Structural Analysis of Viral Replicative Intermediates Isolated from Adenovirus Type 2-Infected HeLa Cell Nuclei

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Received for publication 12 December 1977

Deoxyribonucleoprotein complexes released 17 h postinfection from adenovirus type 2 (Ad2)-infected HeLa cell nuclei were shown by electron microscopy to contain filaments much thicker (about 200 Å [20 nm]) than double-stranded DNA (about 20 Å [2 nm]). The complexes were partially purified through a linear sucrose gradient, concentrated, and further purified in a metrizamide gradient. The major protein present in the complexes was identified as the 72,000-dalton (72K), adenovirus-coded single-stranded DNA-binding protein (72K DBP). Three types of complexes have been visualized by electron microscopy. Some linear complexes were uniformly thick, and their length corresponded roughly to that of the adenovirus genome. Other linear genome-length complexes appeared to consist of a thick filament connected to a thinner filament with the diameter of double-stranded DNA. Forked complexes consisting of one thick filament connected to a genome-length, thinner double-stranded DNA filament were also visualized. Both thick and thin filaments were sensitive to DNase and not to RNase, but only the thick filaments were digested by the single-strand-specific *Neurospora crassa* nuclease, indicating that they correspond to a complex of 72K DBP and Ad2 single-stranded DNA. Experiments with anti-72K DBP immunoglobulins indicated that these nucleoprotein complexes, containing the 72K DBP, correspond to replicative intermediates. Both strands of the Ad2 genome were found associated to the 72K DBP. Altogether, our results establish the in vivo association of the 72K DBP with adenovirus single-stranded DNA, as previously suggested from in vitro studies, and support a strand displacement mechanism for Ad2 DNA replication, in which both strands can be displaced. In addition, our results indicate that, late in infection, histones are not bound to adenovirus DNA in the form of a nucleosomal chromatine-like structure.

A virus-coded single-stranded DNA (ss-DNA)-binding protein (DBP), whose synthesis begins early after adenovirus infection, has been recently implicated in adenovirus type 2 (Ad2) DNA replication. This protein, with a molecular weight of 72,000 (72K), has been purified from cells infected with Ad2 and Ad5, and its preferential binding to ss-DNA has been demonstrated (24, 34, 39). In addition, an Ad5 mutant, temperature sensitive for DNA replication, has been isolated and shown to contain a 72K DBP, thermosensitive in binding to ss-DNA (11, 40). It was also shown that the 72K DBP is required in its active state for both initiation and elongation of DNA synthesis (41, 42). In vivo, the 72K DBP was assumed to bind to the single-stranded regions of replicative intermediates, playing a role in DNA replication and perhaps also protecting

the DNA against nuclease digestion (42).

We have recently described a procedure to release selectively, 17 h postinfection, Ad2 nucleoprotein complexes from infected HeLa cell nuclei (46). These nucleoprotein complexes, which were partially purified by velocity sedimentation through sucrose gradients, contain both transcription and replication adenovirus complexes (5, 46). We have further purified the replication complexes by sedimentation through a metrizamide gradient, and we have analyzed their structure. We report here biochemical and electron microscopic evidence demonstrating that the 72K DBP is bound to the ss-DNA present in replicative intermediates actively engaged in viral DNA replication.

MATERIALS AND METHODS

HeLa cell suspension cultures were maintained, infected, and harvested as previously described (46). Nuclei were purified and extracted with 200 mM am-

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monium sulfate to obtain fractions S200 and PS200, corresponding, respectively, to the supernatant and the nuclear pellet after extraction (5, 46).

Fractionation and concentration of the nucleoprotein complexes present in fraction S200. Fraction S200 was layered onto a 10 to 58% linear sucrose gradient (either 16 or 11.5 ml) containing 200 mM ammonium sulfate–20 mM Tris-hydrochloride (pH 8)–2 mM EDTA and centrifuged at 2°C for either 5 h at 27,000 rpm in an SW27.1 rotor or 4 h at 40,000 rpm in an SW41 Beckman rotor.

Fractions corresponding to peak 1, interpeak, and peak 2 were pooled (see Fig. 1), diluted in the same buffer to 10 to 12% sucrose (as determined from the refractive index), and concentrated by centrifugation at 40,000 rpm for 14 h in SW41 rotor tubes containing a 0.4-ml cushion of 63% sucrose in water. Recovery, using EDTA-treated nitrocellulose tubes, was nearly quantitative. A 10-fold concentration was generally obtained, and the complexes could be stored in 20% sucrose solutions at –20°C or in liquid nitrogen without any visible structural alteration.

Metrizamide gradients. The concentrated interpeak (see above) was dialyzed against 10 mM sodium chloride–10 mM Tris-hydrochloride (pH 7.5)–0.1 mM EDTA until the sucrose concentration was reduced to less than 10%. One-milliliter aliquots were layered onto preformed 1.1- to 1.3-g/ml metrizamide gradients (4 ml) containing 10 mM sodium chloride–10 mM Tris-hydrochloride (pH 7.5)–0.1 mM EDTA. After centrifugation for 20 h (4°C, 35,000 rpm in a Beckman SW65 rotor), fractions were collected from the bottom of the tube, aliquots were counted, and the slope of the density gradient was determined by weighing 150- μ l aliquots in a calibrated micropipette.

Neutral sucrose gradient analysis of DNA present in the nucleoprotein complexes. Sodium dodecyl sulfate (SDS) was added to nucleoprotein complexes to a final concentration of 0.5%. These samples were kept in ice until layered on the gradients. Gradients (16 ml) of 10 to 40% sucrose containing 200 mM NaCl, 20 mM Tris-hydrochloride (pH 7.5), 2 mM EDTA, and 0.1% Sarkosyl were loaded with 2.2 ml of SDS-treated samples and centrifuged in EDTA-treated nitrocellulose tubes for 15 h at 2°C and 27,000 rpm in a Beckman SW27 rotor.

Nuclease assays. S1 nuclease assays were carried out in 0.2 ml containing 100 mM ammonium sulfate, 50 mM sodium acetate (pH 4.5), 4 mM ZnCl₂, about 10 U of nuclease (43), and 1 μ g or less of the labeled DNA to be tested. After incubation at 37°C for 30 min, acid-precipitable counts were determined. Under these conditions more than 90% of Ad2 ss-DNA was rendered acid soluble, whereas no degradation of double-stranded DNA (ds-DNA) was observed.

Neurospora crassa nuclease assays were performed as previously described (5).

Acrylamide gel electrophoresis. Samples containing 20 to 50 μ g of protein, determined by a Coomassie staining procedure (19), were precipitated overnight at 0°C in 15% trichloroacetic acid. After centrifugation for 30 min at 15,000 $\times$ g, the pellets were washed with 80% ethanol and dried in vacuo.

A 5 to 17.5% linear acrylamide gel (12 cm long and 1.5 mm thick) was formed, using the system of Laem-

mli (20), in a vertical slab gel apparatus (Hoefer Scientific Instruments, Inc., San Francisco, Calif.). The precipitated protein samples were dissolved in 40 μ l of a buffer containing 60 mM Tris-phosphate (pH 6.8), 1% SDS, 1% β -mercaptoethanol, 12% glycerol, and 0.05% bromophenol blue. The mixture was heated for 3 min at 100°C, loaded into the slots, and subjected to electrophoresis at 13°C (50 to 100 V) until the dye reached the bottom of the gel. The gel was stained overnight in Coomassie brilliant blue and destained by diffusion as previously described (18).

DNA reassociation kinetics. DNA samples were heated at 100°C for 15 min in 0.3 N NaOH to destroy any traces of RNA and to fragment the DNA (46). The solution was neutralized with HCl at 0°C and adjusted to 1 M NaCl, 50 mM Tris-hydrochloride (pH 7.5), and 1 mM EDTA. Aliquots (5 μ l) containing 1,000 cpm (1 ng) of [³H]DNA were incubated under 0.2 ml of paraffin oil in siliconized tubes for various times at 68°C. After incubation, these aliquots were either treated with *N. crassa* endonuclease in the presence of the paraffin oil (see above) or directly acid precipitated.

Purification of the 72K ss-DNA binding protein. The 72K DBP was purified either from the cytoplasmic fraction of Ad2-infected HeLa cells, which were treated with cytosine arabinoside as described by Shanmugan et al. (32), or from fraction S200 as follows. The soluble proteins present in fraction S200 and remaining on the top of a sucrose gradient (Fig. 1) were concentrated 50 times by ammonium sulfate precipitation (60% saturation). The pellet was resuspended in 20 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA–2 mM β -mercaptoethanol–50 mM NaCl and dialyzed overnight against the same buffer. After dialysis a small precipitate appeared which was removed by centrifugation for 10 min at 8,000 rpm in an HB4 Sorvall rotor. The supernatant was adsorbed (90 μ l/min) on a denatured DNA-cellulose column (1), which was equilibrated in the same buffer (the column contained 3.2 ml of DNA-cellulose with about 1.6 mg of denatured DNA, for 10 mg of loaded protein). Proteins were eluted stepwise with 10 ml of the same buffer containing 0.4 and 1 M NaCl. The 72K DBP was eluted in the last step and was at least 80% pure, as judged by SDS-polyacrylamide gel electrophoresis.

Electron microscopy. Aliquots of gradient fractions or concentrated fractions (see above) or nuclease-treated samples were dialyzed against a large excess of 10 mM NaCl, 10 mM Tris-hydrochloride (pH 7.5), and 0.1 mM EDTA. Recovery after dialysis was at least 70%. The DNA concentration was adjusted to 0.3 to 0.5 μ g/ml (determined at all steps by counting aliquots, since the specific activity of the DNA had been determined in the total fraction S200), and the samples were processed for electron microscopic visualization, using either the method of Dubochet et al. (9), as described previously (25), or the method of Davis et al. (7), as modified by Wellauer et al. (45). In the latter case the material was first dialyzed against 10 mM sodium phosphate buffer (pH 7.4)–0.1 mM EDTA and then fixed in the presence of 0.15% glutaraldehyde (final concentration). After 15 min at 37°C, the glutaraldehyde was neutralized by adding glycine to a final concentration of 15 mM, and the mixture

was further incubated for 5 min at 37°C. Aliquots were then adjusted to 30% formamide, 100 mM Tris-hydrochloride (pH 8.5), 10 mM EDTA, and 50 µg of cytochrome *c* per ml and spread as described in reference 45.

Other methods. Proteins were labeled with [³H]-leucine as follows. After infection (see above), the cells were resuspended in the usual growth medium but without leucine. Two hours later, [³H]leucine (35 Ci/mmol, 4 µCi/ml) was added to the infected cells for 15 or 22 h, depending on the experiment. Viral DNA was labeled with [¹⁴C]thymidine (53.9 mCi/mmol, 0.005 µCi/ml) from 6 to 17 h postinfection. In this low-leucine medium viral DNA synthesis was not significantly affected, as could be judged from the recovery of labeled DNA in fraction S200.

Fluorography of ³H-labeled polypeptides in polyacrylamide gels was essentially performed as described by Laskey and Mills (21). Ad2 virions were purified as described by Doerfler (8).

Materials. [³H]leucine was purchased from the CEA (France). Analytical-grade metrizamide was supplied by Nyegaard and Co. (Oslo, Norway). Cytosine arabinoside hydrochloride was purchased from Sigma Chemical Co. (St. Louis, Mo.). Anti-72K DBP and control immunoglobulins were obtained from rabbits as described by van der Vliet et al. (42) and were kindly provided by P. van der Vliet and L. Philipson. Total histones from calf thymus and S1 nuclease purified by the method of Vogt (43) were gifts of M. Bellard from our laboratory. All other methods and materials have been described previously (5, 46).

RESULTS

Identification of the proteins associated with Ad2 DNA in fraction S200. Treatment of 17-h-postinfection HeLa cell nuclei with 200 mM ammonium sulfate releases 50 to 70% of the nonintegrated Ad2 DNA into fraction S200, with no measurable contamination by cellular chromatin fragments (5, 46). In a typical experiment, fraction S200 contained 10 µg of Ad2 DNA per ml and 200 to 250 µg of protein per ml. Ad2 DNA, labeled from 6 to 17 h postinfection, could be separated on linear sucrose gradients into two distinct peaks (peaks 1 and 2) and a heterogeneous interpeak region (Fig. 1). At the top of the gradient 70 to 75% of the total proteins were soluble and not associated with Ad2 DNA (soluble S200 protein fraction). An SDS-polyacrylamide gel analysis of the proteins in fractions S200 and PS200 and in different portions of a preparative sucrose gradient (Fig. 1) is shown in Fig. 2.

The total fraction S200 (Fig. 2, slot 3) contained a complex mixture of proteins, some of which are known to be adenovirus specific, for instance, the hexon (II = 120K), the 100K protein, and 72K DBP (2), which were all present in a high amount.

When fraction S200 was sedimented through

FIG. 1. Fractionation of the nucleoprotein complexes present in fraction S200 and S1 nuclease sensitivity of the DNA. Infected cells (60 ml at 3×10^6 cells per ml) were labeled with [³H]thymidine (25 Ci/mmol, 1.3 µCi/ml) from 6 to 17 h postinfection. After labeling, fraction S200 (2 ml) was prepared and centrifuged through a 10 to 58% sucrose gradient (.....) in an SW27.1 rotor (see text). Fractions of 0.4 ml were collected from the top, and the acid-precipitable [³H]DNA was determined on aliquots (●). Where indicated, aliquots of fractions were treated with S1 nuclease (see text), and the percentage of DNA sensitive to S1 nuclease was determined (○). The brackets indicate the regions of the gradient referred to as peak 1, interpeak, and peak 2. The letters (A to H) indicate the fractions that were pooled on a parallel gradient for polyacrylamide gel analysis (see Fig. 2).

FIG. 2. SDS-polyacrylamide gel electrophoresis of the proteins present in the nucleoprotein complexes of fraction S200. Fractions from a sucrose gradient similar to that shown in Fig. 1, were pooled as indicated in Fig. 1, acid precipitated, dissolved in sample buffer, loaded into the slots of a polyacrylamide slab gel, and subjected to electrophoresis as described in the text. Slots 1 to 14 contained: 1, 10 μ g of total calf thymus histones; 2, the amount of fraction PS200 corresponding to 200 μ l of fraction S200; 3, the equivalent of 200 μ l of fraction S200; 4, 30 μ g of Ad2 virion proteins; 5-12, fractions A to H indicated in Fig. 1; 13, 30 μ g of Ad2 virion proteins; 14, 10 μ g of purified 72K DBP.

the sucrose gradient shown in Fig. 1, a large number of protein bands were found in the peak 1 region (Fig. 2, slots 6 and 7). These proteins are probably free proteins which contaminate the peak 1 region by trailing down from the top of the gradient. Indeed, when peak 1 fractions were pooled, dialyzed, and recentrifuged through a second sucrose gradient, very little protein cosedimented with the DNA (not shown).

In marked contrast, it was surprising to find a predominant protein in the interpeak region (Fig. 2, slots 8-11) with an apparent molecular weight of 72,000. This protein was identified as the 72K DBP by two criteria: (i) purified 72K DBP comigrated in SDS-polyacrylamide gels, with the major protein associated with the nucleoprotein complexes of the interpeak region (Fig. 2, cf. slots 14 and 8-11); (ii) this protein was specifically immunoprecipitated by anti-72K DBP immunoglobulins, as shown by the follow-

ing experiment. Infected cells were labeled from 2 to 17 h postinfection with [3 H]leucine (see Materials and Methods). Fraction S200 was prepared and sedimented through a sucrose gradient as described above. The labeled proteins present in the interpeak region were immunoprecipitated with an antibody preparation against purified 72K DBP (42) (identical results were obtained using antiserum against Ad2 or Ad5 72K DBP). This immunoprecipitation was carried out either after extensive digestion of the DNA or in the presence of 1 M NaCl, in order to release the proteins from the nucleoprotein complexes. When the immunoprecipitated material was analyzed by electrophoresis on SDS-polyacrylamide gels, a major labeled protein band was observed at the 72K DBP position, after fluorography of the gel, whereas no radioactive 72K DBP was detected when a control serum was used (results not shown).

The peak 2 region contained some 72K DBP in addition to most virion proteins and some precursor polypeptides (Fig. 2, slot 12), suggesting the presence of incomplected virions in peak 2 (see also Fig. 3f).

The possibility of fortuitous artifactual binding of the 72K DBP to Ad2 DNA during the preparation of fraction S200 and its subsequent fractionation was ruled out by the following experiment. [^3H]leucine was added to an infected cell culture at 2 h postinfection (see Materials and Methods), and the labeled cells were harvested at 24 h postinfection. Fraction S200 was prepared from 4×10^6 nuclei and centrifuged on a sucrose gradient as described (Fig. 1). The [^3H]leucine-labeled S200 soluble protein fraction, which remained at the top of the gradient (Fig. 2, slot 5), was dialyzed against a buffer containing 20 mM Tris-hydrochloride (pH 7.9), 2 mM EDTA, 50 mM ammonium sulfate, and 0.1 mM dithiothreitol (TEAD buffer). Infected nuclei (4×10^6), in which only the DNA was labeled ([^{14}C]thymidine), were then mixed with this ^3H -labeled soluble protein fraction. A new fraction S200 (in which the ratio of labeled over unlabeled proteins was about 1:1) was prepared from this mixture and fractionated as before. Only a small amount of labeled protein sedimented down to the interpeak region (not shown) and was estimated by specific activity to represent less than 5% of the total protein associated with the Ad2 DNA nucleoprotein complexes present in this region of the gradient. Since the 72K DBP in this region represents more than 30% of the total protein content of these fractions (see Fig. 2), we can rule out a major nonspecific binding of the 72K DBP to the Ad2 DNA during our extraction procedure.

Electron microscopy of the nucleoprotein complexes present in fraction S200. The nucleoprotein complexes present in the peak 1, peak 2, and interpeak regions of a sucrose gradient (Fig. 1) were examined by electron microscopy either directly or after concentration by centrifugation on a cushion of dense sucrose (see Materials and Methods).

Initial electron microscopy studies were performed on material spread by the method of Dubochet et al. (9; see Materials and Methods). Under these conditions the three regions of a sucrose gradient revealed several predominant

types of structures. Peak 1 contained Ad2 ds-DNA molecules (20 Å [2 nm] in width; sensitive to DNase I but not to nuclease S1), which appeared (Fig. 3a) mostly "naked," although "beads" were occasionally seen on the DNA (see below). The interpeak region contained much thicker nucleoprotein structures (about 200 Å [20 nm] thick). Some of the molecules were entirely "thick" (Fig. 3c). Others contained "thin" (naked ds-DNA) and thick segments arranged in tandem and apparently continuous with each other (Fig. 3b, c, and d). In all cases the total length of the structures varied between 70 to 90% of the length of deproteinized Ad2 DNA ($11.43 \pm 0.40 \mu\text{m}$ as determined under our conditions), depending on the proportion of thin naked versus thick regions in the molecules. This observation suggests a possible compaction of the Ad2 DNA in the thick filaments, and further investigations are presently in progress to ascertain this point. "Forked" structures where a thick arm was branched on an apparently naked ds-DNA molecule were also occasionally seen but appeared to be too entangled to allow unequivocal determination of their configuration. Peak 2 contained a few complexes similar to those seen in the interpeak and appeared to comprise mostly immature virions (Fig. 3f). These virion-like forms have not been further investigated.

In a second series of experiments the formamide-spreading method was used (see Materials and Methods). Under these conditions the same type of structures were visualized in peaks 1 and 2 as with the method of Dubochet et al. (9), but the molecules present in the interpeak region looked slightly different (Fig. 4). First, the thick portion of the molecules appeared as a more feathery filament. This aspect probably resulted from a partial dissociation of the nucleoprotein complexes in the presence of formamide, despite the glutaraldehyde fixation. Second, in contrast to the method of Dubochet et al., the formamide spreading consistently revealed the presence of forked structures where a thick arm was branched on an apparently naked, genome-length ds-DNA molecule.

In the interpeak region of a typical sucrose gradient (see Fig. 1), about 55% of the DNA molecules visualized by this technique corresponded to Ad2 genome-length ds-DNA (10.40

FIG. 3. Nucleoproteins present in fraction S200 and processed for electron microscopy by the technique of Dubochet et al. (9). Aliquots of concentrated peak 1 (a), interpeak (b, c, d, and e), and peak 2 (f) fractions (see Fig. 1) were processed and examined as described in the text. Bar = 1 μm . (a) Entirely thin molecule of 9.40 μm with 17 beads; (b) 3.20- μm thick filament in continuity with a 5.70- μm thin portion with 15 beads; (c) 4.20- μm thick portion in continuity with a 3.90- μm thin portion binding six beads; (d) 6.80- μm thick portion contiguous with a 1.50- μm thin portion binding five beads; (e) entirely thick molecule of 9.10 μm ; (f) adenovirus virion-like particle associated to ds-DNA.

FIG. 4. Nucleoproteins present in fraction S200 and processed for electron microscopy by the modified technique of Davis et al. (7) (see text). Aliquots of the interpeak region of a sucrose gradient (see Fig. 1) were processed and examined as described in the text. (a) Entirely thin molecule of 10.63 μm ; (b) forked molecule with a thick arm of 1.60 μm and two thin arms of 1.50 and 11.70 μm ; (c) forked molecule with a thick arm of 6.70 μm and two thin arms of 6.60 and 4.50 μm ; (d) entirely thick molecule of 10.97 μm ; (e, f, and g) linear molecules with a thin and a thick portion of 1.30 and 10.80 μm , 4.40 and 7.30 μm , and 8.99 and 1.79 μm , respectively. Bar corresponds to 1 μm , and arrows point to the junction of the thick and thin regions of the molecules. Replicative form ($1.74 \pm 0.07 \mu\text{m}$) and ss-DNAs ($1.57 \pm 0.11 \mu\text{m}$) of ΦX174 have been added to the samples as internal length markers and can occasionally be seen.

FIG. 4 d-g.
371

$\pm 0.60 \mu\text{m}$ (Fig. 4a), as compared to deproteinized Ad2 DNA extracted from the virion and spread by the same technique ($11.22 \pm 0.25 \mu\text{m}$). Forty-five percent of the molecules present in the interpeak region contained a thick portion. Among these molecules about 10% corresponded to forked molecules (Fig. 4b and c) with a thick arm branched on an Ad2 genome-length thin filament ($11.10 \pm 1.18 \mu\text{m}$). The length of the thick arm varied from less than 10% up to 70% of the length of Ad2 DNA (Fig. 5B) but was in all cases equal within 10% to the length of one of the thin branches of the molecule. The other 90% corresponded to linear molecules either completely thick (Fig. 4d) or containing a thick portion continuous with thin DNA (Fig. 4e, f, and g). The length of the thick portion varied from less than 10 to 100% of the total length of the molecule (Fig. 4d and 5A), which appears to remain constant and is $12.20 \pm 1.70 \mu\text{m}$.

In almost all regions of the gradient, and especially when the material was spread by the method of Dubochet et al., thin fibers (ds-DNA) were often associated with a variable number of irregularly spaced, beadlike, proteinase-sensitive structures (about $120 \text{ \AA}$ [12 nm] in diameter) (Fig. 3a through d). These structures have not been conclusively identified, but the possibility that they might be nucleosomes (25) has been

FIG. 5. Diagrammatic representation of the replicative intermediates found in fraction S200. (A) Array of partly thick linear molecules; (B) array of forked molecules. In both (A) and (B) the molecules have been normalized to percentage of Ad2 genome length, and the thick and thin lines represent the thick and thin portions of the molecules, respectively. The thick regions have been arbitrarily oriented to the left.

considered. For instance, the molecule shown in Fig. 3a, which corresponds to the material found in the peak 1 region (Fig. 1), has an apparent length of $9.40 \mu\text{m}$, with about 17 beadlike structures. If these "beads" correspond to nucleosomes, one can calculate that the actual DNA length is about $10.60 \mu\text{m}$, assuming that the DNA in a nucleosome is compacted about six times from its extended length (25). It is therefore likely that the beaded structures observed on this DNA molecule are nucleosomes, since it was shown previously that the DNA present in the peak 1 region is indeed ds-Ad2 DNA (46). Supporting this possibility, a small amount of histone (with a much higher proportion of histone H1 versus the four other histones) is seen in fraction S200 by SDS-acrylamide gel electrophoresis (Fig. 2, slot 3). The four histones H2A, H2B, H3, and H4 are also apparent in the protein profiles of the nucleoprotein complexes after sucrose gradient separation (Fig. 2, slots 6–8) and remain associated to the DNA after recentrifugation of peak 1 (not shown). In a control experiment, purified simian virus 40 (SV40) form I DNA ($20 \mu\text{g/ml}$) was mixed with infected HeLa nuclei. Fraction S200 was then prepared and centrifuged through a sucrose gradient. Electron microscopic examination showed that nucleosomes are bound to SV40 DNA at an average of three nucleosomes per DNA molecule. It appears, therefore, that nucleosomes could be formed during the extraction procedure. This is not surprising since at 200 mM ammonium sulfate the stability of the nucleosome is known to be weakened (13) and "exchange of nucleosomes" could occur between the cellular chromatin and the naked Ad DNA. Indeed, when the Ad2 nucleoprotein complexes were extracted by treating the infected nuclei with 100 mM ammonium sulfate, instead of 200 mM, the released Ad2 ds-DNA (in this particular experiment three times less DNA was released than at 200 mM ammonium sulfate) contained much less of these globular structures (an average of about five "nucleosomes" per DNA molecule, instead of about 20) (not shown). Finally, the fact that these Ad DNA-bound, beaded structures, which have the dimension of nucleosomes, are released from the DNA by treatment with 1.2 M NaCl (not shown) further supports their identification as nucleosomes (13). However, the above data cannot exclude the possibility that some of these structures with a diameter similar to that of nucleosomes might be enzyme or protein complexes involved in Ad2 DNA replication or transcription.

The thick filaments correspond to Ad2 ss-DNA coated with 72K ss-DNA binding

protein. Since the 72K DBP is known to bind to ss-DNA, the possibility arose that the thick filaments viewed by electron microscopy could represent ss-DNA coated with 72K DBP. Indeed, as shown by its sensitivity to S1 nuclease treatment, some of the DNA sedimenting in the interpeak region of a sucrose gradient (Fig. 1) is single stranded. Supporting the above possibility, we observed that after the nuclease treatment, the thick structures present in the interpeak fractions disappeared. However, control experiments without nuclease S1 showed that the thick nucleoprotein structure was not stable under the nuclease S1 incubation conditions. Further electron microscopic controls indicated that the nucleoprotein structures in the interpeak were stable when incubated at 37°C for 15 min at pH 7.5 in <50 mM ammonium sulfate. Treatment of the interpeak nucleoprotein complexes with the single-strand-specific *N. crassa* nuclease (see Materials and Methods) resulted in the disappearance of the thick structures, leaving variable lengths of ds-DNA as visualized by electron microscopy (not shown). These results demonstrate that the thick nucleoprotein complexes contain ss-DNA. Furthermore, the thick structures were sensitive to proteinase K but not to pancreatic RNase treatment (not shown).

To demonstrate unequivocally that the thick filaments present in the interpeak region of a sucrose gradient (Fig. 1) correspond to ss-DNA coated with 72K DBP, they were further purified by equilibrium centrifugation in a metrizamide gradient (Fig. 6). Under these conditions, purified native Ad2 DNA bands at 1.14 to 1.16 g/ml, and intact Ad2 virions band at 1.24 to 1.26 g/ml. About 20% of the DNA of the interpeak region banded at an intermediate density of 1.21 g/ml. The DNA at 1.21 g/ml was more than 70% sensitive to single-strand-specific nucleases, compared to less than 5% sensitivity of the DNA at 1.16 g/ml (Fig. 6). Electron microscopic examination of the fractions at a 1.16-g/ml density revealed only thin ds-DNA, but nearly all of the molecules at 1.21 g/ml contained thick nucleoprotein fibers (not shown). The 72K DBP was essentially the only protein observed in these complexes (Fig. 7, slot 2), very little protein being associated with the ds-DNA at 1.16 g/ml (Fig. 7, slot 3).

Characterization of the single-stranded Ad2 DNA present in the interpeak region. As shown above, the use of a metrizamide gradient yields a fraction highly enriched for Ad2 nucleoprotein structures containing ss-DNA. When the nucleoprotein complexes from the denser peak (1.21 g/ml) of the metrizamide gra-

FIG. 6. Metrizamide density gradient analysis of the nucleoprotein complexes present in the interpeak region of the fractionated S200 extract. Infected cells (50 ml at 3×10^6 cells per ml) were labeled with [3 H]thymidine (25 Ci/mmol, 4 μ Ci/ml) from 6 to 17 h postinfection. After labeling, fraction S200 (1.7 ml, 1.9×10^6 cpm of [3 H]DNA with a specific activity of about 10^6 cpm/ μ g) was prepared and centrifuged through a 10 to 58% sucrose gradient in an SW41 rotor (see text). The interpeak fractions were pooled (4.2 ml, 5.5×10^6 cpm of [3 H]DNA) and concentrated by centrifugation on a sucrose cushion (see text). After dialysis, the material (1 ml, 4.7×10^6 cpm of [3 H]DNA) was layered on a metrizamide density gradient and centrifuged as described in the text. After centrifugation, aliquots were counted (total recovery of [3 H]DNA was 65%) and tested for *N. crassa* nuclease sensitivity (see text). Symbols: (●) total [3 H]DNA; (○) [3 H]DNA resistant to *N. crassa* nuclease treatment; (— —) density.

dient (Fig. 6) were treated with SDS and centrifuged on a neutral sucrose gradient, which allowed the separation of full-length ds-DNA (32S) and full-length ss-DNA (70S), the DNA sedimented as a broad peak, heavier than 32S native Ad2 DNA (Fig. 8). The majority of this DNA was sensitive to *N. crassa* nuclease treatment (Fig. 8). The molecules sedimenting at the 70S position were sensitive to 80% to *N. crassa* nuclease and correspond therefore mainly to full-length ss-Ad2 DNA molecules. By contrast, the lighter peak (1.16 g/ml) of the metrizamide gradient contains essentially ds-DNA sedimenting at 32S and insensitive to *N. crassa* nuclease (Fig. 8A).

The reassociation kinetics of the ss-DNA pres-

FIG. 7. SDS-polyacrylamide gel electrophoresis of the nucleoprotein complexes fractionated on a metrizamide density gradient. Fractions of the two DNA peaks of a metrizamide gradient similar to that shown in Fig. 6 were pooled as indicated by the bars in Fig. 6. The lighter peak and half of the denser peak were trichloroacetic acid precipitated and redissolved in sample buffer, and the proteins were analyzed by SDS-polyacrylamide gel electrophoresis as described in Fig. 2. Slots 1 to 4 contained, respectively: 10 μ g of purified 72K DBP, material corresponding to the denser peak, material corresponding to the lighter peak, and 30 μ g of Ad2 virion proteins.

ent in the nucleoprotein complexes are shown in Fig. 9. The DNA sedimenting at the position of full-length Ad2 ss-DNA (70S) on a neutral sucrose gradient (Fig. 8B) reannealed to the same extent as the Ad2 ds-DNA (32S) (Fig. 8A) after denaturation. The same percentage of reannealing was also obtained when reassociation kinetics were performed with the unfractionated DNA from the denser peak (1.21 g/ml) of the

FIG. 8. Neutral sucrose gradient analysis of the labeled DNA present in the nucleoprotein complexes fractionated on a metrizamide density gradient. Fractions of the two peaks of $[^3\text{H}]\text{DNA}$ separated on the metrizamide density gradient were pooled as indicated by the bars in Fig. 6. The pooled fractions were dialyzed against 10 mM Tris-hydrochloride (pH 7.9), 10 mM NaCl, and 10 mM EDTA. After dialysis, (A) 1.1 ml of the lighter peak (9×10^6 cpm of $[^3\text{H}]\text{DNA}$) and (B) 2.1 ml of the denser peak (5.2×10^6 cpm of $[^3\text{H}]\text{DNA}$) were treated with SDS and layered on a 10 to 40% sucrose gradient (.....) as described in the text. The arrows denote the position of full-length native (32S) and denatured (70S) Ad2 DNA, which were used as markers on a parallel gradient. After centrifugation, fractions (0.6 ml) were collected from the top of the tube, and aliquots were counted (●) (total recovery of $[^3\text{H}]\text{DNA}$ was about 75% for each gradient). Fractions were pooled as indicated by the striped bars and precipitated with 2 volumes of ethanol, in the presence of 20 μ g of calf thymus DNA as a carrier. After 2 h at -20°C , the DNA was collected by centrifugation, and each sample was redissolved in 0.1 ml of a buffer containing 10 mM Tris-hydrochloride (pH 7.9), 10 mM NaCl, and 10 mM EDTA. Aliquots were treated with single-strand-specific *N. crassa* nuclease (see text). The height of the striped bars represents the percentage of $[^3\text{H}]\text{DNA}$ that was nuclease sensitive in each fraction pool.

FIG. 9. Reassociation kinetics of the ss-DNA present in the adenovirus nucleoprotein complexes of fraction S200. The fractions of neutral sucrose gradients containing full-length ss-DNA (70S) (Fig. 8B, fractions 21 to 24) and full-length ds-DNA (32S) (Fig. 8A, fractions 12 to 14) were pooled separately, and the DNA was concentrated as described in Fig. 8. Reassociation kinetics were performed on these two DNA samples as described in the text, and DNA reassociation was expressed as the percentage of material resistant to *N. crassa* nuclease treatment at different incubation times. Symbols: (○) Ad2 ds-DNA (32S); (●) Ad2 ss-DNA (70S).

metrizamide gradient (Fig. 6) after deproteinization (not shown). These results indicate that equal amounts of l and r strands (33) are present in the interpeak region.

The deoxyribonucleoprotein complexes containing the 72K ss-DNA binding protein correspond to viral replicative intermediates. We have previously shown that fraction S200 contains active Ad2 replicative intermediates (5). These replication complexes were found in the interpeak region when sedimented on a sucrose gradient similar to that shown in Fig. 1 and synthesized full-length Ad2 DNA when incubated *in vitro* in the presence of the four deoxyribonucleoside triphosphates. We have performed two types of experiments to demonstrate that the nucleoprotein complexes containing the 72K DBP correspond to these replicative intermediates.

Nuclei or fraction S200 was preincubated with increasing concentrations of anti-72K DBP or control immunoglobulins and then tested *in vitro* for DNA polymerase activity (Fig. 10). The control immunoglobulins had no effect on DNA polymerase activity in either nuclei or fraction S200. On the contrary, under the conditions used in this experiment, the anti-72K DBP immunoglobulins inhibited the DNA polymerase activity by about 50% both in nuclei and fraction S200. Since under our incubation conditions (200 mM ammonium sulfate) no DNA chain reinitiations occur (5), this result indicates that the 72K DBP

is probably involved in the elongation process of the viral DNA (see Discussion).

The following experiment clearly demonstrates that the 72K DBP is bound to the replicative intermediates actively engaged in adenovirus replication. Infected cells, labeled from 6 to 17 h postinfection with [14 C]thymidine, were pulse-labeled with [3 H]thymidine at 17 h postinfection, and fraction S200 was prepared. Alternatively, infected cells were labeled from 6 to 17 h postinfection with [3 H]thymidine, and fraction S200 was prepared and incubated *in vitro* for DNA polymerase activity in the presence of a very low concentration of [32 P]dCTP of high specific activity for 10 min. This procedure permits the 32 P labeling of the replication complexes without extensive elongation and termination of the nascent chains. In both cases aliquots of fraction S200 were incubated with anti-72K DBP immunoglobulins and then sedimented through a sucrose gradient. Both *in vivo* and *in vitro* pulse-labeled DNA became very heavy in the presence of anti-72K DBP immunoglobulins, sedimenting at the peak 2 position (Fig. 11). When fraction S200 was incubated in the presence of an identical concentration of

FIG. 10. Effect of anti-72K DBP immunoglobulins on DNA synthesis in nuclei and fraction S200. Aliquots of the nuclei suspension or of fraction S200, respectively, corresponding to 0.45×10^6 and 0.25×10^6 cells, were preincubated at 200 mM ammonium sulfate for 30 min at 0°C in the presence of increasing concentrations of antibodies. At the end of the preincubation, the medium was adjusted to 100 μ M dATP, dGTP, and dTTP, 5 μ M [3 H]dCTP (128 cpm/pmol), and 5 mM $MgCl_2$, and incubations were carried out for 30 min at 30°C. DNA synthesis was measured as previously described (5). In this particular experiment 100% corresponds to 3,970 and 1,380 cpm of [3 H]dCMP incorporated into acid-insoluble material per 10^6 nuclei, respectively, in the nuclei and fraction S200. Symbols: (●, ○) nuclei incubated, respectively, in the presence of control and anti-72K DBP immunoglobulins; (▲, Δ) fraction S200 incubated, respectively, in the presence of control and anti-72K DBP immunoglobulins.

control immunoglobulins (Fig. 11A and C), the sedimentation profiles on sucrose gradients were identical to those obtained with untreated frac-

FIG. 11. Effect of anti-72K DBP immunoglobulins on the sedimentation properties of *in vivo* or *in vitro* pulse-labeled DNA. (A and B) Infected cells were labeled with [^{14}C]thymidine (10 nCi/ml) from 6 to 17 h postinfection. At 17 h postinfection the cells were pulse-labeled with [^3H]thymidine (20 $\mu\text{Ci/ml}$) for 1 min as previously described (5). Nuclei and fraction S200 were prepared as described in the text. A 100- μl portion of fraction S200 (4,200 ^{14}C cpm; 13,000 ^3H cpm) was incubated in the presence of control (A) or anti-72K DBP (B) immunoglobulins (13 mg/ml) for 1 h at 0°C in the presence of 200 mM ammonium sulfate. At the end of the incubation period, the sample was diluted to 1 ml with unlabeled fraction S200 (this was found to be necessary to obtain reproducible sedimentation profiles) and sedimented through a 11.5-ml sucrose gradient as described in the text. Symbols: (●) [^{14}C]DNA; (○) [^3H]DNA. (C and D) Infected cells were labeled with [^3H]thymidine (2 $\mu\text{Ci/ml}$) from 6 to 17 h postinfection. Nuclei and fraction S200 were prepared as described in the text. A 1-ml portion of fraction S200 was incubated for DNA polymerase activity as described in the legend to Fig. 10, but in the presence of [^{32}P]dCTP (0.4 μM , 2.75×10^5 cpm/pmol) for 10 min at 30°C . The reaction was stopped by adding 10 mM EDTA, and the free nucleotides were removed by chromatography on a Sephadex G75 column (1 by 70 cm) equilibrated with TEAD buffer (see Results) containing 200 mM ammonium sulfate. A 50- μl portion (55,000 ^3H cpm; 67,500 ^{32}P cpm) of the peak of the excluded material was incubated in the presence of control (C) or anti-72K DBP (D) immunoglobulins (9 mg/ml), diluted with unlabeled fraction S200, and sedimented through a sucrose gradient as above. Symbols: (▲) [^3H]DNA; (△) [^{32}P]DNA.

tion S200 (cf. Fig. 5 in reference 5). Thus, we can conclude that the 72K DBP-containing nucleoprotein complexes present in fraction S200 are replication complexes actively engaged in Ad2 DNA replication.

It should be stressed that in these experiments we used an antibody preparation raised against pure 72K DBP, as judged by polyacrylamide gel electrophoresis. In addition, we have determined that the 72K DBP was the only viral protein reacting with this antibody preparation. Indeed, when a crude extract of Ad2-infected cells, labeled with [^3H]leucine (see Materials and Methods), was treated with the anti-72K DBP immunoglobulins and the immunoprecipitated material was analyzed by SDS-polyacrylamide gel electrophoresis, the only protein revealed by fluorography was the 72K DBP (not shown).

DISCUSSION

ss-DNA of Ad2 replicating intermediates is coated with the 72K ss-DNA binding protein. The existence of long ss-DNA stretches in Ad replicative intermediates (4, 22, 26, 28, 30, 36, 38) and the purification from infected cells of an adenovirus-coded protein (72K DBP), which binds specifically to ss-DNA *in vitro*, has suggested that this protein could be involved in adenovirus DNA replication. The isolation of an Ad5 mutant coding for a temperature-sensitive 72K DBP and temperature sensitive for DNA replication has supported this possibility (11, 40).

We have previously shown that replicative intermediates, active in adenovirus DNA replication, can be extracted from infected HeLa cell nuclei and partially purified by sedimentation through sucrose gradients (5). Our present electron microscopic and biochemical results demonstrate that the 72K DBP is coating the ss-DNA associated to the replicating Ad2 intermediates to yield the thick filaments visualized by electron microscopy. The association of the 72K DBP to the replicating intermediates is also supported by the inhibitory effect of anti-72K DBP immunoglobulins on DNA synthesis catalyzed by the replicating intermediates present in fraction S200. Analogous results were obtained by van der Vliet et al. (42). These authors have shown that the anti-72K DBP immunoglobulins, when added to Ad5-infected nuclei, inhibit only partially the *in vitro* synthesis of the viral DNA by slowing down the DNA chain elongation rate. That this mechanism also applied in our case was shown by studies of the time course of incubation (not shown). Although our present studies show clearly that the 72K DBP is directly implicated in adenovirus DNA replication, further studies are required to establish whether this protein has an active role in the

elongation of the nascent DNA chains or whether it merely serves to protect the ss-DNA of the replicative intermediates. Furthermore, the precise function of this protein during the initiation of the newly synthesized DNA chains remains an open question.

The association of the 72K DBP with a nuclear membrane complex that synthesized Ad2 DNA *in vitro* has previously been reported by Shanmugam et al. (32) and interpreted as a possible involvement of this protein in the adenovirus DNA replication, although the authors did not eliminate the possibility of a nonspecific binding, whereas in our study we have shown that less than 5% of the proteins present in the interpeak region could be attributed to a nonspecific binding to the viral DNA during the extraction. Clearly, this experiment rules out that all the 72K DBP present in the complexes are due to rearrangement of proteins during our extraction procedure.

Electron microscopic visualization of the replicating intermediates supports a strand displacement mechanism for Ad2 DNA replication. The current model proposed for adenovirus DNA replication (12, 16, 35, 36, 44) has two major implications. (i) Replication starts at or near one end of the molecule and proceeds towards the other end, displacing one of the parental strands. The displaced strand is later replicated in the opposite direction. (ii) The displacement synthesis can start at either end of the ds-DNA molecules, leading to the accumulation of both parental strands after displacement.

According to the first point of this model, essentially three types of structures should be visualized by electron microscopy: (i) forked molecules with two double-stranded arms and one single-stranded arm; (ii) nonbranched, linear molecules, partly double stranded and partly single stranded, with various ratios of double- to single-stranded portions; (iii) purely linear genome-length double- or single-stranded molecules. In all cases, if ss-DNA were covered with the 72K DBP it should appear in the electron microscope as a nucleoprotein filament, whereas ds-DNA should appear as filaments of nearly naked DNA.

Our results fulfill all the above predictions; all the expected types of intermediates can be clearly visualized in the interpeak region using the formamide-spreading technique, and the single-stranded regions are indeed coated with 72K DBP (Fig. 4). Others (10) have observed that when deproteinized adenovirus DNA replicative intermediates were extracted from whole nuclei, 55% were forked molecules and 45% were linear hybrid single- and double-stranded molecules.

In the interpeak preparations, we have visualized only 10% of the replicative intermediates as forked molecules. Perhaps during the homogenization of nuclei with ammonium sulfate the forked replicative intermediates were more sensitive to shearing forces than the linear intermediates. Indeed, we were unable to visualize forked molecules with a single-stranded arm longer than 70% of the genome length and sometimes have observed thick filaments of various lengths but shorter than the genome size.

Girard et al. (15) have recently shown that Ad5 DNA replicative intermediates extracted by Sarkosyl from infected nuclei late in infection appeared in most cases as circular molecules closed by a proteinaceous linker. Under the present extraction conditions, however, neither circular replicative intermediates nor circular adenovirus DNA molecules were observed. As discussed by Girard et al. (15), the circular molecules could be artefactually generated during the Sarkosyl extraction procedure. On the other hand, we cannot exclude that the circular molecules did exist in the nucleus and were open during our extraction procedure.

The visualization of genome-length thick nucleoprotein complexes (Fig. 3d and 4d) and the detection of full-length ss-DNA on a neutral sucrose gradient (Fig. 8B) suggest that late in a productive infection there exists a pool of Ad2 ss-DNA molecules. This pool represents about 4% of the total 6- to 17-h-postinfection labeled DNA in fraction S200 (unpublished data). Accumulation of genome-length adenovirus ss-DNA has also been seen in deproteinized replicative intermediates extracted from intact infected nuclei (36, 38).

The second point of the current model for adenovirus DNA replication, namely, that the replication is initiated at both molecular ends of the native ds-DNA, is supported by our results. Analysis by DNA-DNA reassociation kinetics of the ss-DNA present in the interpeak region shows that equal proportions of both strands were present (Fig. 9). This, together with other results (12, 16, 35, 37), indicates that both strands of the DNA are displaced with equal probability during adenovirus DNA replication.

Ad2 DNA is not packed in a nucleosomal chromatin-like structure in infected HeLa cell nuclei late in infection. Whereas the ss-DNA present in the nucleoprotein complexes is coated with 72K DBP, the ds-DNA of these complexes appears essentially naked. However, globular structures (the presumptive nucleosomes) are sometimes associated with the ds-DNA of both peak 1 and replicating intermediates as discussed in Results. Their presence on Ad2 DNA could result from an exchange with

the nuclear chromatin during the preparation of fraction S200, since nucleosomes appeared on naked SV40 DNA added to the nuclei before the extraction procedure. Furthermore, SV40 minichromosomes containing 20 to 22 nucleosomes per SV40 DNA molecule can be extracted with 200 mM ammonium sulfate from nuclei of infected CV1 cells (unpublished data; P. Gariglio, personal communication). Since SV40 minichromosomes extracted by other procedures also contain 20 to 22 nucleosomes (see reference 3), this observation indicates that, although less stable at this ionic strength, the nucleosomes present on the nuclear SV40 DNA are not released during the extraction procedure. Therefore it seems unlikely that, late in infection, the Ad2 ds-DNA could be packed in nucleosomes inside the nucleus of infected cells, since most are naked DNA in fraction S200. This conclusion is further supported by the fact that the Ad2 nucleoprotein complexes extracted at 100 mM ammonium sulfate contained much less globular nucleosome-like structures than those extracted at 200 mM (see Results). On the other hand, one cannot exclude that during the extraction procedure some other proteins were displaced from the Ad2 ds-DNA. Our conclusion that the adenovirus genome is not packaged into nucleosomes in the nuclei of infected cells is in agreement with the previous work of Corden et al. (6), who concluded that it is unlikely that cellular histones could be associated with the viral DNA. In any case, the scarcity of the nucleosome-like structures on the Ad2 DNA molecules should be emphasized, since it is in marked contrast with the SV40 situation where the viral nuclear DNA is packed in nucleosomes in the form of minichromosomes. This striking difference in the genome configuration of papovavirus and adenovirus genomes inside the nucleus of infected cells is probably related to two distinctive features of the biology of these viruses: (i) SV40 infection stimulates histone (17) and host DNA synthesis (29), whereas Ad2 infection shuts off cellular DNA synthesis (14); (ii) inside the SV40 virion, the viral DNA is associated to histones of cellular origin (27), whereas inside the Ad2 virion the viral DNA is associated with the virus-coded core proteins (23).

ACKNOWLEDGMENTS

We thank P. Chambon for advice, helpful discussions, and critical reading of the manuscript. We are greatly indebted to L. Philipson and P. van der Vliet for gifts of antisera against Ad2 and Ad5 DBP. We also wish to thank P. Oudet for help and advice during the electron microscopic studies. Excellent technical assistance was provided by C. Hauss. We are grateful to K. Dott and L. Thia-Toong for growing our cells and viruses.

This investigation was supported by grants to P. Chambon

from the Centre National de la Recherche Scientifique (CNRS) (ATP Différenciation cellulaire, contract no. 2117), the Institut National de la Santé et de la Recherche Médicale (ASR Virus oncogènes, contract no. 1), the Fondation pour la Recherche Médicale Française, and the Commissariat à l'Energie Atomique. O. Brison is an attaché de recherche of the CNRS.

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