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Alteration in the Simian Virus 40 Maturation Pathway After Butyrate-Induced Hyperacetylation of Histones

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The role of histone acetylation in the replication and maturation pathways of simian virus 40 was assessed. Histones were hyperacetylated by briefly exposing infected cells to sodium butyrate. Viral DNA in cells exposed to butyrate was found to reenter replication to a greater extent and mature to the previrion form to a lesser extent than viral DNA in control cells. Previrions formed in the presence of butyrate had altered sedimentation properties. These data suggest that increased acetylation of histones is not the signal for removal of DNA from the pool of molecules available for replication. It appears, in fact, that hyperacetylation retards entry into and progression along the maturation pathway.

During a permissive infection, simian virus 40 (SV40) DNA molecules can serve as templates for transcription and replication or as genomes for encapsidation. Wang and I have shown that pulse-labeled DNA is used as a template for further DNA synthesis (reenters replication) for 2 to 4 h and then is removed from the pool of molecules available for replication (20). The kinetics of removal from the replication pool correlate temporally with the progression of this DNA out of the chromatin pool and into the maturation pathway. We proposed that the fate of newly synthesized DNA is determined by the relative abundance of replication and maturation proteins and their relative affinity for the chromatin template (20).

Analyses of the level of acetylation of histones in the different SV40 DNA-protein complexes have shown that the histones in chromatin are underacetylated relative to those in previrions and virions (2, 10, 16). It is possible, using a short pulse of radiolabeled acetate, to show that the label first appears in chromatin and then in previrions and virions (2, 10). Wang and I (20) and Coca-Prados et al. (2) have therefore proposed that the level of acetylation of histones may be a critical factor in determining whether the viral DNA remains in the replication pool or enters the maturation pathway.

To examine the role of histone acetylation I exposed infected cells to sodium butyrate. Butyrate inhibits cellular deacetylases, resulting in hyperacetylation of histones (1, 3, 17, 19). If a higher level of acetylation of histones is critical to commitment of the viral DNA to the maturation pathway, exposure to butyrate should accelerate removal from the replication pool and maturation to previrions and virions. Data presented here show that this is not the case. In butyrate-treated cells, viral DNA remains in the replication pool longer than in untreated cells. Thus, an increased level of histone acetylation does not appear to cause removal of DNA from the pool of molecules available for replication. The data also indicate a slight inhibition by hyperacetylation of the maturation pathway.

MATERIALS AND METHODS

Cells and virus. BSC-1 and CV-1 cells were grown in Dulbecco modified Eagle medium containing 10% calf serum and 50 μg of gentamicin per ml. Similar results were obtained with both cell lines. SV40 strain 776 was used in all experiments.

Monitoring of macromolecular synthesis. Confluent cells were infected with SV40 at an input multiplicity of 5 to 10 PFU per cell. At 40 h postinfection, the medium was removed from a series of dishes and replaced with either Dulbecco modified Eagle medium containing 2% calf serum or Dulbecco modified Eagle medium containing 2% calf serum and 15 mM sodium butyrate. At various times, the medium was removed and replaced with similar medium containing either 10 μCi of [^{3}H]thymidine per ml (specific activity, 40 to 60 Ci/mmol) or 50 μCi of [^{3}H]leucine per ml (specific activity, 59.8 Ci/mmol). After 30 min, the labeling medium was removed, and the dish was washed twice with TD buffer (150 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 20 mM Tris [pH 7.4]). The cells were scraped into cold 5% trichloroacetic acid, and duplicate aliquots were applied to filter paper which was washed in trichloroacetic acid, placed in vials with 1 N HCl, and heated to 100°C for 20 min. Radioactivity was determined after the addition of Aquasol.

Analysis of level of acetylation of histones. Histones were extracted from infected-cell nuclei with sulfuric acid and precipitated with 9 volumes of acetone (12). The histones were dissolved in 10 M urea–0.9 M acetic

TABLE 1. Effect of sodium butyrate on macromolecular synthesis

Macromolecular synthesis monitored	Treatment	Trichloroacetic acid-precipitable ^{3}H cpm incorporated at[a]:	
		0 to 30 min	360 to 390 min
DNA	Control	18,839	47,374
	Butyrate	18,434	46,714
Protein	Control	22,290	34,028
	Butyrate	25,815	35,572

[a] Time zero is the time of addition of butyrate.

acid and electrophoresed in a 15% polyacrylamide slab gel (12) at 170 V for 3.5 h at room temperature. Gels were fixed and stained with 0.1% amido black–20% ethanol–7% acetic acid (12).

Labeling and extraction of viral DNA and NPCs. The detailed protocol is published (20) and will be described only briefly. At 42 h postinfection, cells were labeled for 20 min with 80 μCi of [^{3}H]thymidine per ml (specific activity, 40 to 60 Ci/mmol), chased for 30 min in 10^{-6} M thymidine, and then incubated with 5×10^{-5} M 5-bromodeoxyuridine (BUdR), 2×10^{-5} M 5-fluorodeoxyuridine, and 9×10^{-6} M deoxycytidine. At the time of addition of the BUdR mixture and at various times afterwards, DNA was extracted from one set of cells by the method of Hirt (7); nucleoprotein complexes (NPCs) were extracted from a parallel set of plates by the method of Fernandez-Munoz et al. (5). Sodium butyrate, when present, was added 30 min before the [^{3}H]thymidine pulse (t = −80 min) and was present in all subsequent stages.

Determination of maturation kinetics. Chromatin was separated from previrions and virions on 10 to 40% sucrose gradients containing 2 mM Tris, pH 7.4, in an SW41 rotor at 37,000 rpm for 90 min or in an SW60 rotor at 45,000 rpm for 45 min. The percentage of [^{3}H]DNA in previrions and virions (NPC-II+V) was determined as follows: percent (NPC-II+V) = (NPC-II+V counts per minute ÷ total NPC counts per minute) × 100.

Determination of reentry kinetics. An aliquot of the Hirt supernatant was mixed with cesium chloride in TE buffer (10 mM Tris [pH 7.4], 1 mM EDTA) to give a final density of 1.72 g/ml. Samples were centrifuged either in a type 40 rotor at 33,000 rpm for 48 h or in a VTI 65 rotor at 45,000 rpm for 16 h to separate form I DNA with BUdR substitution in one strand (HL DNA) from unsubstituted form I DNA (LL DNA). The percentage of ^{3}H-labeled HL form I DNA [HL DNA counts per minute/(HL DNA counts per minute + LL DNA counts per minute)] was determined. No difference in the percentage of [^{3}H]DNA in form I in the Hirt supernatant was seen in the presence or absence of butyrate; in both cases, at least 85% of the ^{3}H counts per minute were in form I.

RESULTS

Effect of sodium butyrate on macromolecular synthesis. Short-term exposure (less than 1 h) of cells to millimolar amounts of sodium butyrate causes the hyperacetylation of histones (3, 13). Long-term exposure (24 h) has a variety of effects including inhibition of cellular DNA synthesis (6) and induction of erythroid differentiation in erythroleukemic cells (11). To avoid such long-term effects, cells were preincubated for 30 min with 15 mM sodium butyrate before initiation of the experiments. To verify that addition of the drug did not alter macromolecular synthesis, DNA synthesis and protein synthesis in the presence and absence of butyrate were monitored. No perturbation of macromolecular synthesis was detected (Table 1).

Effect of sodium butyrate on histone acetylation. In the experiments to be described below the first time point examined was 80 min after addition of sodium butyrate. To determine whether hyperacetylation of histones had occurred by that time, histones were extracted from butyrate-treated and control cultures and analyzed on acetic acid-urea gels. Acetylation slows the migration of a given histone in these gels (8). As shown in Fig. 1, by 80 min histone H4 from butyrate-treated cells was hyperacetylated relative to the control. Whereas in the control, the two lower bands (corresponding to zero or one acetylysine) predominated, in the presence of butyrate, the relative distribution changed in favor of the upper bands (more-acetylated forms). Although not clearly resolved, it is reasonable to assume that the other histones were similarly hyperacetylated (3).

Kinetics of reentry and maturation of viral DNA in the presence and absence of sodium butyrate. SV40-infected BSC-1 cells with or without butyrate treatment were labeled with [^{3}H]thymidine for 20 min, chased with cold thymidine for 30 min, and then incubated with BUdR. Figure 2 shows the profiles of ^{3}H-labeled NPCs extracted from cells incubated in the presence or absence of sodium butyrate either

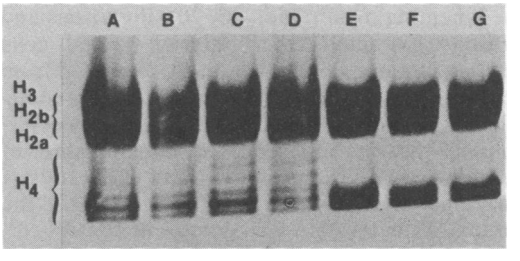

FIG. 1. Degree of histone acetylation in the presence or absence of 15 mM sodium butyrate. Histones were extracted from treated (lanes A to D) or untreated (lanes E to G) SV40-infected cells at 80 min (lanes A and E), 170 min (lanes B and F), 260 min (lane C), and 350 min (lanes D and G) after addition of sodium butyrate and run on a 15% acetic acid–urea gel. The cathode is at the bottom of the figure.

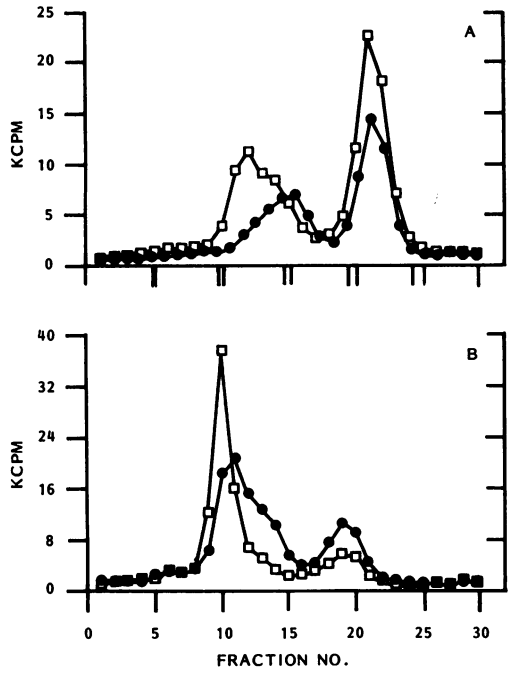

FIG. 2. Separation of chromatin from previrions and virions (NPC-II+V). SV40 NPCs were extracted from treated and untreated infected cells at (A) t = 0 (time of addition of BUdR) or (B) t = 5 (5 h after BUdR addition) and separated on 10 to 40% sucrose gradients containing 2 mM Tris, pH 7.4. Sedimentation is from right to left. Symbols: □, untreated; ●, sodium butyrate present from t = −80 min to termination of experiment. (Double tick marks for the fraction number in panel [A] reflect scaling the gradients to the same distance on the *x* axis.)

immediately after the cold thymidine chase (Fig. 2A, t = 0) or after 5 h in BUdR (Fig. 2B, t = 5). The slow-sedimenting material at the right of the profile is chromatin; the faster-sedimenting material is previrions and virions, with virions sedimenting most rapidly (5, 20). Previrions and virions extracted from butyrate-treated cells sediment more slowly than those extracted from control cells. The progression of [^{3}H]DNA along the maturation pathway was monitored by determining the rate and extent of accumulation of ^{3}H-labeled previrions and virions. The results of a typical experiment are shown in Fig. 3A; the extent of maturation was greater in control cultures than in butyrate-treated cultures. The involvement of [^{3}H]DNA in the replication pathway was assessed by determining the rate and extent of accumulation of ^{3}H-labeled HL DNA (DNA with BUdR substitution in one strand). Such hybrid-density labeled DNA arises if DNA synthesized during the pulse is used as a template for a subsequent round of DNA synthesis (reenters replication) during the BUdR chase (14). The extent of reentry (percent HL DNA) was greater in the butyrate-treated cultures than in the control cultures (Fig. 3B).

DISCUSSION

I was interested in learning what determines whether viral DNA enters the replication or maturation pathway. The specific question addressed here was whether the level of acetylation of histones on the chromatin template was the critical factor. Hyperacetylation of histones was induced by short-term exposure of SV40-infected cells to sodium butyrate. The fate of viral DNA complexed with these hyperacetylated cellular histones was then monitored. The hypothesis was that such altered chromatin

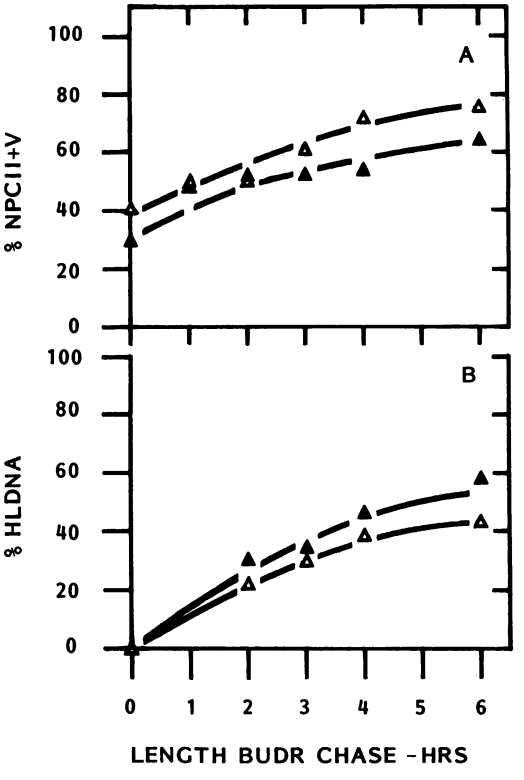

FIG. 3. Effect of sodium butyrate on the participation of pulse-labeled SV40 DNA in the replication and maturation pathways. (A) The percentage of [^{3}H]DNA in previrions and virions (percent NPC-II+V) was determined after separation of chromatin from previrions and virions on neutral sucrose gradients (5, 19). (B) The percentage of labeled DNA which remained in the pool of molecules available for replication and was used as a template for further DNA synthesis (percent HL DNA) was determined after separation from unsubstituted labeled DNA by cesium chloride density gradient centrifugation. Symbols: △, untreated; ▲, 15 mM sodium butyrate was added to cultures.

would be used as the core of encapsidation intermediates (previrions) more rapidly than chromatin from untreated controls. As shown in Fig. 3A, this is not the case. In fact, there is a small but reproducible inhibition of packaging in the presence of butyrate. The maturation experiment has been repeated 10 times, and a paired Student t test comparing the percentage of the ^3H counts per minute in the previrion and virion pool at each time point with and without butyrate shows this difference to be statistically significant ($P < 0.001$). Previous experiments have shown that the replication and maturation pathways are temporally linked and have suggested that DNA was either in the pool of molecules available for replication (and possibly transription) or in the maturation pool (20). Consistent with this, DNA in butyrate-treated cells remained in the replication pool somewhat longer than DNA in control cells (Fig. 3B). Again, a paired Student t test showed this difference to be statistically significant ($P < 0.001$).

That viral DNA synthesis can occur within chromatin containing an elevated level of histone acetylation is consistent with recent publications. Daniell et al. (4) and Kawasaki et al. (9) have shown that SV40 can induce cellular and viral DNA synthesis in the presence of histones hyperacetylated by long-term exposure of cells to sodium butyrate. The fact that we find a higher proportion of the DNA used as a template for further DNA synthesis may simply be a reflection of the fact that it cannot be efficiently used in the maturation pathway. Clearly, acetylation of histones is not the key to removal of DNA from the pool of molecules available for replication. Some other factor(s) must be required to shunt DNA out of the replication pathway.

Daniell et al. (4) found a decrease in SV40 PFU after long-term exposure to butyrate. It was not possible from their data to determine whether this decrease was due to a maturation or infectivity defect. As shown in Fig. 2 and 3A, a maturation defect can be demonstrated by 80 min after exposure to butyrate (the earliest time point analyzed). Several possible reasons for inefficient use in the maturation pathway of the chromatin template formed in the presence of butyrate can be proposed. The altered histone acetylation pattern could either alter transcription and decrease the quantity of viral structural proteins or alter the nucleosome structure and interfere with the addition of structural proteins to the complex. Edenberg and I have previously shown that when protein synthesis is inhibited by UV irradiation or cycloheximide, the amount of previrions and virions is decreased in proportion to the degree of inhibition but the sedimentation profile is similar to that seen in control dishes (15). The altered profile shown in Fig. 2 and the absence of inhibition of protein synthesis shown in Table 1 argue against a simple lack of structural proteins and suggest a qualitative difference in the previrions and virions maturing in the presence of butyrate. Indeed, the observation of a change in DNase I sensitivity (in HeLa cells) after butyrate-induced hyperacetylation of histones indicates that the relationship of histones to DNA is changed (19). In addition, the recent report that in untreated cells some acetylation of histones occurs within previrions (18) suggests that premature hyperacetylation might interfere with protein interactions leading to formation of fast-sedimenting complexes. Finally, it is possible that butyrate also induces modifications of viral structural proteins which are incompatible with formation of compact previrions and virions.

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