Chromosome of the mature virion of simian virus 40 contains H1 histone

S.A.Nedospasov, V.V.Bakayev and G.P.Georgiev

Institute of Molecular Biology, USSR Academy of Sciences, Vavilov str.32, Moscow 117312, USSR

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ABSTRACT

In addition to free SV40 minichromosomes in the compact form, complete virions were obtained from the nuclear extract of productively infected cells. Capsid proteins VP1, VP2, and VP3, as well as histones, were observed on electrophoregrams of proteins prepared from virions. In contrast to the widely accepted view, histone H1 was found in virions in stoichiometric amounts with respect to other histones. The same is true for virions isolated by a conventional method. Free minichromosomes present in infected cells contain all histones and practically no viral proteins.

INTRODUCTION

In cells infected with simian virus 40 (SV40), the viral DNA is simultaneously present in at least two kinds of nucleoprotein complexes: in small free chromosomes, designated as minichromosomes and in mature virions. DNA in free minichromosomes is organized in nucleosomes /1/. Upon encapsulation, DNA remains complexed with histones and thus the mature virions represent the same particle covered with capsid proteins: VP1 (the main component), VP2, and VP3. It was accepted until recently that neither free minichromosome nor virions contained H1 histone. Only four other histones were detected /2-5/. In the previous reports from this laboratory H1 was found in free minichromosomes isolated from SV40 infected cells /6,7/ and seemed to be responsible for compactization of minichromosome /7,8/.

Now we have modified the isolation procedure in order to obtain high amounts of mature virion particles directly from the nuclear extracts of infected cells. Analysis of its protein composition by gel electrophoresis in different buffer

systems revealed the presence of a complete set of histones including H1 histone in usual stoichiometric amounts with respect to other histones. Three capsid proteins were also detected. Virions isolated from nuclear extracts were indistinguishable from the preparation obtained in a conventional way in respect to their electron microscopic appearance, buoyant density and infectivity. Finally, we also found H1 in the latter.

One may conclude that the minichromosome of SV40 being organized in a compact particle of $\sim 300 \text{ \AA}$ in diameter is incorporated into the virion as a whole without a loss of any histone fraction.

MATERIALS AND METHODS

Cell growing and labeling. The green monkey kidney cells CV1 were grown in Eagle's minimal essential medium (MEM) supplemented with 10% calf serum. When cell monolayers became confluent ($2-5 \times 10^7$ cells/flask), they were washed with phosphate-buffered saline (PBS) and then infected with 20 PFU/cell of plaque purified SV40 virus (strain 776). The medium after infection contained 1% dialysed calf serum.

The cells were labeled with $[\text{Me-}^3\text{H}]$ thymidine ($0.25 \text{ \mu Ci/ml}$) and $[\text{}^{14}\text{C}]$ protein hydrolysate ($0.5 \text{ \mu Ci/ml}$) added 20-30 hrs after infection. At 70-75 hrs after infection (this stage was used for preparation of large amounts of virions) only about one-tenth of cells had the SV40 specific cytopathic morphology.

Preparation and fractionation of nuclear extracts. After washing twice with 0.14 M NaCl to remove the radioactive medium, the cells were allowed to swell at 4°C for 10-15 min in 0.25% Triton-X-100, 10 mM Na-EDTA , $10 \text{ mM triethanolamine}\cdot\text{HCl}$ (TEA-HCl), pH 6.8 (10 ml of the buffer per 5×10^7 cells). The lysate obtained upon the addition of 1 M NaCl , $0.5 \text{ M phenyl-methylsulphonyl fluoride}$ (PMSF; Sigma) up to 0.12 M and 1 mM , respectively, was decanted from flasks or gently scrapped with a rubber policeman and centrifuged at 3000 g for 5 min at 4°C . The nuclear pellet was briefly washed with 0.25% Triton X-100, 0.12 M NaCl , 10 mM Na-EDTA , 10 mM TEA-HCl , pH 6.8, and centrifuged. The pellet was resuspended in the same buffer (but at pH 8.0) at a concentration of about 2×10^8 nuclei/ml and extrac-

ted for 3-4 hrs at 4°C with continuous mixing on a magnetic stirrer. The extract was cleared at 5000 g for 10 min, and layered onto linear sucrose gradients (5-25%) in 0.12 M NaCl, 1 mM Na-EDTA, 1 mM TEA-HCl, pH 7.5. The samples were centrifuged in a SW25.2 rotor (Beckman) at 24,000 rpm for 4 hrs at 3°C. Peak fractions were pooled and used for the next experimental procedures.

In these conditions we found that only traces of the [³H]labeled acid insoluble material was lost in the pooled supernatant obtained before extraction.

Polyacrylamide gel electrophoresis of proteins. We have carried out the SDS-gel electrophoresis in 15% gels as described by Laemmli /9/ or in 5% gels as described by Bhoree and Pedersen /10/. The acetic acid-urea electrophoresis was performed according to Paniym and Chalkley /11/ with modifications /12/, two-dimensional electrophoresis was performed according to Orrick et al. /13/.

Electron microscopy. The material from the sucrose gradient was applied to carbon coated grids directly or after fixation with 1% formaldehyde. The grids were extensively washed and stained with 1% uranyl acetate. The samples were examined in a JEM-100B electron microscope (Jeol) at instrumental magnification of 50,000.

Ultracentrifugation in CsCl density gradient. For further purification of the heavy peak obtained from the sucrose gradient, the solution of CsCl ($\rho = 1.9 \text{ g/cm}^3$) was added to pooled fractions to a final density of 1.33 g/cm^3 . The samples were centrifuged in a SW60Ti rotor (Beckman) at 35,000 rpm for 20 hrs at 20°C.

For analytical purposes, the samples before ultracentrifugation were first fixed with 1% formaldehyde for 12 hrs and then extensively dialysed against 0.1 M NaCl, 1 mM Na-EDTA. The samples were centrifuged in the same rotor at an average density of 1.45 at 40,000 rpm for 24-30 hrs at 20°C for banding of free DNA, minichromosomes and virions in the parallel gradients, or alternatively at an average density of 1.35 at 35,000 rpm for 24-30 hrs at 20°C in the same rotor for the accurate

determination of the viral particle density. Sarcosyl was added to the fixed samples to a final concentration of 0.1%.

The fraction density was determined by measuring the refraction index. The acid-insoluble radioactivity profile was determined after counting the appropriate aliquotes on GF/B filters (Whatman) in the toluene scintillator in a SI 30 spectrometer coupled with a multi-20 computer (Intertechnique) with the program for distinguishing the double label.

THE RESULTS

1. Isolation of virions and free minichromosomes from nuclear extracts of the infected cells

The isolated nuclei of CV1 cells taken at different intervals after infection were extracted by 0.12 M NaCl, 10 mM Na-EDTA, 10 mM TEA-HCl, pH 8.0, and the extracts obtained were ultracentrifuged in a sucrose density gradient. In comparison with the previous experiments /6,7/ the time of centrifugation was reduced (to 4 hrs) and that resulted in the appearance of the fast sedimenting peak containing labeled DNA in addition to the $\sim 70S$ peak corresponding to free minichromosomes (Fig.1). At the late stage of infection, the heavy peak was predominant while, at early stages, most of the labeled DNA was concentrated in the light one. Both peaks contained the SV40 DNA mostly in the supercoiled state, form I (data not shown).

To characterize particles present in the heavy (peak I) and light (peak II) components, the material obtained from the sucrose gradient was examined in electron microscope using the negative contrast technique. One can see (Fig.2) that peak I contains particles ca 450 Å in diameter with resolved capsomers indistinguishable from the SV40 virions. Peak II contains typical minichromosomes in the compact form ca 300 Å in diameter /7/.

After analysis, the material of peak I was analysed by ultracentrifugation in a CsCl density gradient (Fig.3). The particles present in peak I had the buoyant density typical of mature virions (1.345 g/cm^3) /14/. The fixed minichromosomes from peak II had a higher buoyant density ($\sim 1.45 \text{ g/cm}^3$), where as unfixed particles dissociated into DNA and protein (not shown).

Fig. 1. Sucrose gradient centrifugation of cleared nuclear extracts taken at different times after infection.

The cells were incubated with [^3H]thymidine since the 20th hr after infection. The nuclear extracts taken 28 hrs (a), 45 hrs (b) or 75 hrs (c) after infection were ultracentrifuged in a 5–25% linear sucrose gradient for 4 hrs (see Materials and Methods).

—○— [^3H]DNA; —●— [^{14}C]protein and —△— optical density; the DNA/protein ratio.

Arrows indicate fractions which were pooled for further analysis.

Finally, we compared the specific infectivity of the particles obtained from peak I with that of viral stocks prepared in a usual way. In our conditions, 1 ml of pooled fractions from peak I corresponded to $\sim 10^9$ plaques, whereas 1 ml of viral stock gave $\sim 10^8$ plaques. The virion concentration (as estimated from DNA yield) in the first case was ~ 5 times higher. In other words, the specific infectivity of the particles from peak I corresponded to that of SV40 virions.

Thus, according to all above mentioned tests, the partic-

Fig. 2. Electron microscopy of particles separated by gradient centrifugation.

Particles from peak I (a) and peak II (b) negatively stained with uranyl acetate (see the text for details); bar corresponds to $0.1 \mu\text{m}$.

les present in peak I can be accounted as mature virions of SV40. Further details of our procedure for preparation of SV40 virions as well as the kinetics of virion formation in the nuclei will be described elsewhere (Bakayev *et al.*, in preparation).

2. The protein composition of SV40 virions and free mini-chromosomes

The protein composition of virions obtained from nuclear extracts was studied. First of all, the SDS gel electrophoresis has been performed (Fig.4). The Laemmli variant of this technique /9/ using 15% gel does not separate histone H1 from VP3 and it is not possible therefore to identify H1 by this method. However, the electrophoresis in 5% gel in phosphate buffer /10/ allows one to separate easily the two aforementioned-

Fig. 3. Buoyant density centrifugation of the fast sedimenting particles (peak I).

Pooled fractions from the sucrose gradient (see Fig. 1) were dialysed overnight against 0.1 M NaCl, 10 mM TEA-HCl pH 7.5, 1 mM Na-EDTA and then mixed with a CsCl solution ($\rho = 1.9 \text{ g/cm}^3$) to a final density of 1.3. The samples were run at 35,000 rpm in a SW60 rotor at 20°C for 20 hrs. The fractions were collected from the bottom and the profiles of the density and the radioactivity were determined.

—○— [3H]DNA; —●— [14C]protein; the DNA/protein ratio; —▲— buoyant density profile.

ed proteins. Therefore, this technique was applied for the analysis of virion proteins. One can see that virions isolated from the nuclei of the infected cells contain three capsid proteins VP1, VP2 and VP3, and all the five histones, H1, H2a, H2b, H3 and H4. Histone H1 is present in the same stoichiometric ratio to the other histones as in the host chromatin (Fig. 5).

The existence of histone H1 in the virion was confirmed in experiments where the electrophoresis was performed in the acetic acid-urea medium according to Paniym and Chalkley /11/ (Fig.6). Again, the location of one of the proteins present in the virions exactly coincided with the position of H1 histone.

Fig. 4. SDS gel electrophoretic analysis of proteins of virions and free minichromosomes.

(a-c) Separation in a 15% discontinuous gel according to Laemmli /9/;
 (a) - virions from a sucrose gradient; (b) - virions purified according to Estes et al. /16/; (c) CV-1 histones.
 (d-m) Separation in a 5% gel in phosphate buffer according to Bhorjee & Pedersen /10/.
 (d) CV-1 histones; (e) virions from a sucrose gradient; (f) minichromosomes; (g) virions from a CsCl density gradient; (h,i) CV-1 histones; (j) minichromosomes; (k) minichromosomes purified by recentrifugation; (l) virions purified according to Estes et al. /16/; (m) virions from a CsCl density gradient.

The slots (d-h) and (i-m) were obtained in two different experiments.

prepared from the host chromatin (Fig.6). One can also see the small but significant changes in the electrophoretic mobilities of histones (particularly, of H4) which may depend on extensive modification of histones in the virion /15/.

Thus, the results obtained in two different electrophoretic systems allow us to identify histone H1 among the proteins present in the virion. In addition, these two systems were combined in the two-dimensional gel electrophoresis and the positions of capsid proteins and histones were determined (Fig.7). Again two spots of the virion proteins are located exactly in

Fig. 5. Densitogram of the virion protein distribution after electrophoretic separation.

The gels were stained with Coomassie Brilliant Blue as described /6/. Densitograms were obtained with the use of Opton apparatus.

(a) CV-1 histones - slot d from Fig.4; (b) virions from a sucrose gradient - slot e from Fig. 4.

Squares under the curves correspond to amounts of H1 (A) and of other four histones (B). The ratio of A to B is equal to 0.24 (a) and 0.22 (b).

Fig. 6.. Acetic acid-urea gel electrophoresis of proteins of virions and free minichromosomes.

(a) Virions from a sucrose gradient; (b) CV-1 histones; (c) free minichromosomes; (d,f) virions from a CsCl density gradient; (e) zone of minichromosomes after centrifugation of disrupted virions (see the text).

The slots (a,b), (c,d) and (e,f) were obtained in three different experiments.

The absence of sharp bands in H3 and H4 zones is due to modification of the viral histones.

the position of two subfractions of histone H1. It also seems from Fig.7 that the material present in the upper left part of the gel represents the oligomers of VP1. Thus, no proteins except three viral specific proteins and histones are present in the mature virion in significant amounts.

To be sure that histone H1 is really present in the virions, the viral particles purified by the CsCl density gradient ultracentrifugation without prefixation. Each treatment with concentrated CsCl led to a disruption of some part (~20-30%) of the virions. However, it did not change the protein composi-

Fig. 7. Two-dimensional gel electrophoresis of viral proteins.

Electrophoresis of the virions purified by sucrose gradient ultracentrifugation. The first dimension (in acetic acid-urea) was from left to right, the second (in SDS phosphate buffer) from top to bottom. The network in the left upper part of the gel corresponds to VP1 oligomers.

tion of the virions surviving the treatment (Figs. 4,6). Further, the proteins of the virions isolated from the nuclear extracts and by the conventional method [16,17] were compared. Again, no difference in the histone composition could be observed (except a small degradation of VP1 in the latter case) (Figs. 4,6). Thus, the presence of H1 in the virion cannot be attributed to differences in the isolation procedure.

The next experiment was performed to check whether H1 histone in the virion is associated with the SV40 chromosome itself. For this purpose, the virions were disrupted by treatment with 10 mM dithiotreitol at pH 7.5 at room temperature [18,19] and fractionated by sucrose gradient centrifugation as described for nuclear extracts (not shown). In this case, H1 as well

as other histones were found in the peak of the minichromosomes (Fig.6).

In most of the above mentioned experiments the proteins of free minichromosomes were also examined (Figs.4,5,6). We have confirmed the previous observation about the existence of a full set of histones in minichromosomes.

After the first ultracentrifugation, a small amount of VP1 cosediments with free minichromosomes. However, after re-centrifugation (and in some experiments even after the first one) the minichromosomes are virtually completely free of the viral proteins (VP1, 2 and 3).

DISCUSSION

This work has succeeded in demonstrating the presence of H1 histone in the chromosome of the mature SV40 virion. The failure to find it claimed in previous reports probably depended on the inadequacy of analytical methods.

Thus, the composition of free and encapsulated SV40 chromosomes is very similar. Put differently the maturation of a virion comprises the covering of a minichromosome being in the compact form with capsid proteins. It is very probable that the spatial organization of the SV40 chromosome does not change in this process although some minor alterations depending on the additional modification of histones may take place. The size of the free SV40 chromosome in the native compact state is ca 300 Å in diameter /7/. Such a particle can be easily packed into an icosahedral virion ca 450 Å in diameter. Several possible models for the structure of the compact minichromosome have been proposed /7,20/. In two of them, the nucleosomes form a dodecahedron. One variant considers the localization of all the DNA organized in nucleosomes on the surface of the dodecahedron. Another variant postulates the existence of a returning DNA strand going inside the dodecahedron. In both cases, the dodecahedral arrangement of nucleosomes of the SV40 chromosome can determine the assembly of the capsid in the form of an icosahedron.

The existence of a minichromosome with a full set of histones within the virion opens interesting possibilities for

studying its organization. In this case the capsid should limit the rearrangement of the chromosomal constituents during the treatment with different agents. On the other hand, experiments with micrococcal nuclease digestion have demonstrated that the capsid does not prevent from the penetrating of some small proteins to the encapsulated chromosome.

The previous communication from this laboratory on the complete stability of the SV40 minichromosome to micrococcal nuclease /7/ depended on the fact that non-optimal conditions were used for the digestion. In fact, although the minichromosome is more resistant to this nuclease than the nuclear chromatin, it may be digested both in the compact configuration and in the encapsulated state, provided the appropriate conditions are used. Studies on the pattern of nuclease digestion of the SV40 chromosome inside the virion are in progress now.

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