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Isolation and Characterization of Adenovirus Core Nucleoprotein Subunits

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Digestion of adenovirus type 2 (Ad2) or Ad5 cores with micrococcal nuclease generated four nucleoprotein species that could be resolved by electrophoresis in low-ionic-strength polyacrylamide gels: these nucleoproteins displayed mobilities equivalent to those of DNA fragments of 900 to 1,025, 775 to 850, 650 to 725, and 525 to 600 base pairs (bp) and thus were readily distinguishable from HeLa cell mononucleosomes. The DNA fragments associated with the core nucleoprotein species were more than 250 to 90 bp long. Nucleoproteins containing 150, 120, or 90 bp of DNA were the most stable. Polypeptide VII was associated with each of the nucleoprotein species liberated from Ad2 cores. These data suggest that polypeptide VII and viral DNA of 90 to 150 bp comprise the unit particle of the Ad2 or Ad5 core nucleoproteins.

The linear, double-stranded DNA genome (almost 36 kilobases [26]) of subgroup C human adenoviruses is packaged as a nucleoprotein structure within the icosahedral capsid. This internal nucleoprotein, termed the core, also contains a 55-kilodalton protein covalently attached to the 5′ termini of the DNA molecule (25, 27) and three basic, arginine-rich proteins, VII, V, and μ (X) (1, 13, 20, 23, 24, 28, 31–33). Despite the wealth of information available about the morphology of the adenovirus core (21) and the properties of its constituents (22), the mechanism of viral DNA packaging is not well understood. Unlike cellular chromatin, which yields discrete, mononucleosomal DNA fragments of some 146 base pairs (bp) upon micrococcal nuclease digestion (for reviews, see references 11, 16, and 18), the adenovirus core nucleoprotein yields a heterogeneous population of DNA fragments ranging from less than 50 to greater than 300 bp in length (2, 6, 17, 31). Moreover, no series of DNA fragments corresponding to those predicted for an oligomeric structure has been reported among the deproteinized products of micrococcal nuclease digestion of adenovirus core nucleoproteins. Here we present the results of attempts to gain a better understanding of adenovirus core structure by isolation and characterization of the nucleoprotein subunits of which the core is built.

Adenovirus type 2 (Ad2) or Ad5 virions purified by repeated banding in CsCl gradients were disrupted by exposure to 10% pyridine, as described previously (31). Cores were prepared in this way as rapidly as possible and only from freshly banded virions: storage, at either 4 or −20°C, of purified virions dialyzed under standard conditions (9) induced sufficient disruption and aggregation of cores to prevent both their isolation as a discrete peak in sucrose gradients and the production of nucleoprotein species upon nuclease digestion.

Cores were digested with micrococcal nuclease for 20 min at 20°C in 10 mM Tris hydrochloride (pH 7.4) containing 0.085 M KCl, 1 mM $MgCl_2$, 5.5% (wt/vol) sucrose and the concentrations of $CaCl_2$ and enzyme given in the figure legends. In initial attempts to identify the unit particles from which the adenovirus core nucleoprotein might be constructed, the nondenatured products of micrococcal nuclease digestion were sedimented in sucrose gradients, a method that has been reported to permit isolation of an 11S particle (17). In our hands, such an experimental approach did not yield material suitable for further elucidation of viral nucleoprotein organization: some nucleoprotein was observed in the 10S region of sucrose gradients but was superimposed upon a background of material dispersed throughout the gradient that contained both adenovirus DNA and core proteins. Moreover, nearly 80% of the core proteins present in micrococcal nuclease-digested adenovirus cores sedimented to the bottom of the tube, displaying sedimentation values of greater than 1,000S (data not shown). Such fast-sedimenting material contained aggregates of core nucleoprotein formed before digestion (data not shown). Despite numerous attempts, conditions suitable for nuclease digestion that alleviated aggregation of cores could not be established. However, viral nucleoprotein species liberated when cores were digested with micrococcal nuclease (or DNase I) could be separated from aggregated material by electrophoresis in polyacrylamide gels (14, 30). The results of one such experiment, in which the products of digestion of adenovirus core nucleoproteins and HeLa cell chromatin were compared, are shown in Fig. 1.

Micrococcal nuclease digestion of HeLa cell chromatin liberated a prominent ethidium bromide-staining product, which migrated with a mobility equivalent to that of a deproteinized DNA fragment of 450 bp and contained DNA fragments of 145 to 160 bp (Fig. 1A, lanes 1 and 2). In contrast, digestion of Ad5 cores with micrococcal nuclease liberated nucleoprotein species that displayed mobilities under these conditions equivalent to those of deproteinized DNA fragments of some 550, 600 to 650, and 825 to 850 bp (Fig. 1B, lanes 1 to 4). Digestion of deproteinized Ad5 DNA with micrococcal nuclease under identical conditions produced a heterogeneous set of DNA fragments of less than 200 bp (data not shown). When the products generated from viral cores by micrococcal nuclease digestion were deproteinized, by phenol extraction or heating in the presence of 0.1% sodium dodecyl sulfate for 5 min at 68°C, before electrophoresis, DNA fragments of less than 300 bp were observed (Fig. 1B, lane 6). We can, therefore, conclude that the ethidium bromide-staining products of digestion of Ad5 cores that displayed mobilities equivalent to those of DNA

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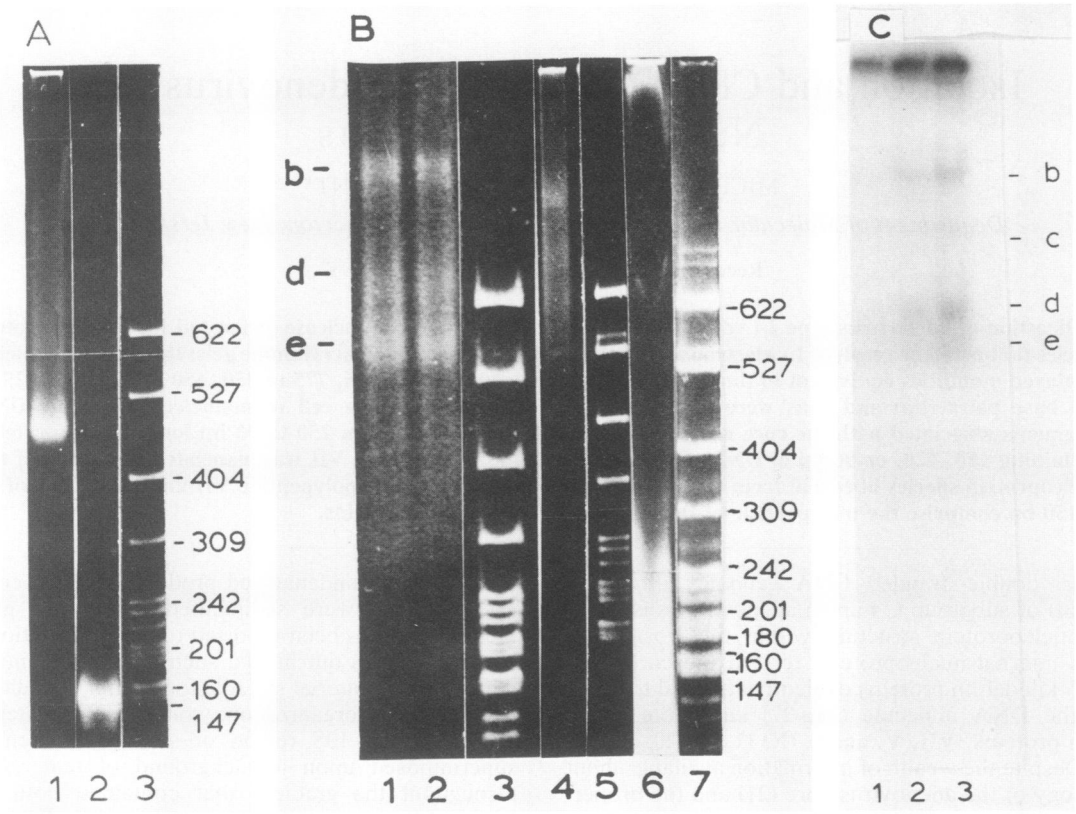

FIG. 1. Resolution of adenovirus nucleoprotein subunits by electrophoresis. Micrococcal nuclease digestions were performed in 0.01 M Tris hydrochloride (pH 7.4) containing 0.085 M KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5.5% (wt/vol) sucrose for 20 min at 20°C. The native products of digestion were resolved by electrophoresis in 5.5% polyacrylamide gels cast and run in 50 mM Tris-borate (pH 8.5) containing 0.5 mM EDTA. (A) HeLa cell nuclei were digested with 50 U of micrococcal nuclease per OD_{260} unit. The products were analyzed directly (lane 1) or after deproteinization (lane 2). The photograph shown is a composite of separate Polaroid exposures of the top and bottom regions of an ethidium bromide-stained gel. Standards *Hpa*II fragments of pBR322 DNA, are shown in lane 3; lengths in base pairs are shown in the margin. (B) Ad5 cores were digested with 150 (lanes 1, 2, and 6) or 200 (lane 4) U of micrococcal nuclease per OD_{260} unit. The products were applied directly to the gel (lanes 1, 2, and 4) or deproteinized before electrophoresis (lane 6). Lanes 1 to 3 and 4 and 5 show digestions of independent preparations of Ad5 cores. Standards are shown in lanes 3, 5, and 7; lengths in base pairs are shown in the margin. (C) Ad2 cores were digested with 75, 150, or 300 U micrococcal nuclease per OD_{260} unit for 20 min (lanes 3, 2, and 1, respectively), and the products were analyzed by electrophoresis as described above. The proteins were then transferred electrophoretically to a nitrocellulose filter, reacted sequentially with anti-protein VII antibodies from the hybridoma cell line P65 (R. Lunt, M. E. Vayda, M. Young, and S. J. Flint, manuscript in preparation) and ^{125}I-protein A as described previously (3). After being washed, the filter was exposed to Kodak X/AR film at −80°C in the presence of an intensifying screen for 7 days. The positions of which nucleoprotein species detected by ethidium bromide staining migrated are indicated on the right.

fragments of 550, 600 to 650, and 825 to 850 bp contained both DNA and protein. Indeed, all nucleoprotein species generated by micrococcal nuclease digestion of adenovirus cores contained protein VII (Fig. 1C), the principal DNA-binding protein of the virion (1, 4, 31). The apparent mobilities of these core nucleoprotein species, relative to those of deproteinized DNA fragments, were dependent upon the conditions of both nuclease digestion and electrophoresis. Therefore, they are hereafter designated nucleoprotein species b to e (Fig. 1 and 2).

Digestion of native Ad5 cores with DNase I released products that migrated in nondenaturing polyacrylamide gels with mobilities comparable to those of the nucleoprotein species generated by micrococcal nuclease digestion of cores, although the former nucleoproteins were less discrete than the latter (data not shown). These products do not, therefore, result from sequence-specific cleavage of core DNA by micrococcal nuclease (8, 10, 12).

To investigate potential precursor-product relationships among the core nucleoprotein species, the nucleoproteins produced during increasing periods of micrococcal nuclease digestion were examined. In the experiments whose results are shown in Fig. 2, samples were removed from a single reaction mixture containing 100 U of micrococcal nuclease per OD_{260} unit of cores and 1 mM $CaCl_2$ after intervals of 5 to 15 min. The more slowly migrating nucleoprotein species (b and c) predominated under the mildest digestion conditions (Fig. 2, lane 3). Between digestion times of 5 and 10 min, the quantities of these two species decreased substantially, while species e appeared (Fig. 2), suggesting that species e was produced by further digestion of nucleoprotein species b and c. More extensive digestion apparently resulted in the appearance of species d and the loss of species c (data not shown), explaining the underrepresentation of species c among the products of most reactions run for 20 min (for example, Fig. 1B and C).

To characterize the viral DNA fragments associated with the core nucleoprotein species, DNA fragments associated with the products of micrococcal nuclease digestion were directly end labeled by using polynucleotide kinase and

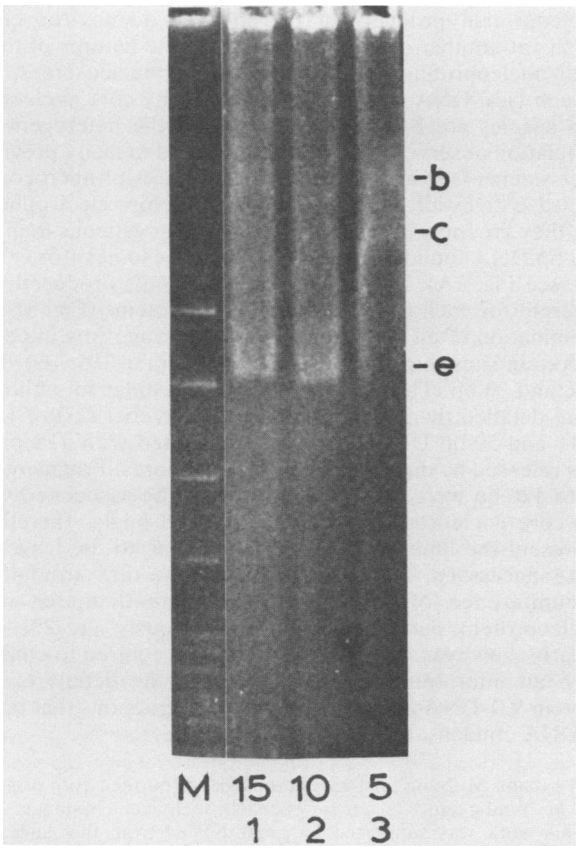

FIG. 2. Core nucleoprotein species produced with increasing periods of micrococcal nuclease digestion of adenovirus cores. Ad2 cores were digested with 100 U of micrococcal nuclease per OD_{260} unit in the presence of 1 mM $CaCl_2$. After the periods (minutes) of incubation shown, samples were removed and the reaction was stopped by the addition of EGTA [ethylene glycol-bis(β-aminobethyl ether)-*N*,*N*,*N*′, *N*′-tetraacetic acid] to 10 mM. The products of digestion were applied to a 6% polyacrylamide gel run as described in the legend to Fig. 1. *Hpa*II fragments of pBR322 DNA are shown in lane M.

[γ-^{32}P]ATP. Such end labeling did not adversely affect the integrity of the nucleoprotein particles (data not shown). The regions of native polyacrylamide gels that contained labeled nucleoprotein species were excised, and the DNA was eluted and deproteinized by phenol and chloroform extraction. The DNA was then analyzed by electrophoresis in 8% polyacrylamide gels. Typical results are shown in Fig. 3. The four nucleoprotein populations designated b to e, produced in this experiment by digestion in the presence of 6 mM $CaCl_2$, each contained DNA fragments of 80 to 120 bp, as well as less abundant, larger fragments (Fig. 3). However, the lengths of both classes of DNA fragments decreased as the mobility of the particle from which they were obtained increased. Thus, for example, nucleoprotein population e contained DNA of 155 to 170 and 190 to 200 bp, whereas nucleoprotein population d contained DNA segments of 165 to 185 and 210 to 225 bp (Fig. 3, lanes 5 and 4, respectively). Similarly, the larger DNA fragments released from nucleoprotein species b or c, although present at low concentrations, ranged to about 240 bp in length. Although the nucleoprotein particle-associated DNA fragments were markedly more discrete than those obtained when deproteinized products of micrococcal nuclease digestion of adenovirus cores were analyzed directly (Fig. 1B, lane 6), they were strikingly more heterogeneous than the DNA constituents of the cellular nucleosome (see, for example, Fig 1A, lane 2). Because the nucleoprotein particles that displayed greater mobility contained shorter DNA fragments, it seemed possible that the heterogeneity of the particles and their associated DNA populations might have been the result of incomplete nuclease digestion, that is, that the faster-migrating nucleoprotein species and DNA fragments were generated from those of lower mobility upon further nuclease digestion. However, the migration of nucleoprotein particles in native polyacrylamide gels was not substantially altered as their DNA components were shortened during increasing periods of micrococcal nuclease digestion (data not shown). Moreover, the differences in the lengths of the most abundant nucleoprotein-associated DNA fragments, at most 50 bp and generally 20 to 30 bp (for example, see Fig. 3), were much less than the differences in apparent mobility displayed by the nucleoprotein species themselves. These results suggest that, although each nucleoprotein class contained DNA fragments of characteristic size and the more slowly migrating nucleoprotein species were associated with longer DNA fragments (Fig. 3), the mobility of the particles in

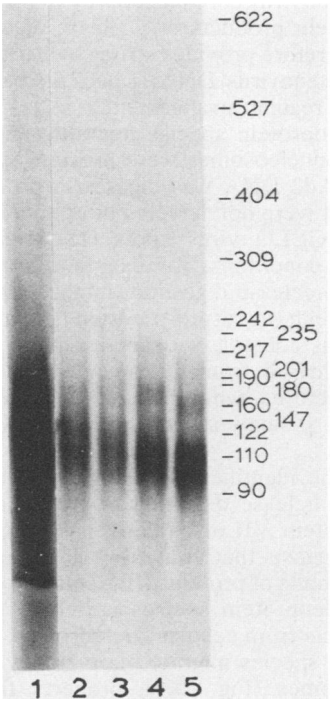

FIG. 3. DNA fragments present in Ad5 core nucleoprotein particles. Ad5 cores were digested with 150 U of micrococcal nuclease per OD_{260} unit in the presence of 6 mM $CaCl_2$ for 20 min at 20°C. The products of the reaction were then end labeled (see the text) before electrophoresis as described in the legend to Fig. 1. The regions corresponding to the material at the top of the gel and nucleoprotein species b to e (Fig. 1B) were excised, and the DNA was eluted and purified as described in the text before electrophoresis in an 8% polyacrylamide gel cast in 0.05 M Tris-borate buffer (pH 8.5) containing 1 mM EDTA. End-labeled DNA was detected by autoradiography with Kodak X/RP film. Lanes 2 and 5, End-labeled DNA fragments recovered from nucleoprotein species b to e, respectively, of an ethidium bromide-stained gel; lane 1, those from aggregated material that failed to enter the first gel. The positions to which *Hpa*II fragments of pBR322 DNA migrated are shown on the right; lengths are in base pairs.

native gels must be largely determined by other parameters, such as protein complement or conformation.

The observation that micrococcal nuclease digestion of adenovirus cores liberates nucleoprotein subunits is consistent with electron microscopic visualization of particles 9 to 10 nm in diameter within the adenovirus core (1, 19, 31). The notion that viral DNA is condensed in some regularly arranged core nucleoprotein also receives support from X-ray scattering patterns obtained with Ad2 virions and cores (7). Unexpectedly, however, a total of four nucleoprotein species, or populations, distinguished by their apparent mobilities relative to those of deproteinized DNA fragments, were detected electrophoretically, using assays for the presence of either DNA or protein VII (Fig. 1). Such nucleoproteins are not artifacts of micrococcal nuclease cleavage, since similar species were produced when cores were digested with DNase I but were absent from the products of micrococcal nuclease digestion of deproteinized Ad5 DNA (Fig. 1). Moreover, similar nucleoprotein species are generated when nuclei isolated from cells infected with Ad2 or Ad5 for various periods are digested with micrococcal nuclease (5, 29; M. E. Vayda and S. J. Flint, submitted for publication). Thus, the nucleoprotein species illustrated in Fig. 1 cannot be artifactual products of the in vitro manipulation of adenovirus core nucleoproteins. The identification of nucleoprotein products of nuclease digestion of Ad2 or Ad5 cores therefore provides strong evidence to support the notion that adenovirus DNA is packaged by the core proteins in some regular arrangement.

Viral nucleoprotein species migrating more slowly than cellular mononucleosomes were previously observed when intracellular Ad2 DNA was digested with micrococcal nuclease (29) but were not detected after micrococcal nuclease digestion of CELO virus cores (15). Differences in the experimental conditions, for example, the purification of cores before nuclease digestion, might account for the differences between the results reported here and those of Li et al. (15). In this context, we cannot emphasize too strongly that cores released from human adenovirions are fragile, unstable structures whose morphology and biochemical properties are strongly influenced by their in vitro environment (20, 31).

Although the identification of nucleoprotein products of micrococcal nuclease digestion of Ad5 cores that contain both core protein VII and relatively discrete fragments of viral DNA suggests that viral DNA is organized in the core by packaging units of protein VII, it must be emphasized that the viral nucleoprotein species we characterized differ in several respects from cellular nucleosomes. The adenovirus nucleoprotein species migrate more slowly than HeLa cell mononucleosomes (Fig. 1), a property that has proved valuable in the investigation of the structure and organization of the viral nucleoproteins present in infected cells (5). Moreover, the relative migration of the adenovirus core nucleoprotein species (Fig. 1), the results of protein blotting experiments (Fig. 1; data not shown), which indicate that only the more slowly migrating species contain protein V, and the analysis of DNA fragments associated with individual core nucleoprotein species discussed previously strongly suggest that the core nucleoproteins do not represent a multimeric series of nucleoproteins like that comprising a typical nucleosomal ladder (11, 16). It therefore seems likely that protein complement or conformation or both are major determinants of the mobilities displayed by adenovirus core nucleoprotein species.

The properties of the viral DNA fragments associated with nucleoprotein products of digestion of adenovirus cores finish yet another example of the disparate natures of these viral nucleoprotein species and cellular nucleosomes. Although viral DNA fragments present in any core nucleoprotein species are far more discrete than the heterogeneous population observed both here (Fig. 1) and in many previous experiments (see above) when the products of micrococcal nuclease digestion were deproteinized before electrophoresis, they are, nevertheless, much less homogeneous than the 146-bp DNA found in cellular mononucleosomes (for example, see Fig. 1A). The exact set of fragments produced was different for each population of nucleoproteins (Fig. 3), but examination of all sets suggests that cleavage was impeded at certain sites to produce fragments of some 185, 160, 150, 120, and 90 bp. These observations are similar to, although more detailed than, those of Mirza and Weber (17) of 150-, 100-, and 50-bp DNA fragments associated with 11S particles released by digestion of adenovirus cores. Fragments of 90 to 120 bp were the smallest found to be associated with the core nucleoprotein units (Fig. 3) and might, therefore, represent the limit product, corresponding to the length of DNA necessary to complete at least one turn around the circumference of the 9.35- to 10.4-nm-diameter viral nucleoprotein particle described previously (1, 20, 31). Clearly, however, additional studies are required to confirm such an interpretation and to provide the details of the protein VII-DNA and protein-protein interactions that result in DNA condensation.

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