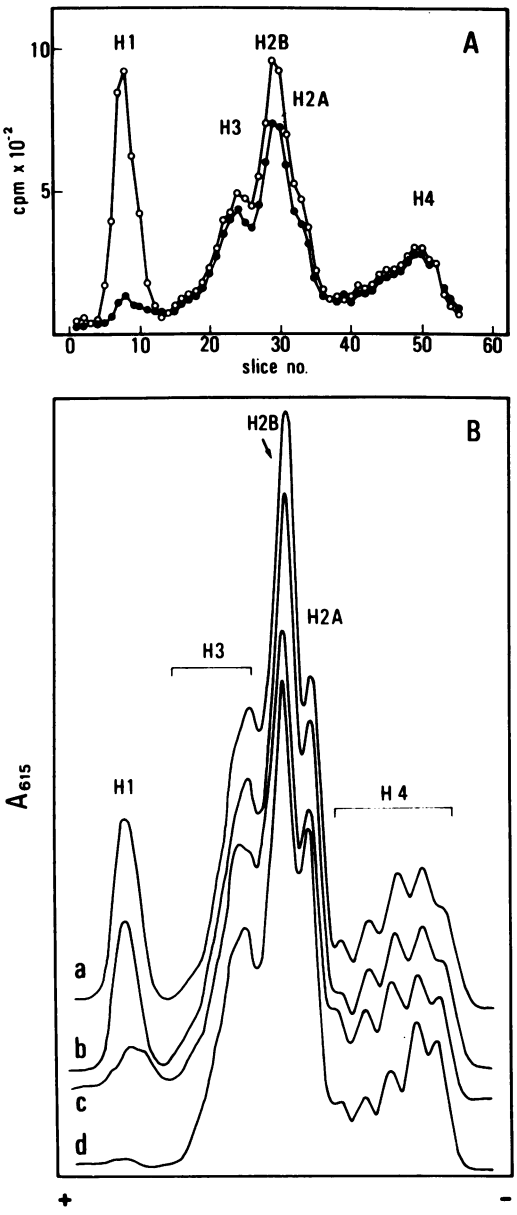

FIG. 7. *Histone H1 presence in intracellular 180S particles (immature virions) and absence in mature virions. (A) Acetic acid-urea gel electrophoresis of a mixed extract of ^{3}H-labeled (immature virions) and ^{14}C-labeled (mature virions) histones. At 24 h postinfection, part of a culture received 20 μCi of [^{3}H]-lysine (specific activity, 63 Ci/mmol) per ml, and part received 0.4 μCi of [^{14}C]lysine (specific activity, 286 mCi/mmol); the first culture was stopped at 62 h postinfection and the second was stopped at 130 h postinfection when lysis was completed. The two types of particles were isolated as described in the legend to Fig. 3, mixed, and extracted for histones. Symbols: ○, [^{3}H]lysine; ●, [^{14}C]lysine. (B) Acetic acid-urea gel electrophoresis of histones from intracellular and released 180S particles. For isolation procedures, see the legend to Fig. 3. a and b, Densitometric tracings of histones from intracellular and released particles at 60 h after infection, respectively; c and d, histone tracing from residual intracellular and released particles at 130 h after infection, respectively. A_{615}, Absorbance at 615 nm.*

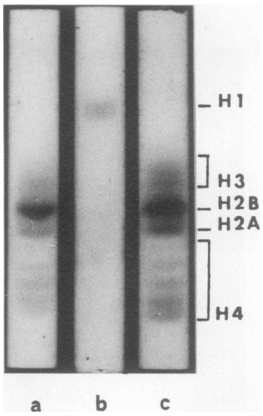

FIG. 8. *Histones from intracellular 180S particles centrifuged in CsCl and from mature virions reextracted from uninfected-cell homogenate. 180S particles labeled with [^{3}H]thymidine for 20 h were extracted from cells at 50 h postinfection and isolated in sucrose gradients as described in the legend to Fig. 1. The 180S peak fractions were pooled, diluted with 0.5 volume of buffer to a sucrose concentration of 15%, and layered over a 34% CsCl solution in SW50.1 tubes (6). Centrifugation was for 90 min at 40,000 rpm. Ninety-four percent of the input radioactivity was recovered in a neat opalescent band halfway down the CsCl column (another 5% was in a barely visible band about 2.5 mm above the main band). Mature virions were pelleted from a cell lysate, suspended, homogenized with appproximately 10^{7} uninfected cells, and isolated in sucrose gradients by the standard procedure used for intracellular 180S particles. Histones were extracted from the main CsCl band of 180S particles (a), from the CsCl layer at the boundary with the sucrose column (b), and from the sucrose gradient peak of mature virions (c). Analysis was carried out as in Fig. 6B.*

In addition, it also showed that histones of the intracellular 180S particles became labeled during short pulses with radioactive acetate and that this radioactivity was fully retained.

An analytical evaluation of the 180S histone labeling, however, is rather complex. The net incorporation detected at any time in these particles may depend on at least two possible mechanisms: the afflux of precursor complexes labeled before their entry and the direct acetylation of the 180S histones minus the 180S particles released from cells and the histone deacetylation,

TABLE 1. *Infectivity of SV40 particles*[a]

Expt	PFU × 10^{-9}/μg of 21S DNA		
	Mature virions	180S (60 h postinfection)	75S
1	1.5	0.32	0.01
2	1.0	0.05	0.001
3	1.1	0.1	

[a] Particles were obtained in each experiment from the same infected culture, part of which was stopped at 60 h postinfection to extract 180 and 75S complexes and part of which was allowed to lyse until 120 h postinfection to obtain mature virus. All particles were isolated from sucrose gradients as described in the legend to Fig. 3, and dilutions were made directly from gradient fractions. 21S DNA was quantitated as described in the legend to Fig. 2.

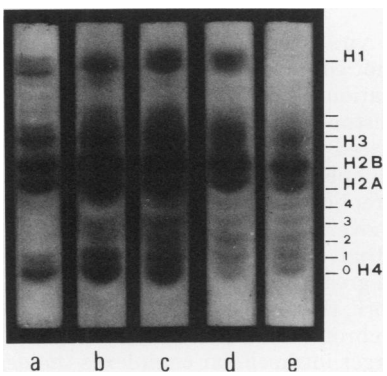

FIG. 9. *Electrophoretic analysis of histones from different SV40 nucleoprotein particles. Infected cells from 20, 10-cm dishes were fractionated at 65 h after infection in a series of sucrose gradients similar to that shown in Fig. 1. Samples from the 75 and 180S peaks were analyzed together with two pools of intermediate gradient fractions (corresponding to fractions 6 to 7 and 9 to 11 of Fig. 1). a, 75S complexes; b, post-75S region; c, pre-180S region; d, 180S particles; e, virions from cells allowed to complete lysis.*

if present. The essential question, however, can be put more simply by asking only whether the load of labeled histones accompanying that fraction of the viral DNA which enters the 180S particles during an acetate pulse is sufficient to account for all of the acetate radioactivity incorporated at the end of the pulse. The answer, with reservations due to the assumptions mentioned below, is that it is not sufficient. The first indication for this comes from the experiment shown in Fig. 10 (see the legend), in which the proportion of labeled histones contained in the 180S particles at the end of a 20-min pulse with [^{3}H]acetate followed by a 20-min chase was about 27% of the total labeled histones in viral chromatin. The rate of 21S DNA entry into the 180S particles can be estimated, albeit very approximately, from the kinetics shown in Fig. 2. This estimate, for the above time interval, falls between 3 and 6% of the total 21S DNA, a figure considerably lower than its equivalent as radioactive acetate incorporation.

An experiment was then carried out to determine more directly the proportions of viral DNA and histones that enter the 180S particles during a radioactive acetate pulse by pulse-labeling the same cells with [^{14}C]thymidine and [^{3}H]acetate. [^{14}C]thymidine was administered first for 20 min; [^{3}H]acetate was administered afterwards for 30 min. The labeling was not simultaneous to allow some time for viral DNA replication and to avoid underestimating the entrance of the ^{14}C label into the 180S particles. At the end of the [^{3}H]-acetate pulse, the viral DNA complexes were fractionated in a sucrose gradient similar to that shown in Fig. 1; the viral DNA was then quantitated by centrifugation in sucrose-SDS gradients, and the histones were quantitated by polyacrylamide gel electrophoresis. The 180S peak was found to contain 2,350 cpm of ^{14}C-labeled 21S DNA and 10,300 cpm of ^{3}H-labeled histones. The 75S peak contained essentially all of the remaining radioactive chromatin, 27,500 cpm as ^{14}C-labeled 21S DNA and 38,500 cpm as ^{3}H-labeled histones. Thus, the ^{14}C radioactivity in the 180S particles, which could derive only from the 75S particles, was 8% of the total, and the ^{3}H radioactivity which might derive both from the 75S particles and from the direct incorporation was 21% of the total; this disproportion strongly suggests that a direct incorporation of [^{3}H]acetate indeed occurs. The conclusion depends on the assumption that the viral chromatin labeled with [^{3}H]acetate moves into the 180S particles at the same rate of the ^{14}C-labeled molecules (or, in any case, that a hypothetical delay of ^{14}C does not exceed the 30-min advantage received). Still, considering the consistency of this result with the first rough estimate, which was based on kinetics following the behavior of total viral DNA, the most objective interpretation of the data is that the [^{3}H]acetate is not only transported but is also directly incorporated into the 180S histones.

DISCUSSION

The main conclusions that can be drawn from the present work are the following: (i) the SV40 chromatin changes during the process of virion assembly, with a progressive increase in histone acetylation and a final loss of histone H1; and (ii) an important stage in such a process is represented by an intracellular particle with the properties of an immature virion.

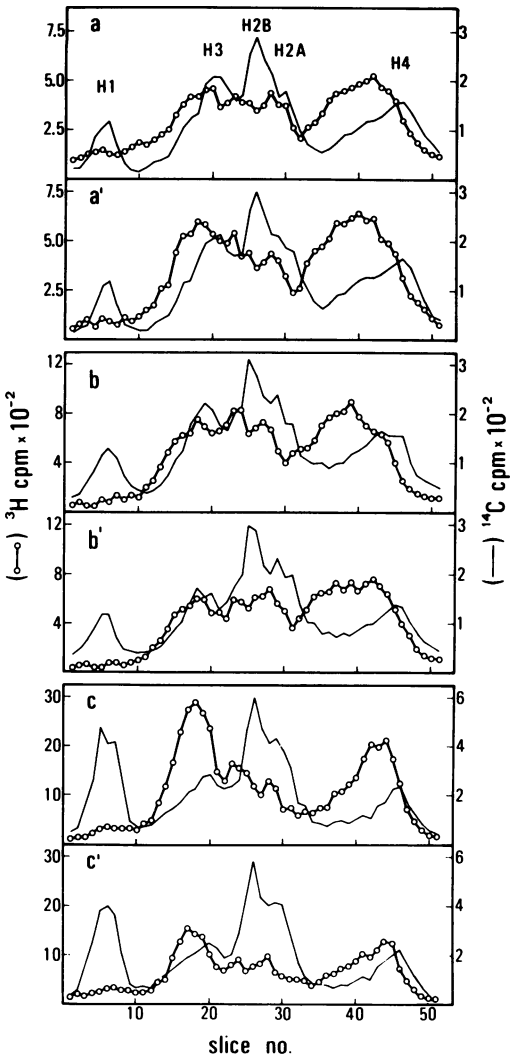

FIG. 10. *[^{3}H]acetate incorporation and retention by histones from 180S particles, 75S particles, and host chromatin. A total of 7 × 10^7 infected cells were labeled from 24 to 46 h postinfection with [^{14}C]lysine and [^{14}C]arginine (each at 0.125 μCi/ml) and then labeled for 20 min with 500 μCi of [^{3}H]acetate per ml. One half of the culture (indicated below as brief chase) was incubated for another 20 min in nonradioactive medium; the other half was incubated for another 90 min (long chase). For particle isolation and histone extraction, see the text. Five percent of cellular histones, 50% of 75S histones, and 100% of 180S histones were electrophoresed in acetic acid-urea gels. a, b, and c (brief chase), a', b', and c' (long chase), histones from 180S, 75S, and cell nuclei, respectively. The ^{3}H radioactivity contained in gels (following the above order, with primed samples given in parentheses) was 16,288 (17,107), 21,405 (20,710), and 53,263 (30,310) cpm. These figures (not the graphs) are corrected for minor ^{14}C differences (all below 10%) between the histones from the same source. The higher ^{3}H/^{14}C ratio in cell histones than in viral histones can be explained by the different content in new and old histones of the two chromatins (16).*

During a lytic cycle, SV40 DNA is detected in different types of intracellular nucleoprotein particles; there is now fairly good evidence that the large majority of viral DNA molecules enter these structures in turn, which represent successive stages is the formation of mature virions (8, 10). The lifetimes of two complexes, one containing only the set of internal proteins (mainly the five histones) and the other containing the full set of internal and external proteins, appear to be the longest on the basis of the viral DNA mass distribution that we observed. Other complexes have been identified by labeling the viral DNA with short pulses (10), and the overall picture obtained with the fractionation of infected cells may actually suggest the existence of a larger number of intermediates, which are too short lived and variable in size to be identified as discrete species (8; unpublished observations).

The viral chromatin, soon after its synthesis, does not contain histones with a high degree of acetylation (only a little higher than that of the host histones), but ends up in virions with highly acetylated histones (16). The initial nucleoprotein complexes, constituted by the 75S and the DNA replicative structures, are characterized by a turnover of acetyl groups already different from that of the host chromatin in that they have a greater acetyl retention (16). In the present work, the data concerning the acetylation of SV40 chromatin show the following: (i) that in the larger intracellular complexes the degree of histone acetylation is higher than that in the 75S complexes and near that of the most mature virions; and (ii) that the increase in histone acetylation, as indicated by [^{3}H]acetate incorporation, continues even in particles that have completed, or nearly completed, the assembly of the external proteins (this again occurs with a valvelike mechanism, allowing the transfer of acetyl groups to histones but not their removal by deacetylases).

These fully assembled particles, sedimenting at 180S like mature virions, are remarkable for another chromatin change that occurs during virion maturation: they represent the latest stage in which viral DNA is still associated with histone H1. We showed that at a later stage in the particles that we consider to be the actual mature virions, H1 was no longer present. The immature forms of virions prevailed during a 5-day productive cycle until around 70 h postinfection, when cell lysis began; at about 120 h

postinfection, the lysis of cells was completed and the virions then had acquired different characteristics. The recovery of particles in this final stage was high enough (at least 80%) to rule out a significant selection. The particles described as immature virions, or provirions, resembled the more mature virions in morphology, sedimentation velocity, and density in CsCl. Their distinct properties were the following: the presence of histone H1, the incorporation of radioactive acetate into histones (until around 60 h postinfection), lower specific infectivity, and instability in some ionic environments. The existence of two similar, but not identical, virionic structures may explain some apparently conflicting data in the literature on the properties of mature SV40. Earlier reports on the protein content of virions (generally collected at the end of cell lysis) did not include histone H1 (6, 17, 22, 23). Besides, in view of the standard purification procedures, it appears that SV40 virions were not considered overly unstable. In recent studies of viral chromatin, the intracellular complexes were fractionated at earlier times in the lytic cycle, and the heavier structures, similar in morphology and sedimentation properties to mature virus, have been identified as such. Our results are thus consistent with those of Nedospasov et al. (19) concerning the presence of histone H1 in assembled virions, but differ in the interpretation. The same can be said about the instability of virions (for us, of provirions), although it should be clear that even the truly mature virus is not resistant to many destabilizing treatments, only more so than immature virions. Our data confirm, of course, that undegraded 75S complexes contain histone H1 (25).

Some technical aspects relevant to the present work deserve more specific consideration. The possibility that the detection of histone H1 in the 75 and 180S particles might be due to cell chromatin contamination was excluded by the fact that when the DNA was allowed to incorporate radioactive thymidine for 22 h or longer, all of the radioactivity in the particle peaks extracted for histones was recovered as 21S DNA, 95% in supercoiled form. Moreover, even rough estimates of the absorbance profiles (Fig. 1) suggest that the amount of viral chromatin contained in the two major peaks is just too large to be substantially altered in its composition by background contamination. Finally, reconstruction experiments in which mature virions were reisolated from uninfected-cell homogenates ruled out an external absorption to virions of histone H1. As to the [^{3}H]acetate-labeled viral chromatin, the divergent behavior of cellular and viral histones in pulse-chase experiments and their consistently different acetate/amino acid labeling ratio (Fig. 9) render the possibility of a significant cellular contamination rather unlikely. Our data agree with those of Garber et al. (10) concerning the artifacts that may be caused by improper extracting procedures; it is quite evident that the use of EDTA in infected-cell extracts should be carefully controlled (see above and Fig. 3 and 4). To this we should add two other technical observations made in preliminary experiments. The first is that the procedures that discard a cytoplasmic fraction almost inevitably select out a nonnegligible fraction of viral chromatin (11, 16), easily underestimated when radioactivity alone is monitored. The second is that the Dounce homogenization squeezes out of nuclei more rapidly and completely the viral nucleoprotein complexes that would leak out spontaneously only upon prolonged incubation. In the absence of detergents, the risk of producing nuclear fragments too small to sediment at 10,000 $\times$ g remains fairly low, since CV-1 nuclei do not appear to be very fragile. On the contrary, prolonged incubations (especially in the presence of detergents) can easily disrupt some viral structures and favor histone H1 and H3 degradation and histone deacetylation in vitro. Perhaps these basic differences of procedure may explain why our data led us to conclude that reduced deacetylation promotes the difference between the viral and cellular histones, rather than increased acetylation, as concluded by Chestier and Yaniv (5). From a physiological point of view, the increase in histone acetylation in viral chromatin may represent an advantage facilitating gene transcription for the infecting virus (1, 27); the disappearance from virions of histone H1 may represent the elimination of a protein whose role in packaging the chromatin has ended; the advantage of an increased stability is obvious. The surprising and difficult-to-explain aspect is that the elimination of histone H1 and the final acetyl transfer to histones occur in structures (the immature virions) in which the process of encapsidation appears to be completed. To some extent, this phenomenon is not really unique in animal virus morphogenesis. The terminal steps in poliovirus maturation involve the formation of a provirion, first discovered as a discrete species sedimenting at 125S. It was eventually found that the natural form of the provirion cosedimented with mature virions at 150S and that the slower-sedimenting form was produced by the unique sensitivity of the provirion to EDTA. This unstable provirion also contains a protein, VP0, which has yet to be cleaved to two final products (9, 12) by the action of a soluble protease or by a protein of the procapsid itself (2, 13). In adenovirus morphogenesis, the transition

from young virions to aged virions (two structures undistinguishable by density) is also accompanied by polypeptide cleavage. This takes place within the young virions and originates one of the core proteins plus other polypeptides, with a loss of molecular weight (4, 15). The enzymatic mechanisms and the structural rearrangements presumably required by this type of morphogenetic change have not yet been elucidated. In the case of SV40, we can only suppose that the instability of the immature particles reflects a capsid structure in which some elemental components are not so tightly connected as to totally isolate the virion internal proteins from the environment. Histone H1 does not necessarily need to be eliminated by proteolysis; it might be expelled when the proper degree of chromatin condensation is reached, such as when "scaffolding" internal proteins of some phages are expelled from unstable proheads upon DNA encapsidation (4). The SV40 capsid might then be sealed into its stable form by forming extra intermolecular disulfide bonds.

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