Vol. 83, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 14, 1978 Pages 814-821

# PROPERTIES OF THE HISTONES ASSOCIATED WITH SIMIAN VIRUS 40 REPLICATIVE FORM NUCLEOPROTEIN COMPLEX

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Received June 9,1978

SUMMARY: Nucleoprotein complexes containing replicative form SV40 DNA were isolated from infected cells. Electrophoretic analysis of purified samples in 15% acrylamide gels revealed the presence of histones H1, H3, H2B, H2A and H4. In addition, the complex electrophoretic patterns of these histones contained a series of protein bands migrating in the expected positions for acetylated subfractions of histones H3 and H4. The identity of these bands was supported by their relatively high specific activities when [$^{14}$C]acetate was incorporated.

## INTRODUCTION

In cells lytically infected with polyoma virus or simian virus 40, progeny viral DNA molecules become associated with proteins forming complexes which can be extracted by cell lysis with nonionic detergents (1-5). The proteins of purified NPCs are primarily histones (6-9). Crude extracts of viral infected cells contain a mixture of viral NPCs consisting of NPCs with closed circular supercoiled viral DNA and NPCs with replicative forms of viral DNA (1,3,4,10,11). We have demonstrated that the sedimentation coefficients of SV40 NPC-SC and NPC-RF are 61S and 70S respectively. However, glutaraldehyde fixed NPC-RF and NPC-SC have identical buoyant densities indicating that protein is bound to all forms of SV40 viral DNA at the same protein to DNA ratio (4).

It has been suggested that histone-DNA interactions are involved in modulation of DNA replication (12). The existence of NPC-RF presents a unique opportunity for analysis of histone-DNA and histone-histone interaction in the replicative metabolic state of the chromosome. Although several of the reports discussed above were concerned with electrophoretic separation and quantitation of NPC-SC histones, similar information has not been reported concerning NPC-RF. Therefore, in the present investigation, we have developed a method for isolation and purification of NPC-RF, and have analyzed their

The abbreviations used are: NPC, nucleoprotein complex; NPC-RF, nucleoprotein complex containing replicative form SV40 DNA; NPC-SC, nucleoprotein complex containing circular supercoiled SV40 DNA.

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0006-291X/78/0833-0814$01.00/0

histone composition by acrylamide gel electrophoresis.

## MATERIALS AND METHODS

Virus and Cells. Simian virus 40 (strain RH911) was propagated in the TC-7 clone of the CV-1 monkey kidney cell line. Cell monolayers were grown to confluence on 10 cm plastic petri dishes containing 10 ml of Eagle medium supplemented with 10% calf serum. Cultures were infected approximately 24 hours after confluence with 0.2 ml of virus (1-2 pfu/cell) and the virus was allowed to adsorb for 90 minutes. After adsorption, 10 ml of Eagle medium containing 2% horse serum was added to each culture.

Purification of Viral Nucleoprotein Complexes. Nucleoprotein complexes containing SV40 DNA were extracted by procedures described previously (3,4) and the crude supernatant fraction was concentrated to approximately one fifth its original volume by vacuum dialysis using a Sartorious collodion bag apparatus. Initial purification of the concentrated extract was accomplished by velocity centrifugation of 0.5 ml samples in 10.0 ml linear 10 to 30% (wt/wt) sucrose gradients prepared in STTE buffer (0.25% Triton X-100, 0.01 M EDTA, 0.15 M NaCl, and 0.01 M Tris-HCl, pH 7.9). Samples were centrifuged at 35,000 rpm for 165 minutes at 4°C in a Spinco SW 41 rotor. Tubes were punctured at the bottom and 35-40 fractions collected. Aliquots (25λ) of each fraction were transferred to filter discs and trichloracetic acid precipitable radioactivity determined. Fractions corresponding to the 70S replicative form nucleoprotein complex (4) were pooled and diluted to approximately 10% sucrose by addition of STTE. The diluted NPC-RF preparations were further purified by layering 2.0 ml samples onto sucrose gradients consisting of 2.5 ml 20% wt/wt sucrose in STTE over a cushion of 0.5 ml 60% wt/wt sucrose in STTE. These gradients were centrifuged at 40,000 rpm for 16 hours at 4°C in a Spinco SW 50.1 rotor. The pellet containing SV40 nucleoprotein complex was either dissolved in STTE or the electrophoresis buffer described in a succeeding paragraph.

Virus Purification and Labeling. Immediately following transfer, 10 ml of Eagle medium containing 10% calf serum and either 5.0 μCi/ml of [$^{3}$H]amino acids (reconstituted protein hydrolysate) or 2.5 μCi/ml [$^{14}$C]acetate was added to the cells and the cultures were incubated until they had been confluent for 24 hours. At 24 hours postconfluence, labeling medium was removed and cells were infected as described above. After infection, cells were overlayed with Eagle medium containing 2% horse serum and either 5 μCi/ml [$^{3}$H]amino acids or 2.5 μCi/ml [$^{14}$C]acetate. Approximately seven days postinfection, when infected monolayers were 80%-90% lysed, virus was isolated and purified by standard techniques (13,14).

Histone Extraction. Confluent TC-7 monolayers were lysed by the procedure previously described for NPC extraction (3,4) with the exception that, after addition of NaCl, plates were incubated for ten minutes rather than three hours. The pellet, containing TC-7 nuclei, was retained and histones were extracted utilizing 0.4 N $H_2SO_4$ as described by Panyim et al. (15). Histone concentrations in selected samples were determined by the method of Lowry et al. (16) using calf thymus histones as standards.

Polyacrylamide Gel Electrophoresis. Proteins were resolved by electrophoresis in 15% acrylamide gels containing 0.9 N acetic acid and 2.5 M urea as described by Panyim and Chalkley (17). Histones, purified virions, or NPC were dissolved in a dissociation buffer consisting of 10 M urea, 2% 2-mercaptoethanol, 5 mg/ml protamine sulfate, 15% sucrose (w/v) and 0.9 N acetic acid. After incubation at 37°C for 16 hours, 50-100 μl samples were mixed with methyl green tracking dye and layered on the gels.

Relative proportions of proteins were determined by either dye content or radioactivity of the protein bands. Gels were scanned at 630 nm by a Gilford spectrophotometer gel scanner with an attached linear transport. Measurement of peak size was then used to determine relative stain content of each band. Alternatively, each band was excised from the gel and the dye removed with 2.0 ml DMSO at 37°C overnight (18). Optical density readings of the DMSO extracts were made at 615 nm in a Beckman Model 25 Spectrophotometer. Since amido black binds to each class of histone with a different affinity, the dye content values were adjusted by binding factors determined by Sonnenbichler and Zetl (19).

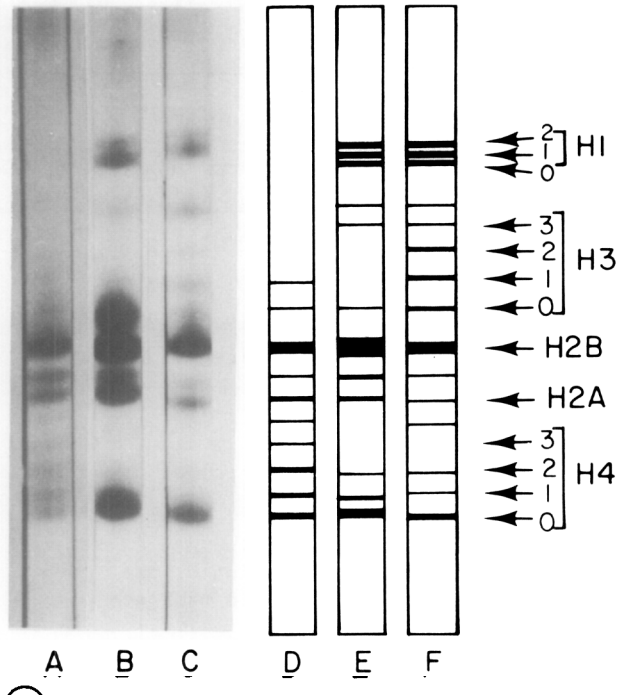
Radioactivity of protein bands was determined by either excising the whole band or slicing an entire frozen gel into 1 mm disks with a Biorad Model 190 gel slicer. The excised bands or the individual gel slices were solubilized in 0.5 ml of 30% $H_2O_2$ (v/v) for a minimum of 24 hours at 37°C and then 10 ml of Yorktown TT21 scintillation fluid was added for scintillation counting.

## RESULTS

Electrophoretic Analysis of Histone Fractions. Histones isolated from NPC-RF, TC-7 cells, and SV40 virions were separated by polyacrylamide gel electrophoresis. Samples were electrophoresed for a minimum of 20 hours on 18 to 20 cm acetic acid-urea gels. Under these conditions, all histone fractions migrated a distance of 12 to 18 cm. The major histone fractions were identified using histone standards (Sigma Chemical Co., St. Louis, Missouri) which were coelectrophoresed in separate gels.

The electrophoretic patterns of histones obtained from SV40 virions, TC-7 cells, and RF complex are shown in Fig. 1A-C. The five major histone fractions and some subfractions are identified in the diagrammatic representations (Fig. 1D-F). These results clearly indicate the histones from each of the sources give different electrophoretic patterns. The SV40 gel (Fig. 1A) shows four distinct H4 subfractions and the H1 fractions are absent. Several slower migrating viral proteins which are not histones are also visible when SV40 virions are electrophoresed (data not shown). The NPC-RF band pattern (Fig. 1C) appears to be quite similar to that of TC-7 histones (Fig. 1B) except the NPC-RF gel contains additional distinct H3 subfractions. These differences between the histone band patterns are even more evident upon comparison of spectrophotometric scans (Fig. 2) of the gels.

Quantitation of Major Histone Fractions. The major histone fractions of TC-7, SV40 and NPC-RF were quantitated by determining dye content or the radioactivity of the histone bands. The dye content values were adjusted by binding factors (19) which took into account affinity differences between the classes of histones and amido black stain. The adjusted dye content values and the radioactivity were used to determine percentages of each histone class (Table 1). It appears that infected TC-7 cells have a 20% lower proportion of fraction H1 than do uninfected TC-7 cells and NPC-RF. Otherwise,

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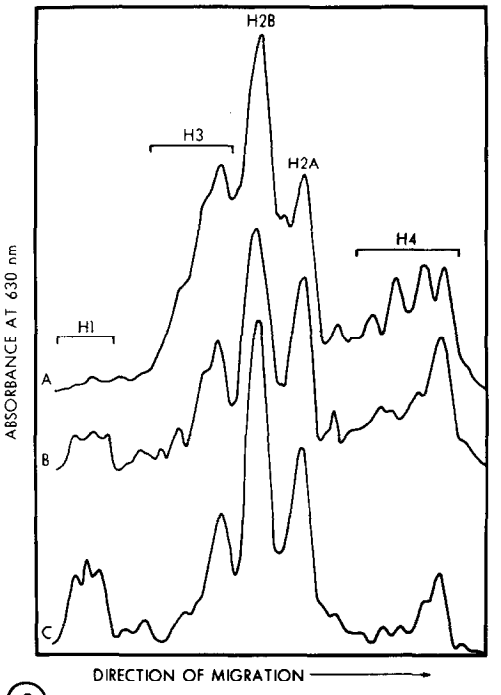


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FIGURE 1. Acetic acid-urea polyacrylamide gel electrophoresis of histones from SV40 virions (A), TC-7 cells (B), and replicative form nucleoprotein complex (C). Diagrammatic representations of each gel are presented to the right. SV40 virions (D), TC-7 cells (E), replicative form nucleoprotein complex (F). Only the histone region of 20 cm gels are shown.

FIGURE 2. Densitometric scans of the histone regions of polyacrylamide gels of SV40 virions, TC-7 cells, and replicative form nucleoprotein complexes. Samples were prepared and electrophoresed as described in Materials and Methods. Proteins were stained with amido black and the histone containing region of each gel was scanned at 630 nm. From top to bottom of the figure are: (A) SV40 virions, (B) TC-7 cells, and (C) replicative form nucleoprotein complexes.

except for the obvious absence of H1 in SV40 virions, there appear to be no patterns of differences of histone fraction content.

Quantitation of Histone Subfraction Acetylation. The electrophoretic patterns in Fig. 1 indicate that there may be quantitative differences of histone subfractions between histones isolated from TC-7 cells, SV40 virions and NPC-RF. By labeling with [$^{14}$C]acetate, it was determined that the subfractions of H3 and H4, in order of decreasing electrophoretic mobility, have increasing acetate specific activities (Table 2). This is in agreement with results obtained by Wangh et al. (20) using cellular histones and Schaffhausen and Benjamin (14) using polyoma histones. Examination of specific activities of the H4 subfractions of NPC-RF, for example, reveal that the band with the

TABLE 1. Relative percentages of major histone fractions from TC-7 cells, SV40 virions and SV40 replicative form nucleoprotein complexes

| Histone Fraction | TC-7[a] (uninfected) | TC-7 (infected) | SV40 | NPC-RF |
|---|---|---|---|---|
| H1 | 12.2 ± 1.5[b] | 7.6 ± 1.3 | 0 | 11.1 ± 1.1 |
| H3 | 17.3 ± 1.0 | 16.3 ± 1.3 | 23.3 ± 1.4 | 20.2 ± 2.3 |
| H2B | 31.9 ± 1.7 | 36.2 ± 1.5 | 37.3 ± 1.7 | 33.7 ± 1.8 |
| H2A | 22.4 ± 1.7 | 24.1 ± 1.6 | 22.6 ± 2.6 | 19.0 ± 2.3 |
| H4 | 16.2 ± 0.6 | 15.8 ± 1.5 | 17.9 ± 3.1 | 16.0 ± 0.8 |

[a]Percentages calculated from at least ten observations

[b]Mean ± S.E.

Histones were separated by electrophoresis as described in Materials and Methods. The major histone fractions were quantitated by integration of the peaks formed by scanning of stained gels or by summing the counts in radioactively labeled fractions from sliced frozen gels.

most protein and also the fastest migrating band has the lowest specific activity. The second fastest migrating subfractions of H4 show larger acetyl specific activity and migrate in the position where one would expect to find a monoacetylated form of H4. The third and fourth bands have even higher specific activities indicating a greater degree of acetylation. The same pattern is seen with the subfractions of H3 in which the slower migrating fractions exhibit successively higher acetyl specific activities. In the RF complex, the subfractions of H1 show much higher isotopic acetyl specific activities than that reported by Wangh et al. (20).

## DISCUSSION

Electrophoretic comparison of the histones isolated from SV40 RF nucleoprotein complexes with those isolated from SV40 virions and TC-7 nuclei revealed no distinct pattern of differences in the major histone fraction content of each source. With the exceptions of the complete lack of H1 in the SV40 virion and a possible decrease in the H1 fraction of TC-7 cells following viral infection, it appears that all five major histone fractions are equally represented among the various histone sources.

The finding of H1 in NPC-RF confirms the recent report by Varshavsky et al. (21) that H1 is present in SV40 nucleoprotein complexes. The presence of histone H1 in the SV40 NPC-RF and its absence from the completed virion

TABLE 2. $^{14}C$-acetate specific activities* of histone subfractions from SV40 replicative form nucleoprotein complexes, SV40 virions and TC-7 cells

| Subfraction | | RF Nucleoprotein | SV40 Virion | TC-7 (Infected) |
|---|---|---|---|---|
| H1 | 2 | 4.6 | ... | 3.9 |
| | 1 | 3.4 | ... | 2.3 |
| | 0 | 3.3 | ... | 2.7 |
| H3 | 3 | 2.2 | ... | ... |
| | 2 | 1.0 | 4.1 | ... |
| | 1 | 0.9 | 1.1 | ... |
| | 0 | 0.5 | 0.6 | 0.6 |
| H2B | | 0.6 | 0.9 | 1.1 |
| H2A | | 0.4 | 0.9 | 0.9 |
| H4 | 3 | 4.0 | 0.9 | ... |
| | 2 | 1.8 | 1.1 | ... |
| | 1 | 1.4 | 1.0 | 0.9 |
| | 0 | 0.9 | 0.9 | 0.7 |

*Specific Activity = $\frac{\text{Subfraction CPM as \% total cpm}}{\text{Subfraction wt. as \% total wt.}}$

Cultures were labeled for 48 hours with [$^{14}C$]acetate prior to infection and for the full incubation period following infection until histones, NPCs or virions were extracted. Histone fractions were separated by electrophoresis as described in Materials and Methods and the quantity of each fraction or subfraction was determined from the spectrophotometric scans of amido black stained gels. After scanning, each stained band was excised from the gel and dye was removed by incubation overnight with DMSO. Radioactivity of each sample was determined by scintillation counting of peroxide solubilized material.

naturally cause speculation as to its function. One simple interpretation might be that H1 interaction with viral DNA facilitates packaging of nucleoprotein into a compact core structure necessary for virion assembly but is not necessary for maintenance of the core structure of completed virions. Support for this model is found in results obtained by Bellard et al. (22) that addition of histone H1 produced a marked condensation of the SV40 minichromosome.

The distinct patterns of subfractions found in virion and NPC-RF histones, and their comparative lack in TC-7 histones seem to indicate some specificity in the binding of histone subfractions to SV40 virus DNA. Isotopic acetate labeling experiments with NPC-RF and SV40 virions yielded specific activities which correlated well with reports of the presence of mono-, di-, tri- and unacetylated forms of H3 and H4 (14,20,23).

Since histone modifications have been related to changes in transcription, replication, and structure of cellular chromatin by many investigators (for detailed reviews see Hnilica (24) and Delange and Smith (25)), it should be of interest to further investigate the differences in histone modifications found in NPC-RF, NPC-SC and completed SV40 virions. Of particular interest will be any modification differences between the histones of NPC-SC and NPC-RF because of the structural and functional differences between them. Variations found in histone fractions could reflect the different metabolic states of the two forms of SV40 DNA in the two nucleoprotein complexes. It is known that NPC-RF contains replicating viral DNA while NPC-SC contains the mature viral DNA (4). In addition, Su and DePamphilis (11) and Edenberg et al. (26) have demonstrated that the SV40 NPC-RF can continue DNA replication in vitro. In one report (11), at least forty percent of the replicative form DNA in NPC-RF was converted into covalently closed superhelical DNA in vitro. Significantly, the DNA products of replication were also found as nucleoprotein complexes. Thus, it appears that correlations established between the histone modification patterns of these complexes might provide a better understanding of the function of histones during DNA replication.

ACKNOWLEDGEMENTS: This investigation was supported by Grant Number NP-189 from the American Cancer Society and by Grant Number CA 23081 awarded by the National Cancer Institute. We acknowledge the excellent technical assistance of Ms. Brenda Kozyra and Mr. Warren Hall.

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