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Biochemistry

Rapid turnover of acetyl groups in the four core histones of simian virus 40 minichromosomes

(chromatin/histone acetylation)

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ABSTRACT **The four core histones (H2a, H2b, H3, and H4) bound to simian virus 40 minichromosomes isolated from infected cells contain rapidly labeled acetyl groups in internal positions of the histone polypeptide chain. Upon chase, these acetyl residues decay with a half-life of less than 15 min. The acetyl groups are incorporated in histones bound to mature chromosomes and not in newly synthesized histones bound to replicating viral chromosomes. The rate of acetate incorporation is not related to the degree of steady state acetylation of the individual viral or cellular histones. This rate is 4-fold higher for the viral chromatin than for its cellular counterpart isolated from the same nuclei. The possible role for histone acetylation in viral genome expression is discussed.**

The circular double-stranded DNA of simian virus 40 (SV40) is associated in virions and in the infected cells with histones H2a, H2b, H3, and H4 (1). The fifth histone, H1, is found only in viral nucleoprotein complexes (minichromosomes) extracted from infected cells (refs. 2 and 3 and the present study). Electron microscopy and biochemical studies have shown that viral and cellular chromatins are very similar, if not identical (4–8). The replicating viral DNA is associated with histones as is the replicating cellular DNA (9, 10). The viral nucleoprotein complexes are the template for viral RNA synthesis in the infected cells (11, *). Hence, the viral DNA–histone complex is a useful model system for the study of the mechanisms of replication and transcription of the cellular chromosome.

Although the knowledge of the basic features of the nucleosome structure is rapidly progressing (12, 13), the possible modifications of this structure during transcription, replication, and mitosis are still obscure. Actively transcribed genes contain modified nucleosomes as revealed by their increased sensitivity to the action of DNase I (14). Electron microscopic observations and biochemical studies indicate that, although histones are present on nonribosomal active genes, RNA polymerase can nevertheless elongate RNA chains *in vivo* and under certain conditions *in vitro* (11, 13, *).

In an attempt to understand the role of histone modifications in gene regulation, we have examined the acetylation of histones bound to viral and cellular DNA in monkey cells infected with SV40. We report here that the core histones H2a, H2b, H3, and H4 contain rapidly turning over acetyl groups. Histones H3 and H4 bound to the viral DNA are found to be more highly acetylated than those bound to cellular DNA. The rate of acetylation of the histones bound to the viral DNA is about 4-fold higher than of those bound to the cellular DNA of the same infected cells.

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MATERIALS AND METHODS

Cells and Virus Infection. African green monkey kidney cells, line CV1, were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum in 88-mm plastic dishes in an air/CO_2 incubator. Plaque-purified SV40 was used to infect the cells at an input multiplicity of 30–50 plaque-forming units per cell. At the end of a 2-hr adsorption period, 10 ml of medium containing 1% serum was added to the infected cultures.

Labeling of Cells. [^{14}C]Thymidine (52.8 mCi/mmol, Commissariat à l'Energie Atomique) (1 Ci = 3.7×10^{10} becquerels), 5 μCi per plate, was used to label DNA for 16 hr; [^{3}H]thymidine (35 Ci/mmol), 500 μCi per plate in 1 ml of medium, was used to label DNA for 10 min. For acetate labeling, sodium [^{3}H]acetate (20 Ci/mmol) was dissolved in fresh medium and 9.3 mCi in 1 ml was added per plate for 10-min labeling.

Purification of Nucleoprotein Complexes and of Cellular Histones. Cultures were pulse-labeled with [^{3}H]acetate at 41 hr postinfection. After labeling, the medium containing the radioisotopes was removed, the monolayer was washed twice with phosphate-buffered saline, the nuclei were isolated in the presence of 0.5% of Nonidet P-40, and the nucleoprotein complex was extracted with 0.2% Triton X-100 and 0.2 M NaCl (7) by two successive treatments for 30 min at 20°C. Approximately 70% of the viral DNA was extracted by this procedure, and the final DNA yield was 5–7 μg per plate. The Triton supernatant was obtained after centrifugation (800 × *g* at 4°C), and the nucleoprotein complexes were partially purified by sedimentation on 5–20% (wt/vol) sucrose gradients in 0.01 M Tris·HCl (pH 7.9)/0.001 M EDTA/0.2 M NaCl for 95 min at 40,000 rpm in an SW 41 rotor at 4°C. Fractions were collected and assayed for radioactivity, and protein was precipitated with 20% trichloroacetic acid in the presence of 100 μg of protamine as carrier. After centrifugation, the protein pellet was washed successively with acidic acetone, acetone, and ether and dissolved in the appropriate sample buffer for polyacrylamide gel electrophoresis. The nuclear pellet remaining after the Triton X-100 extraction was suspended in 0.5 ml of water by brief sonication, and the histones were then extracted with 0.2 M H_2SO_4. After precipitation with 5 vol of ethanol, the histone pellet was dissolved in sample buffer.

Gel Electrophoresis. Three different methods were used for analysis of histones on 14-cm slab gels: 17.5% polyacrylamide gels in the presence of sodium dodecyl sulfate ($NaDodSO_4$) and acid urea, 15% gels containing 2.5 M urea as described (9), or

Abbreviations: SV40, simian virus 40; $NaDodSO_4$, sodium dodecyl sulfate.

* P. Gariglio, R. Llopis, P. Oudet, and P. Chambon, unpublished results.

acid urea gels containing Triton X-100 (15). Gels were stained for 1 hr with Coomassie blue, destained, photographed, impregnated with 2,5-diphenyloxazole (16), dried, and autoradiographed with sensitized films by exposure at −70°C.

RESULTS

Viral Histones H3 and H4 Were More Highly Acetylated than Cellular Histones. Mild nonionic detergent treatment of nuclei isolated from SV40-infected cells permits the preferential solubilization of native viral minichromosomes (4, 7). These nucleoprotein complexes can be purified further by zonal sedimentation on sucrose gradients as shown in Fig. 1. Portions of the gradient were pooled, and their histone content was analyzed on acid/urea/polyacrylamide gels. As shown in Fig. 2*A*, the concentrations of the four core histones (H2a, H2b, H3, and H4) paralleled those of the viral DNA labeled with [^{14}C]thymidine for 16 hr. The H4 pattern reveals that the viral chormatin contained four different species of H4 histone. When compared to cellular histones extracted from normal or butyrate-treated cells (Fig. 2*A*), the viral DNA-bound H4 histone migrated identically to the highly acetylated H4 isolated from butyrate-treated cells (17). Since H4 contains an *N*-acetylserine in its NH_2-terminal position (18, 19), the second, third, and fourth bands correspond to one, two, and three internal acetylations (17, 20). The nuclear pellet remaining after the extraction of viral minichromosomes was sonicated, and the cellular histones were acid-extracted and analyzed on the same polyacrylamide gel. The cellular H4 contained only a monoacetylated species as shown in Fig. 2*A*. Although less well separated than H4 in this gel, the viral H3 histone contained acetylated forms (migrating slower than the main H3 band) similar to highly acetylated H3 extracted from butyrate-treated cells.

Rapid Acetate Incorporation in Nonreplicating Viral Minichromosomes. Short pulse labeling (5–10 min) of infected cells with [^{3}H]thymidine permits the preferential labeling of replicating intermediates. These replicating minichromosomes sediment about 1.3 times faster than the mature viral chromosomes (9, 10). Short labeling of infected cells with [^{35}S]-methionine showed that newly synthesized histones associate preferentially with the replicating intermediates (9). At least 30–50% of the histones synthesized in a 10-min pulse were associated with the fast-sedimenting replicating intermediates. Electron microscopic observations revealed that nucleosome-like beads are present on both the parental DNA and on the daughter double helices (10). To test whether newly synthesized histones are found in higher acetylated forms, we labeled the infected cells for 10 min with [^{3}H]acetate. As shown in Figs. 1 and 2, the [^{3}H]acetate counts cosedimented with the mature viral minichromosomes previously labeled with [^{14}C]thymidine, not in the position of replicating complexes. In addition, acetate was incorporated in soluble nuclear nonhistone proteins found on the top of the gradient. NaDodSO$_4$/polyacrylamide gel analysis of proteins from a similar gradient of viral chromatin labeled with [^{3}H]acetate for 10 min *in vivo* is shown in Fig. 3. Again, little acetate label was found in fraction 2, which contained the replicating complexes. The majority of acetate label was found in fraction 3, which contained the mature complexes. The specific activities of acetate label in replicating and mature complexes were calculated from the stain and the autoradiography of the gel and were found to be roughly equal. To exclude artifactual acetylation arising during the isolation of the viral minichromosomes, we mixed [^{3}H]acetate pulse-labeled noninfected cells with [^{14}C]thymidine-labeled infected cells and extracted the viral DNA histone complex. No acetate label was found associated with the viral histones; only the cellular histones were labeled (results not shown).

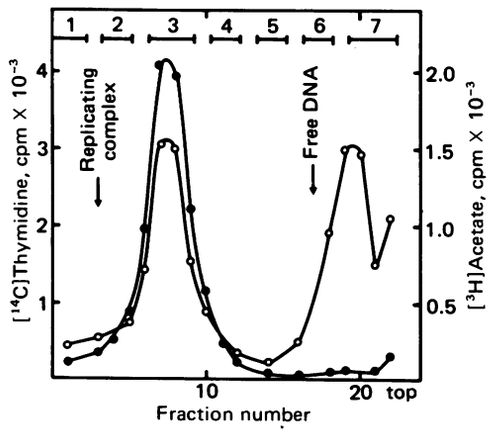

FIG. 1. Sedimentation profile of SV40 minichromosome labeled for 16 hr with [^{14}C]thymidine and pulse-labeled for 10 min with sodium [^{3}H]acetate. The nucleoprotein complex was extracted from three plates and analyzed. ●, [^{14}C]Thymidine; ○, [^{3}H]acetate.

Acetate Incorporation in H2a, H2b, H3, and H4, but Not in H1. The autoradiography of the [^{3}H]acetate-labeled histones separated on the NaDodSO$_4$/acrylamide gel shown in Fig. 3 and the scan of the same autoradiograph (Fig. 4 *A* and *B*) demonstrate that only the four core histones H2a, H2b, H3, and H4 and not H1 are rapidly labeled with acetate. Although the H2a and H2b are not separated in the fluorograph given, the width of the H2a and H2b band is twice that of H3 or H4, suggesting that both H2a and H2b are acetylated. In addition, the scan of an acid urea gel given in Fig. 5 clearly showed acetate labeled in all four core histones. The absence of extensive acetate label in the fastest migrating species of H4 (Fig. 2) and in H1, which both contain stable *N*-acetylserine in their NH_2-terminal positions (18), argues that NH_2-terminal acetylation of histones contributed only a small percentage of the observed rate of histone acetylation in SV40 chromatin. During the short period of labeling with [^{3}H]acetate, almost no radioactive acetate was metabolically incorporated into the amino acid pool as shown by the following observation: the viral structural proteins, VP1, VP2, and VP3, were abundantly synthesized at 40 hr postinfection; however, only trace ^{3}H incorporation was detected in the VP1 band in Fig. 3*B*.

Viral Histones Were Acetylated at a Faster Rate than Were the Cellular Histones. Scans of the stained NaDodSO$_4$ (Fig. 4) and acid urea gels (not shown) and their autoradiographs permitted the calculation of the relative acetate incorporation in the viral and the cellular histones originating from the same infected culture. The specific activity found for the viral histones was about 4 times higher than that of the cellular histones (Table 1). In addition, a certain qualitative difference was observed between the labeled cellular and viral histones. Viral H2b incorporated a higher fraction of total acetate than did cellular H2b (Fig. 4).

Rate of Dissociation of [^{3}H]Acetate Label. When a short pulse of [^{3}H]acetate was followed by a chase with a medium containing nonradioactive acetate, the specific activity of all four core histones decreased. The rapidly labeled internal acetyl groups were displaced by unlabeled acetate with a half-life of about 15 min as deduced from the amount of acetate label remaining after a chase of 5 or 10 min (Fig. 5). This deacetylation rate may be an underestimate of the real rate of exchange of the acetate groups. The large intracellular pool of acetyl-CoA (21) was equilibrated rapidly with the low molarity of the radioactive acetate added to the cells. On the contrary, after the pulse, removal of the radioactive medium and its replacement

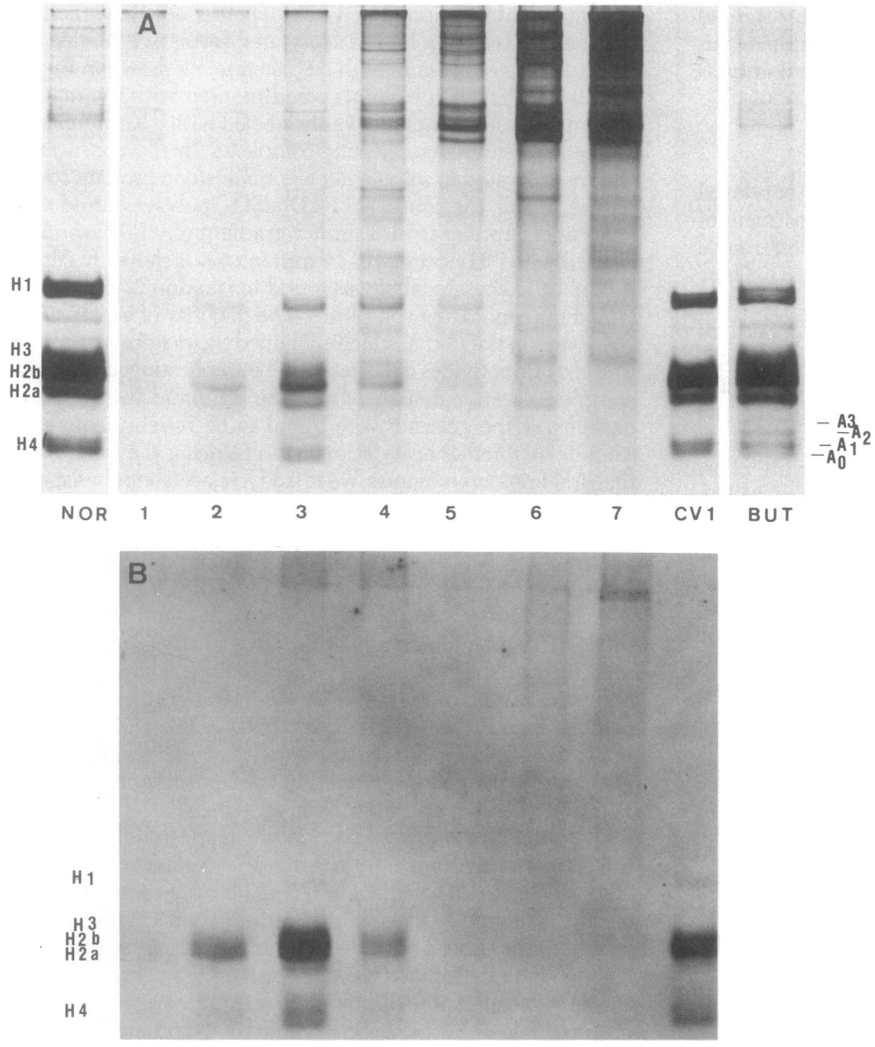

FIG. 2. Acetic acid/urea/polyacrylamide gel analysis of cellular and viral histones. (*A*) Coomassie blue staining. Lanes: NOR, total histones from noninfected CV1 cells; 1–7, proteins of sections 1–7 of the gradient described in Fig. 1; CV1, cellular histones extracted from the nuclear pellet of infected cells; BUT, histones obtained from noninfected CV1 cells pretreated for 24 hr with 3 mM sodium butyrate (17). A_0, A_1, A_2, and A_3 designate position of H4 containing none, one, two, and three internal acetyl groups, respectively. (*B*) Autoradiography of the gel.

with a medium containing nonradioactive acetate may have caused only a slow depletion of the internal pool of labeled acetyl-CoA. The decay rate measured may in fact be the rate of utilization of acetyl-CoA in the different metabolic pathways and not the real exchange rate of the internal acetate groups in the different histone molecules.

Relationship between Rapid Acetate Incorporation and the Number of Stable Acetyl Groups. The content of *N*-ε-acetyllysine in different histones from several sources has been analyzed after enzymatic hydrolysis of the histones. Acetyllysine was found mainly in histones H3 and H4; only a trace was found in H2a and none was found in H2b (18, 22). Radioactive acetate labeling of cellular histone *in vivo* showed that, in addition to the internal acetylation of H3 and H4, some acetylation of histones H2a and H2b occurred (23–25). Comparison of the staining pattern and the autoradiographs of [^{3}H]acetylated viral or cellular histones on acid urea/Triton gels permits the estimation of the fraction of total H2b, H2a, and H4 that was acetylated at any moment. When cellular histones were extracted from infected cells, a visible stained band was seen in the position of monoacetylated H4, but none was seen in the position of monoacetylated H2b and H2a. Similar results were obtained with the viral-bound histones (results not shown). Hence, only a few percent of H2b or H2a can be acetylated at any moment in the cellular or viral chromatin. The scans of the autoradiographs given in Figs. 4 and 5 permitted a rough cal-

Table 1. Quantitation of the degree of acetylation of the cellular and viral histones obtained from the densitometer scans shown in Fig. 5

Type of gel	Source of histones (H3, H2b, H2a, H4)	Histone concentration (staining), arbitrary units	Acetate incorporation (autoradiography), arbitrary units	Acetate specific activity	Ratio, viral to cellular
$NaDodSO_4$	Viral	14	60.6	4.33	5.2
	Cellular	57	47.5	0.83	
Acid urea	Viral	66.2	96.4	1.46	3.6
	Cellular	105.9	42.3	0.4	

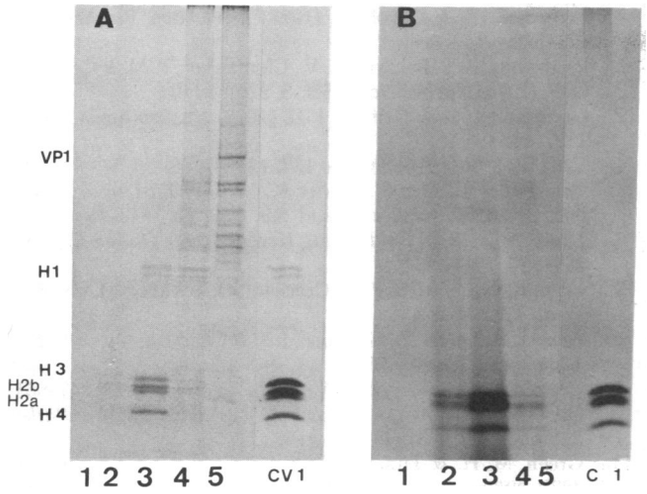

FIG. 3. $NaDodSO_4$/polyacrylamide gel analysis of cellular and viral histones. (*A*) Coomassie blue staining. Lanes: 1–5, proteins of sections 1–5 of a sucrose gradient similar to that in Fig. 1. Lanes 2 and 3 contained histones bound to replicating and mature viral DNA, respectively; CV1, histones extracted from the nuclear pellet after the solubilization of the viral minichromosomes; VP1, viral structural protein. (*B*) Autoradiography of the gel.

culation of the relative acetate incorporation in the four viral core histones: 13%, 40%, 26%, and 20% in H2a, H2b, H3, and H4, respectively. The higher [^{3}H]acetate incorporation into histone H2b than into histone H3 or H4 suggests that rapid acetate incorporation is not related to the degree of steady-state acetylation of the histone molecule. On the contrary, examination of acetate incorporation in the mono-, di-, and triacetylated forms of a single histone, H4, suggests that the rate of acetate incorporation in the different bands of H4 is related to the degree of acetylation—e.g., the concentration of diacetylated H4 (A_2 in Fig. 2) was about half of that of the monoacetylated form (A_1), whereas roughly an equal amount of radioactivity was incorporated into the two forms.

DISCUSSION

Previous sequence studies have shown that mainly the arginine-rich histones H3 and H4 undergo acetylations in several positions of the basic NH_2-terminal portion of their polypeptide chain (17, 22). Rapid incorporation of acetyl residues into histones H3 and H4 and to a lesser extent into H2a and H2b was

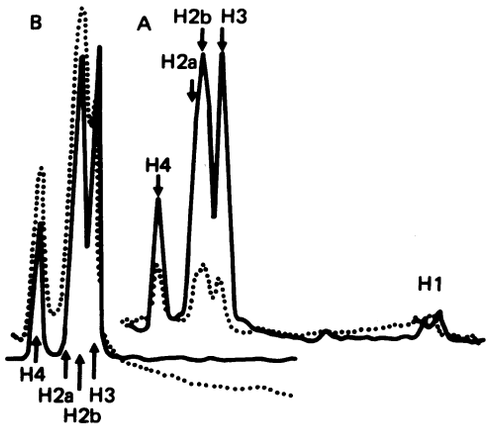

FIG. 4. Densitometric scans of the stained gel and of the autoradiograph of the histone regions of the $NaDodSO_4$ gel shown in Fig. 3. Coomassie blue staining (*A*) and autoradiography (*B*) of viral (lane 3) (- - -) or cellular (—) histones.

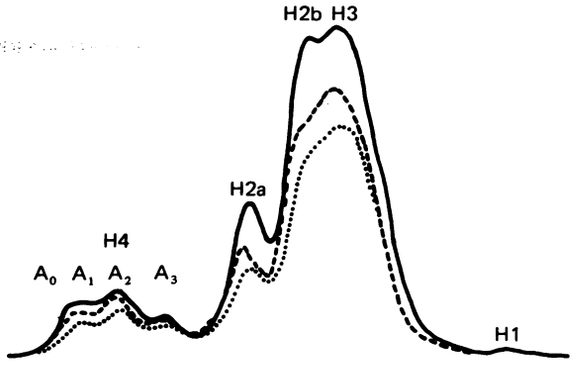

FIG. 5. Densitometric scans of autoradiographs of the viral histones labeled with [^{3}H]acetate for 10 min (—) and chased with nonradioactive acetate for 5 min (- - -) and 10 min (. . .). After labeling for 10 min, the radioactive medium was replaced with 10 ml of fresh medium containing 1 mM sodium acetate. The viral complex was purified by sucrose gradient centrifugation and the viral histones were analyzed on acid urea gels.

observed after incubation of different cell types with radioactive acetate (23–25). The studies on acetate-labeled histones from trout testis cells permitted the identification of the acetylation sites on histones H2a and H2b (23). Recently, Schaffhausen and Benjamin (20) found that polyoma virion histones H3 and H4 are highly acetylated. In agreement with these findings, we observed that histones H3 and H4 bound to SV40 minichromosomes in the nuclei are highly acetylated. As in the case of polyoma (20), the viral histones H3 and H4 are more acetylated than the corresponding cellular histones.

Newly synthesized histone H4 labeled with tritiated amino acids was shown by migration in acidic urea to contain two acetyl groups that are partially removed after the incorporation of these histones in the chromatin (26, 27). Crémisi *et al.* (9) have shown in SV40-infected cells that newly synthesized histones associated preferentially with replicating intermediates. Thus, aiming to study the acetylation of newly synthesized histones, we pulse-labeled cells with [^{3}H]acetate for 10 min. No preferential acetate labeling was observed in replicative minichromosomes; on the contrary, the [^{3}H]acetate incorporation was observed mainly in the mature (nonreplicating) DNA–histone complexes. In addition, comparison of [^{35}S]methionine-labeled histones bound to replicating complexes with the histones bound to mature DNA did not reveal any difference in the degree of acetylation of H3 and H4 (unpublished results). Thus, in the case of SV40 chromatin replication, newly assembled histones do not contain a large fraction of acetate label and are not found in an acetylated state higher than that of histones bound to mature chromosomes.

All four core histone H2a, H2b, H3, and H4 became labeled, whereas almost no radioactivity was incorporated into histone H1. The viral histone H4 contains approximately one internal acetyl residue per mole (Fig. 2), whereas the acid urea/Triton gels show that the fraction of steady-state acetylated H2b is relatively small in both cellular and viral chromatin (less than 0.1 acetyl residue per molecule). On the other hand, more radioactive acetate was incorporated into viral H2b than into viral H4. We conclude that no correlation exists between the degree of steady-state acetylation of the different histones observed by staining and the rate of [^{3}H]acetate incorporation.

The viral minichromosomes in the nuclei can undergo three different processes: replication, transcription, and encapsidation. We have shown that replicating minichromosomes incorporate less than 10% of the pulse-labeled acetyl residues, whereas they incorporate between 30 and 50% of the newly synthesized histones. Virions isolated from infected cells after

pulse labeling with [^{3}H]acetate did not contain any labeled histones (unpublished observations). Thus, the fast decay of the acetyl groups is not due to packaging of acetate-labeled minichromosomes in newly formed virions.

Studies initiated by Weintraub and Groudine (14) and extended to different systems have shown that active genes possess a modified nucleosomal structure. A possible mechanism for gene activation *via* histone acetylation was proposed by Allfrey *et al.* (28). Yamamoto and Alberts extended this model and postulated that gene activation by acetylation may precede specific transcription (29). A model for ribosomal gene activation that includes a change in chromatin structure before the initiation of specific transcription was proposed by Foe (30). It is possible that the rapid acetylation of the core histones is necessary for the maintenance of SV40 minichromosomes as active genes. Although only a small fraction (0.5–3%) of the total minichromosomes can be engaged in transcription at any moment (11, *), it is plausible that a higher fraction of the minichromosomes is kept in an "active" conformation. Rapid acetylation of the different histones could modify the nucleosome structure and permit initiation and elongation of the RNA chains. Alternatively, acetylation of lysine residues in nucleosomes along the viral genome could occur concomitantly with the transcription process. Additional studies are necessary to identify the exact role of histone acetylation in the viral transcription process and to distinguish between the two alternative models. The acetylating enzyme(s) in the infected cell incorporate acetate in the viral chromatin at a 4-fold higher rate than in the cellular chromatin (Table 1). This rate difference may be caused by minor structural variations among the various chromatin fibers in the nuclei (higher order structure, nonhistone proteins). Only active genes in the cell may have a chromatin conformation similar to that of active SV40 minichromosomes. One or more of the minor t antigen species (31–34) which are affected in *hrt* mutants may participate in the regulation of acetylation as suggested by Schaffhausen and Benjamin (20). These authors showed recently that in *hrt* (host range nontransforming) gene mutants of polyoma, the viral H3 and H4 histones are less acetylated than in the wild-type strain.

Note Added in Proof. No difference was observed in the rate and extent of viral histone acetylation among wild-type and small t deletion mutants of SV40 (dl 2102 and dl 2112) kindly provided by J. Feunteun [Feunteun, J., Kress, M., Gardes, M., & Monier R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4455–4459]. SV40 deletion mutants in the 0.54–0.59 region may differ from polyoma *hrt* mutants in the absence of host-range restriction, in their leakiness for transformation, and in their normal level of histone acetylation.

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