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Intracellular Forms of Simian Virus 40 Nucleoprotein Complexes

IV. Micrococcal Nuclease Digestion

M. COCA-PRADOS,† H. Y.-H. YU,‡ AND M.-T. HSU*
The Rockefeller University, New York, New York 10021

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The structures of DNAs present in various intracellular forms of simian virus 40 (SV40) nucleoprotein complexes were analyzed by micrococcal nuclease digestion. The results showed that the 70S SV40 chromatin was completely sensitive to nuclease digestion, whereas CsCl gradient-purified mature virion was completely resistant. Virion assembly intermediates with different degrees of virion maturation showed intermediate resistance, and three products were found: nucleosomal DNA fragments, representing the fraction of intermediates that were sensitive to nuclease; linear SV40 genome-sized DNA, representing the more mature intermediates that contained one or limited defects in the capsid shell; and supercoiled SV40, which was derived from mature virions. These digestion products, however, remained associated with capsid shells after nuclease digestion. These results were consistent with the model in which maturation of the SV40 virion is achieved through the organization of capsid proteins that accumulate around SV40 chromatin. Mild digestion of SV40 nucleoprotein complexes with micrococcal nuclease revealed the difference in nucleosome repeat length between SV40 chromatin and virion assembly intermediates. A novel DNA fragment of about 75 nucleotides was observed early in nuclease digestion.

In our previous studies, we have shown that there are three major classes of intracellular simian virus 40 (SV40) nucleoprotein complexes (NPC) in SV40-infected CV-1 cells (4, 5, 7). The 70S chromatin contains the major enzymatic activities for the synthesis of SV40 RNA and DNA. A portion of the 70S chromatin is converted to virion assembly intermediates, which are the precursors of mature virions. Similar results were also obtained by other laboratories (1, 8, 10). In the last report (5), we presented evidence that, during the conversion from 70S chromatin to virion assembly intermediates, histones become highly modified and the capsid proteins accumulate around SV40 chromatin. Our data favor the interpretation that maturation of the SV40 virion is achieved through the organization of the capsid proteins accumulated around SV40 chromatin. Our data are not consistent with the model in which SV40 virions are formed through the encapsidation of SV40 chromatin by the preformed capsid shells. In the present report, we used micrococcal nuclease to probe the structures of SV40 DNA during the virion maturation process. This study revealed three stages of the maturation process with respect to nuclease sensitivity. We also compared the sizes of the nucleosome DNA repeat present in 70S chromatin and in virion assembly intermediates.

† Present address: Department of Opthalmology, Yale University, New Haven, CT 06510.
‡ Present address: Department of Biochemistry, University of Southern California School of Medicine, Los Angeles, CA 90033.

MATERIALS AND METHODS

SV40 infection and extraction of SV40 NPC. Procedures for the growth of cells, SV40 infection, and isolation of SV40 NPC were described previously (7).

Gel electrophoresis. Agarose gel electrophoresis was performed with a horizontal submerged-type gel apparatus. Electrophoresis was carried out in TEA buffer (40 mM Tris [pH 7.5], 5 mM sodium acetate, 1 mM EDTA) at 60 V. For polyacrylamide gels, a vertical gel apparatus was used, and electrophoresis was carried out in TEA buffer at 100 V. After electrophoresis, the gels were either cut into 2-mm slices and assayed for radioactivity as described previously (4, 5) or fluorographed with En^{3}Hance solution (New England Nuclear Corp.) according to the instructions of the manufacturer.

CsCl gradient centrifugation. The CsCl gradient was composed of a 2-ml layer of 10% sucrose in 10 mM sodium phosphate (pH 6.8)–0.1% Triton X-100, an 8-ml CsCl solution (density, 1.34 g/ml), and a 1-ml Cs_2SO_4 cushion (density, 1.6 g/ml). The gradient was centrifuged at 30,000 rpm for 20 h in an SW40 rotor.

Digestion of SV40 NPC with micrococcal nuclease.

SV40 NPC isolated from sucrose gradients were diluted with micrococcal nuclease digestion buffer (50 mM Tris [pH 7.8], 60 mM KCl, 15 mM NaCl, 2 mM $CaCl_2$) and digested with 40 U of micrococcal nuclease (Worthington Diagnostics) per ml at either 0 or 37°C. The samples were preincubated at the desired temperature for 30 min before the enzyme was added. The reaction was stopped by adding EDTA to a final concentration of 10 mM, and the DNA was immediately extracted with phenol and chloroform as described previously (7).

RESULTS

Accessibility of SV40 NPC to digestion with micrococcal nuclease. Two major forms of SV40 NPC can be extracted from SV40-infected CV-1 cell nuclei at late times after infection (1, 7, 8, 10): 70S chromatin, which contains the major biosynthetic activity of SV40 RNA and DNA, and virion assembly intermediates, which sediment heterogeneously around 180S and contain SV40 NPC at various stages of virus maturation. The final product of virus assembly, i.e., the mature virus particle, could be purified from the rest of the assembly intermediates by banding in CsCl gradients at a density of 1.34 g/ml. The DNA present in the CsCl gradient-purified mature virus particle was completely resistant to micrococcal nuclease digestion, whereas the DNA in the 70S chromatin was converted into nuleosome-sized DNA fragments after extensive nuclease digestion (Fig. 1a and c). The virion assembly intermediates, which contain SV40 NPC of different degrees of virus maturity, showed intermediate sensitivity to micrococcal nuclease digestion. The digestion products consisted of both SV40 genome-sized DNA, which was resistant to further digestion, and low-molecular-weight nucleosomal DNA fragments (Fig. 1b). The ratio of the products of the two size classes depended on the degree of maturity of the assembly intermediates (see discussion of Fig. 3 below); the proportion of closed circular DNA increased with the maturity of the assembly intermediates.

In contrast to CsCl gradient-purified mature virus extracted from nuclei, however, the virion assembly intermediate genome-sized DNA resistant to micrococcal nuclease digestion consisted of both circular and linear SV40 DNAs. The proportion of the linear DNA species also varied according to the maturity of the assembly intermediates. In the early assembly intermediates, only the linear DNA species could be seen (see Fig. 3).

Association of nucleosome core with capsid protein shell in virion assembly intermediates. In an attempt to separate nucleosomes from more mature assembly intermediates, we resedimented virion assembly intermediates in a sucrose gradient after nuclease digestion. To our surprise, most of trichloroacetic acid-precipitable [^{3}H]thymidine label in the DNA still sedimented at about the same rate as the original undigested complex. About 70 and 95% of the digested virion assembly intermediates that originally sedimented at about 180S and 210S, respectively (corresponding to regions designated A and B in Fig. 2a), resedimented at about the same positions in the second sucrose gradient (Fig. 2b and c). DNA was extracted from the 180S and 210S regions shown in Fig. 2b and c and analyzed by agarose gel electrophoresis. The DNA recovered from the fast-sedimenting NPC after micro-

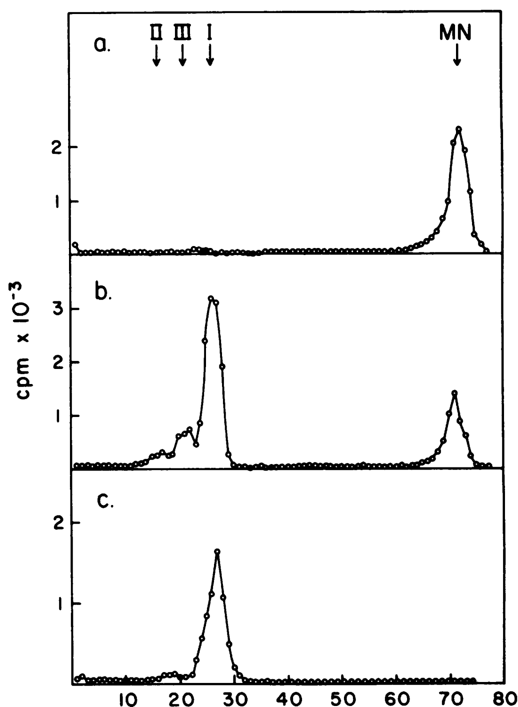

FIG. 1. Agarose gel electrophoresis analysis of the DNA extracted from micrococcal nuclease-digested SV40 NPC. [^{3}H]thymidine-labeled SV40 NPC were extracted from SV40-infected cells at 48 h postinfection, as described previously (7). Mature SV40 virus particles were obtained by banding the 210S SV40 NPC in a CsCl gradient (density, 1.34 g/ml) in a buffer containing 10 mM sodium phosphate (pH 6.8) and 0.1% Triton X-100. The SV40 NPC and mature virus particles were digested with micrococcal nuclease (40 U/ml) at DNA concentrations of about 1 μg/ml for SV40 70S and 180S NPC and about 0.5 μg/ml for mature virions. After 1 h of digestion at 37°C, DNA was extracted as described in the text and analyzed by electrophoresis in a 1% agarose gel. (a) 70S SV40 chromatin; (b) 180S NPC; (c) mature virus particles. The positions of SV40 supercoiled DNA, nicked circular DNA, and full-length linear DNA are indicated by the symbols I, II, and III, respectively. MN, Position of CV-1 cell mononucleosomal DNA.

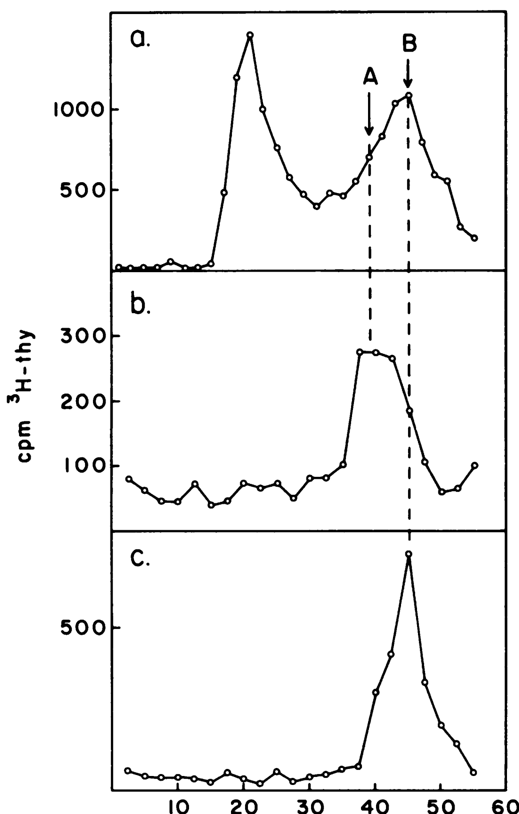

FIG. 2. Sucrose gradient analysis of SV40 NPC after nuclease digestion. (a) Profile of SV40 NPC isolated from SV40-infected cells and analyzed in a 5 to 40% sucrose gradient as described previously (7). Sedimentation is from left to right. The DNA was labeled for 2 h with 10 μCi of [^{3}H]thymidine per ml. (b) SV40 NPC in the fraction designated A (180S) in (a) was digested with 40 U of micrococcal nuclease per ml at 37°C for 1 h and resedimented in a 5 to 40% sucrose gradient. One-tenth of the sample was used to determine radioactivity by trichloroacetic acid precipitation. (c) Same as (b), except that NPC from the fraction designated B (210S) in (a) was used.

coccal nuclease digestion consisted of both SV40 genome-sized DNA and nucleosomal DNA fragments about 140 nucleotides long (Fig. 3). Since free nucleosomes have a sedimentation rate of about 11S, the quantitative recovery of nucleosomal DNA fragments in the fast-sedimenting NPC after nuclease digestion strongly suggested that the nucleosome cores remained associated with the capsid shell after nuclease digestion.

To demonstrate further the association between the nucleosome and the capsid shell after nuclease digestion, we fixed nuclease-digested assembly intermediates with glutaraldehyde before loading them on a CsCl gradient. Essentially all of the trichloroacetic acid-precipitable label in the DNA was recovered in the material banding at the density of mature virus (ρ = 1.34) or lower. No significant DNA was found in the region corresponding to fixed nucleosomes. This result was consistent with the interpretation that most SV40 nucleosome cores remained associated with the capsid shell after nuclease digestion. When unfixed complexes were analyzed in CsCl gradients after nuclease digestion, nucleosome-sized DNA could be recovered from the material banding in the region of 1.32 to 1.34 g/ml, with the DNA profile similar to that shown in Fig. 1b. Thus, at least some nucleosomes after digestion were still associated with the capsid shell and banded at the density of mature virus or at a slightly lower density.

Comparison of the nucleosome DNA repeat in 70S chromatin and virion assembly intermediates.

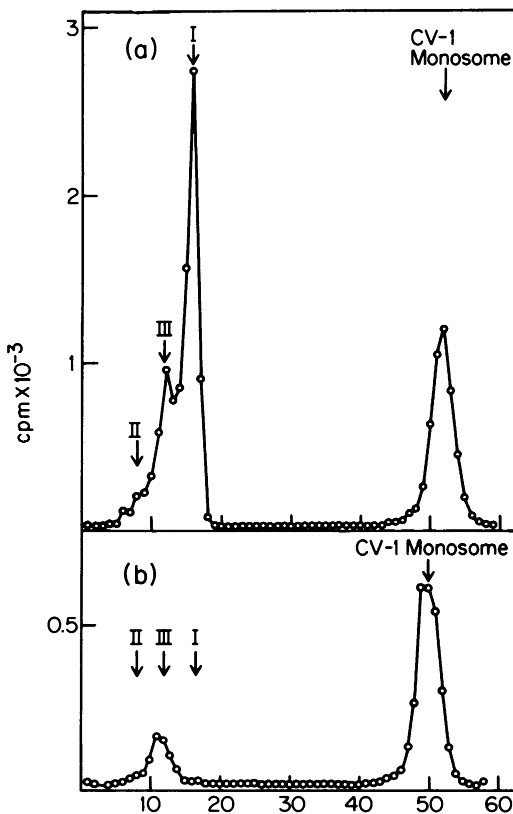

FIG. 3. Agarose gel electrophoresis analysis of DNA extracted from micrococcal nuclease-digested and resedimented SV40 NPC as shown in Fig. 2b and c. (a) DNA isolated from pooled peak shown in Fig. 2c. (b) DNA isolated from pooled peak in Fig. 2b. I, II, and III show the positions of SV40 supercoiled DNA, nicked circular DNA, and full-length linear DNA, respectively.

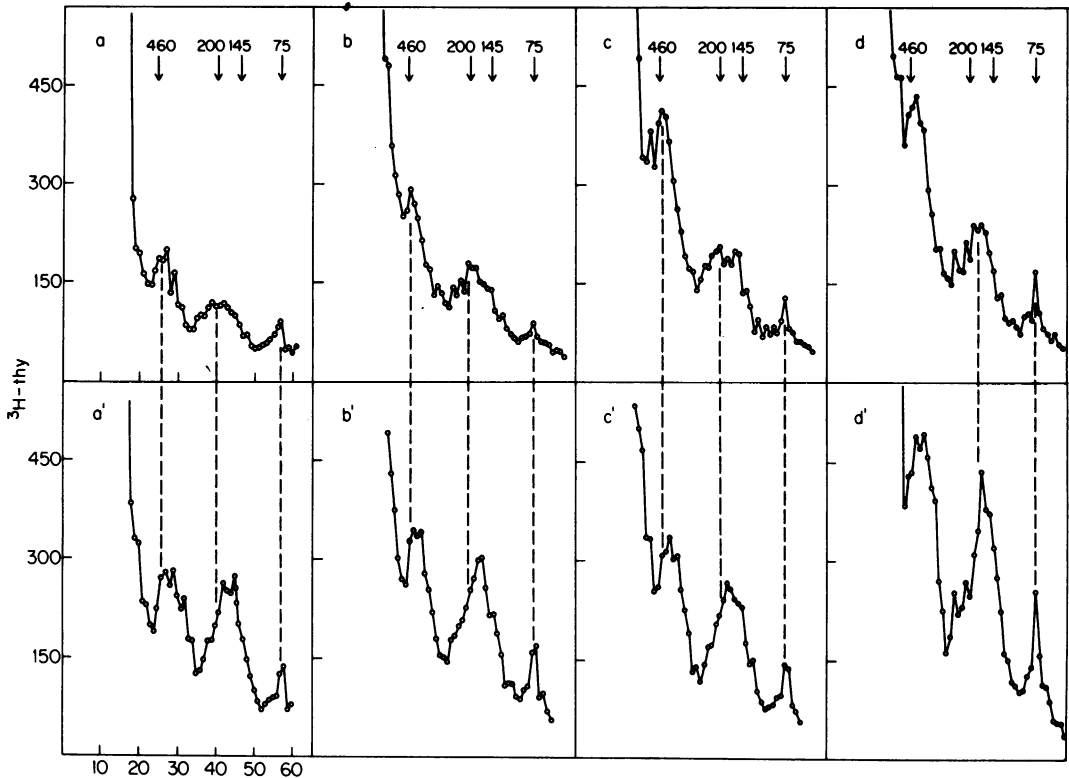

FIG. 4. Polyacrylamide gel electrophoresis analysis of micrococcal nuclease digestion products of SV40 chromatin and virion assembly intermediates. 70S SV40 chromatin (a, b, c, d) and 180S virion assembly intermediates (a', b', c', d') at a DNA concentration of about 1 μg/ml were digested with micrococcal nuclease (40 U/ml) at 0°C. At various times after digestion, the DNA was extracted and analyzed in a 12% polyacrylamide gel. (a, a') 30-s digestion, (b, b') 2-min digestion, (c, c') 5-min digestion, (d, d') 15-min digestion. Electrophoresis was performed for 12 h at 80 V. Gels were sliced into 2-mm slices and treated with Protosol (New England Nuclear) before radioactivity was determined as described previously (4). SV40 DNA products of *Hin*fI and *Alu*I digestion were used as size markers.

To study the DNA repeat length of the nucleosome in SV40 NPC, we found that it was essential to reduce the effect of end trimming by micrococcal nuclease during digestion. As shown by Noll and Kornberg (16), this can be achieved by reducing the temperature of digestion and by calculating the repeat length based on the differences in DNA length between successive nucleosome oligomers. Using this approach, we digested SV40 NPC at 0°C to minimize the degradation of the DNA ends by micrococcal nuclease. DNA fragments present in 180S assembly intermediates were significantly larger than those present in 70S SV40 chromatin (Fig. 4; Table 1). The repeat length, calculated by subtraction of the DNA lengths between successive nucleosome oligomers (16), was also higher in 180S virion assembly intermediates (211 ± 14 base pairs) than it was in 70S SV40 chromatin (194 + 8 base pairs). Furthermore, the length distribution of nucleosomal DNA was found to be more heterogeneous in 180S SV40 virion assembly intermediates. The more heterogeneous length distribution may account for the failure to observe nucleosome DNA fragments larger than tetramers in virion assembly intermediates, whereas in 70S chromatin DNA frag-

TABLE 1. Sizes of nucleosome DNA repeats of SV40 chromatin and virion assembly intermediates

Repeat no.	SV40 chromatin (base pairs)[a]	Virion assembly intermediates (base pairs)[b]
1	193	205
2	385	420
3	580	640
4	780	840
5	970	

[a] Average, 194 ± 8.
[b] Average, 211 ± 14.

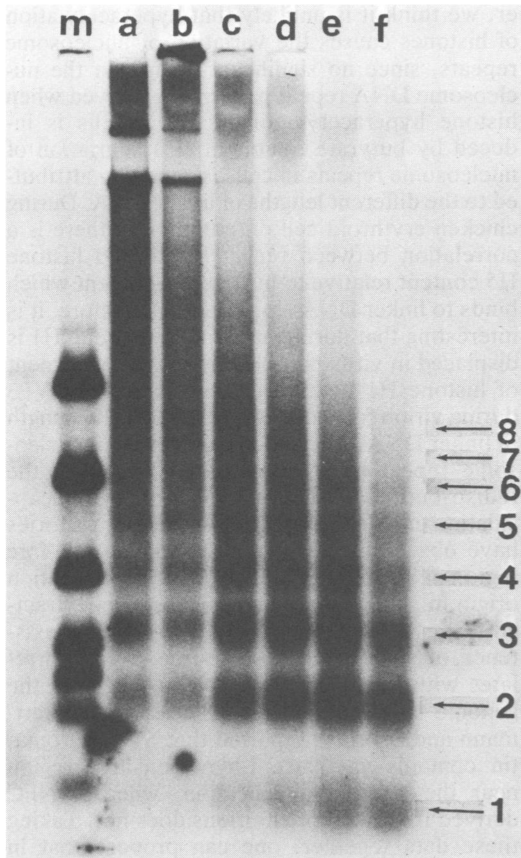

FIG. 5. Agarose gel electrophoresis of nucleosome DNA repeat present in 70S SV40 chromatin. [^{3}H]thymidine-labeled 70S SV40 chromatin (2 μg/ml) purified from a sucrose gradient was digested with micrococcal nuclease (40 U/ml) at 0°C for 30 s (a), 2 min (b), 5 min (c), 10 min (d), 20 min (e), and 40 min (f). At the end of the digestion periods, the DNA was extracted and analyzed in a 1.5% agarose gel. SV40 DNA products of *Hin*dIII and *Kpn*I digestion, which had lengths of 1,768, 1,169, 752, 526, 447, 366, and 215 base pairs (top to bottom), were used as size markers (lane m). Numbers on the right show the positions of the DNA in the monomer, dimer, trimer, etc., of the nucleosome.

ments, up to nine nucleosome lengths could be counted (Fig. 5). Early in the digestion of 70S chromatin, the DNA was cut once to produce full-length linear DNA (Fig. 5, lanes a and b).

Rapid appearance of a novel DNA fragment of half the size of the nucleosome core. A DNA fragment of about 75 nucleotides was observed repeatedly even at the earliest time of digestion at 0°C of sucrose gradient-purified 70S SV40 chromatin and 180S assembly intermediates (Fig. 4). Similar DNA fragments were observed when CV-1 cell nuclei were digested briefly at 0°C. However, no such fragments could be observed when digestion was performed at 37°C, even under limited digestion conditions.

DISCUSSION

We have presented evidence that the DNA present in some of the virion assembly intermediates was accessible to micrococcal nuclease digestion and that the DNA fragments produced remained associated with capsid shells after digestion. This was demonstrated by the recovery of nucleosome-sized DNA fragments from the fast-sedimenting NPC after nuclease digestion and from NPC banding in CsCl density gradients at a density slightly lower than that of mature virus. These results were consistent with the interpretation that, before maturation of viral particles, the capsid shell surrounding SV40 chromatin is of a rather loose structure and can be penetrated by micrococcal nuclease. However, since only full-length SV40 DNA (linear and circular) and nucleosomal-sized DNA fragments were produced and no intermediate-size fragments were detected, we envisioned that the structure of the capsid shell must have undergone a cooperative change during the virion maturation process, from a state that was totally accessible to a state that was almost (production of full-length linear DNA) or totally resistant to the nuclease (Fig. 6).

The mechanism of the cooperative organization of the capsid shell is still unknown. We have performed some preliminary experiments and have observed that prolonged dialysis of mature. virions against a 10 mM EDTA solution results in a change in the sedimentation property as well as a change in accessibility to micrococcal nuclease digestion. Thus, divalent ions probably play an important role in the organization of the capsid shell, as suggested by previous studies (2).

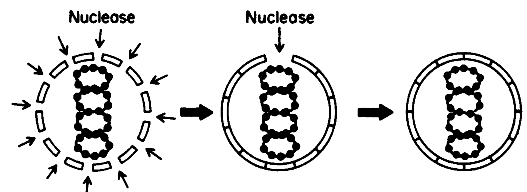

FIG. 6. Model for the packaging of the SV40 virion. The early-assembly intermediates are depicted as a structure that contains a porous capsid shell which allows nucleases to penetrate into the particle to digest the SV40 DNA-histone complex. The digested DNA remains associated with the capsid shell through protein-protein interaction. In the final stage of virus maturation, the capsid shell is organized in such a way that it becomes inpenetrable to micrococcal nuclease, except at limited defect sites still present.

The presence of linear SV40 DNA resistant to further nuclease digestion suggested that, in the final stage of virion formation, a defect was present in the capsid shell. Digestion of the linear DNA by *Eco*RI and *Bam*HI endonucleases showed that there were two preferential sites in the SV40 genome that were sensitive to micrococcal nuclease digestion. They were mapped around coordinates 65 and 43 (M. Coca-Prados and M.-T. Hsu, unpublished data). These results suggested that these two regions of the SV40 genome were frequently but not exclusively located near the final defect in the capsid shell.

Waldeck et al. (19) reported the observation of linear SV40 DNA isolated from SV40 virion particles. These molecules are probably produced by digestion of the almost-mature virus by endogenous endonuclease present in infected cells (Fig. 6). The end of the linear DNA was reported to be near coordinate 69, similar to one of our major micrococcal nuclease digestion sites in incomplete virion particles.

The results of digestion of 70S SV40 chromatin at 0°C showed that the regular nucleosome repeat pattern could be obtained at least up to the nonamer (Fig. 5). Higher oligomers were more difficult to resolve by gel electrophoresis, even with cellular chromatin. Previously, other laboratories showed that only sizes up to tetramers can be visualized in the digestion product of SV40 chromatin when digestion is carried out at 37°C (2, 5). We confirmed this observation. Our results therefore suggest that the nucleosomes in 70S SV40 chromatin are arranged in a more or less regular pattern. The failure to observe higher oligomers of nucleosomes at 37°C is therefore most likely due to the sliding of nucleosomes on 70S SV40 chromatin at the higher temperature. At the early stage of digestion of 70S SV40 chromatin at 0°C with micrococcal nuclease, full-length linear SV40 DNA was produced (Fig. 5). The initial cut sites were mapped to locations similar to those obtained by Sundin and Varshavsky (18). However, in contrast to their results, we found that 70S SV40 chromatin was rapidly degraded into nucleosomes at 0°C. Whether this was due to different digestion conditions is under investigation.

Digestion of 70S SV40 chromatin and 180S virion assembly intermediates with micrococcal nuclease revealed a significant difference in the nucleosome repeat length and length distribution. The change in nucleosome structures in these two forms of viral NPC could be due either to the change of histone composition or to the redistribution of nucleosomes on the SV40 genome. During the conversion of SV40 chromatin into virion assembly intermediates, histones become highly acetylated (5, 12). However, we think it is unlikely that hyperacetylation of histones causes the variation of nucleosome repeats, since no significant change in the nucleosome DNA repeat pattern is observed when histone hyperacetylation in HeLa cells is induced by butyrate treatment (13). Variation of nucleosome repeats in cells is generally attributed to the different lengths of linker DNA. During chicken erythroid cell differentiation, there is a correlation between repeat length and histone H5 content relative to histone H1 content which binds to linker DNA (14, 15, 20). Therefore, it is interesting that during maturation, histone H1 is displaced in virus particles (3, 11). Replacement of histone H1 by capsid proteins such as VP3 during virion formation may thus alter the length of linker DNA. Alternatively, changes in nucleosome repeat length may be the result of the redistribution of nucleosomes during the encapsidation of SV40 chromatin. Several laboratories have observed that there is a nucleosome-free region of SV40 DNA located near the replication origin in some SV40 minichromosomes extracted from SV40-infected cells (11, 17). The existence of such a nucleosome-free region correlates with hypersensitivity of this region of the genome to digestion with DNase I (17). Hartmann and Scott (9) reported that SV40 chromatin contains a DNase I-hypersensitive region near the origin of replication, whereas NPC derived from disrupted virions does not. Taking these data together, one can propose that in most of the 70S SV40 chromatin, there is a nucleosome-free region about 350 nucleotides long near the replication origin, whereas the rest of the genome is packaged into regular nucleosomes. This would result in a nucleosome size of about 188 to 200 nucleotides, assuming that there are 24 to 26 nucleosomes (17). During encapsidation, factors that maintain the nucleosome-free region in SV40 chromatin (e.g., a regulatory protein that binds to this region of the SV40 genome) are dissociated from SV40 chromatin, and nucleosomes in other regions of the genome are then allowed to move into this region. This would result in an increase in average nucleosome size of about 13 to 14 nucleotides. The increase in the average nucleosome repeat length in a fixed-length region would also result in an increase in the heterogeneity of repeat length, according to the model of Feldman et al. (6). Thus, redistribution of nucleosomes when the nucleosome-free region of SV40 chromatin is eliminated during encapsidation can account for both the increase in the repeat length and the increase in length distribution of virion assembly intermediates relative to those of SV40 chromatin. Further experiments are required to test these hypotheses.

During the mild digestion of SV40 chromatin

and virion assembly intermediates, we also observed a novel DNA fragment about 75 base pairs long. Since this fragment appeared at the earliest time of digestion, when less than 0.1% of DNA was digested, it was distinguishable from the subnucleosome fragments that appeared in extensively digested chromatin. Similar DNA fragments were also observed when uninfected CV-1 cell nuclei were digested. However, when viral or cellular chromatin was digested at 37°C under limited digestion conditions, the 75-base-pair fragment was never observed. It is interesting that the size of this novel DNA fragment was about half the size of the nucleosome core. Whether it represents the digestion product of a novel chromatin structure remains to be determined.

ACKNOWLEDGMENTS

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