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Intracellular Forms of Adenovirus DNA

II. Isolation in Dye-Buoyant Density Gradients of a DNA-RNA Complex from KB Cells Infected with Adenovirus Type 2

WALTER DOERFLER, ULLA LUNDHOLM, UTE RENSING, AND LENNART PHILIPSON

The Rockefeller University, New York, New York 10021, The Wallenberg Laboratory, University of Uppsala, Uppsala, Sweden, and Institute of Genetics, University of Cologne, Cologne, Germany

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DNA-RNA complexes with different DNA/RNA ratios have been isolated from KB cells productively infected with human adenovirus type 2 by the dye-buoyant density procedure by using propidium iodide. Both the DNA and RNA components of these complexes are virus specific, and parental as well as newly synthesized viral DNA can be recovered from these complexes. The RNA component is susceptible to digestion with pancreatic ribonuclease at low and high salt concentrations. The RNA is in part liberated from the complex during purification over several cycles of equilibrium centrifugation in dye-buoyant density and Cs_2SO_4 gradients. Analysis in the latter gradients reveals at least four classes of virus-specific nucleic acid: (i) RNA loosely bound and released during purification, (ii) and (iii) two distinct classes of RNA-DNA complexes with different DNA/RNA ratios, and (iv) DNA apparently not associated with RNA. The size of the RNA molecules is large but varying in the different classes of complexes. The biological function of these complexes is still uncertain, but they may be transcription complexes.

In a series of investigations, the intracellular forms of the DNA of adenovirus type 12 (Ad12) in abortively infected BHK cells have been analyzed. Apart from free viral DNA of mol wt 20×10^6, fragments of adenovirus DNA of approximately 5×10^6 daltons (3) and the integrated state of viral DNA (6) have been observed. Moreover, there is viral DNA in a complex with RNA and protein (8), which can be isolated in dye-buoyant density gradients. The systematic investigation of the intracellular forms of viral DNA in KB cells productively infected with adenovirus type 2 (Ad2) may reveal details of the mechanism of viral DNA replication. Viral DNA sedimenting faster than parental DNA at a rate of 50 to 90*S* in alkaline sucrose density gradients has been described (H. Burger, M. Hirsch-Kauffmann, and W. Doerfler, Fed. Proc. **31**:407, 1972).

From cells productively infected with Ad2 and actively synthesizing viral DNA, a DNA-protein complex has been isolated (22, 32). This complex may be involved in viral DNA replication.

A number of laboratories have reported that partially single-stranded DNA of adenoviruses is found in cells active in viral DNA replication (27, 29, 30; G. D. Pearson; U. Pettersson and H. Delius, personal communications).

In this paper, evidence for the presence of DNA-RNA complexes in Ad2-infected cells will be presented. When analyzed on polyacrylamide agarose gels the RNA component of such complexes is as large as 1.6×10^6 daltons, and is probably larger. It is likely that these complexes are involved in the transcription of viral DNA.

MATERIALS AND METHODS

Cell culture and media. KB cells (10) were grown in monolayers on 60-mm-diameter plastic petri dishes in Eagle medium (11) supplemented with 10% calf serum (MEMC). For the propagation of human Ad2, KB cells were grown in suspension culture in Eagle medium for suspension cultures with 10% calf serum.

Virus growth and purification. Ad2 was grown and purified as described previously (5). The preparation of Ad2 labeled with ^{3}H-thymidine or ^{14}C-sodium formate and the isolation of viral DNA was described earlier (6, 8).

Chemicals and radioisotopes. Most chemicals used had analytical grade purity. CsCl (optical grade) was purchased from the Harshaw Chemical Co.; Cs_2SO_4 was purchased from Merck, Darmstadt, Germany; ethidium bromide was a gift of the Boots Pure Drug Co., Nottingham, England; and propidium

iodide was obtained from Calbiochem, Los Angeles, Calif. Acrylamide and *N,N'*-methylenebisacrylamide were purchased from the Eastman Kodak Co., Rochester, N.Y., and were recrystallized from chloroform and acetone, respectively, prior to use. Phenol was redistilled at least once and was saturated at 25 C with 1 M Tris-hydrochloride before it was used for the extraction of nucleic acid. Sodium dodecyl sulfate (SDS) was purchased from Schuchardt, Munich, Germany; lauryl sarcosine (Sarkosyl) was obtained from Geigy, Industrial Chemicals. Agarose, Indubiose A37, was purchased from L'Industrie Biologique Française, S.A. Gennevilliers, France; *N,N,N',N'*-tetramethylethylenediamine was purchased from the Eastman Kodak Co.; polyethylene glycol (PEG 6000) and dextran 500 were obtained from Pharmacia, Uppsala, Sweden. Ribonuclease-free sucrose was obtained from Schwarz/Mann, Orangeburg, N.Y.; dimethylsulfoxide was obtained from Serva GmbH, Heidelberg, Germany; formamide and paraffin oil were from Merck, Darmstadt, Germany. Pronase B was purchased from Calbiochem, Los Angeles, Calif.; benzoylated-naphthoylated DEAE-cellulose (BND-cellulose) was obtained from Gaillard-Schlesinger Co., New York, N.Y. The following radioisotopes were employed: thymidine-6-^3H (25 Ci/mmol), uridine-5-^3H (30 Ci/mmol), thymidine-2-^{14}C (62 mCi/mmol), formic acid-^{14}C-Na (56 mCi/mmol), uridine-2-^{14}C (60 mCi/mmol), and L-methionine-^{35}S (16 Ci/mmol), all from Radiochemical Centre, Amersham, England.

Solutions. TE is 0.01 M Tris-hydrochloride (pH 7.2) and 0.001 M EDTA; SSC is 0.15 M NaCl and 0.015 M sodium citrate (19); PBS is phosphate-buffered saline (9); PBSd is PBS deficient in Ca^{2+} and Mg^{2+}; STE is 0.5% SDS in 0.1 M Tris-hydrochloride and 0.02 M EDTA.

Inoculation of KB cells with Ad2. KB cells growing in monolayers were inoculated with ^{3}H-labeled or unlabeled Ad2 in PBS (1 ml). The ^{3}H-labeled virus was purified by three cycles of equilibrium centrifugation in CsCl density gradients. Unlabeled Ad2 was purified once by equilibrium sedimentation in CsCl density gradients and was diluted in PBS. Adsorption was allowed to proceed for 2 h. After adsorption, the cells were washed with PBS and 5 ml of MEMC was added. The newly synthesized DNA or RNA was labeled as indicated in the experiments described in Results.

In some of the experiments, the newly synthesized proteins in Ad2-infected KB cells were labeled with ^{35}S-methionine (10–12 μCi of medium per ml) between 23 and 30 h postinfection (i.e., at the same time that the newly synthesized DNA was labeled with ^{3}H-thymidine). Extracts of these cells were analyzed in dye-buoyant density gradients (see below).

Titration of adenovirus by plaque assay. Ad2 was titrated on human embryonic kidney cells as described previously (18).

Extraction of the intracellular nucleic acid. Ad2-infected and uninfected KB cells were lysed in 1 ml of STE containing 500 μg of Pronase per ml. The extracts were incubated for 30 min at 37 C, and the intracellular nucleic acid was extracted by treatment with phenol as previously described (6, 8). Phenol was removed by ether, and finally the sample was flushed with N_2 to evaporate the ether.

Equilibrium sedimentation in dye-buoyant density gradients. The method of Hudson et al. (14) was applied. The experimental details were precisely the same as described in an earlier communication (8). In the experiments illustrated in Fig. 1, 2, 3, 9, and 10, propidium iodide (660 μg/ml) was used; in a few experiments (not described in detail) ethidium bromide was the dye component. The dye-CsCl solutions were overlayed with paraffin oil and centrifuged to equilibrium at 20 C for 36 to 48 h at 40,000 rpm in the SW56 rotor of the L2-65B Spinco ultracentrifuge. After centrifugation, samples of the fractions collected were either precipitated with trichloroacetic acid and the ^{3}H and ^{14}C activities were determined on GF/C glass fiber filters, or samples were counted directly in a 1:1 mixture of toluene and methanol containing Liquifluor (New England Nuclear Corp.).

Removal of dye from DNA solutions. Propidium iodide was removed by extensive dialysis against 5 M NaCl in TE, followed by dialysis against TE. Dye which remained after this treatment was adsorbed to a mixed bed resin, Bio-Rex RG-501-X8 (BioRad Laboratories, Richmond, Calif.), and the sample was dialyzed subsequently against TE. In some experiments the dye was removed by repeated extraction with isopropanol, followed by dialysis against TE.

Equilibrium sedimentation in Cs_2SO_4 density gradients. After removal of the dye, samples of intracellular nucleic acid previously fractionated in dye-buoyant density gradients were mixed in a total volume of 2.5 ml with 1.51 g of Cs_2SO_4 powder to yield a buoyant density of 1.42 g/cm^3. This solution was filled into nitrocellulose tubes of the Spinco SW56 rotor, overlayed with paraffin oil, and centrifuged to equilibrium at 15 to 20 C for 48 h at 35,000 rpm. After centrifugation, five-drop fractions were collected and the ^{3}H and ^{14}C activities were determined in 10- to 20-μliter samples after trichloroacetic acid precipitation of each fraction.

Equilibrium centrifugation in Cs_2SO_4-dimethylsulfoxide density gradients (34). In some experiments the Cs_2SO_4 solution was made 5 or 20% in dimethylsulfoxide. Otherwise, the conditions were as described in the preceding section.

Zone velocity sedimentation experiments in sucrose density gradients. These experiments were performed by methods previously described (3). The gradients were 5 to 20% in sucrose and 1 M NaCl, 0.001 M EDTA, pH 12.5, and 0.01 M Tris-hydrochloride, pH 7.6 (neutral sucrose density gradients), or 0.7 M NaCl, 0.3 M NaOH, and 0.001 M EDTA (alkaline sucrose density gradients).

DNA-DNA hybridization. The procedure of Denhardt (4) was employed as described in an earlier report (6).

RNA-DNA hybridization. The method of Gillespie and Gillespie (12) was followed as described by Lindberg et al. (17). Each filter carried 5 μg of unlabeled or ^{14}C-labeled, denaturated Ad2 DNA. Hybridization was carried out in 0.25 M NaCl, 0.0125 M Tris-hydrochloride, pH 7.5, 0.0005 M EDTA,

0.125% Sarkosyl, and 50% formamide at 37 C for 3 days. Subsequently, the filters were washed in 2 × SSC at 37 and 65 C; RNase was not used in the washing procedure. It had been shown (17) that 25 to 30% of the RNA hybridized under these conditions was RNase sensitive. In some of the experiments (Table 2, experiment 1), the DNA-RNA complexes were added to the hybridization mixture; in other experiments (Table 2, experiment 2), the DNA and RNA components were separated by equilibrium centrifugation in Cs_2SO_4 density gradients after heat denaturation. The purified RNA was then added to the hybridization mixture.

Polyacrylamide gel electrophoresis of RNA samples. Previously described methods were used (17, 21). The RNA or DNA-RNA sample to be analyzed was dialyzed into 0.1 × SSC and was made 0.5% in Sarkosyl and 10% in glycerol. A small amount of ^{14}C-labeled rRNA from KB cells was added as marker. The samples were carefully layered on gels consisting of 2.2% acrylamide (acrylamide-*N*,*N*'-bisacrylamide, 19:1), 0.4% dimethylaminopropionitril, 0.05% ammonium persulfate, and 0.8% agarose in Tris-EDTA-boric acid (TEB) propylamine buffer (17). The TEB propylamine buffer used for electrophoresis contained 0.089 M Tris, 0.089 M boric acid, 0.0025 M EDTA, 0.01 M propylamine, and 0.5% Sarkosyl. Electrophoresis was for 4 h at 100 V and 4 C. The gels were sliced, each slice was solubilized in 0.5 ml of NCS (Amersham, England), and the ^{3}H and ^{14}C activities were determined after the addition of 10 ml of toluene-Liquifluor in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc.).

Preparation of ^{14}C-uridine-labeled RNA from KB cells. The method of Scherrer (25) was modified. KB cells were grown for 3 to 4 days in MEMC in the presence of uridine-2-^{14}C (0.2 μCi/ml). The cells were washed extensively with PBSd and were resuspended in reticulocyte standard buffer (0.01 M NaCl, 0.01 M Tris-hydrochloride, pH 7.4, and 0.0015 M $MgCl_2$). The cells were allowed to swell for 10 min at 0 C and were then broken by homogenization in a Dounce homogenizer. The nuclei were pelleted and the supernatant fluid was made 0.5% in Sarkosyl. The supernatant fluid was next treated three times with phenol (redistilled, saturated with 0.01 M Na-acetate, pH 6.5). The aqueous phase was made 0.1 M NaCl, two volumes of −20 C ethanol were added, and the mixture was stored at −20 C for 18 h. The RNA was pelleted by centrifugation and resuspended in 0.01 M Na-acetate, pH 5.2. This solution was kept at −20 C. The ^{14}C-labeled RNA was analyzed by rate zonal sedimentation in a sucrose density gradient in 0.1 M NaCl, TE with ^{3}H-labeled Ad2 DNA (30.5*S*) as marker. The RNA sedimented in three peaks at 29.6, 18.1, and 6.7*S*.

Phase distribution in 5% dextran and 4% polyethylene glycol. The method of Albertsson (1) was used as modified by Pettijohn (23). DNA samples from various buoyant density strata (HHP, HP, LP; see legend, Fig. 3) from dye-buoyant density experiments were diluted in sodium phosphate buffer of different pH values in 5% dextran 500 and 4% polyethylene glycol (PEG 6000). A small amount of ^{14}C-labeled Ad2 DNA was added to each sample as internal control, and the mixture was shaken vigorously. After separation of the phases by low-speed centrifugation, the ^{3}H and ^{14}C activities were determined in samples of each phase after precipitation with trichloroacetic acid. The partition coefficients were calculated by the equation: K = counts/min in upper phase ÷ counts/min in lower phase.

Chromatography on BND-cellulose. The method of Kiger and Sinsheimer (15) was applied in the modification described by Levine et al. (16). The ^{3}H-labeled HHP, HP, and LP fractions (see legend, Fig. 3) and 6.4 μg of ^{14}C-labeled Ad2 DNA were applied in 0.3 M NaCl in TE to a BND-cellulose column (0.8 cm diameter by 3–4 cm) equilibrated with the same buffer. The DNA was eluted first with a continuous gradient ranging from 0.3 M to 1.0 M NaCl, followed by a continuous gradient from 0 to 2% caffeine (Merck, Darmstadt) in 1.0 M NaCl. The ^{3}H and ^{14}C activities in each fraction were determined after trichloroacetic acid precipitation.

Determination of radioactivity. A Tri-Carb liquid scintillation spectrometer model 3380 (Packard Instrument Co., Inc.) was used for all measurements. Liquid samples (direct determinations were dissolved in a 1:1 mixture of toluene-methanol containing 42 ml of Liquifluor (New England Nuclear Corp.) per liter. Samples precipitated by trichloroacetic acid and collected on GF/C filters (Whatman) were counted in toluene-Liquifluor.

Gel slices were processed as described above. In some of the experiments, 5 g of 2,5-diphenyloxazole and 0.3 g of 2,2'-*p*-phenylen-bis-(5-phenyloxazol) were used per liter of toluene or toluene-methanol instead of Liquifluor.

RESULTS

Analysis in dye-buoyant density gradients of parental viral DNA in Ad2-infected KB cells. The data presented in Fig. 1 demonstrate that up to 52% of the parental viral DNA from KB cells productively infected with Ad2 has a buoyant density in dye-buoyant density gradients which is higher than that of Ad2 DNA extracted from CsCl-purified virions. Similar results have been obtained for the DNA of human Ad12 in abortively infected BHK cells (8). In the latter system, it has been shown that the DNA of higher buoyant density in dye-buoyant density gradients represents a complex form of viral DNA which is tightly associated with protein and RNA. In contrast to the findings in the BHK-Ad12 system, the parental viral DNA in Ad2-infected KB cells is distributed over a wide range of density strata in dye-buoyant density gradients (Fig. 1).

Analysis in dye-buoyant density gradients of the newly synthesized DNA in Ad2-infected KB cells. At late times after infection, the DNA synthesized in cells productively in-

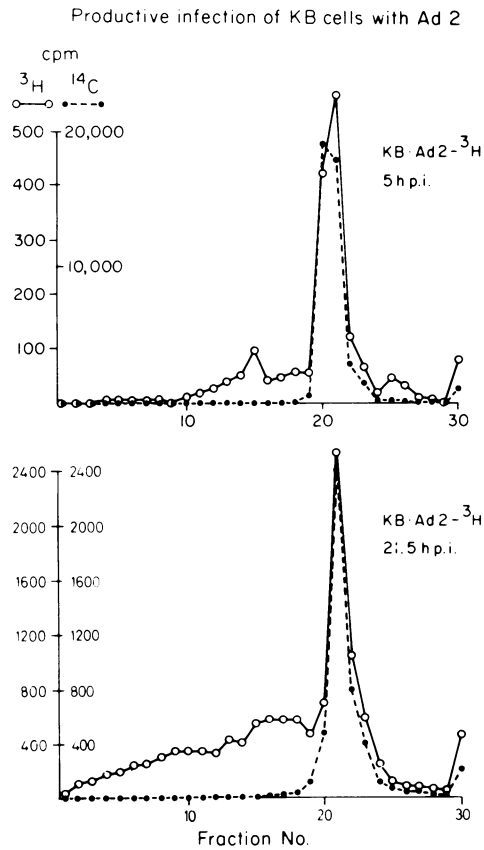

FIG. 1. *Equilibrium sedimentation in dye-buoyant density gradients of the intracellular DNA from KB cells infected with ^3H-labeled Ad2 (8.42 optical density at 260 nm [OD_{260}] units; 8.6 × 10^5 counts per min per OD_{260} unit). Adsorption was allowed to occur for 2 h. The inocula were then removed, the cell sheets were extensively washed with PBS, and 5 ml of MEMC was added to each plate. At 5 and 21.5 h postinfection, the total intracellular DNA was extracted and analyzed in dye-buoyant density gradients as described in Materials and Methods. As a marker, ^{14}C-labeled Ad2 DNA was added to each gradient. Centrifugation was performed in an SW56 rotor at 40,000 rpm for 66 h at 20 C. After centrifugation, five-drop fractions were collected, and the ^3H and ^{14}C activities were determined in samples of each fraction after trichloroacetic acid precipitation.*

fected with Ad2 is almost exclusively viral (5, 13, 24, 28). Thus, it is possible to label predominantly viral DNA or intracellular forms of viral DNA involved in the replication of adenovirus DNA when Ad2-infected KB cells are maintained between 24 and 30 h postinfection in medium supplemented with ^3H-thymidine. Since in cells productively infected with Ad2 a large number of copies of viral DNA are synthesized (5, 13), even a minor, metabolically important component of viral DNA may be detectable in this system.

The newly synthesized DNA in Ad2-infected KB cells has been analyzed in dye-buoyant density gradients (Fig. 2). A relatively small fraction (up to 15.2%; see Fig. 9) of the newly synthesized DNA bands in a higher buoyant density stratum in dye-buoyant density gradients than the bulk of the DNA which cosedi-

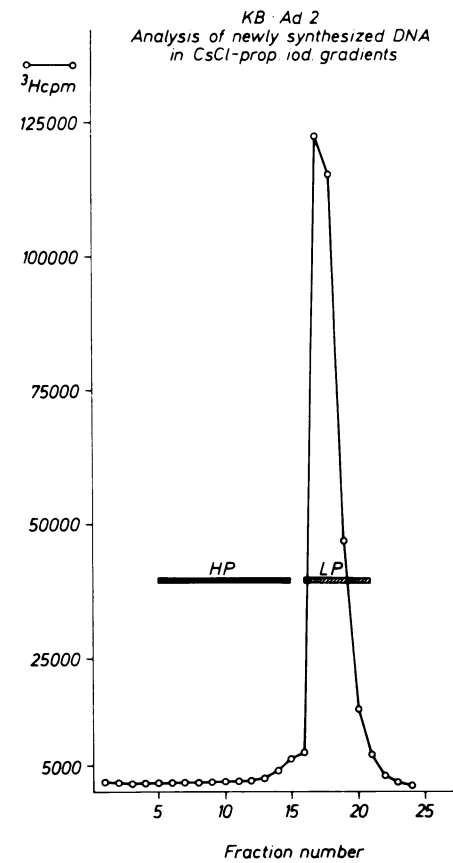

FIG. 2. *Analysis of the newly synthesized DNA in Ad2-infected KB cells in dye-buoyant density gradients. Monolayers of KB cells were inoculated with a 1:50 dilution in PBS of a crude extract of Ad2-infected KB cells. After a 2-h adsorption period at 37 C, the inoculum was removed and 5 ml of MEMC was added. At 24 h postinfection, ^3H-thymidine (20 μCi/ml) was added to the medium, and at 30 h postinfection, the total intracellular nucleic acid was extracted and analyzed in dye-buoyant density gradients. Centrifugation was performed in an SW56 rotor at 40,000 rpm for 41 h at 20 C. The abbreviations HP and LP refer to the heavy and light peak fractions, respectively. The fractions indicated by the bars were pooled and analyzed by recentrifugation in a dye-buoyant density gradient (see Fig. 3).*

ments with the ^{14}C-labeled Ad2 marker DNA (not shown in Fig. 2, but see Fig. 3, bottom).

The DNA fractions of high buoyant density are designated HP (heavy peak); those cosedimenting with marker DNA are referred to as LP (light peak). The HP DNA is observed also when ^{3}H-thymidine is added to the medium between 8 and 13 or between 16 and 22 h postinfection. The HP DNA is found also when extracts of uninfected KB cells are analyzed in dye-buoyant density gradients.

In the experiment described in Fig. 3, the HP and LP fractions from the experiment shown in Fig. 2 have been pooled and resedimented in dye-buoyant density gradients. It is apparent that each DNA fraction resediments in the proper density stratum; the HP fraction resediments in a relatively wide region which has been subdivided arbitrarily into the HHP and HP fractions.

It has been shown in an earlier report (Fig. 1 of ref 8) that the DNA in the HP density region is not due to an artifactual binding of viral DNA to cellular structures during the extraction of the total intracellular DNA. When intact virus is added to cells growing in monolayers and the total DNA is then extracted and analyzed in dye-buoyant density gradients by the techniques described in Materials and Methods, viral DNA is found not in the HP region but exclusively in the LP density stratum cosedimenting with the viral marker DNA. Furthermore, it has been demonstrated (Fig. 6 of ref 8) that the DNA in the HP region is not found when macromolecular synthesis (DNA, RNA, or protein syntheses) are inhibited chemically. This finding argues against artifactual binding of RNA or protein to viral DNA as an explanation for the increased density in dye-CsCl gradients.

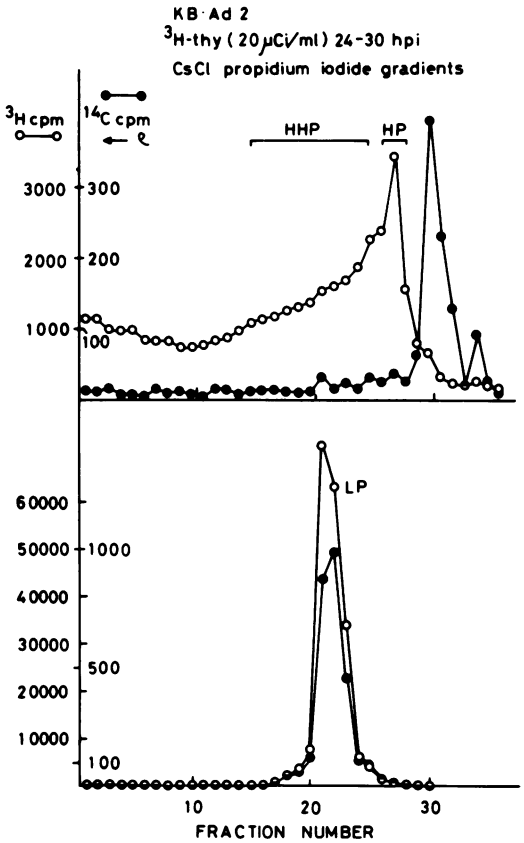

FIG. 3. *Resedimentation of ^{3}H-labeled DNA in dye-buoyant density gradients. In the experiment described in the legend to Fig. 2, fractions 5 to 15 (HP) (top frame) and 16 to 21 (LP) (bottom frame) were combined and resedimented in dye-buoyant density gradients under the conditions described in Materials and Methods. To each gradient ^{14}C-labeled Ad2 DNA was added as density marker. HHP, HP, and LP refer operationally to heavy-heavy, heavy, and light peaks, respectively, indicating DNA fractions of increasing buoyant density relative to LP which has the buoyant density of viral (Ad2) DNA complexed with propidium iodide. The limit between the HHP and HP fractions was drawn arbitrarily.*

Further characterization of the HHP and HP DNA fractions. (i) Rate zonal sedimentation. The combined HHP and HP DNA and the LP fractions have been dialyzed extensively (legend, to Fig. 7). Subsequently, the DNA from these fractions has been analyzed further by rate zonal sedimentation experiments in neutral (pH 7.6) and alkaline (pH 12.5) sucrose density gradients (Fig. 4). At pH 7.6, the HHP/HP fractions sediment in a double peak at 59 and 39*S* (Fig. 4A), and the LP fractions sediment in a double peak of 45 and 32*S* (Fig. 4B). At pH 12.5, the HHP/HP fractions sediment in a single peak at 34*S* (Fig. 4C), i.e., together with the ^{14}C-labeled Ad2 DNA used as marker.

(ii) DNA-DNA hybridization. The data in Table 1 demonstrate that the DNA in the HHP, HP, and LP fractions is predominantly viral DNA. The extent of the hybridization of these DNA fractions to cellular (KB) DNA lies significantly above background. In one set of experiments (Table 1, experiment 1), the ^{3}H-DNA from the HHP, HP, and LP fractions has been hybridized to viral DNA without pretreatment of the fractions. There is some variation in the extent of hybridization of the LP DNA to viral DNA. This variation may be due to the presence of varying amounts of cellular DNA in different preparations. In a second experiment (Table 1, experiment 2), the ^{3}H-DNA from the HP and LP fractions has been boiled in alkali and

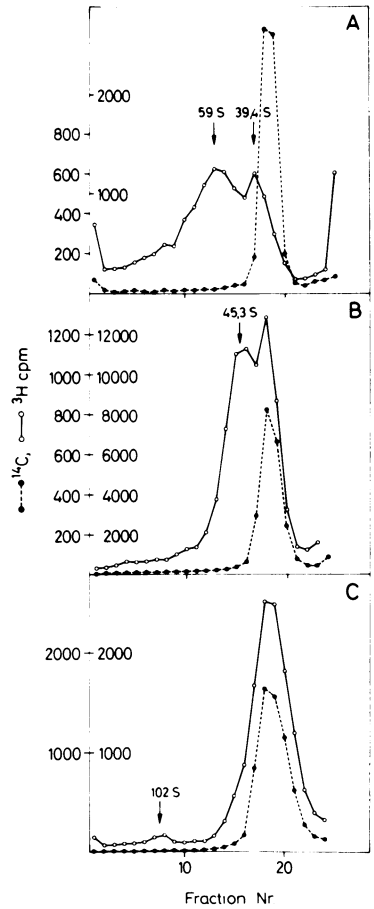

FIG. 4. *Zonal sedimentation of HP DNA and LP DNA in neutral and alkaline sucrose density gradients. KB cells were infected with unlabeled Ad2, and the infected complexes were labeled with ^{3}H-thymidine between 12 and 24 h postinfection as described in Methods. The HP and LP DNA fractions were isolated by two cycles of equilibrium centrifugation in dye-buoyant density gradients as described in the legends to Fig. 2 and 3. Subsequently, the dye was removed (see Materials and Methods) and the fractions were dialyzed against TE and analyzed by zonal sedimentation in neutral or alkaline sucrose density gradients as described in Materials and Methods. A small amount of ^{14}C-labeled Ad2 DNA was used as marker. The samples were centrifuged in an SW56 rotor at 45,000 rpm for 80 min at 4 C. At the end of centrifugation, six-drop fractions (0.2 ml) were collected and counted as described in Materials and Methods. A, HP in neutral sucrose density gradient; B, LP in neutral sucrose density gradient; C, HP in alkaline sucrose density gradient.*

neutralized before hybridization to eliminate RNA from the complex in the HP fraction. The extent of hybridization to viral DNA of the ^{3}H-DNA in the HP fraction is identical in both experiments. Hence, the RNA component in complexes is effectively removed from the DNA by heat denaturation alone (Table 1, experiment 1).

It is concluded that the DNA in the HHP, HP, and LP fractions is predominantly Ad2 DNA and sediments at 59 and 39*S* in neutral and at 34*S* in alkaline sucrose density gradients (7). Cellular DNA synthesis is not completely shut off between 24 and 30 h postinfection, and cellular DNA is present both in the HHP/HP and LP fractions.

(iii) Chromatography on BND-cellulose. The ^{3}H-labeled HP fractions have been chromatographed, together with a sample of ^{14}C-labeled Ad2 DNA, on a BND-cellulose column (Fig. 5). The bulk of the ^{3}H label elutes simultaneously with the viral marker DNA. A small amount of ^{3}H label eluting from the column

TABLE 1. *Characterization of the DNA from the HHP, HP, and LP fractions (Fig. 3) by DNA-DNA hybridization*

Expt no.	^{3}H DNA from	Input (counts/min)	Counts/min hybridized to		% of input hybridized to	
			KB DNA	Ad2 DNA	KB DNA	Ad2 DNA
1[a]	HHP	8,224	111	3,039	1.3	37.0
	HP	10,209	148	5,008	1.4	49.1
	LP	61,761	2,402	57,657	3.9	93.4
	Ad2 virus (control)	125,278	161	56,460	0.1	45.1
2[b]	HP	36,730		16,586		45.1
	LP	301,152		124,733		41.4
	Ad2 virus (control)	12,020		5,870		48.8

[a] Samples of the fractions designated HHP, HP, and LP were dialyzed against 5 M NaCl in TE and against TE, were treated with a mixed bed resin (Bio-Rex RG 501-×8), and were hybridized to KB DNA and Ad2 DNA (each 5 μg) fixed to nitrocellulose filters (see Materials and Methods). The figures represent the mean of double determinations. Control experiments were performed with ^{3}H-labeled Ad2 DNA.

[b] KB cells were infected with unlabeled Ad2 and were labeled with 20 μCi of ^{3}H-thymidine per ml from 15 to 20 h postinfection. The HP and LP fractions were purified over two cycles of equilibrium centrifugation in dye-buoyant density gradients. The dye was removed by extensive dialysis versus 5 M NaCl in TE and TE. Samples of the ^{3}H-labeled HP and LP fractions and of the ^{3}H-labeled Ad2 DNA were boiled in 0.27 N NaOH for 10 min to denature the DNA and destroy the RNA. Subsequently, the samples were chilled, rapidly neutralized, and added to the hybridization mixture. Each value represents the mean of double determinations.

after application of the caffeine gradient (between fractions 20 and 30) may be due to partly single-stranded DNA. The same results have been obtained with the DNA from the HHP fractions. These data indicate that the DNA in the HHP and HP fractions represents mainly double-stranded nucleic acids. In control experiments it has been established that single-stranded viral DNA elutes only after application of the caffeine gradient.

(iv) Analysis by phase distribution. The distribution of the HHP, HP, and LP fractions, each mixed with ^{14}C-labeled Ad2 DNA as internal control, has been determined in a polyethyleneglycol-dextran phase system at increasing pH values (Fig. 6). The partition coefficients (K) for all pH values are highest for the Ad2 marker DNA and are decreasing in the order LP, HP, and HHP. Although the differences between the LP, HP, and HHP fractions are not excessive, they have been very reproducible. The results are compatible with the hypothesis that the viral DNA in the HP and HHP fractions is associated with increasing amounts of RNA (23).

Is the DNA from the HHP and HP fractions associated with RNA? The possibility has to be considered that the DNA banding in the HHP and HP regions in dye-buoyant density gradients represents supercoiled circular molecules (14). However, the results of the rate zonal sedimentation experiments in neutral and alkaline sucrose density gradients are not consistent with the presence of supercoiled DNA molecules. Moreover, electron microscope examination of the DNA in the HHP and HP fractions does not reveal circular structures. The details of the electron microscope analysis are complex and require further studies. It has been observed previously in the BHK-Ad12 system that a viral DNA complex in association with RNA and protein assumes a buoyant density in CsCl-propidium iodide gradients which is identical to that of supercoiled circular DNA (8).

Analysis in Cs_2SO_4 density gradients. The ^3H-labeled DNA from the HHP, HP, and LP fractions has been centrifuged to equilibrium in Cs_2SO_4 density gradients before and after treatment with pancreatic ribonuclease at low (0.005 M Tris-hydrochloride, pH 7.5) and high salt concentrations (0.2 M NaCl in 0.01 M Tris-hydrochloride, pH 7.5, and 0.01 M Mg^{2+}) to determine whether the viral DNA in these fractions is associated with RNA. The results of this experiment clearly demonstrate (Fig. 7)

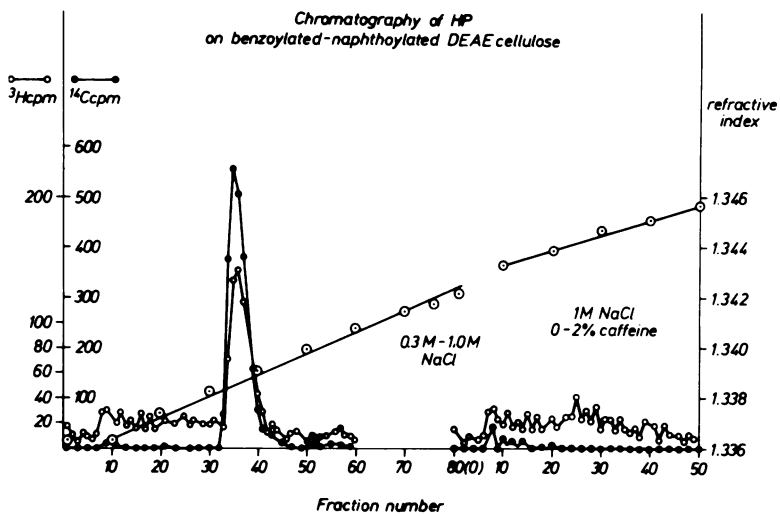

FIG. 5. *Chromatography of a sample of the HP fraction on BND-cellulose. The ^3H-labeled DNA from the HP fraction was isolated by two cycles of equilibrium sedimentation in CsCl-propidium iodide density gradients, as described in the legend to Fig. 3, and was dialyzed against 5 M NaCl-TE for 24 h and subsequently against TE. The HP DNA sample and 6.4 μg of ^{14}C-labeled Ad2 DNA in 0.3 M NaCl-TE in a total volume of 300 μliters were applied to a BND-column, as described in Materials and Methods. The column was eluted with a continuous gradient of 0.3 M to 1.0 M NaCl in TE, followed by a continuous gradient of 0 to 2% caffeine in 1.0 M NaCl in TE. Refractive indices were measured in every tenth fraction, and the ^3H and ^{14}C activities were determined in each fraction after trichloroacetic acid precipitation. The radioactivity in samples of fractions 60 to 79 was determined directly in a toluene-methanol mixture. Only background activities were observed. These results are not included in the graph since they are not comparable to those measured after trichloroacetic acid precipitation.*

that, prior to digestion with ribonuclease, the DNA in the HHP and HP fractions from dye-buoyant density gradients sediments in Cs_2SO_4 gradients in a density stratum characteristic for DNA-RNA complexes. The data indicate that the DNA from the HHP region is associated with a larger amount of RNA than the DNA from the HP region. Both fractions of DNA contain structures which apparently lose their RNA components during removal of the propidium iodide prior to equilibrium centrifugation in Cs_2SO_4 density gradients, since some of the DNA cobands with the ^{14}C-labeled Ad2 marker DNA in Cs_2SO_4 density gradients. As expected, the DNA from the LP region in the dye-buoyant density gradients co-bands with the marker DNA in Cs_2SO_4 density gradients. When the DNA in the HHP, HP, and LP fractions is treated at high or low salt concentrations with pancreatic ribonuclease (previously heated to 90 C for 5 min) and then sedimented to equilibrium in Cs_2SO_4 density gradients, all of the ^3H activity co-bands with the marker DNA (Fig. 7B).

These results suggest that a fraction of the newly synthesized viral DNA in Ad2-infected KB cells is associated with RNA in such a way that the RNA component of the complex becomes susceptible to digestion by pancreatic ribonuclease at high or low salt concentration. This finding can be explained best by assuming that an RNA chain is hydrogen bonded to DNA by only part of its sequences and that the major part of the RNA chain remains free. The DNA complex can be separated from "free" viral DNA by equilibrium centrifugation in CsCl-propidium iodide density gradients.

In a control experiment, ^{14}C-labeled rRNA from uninfected KB cells was centrifuged to equilibrium in a dye-buoyant density gradient identical to the gradients used for the isolation of the DNA-RNA complex. All the RNA was recovered on the bottom of the centrifuge tube. In the density regions corresponding to the HHP and HP regions, ^{14}C label was not detected. It is concluded that the label appearing in the HHP and HP regions of the dye-buoyant density gradients cannot be due to contamination with free RNA.

The RNA component of the HHP and HP fractions was also labeled with ^3H-uridine. The results of an experiment in which ^3H-uridine has been used to label Ad2-infected KB cells between 22 and 28 h postinfection are illustrated in Fig. 8. After two cycles of equilibrium centrifugation in CsCl-propidium iodide density gradients, the ^3H-uridine label from the HP region bands in Cs_2SO_4 density gradients in the

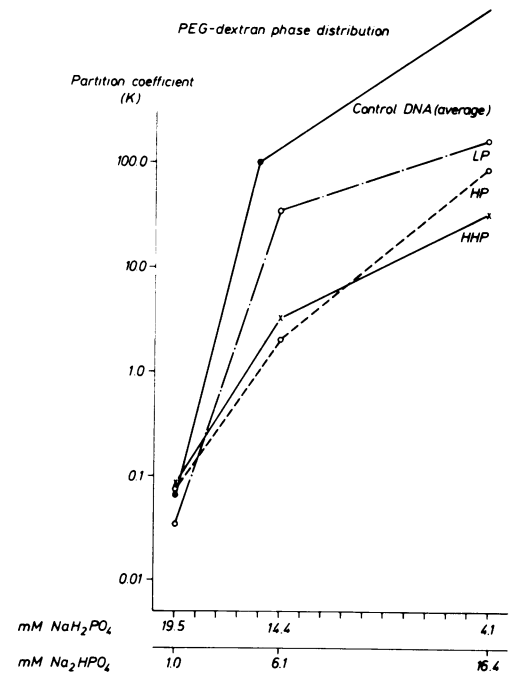

FIG. 6. *Distribution in a polyethylene glycol (PEG)-dextran phase system of DNA samples from dye-buoyant density gradients. The ^3H-labeled HHP, HP, and LP fractions were prepared in experiments similar to the ones described in the legends of Fig. 2 and 3. The partition coefficients (K) (ordinate) of the ^3H-labeled HHP, HP, and LP fractions and of the ^{14}C-labeled Ad2 DNA used as internal control in each experiment were determined as described in Materials and Methods. The relative molarities of NaH_2PO_4:Na_2HPO_4 in the buffer systems were plotted on the abscissa.*

density strata of free RNA and of a DNA-RNA complex. The label from the LP region cosediments exactly with the marker and represents ^3H-uridine incorporated into DNA. Identical results are obtained with the corresponding nucleic acid fractions from uninfected KB cells.

In a control experiment, Ad2-infected KB cells were double labeled with ^3H-uridine and ^{14}C-thymidine (see Fig. 9–11), and the HP and LP fractions were separated by equilibrium centrifugation in CsCl-propidium iodide and Cs_2SO_4 density gradients. The ^3H and ^{14}C activity profiles of the LP fractions coincide exactly, both in CsCl-propidium iodide (Fig. 9) and in Cs_2SO_4 density gradients (Fig. 10, bottom). A portion of the LP material (see Fig. 10, bottom) was dialyzed into TE, made 1.0 M KOH, and heated to 100 C for 10 min prior to neutralization and recentrifugation in a CsCl density gradient. A control sample was treated

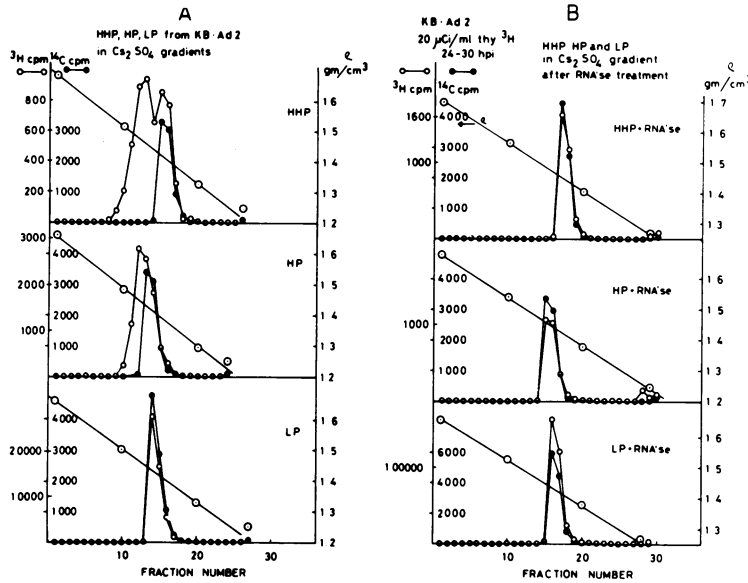

FIG. 7. *Analysis of the HHP, HP, and LP fractions by equilibrium sedimentation in Cs_2SO_4 density gradients. A, Samples of the HHP, HP, and LP fractions, as described in the legend to Fig. 3, were dialyzed against 5 M NaCl in TE and against TE for a total of 54 h and were subsequently centrifuged to equilibrium in Cs_2SO_4 density gradients (see Material and Methods). Each gradient contained 6.4 μg of ^{14}C-labeled Ad2 DNA as density marker. The gradients were centrifuged for 44 h at 35,000 rpm and at 20 C. In every tenth fraction, the refractive indices were measured and the densities were calculated by the equation of Vinograd and Hearst (31). B, In this series of experiments the samples from the HHP, HP, and LP fractions were incubated with pancreatic ribonuclease prior to equilibrium sedimentation. Samples (0.1 ml) of the HHP, HP, and LP fractions were diluted with 0.4 ml of 0.01 M Tris-hydrochloride, pH 7.5, 0.2 M NaCl, and 0.01 M Mg^{2+}, or with 0.4 ml of 0.005 M Tris-hydrochloride and were incubated with pancreatic ribonuclease (18 ug/ml) at 37 C. After 80 min, the mixtures were extracted with twice the volume of phenol saturated with 1 M Tris-hydrochloride, pH 7.5. The phenol was removed by diethylether. Finally, the samples were flushed with N_2 and prepared for equilibrium sedimentation in Cs_2SO_4 density gradients.*

in the same way except that it was heated in TE and not in KOH. The ^3H-^{14}C ratios over the peak of DNA after recentrifugation in CsCl density gradients were determined and found to be identical for the samples heated in 1.0 M KOH and in TE. This result confirms that the ^3H-uridine label found in the LP region is incorporated into DNA.

The data in Fig. 8 demonstrate that the RNA moiety of the HP fraction is partly liberated before or during the final centrifugation procedure, since there is free RNA present in the gradient even prior to thermal denaturation of the HP fraction. This RNA is possibly liberated from the complex because of the shift from solutions of high to low to high salt concentration. After heat denaturation of the HP fraction and banding in Cs_2SO_4 density gradients, the label in the density stratum characteristic for a DNA-RNA complex shifts to the density position of free RNA.

Characterization of the RNA component associated with viral DNA. (i) Isolation by Cs_2SO_4 density gradient centrifugation. The DNA-RNA complex has been labeled both with ^{14}C-thymidine and ^3H-uridine. After equilibrium sedimentation in dye-buoyant density gradients, both the HP region (fractions 6–14) and the LP fractions (fractions 16–17) in Fig. 9 contain ^{14}C and ^3H activities. The results of an experiment in which the HP and LP fractions have been recentrifuged in a second CsCl-propidium iodide gradient are shown in Fig. 10. The HP fraction gives rise to a peak of free RNA (fractions 1–5) and to at least two peaks of RNA-DNA (fractions 7–11) and DNA-RNA (fractions 13–18) complexes containing different amounts of DNA, whereas the LP fractions resediment as a sharp, symmetrical peak of DNA in which the ^3H and ^{14}C activity profiles are matched exactly (see above).

The fractions characterized by the experiment in Fig. 10 have been purified further by equilibrium centrifugation in Cs_2SO_4 density gradients (Fig. 11). It can be shown that the free RNA component (Fig. 11A) and the RNA-DNA complex (Fig. 11C) can be clearly resolved in these gradients. Again, it is observed that the

^3H and ^{14}C activity profiles in the free DNA component are completely congruent. Identical results are obtained in Cs_2SO_4 gradients containing low concentrations of dimethylsulfoxide (34).

(ii) RNA-DNA hybridization. The RNA components of the RNA-DNA and DNA-RNA fractions as well as free RNA as characterized by the experiment described in Fig. 10 have been analyzed by RNA-DNA hybridization (Table 2, experiment 1). In one experiment (Table 2, experiment 2), the RNA component of the complexes was isolated in Cs_2SO_4 density gradients after heat denaturation of the complexes and then used in RNA-DNA hybridization experiments. All RNA fractions hybridize predominantly to viral DNA. There is no difference in the extent of hybridization to viral DNA between the RNA isolated from the complexes and the RNA still in the complex. There is little hybridization to cellular DNA. When the RNA hybridized to viral DNA is eluted (2) from the filters and annealed to cellular DNA in a second hybridization reaction, hybridization to cellular DNA cannot be detected. These data demonstrate that the RNA in the complex is predominantly virus specific.

(iii) Analysis by electrophoresis on polyacrylamide agarose gels. The size of the RNA components of the three fractions (RNA, RNA-DNA, and DNA-RNA) characterized in Fig. 10 has been determined by the method of Peacock and Dingmann (21). By using rRNA from uninfected KB cells as markers (28*S* and 18*S*, corresponding to mol wt of 1.9×10^6 and 0.7×10^6, respectively [20]), molecular weight estimates (21) are obtained. The RNA fraction contains one species of RNA of around 10^5 daltons (Fig. 12A), the RNA-DNA fraction species of approximately 10^6 daltons (Fig. 12B), and the DNA-RNA fraction components as large as 7×10^6 daltons (Fig. 12C). The apparent heterogeneity of some of the minor RNA components in each fraction is probably nuclease action. However, the major components gave sharp peaks in the electropherograms. The molecular weight of the RNA component is highest in those fractions which, judging from the buoyant density, contain the least amount of RNA. Each of the RNA fractions characterized

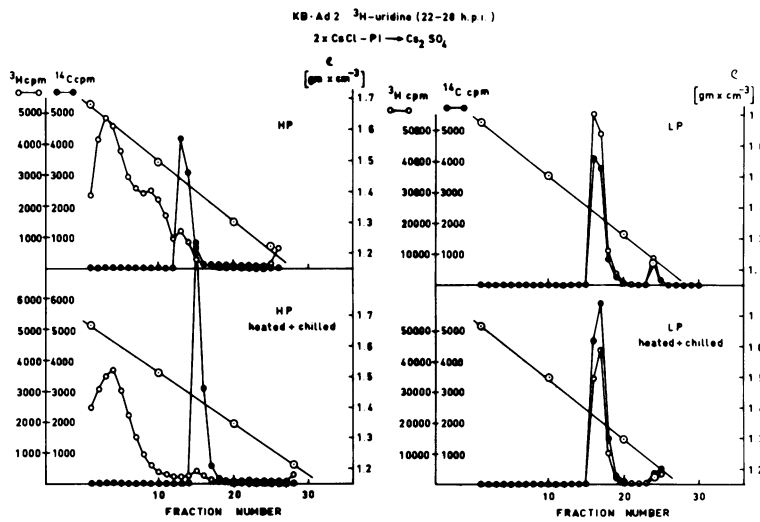

FIG. 8. *Analysis in Cs_2SO_4 density gradients of extracts of Ad2-infected KB cells labeled with ^3H-uridine. KB cells growing in monolayers were inoculated with Ad2 as described in the legend to Fig. 2. At 22 h postinfection, ^3H-uridine (20 μCi/ml) was added to the medium, and at 28 h postinfection, the total intracellular nucleic acid was extracted and centrifuged to equilibrium in dye-buoyant density gradients. The HP and LP fractions were resedimented in dye-buoyant density gradients as described in the legend to Fig. 3. Finally, the HP and LP fractions from the second cycle of equilibrium sedimentation in dye-buoyant density gradients were extensively dialyzed as described in the legend to Fig. 4 and were centrifuged to equilibrium in Cs_2SO_4 density gradients containing 13.4 μg of ^{14}C-labeled Ad2 DNA as a density marker. In the experiments illustrated in the top frames (HP and LP), the samples were unheated; in the experiments illustrated in the bottom frames (HP and LP), the samples were heated to 100 C for 5 min and were chilled in an ice-water bath prior to equilibrium sedimentation. The densities in every tenth fraction were calculated as described in the legend to Fig. 7.*

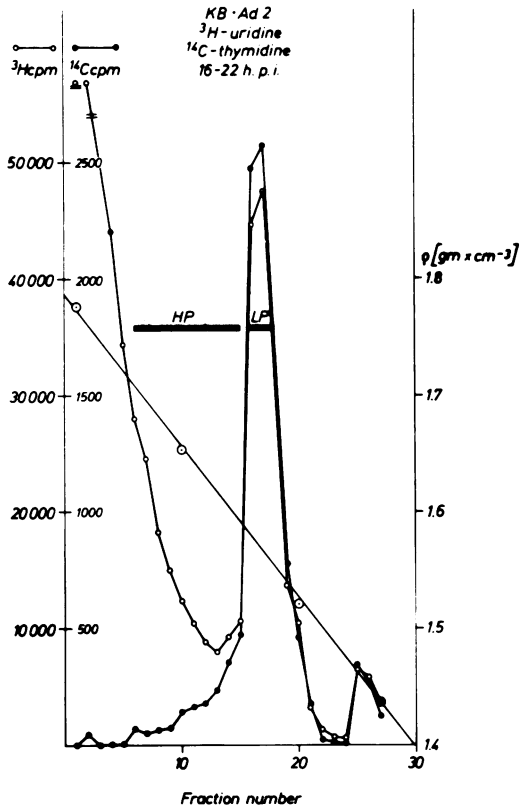

FIG. 9. *Analysis of DNA from Ad2-infected KB cells labeled with ³H-uridine and ¹⁴C-thymidine. KB cells (4.9 × 10⁶ cells per petri dish) growing in monolayers were inoculated with CsCl-purified Ad2 (approximately 10³ PFU/cell) as described in the legend to Fig. 2. At 16 h postinfection, 40 μCi of ³H-uridine and 0.5 μCi of ¹⁴C-thymidine were added per ml of medium. At 22 h postinfection, the cells were washed with PBSd, and the intracellular DNA was extracted and analyzed in a dye-buoyant density gradient by using CsCl and propidium iodide. Samples of all fractions were precipitated with trichloroacetic acid, and the radioactivity was determined to yield the distribution of the ³H and ¹⁴C activities. Fractions 6 to 15 were combined to the HP fraction, and fractions 16 to 18 were combined to the LP fraction. Densities were calculated from the refractive indices as described by Vinograd and Hearst (31).*

in the experiments described above was also subjected to electrophoresis without ¹⁴C-labeled marker RNA. In these experiments, ¹⁴C-labeled material which could be derived from viral DNA only is absent from the gels, indicating that DNA molecules did not enter the gels.

DNA-RNA complexes are not generated by binding of the RNA during extraction. The ominous possibility has to be ruled out that the DNA-RNA complexes described are generated by artifactual association of viral mRNA with viral DNA, possibly with single-stranded stretches in viral DNA, during the extraction and purification procedures. This possibility is highly unlikely because of the following findings: (i) in the absence of macromolecular syntheses, the complexes are not found (8). (ii) Viral marker DNA present during extraction and/or during repeated centrifugation steps in CsCl-propidium iodide and Cs_2SO_4 density gradients is never found in the HP region (Fig. 1, 3, 7, 8). Finally, artifactual binding of viral mRNA was directly ruled out by the following set of experiments. Ad2 mRNA labeled with ³H-uri-

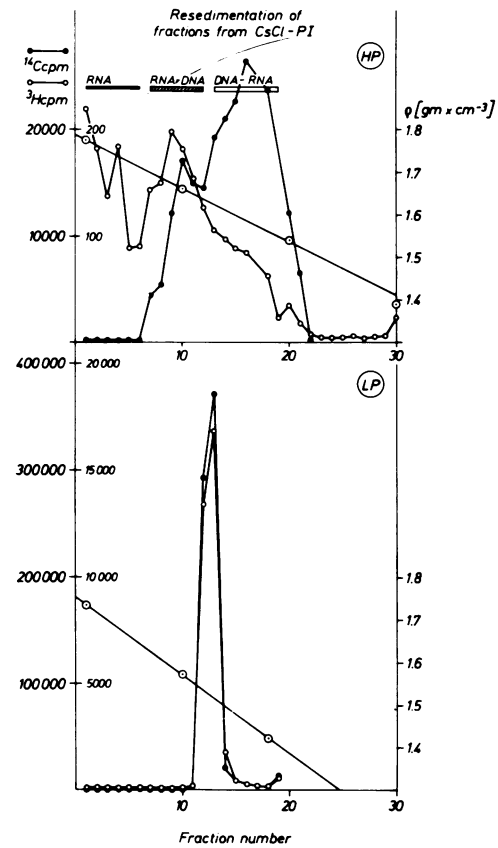

FIG. 10. *Resedimentation of the HP and LP fractions in CsCl-propidium iodide gradients (Fig. 9) in dye-buoyant density gradients. The HP material (top frame) can be resolved into the RNA, RNA-DNA, and DNA-RNA fractions. Judging from the buoyant densities of the fractions, free viral DNA would be expected to band between fractions 20 and 22 in the HP resedimentation experiment. The LP material resediments in the density stratum of free DNA (bottom frame). The experimental conditions are the same as described in the legend to Fig. 9.*

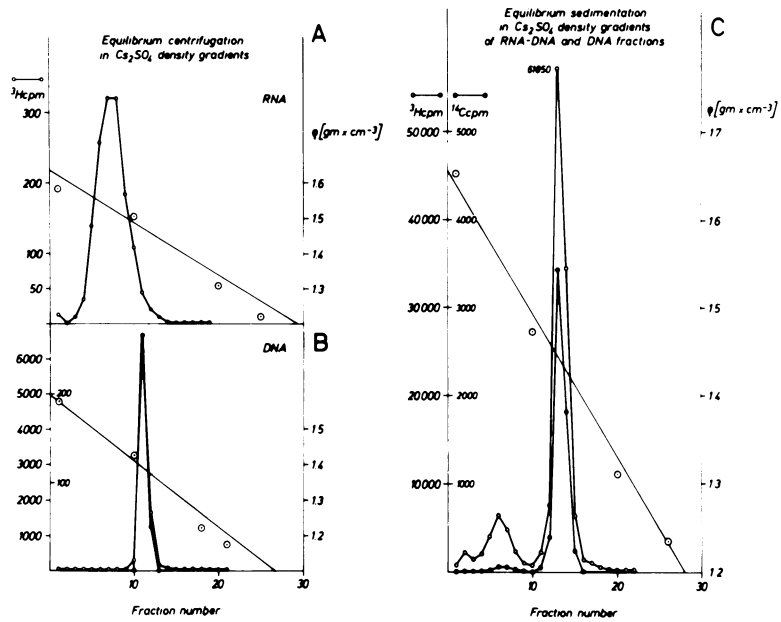

FIG. 11. *Resedimentation in Cs_2SO_4 density gradients. The fractions characterized in Fig. 9 were analyzed further in Cs_2SO_4 density gradients. Fractions from the experiment described in the legend to Fig. 10 were pooled as indicated, and the dye was removed from each fraction as described in Materials and Methods. The density in each fraction was then adjusted to 1.42 g/cm³ by the addition of Cs_2SO_4 powder. The solutions were centrifuged at 35,000 rpm for 40 h at 20 C in the SW56 rotor of the Spinco L2-65B ultracentrifuge. After centrifugation, five-drop fractions were collected, and the ³H and ¹⁴C activities were determined after trichloroacetic acid precipitation of 25-μliter samples of each fraction. The buoyant densities were measured as described in the legend to Fig. 7. A, RNA fraction; B, DNA fraction; C, mixture of the RNA-DNA and DNA fractions.*

dine and isolated from the cytoplasmic fraction of Ad2-infected KB cells was mixed with (i) ¹⁴C-labeled Ad2 DNA, or (ii) ¹⁴C-labeled Ad2 DNA and uninfected KB cells, or (iii) ¹⁴C-labeled Ad2 DNA and Ad2-infected KB cells. Subsequently, each of the mixtures was extracted with STE-pronase-phenol and analyzed by equilibrium sedimentation in dye-buoyant density gradients as described in Materials and Methods. In none of the experiments was ³H activity derived from viral mRNA found in the HP region; all the RNA banded at the bottom of the gradients. The ¹⁴C-labeled viral DNA banded in a sharp symmetrical peak. These controls rule out conclusively the possibility that the complexes are due to artifactual binding of viral mRNA to viral DNA during the extraction and purification of the complexes. The complexes probably originate during transcription of viral DNA.

DISCUSSION

The intracellular DNA of KB cells productively infected with Ad2 has been analyzed by equilibrium sedimentation in dye-buoyant density gradients to investigate whether Ad2 DNA can become circularized in the process of viral DNA replication. Although as much as 15% of the newly synthesized viral DNA has a buoyant density in CsCl-propidium iodide gradients which is characteristic of supercoiled circular DNA, further analysis of this fraction of viral DNA does not support the notion that Ad2 DNA becomes circularized in the cell. Electron microscope examination of the material from the higher buoyant density regions does not reveal circular structures. Furthermore, the results of rate zonal analysis in sucrose density gradients at pH 7.6 and 12.5 are not compatible with the presence of supercoiled circular Ad2 DNA molecules (Fig. 4).

The experimental evidence presented is in agreement with the conclusion that an Ad2-specific DNA-RNA complex can be separated from the bulk of the newly synthesized DNA because of the increased buoyant density of the complex in CsCl propidium iodide density gradients. The complex is sensitive to treatment with pancreatic ribonuclease (Fig. 7), it can be double labeled with ¹⁴C-thymidine and ³H-uri-

dine (Fig. 9), and in Cs_2SO_4 density gradients it has the buoyant density characteristic of a DNA-RNA hybrid (Fig. 8, 11). Furthermore, the ^{3}H-uridine label can be displaced from the hybrid density position towards the density position of free RNA by melting the complex (Fig. 8). The RNA component of the complex is of high molecular weight, as demonstrated by electrophoresis in polyacrylamide-agarose gels (Fig. 12). Both the DNA and RNA components of this complex are virus specific, as can be shown by nucleic acid annealing experiments (Tables 1, 2).

Preliminary data (F. Ortin and W. Doerfler, unpublished results) indicate that the bulk of the RNA isolated from the complexes carries sequences of polyadenylic acid, since more than 80% of the RNA binds to polyuridylic acid-sepharose under suitable conditions (17). This finding further supports the notion that the DNA-RNA complex is involved in transcription. Extensive control experiments described in the last section of Results rule out the possibility that viral mRNA binds artifactually to viral DNA during extraction and purification of the intracellular DNA.

It might be argued that the presence of ^{3}H-uridine label in the density stratum of the complex was due to contamination with free RNA from the bottom of the gradient. However, the data presented in Fig. 9 demonstrate that free RNA is pelleted on the bottom of a dye-buoyant density gradient. This observation is corroborated by the results of a control experiment in which ^{14}C-labeled rRNA from uninfected KB cells is found in the pellet region of a CsCl propidium iodide gradient.

Both parental (Fig. 1) and newly synthesized viral DNA are involved in the formation of the complex. Parental DNA is found as early as 5 h postinfection in the complex, and newly synthesized viral DNA appears in the complex starting 8 h postinfection. The most likely role for the DNA-RNA complex is thought to be in the transcription of viral DNA.

The data obtained so far do not provide information on the question of whether some of the RNA in this complex is covalently linked (26, 33) to viral DNA. It has to be emphasized that the ^{3}H-uridine label incorporated into DNA (in the LP structure) is alkali stable and hence not in RNA. A number of laboratories have reported that a partly single-stranded DNA structure may play a role in the replication of adenovirus DNA (27, 29, 30; G. D. Pearson; U. Pettersson, and H. Delius, personal communications) and that such an intermediate structure may contain RNA (G. D. Pearson, personal communication).

In short pulses of ^{3}H-thymidine of 5-, 10-, and 15-min duration given at 16 h postinfection, 36.4, 43.4, and 32.6%, respectively, of the total

TABLE 2. *Characterization of the RNA moiety from the RNA, RNA-DNA, and DNA-RNA fractions by RNA-DNA hybridization*

Expt no.	^{3}H-labeled RNA	Input (counts/min)	Hybridized counts/min		% of input hybridized	
			KB DNA	Ad2 DNA	KB DNA	Ad2 DNA
1[a]	RNA fraction	41,131	102	4,639	0.25	11.3
	RNA-DNA fraction	9,582	134	3,745	1.4	39.1
	DNA-RNA fraction	1,026	6	1,430	0.6	100
2[b]	HP heated	3,159		473		15.0
	HP unheated	673		325		48.3

[a] The fractions after the second cycle of equilibrium sedimentation in dye-buoyant density gradients (Fig. 9) were used for this analysis. The details of the RNA-DNA annealing procedure are described in Materials and Methods. To each filter, 5 μg of ^{14}C-labeled Ad2 DNA or KB DNA were fixed. By using ^{14}C-labeled DNA on the filter, it was possible to standardize the results to a unit amount of DNA on the filter. In these experiments, the RNA-DNA and DNA-RNA fractions were added directly to the hybridization mixture without previous denaturation. The values represent the mean of double determinations.

[b] Ad2-infected KB cells were labeled with ^{3}H-uridine (20 μCi/ml) and ^{14}C-thymidine (0.1 μCi/ml) from 16 to 23 h postinfection. The HP and LP fractions were isolated by two cycles of equilibrium sedimentation in dye-buoyant density gradients. The dye was removed by dialysis and the HP fractions were resedimented in Cs_2SO_4 density gradients. Prior to equilibrium sedimentation in Cs_2SO_4 density gradients, one-half of the HP material was heat denatured (100 C for 5 min and then chilled). For the DNA-RNA hybridization reaction, the ^{3}H-labeled material from the density position of free RNA (HP heated) and of the DNA-RNA hybrid (HP unheated) was used.

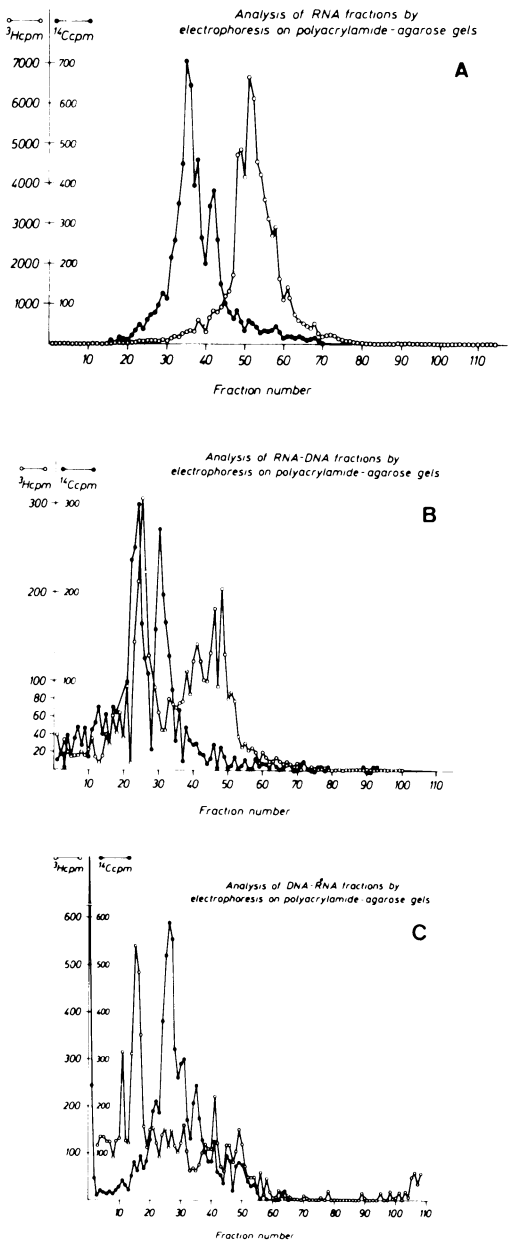

FIG. 12. *Analysis by electrophoresis on polyacrylamide-agarose gels of RNA fractions. Fractions from the experiment described in Fig. 10 (top frame) were pooled as indicated by the horizontal bars, dialyzed against 0.1 × SSC, and analyzed on polyacrylamide-agarose gels as described in Materials and Methods. Electrophoresis is to the right. To each sample ^{14}C-labeled rRNA was added as a marker. A, RNA fraction; B, RNA-DNA fraction; C, DNA-RNA fraction.*

label incorporated into DNA is found in the HP fraction. A high proportion of this label can be chased (30 min, 100 μg of cold thymidine per ml) into the LP position of free viral DNA. Thus, it is likely that a part of the label found in the HP position after short pulses represents partly single-stranded DNA which may be involved in viral DNA replication. When a 6-h labeling period is used, the bulk of the ^{3}H label in the HP region is due to the DNA-RNA complex.

Experiments not presented here have shown that a fraction of the ^{3}H-thymidine incorporated into the DNA of mock-infected KB cells can be separated from the bulk of the newly synthesized DNA in dye-buoyant density gradients and that this fraction is comparable to that incorporated into DNA in Ad2-infected cells. This complex has not been characterized further. If it proved to be of a similar nature as that found in Ad2-infected cells, it may have a general function both in infected and uninfected cells and it may represent an intermediate in transcription.

The results obtained by chromatography of the DNA-RNA complex on BND-cellulose suggest that the complex does not carry exposed single-stranded regions. The RNA component must be associated with DNA in such a way that the RNA remains sensitive to RNase and that the RNA is released from the complex during repeated recentrifugation (Fig. 7) and/or changes in salt concentration. It is significant that the RNA moieties isolated from the various forms of the DNA-RNA complex are rather large in size; hence, extensive nucleolytic degradation cannot have occurred.

Part of the RNA seems to be bound to DNA by hydrogen bonds, since some of the RNA is released from the complex after melting. In an operational sense, the complex differs from double-stranded linear DNA in that the complex binds less propidium iodide and thus can be separated from double-stranded DNA. When ethidium bromide is used instead of propidium iodide in an otherwise identical set of experiments, HP material cannot be detected. There is no evidence that protein is part of the complex, as has been described for a fraction of the intracellular viral DNA in BHK 21 cells abortively infected with Ad12 (8). Experiments, in which Ad2-infected KB cells were labeled with ^{35}S-methionine before or after infection with Ad2, did not reveal any ^{35}S label in the density regions of the HP and LP materials.

The elucidation of the exact structure of the DNA-RNA complex will have to await further analysis, especially by electron microscopy.

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