View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

SV 40 NUCLEOPROTEIN COMPLEXES: STRUCTURAL MODIFICATIONS AFTER ISOPYCNIC CENTRIFUGATION IN METRIZAMIDE GRADIENTS

P. F. PIGNATTI*, C. CREMISI*, O. CROISSANT** and M. YANIV*

*Department of Molecular Biology, and **Department of Virology,*
Institut Pasteur, 25 rue du Dr. Roux, 75015 Paris, France

Received 17 October 1975

1. Introduction

Nucleoprotein complexes (NPCs) containing viral DNA and cellular histones except H1 can be isolated from permissive cells infected with SV 40 [1] or polyoma virus [2]. Similar complexes can be obtained by mild dissociation of SV 40 or polyoma virions [3,4]. Electron microscopic studies of these complexes showed the presence of 21 ± 2 ν bodies or nucleosomes interconnected by naked DNA filaments [5,6]. This beaded structure is very similar to that of cellular chromatin [7,8]. The purification of viral NPCs, essentially limited to sucrose density gradients, is insufficient. Buoyant density centrifugations of irreversibly fixed NPCs have been described [9–11], but the fixation process might introduce artifacts, and the material so purified is of little use for further biochemical studies.

Metrizamide, a new density gradient medium [12], was recently used for the purification of chromatin in the absence of aldehyde fixation [13], for the isolation of reconstituted DNA–histone complexes [14], for the purification of ribonucleoprotein particles [15], and for biochemical studies on polyoma NPCs [16].

We have isolated SV 40 nucleoprotein complexes by sedimentation on sucrose gradients, and further purified them by isopycnic centrifugation in metrizamide gradients. Detailed electron microscopic studies of SV 40 NPCs show that although the beaded circular structure is generally well preserved after metrizamide centrifugation, several differences appear when compared to complexes partially purified by sedimentation in sucrose gradients. The NPC contour length is increased by about 45%, each complex has lost on average two nucleosomes and each remaining nucleosome contains less DNA. These changes which occur in the SV 40 nucleoprotein complexes after isopycnic centrifugation in metrizamide gradients point out the necessity of careful consideration in the use of this product as an analytical reagent.

2. Materials and methods

Detailed experimental procedures for the preparation of NPCs and for electron microscopic analysis have been previously given [6].

2.1. *NPC preparation*

African green monkey kidney cells (HP8, a subclone of CV1) were infected at 50 pfu/cell with originally plaque purified, low multiplicity propagated SV 40. Infected cultures were labelled with [^{14}C]- or [^{3}H]thymidine at 25 h post-infection. At 30 h the cells were collected, the nuclei were isolated [17] and the NPCs extracted [2]. Partial purification of NPCs was obtained by sedimentation on 5 to 20% sucrose gradients.

2.2. *Metrizamide gradients*

Metrizamide, a tri-iodinated glucose derivative (2(3-acetamido-5-*N*-methylacetamido-2,4,6-triiodobenzamido)-2-deoxy-D-glucose) was obtained from

* Present address: Instituto di Genetica dell'Università, Via Sant'Epifanio 14, 27100 Pavia, Italy.

Nyegaard and Co., Oslo. Metrizamide solutions were prepared in a buffer containing 0.01 M Hepes (Calbiochem.) pH 7.9, 0.001 M EDTA, 0.01 M β-mercaptoethanol, and 0.2 M NaCl. Three different procedures were used for the formation of metrizamide gradients: (a) Discontinuous gradients were made up of 1.1 ml cushions of metrizamide solutions of a density of 1.31, 1.24 and 1.18 g/cm^3. NPC samples (0.5 ml) isolated by sedimentation in 5 to 20% sucrose were layered on the gradients, and centrifuged at 50 000 rpm for either 3 h or 12 h at 4°C in a SW 56 rotor; (b) Continuous gradients were prepared utilizing solutions of 1.3 and 1.16 g/cm^3 (4 ml each). Samples of 4 ml each were layered on the gradients, and centrifuged at 40 000 rev/min for 14 h at 4°C in a SW 41 rotor; (c) Density gradients were generated by centrifugation of a solution of NPCs in metrizamide with an initial density of 1.186 g/cm^3 for 72 h in fixed angle rotors. Fractions were collected, and density was determined according to the formula $\rho = 3.350\ c - 3.462$ [14], where c is the refraction index. The radioactivity was measured after precipitation of aliquots from the different fractions to determine the position of NPCs. Addition of a protease inhibitor (phenylmethyl sulfonylfluoride, Sigma) during extraction and purification procedures, or omission of 0.2 M NaCl in the metrizamide solutions during centrifugation, did not change the appearance of the complex.

2.3. *Electron microscopy*

NPCs were visualised by the technique developed by Dubochet [18]. Samples were diluted 20 fold with a buffer containing 0.01 M Tris–HCl pH 7.9 and 0.001 M EDTA. A drop of the solution was applied to a carbon-coated grid which was previously activated by discharge in amylamine vapor. After 2 min, excess liquid was removed from the grid, the absorbed material was stained with 2% uranyl acetate in water, and dried on filter paper. The grids were then rotary shadowed with platinum–palladium (80:20) at an angle of 8°. Samples were observed with a Siemens Elmiskop 101 electron microscope and pictures were taken at a magnification of 16 000. Measurements were made on photographic enlargements with a computer-connected coordinatometer. An internal marker for enlargement calculations was often used (φX-174 RF DNA). Calibration was based on examination of carbon grating replicas.

3. Results

SV 40 NPCs extracted from nuclei of infected cells and partially purified by sedimentation in sucrose

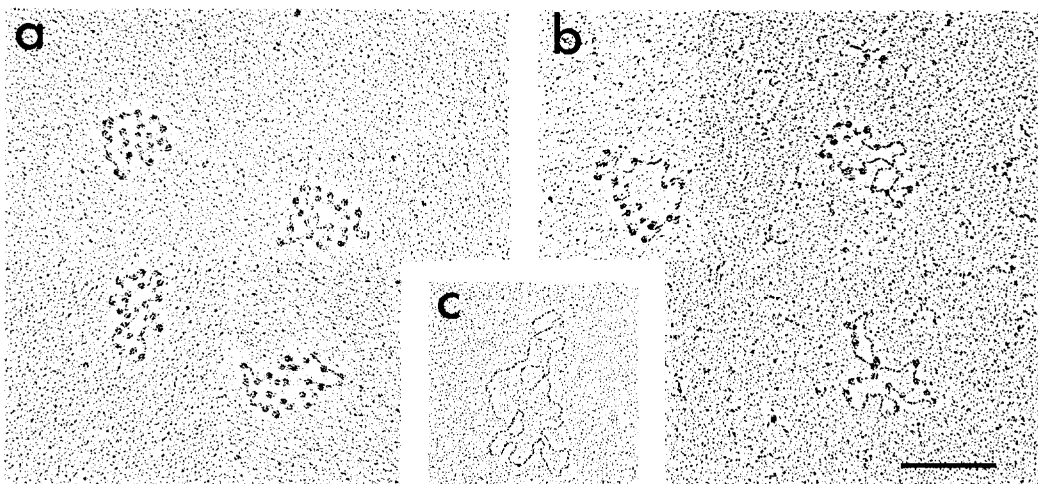

Fig.1. Electron micrographs of SV 40 NPCs. (a) NPCs after sucrose gradient purification; (b) NPCs after further purification on metrizamide gradients; (c) SV 40 DNA obtained by treatment of NPCs with 1 M NaCl for 30 min at 20°C. Samples for electron microscopy were prepared, stained and shadowed as described in Materials and methods. Further details on the technique employed have been given elsewhere [6]. The bar indicates 200 nm.

Table 1
SV 40 NPC dimensions after sucrose and metrizamide centrifugation

SV 40 dimensions	Sucrose	Metrizamide
(a) Deproteinized DNA length (nm)	1560 ± 89 (95)	same
(b) NPC length (nm)	605 ± 131 (42)	875 ± 155 (77)
(c) Condensation ratio (a/b)	2.6	1.8
(d) Nucleosomes/molecule	21 ± 2 (112)	19 ± 3 (78)
(e) Nucleosome diameter (nm)	11.8 ± 1.8 (43)	10.7 ± 1.7 (108)
(f) Base pairs/nucleosome	175	143
(g) Internucleosomal filament length (nm)	17.6 ± 11.8 (236)	36.2 ± 26 (227)
(h) Base pairs/filament	55	112
(i) Nucleosome/packing ratio (nucleosomal DNA length/e)	4.8	4.3

The values given for diameters and widths have been corrected for the fractional increase by deposited platinum–palladium. The average number of base pairs was calculated by dividing the length of DNA by 0.322, taken as the distance in nm between the plains of neighbouring base pairs [6]. The length of DNA packed in a nucleosome was calculated by subtracting from SV 40 deproteinized DNA length the sum of the internucleosomal filament lengths, and by dividing the result by the number of nucleosomes per molecule. Number of observations in brackets.

gradients appear as a circular series of globular structures called ν bodies or nucleosomes connected by thin filaments which have the same width as naked DNA (fig.1a). The fractions from the sucrose gradient containing viral NPCs were pooled and applied on to a preformed metrizamide density gradient or centrifuged to equilibrium as described in Materials and Methods. In agreement with previous observations, the NCPs band at a density of 1.164 g/cm^3, and are separated from free DNA [6]. Samples from the peak fractions were diluted and analysed by electron microscopy (fig.1b). The three different procedures used for the preparation of metrizamide gradients gave the same electron microscopic results. A molecule of SV 40 DNA obtained by 1 M NaCl treatment of SV 40 NPCs is shown at the same magnification for comparison (fig.1c). The measurements of NPCs after sucrose and metrizamide gradients are given side by side in table 1. As can be seen, the NPC length in metrizamide increases on the average 45%. The distribution of the length measurements of the complexes isolated by sedimentation in sucrose gradients and by further isopycnic centrifugation in metrizamide gradients are given in fig.2. It should be noted that the two histograms have the same shape, while being shifted along the length axis. Histogram b shows the presence of a few complexes of a length which is the double of the sucrose NPC length.

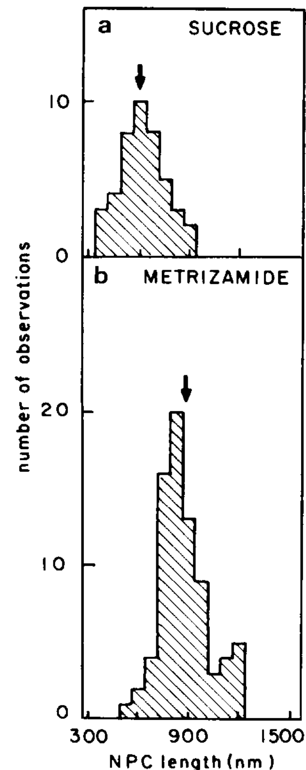

Fig.2. NPC length after (a) sucrose and (b) metrizamide gradients. Length was measured on photographic enlargements as explained in Materials and methods. The arrows indicate the means of the distributions which are given in table 1a.

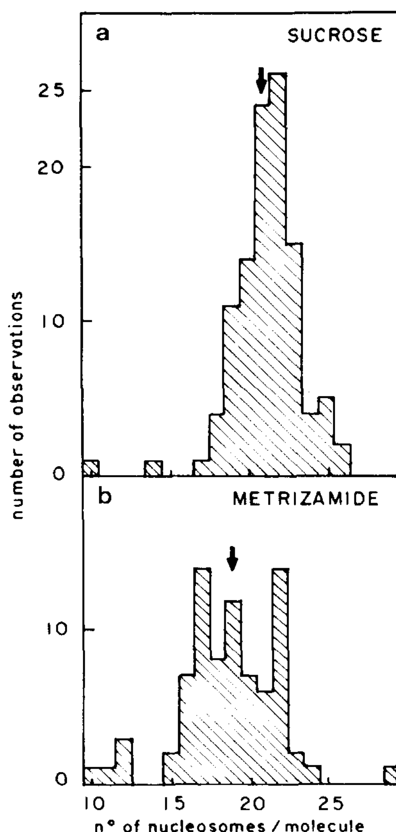

Fig.3. Number of nucleosomes per molecule after (a) sucrose and (b) metrizamide purification of SV 40 NPCs. The nucleosomes were counted on photographic enlargements. The arrows indicate the position of the means, given in table 1d.

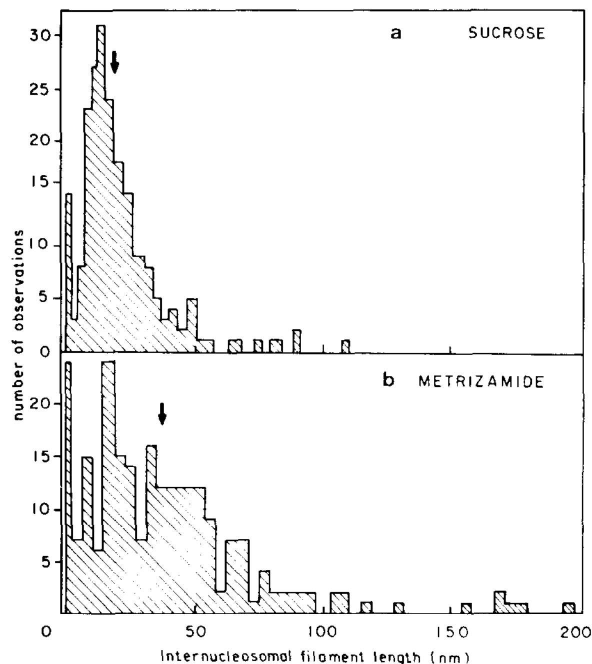

Fig.4. Distance between two successive nucleosomes on (a) sucrose and (b) metrizamide purified NPCs. The distances were measured on photographic enlargements. The column of 0 distance indicates the observations where no internucleosomal filament could be seen. The arrows indicate the position of the means, which are given in table 1g.

With regard to the number of nucleosomes per molecule, the metrizamide NPC contains on average 10% less nucleosomes of a slightly reduced diameter (table 1 d). Fig.3b shows that the small percentage difference is actually the expression of an heterogeneous metrizamide population. Thus, a class of molecules still containing 22 nucleosomes (as in the sucrose NPCs shown in fig.3a) is present, and two new important classes show up at 19 and 17 nucleosomes per molecule.

A similar even more irregular distribution is shown by the variation of the internucleosomal filament length measurements, given in fig.4a, b. While for sucrose NPCs a symmetric bell-shaped curve is clear, for metrizamide NPCs very frequent longer lengths are observed, which shift the average of all observations to twice that given in fig.4a. It should be also pointed out that a class of observations representing the average sucrose NPC internucleosomal distance is still present.

From the direct measurements given above, the number of DNA base pairs contained in the internucleosomal filaments and in the nucleosomes can be calculated, as described in the legend to table 1. These calculations showed that the loss of nucleosomes only partly accounted for the increase in contour length of metrizamide NPCs. Quantitatively more important is the contribution of a small average (18%) loss of DNA from each nucleosome (table 1).

4. Discussion

Metrizamide has been recently widely used for the purification of nucleic acids, proteins, and nucleic acid-protein complexes of various origins. The use

of a very well characterized sample for metrizamide gradients, i.e. the SV 40 NPC, was instrumental in determining important modifications taking place in the NPC structure.

An homogeneous NPC population was transformed by exposure to metrizamide into an heterogeneous one, with the loss of a few nucleosomes, and the appearance of longer stretches of naked DNA between them. The DNA content of the nucleosomes was redused down to an average number of base pairs per nucleosome (table 1, line f) similar to that obtained after prolonged nuclease digestion of chromatin [19]. About 50 base pairs could then be removed from the nucleosomes by nuclease or metrizamide treatment more easily than the remaining base pairs. It should be pointed out, in this respect, that the NPCs are otherwise quite stable structures. They can, for example, be kept at 4°C for several days after isolation on sucrose gradients, without appreciable changes, as well as for at least a few hours at 37°C.

Fig.4b shows that exposure to metrizamide produces a wide variation in the length of internucleosomal DNA with an increase in longer distances and the appearance of several well represented classes on the histogram. This finding suggests that in these conditions, no histone migration along the DNA molecule, and no rearrangement of the nucleosomes occurs, otherwise a normal symmetric distribution of observations (as in fig.4a) might be expected, and of a higher mean length, as in the case of fig.2. The hypothesis that the nucleosomes occupy random but stable positions in NPCs is in agreement with our recent observations on the partial sensitivity of sucrose purified NPCs to cleavage by Eco-R1 nuclease [6]. In that case, even after prolonged incubations, a plateau in the quantity of cleaved linear NPCs counted was reached, and the percentage of sensitive molecules was similar to the fraction of DNA which is contained in the internucleosomal filaments.

Acknowledgements

This work was supported by grants from the Centre National de la Recherche Scientifique and the Jane Coffin Childs Foundation for Medical Research. P.F.P. was supported by a long term EMBO fellowship.

We thank Dr D. Rickwood for valuable discussions, C. Dauguet and D. Cany for expert technical assistance.

References

[1] White, M. and Eason, R. (1971) J. Virol. 8, 363–371.
[2] Green, M. H., Miller, H. I. and Hendler, S. (1971) Proc. Nat. Acad. Sci. USA 68, 1032–1036.
[3] Huang, E. S., Estes, M. K. and Pagano, J. S. (1972) J. Virol. 9, 923–929.
[4] Friedmann, T. and David, D. (1972) J. Virol. 10, 776–782.
[5] Griffith, J. D. (1975) Science 187, 1202–1203.
[6] Cremisi, C., Pignatti, P. F., Croissant, O. and Yaniv, M. (1976) J. Virol., January, in the press.
[7] Olins, A. L. and Olins, D. E. (1974) Science 183, 330–332.
[8] Oudet, P., Gross-Bellard, M. and Chambon, P. (1975) Cell 4, 281–300.
[9] Mc Millen, J. and Consigli, R. A. (1974) J. Virol. 14, 1326–1336.
[10] Meinke, W., Hall, M. R. and Goldstein, D. A. (1975) J. Virol. 15, 439–448.
[11] Sen, A., Hancock, R. and Levine, J. (1974) Virology 61, 11–21.
[12] Rickwood, D. and Birnie, G. D. (1975) FEBS Lett. 50, 102–110.
[13] Rickwood, D., Hell, A. and Birnie, G. D. (1973) FEBS Lett. 33, 221–224.
[14] Birnie, G. D., Rickwood, D. and Hell, A. (1973) Biochim. Biophys. Acta 331, 283–294.
[15] Buckingham, M. E. and Gros, F. (1975) FEBS Lett. 53, 355–359.
[16] Louie, A. J. (1974) Cold Spring Harbor Symp. Quant. Biol. 39, 259–266.
[17] Burgoyne, L. A., Wagar, M. A. and Atkinson, M. R. (1970) Biochem. Biophys. Res. Comm. 39, 254–259.
[18] Dubochet, J., Ducommun, M., Zollinger, M. and Kellenberger, E. (1971) J. Ultrastruct. Res. 35, 147–167.
[19] Noll, M., Thomas, J. O. and Kornberg, R. D. (1975) Science 187, 1203–1206.