Intracellular Forms of Simian Virus 40 Nucleoprotein Complexes

III. Study of Histone Modifications

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The modification patterns of histones present in various forms of intracellular simian virus 40 nucleoprotein complexes were analyzed by acetic acid-urea-polyacrylamide gel electrophoresis. The results showed that different viral nucleoprotein complexes contain different histone patterns. Simian virus 40 chromatin, which contains the activities for the synthesis of viral RNA and DNA, exhibits a histone modification pattern similar to that of the host chromatin. However, virion assembly intermediates and mature virions contain highly modified histones. Pulse-chase experiments with [3H]lysine showed that the newly incorporated histones in the virion assembly intermediates were already highly modified. The majority of in vivo acetylation activity of histones occurred on the 70S simian virus 40 chromatin as analyzed by pulse-labeling with [3H]acetate. These results and our previous analysis of the virion assembly pathway suggest that three stages are involved in the packaging of simian virus 40 chromatin into the mature virion: (i) modification of histones, (ii) accumulation of capsid protein around the chromatin with highly modified histones, and (iii) organization of capsid proteins into salt-resistant shells. The role of histone modification in virion assembly is discussed.

In simian virus 40 (SV40) and polyoma virus, viral DNAs are associated with histones derived from the host cells (6). The histones in the mature virion, however, show both qualitative and quantitative differences from the histones in the host chromatin. Besides the absence of H1 histones, the histones in mature SV40 and polyoma virus were found to be acetylated to a much higher degree than the cellular histones (2–4, 9, 11, 17). Recently, the histones in the intracellular viral nucleoprotein complexes (NPC) were also found to be highly modified (2, 3). However, the viral NPC used in these studies were obtained by extraction from the infected cells with Triton buffer, which we have shown to cause disruption of mature virions and virion assembly intermediates. Since virions and virion assembly intermediates accumulate late in infection, the 70S NPC studied by previous investigators are probably composed of both the genuine 70S viral chromatin and the disrupted virions. Thus, it is rather unclear whether the highly modified histone patterns observed in the 70S NPC represent the true 70S viral chromatin.

In our earlier report we developed a gentle extraction method for isolating intact intracellular forms of viral NPC (7). Three forms of viral NPC could be obtained by this extraction method: (i) mature virus (V), (ii) virion assembly intermediate (NPII), and (iii) the 70S viral chromatin (NPI) which is the precursor to NPII complex and contains the major activities for synthesizing viral RNA and DNA. The purpose of the present communication was to reinvestigate the histone modification patterns in the various forms of intracellular SV40 NPC extracted by the gentle procedure. The results showed that the histones in mature virions and virion assembly intermediates (NPII) were modified to a much higher extent than those of SV40 and cellular chromatins. Furthermore, we performed pulse-labeling experiments and showed that extensive modification of histones in virion assembly intermediates occurred before the accumulation of capsid proteins around SV40 chromatin.

MATERIALS AND METHODS

Cells and growth of SV40 virus. SV40 virus strain SWS was grown on CV-1 cells as described previously (7).

Radioactive pulse-labeling of SV40-infected cells. All pulse-labeling experiments were performed in a 37°C room. For pulse-labeling with [3H]lysine, cells were first incubated for 10 min in lysine-free medium (GIBCO Laboratories) supplemented with 2% dialyzed fetal calf serum. The medium was then replaced by a medium containing 200 to 400 μCi of [3H]lysine (60 to 80 Ci/mmol; Amersham Corp.) per ml for the period desired. At the end of the pulse
period, the petri dish was immediately placed on ice, and the radioactive medium was replaced by ice-cold Tris-buffered saline. For chase experiments with lysine, the radioactive medium was withdrawn, and the cells were washed once with 10 ml of medium containing 15 mg of unlabelled lysine-HCl per ml and 2% fetal calf serum and overlaid with the same medium for the chase period desired.

Cells were pulse-labeled with 5 mCi of [3H]acetate (2 to 5 Ci/mmol; Amersham Corp.) per ml for 10 or 30 min. Procedures for terminating pulse-labeling follow those described for [3H]lysine pulse-labeling.

Extraction of intracellular SV40 NPC. SV40 NPC were extracted from the nuclei of SV40-infected cells, as described previously (7). Briefly, infected cells were washed with Tris-buffered saline and lysed with 0.5% Nonidet P-40. The nuclei were suspended in TD buffer (25 mM Tris-hydrochloride, pH 7.4, 0.136 M NaCl, 7 mM KCl, 0.7 mM Na2HPO4) and homogenized in a tight-fitting Dounce homogenizer for about 30 strokes. Cell nuclei were pelleted by centrifugation (1,000 x g), and the extract was layered on a 5 to 40% sucrose gradient containing 10 mM Tris, pH 7.4-0.5% phenylmethylsulfonyl fluoride and spun in an SW40 rotor at 37,000 rpm for 70 min.

Gel electrophoresis analysis of histone. Discontinuous sodium dodecyl sulfate-gel electrophoresis was performed by the method of Laemmli (10). Polyacrylamide gels, 12% (13 by 22 cm by 1.5 mm), were used to analyze histones present in SV40 NPC. For studying the histone modification pattern, the acid-urea-polyacrylamide gel electrophoresis system as modified by Shmatchenko and Varshavsky was used (15). SV40 NPC were dissolved in 9 M urea-3% mercaptoethanol-0.9 M acetic acid-0.5% cetyltrimethylammonium bromide and electrophoresed in a 15% polyacrylamide gel (13 by 33 cm by 1.5 mm) at 17 V for 34 h at 4°C. Gels were fixed in 50% trichloroacetic acid overnight at 4°C and stained with 0.1% Coomassie blue in 50% trichloroacetic acid. Destaining was achieved by repeated washings with a solution containing 5% methanol and 7% acetic acid. Gels were spliced into 1-mm slices for radioactive counting.

RESULTS

Electrophoretic patterns of histones in SV40 NPC. SV40 NPC were isolated from SV40-infected CV-1 cells at 48 h postinfection as described previously (7). The extract was further fractionated in a 5 to 40% sucrose gradient into three subspecies of viral NPC: (i) SV40 chromatin (70S), (ii) replication intermediates (100S), and (iii) virion and virion assembly intermediates (150 to 210S). The histones present in these various fractions of intracellular NPC were analyzed by acetic acid-urea-polyacrylamide gel electrophoresis as described by Panym and Chalkley (12). This gel system is particularly useful in analyzing histone modification patterns in which modified species migrate as slower bands (8, 12). Such analysis reveals a rather distinctive modification pattern of histones present in SV40 chromatin and in mature virus purified from CsCl gradients (Fig. 1B and C). The histones present in SV40 chromatin are less modified and resemble cellular histone profile (Fig. 1A and B), whereas purified virus lacks...
The most prominent differences between the histones in mature virus and those in viral chromatin are the presence of highly acetylated forms of histone H4 subfractions and the relative reduction of the H2a-H2b peak in mature virus (Fig. 1C). The reduction of the H2a-H2b peak in mature virus is not due to the deficiency of these histones in mature virus, since sodium dodecyl sulfate-polyacrylamide gel analysis failed to reveal any quantitative differences of these histones between mature virus and viral chromatin (unpublished data). Thus, these results indicate that histones H2a or H2b in SV40 virion, or both, are modified to a higher extent than those in viral or cellular chromatin.

Virion assembly intermediates isolated from sucrose gradients contain the same histone pattern as that in mature virus except that histone H1 is present in these complexes. The extensive modification pattern of virion assembly intermediates as compared with that of viral chromatin can be seen in Fig. 2.

The high level of histone acetylation in SV40 virion and virion assembly intermediates relative to the viral chromatin was also confirmed by the analysis of the content of ε-acetyllysine in these complexes. The content of ε-acetyllysine in SV40 virions and virion assembly intermediates is about five times higher than that in SV40 chromatin (Table 1).

Thus, these results demonstrate that there are two histone modification patterns in intracellular SV40 NPC: (i) a less modified pattern associated with viral chromatin which is involved in the biosynthesis of viral RNA and DNA (7), and (ii) an extensively modified pattern in the virions and virion assembly intermediates.

Electrophoretic pattern of newly incorporated histones in SV40 NPC. The results described above show that the histones in SV40 chromatin and virion assembly intermediates are modified to different extents. Previously we have shown that virion assembly intermediates are derived from SV40 chromatin (4, 7). Thus, the conversion of SV40 chromatin into virion assembly intermediates is associated with the modification of histones. However, it is not clear whether the change in histone modification pattern occurs early during the assembly or during the maturation of virions. To analyze this problem we pulse-labeled SV40-infected CV-1 cells at 48 h postinfection with [3H]lysine for 90 min. During this relatively short pulse period, labeled histones were observed only in early virion assembly intermediates (4), as most of the labeled protein could be disassociated from SV40 DNA in the CsCl gradient. The histones present in the pulse-labeled SV40 chromatin and virion assembly intermediates were analyzed in acid-urea gel. The results are shown in Fig. 3. The labeled histones which just become detectable in the newly formed virion assembly intermediate (150 to 180S in the sucrose gradient) are already extensively modified as in the mature virus. In a separate experimental protocol, the histones in SV40 chromatin were briefly pulsed and then chased into virion assembly intermediates. During a short pulse (2 to 5 min) labeled histones were observed in the positions of SV40 chromatin and replication intermediates (5), but no radioactivity in the position of virion assembly intermediates could be detected (Fig. 4A and B). During a brief pulse for 2 min followed by a chase with a 100-fold excess of unlabeled lysine

FIG. 2. Acid-urea-polyacrylamide gel electrophoresis of histone modification patterns in SV40 chromatin (left column) and SV40 virions and virion precursors (right column) isolated from a sucrose gradient as described previously (6).
for 1 h, labeled histones began to be observed in the virion assembly intermediates (complete conversion of newly formed SV40 chromatin into virion assembly intermediate requires at least 4 h of chase, depending on the period of viral infection). Again, the newly incorporated histones present in the virion assembly intermediates were highly modified as compared with SV40 chromatin (Fig. 4C, and C3) which is similar to the steady-state pattern shown in Fig. 1.

The pulse-chase experiment also revealed a subtle change of histone pattern in the newly synthesized SV40 chromatin. Histone H4 is more highly acetylated in the briefly pulsed chromatin than in the chased steady-state labeled chromatin (Fig. 4A1-A3). On the other hand, histone H3 is less modified in the newly synthesized chromatin than in the chased chromatin. These results are similar to those observed in cellular chromatin (8).

**Analysis of rate of acetylation of histones in SV40 NPC.** The results described above show that newly incorporated histones in young virions are already highly modified. These data, however, do not tell us when the modification of histones in virion assembly intermediates occurs. Modification could occur in a fraction of SV40 chromatin before the accumulation of capsid proteins onto these modified chromatin. Alternatively, modification of histones could happen after the formation of virion assembly intermediates. To distinguish these possibilities we studied the kinetics of one of the major histone modifications, acetylation, by pulse-labeling with [3H]acetate (a pulse-labeling study of histone phosphorylation was not carried out because of the long equilibration time of the 32P-labeled pool). A short pulse with [3H]acetate should label the complex. On the other hand, if acetylation of histone occurs in the 70S chromatin and then accumulates in the virion assembly intermediates, then only 70S chromatin will be preferentially labeled during a short pulse with [3H]acetate. When SV40-infected CV-1 cells were pulse-labeled with [3H]acetate for 10 min, the majority of histone acetylation was observed in the 70S SV40 chromatin fraction instead of in the virion assembly intermediates (Fig. 5). A longer labeling period results in the accumulation of label in virion assembly intermediates (unpublished data). These results suggest that the hyperacetylation of histones in the SV40 virion probably takes place in SV40 chromatin before the accumulation of capsid proteins. The distribution of [3H]acetate in the pulse-labeled histones in SV40 chromatin was also analyzed in acid-urea-polyacrylamide gel. The result is shown in Fig. 6. As expected, histone H1 is not labeled. Since H1

**Table 1. e-Acetyllysine content in SV40 virion, virion assembly intermediates, and viral chromatin**

<table>
<thead>
<tr>
<th>Complex</th>
<th>H2a, H2b, H3, H4 (input cpm)</th>
<th>e-Acetyllysine (cpm)</th>
<th>Lysine as e-acetyllysine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70S chromatin</td>
<td>1.70 x 10^6</td>
<td>2,310</td>
<td>1.36</td>
</tr>
<tr>
<td>Assembly intermidates</td>
<td>3.84 x 10^6</td>
<td>2,737</td>
<td>7.12</td>
</tr>
<tr>
<td>Virion</td>
<td>1.98 x 10^4</td>
<td>1,203</td>
<td>6.08</td>
</tr>
</tbody>
</table>

* SV40 nucleoprotein complexes were labeled for 24 h with [3H]lysine and fractionated in the sucrose gradient as described previously (7). Analysis of e-acetyllysine was performed as described by La Bella et al. (9).
has an acetyl group at its amino termini added during its synthesis in the cytoplasm (13), this result indicates that the majority of labels observed in Fig. 5 and 6 are due to the internal lysine acetylation. The data in Fig. 5 and 6 show that histones H3, H4, and H2a in 70S chromatin are the major histone species acetylated during a short pulse.

DISCUSSION
We have presented evidence that the histone modification patterns in SV40 chromatin are strikingly different from those of SV40 virion and virion assembly intermediates. Whereas the histones present in the latter NCP are unusually highly modified, the SV40 chromatin contains essentially unmodified histones. In some of the previous reports (2, 3), histones present in the 70S SV40 chromatin extracted by the Triton method were found to be highly modified. The discrepancy between the present data and the previous report is most likely due to the contamination of 70S SV40 chromatin by the disrupted virions or virion assembly intermediates when SV40 complexes were prepared by Triton extraction. Disruption of virions during Triton extraction was shown by our laboratory previously (7). Thus, the present results show that there are two distinct histone modification patterns in the intracellular SV40 NCP during lytic infection of CV-1 cells, an essentially unmodified histone pattern in the bulk of SV40 chromatin and a very highly modified pattern in SV40 virions and virion assembly intermediates.

Since we have shown in the previous reports (4, 7) that SV40 virion assembly intermediates and mature virions are derived from the 70S SV40 chromatin, the differences in the histone
modification patterns between these complexes suggest that changes of histone modification patterns accompany the transition from SV40 chromatin to virion assembly intermediates. Our results with \(^{3}H\)lysine pulse and chase suggest that the changes in histone patterns occur early during the genesis of SV40 virion. Pulse-labeling experiments with \(^{3}H\)acetate indicate that there is no major acetylation activity of histones in the virion assembly intermediate. Instead, most of the acetylation activity was detected in the 70S SV40 chromatin fraction. This result suggests that the hyperacetylation of histones in virion assembly intermediates occurs before the accumulation of capsid proteins. Although we cannot address the question of when histone phosphorylation might occur because of experimental difficulties, our data of the steady-state \(^{32}P\)-labeling pattern of histones in the different fractions of SV40 NCP do not show a significantly higher degree of phosphorylation of histone in virion as compared with SV40 chromatin (unpublished data). These results and our previous analysis of the SV40 virion maturation pathway indicate that transformation of SV40 chromatin into mature virus involves at least three stages in the following order: (i) hyperacetylation of histones, (ii) accumulation of capsid proteins, and (iii) organization of capsid proteins into a salt-resistant shell.

The biological function of the unusually extensive modification of histones in the SV40
virion is unknown. The presence of very highly modified histones in SV40 virion and virion assembly intermediates but not in SV40 chromatin suggests that modification of histones is necessary for the efficient interaction between nucleosomes and capsid proteins for packaging SV40 DNA-histone complexes. This may be the mechanism by which the pool of SV40 chromatin destined for metabolic processes (synthesis of RNA and DNA) and the pool of chromatin for the production of progeny virus can be distinguished. These two pools of chromatin are presumably equilibrated by the action of histone modification and demodification enzymes. The hypothesis that hypermodification of histones is necessary for the efficient packaging of SV40 chromatin is supported by the following observations: (i) nucleosome in virion assembly intermediates remained associated with capsid structures after micrococcal nuclease digestion, suggesting strong interaction between the highly modified nucleosome and capsid proteins; (ii) preliminary results of in vitro assembly using native 70S SV40 chromatin and the 70S minichromosome derived from mature virus showed that the latter is more efficient in partial reassembly with capsids (Coca-Prados and Hsu, unpublished data); (iii) host range mutants of a related virus, polyoma, are deficient in modified histones (14), and the efficiency of virus production is also suppressed, in spite of apparently normal synthesis of capsid proteins (16). Further studies are necessary to characterize the role of histone modifications in the interaction between the SV40 chromatin and capsid proteins for the formation of SV40 virion.

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ADDITION

After the completion of this manuscript F. La Bella and C. Vesco (J. Virol. 33:1138-1150, 1980) published similar results.

LITERATURE CITED