Intracellular Forms of Adenovirus Deoxyribonucleic Acid

I. Evidence for a Deoxyribonucleic Acid-Protein Complex in Baby Hamster Kidney Cells Infected with Adenovirus Type 12

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The total intracellular deoxyribonucleic acid (DNA) from baby hamster kidney cells abortively infected with ³H-adenovirus type 12 was analyzed in dye-buoyant density gradients. Between 10 and 20% of the cell-associated radioactivity derived from viral DNA bands in a density position which is 0.043 to 0.085 g/cm³ higher than that of viral DNA extracted from purified virions. The DNA in the high-density region (HP-fraction) is almost completely absent when DNA, ribonucleic acid (RNA) or protein synthesis is chemically inhibited in separate experiments. The HP-fraction is not found when the virus does not adsorb to and enter the cell. The DNA in the HP-fraction appears as early as 2 hr after inoculation. At 2 hr after infection, the HP-fraction is present both in the nucleus and the cytoplasm. This DNA hybridizes exclusively with viral DNA and sediments at approximately the same rate in both neutral and alkaline sucrose density gradients. Electron microscopy has revealed no circular DNA molecules in this fraction. Evidence indicates that the viral DNA in the HP-fraction exists in a complex with protein and possibly RNA. The protein component of the complex is resistant to enzymatic digestion, whereas the complex is susceptible to ribonuclease treatment. Digestion with deoxyribonuclease reduces the amount of DNA found in the HP-fraction. The structure and biological function of this complex are currently being investigated.

In baby hamster kidney (BHK-21) cells, adenovirus type 12 (Ad12) cannot replicate its deoxyribonucleic acid (DNA) (9, 13), and fragments of the viral DNA (10) become integrated into the host chromosome (8, 10, 33). Apparently, integration of the DNA of Ad12 can occur even though DNA synthesis is blocked more than 96% in infected cells (10). Similar observations have been reported with Chinese hamster cells infected with simian virus 40 (20). The mechanism of integration of viral DNA into the host chromosome is not understood. In BHK-21 cells abortively infected with Ad12, the parental viral DNA is fragmented to pieces of approximately 5×10^6 daltons (4), presumably by the adenovirus endonuclease (5). It has been proposed that some of these fragments of viral DNA may become integrated into the cellular DNA (4, 10). We have started to analyze the intracellular forms of viral DNA in order to investigate whether the viral DNA has to be in a specific conformation before

it can become integrated. We have begun a similar study to search for intermediates in viral DNA replication in KB cells productively infected with adenovirus type 2 (Ad2; Burger, Hirsch-Kauffmann, and Doerfler, *Fed. Proc.* 1972, *in press*).

Evidence will be presented that, in abortively infected BHK-21 cells, a form of Ad12 DNA occurs which can be distinguished from the bulk of the parental viral DNA by its higher density in dye-buoyant density gradients. The results suggest that this new form of viral DNA exists in a complex with (cellular) protein and possibly with ribonucleic acid (RNA). The amount of DNA in the complex is reduced when DNA, RNA, or protein synthesis is chemically inhibited before and during infection of BHK-21 cells with Ad12.

MATERIALS AND METHODS

Cell culture media. The composition of Eagle spinner medium (16) and of reinforced Eagle (ETC) medium (1) has been described (9). Lipostabilized calf serum was purchased from the Grand Island Biolog-

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ical Co. Tryptone broth was supplemented with, per milliliter: 10 mg each of L-threonine and L-leucine, 1 mg of thiamine, and 0.1 mg of thymidine.

Solutions. PBS is phosphate-buffered saline (14). TE is 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.2 to 7.5, and 0.001 M ethylenediaminetetraacetate (EDTA). STE is 0.5% sodium dodecyl sulfate (SDS) in 0.1 M Tris-hydrochloride and 0.02 M EDTA. The composition of the neutral CsCl solution has been published elsewhere (8).

Cells and viruses. Origins of the human epidermoid carcinoma (strain KB) (15) and BHK-21 (29) cells, Ad2, and Ad12 have been given (8, 9). *Escherichia coli* B cells, grown to mid-log phase, were obtained from the Grain Processing Corp., Muscatine, Iowa. *E. coli* strain CR34 (λ C_I 857 S7) was a gift of W. F. Dove, University of Wisconsin.

Chemicals and radioisotopes. The source of most of the chemicals used has been reported (8-10). Sepharose 4B for gel filtration was purchased from Pharmacia, Uppsala; propidium iodide was from Calbiochem, Los Angeles, Calif.; the nonionic detergent P-40 was from Shell Chemical Co., a Division of Shell Oil Co., New York, N.Y.; and acrylamide and *N,N'*-methylenebisacrylamide were from Canal Industrial Corp., Rockville, Md.

The specific activity and the source of the radioisotopes used were as follows: thymidine-6-³H, 20 to 26 Ci/mmmole, Amersham/Searle Corp.; sodium salt of ¹⁴C-formic acid, 50 to 55 mCi/mmmole, Schwarz BioResearch Inc., Orangeburg, N.Y.; D-glucosamine-6-³H, 3.6 Ci/mmmole, New England Nuclear Corp.; L-methionine-³⁵S, 1.6 Ci/mmmole, Schwarz BioResearch, or 18 Ci/mmmole, Amersham/Searle Corp.; reconstituted protein ³H-hydrolysate, 5.0 mCi/ml, Schwarz BioResearch.

Enzymes. The preparations of Pronase, deoxyribonuclease, and ribonuclease were as reported previously (10).

Methods for the growth, purification and radioactive labeling of Ad2 and Ad12. Methods for the growth, purification, and labeling of Ad2 and Ad12 have been described (8-10). When Ad12 was labeled with both thymidine-6-³H and L-methionine-³⁵S, spinner medium, containing only 13.3% of the amount of L-methionine present in regular medium and 0.7 μ Ci of L-methionine-³⁵S per ml of medium, was used. At 10 hr after infection, 1 μ Ci of thymidine-6-³H per ml of medium was also added. The specific radioactivity of such a preparation was 1,120 counts/min of ³⁵S per μ g of viral protein and 3.2×10^4 counts per min of ³H per μ g of viral DNA. Ad2 was labeled by adding 1 μ Ci of ³H-labeled reconstituted protein hydrolysate per ml of culture medium 2 hr after infection.

E. coli strain CR34 (λ C_I 857 S7) was grown in supplemented tryptone broth and was induced (19). Bacteriophage λ C_I 857 S7 was purified as described elsewhere (11).

Extraction of viral DNA. The published procedure (9) for the extraction of DNA from Ad2 and Ad12 was modified: Ad2 and Ad12 were purified from extracts of infected KB cells by three cycles of equilibrium sedimentation in CsCl density gradients in 0.02

M Tris-hydrochloride, pH 7.2 to 7.5. The samples were then dialyzed into 0.02 M Tris-hydrochloride, pH 7.2 to 7.5. The DNA was extracted immediately after dialysis by adding 500 μ g of Pronase per ml and by making the solution 0.4% in SDS, 0.03 M Tris-hydrochloride (pH 7.2 to 7.5), and 0.002 M EDTA. The mixture was incubated at 37 C for 30 to 60 min, and the DNA was further purified by three extractions with two volumes of twice-distilled phenol which were saturated with 1 M Tris-hydrochloride, pH 7.2 to 7.5. After the last phenol extraction, the aqueous phase was recovered, and the phenol was removed by extensive dialysis against TE.

The DNA of bacteriophage λ was extracted from the purified phage as described earlier (11).

Labeling of cellular proteins and cellular DNA. Growth of BHK-21 cells and specific labeling of cellular proteins with ³⁵S-L-methionine proved to be optimal at a methionine concentration of 20% of the amount present in regular ETC medium. In these experiments, between 1 and 22 μ Ci of ³⁵S-L-methionine per ml of medium was added, and cell replication proceeded for three to four days. In several experiments, the specific radioactivity of cellular proteins ranged between 11,000 and 91,000 counts of ³⁵S per min per μ g of cellular protein. The specific activity was determined by measuring the protein concentration (25) and the ³⁵S activity.

In some experiments, the cellular DNA was also labeled by adding 1 μ Ci of thymidine-6-³H per ml of medium.

Infection of monolayers of cells and extraction of intracellular DNA. Monolayers of BHK-21 cells (8.8×10^5 to 2.67×10^7 cells per 60-mm petri dish) were infected with CsCl-purified (three cycles) ³H-Ad12 [1.38 to 4.66 optical density (OD)₂₆₀ units, 4.9×10^5 to 2.3×10^6 counts per min per OD₂₆₀ unit] or with unlabeled Ad12 (0.96 to 8.82 OD₂₆₀ units).

At the end of the adsorption period or at various times after infection, each cell sheet in a 60-mm petri dish was extensively washed and then lysed by the addition of 1 ml of STE containing 500 μ g of Pronase. The lysate was incubated at 37 C for 30 min and was extracted two to three times with phenol. After the initial phenol extractions, the phenol phase was removed, and the aqueous phase and the interphase were reextracted. After the final phenol extraction, the phenol phase was removed, and the phenol from the aqueous phase was removed by extensive dialysis against TE or by ether extraction followed by evaporation of the ether with N₂. The analysis of intracellular viral DNA by the dye-buoyant density gradient method (21) gave identical results, regardless of which method for the removal of phenol was used. All other conditions were as described (8, 10). The recovery of intracellular viral DNA by this method was between 95 and 100%.

Growth of BHK-21 cells in 5-BUDR-containing medium. Growth conditions of BHK-21 cells in 5-bromodeoxyuridine (5-BUDR)-containing medium were described (8).

Preparation of nuclei and cytoplasm. A modification of a previously published procedure (24) was used. Monolayers of infected or uninfected BHK-21 cells

were washed five times with 0.14 M NaCl, 2.7 mM KCl, 0.045 M sodium phosphate (pH 7.2), and 5 mM MgCl₂ (buffer A). The cells were then scraped off and washed once more in buffer A. Next, the cells were suspended in 0.14 M NaCl, 0.01 M Tris-hydrochloride (pH 8.4), and 1.5 mM MgCl₂ (buffer B). The suspension was then made 0.5% with the nonionic detergent P-40. The nuclei were pelleted by centrifugation at 2,000 rev/min for 5 min in an International centrifuge (model PR-6). The supernatant fluid was decanted, and the nuclei were suspended in buffer B which contained 0.5% P-40. The nuclei were pelleted and suspended again, giving the nuclear fraction. The supernatant fractions were combined into the cytoplasmic fraction.

Sedimentation experiments. The methods used in equilibrium sedimentation in neutral and alkaline CsCl density gradients and in zonal sedimentation in neutral and alkaline sucrose density gradients have been described (8, 9, 10). All sucrose density gradients were 5 to 20%. The neutral gradients were in 1 M NaCl and TE; the alkaline gradients were in 0.3 M NaOH, 0.7 M NaCl, and 0.001 M EDTA.

Equilibrium sedimentation in dye-buoyant density gradients. The procedure of Hudson et al. (21) was used with minor modifications. Each reaction mixture contained the extracts of adenovirus-infected or uninfected cells (each derived from one petri dish 60 mm in diameter) in a total volume of 3.0 ml of 3 mM Tris-hydrochloride (pH 7.2 to 7.5), 3.6 mM EDTA, 2.3 g of CsCl, and 580 to 670 μ g of propidium iodide per ml. In many of the experiments, 1 to 2 μ g of ¹⁴C-labeled DNA extracted from purified Ad12 was added as a marker. The buoyant density of the solution was adjusted to 1.576 to 1.582 g/cm³. The mixtures were filled into nitrocellulose tubes, were overlaid with mineral oil, and were centrifuged in an SW 56 rotor in a Spinco ultracentrifuge (model L2-65B) at 40,000 rev/min for 36 to 48 hr at 20 C. At the end of the centrifugation, five-drop fractions (approximately 0.10 ml) were collected, and the distribution of the ³H, ¹⁴C, or ³⁵S activity was determined after acid precipitation of each fraction (10).

DNA-DNA hybridization. The method of Denhardt (7) was used.

Enzymatic digestion. Deoxyribonuclease (ribonuclease-free) was dissolved at a concentration of 500 μ g/ml in 0.01 M Tris-hydrochloride (pH 7.2), 0.01 M CaCl₂, and 0.01 M MnCl₂ (28). A solution of ribonuclease A (phosphate-free) was prepared in 0.01 M Tris-hydrochloride (pH 7.2), 0.01 M MgCl₂ at a concentration of 500 μ g/ml. This solution was heated for 10 min until it reached 95 C to destroy any contaminating deoxyribonuclease. Pronase was dissolved in 0.01 M Tris-hydrochloride (pH 7.2) at a concentration of 5 mg/ml. The extracts of infected cells (see above) were dialyzed for 20 hr against two changes (2 $\times$ 1,000 ml) of 0.02 M Tris-hydrochloride, pH 7.2. For enzymatic digestion, deoxyribonuclease and ribonuclease were used at a concentration of 50 μ g/ml and Pronase was used at a concentration of 500 μ g/ml, in the respective solutions. The mixtures were incubated for 60 min at 37 C and extracted twice with phenol. The phenol was removed by ether extraction, and the ether was evaporated with N₂. The extracts were then pre-

pared for equilibrium sedimentation in dye-buoyant density gradients.

Polyacrylamide-SDS gel electrophoresis. The intracellular viral DNA was fractionated into the heavy (HP) and light (LP) peak fractions by equilibrium centrifugation in dye-buoyant density gradients. The fractions comprising the HP were pooled, dialyzed, and concentrated by flash evaporation. For gel electrophoresis, 0.07 ml of the concentrated HP fractions, 0.05 ml of Ad2 labeled with ³H-amino acids, 0.03 ml of a 10% solution of SDS in water, 2 μ liters of 2-mercaptoethanol, 0.02 ml of glycerol, 2 μ liters of 10 M urea, and 0.1 ml of 0.01 M potassium phosphate (pH 7.0) were mixed, placed in a boiling-water bath for 2 min, and subjected to electrophoresis in polyacrylamide-SDS gels by the method of Maizel (26). The gels and all buffers contained 0.1% urea. At the end of the electrophoresis, the gels were frozen on a block of solid CO₂ and sliced into 60 to 70 fractions. Each fraction was processed and counted.

Methods for electron microscopy of DNA. These methods were reported elsewhere (12).

Supercoiled forms of the DNA of bacteriophage λ . These forms were prepared by the procedure developed by Gellert (17).

Physical methods. The physical methods were described in an earlier paper (10).

RESULTS

In the course of a systematic investigation of the intracellular forms of adenovirus DNA in abortively infected cells, the question arose as to whether adenovirus DNA or fragments of the viral DNA (4) become circularized inside infected cells. The total intracellular DNA from Ad12-infected BHK-21 cells was extracted and analyzed by the dye-buoyant density method with propidium iodide (21).

High-density DNA fraction in dye-buoyant density gradients. The data presented in Fig. 1 and Table 1 demonstrate that, between 2 and 34 hr after infection of BHK-21 cells with ³H-Ad12, 8.2 to 20.4% of the ³H-labeled, intracellular DNA had a density in dye-buoyant density gradients which was 0.043 to 0.085 g/cm³ greater than the density of ¹⁴C-labeled Ad12 marker DNA extracted from purified virions. In some of the experiments, an intermediate density peak was observed. The fraction of DNA in the high and intermediate density regions will be referred to as HP (heavy peak). Most of the ³H-labeled viral DNA assumed an equilibrium position identical to that of the marker DNA (LP = light peak). Apparently, Ad12 has to adsorb to and enter into BHK-21 cells before the HP fraction can be produced. The HP fraction was absent in a control experiment (Fig. 1, control) in which the total intracellular DNA was extracted immediately after the addition of ³H-Ad12 to a monolayer of BHK-21 cells.

FIG. 1. Equilibrium centrifugation in dye-buoyant density gradients of the total intracellular DNA extracted from BHK-21 cells infected with ^3H -Ad12. Monolayers of BHK-21 cells (2.1×10^6 cells/plate) were inoculated with 1 ml of ^3H -Ad12 ($\text{OD}_{260} = 3.0$; 1.9×10^6 counts per min per OD_{260} unit). After a 2-hr adsorption period at 37°C , the cell sheets were washed extensively with PBS. The cells were either immediately lysed by adding 1 ml of STE containing 500 μg of Pronase (2 hr postinfection), or 5 ml of ETC medium was added to the monolayers (for later time points), and incubation was continued at 37°C . At indicated intervals, the cells were washed again with PBS and lysed, and the intracellular DNA was extracted and analyzed in dye-buoyant density gradients. To each gradient was added 3 μg of ^{14}C -labeled Ad12 DNA as a density marker. The percentage figures between the vertical broken lines indicate the relative amounts of ^3H activity recovered in the respective areas of the gradient. In the control

TABLE 1. Distribution of parental viral DNA in dye-buoyant density gradients^a

Time after infection (hr)	^3H activity (%) recovered in		
	HP ^b	LP ^b	$\Delta\rho^c$ (g/cm^3)
2	17.6	74.2	0.072
6	12.2	80.1	0.073
10	8.2	85.3	0.085
19	20.4	69.5	0.058
24	18.1	52.1	0.043
34	14.8	75.9	0.063

^a BHK-21 cells were infected with ^3H -Ad12, and, at various times after infection, the total intracellular DNA was analyzed in dye-buoyant density gradients.

^b Percentage of ^3H activity recovered in the gradients in the heavy (HP) and light peaks (LP) was calculated as indicated in Fig. 1. A minor portion of the ^3H activity was found in the gradient in a region slightly less dense than that of the LP.

^c Difference in buoyant density between HP and the peak of the ^{14}C -labeled marker DNA was calculated. The density of the fractions was derived from the refractive indexes by using the equation of Vinograd and Hearst (31).

The DNA extracted from uninfected BHK-21 cells formed one peak in dye-buoyant density gradients (Fig. 2a). A minute amount (0.17%) of the ^3H activity was found in the HP region (not discernible at the scale used in Fig. 2a) and is presumably due to mitochondrial DNA.

When the DNA extracted from purified Ad2 or Ad12 was analyzed in dye-buoyant density gradients, only one peak was observed (Fig. 2b). Two possibilities were considered: that the viral DNA may be a supercoiled circular molecule inside the virion and that the endonuclease associated with adenoviruses (5) may convert the supercoiled molecule to a linear molecule before or during the extraction. It has been shown that the adenovirus endonuclease can be completely inhibited by 1 mM 2-mercaptoethanol (5). Ad2 was purified, and the viral DNA was extracted under conditions such that all solutions contained 1 mM 2-mercaptoethanol. The data presented in

experiment (graph on the bottom of figure), 0.2 ml of ^3H -Ad12 ($\text{OD}_{260} = 3.1$; 6.2×10^6 counts per min per OD_{260} units) was added to a monolayer of BHK-21 cells (2.7×10^6 cells/plate). Adsorption of the virus was not allowed to occur. The cells were lysed immediately, the DNA was extracted and analyzed by the dye-buoyant density gradient technique. Refractive indexes were determined in the fractions indicated, and the densities were calculated by the equation of Vinograd and Hearst (31).

FIG. 2. Equilibrium centrifugation of cellular DNA from uninfected cells and of viral DNA from purified virus in dye-buoyant density gradients. (a) ^3H -labeled DNA from uninfected BHK-21 cells was analyzed in a dye-buoyant density gradient. (b) Ad2 was purified as described previously (9), except that 1 mM 2-mercaptoethanol was present in all solutions during purification and dialysis of the virus preparation. The viral DNA was extracted as described in Materials and Methods, and all solutions contained 1 mM 2-mercaptoethanol. The purified DNA was then analyzed in a dye-buoyant density gradient which contained ^{14}C -labeled Ad2 DNA as a marker. The ^{14}C -labeled DNA was prepared without the use of 2-mercaptoethanol. Densities were calculated as described in the legend of Fig. 1.

Fig. 2b give no evidence for the existence of any form of viral DNA other than a linear double-stranded molecule, regardless of the presence or absence of 2-mercaptoethanol before or during extraction.

The HP fractions from several experiments were pooled and centrifuged to equilibrium in a dye-buoyant density gradient. The ^3H activity from the HP regions exhibited a density that is 0.044 g/cm^3 greater than that of the ^{14}C -labeled marker DNA (Fig. 3).

In another type of control experiment, the DNA of BHK-21 cells was made heavy by growing the cells in the presence of 5-BUdR (8). These cells were infected with ^3H -Ad12. Twenty-four hours after infection, the total intracellular DNA was extracted, and the viral and cellular DNA were separated by equilibrium centrifugation in neutral CsCl density gradients (8). The viral DNA

FIG. 3. Resedimentation of the HP-fractions in a dye-buoyant density gradient. BHK-21 cells were infected with ^3H -Ad12, and the total intracellular DNA was extracted 2 hr after infection and was analyzed in dye-buoyant density gradient. The HP fractions were pooled and centrifuged in a dye-buoyant density gradient. To the gradient was added $0.26 \mu\text{g}$ of ^{14}C -labeled Ad12 DNA as a marker. The buoyant densities were determined as described in the legend of Fig. 1.

was recentrifuged in a dye-buoyant density gradient. More than 27% of the ^3H activity from the viral DNA peak banded in the HP region in two peaks which have buoyant densities 0.131 and 0.086 g/cm^3 higher than the ^{14}C -labeled Ad12 DNA used as a marker (Fig. 4).

These data indicate that the ^3H -labeled DNA in the HP fractions derives from a portion of the viral DNA which binds less propidium iodide and, hence, bands in a higher buoyant density position than linear, double-stranded DNA.

Characterization of HP by DNA-DNA hybridization. The DNA in both the HP and LP regions was analyzed by DNA-DNA hybridization to cellular and viral DNA. The data in Table 2 demonstrate that the ^3H -labeled DNA in the HP and LP-fractions hybridized exclusively with viral DNA. This finding rules out the possibility that the HP fraction results from a form of viral DNA that has been integrated into cellular DNA (8, 10).

Zonal sedimentation in sucrose density gradients at pH 7.6 and 12.5. Supercoiled circular DNA from bacteriophage λ sediments at rates 3.8 and 1.5 times faster than the same molecule in the

FIG. 4. Resedimentation of intracellular viral DNA in a dye-buoyant density gradient. Monolayers of BHK-21 cells were grown for 72 hr in ETC medium containing 5 μ g of 5-BUDR per ml. These cells were inoculated with ^3H -Ad12 (3.0 OD_{260} units/petri dish; 1.89×10^6 counts per min per OD_{260} unit). After a 2-hr adsorption period, the cells were washed extensively, and ETC medium containing 5 μ g of 5-BUDR per ml was added. At 24 hr postinfection, the total intracellular DNA was extracted and analyzed. To each gradient, ^{14}C -labeled Ad12 DNA was added as a marker. Buoyant densities were determined as described in the legend of Fig. 1.

linear configuration in alkaline and neutral sucrose density gradients, respectively (2). The corresponding values for the DNA of polyoma virus are 3.3 and 1.43 (32).

The data in Fig. 5a demonstrate that the DNA from the HP region in dye-buoyant density gradients sedimented in alkaline sucrose density gradients in two peaks, 3.3 and 2.4 times faster than the marker DNA, whereas the DNA from the LP region cosedimented with the marker. Similar results were obtained in seven independent zonal sedimentation experiments in alkaline sucrose density gradients: the DNA from the HP region sedimented in two peaks with S values of 100S (mean of seven determinations, range 73 to 134S) and 76.5S (mean of four determinations, range 66 to 93S).

In neutral sucrose density gradients, the DNA from the HP regions sedimented in three peaks, 3.76 to 2 times faster than the marker DNA (Fig. 5b). The mean S values for the two fastest peaks were determined in three independent experiments to be 102S (range 98 to 109S) and 75S (range 70 to 80S). The DNA from the LP regions also cosedimented with the marker DNA in neutral sucrose density gradients.

Hence, the DNA from the HP region in dye-buoyant density gradients sedimented in at least two size classes in neutral and alkaline sucrose

TABLE 2. Characterization of the DNA in the HP and LP fractions by DNA-DNA hybridization^a

Unlabeled DNA on filter	^3H -Labeled DNA from	Hybridization (%)
Ad12 DNA	HP	100
	LP	72.7
	Ad12	85.5
	BHK-21 cells	0.56
BHK-21 DNA	HP	0
	LP	1.2
	Ad12	0.76
	BHK-21 cells	10.6

^a 5-BU-BHK-21 cells (6.9×10^6 cells/plate) were inoculated with ^3H -Ad12 (3.86 OD_{260} units; 4.93×10^5 counts per min per OD_{260} unit). At 2.5 hr after infection, the total intracellular DNA was extracted and analyzed by equilibrium sedimentation in a dye-buoyant density gradient (CsCl-propidium iodide). The fractions in the high-density peak (HP) and the low-density peak (LP) were pooled, dialyzed against TE, and hybridized to Ad12 DNA and BHK-21 DNA. The figures give the mean of two determinations. Control experiments were carried out with ^3H -labeled DNA from Ad-12 and BHK-21 cells.

density gradients. The S values of these classes of DNA were 3.52 and 2.58 times higher than the S value of linear marker DNA in neutral (pH 7.6) sucrose density gradients and 3.0 and 2.32 times higher in alkaline (pH 12.5) sucrose density gradients. This result is not consistent with the notion that the DNA in the HP region is a supercoiled circular molecule.

Examination of intracellular viral DNA by electron microscopy. The DNA in the HP fraction was also analyzed by electron microscopy. Only linear molecules were observed. To be certain that a small amount of circular DNA might not have been lost, the total intracellular viral DNA was isolated from 5-BUDR-prelabeled BHK-21 cells. The viral DNA was once more centrifuged to equilibrium in a CsCl density gradient to minimize contamination with cellular DNA. After the second equilibrium centrifugation experiment, the viral DNA was dialyzed against TE and was examined in the electron microscope. In three independent experiments, only linear molecules were observed.

When supercoiled circular λ DNA, which was synthesized by the method of Gellert (17), was examined in the electron microscope under identical conditions, circular molecules were readily detected.

Distribution of HP fraction between cytoplasm and nucleus. The data in Table 3 indicate that, at

FIG. 5. Zonal sedimentation at pH 7.6 and 12.5 of DNA from the HP and LP regions in sucrose density gradients. (a) Alkaline sucrose density gradients. BHK-21 cells (1.1×10^6 cells/plate) were inoculated with ^3H -Ad12 (4.4 OD_{260} units; 7×10^5 counts per min per OD_{260} unit). At 2 hr postinfection, the cells were washed extensively, and the total intracellular DNA was extracted and fractionated into the HP and LP fractions in dye-buoyant density gradients. The dye was removed from the combined HP and LP fractions by extensive dialysis against 5 M NaCl in TE, followed by dialysis against TE (21). To 0.2-ml samples of the HP and LP fractions were added approximately 1 μg of ^{14}C -labeled Ad12 DNA as a marker and 0.02 ml of 1 N NaOH. The samples were then layered on top of alkaline sucrose density gradients. Sedimentation was performed in an SW 56 rotor at 4 C and at 45,000 rev/min for 75 min in an L2-65B ultracentrifuge (Spinco). At the end of the run, six-drop fractions were collected and the DNA in each fraction was acid-precipitated (10). (b) Neutral sucrose density gradients. The conditions were similar to the ones described in (a), with the following exceptions. For HP, the intracellular DNA was extracted from BHK-21 cells. ^3H -Ad12 24 hr postinfection. The HP fraction was sedimented into a neutral sucrose density gradient in an SW 56 rotor at 4 C and at 40,000 rev/min for 70 min. For LP, BHK-21 cells (2.67×10^6 cells/plate) were inoculated with ^3H -Ad12 (3.1 OD_{260} units; 6.2×10^5 counts per min per OD_{260} unit). The intracellular DNA was extracted at 24 hr postinfection, and the LP fraction was isolated and dialyzed as described. Sedimentation was performed in an SW 56 rotor at 4 C and at 45,000 rev/min for 100 min. The S values were calculated relative to the ^{14}C -labeled marker Ad12 DNA assuming S values of 29 and 33S for Ad12 DNA at neutral and alkaline pH values, respectively (9), and assuming isokinetic conditions of centrifugation in the SW 56 rotor (B. T. Burlingham, Ph.D. thesis, Rockefeller Univ., New York, N. Y., 1970). The figures in brackets under the S values indicate the factor of increase in S value over that of the marker DNA.

2 hr postinfection, 43% of the cell-associated viral DNA could be recovered from the nuclear fraction and that about equal amounts of the HP fraction were found in the nucleus and in the cytoplasm.

Effect of inhibition of macromolecular synthesis. When DNA, protein, or RNA synthesis was chemically inhibited before and during inoculation of BHK-21 cells with ^3H -Ad12, the amount

of cell-associated ^3H activity which banded in dye-buoyant density gradients in the HP region was reduced 10- to 20-fold (Fig. 6). In the control experiment in which no inhibitors were used, 18.5% of the cell-associated ^3H activity appeared in the HP region; when DNA, protein, or RNA synthesis was inhibited, the corresponding values were 1.7, 1.2, and 0.9%, respectively. The amount of ^3H -Ad12 becoming associated with the cells

TABLE 3. Occurrence of HP and LP in the nuclear and cytoplasmic fractions of BHK-21-³H-Ad12^a

Determination	³ H activity (%) in	
	Nucleus	Cytoplasm
Cell-associated parental viral DNA (2 hr PI ^b).....	43.3	56.7
³ H activity in HP.....	23.2	17.6
³ H activity in LP.....	76.8	82.4

^a BHK-21 cells growing in monolayers (8.8×10^5 cells/plate) were infected with ³H-Ad12 (2.26 OD₂₆₀ units; 1.66×10^6 counts per min per OD₂₆₀ unit). At 2 hr postinfection, the inoculum was removed, and the cell sheets were washed with PBS. The nuclear and cytoplasmic fractions were prepared as described in Materials and Methods, and to each fraction was added one-tenth the volume of STE and of Pronase solution (5 mg/ml) in 0.01 M Tris, pH 7.5. The mixtures were incubated at 37 C for 30 min and then extracted with phenol for the preparation of intracellular DNA. The nuclear and cytoplasmic extracts were then analyzed by the dye-buoyant density method in the presence of propidium iodide, and the proportion of the ³H activity in the heavy (HP) and light (LP) density regions was determined.

^b Postinfection.

was not reduced when chemical inhibitors were present in the virus suspension during adsorption.

It is concluded that DNA, protein, and RNA syntheses are necessary for the generation in BHK-21 cells of the fraction of viral DNA that appears in the HP region in dye-buoyant density gradients.

Effect of enzymatic digestion of intracellular DNA on occurrence of HP. The experimental data presented thus far suggest that between 10 and 20% of the cell-associated viral DNA was converted, shortly after infection, into a form of viral DNA which binds less propidium iodide and, hence, bands in a higher density stratum in dye-buoyant density gradients than the DNA extracted from purified virions. DNA, RNA, and protein syntheses are required for this conversion. Although the occurrence of supercoiled circular molecules cannot be ruled out entirely, examination by electron microscopy has not revealed any circular structures but only linear molecules. We propose as a model that a fraction of the cell-associated viral DNA forms a tightly packed complex, possibly also containing protein and RNA. Because of steric restriction, this fraction of viral DNA cannot bind as much propidium iodide as free viral DNA, and therefore has a higher buoyant density in dye-CsCl gradients.

The protein component in this complex is protected from the action of Pronase and phenol during the extraction of intracellular viral DNA.

This model was further tested by subjecting the extracted intracellular DNA to digestion with ribonuclease or deoxyribonuclease or to a second incubation with Pronase, under conditions described above. After ribonuclease digestion, the amount of ³H activity in the HP fraction in dye-buoyant density gradients was markedly reduced, from 11.5% in the control to 2.7%. This result suggests that RNA may be a component of a complex involving viral DNA and that the RNA is accessible to enzymatic digestion. Treatment of the intracellular DNA with deoxyribonuclease led to a slight reduction in the amount of the HP fraction, from 11.5% in the control to 7.0%. A second incubation with Pronase had little or no effect on the amount of ³H label in the HP fraction.

Prelabeling of cellular protein. If the model described above is correct, cellular proteins might be found associated with the HP fraction. BHK-21 cells were grown for several days in medium containing ³⁵S-L-methionine and were then inoculated with ³H-Ad12. The total intracellular DNA was extracted, and the distribution of the ³H and ³⁵S labels was determined in dye-buoyant density gradients. The ratio of the ³⁵S activity to the ³H activity in the HP region was at least ten times higher than in the LP region (Fig. 7). This result has been obtained in four independent experiments, with DNA extracted as early as 2 hr after infection. Considering the fact that the DNA concentration in the LP region, which contains practically all of the cellular DNA, was approximately 10^3 times higher than in the HP region, the amount of ³⁵S label per unit amount of DNA was higher in the HP region by a factor of about 10^4 . This result indicates that the ³⁵S label in the HP region is bound specifically to a certain viral DNA fraction and not unspecifically to any DNA molecule. Since the ³⁵S label in the HP region is observed as early as 2 hr after infection, it is unlikely that the ³⁵S label has been incorporated into a viral protein. Rather, the data indicate that cellular protein binds to viral DNA in a complex fashion. From a knowledge of the specific radioactivity of the cellular proteins, it can be estimated that only a fraction of a nanogram of cellular protein is associated with the DNA in the HP fraction.

In the first of two control experiments, Ad12 virus labeled with ³⁵S-L-methionine and ³H-thymidine was prepared. BHK-21 cells were inoculated with such virus preparations, and the intracellular DNA was extracted at 2, 7.5, and

FIG. 6. Effect of inhibition of macromolecular syntheses on the occurrence of HP. To the medium of BHK-21 cells (2.5×10^6 cells/plate) was added one of the following inhibitors: $30 \mu\text{g}$ of 1- β -D-arabinofuranosylcytosine (ara C), $75 \mu\text{g}$ of cycloheximide, or $20 \mu\text{g}$ of actinomycin D in PBS, per ml of medium. Controls received an identical volume of PBS. The chemical inhibitors were added 4.5 hr before infection with ^3H -Ad12 (2.1 OD_{260} units; 1.52×10^6 counts per min per OD_{260} unit). The virus inoculum contained inhibitor at the same concentration. It has been shown that, at the inhibitor concentration used, DNA, protein, and RNA syntheses are inhibited more than 96%, respectively (10). At 2 hr postinfection, the intracellular DNA was extracted and analyzed in dye-buoyant density gradients, each of which contained $1.28 \mu\text{g}$ of ^{14}C -labeled Ad12 DNA as a marker. The percentage of cell-associated ^3H -activity in the HP and LP regions was calculated.

21 hr after infection and was analyzed in dye-buoyant density gradients. Significant amounts of ^{35}S label were not detected in the gradients, whereas the ^3H label in the HP and LP fractions was apparent. This result indicates that viral structural proteins do not remain associated with the HP or LP fractions.

In the second control experiment, BHK-21 cells were labeled with ^{35}S -L-methionine and ^3H -thymidine and the cellular DNA was extracted and analyzed in dye-buoyant density gradients. Only one peak of cellular DNA was observed in the LP position (cf. Fig. 2a) which also contained a small amount of ^{35}S label. There was no evidence for a peak of either isotope in the HP position. It is concluded that a DNA-protein complex does not preexist in uninfected BHK-21 cells.

Analysis of the proteins associated with the HP

fraction by SDS-polyacrylamide gel electrophoresis. BHK-21 cells were labeled with ^{35}S -L-methionine and infected with ^3H -Ad12, and, 2 hr after infection, the intracellular DNA was extracted, and the HP fraction was isolated in dye-buoyant density gradients. The HP fractions were pooled, concentrated by flash evaporation, and coelectrophoresed in an SDS-polyacrylamide gel (26) with a sample of Ad2 which had been labeled with ^3H -amino acids. The ^{35}S -labeled material, presumably cellular protein associated with the HP fraction, could be dissociated from the DNA moiety and, when subjected to electrophoresis, gave rise to a broad peak in an SDS-polyacrylamide gel in the region of the Ad2 structural proteins having a low molecular weight [7,500 to 24,000 (27)]. These values must be taken with caution, since, during the preparation of the intracellular DNA, the HP fraction had been exposed

FIG. 7. Association of protein with the DNA in the HP region. BHK-21 cells were grown for 3 days in ETC medium containing 20% of the regular amount of L-methionine and 33 μ Ci of 35 S-L-methionine (0.37 Ci/mmole) to a cell density of 10^6 to 2×10^6 cells per plate. These cells were inoculated with 3 H-Ad12 (4.5 OD_{260} units; 1.72×10^6 counts per min per OD_{260} unit). After a 2-hr adsorption period, the cells were washed five times with PBS, and then 5 ml of ETC medium containing the full amount of methionine was added. At 22.5 hr postinfection, the cells were again washed extensively, and the total intracellular DNA was extracted and analyzed in a dye-buoyant density gradient. After equilibrium centrifugation, all fractions were precipitated with trichloroacetic acid and washed five times with 5% trichloroacetic acid. The figures on top of the brackets indicate the 35 S to 3 H activity ratios in the respective fractions. The amount of DNA in the HP fraction (fractions 14 to 16) was in the nanogram range as calculated from the specific radioactivity of the DNA extracted from the inoculum; the amount in the LP fraction (fractions 21 to 23) was in the microgram range as determined from the number of cells used in the experiment and from the amount of DNA per BHK-21 cell (10).

to Pronase and phenol. Hence, it is not possible to give a truly reliable estimate of the size of the protein or proteins associated with viral DNA in the HP fraction in its actual intracellular state. The HP complex will have to be isolated by different techniques. Such experiments are now in progress.

Can 3 H-glucosamine label be detected in the HP fraction? The carbohydrate components of cell membranes can be labeled with D-glucosamine-6- 3 H. It has been shown that the 3 H-glucosamine label incorporated into membranes stays in glu-

cosamine and galactosamine when simian virus 5 (22) or Sindbis virions (30) are labeled with D-glucosamine-6- 3 H.

BHK-21 cells were grown in the presence of this compound and were infected with unlabeled Ad12 or mock-infected with PBS. The intracellular DNA was extracted 2 hr after infection and was analyzed in dye-buoyant density gradients. The data in Fig. 8 show that there was no difference between infected and uninfected cells in the distribution of the 3 H-glucosamine label in dye-buoyant density gradients. The nature of the very heavy peak of 3 H label in the gradients has not been investigated.

It is concluded that significant amounts of glucosamine are not associated with the HP fraction.

FIG. 8. Distribution of D-glucosamine-6- 3 H in dye-buoyant density gradients. BHK-21 cells were grown to confluence in ETC medium containing 10 μ Ci of D-glucosamine-6- 3 H per ml and were inoculated with unlabeled Ad12 (8.82 OD_{260} units) or mock-infected with 1 ml of PBS. After a 2-hr adsorption period, the cells were washed, and the intracellular DNA was extracted and analyzed in dye-buoyant density gradients. To each gradient was added 1.3 μ g of 14 C-labeled Ad12 DNA as a density marker. (a) BHK-21-Ad12; (b) BHK-21-PBS, control.

DISCUSSION

Properties of HP fraction. In this report, evidence was presented that, in BHK-21 cells infected with Ad12, a fraction (10 to 20%) of the cell-associated viral DNA bands in a density stratum in dye-buoyant density gradients which is 0.043 to 0.085 g/cm³ higher than that of Ad12 DNA extracted from the purified virion. This type of viral DNA has been termed HP fraction (heavy peak). The variability in the amount and buoyant density of the HP complex may be due to the instability of the complex during extraction. The HP fraction is observed as early as 2 hr after infection and is present in equal amounts in the cytoplasmic and nuclear fractions. It is absent or largely reduced in amount when DNA, RNA, or protein synthesis is inhibited before and during adsorption of the Ad12. The HP fraction does not occur when adsorption of Ad12 to BHK-21 cells is not allowed. It is absent in cellular DNA and in the DNA extracted from purified virus.

The DNA in the HP fraction hybridizes exclusively to viral DNA. The DNA in the HP fraction sediments at approximately the same rate in neutral and alkaline sucrose density gradients containing 1.0 M Na⁺. By electron microscopy, circular DNA molecules cannot be detected. It is conceivable that a complex form of DNA does not adhere to the cytochrome *c* film.

Evidence has been presented that protein, probably of cellular origin, is bound in a complex to the viral DNA in the HP fraction. The sensitivity of the HP fraction to digestion with ribonuclease suggests that RNA may also be involved in the formation of this complex. The DNA in the HP fraction is only partly sensitive to digestion with deoxyribonuclease.

The buoyant density in regular CsCl gradients of such a complex containing DNA, small amounts of protein, and possibly RNA could be the same as that of free viral DNA if the density contributions of the protein and RNA moieties balanced each other. The data in Fig. 4 indicate that the HP fraction bands together with free viral DNA in regular CsCl density gradients. The buoyant density of the HP fraction in dye-buoyant density gradients is considerably lower than that of free viral DNA in regular CsCl density gradients, which is 1.706 g/cm³ (Fig. 1). In alkaline CsCl density gradients (10), one peak of viral DNA is observed.

All these results indicate that the viral DNA in the HP fraction is a complex form of intracellular viral DNA which has not been described previously. The HP fraction is probably not an artifact of the extraction or purification procedure.

Models for the structure of the HP complex. A fraction of the intracellular parental Ad12 DNA

is involved in the formation of a complex with protein and possibly with RNA. In such a complex, the viral DNA is arranged in such a way that it cannot bind either propidium iodide or free viral DNA. Hence, the amount of dye bound per unit length of DNA is reduced, and thus, this type of viral DNA can be separated from the bulk of the free viral DNA in the cell.

It cannot be ruled out entirely that the HP fraction contains supercoiled circular Ad12 DNA; however, none was detected on intensive examination. It is also conceivable that the viral DNA in the complex is partly denatured. Experiments are in progress to investigate this possibility.

The exact configuration of the viral DNA in the complex and the nature of its association with protein and RNA are not known. The protein component is protected from the action of proteolytic enzymes and may be located in the interior part of the complex. In part, the DNA is also protected from digestion with deoxyribonuclease. On the other hand, the RNA moiety is accessible to ribonuclease digestion, and its presence is necessary for the stabilization of the complex. Could the DNA molecule be coiled up around a protein core? The size of such a core could vary. In this way, it would be possible to explain the multiple peaks that were observed in the heavy density region (Fig. 1 and 4).

At the present stage of the analysis, it is not possible to decide whether the viral DNA in the HP fraction is associated with membranous elements of cytoplasmic or nuclear origin. Preliminary experiments indicate that D-glucosamine-6-³H label does not appear in the HP fraction (Fig. 8).

Unknown biological function of the HP fraction. Since the HP fraction is also observed, both for the parental and the newly synthesized DNA, in KB cells productively infected with Ad2 (Burger, Hirsch-Kauffman, and Doerfler, Fed. Proc., *in press*), it is conceivable that the viral DNA in the HP fraction represents a stage in the replication of the viral DNA. If this speculation were correct, an interesting consideration would be that the Ad12 DNA in abortively infected cells enters into this initiation stage but that later steps in the replication of the viral DNA are blocked, since incorporation of ³H-thymidine into viral DNA was not detectable in this system (9, 13).

Apparently, the HP fraction contains protein. Hence, it will be interesting to search for enzymatic activities associated with the HP fraction. Thus far, experiments in this direction have given negative results. Particularly, it has not been possible to detect endonuclease activity (5) in the HP fraction. However, it will be necessary to isolate the HP fraction by a technique which avoids the use of Pronase and phenol.

Complex forms of viral DNA in other systems. In cell systems productively infected with viruses, complex forms of viral DNA have been observed: λ DNA in *E. coli* (18); T7 DNA in *E. coli* (Knippers, R., 2nd Int. Congr. Virol., Budapest, 1971); simian virus 40 DNA in primary African green monkey kidney cells (23); polyoma DNA in mouse embryo cells (3); and Ad2 DNA in HeLa cells (27a)).

A DNA-protein relaxation complex has been described for the bacterial plasmid DNA (6).

Before a comparison of the HP fractions found in cells abortively or productively infected with adenoviruses can be made with any of the complex structures cited, the molecular structure of the HP fraction must be studied in more detail.

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