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Characterization of Adenovirus Type 2 Transcriptional Complexes Isolated from Infected HeLa Cell Nuclei

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HeLa cell nuclei, isolated 17 h after infection with human adenovirus type 2 (Ad2), were treated with 200 mM ammonium sulfate. The extract (S200 fraction) contained 50 to 70% of the nonintegrated Ad2 DNA, which was in the form of nucleoprotein complexes. These complexes contained native, intact Ad2 DNA (with the exception of replicative intermediates) and could be partially purified and resolved by velocity gradient centrifugation. Using high-salt (200 mM ammonium sulfate) incubation conditions, more than 95% of the nuclear RNA polymerase activity belonged to class B. About 45% of the class B enzyme molecules bound to DNA in the nuclei (those "engaged" in RNA synthesis) were released from the nuclei in the form of Ad2 transcriptional complexes by treatment with 200 mM ammonium sulfate. At least 90% of the RNA synthesized in high salt in the nuclei or in the S200 fraction was Ad2 specific, and essentially all of this RNA was complementary to the *l* strand of Ad2 DNA. These findings are compatible with what is known about Ad2-specific RNA synthesis in vivo. The analysis of the RNA synthesized from partially purified transcriptional complexes supports the contention that its transcription is almost entirely asymmetric, and that the asymmetry observed in vivo is not a consequence of the rapid degradation of *h*-strand transcripts. The RNA synthesized in vitro in the absence of detectable RNase activity sedimented with a maximum size of 35 to 40S. Less than 5% of the nuclear or the S200 fraction RNA polymerase activity was class C when assayed under non-reinitiating conditions. Although much of the RNA synthesized by the class C enzyme was Ad2 specific, 5.5*S* virus-associated RNA was not the predominant product. The isolation of Ad2 DNA transcriptional complexes provides an attractive system for further characterizing the Ad2 DNA template used for transcription and for studying the regulation of the expression of the Ad2 genome during the productive infection cycle.

The study of the factors that regulate the expression of the genome in eukaryotic cells is facilitated when the DNA template, the DNA-dependent RNA polymerase, and the primary transcription product (RNA) are well characterized (for discussion see reference 8). These criteria are at present well satisfied in the somewhat simplified (compared to total eukaryotic chromatin) system of the transcription of human adenovirus type 2 (Ad2) DNA in productively infected HeLa cells. The Ad2 genome is physically well characterized, and significant progress has been made to locate the origin of different mRNA species on the DNA (10, 47, 50, 52) and even to identify the translation products of the different mRNA's (32).

Ad2-specific late mRNA seems to be synthesized in the nucleus as part of a high-molecular-weight precursor, which is subsequently cleaved and polyadenylated before transfer to the cytoplasmic polysomes (2, 33, 35, 43, 69). It has been estimated that the largest Ad2 nuclear RNA might represent the transcript of one entire strand of the Ad2 genome (2, 52). Ad2 mRNA is derived from both strands of the DNA genome (7, 10, 66), and RNA transcribed symmetrically from 60 to 90% of the Ad2 genome exists in nuclei late after infection (47, 59). These results tend to support the hypothesis that there exist limited sites of initiation (possibly one for each strand) for RNA polymerase on the Ad2 DNA, and that mRNA arises from the processing of genome-length precursors.

Two lines of evidence suggest that the transcription of the Ad2 genome is regulated to a certain extent during the productive infection cycle and that specific initiation and termination sites exist in the interior sequences of the genome. (i) The expression of the *l* strand of

Ad2 DNA relative to the *h* strand is increased at late times postinfection (16 to 18 h). The ratio of the quantity of mRNA derived from the *l* strand at early times (4 to 6 h postinfection) to that derived from the *h* strand is about 2 to 1 (7, 52); at late times this ratio becomes 220 to 1 for newly synthesized mRNA (52). Also, pulse-labeled late Ad2 nuclear RNA is only minimally self-complementary, suggesting an asymmetric transcription of RNA (47). (ii) The 5.5*S* virus-associated (VA) RNA has been mapped near 0.3 on the Ad2 genome (36, 48, 61), which is inside a large region identified to code for late mRNA (52). Since the VA RNA is synthesized by a different class of RNA polymerase than the majority of mRNA (see below) and since it is not derived from a high-molecular-weight precursor (61), one concludes that specific initiation and termination sites exist in this region of the Ad2 genome.

Host cell RNA polymerases are thought to be responsible for the transcription of the Ad2 genome during infection. This conclusion was reached by treating nuclei isolated late after infection with α-amanitin and observing an inhibition of large, nuclear, Ad2-specific RNA synthesis at low concentrations of α-amanitin (indicative of class B enzyme; for polymerase nomenclature see reference 8) (54, 70) and an inhibition of VA RNA synthesis at high concentrations of the drug (indicative of class C enzyme activity) (53, 71). It is not excluded that the normal host cell polymerases are modified by viral gene products to alter their template specificity during the infectious cycle. However, up to now no differences have been found when comparing the transcriptional properties of RNA polymerases partially purified from normal or Ad2-infected cells (24, 72, 73). Of course, modifying factors may well have been lost during purification.

The isolation of viral transcriptional complexes from infected cells represents a different approach to study the factors responsible for the specificity of interaction between RNA polymerases and the Ad2 DNA and to facilitate the search for virus-specific or cellular factors that might regulate viral genome expression during infection. It can be anticipated that the purification of such intermediates will allow the identification of the proteins associated with the template in vivo and the determination of the physical state of the DNA template (e.g., intact, nicked, single-stranded, or circular). In addition, they may represent a more physiological template for purified HeLa cell RNA polymerases than purified, deproteinized Ad2 DNA.

This paper describes the release from HeLa cell nuclei, 17 h postinfection, of Ad2 DNA-nucleoprotein complexes and their partial purification and characterization. These complexes contain endogenous RNA polymerase activity, which synthesizes RNA with many similarities to that synthesized in vivo late in infection.

MATERIALS AND METHODS

Growth of HeLa cells and infection with Ad2. HeLa cells were maintained in suspension cultures as previously described (24). The cells were infected after concentration by centrifugation to 10^7 cells/ml (33). Ad2 virions were purified as described by Doerfler (11). One hundred PFU (5, 24) of Ad2 per cell was added, and the cells were diluted to 3×10^5 cells/ml after 30 min at 37 C. Infections were stopped 17 h postinfection.

Labeling of cells. To label the Ad2 DNA synthesized during the infection, [^{14}C]thymidine (53.9 mCi/mmol) was added to the cell culture at 6 h postinfection. Depending on the experiment, between 0.005 and 0.05 μCi/ml was used. At 16.5 h postinfection the cells were pelleted and resuspended at 3×10^6 cells/ml in warm, unlabeled medium from a control infected cell culture. At 17 h postinfection the cell suspension was cooled to 0 C and centrifuged. The cell pellet was resuspended in phosphate-buffered saline (about 10^6 cells/ml) and recentrifuged.

Cell cultures to be pulse-labeled at 17 h postinfection were prepared as above, and [^{3}H]thymidine (25 Ci/mmol, 17 μCi/ml) was added. At the end of the pulse the cells were diluted into 10 volumes of cold (0 C) medium minus serum and then centrifuged and washed with phosphate-buffered saline as above. With these labeling conditions, the specific activity of total DNA was approximately 3,600 counts/min of [^{14}C]thymidine (0.05 μCi/ml; 6 to 16.5 h postinfection) and 5,400 counts/min of [^{3}H]thymidine (10 min at 17 h postinfection) per μg of DNA.

Buffers. Double-distilled sterile water was used for all solutions and pH values were determined at 0 C. TITE buffer contained 20 mM Tris-hydrochloride, pH 7.3, 140 mM NaCl (isotonic), 0.05% Triton X-100, 2 mM EDTA. TEAD buffer contained 20 mM Tris-hydrochloride, pH 7.9, 2 mM EDTA, 50 mM ammonium sulfate, 1 mM dithiothreitol. STE buffer contained 1% sodium dodecyl sulfate (SDS), 50 mM Tris-hydrochloride, pH 7.5, 10 mM EDTA. TNE buffer contained 10 mM Tris-hydrochloride, pH 7.9, 10 mM NaCl, 0.1 mM EDTA. SSC (1×) was 150 mM NaCl, 15 mM sodium citrate, pH 7.

Sucrose solutions are expressed in a weight/weight percentage.

Preparation of nuclei. The washed cell pellet was resuspended at 3×10^6 cells/ml in TITE buffer. After 10 min at 0 C the cells were broken by homogenization with a tight-fitting Dounce B pestle (Kontes Glass Co.). Usually 4 to 6 strokes were required to obtain complete cell breakage without nuclear disintegration, as judged by light microscopic examination. The homogenate was centrifuged at 1,000 × *g*

for 10 min, and the crude nuclear pellet was immediately suspended in TEAD buffer at a concentration of 3×10^6 nuclei/ml in a Dounce homogenizer using the loose-fitting A pestle. Alternatively, the nuclear pellet was stored in liquid nitrogen after resuspension of the nuclei at 60×10^6/ml in TEAD buffer containing 25% sucrose. Results obtained with fresh or frozen nuclei were identical in terms of Ad2 DNA release, RNA polymerase activity, etc.

Extraction of nuclei. Ammonium sulfate (2 M) was added drop by drop, up to a final concentration of 200 mM, to the nuclei (3×10^6/ml) suspended in TEAD buffer at 0 C while mixing gently with the Dounce A pestle. The Dounce homogenizer was then held in a 37 C water bath for 1 to 2 min, and the suspension was mixed gently with the A pestle. At this point the nuclei did not become viscous but were aggregated. By light microscopic observation they were intact. The 200 mM ammonium sulfate nuclear suspension was cooled to 0 C and centrifuged for 10 min at $10,000 \times g$, yielding a supernatant fraction (S200 fraction) and a nuclear pellet (PS200 fraction) (see Fig. 1). As discussed in the text and in Table 3, this original procedure was modified in some experiments.

DNA isolation. Ad2 DNA was labeled and purified as previously described (47). In place of the final dialysis, the DNA was precipitated with 2 volumes of 100% ethanol and redissolved in TNE buffer. Radioactive HeLa cell DNA was prepared from uninfected cells grown for 2 days in the presence of [^{3}H]- or [^{14}C]thymidine.

DNA (about 25 μg/ml) was purified from cells, nuclei, fraction S200, and fraction PS200 by digestion with 50 μg of proteinase K per ml (5 h at 37 C; 18) in the presence of 0.5% SDS, 20 mM Tris-hydrochloride, pH 7.9, and 10 mM EDTA. The digest was then extracted with 0.5 volume of distilled phenol saturated with 0.5 M Tris-hydrochloride, pH 7.9; then 0.5 volume of chloroform was added and the mixture was centrifuged. The aqueous phase was reextracted with 1 volume of chloroform, the mixture was centrifuged, and the aqueous phase was precipitated with 2 volumes of 100% ethanol at −20 C. The DNA was recovered by centrifugation, the pellet was dried in a stream of air, and the DNA was dissolved in an appropriate volume of TNE buffer. Recovery was at least 80%.

Separation of free and DNA-bound nuclear RNA polymerase molecules. Nuclei (corresponding to about 10^7 infected HeLa cells), labeled with [^{14}C]thymidine and prepared as described above, were lysed by gentle mixing with a Dounce homogenizer (A pestle) in 1 ml of a hypotonic buffer containing 0.1 mM sodium phosphate, pH 7.5, 0.1 mM EDTA, and 0.25% Nonidet P-40, as described by Hancock (20). Tris-hydrochloride, pH 7.9, EDTA, and ammonium sulfate were added and adjusted to 20, 2, and 200 mM, respectively, while mixing gently with the Dounce A pestle. This lysate was then sedimented through a sucrose gradient (4 ml of 6 to 10% sucrose in 200 mM ammonium sulfate, 20 mM Tris-hydrochloride, pH 7.9, 2 mM EDTA, underlaid with 0.3 ml of 20% sucrose in the same buffer) for 2.5 h at 64,000 rpm (4 C) in an SW65 Spinco rotor. After centrifugation 0.5-ml fractions were collected. Under these conditions two distinct peaks of class B polymerase were separated (as concluded from labeled amanitin binding assays performed on the fractions; see below): one peak was found in the sucrose cushion and was associated with the DNA, whereas the other peak remained in the upper part of the gradient and was completely free of DNA, as determined by counting aliquots of the fractions for their labeled DNA content.

Determination of RNA polymerase activity and of the number of class B RNA polymerase molecules. RNA polymerase activity was assayed, for 10 min at 37 C, in a standard incubation medium (usual volume, 0.2 ml) containing 200 mM ammonium sulfate, 80 mM Tris-hydrochloride, pH 7.9, 2 mM Mn^{2+} (final concentration was corrected for the amount of EDTA present in the samples), 0.05 mM dithiothreitol, and the four nucleoside triphosphates (1 mM each, except for the labeled nucleotide, either [^{3}H]UTP or [α-^{32}P]CTP, which was 0.1 mM and had a specific activity between 100 and 50,000 counts/min per pmol, depending on the experiment). RNA synthesis was determined by measuring the incorporation of labeled nucleotide in acid-insoluble material as previously described (27). One unit of enzyme activity incorporated 1 pmol of the labeled nucleotide in 10 min of incubation under these incubation conditions. Unless otherwise stated, blank incubations were run under the same conditions but in the presence of 50 μg of actinomycin D per ml.

The number of class B RNA polymerase molecules was determined by the [^{3}H]amanitin binding assay (nitrocellulose filter technique) previously described by Cochet-Meilhac et al. (9).

Purification of RNA synthesized in vitro. Preparative in vitro RNA synthesis was carried out in 1 ml under standard incubation conditions for 30 min at 37 C (see above), and the reaction was stopped by adding 10 mM EDTA to the incubation mixture. The sample was then diluted two times with water, adjusted to 0.5% SDS, and treated overnight at 37 C with 100 μg of proteinase K per ml. After a further threefold dilution with water, 100 μg of yeast RNA was added as carrier, and the mixture was extracted with 1 volume of phenol saturated with 0.5 M Tris-hydrochloride, pH 7.9. The aqueous phase was reextracted with 1 volume of a chloroform-isoamyl alcohol mixture (96:4) and precipitated overnight at −20 C with 2 volumes of ethanol.

After centrifugation (30 min, $16,000 \times g$), the RNA pellet was dissolved in 1 ml of 20 mM Tris-hydrochloride, pH 7.9, 10 mM $MgCl_2$ and incubated for 30 min at 37 C with 50 μg of RNase-free DNase per ml (O. Brison and P. Chambon, submitted for publication). The mixture was extracted with 1 volume of chloroform-isoamyl alcohol, and the aqueous phase was loaded on a G-75 Sephadex column equilibrated and washed with STE buffer. Fractions containing the RNA were pooled and precipitated overnight at −20 C with 2 volumes of ethanol in the presence of 220 mM ammonium acetate (41).

The concentration and the specific activity of the purified RNA were calculated from the specific activity of the labeled nucleotide that was used for the

synthesis, assuming that the incorporation of 1 pmol of the labeled nucleotide corresponds to the synthesis of 1.2 ng of RNA.

Hybridization with filter-bound Ad2 DNA. Nitrocellulose filters (6 mm; Sartorius) were loaded with 5 μg of alkali-denatured [^{14}C]Ad2 DNA (40 to 50 counts/min per μg) according to Gillespie and Spiegelman (15). Ad2 DNA was denatured just before binding to nitrocellulose filters in 0.3 N NaOH for 5 min at room temperature. The mixture was then neutralized by the addition of 3 volumes of 0.1 N HCl and adjusted to 0.3 M NaCl, 24 mM Tris-hydrochloride, pH 7.9, and 3 mM $MgCl_2$ (14). Before hybridization, the RNA was treated for 5 min at 50 C in 95% formamide. Hybridizations were carried out in siliconized hemolysis tubes, each one containing a DNA-loaded filter and about 1,000 counts/min of RNA (0.4 ng of [^{3}H]UMP RNA) in 0.1 ml of the following mixture: 50% deionized formamide, 0.3 M NaCl, 50 mM Tris-hydrochloride, pH 7.5, 10 mM EDTA, 0.2% SDS. Blanks were treated in the same way but without DNA on the filters. Each assay was run in duplicate. The hybridization mixtures were overlaid with 0.5 ml of paraffin oil and incubated at 37 C. The total amount of virus-specific RNA in a sample was determined by hybridization to three Ad2 DNA-loaded filters added consecutively to the RNA solution. The RNA was allowed to hybridize for 48 h with each filter. After each 48-h incubation period, the filter was washed twice in 2 ml of 2× SSC, 0.1% SDS and three times in 2 ml of 2× SSC, treated for 1 h at room temperature with 0.5 ml of RNase A (20 μg/ml) in 2× SSC, and washed four times in 2 ml of 2× SSC. Filters were dried and counted in toluene scintillant. The amount of virus-specific RNA was estimated as the percentage of input RNA radioactivity that hybridized to the three filters (33).

Hybridization with separated strands of Ad2 DNA (*h* and *l*). Separated strands of [^{14}C]Ad2 DNA were purified according to Tibbetts et al. (66). The specific activity of each strand was about 29,000 counts/min per μg. Before hybridization the size of DNA and RNA molecules was reduced by alkali treatment as follows. The separated *h* and *l* strands of Ad2 DNA were treated for 15 min at 100 C in 0.3 N NaOH. The mixture was then neutralized with HCl and adjusted to 20 mM Tris-hydrochloride, pH 7.9 (65). The purified in vitro synthesized RNA was treated with 0.1 N NaOH at room temperature for 15 s, and the mixture was then neutralized with HCl and adjusted to 20 mM Tris-hydrochloride, pH 7.9 (49).

Hybridizations were performed in siliconized hemolysis tubes containing 50 μl of the following mixture: 1 M NaCl, 1 mM EDTA, 10 mM Tris-hydrochloride, pH 7.9, 1,200 ng of strand *h* or *l* per ml, and 6 ng of labeled RNA per ml. Each assay was run in duplicate. The mixture was overlaid with 0.2 ml of paraffin oil and incubated at 68 C for 24 h.

After hybridization, the incubations were diluted with 2 ml of 2× SSC containing 20 μg of RNase A and 20 units of RNase T_1 per ml (RNase T_1 did not contain any detectable DNase activity under these incubation conditions). After 1 h at room temperature, the mixture was acid precipitated, and the precipitate was counted as described below. Parallel incubations were treated as follows. At the end of the hybridization period the medium was diluted 10 times with 0.45 ml of RNase H (3 units/ml) in 40 mM Tris-hydrochloride, pH 7.9, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 4% glycerol, and 30 μg of RNase-free bovine serum albumin per ml and incubated for 30 min at 37 C. The medium was then adjusted to 2× SSC and treated with RNases A and T_1, as described above.

RNA self-hybridization assays. The RNA treatment, the incubation conditions, and the RNase A and T_1 treatments were the same as for the hybridization with the separated strands (above), but no DNA was added to the hybridization mixture. Zero time values were obtained by heating the RNA for 5 min at 100 C before RNase treatment.

Polyacrylamide gel electrophoresis. RNA was analyzed on 3.4% polyacrylamide gels (10 by 0.5 cm) containing 98% formamide, as described by Haines et al. (19) but buffered with 10 mM sodium phosphate, pH 6 (57). Electrophoresis was carried out at room temperature and at 300 V until the tracking dye (bromophenol blue) had reached the bottom of the gel. The gel was then frozen, and 2-mm slices were cut, incubated overnight at 37 C in 0.5 ml of 0.3 N ammonium hydroxyde, and counted in a scintillation mixture (25% Triton X-114, 75% xylene, 0.4% Omnifluor).

Other methods. (i) Acid-precipitated (5% trichloroacetic acid, final concentration) aliquots were collected on Whatman GF/C glass-fiber filters and washed as previously described (27). The dried filters were counted in toluene containing 0.4% Omnifluor (NEN Chemicals; toluene scintillant). Filters containing heavy precipitates were treated with 0.3 ml of Soluene 350 (Packard) and counted in 5 ml of toluene scintillant.

(ii) RNase activity was estimated by incubating, for 30 min at 37 C, aliquots of the S200 fraction (0.15 ml) or of the "peak 1 + interpeak" fraction (0.5 ml, see Fig. 6) with 1 μg of intact [^{3}H]uridine 28*S* rRNA (20,000 counts/min per μg). The presence of RNase was detected by examining the RNA profile after polyacrylamide gel electrophoresis under denaturing conditions, as described elsewhere (Brison and Chambon, submitted for publication).

(iii) Formamide was deionized by stirring for 2 h with Amberlite-mixed bed resin (AG 501-X8[D], Bio-Rad; 2 g/60 ml of formamide). The resin was removed by filtering the suspension through a Whatman GF/C glass-fiber filter.

(iv) Highly polymerized calf thymus DNA was purified as previously described (27). Denatured calf thymus DNA was obtained by heating for 10 min at 100 C in a buffer containing 10 mM Tris-hydrochloride, pH 7.9, and 10 mM NaCl. After rapid cooling in ice, the DNA was stored at 4 C.

(v) DNA was estimated by the procedure of Burton (6).

(vi) Contaminating RNase activity was removed from DNase and from bovine serum albumin by

affinity chromatography on agarose-UMP as described elsewhere (Brison and Chambon, submitted for publication).

(vii) RNase A was treated for 10 min at 80 C in 2× SSC, before use.

(viii) Simian virus 40 was grown and purified as described by Swetly et al. (64).

(ix) HeLa cell ribosomes were prepared according to Penman et al. (46).

Materials. Density gradient-grade sucrose (RNase free) was purchased from Schwarz/Mann (Orangeburg, N.Y.). Cesium chloride and Triton X-114 were supplied by C. Roth (Germany). Nonidet P40 and SDS (specially pure) were purchased from BDH Chemicals (England). Sarkosyl (NL 97) was purchased from Ciba-Geigy (Switzerland). Triton X-100, dithiothreitol, RNase A (from bovine pancreas, type II A), RNase T_1 (from *Aspergillus oryzae*, grade III), and bovine serum albumin (crystallized) were supplied by Sigma Chemical Co. (St. Louis, Mo.). Paraffin oil, formamide, and proteinase K (fungal) were purchased from Merck (Germany). Highly polymerized calf thymus DNA and DNase (DNase I, RNase free, DPFF) were purchased from Worthington Biochemicals Corp. (Freehold, N.J.). Yeast RNA was purchased from P-L. Biochemicals (Milwaukee, Wis.) and reextracted with phenol before use. Sartorius nitrocellulose filters (pore size, 0.45 μm) were supplied by Sartorius-Membranfilter GMBH (Germany). [^{14}C]thymidine was purchased from C.E.A. (France), and [^{3}H]UTP (14 Ci/mmol) was purchased from Amersham (England). [α-^{32}P]CTP was synthesized in our laboratory as previously described (55). All other chemicals were obtained from Merck or as previously indicated (26). RNase H was a gift of H. Stein (Tübingen, Germany). Rifamycin AF/013 was a gift of L. Silvesti (Gruppo Lepetit, Milano, Italy). α-Amanitin and *O*-methyl-demethyl-γ-[^{3}H]amanitin were gifts of Th. Wieland and H. Faulstich (Heidelberg, Germany). Actinomycin D was a gift of Jolles (Rhône-Poulenc). Poly(U-G) was given to us by L. Philipson (Uppsala, Sweden).

RESULTS

Cell fractionation and release of Ad2 DNA from nuclei. Our first objectives were to develop procedures to isolate nuclei without the loss of Ad2 DNA from within the nuclei and to release the Ad2 DNA from the nuclei with minimal contamination by cellular DNA. Initial experiments (not shown) were performed using cells pulse-labeled at 17 h postinfection with [^{3}H]thymidine for 10 min or 1 h. When the cells were disrupted at 0 C by gentle Dounce homogenization in reticulocyte standard buffer (45), an irreproducible 10 to 30% of the acid-precipitable [^{3}H]thymidine was found in the postnuclear supernatant (1000 × *g* for 10 min). This loss, identified as Ad2 DNA on CsCl density gradients, being unacceptable, an isotonic buffer similar to that of Wall et al. (69) was tried (TITE buffer, see above). The inclusion of 0.05% Triton X-100 in this buffer allowed the preparation of nuclei containing few cytoplasmic tags after a one-step homogenization-centrifugation. Further purification of the nuclei was not possible because rehomogenization of the nuclei in TITE buffer resulted in aggregated nuclei, which could not be easily resuspended after a second centrifugation. The pH of the original isotonic buffer was lowered to 7.3 to assure good resuspension of the nuclear pellet in TEAD buffer. Using these conditions, less than 1% of a 10-min pulse of [^{3}H]thymidine was lost from the nuclei into the postnuclear supernatant (Table 1). However, when cells were labeled from 6 to 16.5 h postinfection, a condition under which all the Ad2 DNA synthesized during the infection will be labeled, about 17% of the incorporated counts were released into the postnuclear supernatant (Table 1). This material has been identified as completed Ad2 virions by purification and banding in CsCl (not shown) (11).

To release Ad2 DNA from the nuclei, the crude nuclear pellet was resuspended in TEAD at 0 C, and the concentration of ammonium sulfate was slowly raised to 200 mM (Fig. 1). The release of labeled DNA could be increased either by adding Triton X-100 (≥0.1%) to the 200 mM ammonium sulfate nuclear suspension at 0 C or by warming the suspension to 37 C for

TABLE 1. *Recovery of labeled DNA during cell fractionation*[a]

Fraction[b]	% of acid-precipitable counts[c]	
	[^{14}C]DNA (6-16.5 h)	[^{3}H]DNA (10 min at 17 h)
Whole cell		
Whole cells	100	100
Postnuclear supernatant	17 (12-20)[d]	0.9 (0.5-1.0)
Nuclei	76 (70-80)	94 (85-95)
Nuclear		
Nuclei	100	100
S200	30 (20-40)	55 (50-65)
PS200	57 (50-70)	35 (25-40)

[a] HeLa cell cultures were infected with Ad2, labeled with [^{14}C]thymidine (0.05 μCi/ml) at 6 h, and pulse-labeled with [^{3}H]thymidine at 17 h postinfection as described in the text.

[b] The fractionation procedure is described in the text. Abbreviations are shown in Fig. 1.

[c] Aliquots of the different fractions were acid precipitated and counted as described in the text.

[d] The numbers represent a typical experiment. Numbers in parentheses give the range of values obtained with many experiments.

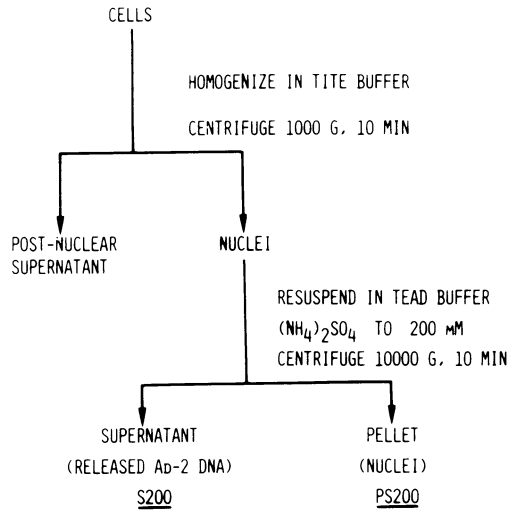

FIG. 1. *Outline for preparation of nuclei and the S200 and PS200 fractions.*

1 to 2 min before the centrifugation to separate the released material in the 200 mM ammonium sulfate supernatant (S200 fraction) from the nuclear residue (PS200 fraction) (Fig. 1). The following experiments utilized the 37 C step; similar results were obtained by Triton X-100 treatment at 0 C.

Table 1 shows that 50 to 65% of pulse-labeled (10 min at 17 h postinfection) nuclear DNA is released into the S200 fraction, as compared to only 20 to 40% of the nuclear DNA labeled between 6 and 16.5 h postinfection. The reason for this discrepancy was found after the DNA purified from different cell fractions was analyzed by CsCl density equilibrium centrifugation (Fig. 2). It is apparent that a significant amount of labeled thymidine was incorporated into whole-cell DNA banding at the density of cellular DNA (1.698 g/ml) (Fig. 2a), although no measurable amount of this cellular DNA-associated label was released into the S200 fraction (Fig. 2b).

It was striking that during long-term labeling a considerable amount of labeled thymidine was incorporated into DNA banding at the density of cellular DNA (Fig. 2a). Although Ad2 DNA synthesis begins at 6 to 8 h postinfection, cellular DNA synthesis is not maximally inhibited until 10 h postinfection in KB cells (16). Therefore, part of the apparent cellular DNA synthesis in cells labeled from 6 to 16.5 h postinfection can be ascribed to residual cellular DNA synthesis from 6 to 10 h postinfection. The incorporation of labeled thymidine into DNA banding at the cellular density (Fig. 2a) at late times after infection has also been reported and characterized by Burger and Doerfler (4) and Doerfler et al. (12). They concluded that a large proportion of this apparent cellular DNA synthesis actually represents Ad2 DNA sequences integrated into the host genome. Our preliminary results, based on the renaturation kinetics of DNA banding at the cellular DNA density, support this interpretation and will be discussed later.

The percentage of labeled, nonintegrated Ad2 DNA present in the different cell fractions is summarized in Table 2. In this experiment

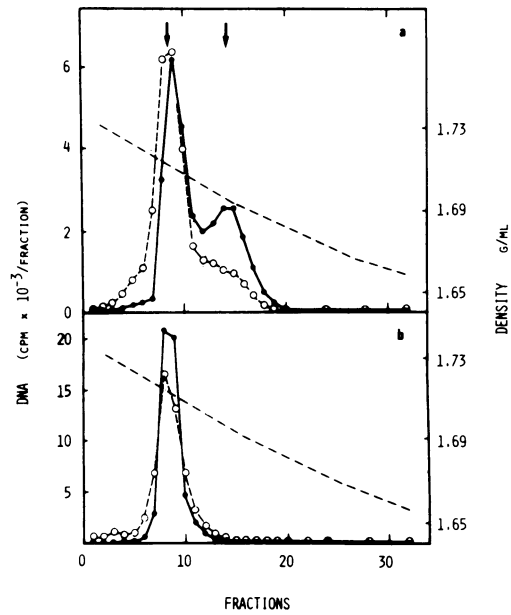

FIG. 2. *CsCl density gradient analysis of labeled DNA. The DNA of aliquots of whole cells (a) and of the S200 fraction (b) from the experiment presented in Table 1 was purified as described in the text. The purified DNA was dissolved in TNE buffer, and an aliquot containing a maximum of 15 μg of DNA was diluted to 4 ml with 50 mM Tris-hydrochloride, pH 7.9, 1 mM EDTA, and 0.1% Sarkosyl, and 5 g of solid CsCl was added. The density was adjusted to 1.704 ± 0.001 g/ml by weighing in a calibrated 100-μl micropipette, and the final volume was brought to 6.0 ml. The tubes were filled with paraffin oil and centrifuged at 35,000 rpm for 42 h at 15 C in a type 50 Spinco rotor. The gradients were collected from the bottom, aliquots were weighed, and the fractions were precipitated and counted. DNA recovery was 75 to 85%. On separate gradients (not shown) Ad2 DNA and cellular DNA markers banded at 1.716 and 1.698 g/ml, respectively, as indicated by the arrows. The labeling of Ad2 and cellular DNA was estimated by summing the counts under the peaks as shown in (a) (dotted line). Symbols: ●, DNA labeled with [^{14}C]thymidine from 6 to 16.5 h postinfection; ○, DNA pulse-labeled with [^{3}H]thymidine for 10 min at 17 h postinfection; ·-·-·, CsCl density.*

TABLE 2. *Proportion of labeled Ad2 DNA in different cell fractions determined by CsCl density gradient analysis*[a]

Fraction	% of nonintegrated Ad2 DNA	
	[^{14}C]DNA (6-16.5 h)	[^{3}H]DNA (10 min at 17 h)
Whole cells	58 (48-63)[b]	80 (70-82)
Postnuclear supernatant	100	ND
Nuclei	42	80
S200	100	100
PS200	20	43

[a] Aliquots of DNA purified from different cell fractions of the experiment presented in Table 1 were analyzed in CsCl gradients as shown in Fig. 2. The percentage of [^{14}C]thymidine (6 to 16.5 h postinfection) and [^{3}H]thymidine (10 min at 17 h postinfection) banding at the density of Ad2 DNA (1.716 g/ml) was estimated as shown in Fig. 2a.

[b] The numbers represent a typical experiment. Numbers in parentheses represent the range of five experiments. ND, Not determined.

58% of the long-term (6 to 16.5 h postinfection) labeled DNA was estimated to be nonintegrated Ad2 DNA. Since some of the Ad2 DNA is released into the postnuclear supernatant in the form of virions, as noted earlier, the nuclei contain only 42% of nonintegrated Ad2 DNA (Table 2). Therefore, since 100% of the labeled DNA released into the S200 fraction is nonintegrated Ad2 DNA (Fig. 2b) and 30% of the total long-term labeled DNA in the nucleus was released (Table 1), the percentage of nonintegrated Ad2 DNA released from the nuclei is 30/42 or 69%. A parallel calculation can be made for pulse-labeled DNA (10 min at 17 h postinfection): in this case 80% of the labeled nuclear DNA was nonintegrated Ad2 DNA. The labeled DNA released into the S200 fraction represented 55% of the total nuclear labeled DNA (Table 1) and was all nonintegrated Ad2 DNA (Fig. 2b). Therefore, 70% (55/80) of the nuclear, pulse-labeled, nonintegrated Ad2 DNA was released into the S200 fraction. It is concluded that a high and equal proportion of both long-term and pulse-labeled nonintegrated Ad2 DNA complexes has been released from the nuclei.

Estimation of contamination by cellular DNA of the DNA released from nuclei into the S200 fraction. The relatively high proportion of [^{14}C]thymidine incorporated into cellular DNA (density, 1.698 g/ml; Fig. 2a) during a long-term labeling (6 to 16.5 h postinfection) is fortunate, since it allows an estimation of the amount of cellular DNA released into the S200 fraction. For example, 53,000 counts/min of [^{14}C]thymidine was recovered at the density of Ad2 DNA in the CsCl gradient in Fig. 2b. Since nonintegrated Ad2 DNA represents only 42% of the labeled DNA in the nuclei from which this S200 fraction was made (Table 2), the corresponding amount of [^{14}C]thymidine at the density of cellular DNA was 73,000 counts/min. Just 2% of these counts released into the S200 fraction would have 1,460 counts/min at a density of 1.698 g/ml (Fig. 2b). Since no counts per minute above background were detected in this region, the contamination would seem to be less than 2% by total counts. Since nonintegrated Ad2 DNA represented about 20% of the total intranuclear DNA (on a weight basis) by 17 h postinfection (unpublished data), the maximum limit of cellular DNA contamination in the S200 fraction would be about 10%, on a weight basis.

Since the specific activity of the cellular DNA was much lower than that of nonintegrated Ad2 DNA, the total cellular DNA may not be well represented by the fraction of counts found at the cellular density (Fig. 2a). Another experiment was therefore performed in which 50 μg of fraction S200 DNA was banded in a CsCl gradient (not shown). Such a quantity of DNA overloads the gradient somewhat and broadens the peak of Ad2 DNA (density, 1.716 g/ml), so that some Ad2 DNA overlaps the region of cellular DNA (density, 1.698 g/ml). The DNA in the lighter half of the cellular DNA region was observed by electron microscopy (18), and it was apparent that, in addition to a few full-length molecules of Ad2 DNA (about 11 μm in length), there were many short pieces of DNA (from 0.5 to 7 μm in length) present. The peak of Ad2 DNA at a density of 1.716 g/ml contained only full-length Ad2 DNA, indicating that the smaller fragments observed at the cellular DNA density were of cellular DNA origin. Calculated on a weight basis, the cellular DNA contamination was estimated to be $\leq$10% of the total fraction S200 DNA.

It must be stressed that the procedure presented above for the selective release of Ad2 DNA from 17-h-postinfection HeLa cell nuclei depends in part on the fact that the nuclei are not physically broken or lysed. Excessive homogenization or other mechanical shearing increases the amount of cellular chromatin released into the S200 fraction. Higher ionic strength (either ammonium sulfate or sodium chloride) or stronger detergents, which cause even partial nuclear lysis, will prevent separation of Ad2 DNA from cellular chromatin in subsequent attempts at velocity gradient separations. Conversely, lower ionic strength de-

creases the release of Ad2 DNA complexes significantly (see Table 3, D).

Intactness of the Ad2 DNA released from nuclei into the S200 fraction. The long-term and pulse-labeled DNA released into the S200 fraction has been shown to be Ad2 DNA by its density in CsCl gradients (Fig. 2b). When aliquots of the total S200 fraction were denatured and centrifuged through alkaline sucrose gradients, the labeled DNA sedimented as shown in Fig. 3. Not more than 5% of the radioactivity of Ad2 DNA isolated from virions sedimented lighter (fractions 9 to 12) than the main peak (fraction 13 to 20). Nine percent of the S200 fraction DNA, labeled from 6 to 16.5 h and chased for 0.5 h, was found in the region lighter than the main peak, whereas 25% of a 10-min pulse-labeled DNA sedimented slower than the main peak. These results indicate that the small DNA pieces found in alkaline gradients originated from replicating Ad2 DNA molecules. Replicating Ad2 DNA is indeed known to contain many pieces shorter than single-strand genome length, which are joined to make a full-length strand (31, 44, 68). Thus, except for DNA in replicative intermediates, essentially all of the Ad2 DNA found in the S200 fraction was intact.

TABLE 3. *Effect of various extraction conditions on the RNA polymerase activity and DNA present in fraction S200*[a]

Extraction conditions	RNA polymerase activity in fraction S200 (units/10^6 nuclei)	% of total nuclear DNA released (^{14}C counts/min)	% of nonintegrated Ad2 DNA released[b]
(A) Nuclei at 3 × 10^6/ml, 2 min at 37 C, in 200 mM $(NH_4)_2SO_4$	4.3	28	67
(B) Nuclei at 9 × 10^6/ml, 2 min at 37 C, in 200 mM $(NH_4)_2SO_4$	5.5	30	72
(C) Nuclei at 9 × 10^6/ml, 15 min at 0 C, in 200 mM $(NH_4)_2SO_4$	13.5	20	48
(D) Nuclei at 9 × 10^6/ml, 15 min at 0 C, in 100 mM $(NH_4)_2SO_4$	1.5	0.5	1.2

[a] Infected HeLa cells were labeled with [^{14}C]thymidine (0.005 μCi/ml) at 6 h postinfection. At 17 h postinfection, the cells were harvested and the nuclei were prepared as described in the text. Four different extraction conditions were tested for the release of RNA polymerase activity and [^{14}C]DNA into fraction S200 (RNA polymerase activity and [^{14}C]DNA content of fraction S200 were determined as described in the text). (A) The nuclei were suspended in TEAD buffer at a concentration of 3 × 10^6 nuclei/ml, and ammonium sulfate was added up to 200 mM. The nuclear suspension was then incubated for 2 min at 37 C before centrifugation. (B) Same conditions as in (A), but the nuclei were three times more concentrated (9 × 10^6 nuclei/ml) during the extraction procedure. (C) Same conditions as in (B), but the 37 C step was omitted; instead, the nuclei were left for 15 min at 0 C before centrifugation. (D) Same conditions as in (C), but the ammonium sulfate concentration was only 100 mM.

[b] Values were calculated from the percentage of total nuclear DNA released, knowing that 42% of the labeled nuclear DNA was nonintegrated Ad2 DNA (Table 2).

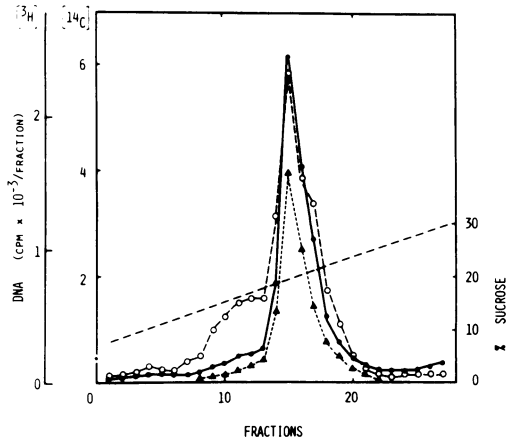

FIG. 3. *Alkaline sucrose gradient analysis of the S200 fraction labeled DNA. Infected HeLa cells were labeled as described in Table 1. The S200 fraction was prepared as described in the text. NaOH (75 μl of 2 N) was added to 0.5 ml of the S200 fraction. This aliquot was layered on a 10.5-ml 10 to 30% sucrose gradient that contained 0.1 N NaOH, 0.9 M NaCl, and 10 mM EDTA. An aliquot of purified [^{14}C]Ad2 DNA was denatured and layered on a separate gradient. The gradients were centrifuged for 18 h at 27,000 rpm in an SW41 Spinco rotor at 4 C. Fractions (0.4 ml) were collected from the top of the tube, and the acid-precipitable radioactivity was determined. Background (40 counts/min) has not been substracted. Symbols: ●, [^{14}C]DNA labeled 6 to 16.5 h postinfection; ○, [^{3}H]DNA labeled 10 min at 17 h postinfection; ▲, [^{14}C]Ad2 DNA control, purified from virions; ·-·-·, percentage of sucrose.*

Sedimentation properties of the Ad2 DNA present in the S200 fraction. Long-term and pulse-labeled Ad2 DNA present in the S200 fraction formed multiple peaks when centrifuged in velocity sedimentation gradients (Fig. 4). The long-term labeled [^{14}C]Ad2 DNA separated into two main peaks, called peak 1 and peak 2, with a disperse region between them, henceforth called the interpeak (see Fig. 4). Under these centrifugation conditions, intact virions were found in the pellet. The Ad2 DNA in peak 1 sedimented somewhat faster than purified Ad2 DNA and has an apparent *S* value of 40 to 50. Treatment of peak 1, peak 2, or the

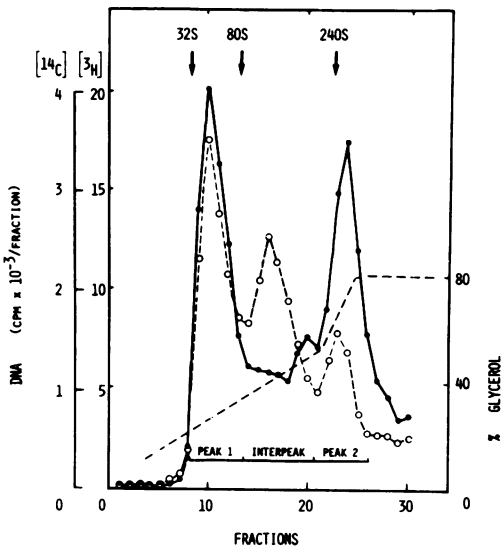

FIG. 4. *Velocity sedimentation of Ad2 DNA complexes present in the S200 fraction. Infected cells were labeled as described in Table 1. The S200 extract was prepared including the 1-min, 37 C heat step, as described in the text. A 2.4-ml portion containing about 12 μg of DNA was layered on a 7.5-ml 10 to 40% glycerol gradient with a 7-ml 80% glycerol cushion [glycerol solutions contained 200 mM $(NH_4)_2SO_4$, 2 mM EDTA, and 10 mM Tris-hydrochloride, pH 7.9). The gradient was centrifuged in a Spinco SW27.1 rotor at 26,000 rpm for 5 h at 2 C. Fractions of 0.4 ml were collected by pumping from the top of the tube. The glycerol concentration was determined with a refractometer and trichloroacetic acid-precipitable radioactivity was counted. The bars indicate the regions of the gradient referred to as peak 1, the interpeak, and peak 2. The arrows denote the position of sedimentation markers as follows: purified native Ad2 DNA (32S); HeLa cell ribosomes (80S); and simian virus 40 virions (240S). Symbols: ●, DNA labeled with [^{14}C]thymidine from 6 to 16.5 h postinfection; ○, DNA labeled with [^{3}H]thymidine for 10 min at 17 h postinfection; ·–·–·, percentage of glycerol.*

total S200 fraction with detergent (Sarkosyl or SDS) and proteinase K reduced the sedimentation of the labeled DNA to that of purified Ad2 DNA, indicating that the Ad2 DNA present in the multiple peaks (Fig. 4) was associated with protein.

About 70% of the DNA labeled during a 2-min pulse with [^{3}H]thymidine was found in the interpeak region (not shown). The 10-min pulse-labeled Ad2 DNA complexes in the S200 fraction sedimented in two major peaks: one coincident with peak 1 and the other in the interpeak region (Fig. 4). All of these findings suggest that replicative intermediate forms of Ad2 DNA sediment in the interpeak region, that newly replicated molecules are found in peak 1, and that these peak 1 molecules later become the faster sedimenting forms found in peak 2. Neither the timing nor the process of these transformations is known at present.

Preliminary electron microscopic evidence supports some of the results presented above concerning the nature of the Ad2 DNA complexes in the S200 fraction (P. Oudet et al., manuscript in preparation). The peak 1 region (Fig. 4) was observed to contain DNA molecules with the length of the Ad2 genome. Peak 2 consists of some partially completed virions plus a large amount of linear structures shorter and much thicker than Ad2 DNA but containing full-length Ad2 DNA after deproteinization. The interpeak region contains a mixture of almost naked Ad2 DNA molecules, plus some thicker and shorter molecules like those seen in peak 2 and other structures not yet systematically investigated.

The Ad2 DNA complexes that sedimented in the interpeak and peak 2 regions (Fig. 4) could be stored at −20 C and then observed to resediment to their respective relative positions on similar velocity gradients with minimal (less than 10%) "degradation" to peak 1 size (unpublished data). This result indicates that the different Ad2 DNA-protein complexes are quite stable after they have been isolated on velocity gradients.

RNA polymerase activity is associated with fraction S200. Having established that a heterogeneous population of Ad2 DNA-protein complexes was present in the S200 fraction, we looked for RNA polymerase activity associated with these complexes. Assays were performed at 200 mM ammonium sulfate, where only RNA synthesis catalyzed by RNA polymerase molecules engaged in transcription is measured (see below). RNA polymerase activity was indeed detected in the S200 fraction under these assay conditions (Table 3, A), but the recovery of activity could be increased by introducing two modifications in the original preparation of fraction S200. First, the nuclei were resuspended at a concentration three times higher than in the original method (refer to Materials and Methods for details and see Table 3, B). Second, the 37 C step was omitted and the nuclei were left at 0 C for 15 min before centrifugation (Table 3, C). Under these conditions the recovery of RNA polymerase activity was increased three times, but the release of labeled Ad2 DNA was about 30% lower. This diminished release was, however, not selective for any certain fraction of Ad2 DNA, as best as could be judged from gradient profiles.

Using this modified extraction procedure, we

determined the ionic incubation conditions that gave maximal RNA synthesis for the S200 fraction. The optimal ammonium sulfate concentration was 200 mM. At lower ionic strengths (50 or 100 mM) the incorporation was markedly reduced, but ammonium sulfate concentration up to 400 mM did not inhibit the RNA polymerase activity (Table 4). The optimal Mn^{2+} concentration was 2 mM; the optimal Mg^{2+} concentration was 6 to 10 mM and gave roughly the same activity when used in place of Mn^{2+} (not shown). Under the optimal conditions, the time course for RNA synthesis was linear during the first 15 min and increased for at least 60 min (Table 4).

RNA synthesis was completely abolished in the presence of actinomycin D or EDTA and was dependent on the presence of the four nucleoside triphosphates (Table 4). On the other hand, the RNA synthesis was resistant to addition of either polyriboinosinic acid, Sarkosyl, or rifamycin AF/013, substances known to inhibit RNA chain initiation by RNA polymerase (13, 17, 37). These observations indicate that under our incubation conditions (i.e., in the presence of 200 mM ammonium sulfate) we are essentially measuring RNA synthesis catalyzed by RNA polymerase molecules engaged in ternary transcriptional complexes ("engaged" RNA polymerase). This conclusion was further supported by the observation that the RNA polymerase activity did not increase significantly when native calf thymus DNA was added to the incubation mixture (Table 4).

TABLE 4. *Properties of the RNA polymerase activity present in the S200 fraction*[a]

Assay conditions	pmol of [^{3}H]UMP incorporated/ 10^6 nuclei
Standard	12.8
Standard, but 50 mM AS	3.3
Standard, but 100 mM AS	6.7
Standard, but 400 mM AS	12.6
Standard, but 30 min	25.8
Standard, but 60 min	31.6
Standard + Sarkosyl (1%)	11.8
Standard + polyriboinosinic acid (80 μg/ml)	12.6
Standard + rifamycin AF/013 (280 μg/ml)	12.1
Standard, but omit CTP	0.1
Standard + EDTA (20 mM)	0
Standard + actinomycin D (50 μg/ml)	0
Standard + α-amanitin (10 μg/ml)	0.3
Standard + native CT DNA (250 μg/ml)	14.0
Standard, but 100 mM AS and denatured CT DNA (250 μg/ml)	35.0
+ α-amanitin (10 μg/ml)	12.2
+ α-amanitin (500 μg/ml)	4.1

[a] Fraction S200 was prepared using condition (C) described in Table 3. The standard conditions for RNA polymerase assay were as described in the text. Data from triplicate assays (which agreed within 10%) were averaged after substracting zero time blanks. AS, Ammonium sulfate; CT, calf thymus.

About 95% of the S200 fraction RNA polymerase activity was inhibited by 0.1 μg of α-amanitin per ml, a concentration known to block class B RNA polymerase activity completely (Fig. 5) (28). The residual activity was progressively inhibited at higher amanitin concentration (see inset, Fig. 5), indicating the presence of class C RNA polymerase activity (24, 71). No significant amount of class A polymerase was detectable in fraction S200 under conditions where only the activity of engaged polymerases was measured.

However, when fraction S200 was incubated at lower ionic strength (100 mM ammonium sulfate) and in the presence of an excess of denatured calf thymus DNA, on which free polymerase molecules can initiate (17), about 35 and 12% of the activity was resistant to 10 and 500 μg of amanitin per ml, respectively (Table 4). This result indicated that free RNA polymerase molecules belonging to classes A, B, and C were present in the S200 extract. Since the incubation conditions were not optimized for each polymerase class, these values do not necessarily reflect the relative proportion in frac-

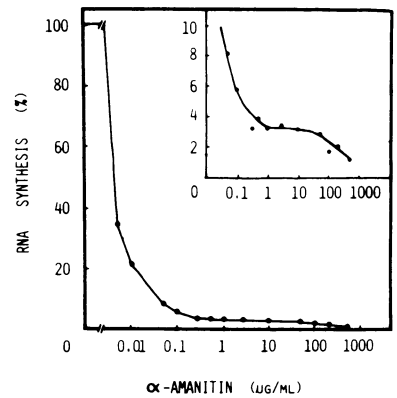

FIG. 5. *Effect of α-amanitin on the RNA polymerase activity present in fraction S200. Fraction S200 was prepared under condition (C) described in Table 3. RNA polymerase assays were run for 30 min at 37 C. [^{3}H]UTP specific activity was 600 counts/min per pmol. α-Amanitin was added at 0 C before the enzyme, as indicated in the figure. Maximal activity (100%) represents 29 pmol incorporated. The inset represents, with different scales, the values corresponding to the higher α-amanitin concentrations used in the same experiment.*

tion S200 of free enzyme molecules of the different classes.

Comparison of engaged RNA polymerase activity in nuclei and fraction S200. To compare the engaged RNA polymerase activity present in nuclei and in the S200 and PS200 fractions, we have first optimized the incubation conditions for each fraction: the optimal ammonium sulfate and Mn^{2+} concentrations were the same as for fraction S200 and the time course of RNA synthesis was identical for all three fractions (data not shown). However, it appeared (Table 5) that only about 20% of the initial nuclear polymerase activity was recovered by summing up the activities found in the S200 and PS200 fractions.

One way to explain the loss of activity after the treatment of nuclei with 200 mM ammonium sulfate would be the release of engaged polymerase molecules from the DNA template. The recovery of class B polymerase molecules was therefore estimated by amanitin binding during the extraction procedure (Table 5). Since class B polymerase molecules are responsible for 95% of the activity (Fig. 5, Table 5), the RNA polymerase activity and the number of class B RNA polymerase molecules determined by labeled-amanitin binding measurements are directly comparable. Assuming that the class B molecules that remain bound to the DNA in 200 mM ammonium sulfate are engaged in transcriptional complexes, as suggested by Hossenlopp et al. (23), DNA-bound engaged molecules were isolated by differential centrifugation through a sucrose gradient containing 200 mM ammonium sulfate. The "nonengaged" (free) molecules remained in the upper part of the gradient, whereas the bound engaged molecules sedimented with the DNA. Amanitin binding experiments shown in Table 5 indicate that, in 200 mM ammonium sulfate-treated nuclei, 56% of the total B polymerase molecules was bound to the DNA. In terms of amanitin binding, 55% of these bound molecules was found in the PS200 fraction, whereas 45% was recovered in a DNA-complexed form in the S200 fraction. On the other hand only 12% of the nuclear polymerase activity was recovered in the latter fraction. There was therefore a loss of activity without concomitant loss of engaged polymerase molecules during the preparation of fraction S200.

To try to explain this loss of activity, the following experiments were performed (not shown). (i) The PS200 fraction was resuspended in the corresponding S200 fraction, and the polymerase activity of the mixture was determined. It still equaled 20% of the initial nuclear activity, indicating that no selective distribution of stimulating factor(s) occurred during the separation of the S200 and PS200 fractions. (ii) The salt concentration of a nuclear suspension in TEAD buffer was first raised to 200 mM ammonium sulfate, and, after 5 min at 0 C, aliquots of the nuclear suspension were assayed under the standard conditions. After this treatment only 20 to 30% of the polymerase activity was recovered in these nuclei as compared to the activity of nuclei kept in TEAD buffer at 0 C. There-

TABLE 5. *Engaged RNA polymerase activity and number of class B polymerase molecules in nuclear, S200, and PS200 fractions*[a]

Fraction	α-Amanitin	RNA polymerase activity (units/10^6 nuclei)[b]	No. of RNA polymerase B molecules/nucleus[c]		
			Total	Free	Bound
Nuclei	−	95 (60-130)	100,000	44,000	56,000
	+	3			
S200	−	12 (4-20)	66,000	42,000	24,000
	+	0.3			
PS200	−	7 (2-8)	34,000		

[a] Infection was as described in Table 3. RNA polymerase activities of nuclear (suspended in TEAD buffer), S200, and PS200 fractions (resuspended in TEAD buffer) were assayed under standard conditions. Where indicated, α-amanitin was added to the reaction mixture at a final concentration of 10 μg/ml. The number of RNA polymerase class B molecules was calculated from [^{3}H]amanitin binding measurements carried out on aliquots of the different fractions. Nonengaged (free) and engaged (bound) nuclear RNA polymerase molecules were separated by differential centrifugation in 200 mM ammonium sulfate. For the S200 fraction, the separation of free and DNA-bound RNA polymerase molecules was achieved as described in Fig. 7b, and the values given in this table were calculated from the labeled amanitin binding measurements performed in that experiment. The PS200 pellet was simply resuspended in TEAD buffer and aliquots were taken for amanitin binding measurements.

[b] The numbers represent a typical experiment. Numbers in parentheses correspond to extreme values obtained in some experiments.

[c] Averages of three independent experiments, which agreed within 10%.

fore, the decrease in engaged RNA polymerase activity that occurs during the preparation of fraction S200 is related to the salt treatment step. It is worth mentioning that this loss of engaged RNA polymerase activity was not specific to adenovirus-infected cells, since a very similar loss was observed when nuclei from uninfected cells were treated in the same way, although no engaged RNA polymerase activity was found in the S200 fraction of uninfected cells.

Fractionation of the RNA polymerase activity present in fraction S200. Figure 6 compares the sedimentation profiles of [^{14}C]Ad2 DNA and of the engaged RNA polymerase activity of fraction S200 through a linear sucrose gradient containing 200 mM ammonium sulfate. The profiles of labeled Ad2 DNA (6 to 16.5 h postinfection) were similar whether glycerol or sucrose gradients were used (not shown). Sucrose gradients were preferred because sucrose, at high concentration in the incubation mixture, was found to be less inhibitory for the RNA polymerase activity than glycerol. Figure 6 shows that the polymerase activity was mainly associated with the heavy side of peak 1 and with the interpeak region. The highest specific activity (expressed on a DNA basis) sedimented slightly faster than peak 1. Very little activity was detected in peak 2. The RNA polymerase activity found in peak 1 and in the interpeak region corresponded very likely to the engaged enzyme molecules that have initiated RNA synthesis in vivo, because there is almost no RNA chain initiation under our high-salt incubation conditions (see above). In addition, the free RNA polymerase molecules were found on the top of the gradient under these centrifugation conditions (see below). To further support the conclusion that the transcriptional complexes present in peak 1 and the interpeak regions were actually formed in vivo and not artefactually generated during the extraction procedure from free RNA polymerase molecules and Ad2 DNA, the following experiment was performed (not shown). Purified [^{14}C]Ad2 DNA was added to uninfected HeLa cell nuclei suspended in TEAD buffer at a concentration (50 μg/ml) about fivefold higher than the Ad2 DNA concentration in a standard S200 extract. Ammonium sulfate was then added up to 200 mM, and the S200 fraction was prepared as usual. Although all the added Ad2 DNA was recovered, almost no RNA polymerase activity was detected in our standard incubation conditions in this "mock" S200 fraction (about 5% of the activity present in an S200 extract prepared from the same amount of infected nuclei). When this "mock" S200 fraction was centrifuged through a sucrose gradient, no significant RNA polymerase activity (less than 1% of the activity recovered in the gradient shown in Fig. 6) was detected in any region, and the added DNA sedimented as purified Ad2 DNA (32S). It is therefore very unlikely that the transcriptional complexes released in the S200 fraction and found in peak 1 and the interpeak regions could have been formed in vitro during the nuclei treatment.

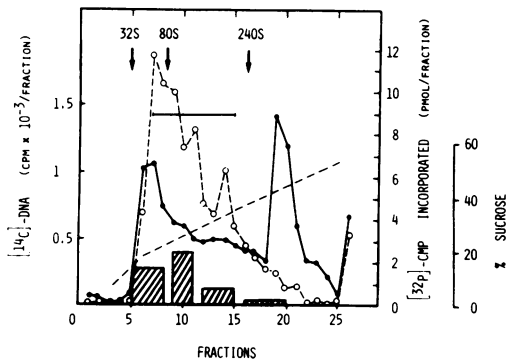

FIG. 6. *Sucrose gradient sedimentation analysis of the engaged RNA polymerase activity present in fraction S200. Infected HeLa cells were labeled as indicated in the legend to Table 3, and fraction S200 was prepared under condition (C) of Table 3. A 1.4-ml portion of the extract was sedimented through a 10.5-ml sucrose gradient (·-·-·-·) (10 to 58% sucrose, 20 mM Tris-hydrochloride, pH 7.9, 200 mM ammonium sulfate, 2 mM EDTA) for 3 h at 41,000 rpm (4 C) in an SW41 Spinco rotor. Fractions of 0.45 ml were collected by pumping from the top of the tube, and aliquots were analyzed for acid-precipitable [^{14}C]DNA (●) and for RNA polymerase activity by incorporation of [α-^{32}P]CMP (650 counts/min per pmol) into acid-precipitable RNA (○). The incubation conditions were as described in the text, but the sucrose concentration was brought to 22% and the incubation time was 30 min. In this particular experiment, 90% of the RNA polymerase activity was recovered in the gradient after centrifugation. The lined boxes represent, at a 10-fold expanded scale, the RNA synthesis, which was resistant to α-amanitin, when aliquots of pooled fractions 5 to 8, 9 to 11, 12 to 15, and 16 to 20 (see figure) were incubated in the presence of 10 μg of amanitin per ml. The bar indicates the fractions (7 to 15) of the gradient (peak 1 + interpeak) that were pooled for further RNA synthesis (see Table 6). The arrows indicate the position of purified native Ad2 DNA (32S), HeLa cell ribosomes (80S), and simian virus 40 virions (240S), which were used as sedimentation markers in parallel gradients.*

In fact, it was possible to completely separate the free RNA polymerase molecules from those engaged in viral transcriptional complexes by sedimenting the S200 fraction prepared from infected cells through a shallow sucrose gra-

dient overlaid on a dense sucrose cushion. Under these centrifugation conditions, part of the interpeak material was accumulated with the peak 2 region on the sucrose cushion, but the material remaining on the top of the gradient was well resolved from peak 1 (Fig. 7). When each fraction of such a gradient was incubated at 100 mM ammonium sulfate in the presence of denatured calf thymus DNA, on which free polymerase could initiate (17), a large peak of polymerase activity was found on the top of the gradient (Fig. 7a). In agreement with the results presented in Fig. 6, no activity was found in the upper part of the gradient in the absence of added DNA (not shown). A very similar profile was obtained when RNA polymerase class B molecules were localized in the gradient by [^{3}H]amanitin binding assays (Fig. 7b).

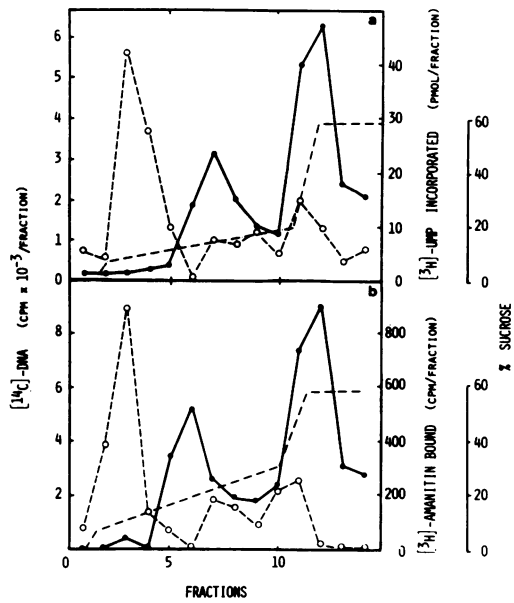

FIG. 7. *Distribution of free and DNA-bound RNA polymerase molecules in fraction S200. Fraction S200 was prepared as described in the legend to Fig. 6. (a) A 1.4-ml portion of fraction S200 was sedimented through a sucrose gradient (·–·–·) (8 ml of 6 to 20% sucrose in 200 mM ammonium sulfate, 20 mM Tris-hydrochloride, pH 7.9, 2 mM EDTA, underlaid with 2.5 ml of 58% sucrose in the same buffer) for 4 h at 41,000 rpm (4 C) in an SW41 Spinco rotor. Fractions were collected and analyzed for [^{14}C]DNA (●) and for RNA polymerase activity by incorporation of [^{3}H]UMP (100 counts/min per pmol) into acid-precipitable RNA (○), as described in the legend to Fig. 6, but 250 μg of denatured calf thymus DNA per ml and 100 mM ammonium sulfate were present in the incubation mixture. (b) In a different experiment 1.4 ml of fraction S200 was sedimented through a sucrose gradient (·–·–·) (8 ml of 10 to 30% sucrose underlaid with 2.5 ml of 58% sucrose, in the same buffer as above) for 3.5 h at 41,000 rpm (4 C) in an SW41 Spinco rotor. The [^{14}C]DNA profile (●) was determined as in (a) and the [^{3}H]amanitin binding (○) was measured as described in the text.*

RNA synthesized in vitro by the transcriptional complexes released in fraction S200 is transcribed mainly from the *l* strand of Ad2 DNA. The RNA synthesized in vitro was first characterized by exhaustive hybridization with filter-bound Ad2 DNA (Table 6). The results show that 88% of the RNA synthesized by the S200 fraction was complementary to Ad2 DNA. Furthermore, self-hybridization experiments performed with the RNAs synthesized by fraction S200 and by "peak 1 plus interpeak" fractions resulted in 8% RNase resistance (Table 6). These values correspond to maximum values, since identical results were obtained when self-hybridization was carried out for longer time periods and at higher RNA concentrations. The in vitro transcription was thus mainly asymmetrical. The RNA resistant to RNases A and T_1 after self-hybridization was also resistant to RNase H (Table 6), which degrades specifically the RNA moiety of an RNA-DNA hybrid (21), indicating that the RNA preparations were free of contaminating DNA.

To further study asymmetry of transcription, liquid DNA-RNA hybridization experiments have been performed with the separated strands (*h* and *l*) of Ad2 DNA. The results (Table 6) show that almost all of the RNA synthesized in vitro by the nuclei and by the S200 fraction was complementary to the *l* strand of Ad2 DNA. On the other hand, it appears that there was very little complementarity to the *h* strand. An important control was to treat the samples first with RNase H and then with RNases A and T_1 (see Materials and Methods). The results demonstrate clearly (Table 6) that the RNA was in true DNA-RNA hybrids with the *l* strand of Ad2 DNA but that the proportion of RNA hybridized to the *h* strand (calculated as the difference between RNase A and T_1 resistance before and after RNase H treatment) did not exceed 1%. It is interesting that, although the RNA synthesized by fraction S200 represented quantitatively only about 15% of the RNA synthesized under the same incubation conditions by the corresponding nuclei (Table 5), both RNAs exhibited the same *l* strand specificity (Table 6).

A possible explanation for the asymmetry of the RNA is that some processing nuclease, present in the nuclei and in the S200 fraction, could specifically degrade the RNA molecules transcribed from the *h* strand. Although not excluded, such a possibility is not likely since

TABLE 6. *Hybridization properties of RNA synthesized in vitro by nuclei, S200 fraction, and peak 1 plus interpeak fractions*[a]

Source of RNA	% of input RNA resistant to RNases			
	Self-hybridization[b]	Hybridization to total Ad2 DNA	Hybridization to separated strands	
			h	*l*
Nuclei	7	88	3	84
	(7)[c]	(ND)	(3)	(4)
S200	8	88	4	92
	(ND)	(ND)	(3)	(3)
Peak 1 + interpeak	8	ND	6	98
	(8)		(4)	(2)
cRNA[d]	23	94	38	88
	(23)	(ND)	(15)	(19)
cRNA[e]	50	ND	40	43
	(ND)		(4)	(2)

[a] [^{3}H]uridine-labeled RNA (2.3×10^6 counts/min per μg) was synthesized by incubating nuclei or the S200 fraction under standard conditions for 30 min at 37 C. [^{3}H]UTP specific acitivity was 2,800 counts/min per pmol. [^{32}P]CMP-labeled RNA (1.6×10^7 counts/min per μg) was synthesized from the peak 1 + interpeak fraction as follows. Fractions from a sucrose gradient were pooled as indicated in Fig. 6, and an aliquot was incubated under standard conditions for 30 min at 37 C. [^{32}P]CTP specific activity was 36,000 counts/min per pmol. Labeled RNAs were purified as described in the text. Hybridizations to total Ad2 DNA were carried out with filter-bound DNA, and input RNA was about 1,000 counts/min per incubation. Hybridization to separated strands and self-hybridizations were performed as described in the text (input RNA was equivalent to 0.3 ng of labeled RNA per incubation).

[b] Zero time values for self-hybridization were not substracted and correspond to about 5% of the input RNA.

[c] Numbers in parentheses represent the percentage of input RNA resistant to RNases A and T_1 in 2× SSC after treatment with RNase H (see text). ND, Not determined.

[d] Hybridization experiments with ^{3}H-labeled complementary RNA (cRNA) (1.8×10^6 counts/min per μg) synthesized with *E. coli* RNA polymerase (25) on native Ad2 DNA under the conditions described by Pettersson et al. (49) were carried out to control the efficiency of hybridization. Input RNA was about 1,700 counts/min per incubation for hybridization to filter-bound Ad2 DNA and was equivalent to 0.3 ng of labeled RNA per incubation for hybridization to separated strands.

[e] Hybridization experiments with [^{3}H]cRNA (2×10^6 counts/min per μg) synthesized with *E. coli* RNA polymerase on denatured Ad2 DNA under the conditions used in footnote *d* were carried out to control the efficiency of hybridization. Self-hybridization was carried out at an RNA concentration of 200 ng/ml for 48 h under the conditions described in the text. Hybridizations with separated strands were carried out as described in the text.

the same strand specificity was exhibited by the RNA synthesized by the peak 1 plus interpeak fractions (Table 6), from which such a nuclease might have been removed during the centrifugation through the sucrose gradient. Indeed, the percentage of in vitro synthesized RNA hybridized with the *h* strand did not exceed 2%, as judged from the effect of RNase H (Table 6).

Length of the synthesized RNA molecules. It is difficult to analyze the lengths of RNA molecules if the fractions used for RNA synthesis are contaminated by RNase. Therefore, the size of the RNA synthesized only by the peak 1 plus interpeak fractions (Fig. 6) was analyzed, since these fractions were free of RNase as judged from the very sensitive assay described above, whereas fraction S200 was contaminated by a minute amount of RNase (about 70% of the 28*S* RNA remained intact after incubation with an aliquot of the S200 fraction when analyzed under the conditions described above). The size of the synthesized RNA was analyzed on sucrose gradients under denaturing conditions (Fig. 8). After 30 min of synthesis the RNA was extremely heterogeneous, with sizes ranging from about 6*S* to more than 28*S*. It is also apparent from Fig. 8 that chain elongation occurred between 3 and 30 min, since the entire profile was displaced toward higher *S* values during that period of time.

Characterization of the class C RNA polymerase activity present in the S200 fraction. As already pointed out, fraction S200 contains a low, but definite, level of RNA polymerase activity belonging to class C. This activity did not seem to be confined to a particular Ad2 DNA nucleoprotein complex, since its distribution paralleled roughly the engaged RNA polymerase class B peak when sedimented through a sucrose density gradient (Fig. 6). DNA-RNA hybridization indicates that about 30% of the sequences synthesized under the standard conditions, but in the presence of 1, 10, or 100 μg of a α-amanitin per ml, hybridized with Ad2 DNA (Table 7). Since the hybridization conditions were not exhaustive in these experiments (only 49% of the complementary RNA synthesized with *Escherichia coli* polymerase has hybridized to Ad2 DNA under these hybridization conditions; cf. Table 6), more than 30% of the RNA synthesized in the presence of amanitin was transcribed from the adenovirus genome. It is therefore very likely that a large part of the class C activity measured under the standard conditions corresponds to RNA polymerase molecules, which were engaged in vivo in Ad2 DNA transcription, since Hossenlopp et al. (24) have previously reported that enzymes belonging to class C could not initiate RNA synthesis

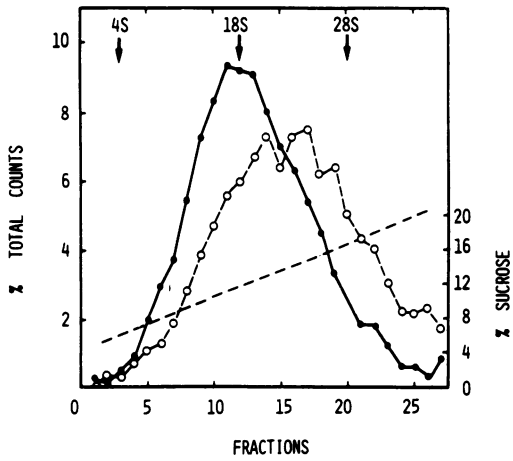

FIG. 8. *Size of the RNA synthesized in vitro by the peak 1 plus interpeak fractions. RNA was synthesized by incubating the pooled fractions corresponding to the peak 1 + interpeak regions (see Fig. 6) in the standard incubation medium in the presence of [α-^{32}P]CTP (800 counts/min per pmol). After a 3-min (●) or 30-min (○) incubation period, RNA was purified as described in the text. After ethanol precipitation, the RNA was dissolved by heating for 5 min at 80 C in a buffer containing 25 mM sodium phosphate, pH 7.7, 1 mM EDTA, and 1.1 M formaldehyde. Centrifugation through formaldehyde-sucrose gradients (1.1 M formaldehyde, 5.5 to 21% sucrose, 0.1 M sodium phosphate, pH 7.7, and 1 mM EDTA) were run at 28,000 rpm for 14 h at 20 C in an SW41 Spinco rotor, as described by Mandel and Chambon (35). Fractions were collected from the top of the tubes, and acid-precipitable radioactivity was collected on GF/C glass filters and counted. The arrows indicate the position of [^{3}H]uridine-labeled 4S, 18S, and 28S cellular RNAs, which were used as markers in the same gradient.*

TABLE 7. *Hybridization properties of RNA synthesized by the S200 fraction in the presence of α-amanitin*[a]

α-Amanitin concn (μg/ml)	Total amount of RNA synthesized (counts/min)	Input RNA (counts/min)	Hybridization to total Ad2 DNA (% of input)
1	37,000	2,150	32
10	28,500	1,500	31
100	20,000	1,900	33

[a] [^{32}P]CMP-labeled RNA (3 × 10^{7} counts/min per μg) was synthesized by incubating the S200 fraction under standard conditions for 30 min at 37 C, as described in the text, but in the presence of the indicated α-amanitin concentration. [^{32}P]CTP specific activity was 50,000 counts/min per pmol. Hybridizations were carried out with filter-bound Ad2 DNA as described in the text, but only two filters were added consecutively.

on intact Ad2 DNA at an ionic strength higher than 100 mM ammonium sulfate.

Since, in vivo, a class C RNA polymerase activity is responsible for the synthesis of the 5.5*S* VA RNA (53, 71), we have analyzed by polyacrylamide gel electrophoresis the RNA molecules synthesized in vitro in the presence of 10 μg of α-amanitin per ml (Fig. 9). The size of these RNA species ranges from 4*S* to more than 18*S* but with no major peak coincident with an authentic 5.5*S* VA RNA marker. The same RNA profile was obtained when RNA was synthesized in the presence of 1 or 100 μg of α-amanitin per ml (not shown). It should, however, be kept in mind that fraction S200, which was used for RNA synthesis in these experiments, contains traces of RNase contamination (see above).

DISCUSSION

Viral transcription complexes are present in fraction S200. The method described in this paper allows the release of viral transcriptional complexes from Ad2-infected HeLa cell nuclei (17 h postinfection). Under incubation conditions where only engaged RNA polymerase molecules are active (that is, in the absence of

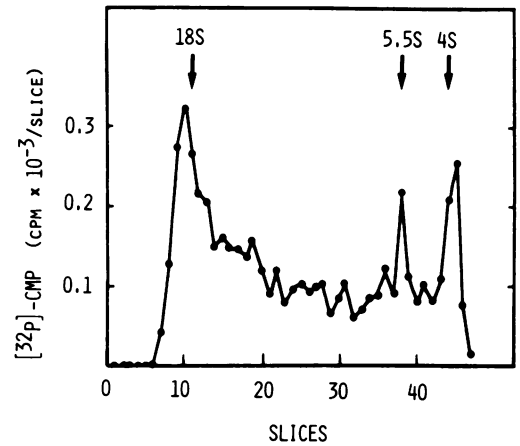

FIG. 9. *Size of the RNA synthesized in vitro by fraction S200 in the presence of 10 μg of α-amanitin per ml. RNA was synthesized by incubating fraction S200 in the standard incubation medium for 30 min at 37 C but in the presence of 10 μg of α-amanitin per ml; the labeled nucleotide was [α-^{32}P]CTP (50,000 counts/min per pmol). The RNA was then purified as described in the text. After ethanol precipitation, the RNA was dissolved by heating for 5 min at 37 C in 99% formamide containing 10 mM sodium phosphate, pH 6. An aliquot was loaded, in a final volume of 130 μl, on a 3.4% polyacrylamide gel containing 98% formamide. The arrows indicate the position of [^{3}H]uridine-labeled marker RNAs (18S rRNA, 4S cytoplasmic RNA, and 5.5S VA RNA) run on the same gel.*

exogenous DNA and at high ionic strength), only Ad2-specific RNA is synthesized in this extract (Table 6), as required for true viral transcriptional complexes. Control experiments indicate that these complexes are not generated by the extraction procedure. When the extract is sedimented through a high-salt sucrose gradient, under conditions where free RNA polymerase molecules remain on the top of the gradient, RNA polymerase activity is found in the gradient, sedimenting heterogeneously from 40 to about 200S (Fig. 6). A transcriptional complex, with a sedimentation coefficient of about 73S, has been isolated by Wallace and Kates (70), but no further comparison can be made with our present results since neither the RNA polymerase activity nor the product of the in vitro synthesis was characterized.

Under our extraction conditions, which were optimized for RNA polymerase activity recovery, 20% of the long-term labeled Ad2 DNA (6 to 16.5 h postinfection) is released (Table 3). Since only 42% of such a long-term labeled DNA is nonintegrated Ad2 DNA in the nuclei (Table 2), this 20% release actually corresponds to about 50% of the nonintegrated intranuclear Ad2 DNA molecules (Table 3). Furthermore, as already discussed in Results, the results presented in Table 5 indicate that about 45% of the total nuclear viral transcriptional complexes is released into the S200 fraction by the 200 mM ammonium sulfate treatment. Thus, Ad2 DNA molecules and Ad2-specific transcriptional activity are released in similar proportions, suggesting that the extraction procedure does not select for certain classes of Ad2 DNA complexes involved in RNA transcription. This conclusion is further substantiated by the finding that the same specificity of transcription (i.e., little or no RNA transcribed form the *h* strand) is exhibited by the nuclei and by the S200 and the peak 1 plus interpeak fractions (Table 6).

If all the released transcriptional complexes remained equally active in fraction S200, one would expect 45% of the nuclear polymerase activity to be present in fraction S200. In fact, only 12% of the total nuclear polymerase activity is recovered in fraction S200. A possible explanation for this decrease in polymerase activity would be that about 70% of the released engaged polymerase molecules are "frozen" but remain bound to the DNA, and the other 30% of the molecules are active, with the same elongation rate as in the intact nuclei. An alternative possibility to explain this loss of activity during the extraction would be a general slowdown by a factor of about 3.5 of the elongation rate of essentially all the "engaged" RNA polymerase molecules. An apparent elongation rate can be estimated from the RNA polymerase activity as measured under our high-salt conditions, where no RNA chain initiations occur (Tables 4 and 5), and from the number of RNA polymerase molecules that remain associated to the DNA under the same ionic conditions (Table 5). Assuming that all these DNA-bound molecules are equally active for RNA synthesis, one calculates an elongation rate of about seven nucleotides incorporated per second and per polymerase molecule in the nuclei and a rate of only about two in the S200 extract. The validity of such a determination of the rate of chain elongation in nuclei is supported by the results of Udvardy and Seifart (67) who, using a different technique, also found a rate of seven nucleotides per second in HeLa cell nuclei incubated in vitro. The apparent rate of elongation in peak 1 plus interpeak fractions (Fig. 6) is also about two nucleotides per second, since there is a recovery of 90% of the polymerase activity and the RNA polymerase molecules during the fractionation of the S200 fraction (Fig. 6). Using another approach, an elongation rate of about two nucleotides per second in the peak 1 plus interpeak fraction can be estimated by the shift of the mean lengths of the synthesized RNA, from 18S (about 2,000 nucleotides) to about 23S (about 3,500 nucleotides), between 3 and 30 min of incubation (Fig. 8; for this calculation one has to take into account that the amount of RNA synthesized between 10 and 30 min is only doubled). These results strongly support the hypothesis of a general slackening of the elongation rate of all engaged polymerase molecules in fraction S200. Indeed, the hypothesis in which 70% of the polymerase molecules might remain frozen on the DNA, but where the remaining 30% would transcribe at an elongation rate of seven nucleotides per second (the same as inside the nuclei), would anticipate an average shift of the RNA peak (Fig. 8) of at least 8,000 nucleotides, which is obviously not the case. One explanation for this drastic drop in the elongation rate in the S200 extract could be the alteration of a critical organization in the transcriptional machinery and/or the release of elongation factors from the DNA-bound polymerases during the salt treatment.

RNA synthesized by class B polymerase in nuclei, S200 fraction, and the partially purified transcriptional complexes is transcribed from the *l* strand of the adenovirus genome. More than 95% of the RNA polymerase activity detected under our incubation conditions in both nuclei and the S200 fraction belongs to class B (Tables 4 and 5). This finding is similar to those of Wallace and Kates (70) and Price

and Penman (54), who showed, respectively, that 80 and 85% of the nuclear RNA synthesis was inhibited by low concentrations of α-amanitin. In the latter case, however, precursor rRNA synthesis had been inhibited by treating the cells with a low dose of actinomycin D, thus suppressing any class A activity that might have been present. We find, however, essentially no engaged class A polymerase activity under our incubation conditions, in agreement with Weinmann et al. (71, 72). The remaining RNA polymerase activity is attributable to class C polymerase, as discussed below.

At least 90% of the RNA transcribed in intact nuclei and in the S200 fraction is Ad2 specific. Other reports have shown between 18 and 30% of nuclear RNA synthesis in isolated infected HeLa cell nuclei to be Ad2 specific (54, 70). The reasons for this discrepancy are not entirely clear but could stem in part from procedural differences in the preparation and incubation of the nuclei (ionic strength, presence of divalent cations during isolation, etc.) and also from the hybridization analysis used. Since large amounts of unlabeled Ad2 RNA are present in the nuclei late in infection, we found it necessary to use rigorously saturating hybridization conditions, employing successive filters. Nevertheless, our results are in agreement with those of Philipson et al. (52), who concluded that there is very little, if any, host-specific heterogeneous nuclear RNA being synthesized late in infection. Their observation that, after a pulse labeling with uridine late in infection, about 60% of the in vivo labeled nuclear RNA was of ribosomal origin is not incompatible with our results, because our high-salt incubation conditions are inhibitory for rRNA synthesis in isolated nuclei (56).

The RNA synthesis catalyzed by the transcriptional complexes appears to be highly asymmetrical as judged from the self-hybridization experiments (8% is RNase resistant, but about 5% corresponds very likely to the secondary structure of the RNA; see zero time values in Table 6). This result is in agreement with the in vivo finding of Pettersson and Philipson (47) that less than 2% of the pulse-labeled late nuclear RNA can self-hybridize. The asymmetry of the in vitro transcription is confirmed by hybridization to the separated *h* and *l* strands, since, at most, 2% of the synthesized RNA forms a true DNA-RNA hybrid with the *h* strand (peak 1 plus interpeak, Table 6). In this respect it is interesting to mention that Vennström and Philipson (personal communication) found little, if any, transcription of the *h* strand when incubating HeLa cell nuclei in vitro at low ionic strength. Our results are also in agreement with previous studies that concluded that most of the RNA synthesized in vivo late in infection is transcribed from the *l* strand (10, 52, 59). In summary, all of our results suggest that most of the known in vivo specificity of transcription by the class B RNA polymerase is retained in the transcriptional complexes isolated from nuclei of infected cells. Further studies are required, however, to determine whether the same regions of the *l* and *h* strands are transcribed in vitro and in vivo.

Late in infection nuclei contain complementary RNA sequences corresponding to between 60 and 90% of the genome (52, 59). This suggests the possibility that extensive symmetrical transcription, followed by a rapid degradation of the RNA coded by the *h* strand, could actually occur in vivo. Symmetrically transcribed RNA has also been characterized in several other eukaryotic systems (1, 29, 30, 63). Our present data do not support the view that the Ad2 genome is symmetrically transcribed late in infection, since very little RNA complementary to the *h* strand was synthesized by the transcriptional complexes purified through a high-salt gradient (peak 1 plus interpeak fraction). Indeed, one would expect that such a purification would remove the processing nuclease responsible for the degradation of the *h* strand RNA transcript, unless it is an integral part of the transcriptional machinery.

The RNA synthesized in vitro by the partially purified transcriptional complexes has a maximum size estimated at 35 to 40*S* on sucrose gradients under denaturing conditions (Fig. 8). Much previous evidence has suggested that viral mRNA could be transcribed in the nucleus as part of a high-molecular-weight precursor (viral heterogeneous RNA) (2, 43, 52, 69). This heterogeneous nuclear RNA sediments at an estimated 70 to 80*S* and may then represent a transcript of all or of a very large segment of the Ad2 DNA. The discrepancy between this large in vivo RNA and that synthesized in vitro may well result from a nuclease activity that would cleave the larger RNA molecules associated with the transcriptional complexes during the preparation of the nuclei and/or fraction S200. In fact, such a possibility is supported by the presence of a very low, but definite, level of RNase activity in fraction S200.

Class C polymerase—is it responsible for the synthesis of 5.5*S* VA RNA in transcriptional compleses? Studies using isolated, infected HeLa cell nuclei have shown that a class C RNA polymerase is responsible for the synthesis of the adenovirus-coded 5.5*S* VA RNA (53, 71). We find less than 5% class C RNA polymerase activity both in isolated nuclei and

in the S200 fraction (Table 4, Fig. 5) under conditions (200 mM ammonium sulfate) that do not permit reinitiation. In contrast, Weinmann et al. (71) measured about 40% class C activity in isolated infected nuclei. This discrepancy is certainly due to their low-salt incubation conditions, which are not optimal for class B RNA polymerase activity but which allow reinitiation of class C RNA polymerase (24). In fact, the two VA RNA genes do not represent more than 1% of the total Ad2 genome (39, 40). One would not expect, therefore, that class C activity would represent more than a few percent of the total RNA polymerase activity under nonreinitiating conditions, unless class C enzymes transcribe genes other than those coding for VA RNA.

Very little, if any, 5.5S RNA was seen when the RNA synthesized by the class C RNA polymerase present in the S200 fraction was analyzed by electrophoresis on polyacrylamide gels (Fig. 9). Although there is a low level of RNase activity in the S200 fraction, it is highly unlikely that the absence of 5.5S RNA is due to its degradation during the incubation period. In fact, RNA molecules of much higher molecular weight (around 18S) were synthesized, and there is little RNA migrating faster than 5.5S. One has then to consider the possibility that the absence of 5.5S RNA might be due to the loss of proper termination in the S200 fraction, allowing read-through by the polymerase and resulting in much longer RNA molecules. On the other hand, some of the longer RNAs could correspond to transcripts not initiated on VA RNA genes, since it has been suggested that class C polymerase may be responsible for the synthesis of other Ad2 RNAs (71).

Nature of the template in the transcriptional complexes. From the present study and from the consideration of previous work, some suggestions concerning the nature of the template used late in infection for the synthesis of Ad2 RNA can be proposed.

Most (>95%) of the Ad2 DNA in the S200 fraction is intact and unnicked (Fig. 3). It cannot be necessarily concluded that the template consists of unnicked double-stranded DNA. The number of Ad2 DNA molecules in the S200 fraction that are actually engaged in transcription is indeed unknown, because the number of polymerase molecules transcribing one DNA molecule has not been determined. The molecules containing single-strand fragments of less than genome length represent replicative intermediates and are found mainly in the interpeak region (Fig. 4). Therefore, the possibility is raised that replicative intermediates may be involved in transcription, because endogenous RNA polymerase activity is localized in the peak 1 and interpeak regions (Fig. 6). This possibility is supported by the observation that early mRNA is synthesized in vivo in the absence of DNA replication, whereas viral DNA synthesis seems to be a prerequisite for the appearance of late mRNA (51), suggesting at least a temporal link between DNA replication and late mRNA production. Further characterization of the transcriptional complexes is required to ascertain if transcription and replication are physically linked.

Recent evidence has shown that Ad2 DNA can be isolated from virions in a circular configuration where the ends are held together by a Pronase-sensitive, presumably proteinaceous linker (3, 12, 58). The role, if any, of the circularized molecule in the infected cell is unknown; up to now it has been isolated only from intact virions. Preliminary electron microscopic results (P. Oudet, unpublished data) have suggested the presence of circular forms in the S200 fraction, but no preferential localization of these forms in the interpeak region has been found. Therefore, there is presently no evidence to assign circular DNA molecules a role in transcription.

At 17 h postinfection, as much as 15% of the total Ad2 DNA may be integrated into the host cell DNA (4, 12). A similar observation was made in the course of our experiments, since rebanding of the labeled DNA from the cellular density (Fig. 2a), followed by the analysis of its reassociation kinetics, indicated the presence of Ad2 DNA sequences in this fraction (unpublished data). It can be imagined that this integrated DNA may also serve as a template for RNA synthesis, since about 50% of the Ad2 RNA synthetic activity is not released into the S200 fraction, as discussed previously. Two observations argue against such a possibility. (i) As discussed previously, nonintegrated Ad2 DNA and engaged RNA polymerase are released from the nuclei into the S200 fraction in similar quantities (Tables 2 and 5). Since the release of Ad2 DNA complexes from nuclei seems not be selective for a particular type of complex (see above), and since labeled cellular density DNA is not released into the fraction S200 (Fig. 2b), we conclude that the engaged RNA polymerase molecules remaining in the nuclei are most likely associated with nonintegrated Ad2 DNA. (ii) The integration of Ad2 DNA into the cellular genome does not appear to be a prerequisite for the success of the lytic infection cycle, as can be inferred from the results of Hodge and Scharff (22) and Simmons et al. (60). Using synchronized cell cultures in which Ad2 DNA synthesis occurred during the

G_1 phase in the absence of cellular DNA replication, they found that labeled thymidine was incorporated only into Ad2 DNA. Thus, it can be hypothesized that irregularities in the cellular DNA replication process (in infected cells in which the S phase is not completely inhibited due to the timing of the infection in random cell populations) permit the integration of viral genes into the host genome. This argument does not, of course, exclude the possibility of the integration of a limited number of Ad2 DNA molecules into cellular DNA, but at least it seems that the integration of as much as 15% of the newly synthesized viral DNA late in infection is not required for late viral RNA transcription.

It is apparent that the Ad2 DNA released into the S200 fraction is associated with protein, since deproteinization of the S200 fraction yields Ad2 DNA sedimenting as purified Ad2 DNA. Furthermore, it seems that some protein is associated with every Ad2 DNA molecule released, since purified Ad2 DNA (32*S*) is slower sedimenting than peak 1 (40 to 50*S*) (Fig. 4 and 6). Preliminary analyses by SDS-acrylamide gel electrophoresis of the Ad2 DNA-protein complexes purified on velocity gradients confirm the existence of protein associated with the Ad2 DNA in the S200 fraction. It is worth mentioning that peak 2 and the interpeak region (see Fig. 4) contain Ad2 DNA protein complexes that are shorter and much thicker than purified Ad2 DNA (Oudet et al., in preparation). The structure of these compacted Ad2 DNA-protein complexes does not resemble that of nucleosomes associated with DNA (42), and histone bands are not seen on acrylamide gels of the S200 fraction or of the purified complexes (unpublished data). Although histone H1 might be solubilized from chromatin in 200 mM ammonium sulfate, the other histones (H2a, H2b, H3, and H4), which are primarily responsible for the formation of nucleosomes, would not be removed (62). These results suggest that the compaction of Ad2 DNA in infected nuclei is effected by means other than the association with histones. Similar thick Ad2 DNA protein complexes have also been visualized recently by a different technique (38). Work is in progress to more fully characterize the Ad2 DNA-protein complexes.

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