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Intracellular Forms of Simian Virus 40 Nucleoprotein Complexes

II. Biochemical and Electron Microscopic Analysis of Simian Virus 40 Virion Assembly

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The simian virus 40 virion assembly process was studied with pulse-labeling kinetics of virion proteins, CsCl gradient analysis, electron microscopy, and low-salt gel electrophoresis. The results obtained are consistent with the model of gradual addition and organization of capsid proteins around simian virus 40 chromatin. Empty virions, as observed in the CsCl gradient by previous workers, were found to be the dissociation product of immature virus. Histone H1 was found in simian virus 40 chromatin and virion assembly intermediates but not in the mature virion banding at 1.34 g/ml in the CsCl gradient.

Little is known about the mechanism of simian virus 40 (SV40) virion assembly. Ozer (12) and Ozer and Tegtmeyer (13), using pulse-chase radioactive labeling of viral capsid proteins, suggested that capsid proteins first form an empty shell to which viral genome is encapsidated. Since the CsCl gradient procedure used by these authors is known to disrupt many nucleoprotein complexes, including adenovirus assembly intermediates (2, 3), it is not clear whether the empty virion observed in the CsCl gradient is an entity independently synthesized from capsid proteins or whether they are derived from immature virions whose DNA-histone core is dissociated in a high-salt condition. To distinguish these two possibilities, we analyzed SV40 virion assembly intermediates by a mild extraction procedure that we developed (4). Our results do not agree with the model that empty virion is synthesized as a separate entity from capsid proteins. Rather, they are consistent with the model that capsid proteins are added gradually onto SV40 chromatin and subsequently organized through a still unknown mechanism into mature virus. Empty virion, we believe, is an artifact generated from assembly intermediates in the CsCl gradient.

MATERIALS AND METHODS

Virus. The description of SV40 and the infection procedure have been made previously (4).

Extraction and fractionation of SV40 complexes. SV40 nucleoprotein complexes were extracted as described previously (4). Briefly, SV40-infected cells were scraped off a tissue culture plate with a rubber policeman. The cells were lysed in 0.5% Nonidet P-40 in TD buffer (25 mM Tris [pH 7.4], 0.136 M NaCl, 7 mM KCl, 0.7 mM Na_3PO_4). The nuclei were suspended in TD buffer and homogenized 30 to 40 strokes with a tight-fitting homogenizer. The nuclear extract was layered on top of 12 ml of a 5 to 40% sucrose gradient and centrifuged for 70 min at 37,000 rpm in an SW40 rotor.

Radioactive labeling of SV40 nucleoprotein complexes. For pulse-labeling experiments with [^{3}H]lysine, SV40-infected cells were washed and preincubated with lysine-free medium for 10 min and then labeled with the warm lysine-free medium containing 2% dialyzed serum and 100 μCi of [^{3}H]lysine (Amersham Corp.; 60 to 80 Ci/mmol) per ml for the period desired. For chase experiments, the radioactive medium was replaced with medium containing 2% fetal calf serum and 7.3 mg of unlabeled lysine per ml (a 100-fold excess of lysine over the concentration in normal medium).

Fixation of nucleoprotein complexes with glutaraldehyde. SV40 nucleoprotein complexes isolated from the sucrose gradient were fixed with glutaraldehyde by the procedure of Baltimore and Huang (1).

CsCl density gradient analysis of SV40 nucleoprotein complexes. Fixed or unfixed SV40 nucleoprotein complexes were analyzed with a preformed CsCl gradient (1.2 to 1.6 g/ml) as described previously (4).

Electron microscopy. Sample preparation for electron microscopy was described previously (4).

SDS- and acid-urea-polyacrylamide gel electrophoretic analysis of proteins. Sodium dodecyl sulfate (SDS)-gel electrophoresis analysis was performed by the procedure of Laemmli (7). A modified acid-urea-gel electrophoresis technique (18) which allows direct analysis of protein components of nucleoprotein complexes without removing DNA was used to study the protein compositions of SV40 nucleoprotein complexes. Electrophoresis was carried out in 15% polyacrylamide at 4°C for 30 h.

RESULTS

Study of SV40 virion assembly by pulse-chase experiments with [^{3}H]lysine. In our previous study using a new extraction procedure, we showed that there are three groups of SV40 nucleoprotein complexes in the nuclei of infected CV-1 cells (4). The three nucleoprotein complexes, NP-I, NP-II, and virion, can be separated in sucrose gradient as shown in Fig. 1 (lower graph). A pulse-chase experiment with [^{3}H]thymidine demonstrated the following biochemical pathway for the DNA component of the nucleoprotein complexes:

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

In the present study we analyzed the biochemical pathway of the protein components of SV40 nucleoprotein complexes. A short pulse with [^{3}H]lysine for 5 min at 48 h postinfection labeled preferentially NP-I (Fig. 1, upper graph). Pulse-labeled proteins present in different regions of the sucrose gradient were analyzed by SDS-gel electrophoresis (Fig. 2). Proteins present on the top of the gradient migrated only on the high-molecular-weight region of the gel. No histone protein was observed in this part of the gradient. They probably represented ribonucleoproteins and free protein pools in the nucleus. The major protein species labeled in NP-I during a 5-min pulse were VP-1, VP-3, and histones. The protein barely detectable in NP-II during this short pulse period was VP-1 capsid protein. The presence of both VP-3 viral protein and H1 histone in pulse-labeled NP-I was confirmed by using the acid-urea-gel electrophoresis technique which resolves H1 from VP-3 (data not shown). When the pulse-labeling period was increased to 10 min, we began to see the appearance of labeled VP-1 capsid protein in NP-II and virion positions in the sucrose gradient (Fig. 3). The histones in NP-II and virion were still not well labeled during a 10-min label. During the chase with a 100-fold excess of unlabeled lysine, NP-II and virion became substantially labeled. The majority of label accumulated in NP-II and virion appeared in the capsid protein VP-1 (Fig. 4). These pulse-labeling data suggested to us that newly synthesized histones and capsid proteins are first added to NP-I, which is active in synthesizing SV40 DNA and RNA (4). Subsequently, during the conversion from NP-I to NP-II, more capsid proteins are added onto SV40 chromatin. This result, therefore, is consistent with the model shown in Fig. 12 that SV40 virion is assembled through the addition of capsid proteins to SV40 chromatin.

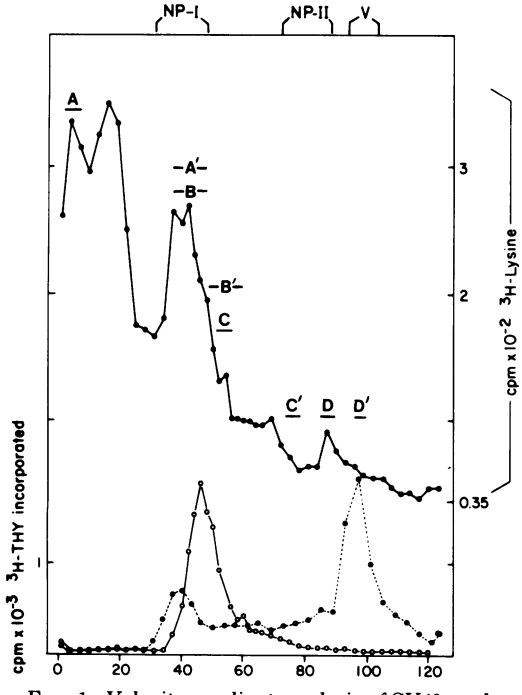

FIG. 1. *Velocity gradient analysis of SV40 nucleoprotein complexes pulse-labeled with [^{3}H]lysine for 5 min (●——●), [^{3}H]thymidine for 5 min (○——○), and with [^{3}H]thymidine for 24 h (●- - - -●). The SV40 nucleoprotein complexes were extracted from nuclei of SV40-infected CV-1 cells at 48 h postinfection and analyzed in a 5 to 40% sucrose gradient. Sedimentation is from left to right. Three groups of SV40 nucleoprotein complexes are marked on the top of the panel.*

CsCl gradient analysis of pulse-labeled SV40 nucleoprotein complexes. An empty capsid of polyoma virus has a sedimentation coefficient of about 140S (10). The appearance of labeled capsid proteins sedimenting around 140S (corresponding to about the fraction 70 to 80 region in Fig. 1 and 2) during a 10-min [^{3}H]lysine pulse can be interpreted to be either in the form of "empty virions," as suggested by previous workers (12, 13), or in the form of nucleoprotein complexes, as suggested above. These two possibilities can be distinguished by CsCl gradient analysis. If labeled capsid proteins are in the form of empty virions, then they should band at a density of 1.30 g/ml in the CsCl gradient. Figure 5a shows that the majority of capsid proteins labeled in a 10-min pulse (see also Fig. 3C) sedimented in the 140S bands at a density of less than 1.25 g/ml. During the chase, two-thirds of the label banded at a density of 1.30 g/ml, whereas one-third banded at the top of the gradient (Fig. 5b). However, upon fixation with glutaraldehyde, more than 90% of the label

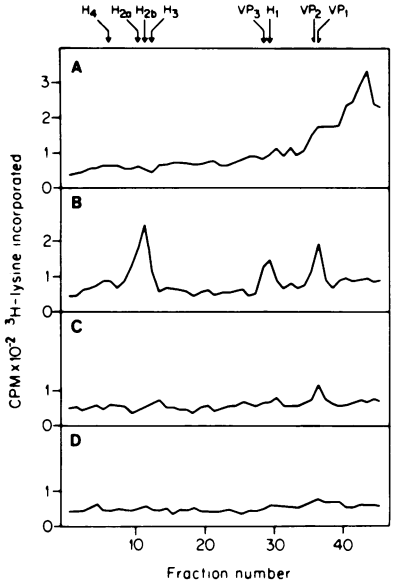

FIG. 2. *SDS-polyacrylamide gel electrophoretic analysis of pulse-labeled SV40 nucleoprotein complexes. SV40 nucleoprotein complexes pulse-labeled in vivo for 5 min with [^{3}H]lysine were extracted and fractionated in a 5 to 40% sucrose gradient as shown in Fig. 1. Fractions in Fig. 1 corresponding to A, B, C, and D were analyzed further in an SDS-gel.*

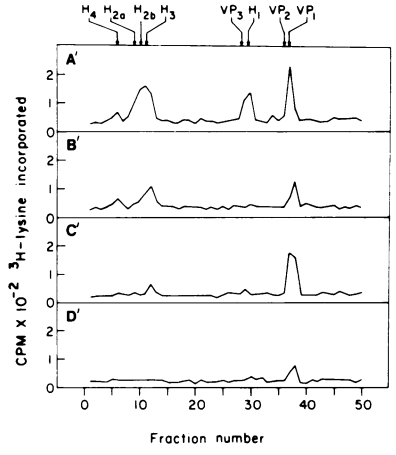

FIG. 3. *SDS-polyacrylamide gel electrophoretic analysis of SV40 nucleoprotein complexes pulse-labeled for 10 min with [^{3}H]lysine, Fractions A', B', C', and D' in Fig. 1, corresponding to the peak of NP-I replicating intermediates, NP-II, and virion, were analyzed.*

banded at a density of 1.34 g/ml (Fig. 5c), as expected for a nucleoprotein complex with a protein-to-DNA ratio similar to that of mature virus (about 90:10), whereas no material banding at a density of 1.30 g/ml could be seen. This result indicates that capsid protein sedimenting in the 140 to 180S region in the sucrose gradient is in the form of nucleoprotein complex and not in the form of empty virions. Empty virions, as observed in the unfixed sample in the CsCl gradient, are most likely the artifact formed from incompletely assembled virus by dissociating its

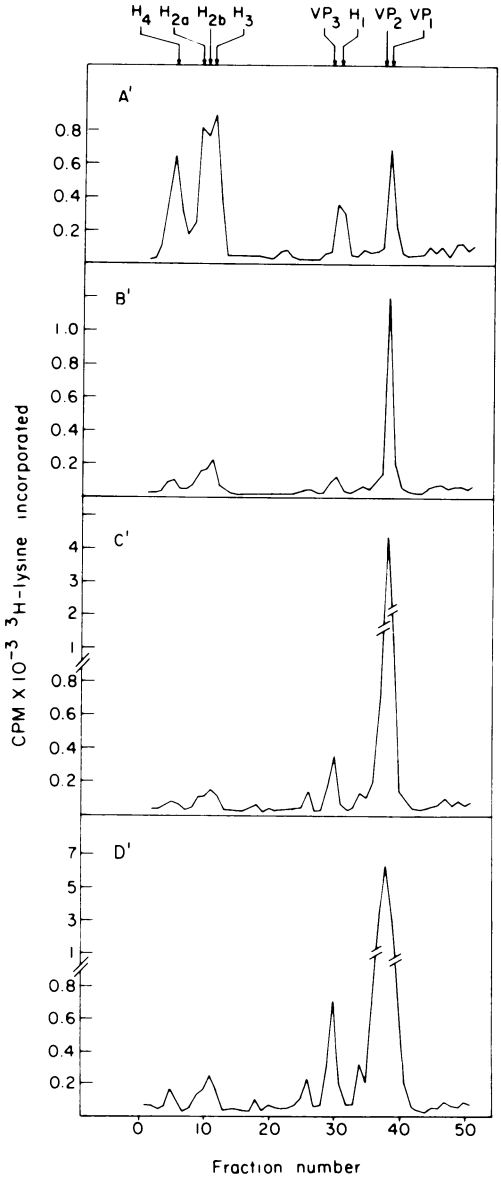

FIG. 4. *SDS-gel profile of the proteins in SV40 nucleoprotein complexes pulse-labeled for 10 min with [^{3}H]lysine and chased for 1 h with a 100-fold excess of unlabeled lysine. The same fractions in the sucrose gradient, as shown in Fig. 3, were analyzed.*

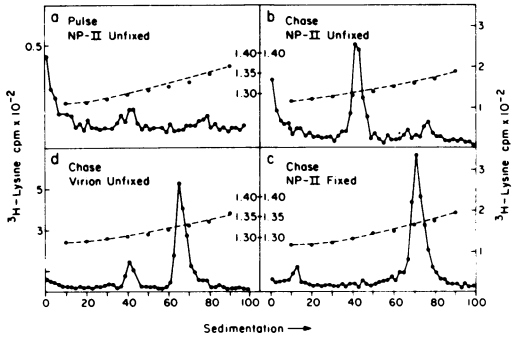

FIG. 5. *CsCl density gradient analysis of fixed and unfixed SV40 nucleoprotein complexes. (a) NP-II fraction pulse-labeled for 10 min with [^{3}H]lysine in vivo; (b) unfixed NP-II complexes pulsed for 10 min and chased for 1 h; (c) NP-II complexes, fixed with glutaraldehyde, pulsed for 10 min and chased for 1 h; (d) chased virion isolated from the sucrose gradient was analyzed in a 1.2- to 1.6-g/ml preformed CsCl gradient, as described previously (4). Symbols: ●, [^{3}H]lysine incorporated counts; ○, density of CsCl gradient as determined by refractive index.*

DNA and histone components from the capsid shell in high salt. This conclusion is supported by the electron microscopic and gel electrophoretic analyses of SV40 nucleoprotein complexes as discussed below.

Electron microscopic analysis of SV40 virion assembly intermediates. During the late cycle of infection, the amount of SV40 capsid proteins in the form of empty virions as analyzed in a CsCl gradient is abundant (6). Therefore, if empty virions are synthesized independently in the infected cells and are not the artifact generated in the CsCl gradient, one should be able to observe empty virions in the nuclear extract before CsCl gradient analysis. Empty virions purified from a CsCl gradient had an empty space in the center when positively stained with uranyl acetate and observed in an electron microscope (Fig. 6C). Such a structure was, however, rarely seen in the SV40 nuclear extract. Instead, a structure (Fig. 6B) with an empty shell was seen associated with SV40 chromatin. This structure was observed predominantly in the sucrose gradient fractions 70 to 80 in Fig. 1. When the SV40 nucleoprotein complexes in fractions 70 to 80 were first exposed to high salt (3 M ammonium acetate) and then examined in an electron microscope, the frequency of the structure shown in Fig. 6B decreased, whereas that of empty virions increased. These observations are consistent with the interpretation from pulse-chase experiments that most, if not all, empty virions are the dissociation products of assembly intermediates containing SV40 chromatin.

A series of possible virion assembly intermediates were observed in an electron microscope from sucrose fractions 70 to 80 (Fig. 7). All of them contain partially encapsidated SV40 genome. (DNA extracted from this region of the gradient consisted of only SV40 circles which comigrated with purified SV40 DNA in the agarose-gel.) Some of them (A to N) had the capsid only partially organized. These complexes were observed preferentially in the slower-sedimenting part of the fraction 70 to 80 region. Structures shown in Fig. 7 (O to T) had most of the capsid organized, whereas SV40 DNA was still not completely encapsidated. These structures were consistent with the pulse-chase experiment in which capsid proteins were gradually added onto and organized around SV40 chromatin. Pulse-labeling experiments with [^{3}H]thymidine showed that the slower-sedimenting part of the fraction 70 to 80 region was more preferentially labeled than that of the faster-sedimenting region, suggesting that the structures shown in Fig. 7A to N are possible precursors to those of Fig. 7O to T. If structures shown in Fig. 7A to N are indeed early precursors of SV40 virion, then the dissociation of newly added and still not organized capsid proteins in the CsCl gradient could account for the salt sensitivity of nascent labeled capsid proteins (Fig. 5a) and for the free proteins observed in Fig. 11A. Subsequent organization of capsid proteins into shell-like structures without full encapsidation of SV40 genome (Fig. 7O to T) would result in the for-

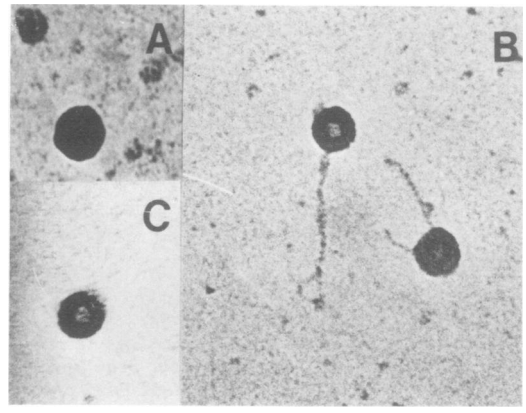

FIG. 6. *Electron micrographs of SV40 virion (A), empty virion purified in the CsCl gradient (C), and empty virion-like structure associated with SV40 chromatin as observed in NP-II nucleoprotein complexes isolated from sucrose gradient (B).*

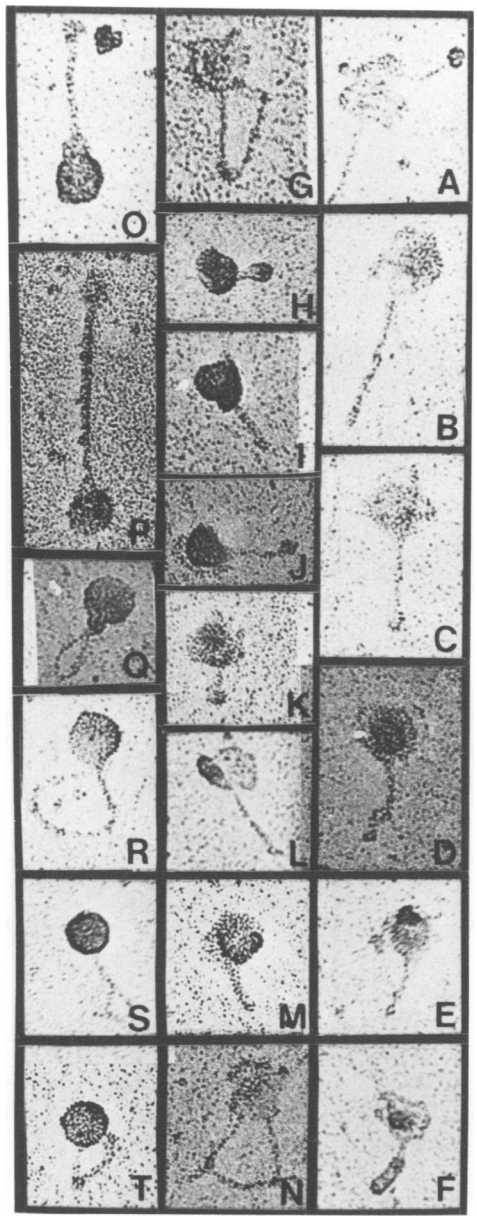

FIG. 7. *Electron micrographs of partially assembled SV40 virions as observed in NP-II fractions. The capsid is still not organized in (A) through (C). Partial organization is observed in structures (D) through (N). Uncapsidated SV40 chromatin fiber can be seen in each of these structures.*

mation of an empty capsid in the CsCl gradient (Fig. 5 and 11A).

Material sedimenting in the peak at fraction 96 consisted mostly of intact virion, as expected. A shoulder on the faster side of the peak was reproducibly observed. In this part of the gradient, a dimer, trimer, and polymer of SV40 virion could be observed. Some have only part of the capsid proteins organized (Fig. 8A to G and 9A to G).

In the bottom of the gradient, a polymer of virion attached to a long strand of chromatin, such as that shown in Fig. 9H, could be observed. Virus aggregates associated with membrane-like structures (Fig. 10A to E) and with unknown helical structures (Fig. 10F) could also be seen (see below).

Low-salt agarose-gel electrophoretic analysis. The presence of empty virions in the nuclear extract of SV40-infected cells was also analyzed by the low-salt agarose-gel electrophoresis technique of Varshavsky et al. (20). SV40-infected cells were double labeled with [^{3}H]lysine and [^{14}C]thymidine. Nuclear extracts were prepared and fractionated in a 5 to 40% sucrose gradient as described in the legend to Fig. 1. Materials present in different regions of the sucrose gradient were analyzed in a 0.4% agarose-gel. In all of the regions analyzed, the ^{3}H-label pattern followed that of the ^{14}C-label pattern (data not shown). (For brevity, these data are not shown, but will be supplied upon request.) The ^{3}H/^{14}C ratio remained more or less constant throughout the gel. In particular, no material labeled only with [^{3}H]lysine and devoid of [^{14}C]thymidine label was observed. Staining with both Coomassie brilliant blue (for proteins) and ethidium bromide (for DNA) yielded the same conclusion. Since empty virions could be isolated in large quantity in CsCl from the same nuclear extract, the absence of any significant quantity of material devoid of DNA indicated that there were no empty virions present in the nuclear extract. This result thus supports the conclusion obtained from pulse-labeling and electron microscopic analyses.

Comparison of protein composition of assembly intermediates, empty virion, and virions. A way to gain an understanding of the virus maturation step was to compare the difference in protein compositions between assembly intermediates and the mature virus. To this end we separated the mature virus from assembly intermediates by using a CsCl gradient. [^{3}H]lysine-labeled SV40 nucleoprotein complexes that cosedimented with mature virus (201S, from fractions 90 to 100 in Fig. 1) were analyzed in the preformed CsCl gradient. Electron microscopic examination showed that the material present in this part of the sucrose gradient was mostly mature virion and assembly intermediates (Fig. 7).

Three components, corresponding to free pro-

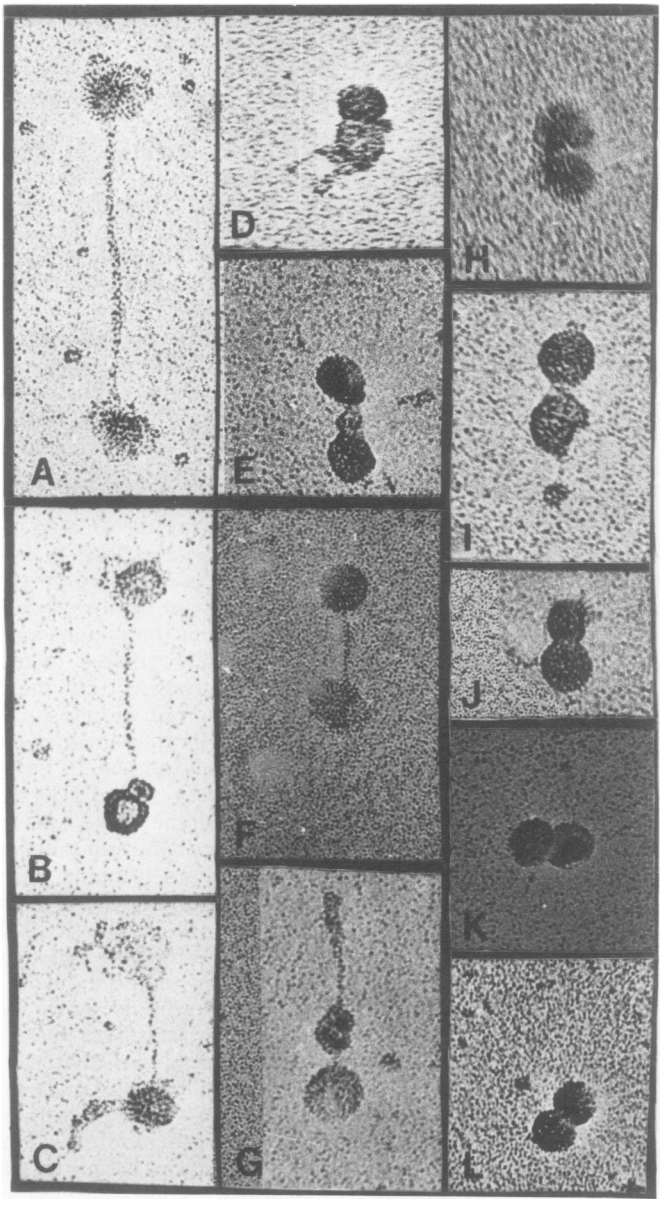

FIG. 8. *Electron micrographs of partially assembled SV40 virion dimers. A completely assembled dimer is shown in (L).*

teins, empty virion, and mature virion (Fig. 11A), were observed in the CsCl gradient. The protein compositions were subjected to analysis in the acid-urea-gel system of Panyim and Chalkley (14). The choice of this gel system was important for distinguishing between histone H1 and VP-3 protein which migrated at similar rates in the SDS-gel system. As shown in Fig. 11C, proteins dissociated from SV40 virion assembly intermediates contained all of the major histones and viral capsid proteins. (The presence of VP-1 and VP-2 proteins could not be demonstrated with this gel system. The presence of these two proteins was confirmed with the SDS-gel system [data not shown].) Empty virion, as expected, contained all of the capsid proteins, but without histones (Fig. 11D). The protein composition of mature virus that banded at a density of 1.34 g/ml was similar to that of dissociated proteins, except H1 histone protein. This result does not

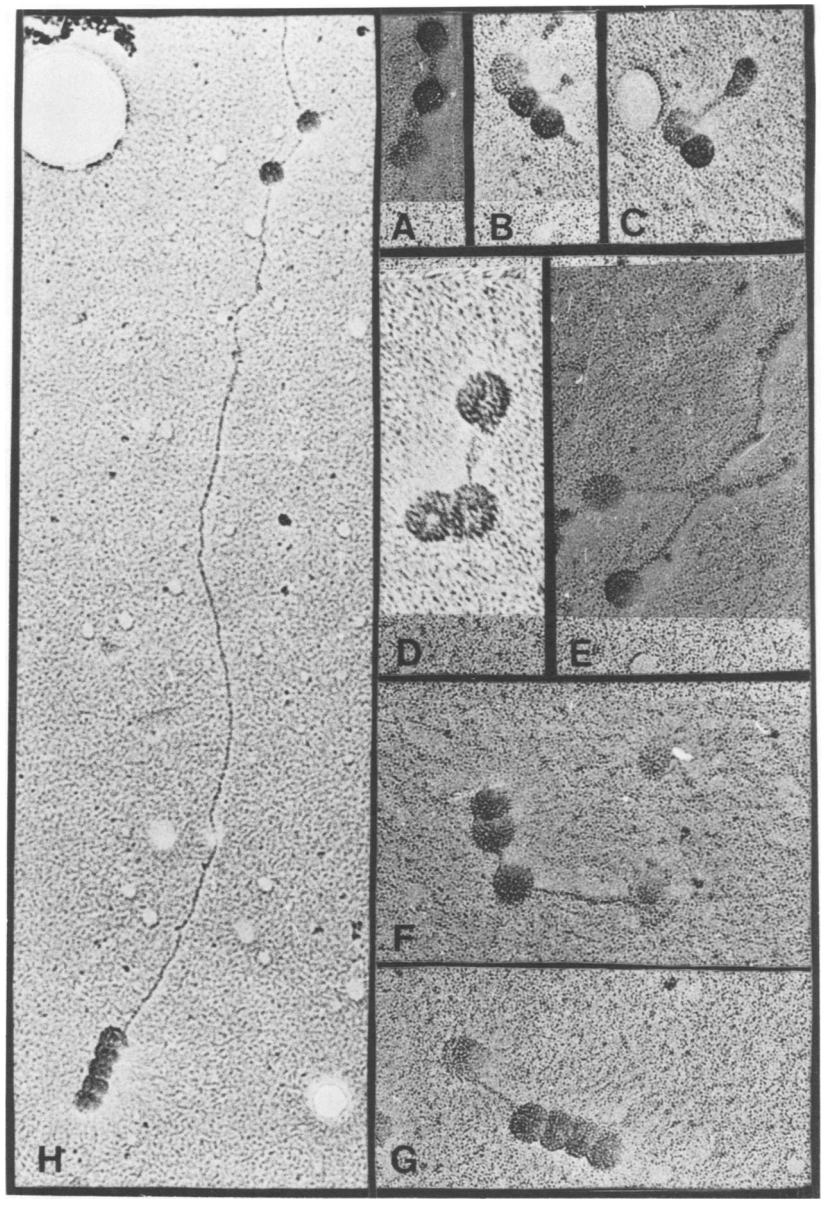

FIG. 9. *Structures of SV40 virion polymers. (A) through (E) correspond to partially assembled trimers, (F) corresponds to a partially assembled tetramer, and (G) corresponds to a partially assembled pentamer. A pentamer linked to a long chromatin (H) was observed in the pelleted fraction in the sucrose gradient.*

agree with the recent report by Nedospasov et al. that purified SV40 virus contains H1 histone (11). The absence of H1 in mature virus and its presence in virion assembly intermediates suggest that during the encapsidation step H1 histone becomes dissociated from SV40 chromatin.

A new protein species associated with virions migrating between H1 and VP-3 was observed in purified virion and dissociated proteins, but was absent in empty virion. The absence of this protein in empty virion makes it unlikely that it is either a contamination of dissociated protein in virion in the CsCl gradient or a host protein that binds nonspecifically to SV40 capsid.

DISCUSSION

The data presented in this report strongly suggest that empty virions are the dissociation

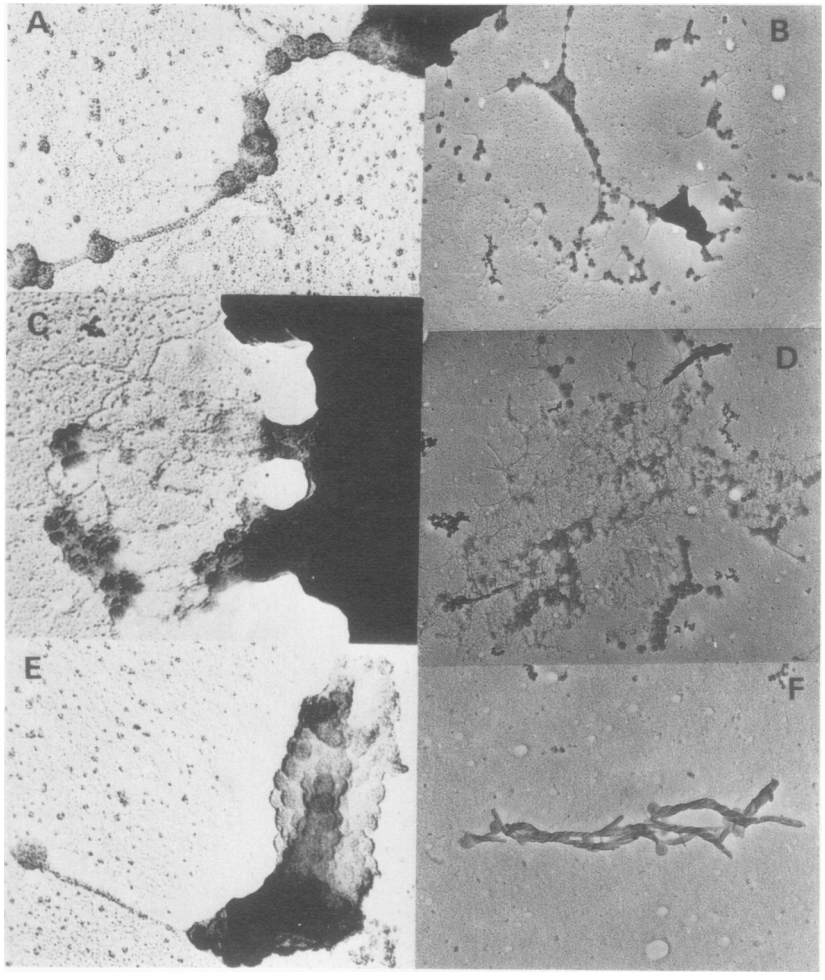

FIG. 10. *Various virion-associated structures observed in the pelleted fraction in the sucrose gradient. (A) Long chromatin fiber with virion-like structure distributed along the fiber; (B) SV40 virion aggregate associated with long chromatin fiber (top) and membrane (right corner); (C) SV40 virion released from nuclear pore of a partially lysed nucleus; (D) SV40 virion associated with a membrane-like structure; (E) SV40 virion crystal associated with membrane and a partially assembled virion; and (F) SV40 virion associated with unknown helical structure.*

products of partially assembled SV40 virions in the CsCl gradient and that SV40 virion formation is achieved by gradual addition to and organization of capsid proteins around SV40 chromatin. This conclusion rests on the following experimental evidence.

(i) Pulse-chase experiments with [^{3}H]lysine and [^{3}H]thymidine suggested that newly synthesized capsid protein was first added on SV40 chromatin and accumulated in NP-II and virion.

(ii) CsCl gradient analysis of glutaraldehyde-fixed material showed that all of the material sedimenting in the 140S region is in the form of nucleoprotein complex and not in the form of empty virion. Empty virions could be generated in the CsCl gradient if the sample was not fixed with glutaraldehyde.

(iii) Electron microscopic analysis of the nuclear extract of SV40-infected cells failed to reveal a structure corresponding to the empty virions purified from the CsCl gradient. In fact, a structure similar to empty virions could be generated by raising the salt conditions during the sample preparation for electron microscopy.

(iv) Agarose-gel electrophoresis of double-labeled material sedimenting around 140S in the sucrose gradient failed to reveal any significant material devoid of DNA. However, we could not completely exclude the possibility that empty capsid was formed in vivo but became immedi-

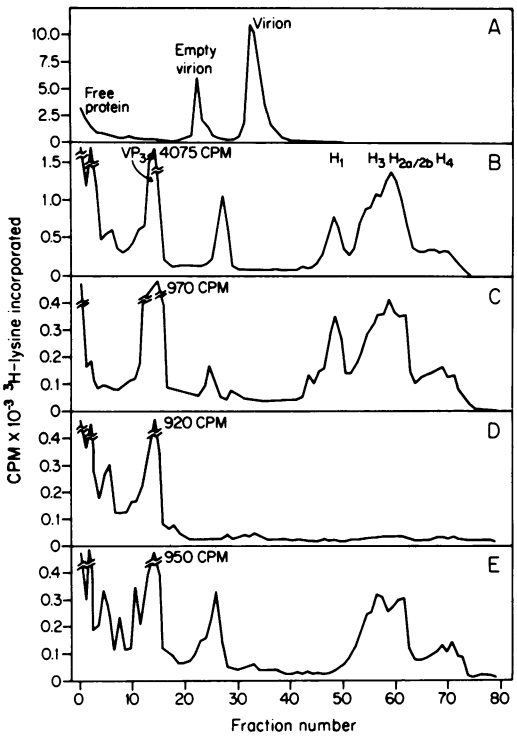

FIG. 11. *Acid-urea-gel analysis of SV40 virion and empty virion. SV40 nucleoprotein complexes labeled for 24 h with [^{3}H]lysine were extracted at 48 h postinfection and fractionated in the sucrose gradient. Fractions that cosedimented with SV40 virion purified from the CsCl gradient were pooled and analyzed in a preformed CsCl gradient (A). Three labeled components, corresponding to free proteins, empty virions, and mature virions, were observed. Materials present in the pooled sucrose gradient fractions (B), free proteins (C), empty virions (D), and mature virions (E) were dialyzed against buffer (0.01 M Tris [pH 7.5], 0.15 M NaCl), lyophilized, and analyzed by a modified acid-urea-gel electrophoresis technique (18).*

ately associated with SV40 chromatin. That the pulse-labeled capsid proteins in NP-II complexes were dissociated into free capsomeres in the CsCl gradient (Fig. 5a) renders it unlikely. It is also possible that SV40 empty virions were synthesized independently from capsid proteins, but that during extraction they became associated with SV40 chromatin. We do not think that this is likely because small amounts of labeled SV40 chromatin (NP-I), when coextracted with unlabeled SV40-infected cells, did not change their sedimentation velocity. Furthermore, empty virions were not observed in freeze-thawed nuclei with an electron microscope.

The disruption of immature virions in the CsCl gradient was also observed with adenoviruses (2, 3). Therefore, the interpretation of the CsCl gradient analysis of virion assembly must be viewed cautiously. Our results, using other analytical procedures to study SV40 virion formation, do not necessarily contradict the results obtained by Ozer and Tegtmeyer (13), using CsCl gradient analysis alone. The empty virions, as we have shown, are only part of the precursors to SV40 virions.

The structures of virion assembly intermediates observed in an electron microscope (Fig. 7) suggest that the partially packaged SV40 chromatin should be sensitive to nuclease digestion. Such an analysis is being studied with micrococcal nuclease. Our preliminary results indicate that SV40 DNA in the assembly intermediates can be digested with micrococcal nuclease into fragments corresponding to the length of DNA present in the nucleosome. Most of the fragmented DNAs, however, are still held together by the capsid proteins because they still sediment in the 140S region in the sucrose gradient.

Based on the data that we obtained, we propose a model (Fig. 12) that SV40 virion is assembled by a gradual addition of capsid proteins to SV40 chromatin and subsequent organization of the capsid into a salt-resistant shell. Several interesting questions arise from this model. (i) Is there a region of SV40 genome that the capsid proteins first deposit to serve as the initiation site for further addition of capsid proteins? It has been reported previously (17) that there are VP-1 proteins tightly bound to SV40 DNA isolated from SV40 chromatin. Whether these VP-1 proteins serve as the center of capsid formation will be the subject of future analysis. (ii) How are the capsid proteins organized into a salt-resistant shell? We believe that phosphorylation of VP-1 proteins per se probably is not the cause of such a process because the VP-1 proteins in all of the nucleoprotein fractions are all phosphorylated (M. Coca-Prados, G. Vidali, and M.-T. Hsu, manuscript in preparation). The possibility that capsid is organized through an intermolecular disulfide bond between VP-1 proteins, as suggested by Walter and Deppert (21), remains to be determined.

Polymers of SV40 virion and their assembly intermediates were observed in an electron microscope (Fig. 8 through 10). They were seen both in the nuclear extract and in freeze-thawed

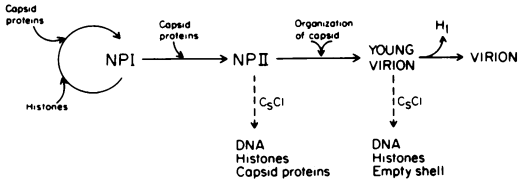

FIG. 12. *Model of SV40 virion assembly.*

nuclei. The significance of such structures in the formation of mature SV40 virion is not clear at the moment. Recently, Rigby and Berg (16) have demonstrated the presence of large amounts of linear polymers of SV40 DNA late in the infectious cycle. The structures that we observed in Fig. 9H and 10A and B may represent the assembly intermediates of these linear polymers. A headful mechanism of bacteriophages may be operative to package polymer DNA into SV40 virion. Enzymes that cleave polymer DNA into unit length and circularize the free ends generated are required for such a process. Failure of circularization may account for the observation by Gruss and Sauer (5) of linear unit length SV40 DNA accumulated late in infection and the encapsidation of SV40-sized host DNA to form pseudovirions (17).

We found that histone H1 was present in virion assembly intermediates but not in purified virion. The observation that H1 was not present in mature SV40 virions agrees with most investigators (8, 9, 15, 19; G. Vidali, personal communication), but not with Nedospasov et al. (11). The cause of such a discrepancy is still unknown. We believe that histone H1 is present in virion assembly intermediates. [^{3}H]lysine- or [^{3}H]thymidine-labeled material sedimenting in the region of NP-II and purified SV40 virion bands in a single peak at a density of 1.35 g/ml in the CsCl gradient after fixation with glutaraldehyde. This result rules out the possibility that H1 histone observed is derived from the contaminating chromatin (which bands at 1.45 g/ml) that happens to sediment in that part of the sucrose gradient. The presence of H1 histone in assembly intermediates but not in the mature virion led us to speculate that replacement of histone H1 by VP-3 is necessary to condense SV40 DNA inside the virion and to stabilize the interaction between the capsid shell and the SV40 DNA-histone complex. Such a model will be the subject of our further analysis of SV40 virion assembly.

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