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Histones: Metabolism in simian virus 40-infected cells and incorporation into virions

(DNA synthesis/histone phosphorylation/Triton–acid–urea gels)

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ABSTRACT **The infection of confluent monkey cells with simian virus 40 induced the synthesis of both cellular DNA and histones. However, during the course of infection, cellular histone synthesis was uncoupled from cellular DNA replication and became coupled to viral DNA replication. The synthesis of all five host histone species was induced after virus infection and they appeared to be more highly phosphorylated than their counterparts in uninfected cells. At late times after infection, the cells contained twice as much histones as did uninfected cells. All the histone species except H1 were incorporated into virions. Compared to cellular histones, virion histones were enriched in the arginine-rich species H3 and H4. Although both old and newly synthesized cellular histones were incorporated into virions, there were about 5 times more newly synthesized than old histone polypeptides in virions.**

Papovaviruses are unique in possessing histones as internal core proteins (1). The histones are of cellular origin because the viral DNA contains only enough genetic information to code for gene A protein and for nonhistone capsid proteins (1), and preformed cellular histones have been detected in virions (2). Furthermore, virion histones have the same tryptic fingerprints as cellular histones (3). Replicating viral DNA is also complexed with histones in the infected cell (4–6). The viral DNA–histone complex extracted from either purified virions or infected cells resembles "minichromosomes" and about 20 nucleosome-like structures are bound to the circular viral DNA (7–9).

Although histones probably play an important role in the replication, maturation, and structure of papovaviruses, little is known about the synthesis and metabolism of these proteins in the infected cell. The viral chromatin resembles cellular chromatin in a number of respects and has been used as a model for the study of chromatin structure (7–9). For the reasons stated above, this study was done to determine the pattern of histone metabolism in cells lytically infected with simian virus 40 (SV40) and the histone composition of virions.

MATERIALS AND METHODS

Cells and Virus. Secondary cultures of African green monkey kidney (AGMK) cells prepared as described (10, 11) were used 2–3 days after reaching confluence. Stock SV40 (large-plaque strain) suspension was prepared in CV-1 (monkey) cells (10). AGMK cells were infected with 50 plaque-forming units of virus per cell in the presence of 0.04 M $MgSO_4$ for 60 min (11). For mock infection, cells were inoculated with uninfected CV-1 cell extract. The cultures were incubated at 37° in Eagle's minimal essential medium containing 0.3 times the regular amount of lysine and arginine and 2% (for virus-infected cells) or 10% (for mock-infected cells) dialyzed calf serum.

Virus Purification. SV40 grown in AGMK cells was purified as described (4, 10). The fully infectious virion fraction (density of 1.35 g/cm^3 in CsCl) was used in this study.

Abbreviations: SV40, simian virus 40; AGMK, African green monkey kidney; $NaDodSO_4$, sodium dodecyl sulfate.

Histone Extraction. Cells were washed twice with phosphate-buffered saline and three times with 0.15 M NaCl/0.05 M $NaHSO_3$/0.05 M Tris·HCl, pH 7.9; they then were scraped off the glass and extracted twice with 0.2 M H_2SO_4/0.14 M 2-mercaptoethanol/0.05 M $NaHSO_3$/phenylmethylsulfonyl fluoride, 0.3 mg/ml over a 20 hr period at 4° with gentle agitation. This long extraction was necessary because the standard short acid extraction resulted in a lower and a variable yield of histones. Histones were precipitated with 20% trichloroacetic acid, washed with acetone/0.1% HCl, and washed finally with acetone.

Polyacrylamide Gel Electrophoresis. (i) Triton Gels. Histones were analyzed in Triton gels as described by Alfageme *et al.* (12) with slight modifications. The gels contained 15% acrylamide, 0.2% *N,N'*-methylene-bis-acrylamide, 0.25% *N,N,N',N'*-tetramethylenediamine, 0.14% ammonium persulfate/0.9 M acetic acid, 4.5 M urea, and 0.04% (vol/vol) of the nonionic detergent Triton-DF 16. The gels, 6 mm in diameter by 12 cm in length, were cast in glass tubes in which the bottom ends were slightly constricted to prevent the gels from slipping out during electrophoresis. Each gel was loaded with 0.1 ml of cysteamine and run overnight (about 19 hr) at 120 V until the current was constant. Histone samples in 0.9 M acetic acid/10% (vol/vol) glycerol/0.14 M 2-mercaptoethanol with a trace of Pyronine Y as tracking dye were electrophoresed for 9 hr at 120 V. The gels were stained overnight with 0.1% amido black in 20% (vol/vol) ethanol/7% (vol/vol) acetic acid and were destained by diffusion. The gel profile of AGMK histones is similar to that of HeLa cells (13) and the histone bands were identified by parallel electrophoresis of purified calf thymus and AGMK cell histone fractions.

(ii) Sodium Dodecyl Sulfate (NaDodSO$_4$) Gels. Electrophoresis in sodium phosphate-buffered (pH 7.2) 9% acrylamide gels containing 0.1% $NaDodSO_4$ and 6 M urea was performed as described (10).

Radiochemicals. [^{3}H]Thymidine (61 Ci/mmol) and [^{3}H]-lysine (63 Ci/mmol) were from New England Nuclear, MA; [^{3}H]arginine (7 Ci/mmol), [^{3}H]leucine (51 Ci/mmol), and ^{14}C-labeled amino acid mixture (algal profile) were from Schwarz/Mann, NY, and [^{32}P]phosphate (carrier-free) was from Amersham/Searle, IL.

RESULTS

DNA and Overall Histone Synthesis. The patterns of DNA and histone synthesis in AGMK cells were determined as follows. Confluent monolayers mock infected or infected with SV40 were incubated at 37° in Eagle's minimal essential medium containing 2% calf serum and labeled with [^{3}H]thymidine at different times after infection to monitor DNA replication. Because little or no cellular DNA replication occurred in mock-infected cells incubated in Eagle's minimal essential medium containing 2% calf serum (data not shown), mock-

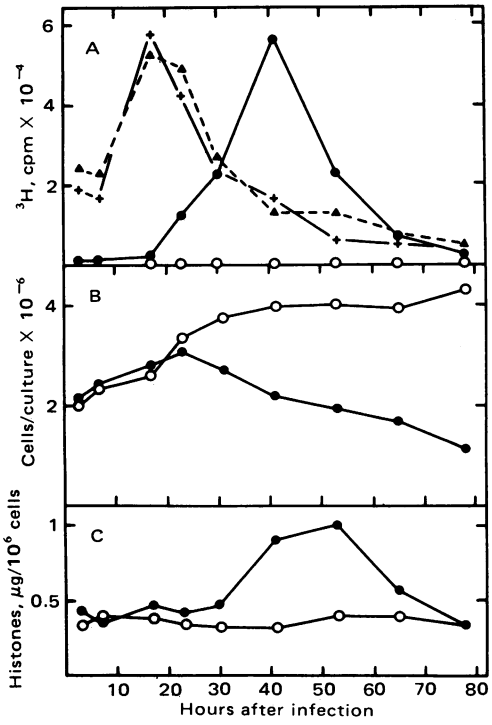

FIG. 1. DNA synthesis, cell doubling, and histone content. Mock-infected or virus-infected cells grown in 5-cm petri dishes were refed with fresh medium containing 10% or 2% calf serum, respectively, and processed as follows: (*A*) DNA synthesis. Cells were labeled with [^{3}H]thymidine (4 μCi/ml) for 30 min and fractionated by the method of Hirt (14) to determine acid-precipitable radioactivity. High-molecular-weight or cellular DNA from mock- (▲) or virus-infected (+) cells; low-molecular-weight or viral DNA from mock- (○) or virus-infected (●) cells. (*B*) Cell doubling. Duplicate mock- (○) or virus-infected (●) monolayers were dispersed with trypsin and the cells were counted in a hemocytometer. (*C*) Histone content. The amount of histone extracted from mock- (○) or virus-infected (●) cultures was determined by the method of Lowry *et al.* (15) and normalized for recovery from 10^6 cells.

infected cells in the experiments described below were incubated in medium containing 10% calf serum to stimulate cell proliferation. Duplicate cultures were harvested for histone extraction. The results showed that cellular DNA replication in both mock- and virus-infected cells reached a maximum at about 17 hr and was declining at 41 hr, the time of maximum viral DNA replication (Fig. 1*A*). The viral DNA fraction obtained from infected cells was found to contain mostly viral DNA components I and II when analyzed in neutral sucrose gradients; cellular DNA, if present, was in insignificant amounts (4). Comparable amounts of ^{3}H-labeled cellular DNA were recovered from both mock- and virus-infected cells. It should be noted that cellular DNA replication in confluent mock-infected monolayers was stimulated by a high concentration (10%) of fresh serum whereas cellular DNA replication in SV40-infected cells (incubated in medium containing 2% serum) was virus-induced.

After mock infection, refeeding of the contact-inhibited monolayer with fresh medium resulted in a doubling of the cell number (Fig. 1*B*). However, only about 50% of the virus-infected cells entered mitosis and cells were progressively lost from the monolayer after 30 hr. Cytopathic effects were first observed at 40 hr and were intense at 80 hr, involving 100% of the cells. Because the mock- and virus-infected cultures differed in cell number, the amount of histones extracted was normalized for recovery from the same number of cells (Fig. 1*C*). As

Table 1. Composition of cellular and virion histones*

Histone source	Relative amount of histone†					
	H2A	H2B	H3.1	H3.2	H4	H1
SV40‡	1.00	1.85	1.24	1.79	3.71	0
SV40§	1.00	2.78	1.56	1.22	4.07	0
AGMK§	1.00	1.77	1.23	0.69	1.20	0.25

* Data are derived from experiments shown in Fig. 3.
† The amount of each histone, determined by measuring the area under the histone peak of the gel scan, was compared to that of H2A in the same gel.
‡ Purified virions treated with urea and protamine sulfate.
§ Histones extracted from purified virions or AGMK cells with acid.

expected, the histone content per cell of mock-infected cultures essentially remained constant. The histone content of virus-infected cells increased dramatically at late times after infection and, between 41 and 53 hr, each cell contained about 2.7 times (2.0, 2.1, and 3.4 times in other experiments) as much histones as did each mock-infected cell. At times later than 60 hr after infection, the cells showed severe cytopathic effects and a loss of histones.

Kinetics of Histone Synthesis and Phosphorylation. Histones extracted from cells labeled with [^{3}H]lysine, [^{3}H]arginine, and [^{32}P]phosphate were analyzed in Triton gels. The different histone fractions varied in their affinity for Triton (12) and were well-separated in the Triton gels (see Fig. 3). After a correction for the amount of histone recovered in the gel, the results showed that the synthesis of all five histone species was induced after virus infection (Fig. 2*A*). During the course of the infection, the different histones were synthesized in the same relative amounts. Furthermore, the different histone fractions were synthesized in proportions similar to those obtained from mock-infected cells. For example, at 30 hr, the ratios of ^{3}H-labeled H2A:H1:H3.1:H3.2:H4 (calculated from data of Fig. 2*A*) were 1.00:0.19:0.93:1.42:0.65:0.82, respectively, for virus-infected cells and 1.00:0.20:0.83:1.41:0.65:0.70, respectively, for mock-infected cells. During the later stages of infection, the synthesis of histones and viral DNA in the virus-infected cells was coordinated (Figs. 1*A* and 2*A*). The maximum rate of histone synthesis either preceded slightly or was coincident with the maximum rate of viral DNA synthesis (data not shown). The incorporation of radioactivity can be used as a measure of histone synthesis because we have previously shown that the [^{3}H]lysine pools in mock- and virus-infected cells are similar in size (16).

The rate of [^{32}P]phosphate incorporation into histones paralleled that of synthesis in virus-infected cells (Fig. 2*B*). All the histone fractions in virus-infected cells were more highly labeled than their counterparts in mock-infected cells; the greatest difference was seen for H1 and H2A.

Analysis of Virion Histones. H1 was not detectable in the histones obtained from purified virions with either acid or protamine sulfate extraction procedures (Fig. 3). Because H1 can bind to both viral DNA and viral "minichromosomes" isolated from infected cells (9, 17), the protamine sulfate extraction procedure was adopted to eliminate the possibility that H1 could have been lost during the standard acid extraction procedure. H4 was heterogeneous and five bands (probably representing different acetylated forms) were seen in acid-extracted histones (Fig. 3*B*). Polyoma virus H4 and H3 have been reported to be acetylated (18). Compared to cellular histones, virion histones were enriched in H3.1 and H4 (Table 1).

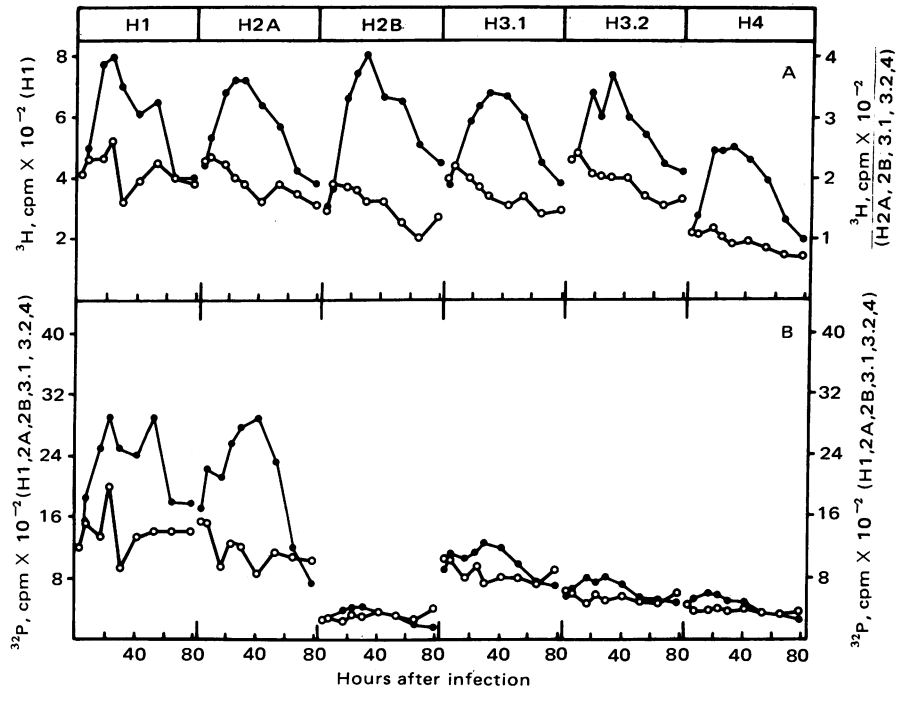

FIG. 2. Histone synthesis and phosphorylation. Mock- (○) or virus-infected (●) cells grown in 9-cm petri dishes were refed with fresh medium (identical but lacking phosphate) containing 12 μCi of [^{3}H]lysine, 4 μCi of [^{3}H]arginine, and 56 μCi of [^{32}P]phosphate per ml. After 1 hr, histones were extracted and 200-μg samples were analyzed in Triton gels. The amount of each histone fraction in the gel was determined by measuring, with a planimeter, the area under each peak of a densitometric tracing obtained by scanning the stained gels at 615 nm. The segment of the gel containing the histone band was excised and solubilized for determination of radioactivity (10). The ^{3}H values were corrected for the ^{32}P spillover. The data are presented as cpm per unit area of the gel scan. (*A*) Histone synthesis; (*B*) histone phosphorylation.

The amount of preformed (or old) and newly synthesized histones in SV40 was determined by infecting ^{3}H-labeled cells and labeling newly synthesized histones with ^{14}C-labeled amino acids. The cells were harvested at 50 hr and the virus was purified. To eliminate possible artifacts resulting from histone extraction and handling, we solubilized samples of whole cell extract and virions with $NaDodSO_4$ under reducing conditions and analyzed them in $NaDodSO_4$ gels (10). In these gels, the histones migrate as three bands—H1, an H2B–3–2A complex, and H4, in order of decreasing electrophoretic mobility. The higher ^{14}C/^{3}H ratio of radioactivity in virion histones compared to total cell histones suggests that virions preferentially incorporate new histones (Table 2). Knowing (*i*) the ^{3}H specific radioactivity (^{3}H cpm per optical density unit of stained histone bands in the gel) of the old histones extracted from cells harvested just before infection, and (*ii*) the ^{3}H radioactivity and

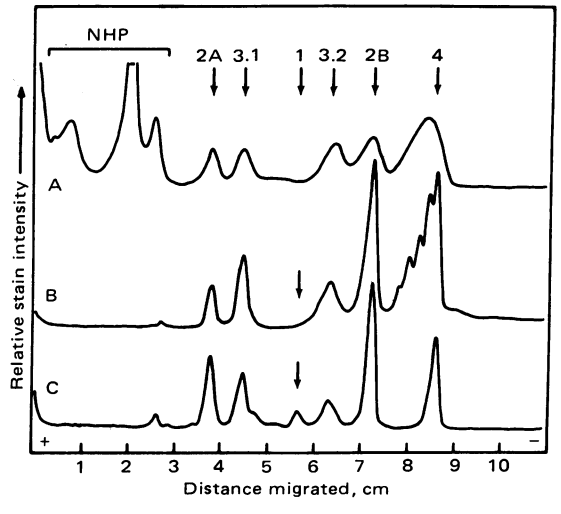

FIG. 3. Analysis of viral and cellular histones in Triton gels. (*A*) Purified virions were incubated overnight at 25° in 10 M urea/0.14 M 2-mercaptoethanol/0.9 M acetic acid/protamine sulfate, 5 mg/ml, and the mixture was subjected to electrophoresis. Virion structural nonhistone polypeptides (NHP) are located in upper portion of the gel. No bands were detected in stained gel containing only protamine sulfate. Histones from purified virions (*B*) and AGMK cells (*C*) were extracted with acid as described in *Materials and Methods*. Virions were purified from AGMK cells infected with 1 plaque-forming unit of stock virus per cell. The positions of the histone fractions are indicated and electrophoretic migration is from left to right.

Table 2. Proportion of old and new histones in infected cells and virions*

Histone source	Ratio ^{14}C:^{3}H†	
	H2B–3–2A	H4
Mock-infected cell	0.97	0.96
Virus-infected cell	2.00	1.88
Purified virions	11.73	7.78

* Cultures at 14 hr after seeding were labeled with 2 μCi (or 5 ng) of [^{3}H]leucine per ml. Monolayers were confluent on day 4. On day 5, they were washed twice, incubated for 1 day in fresh medium to deplete the intracellular [^{3}H]leucine pool, and then infected (50 plaque-forming units/cell). Cells were then incubated for 50 hr in medium containing 2.1 mM (or 267 μg/ml) unlabeled leucine and 1.2 μCi of ^{14}C-labeled amino acids per ml. An aliquot of infected cells was saved and virus was purified from the remainder of the sample.

† Samples were solubilized in $NaDodSO_4$ and analyzed on 9% $NaDodSO_4$ gels that were stained with Coomassie brilliant blue, destained, scanned, and sliced for determination of radioactivity (10). The ^{3}H values were corrected for the ^{14}C spillover. H2B, 3, and 2A migrated together as a broad band and were analyzed as such.

amount of stained virion histones, we estimated 11% of the H2B–3–2A complex and 17% of H4 to represent old histones. These values are overestimations because ^3H radioactivity was detected in VP1, the major viral structural polypeptide, indicating that amino acids derived from degraded prelabeled proteins were utilized for protein synthesis occurring after virus infection. Based on the recovery of histones from the virus particles and from the infected cells, 25–30% of the total (both old and new) intracellular histones (excluding H1) between 40 and 80 hr after infection was estimated to be within virus particles.

DISCUSSION

It has been known for a long time that SV40 induces the synthesis of both cellular DNA and histones and that histones are integral components of virions (1, 19–21). Some of the questions generated by these studies include the following: (*i*) What is the extent and specificity of histone synthesis? (*ii*) Is histone synthesis coupled with viral DNA replication? (*iii*) Does the viral chromatin contain the same histone species in the same proportions as cellular chromatin? I have attempted to answer these questions in this paper.

The synthesis of histones in SV40-infected cells resembles that in uninfected cells in that: (*i*) all histone species are synthesized; (*ii*) histones are synthesized in the same relative amounts; and (*iii*) histone synthesis is coordinated with viral DNA replication. The synthesis of histones in uninfected cells is coordinated with cellular DNA replication (22). Since virus-induced cellular DNA replication is essentially over at the time of maximum viral DNA synthesis (Fig. 1*A*), and the overall histone synthesis continues during viral DNA replication (Figs. 1*C* and 2*A*), this implies that, when cellular DNA replication is shut off, histone synthesis (and phosphorylation) is not and it becomes coupled to viral DNA replication.

Preliminary studies on histone phosphorylation revealed that the histones from virus-infected cells incorporated more [^{32}P]phosphate than their counterparts from mock-infected cells and the rate of H1 and H2A phosphorylation paralleled that of synthesis (Fig. 2). H1 phosphorylation occurs at different stages of the cell cycle and may be involved in regulating DNA replication, chromatin structure, and the initiation of mitosis (23). In SV40-infected cells, H1 appears to be highly phosphorylated; whether this is related to viral DNA replication remains to be determined.

The weight ratio of histones:DNA in both mammalian cells (24) and SV40 (1) is about 1.0. The histones incorporated into virions were enriched with the arginine-rich species H3 and H4 (Table 1, Fig. 3), in agreement with the results of Polisky and McCarthy (6). Whereas it has been reported that arginine-rich histones are associated with GC-rich regions of DNA (25), the enrichment of these histones in virions cannot be attributed solely to this phenomenon because both viral and cellular DNA have the same GC content, 40–42% (26). The histone composition of virions is probably determined by factors operating during virus assembly and by the tightness with which the histone species bind to each other and to DNA. Because H3/H4 have the strongest interaction (27) and bind to DNA tighter than the other histones (28), their incorporation into virions would be favored over the incorporation of other histones.

The association of old and newly synthesized histones with cellular DNA has been studied by several investigators and two models have been proposed: (*i*) both old and new histones are distributed randomly onto the newly synthesized DNA (29, 30), and (*ii*) newly synthesized histones are selectively associated with newly synthesized DNA (31, 32). It is interesting to note that virion DNA, which represents a newly synthesized DNA moiety in the infected cell, is associated predominantly with new histones (Table 2). This finding supports model (*ii*) stated above but only if one assumes that the replication of viral DNA is similar to that of cellular DNA. The induction of histone synthesis in SV40-infected cells is therefore important for providing new histones for virion formation. The old histones in SV40 may represent: (*i*) recycled histones (29, 30), (*ii*) histones derived from degraded cellular chromatin (33), or (*iii*) histones associated with cellular DNA fragments that may be incorporated into virions. Lavi and Winocour (34) have demonstrated that viral DNA can acquire host DNA sequences if the cells are infected with greater than 4 plaque-forming units of virus per cell.

SV40-associated H4 is heterogeneous and this may reflect different degrees of acetylation of H4 molecules. The arginine-rich histones in polyoma virus are reported to be acetylated (18). Mutants that are unable to transform cells contain histones that are acetylated to a lesser extent than are those of the parental wild-type virus (18). Thus, modification of virion histones, for example, by phosphorylation (10) or by acetylation (18), may play an important role in regulating the events that follow infection of cells with papovaviruses.

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