THE FATE OF THE DNA OF ADENOVIRUS TYPE 12 IN BABY HAMSTER KIDNEY CELLS*

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There is evidence that the viral genome, or part of it, persists and continues to be transcribed[1] in cells derived from tumors induced by human adenovirus type 12 (ad 12) and in cells transformed *in vitro* by ad 12. It is not clear whether the viral genome is covalently linked to cellular DNA (integration) or replicates as free DNA in phase with cellular DNA.

In the studies reported here, it is proposed that the decisive events leading to transformation are most likely to occur soon after infection of cells susceptible to transformation by ad 12. A system was developed that permitted an investigation of the fate of the DNA of ad 12 in such cells. Baby hamster kidney cells (BHK21),[2] which can be transformed by ad 12 *in vitro*,[3] were chosen for these studies.

In this communication, evidence will be presented for the first time that the DNA of ad 12, or part of it, can become linked to the DNA of BHK21 cells. The significance of these findings for the phenomenon of transformation remains to be elucidated. A preliminary report of this work has been given.[4]

Materials and Methods.—Cell culture media: Spinner medium is Eagle's medium[5] without $CaCl_2$ enriched with each of the nonessential amino acids (100 μM) and 10% calf serum. ETC is reinforced Eagle's medium[6] with 10% tryptose phosphate broth (TPB) and 10% calf serum.

Solutions: PBS is phosphate-buffered saline;[7] TE is 0.01 *M* Tris, pH 7.2, 0.001 *M* ethylenediaminetetraacetate (EDTA); and STE is 0.5% sodium lauryl sulfate, 0 05 *M* Tris, pH 7.5, and 0.01 *M* EDTA.

Cells and virus: KB cells[8] (CCL17) were obtained from the American Type Culture Collection. BHK21 cells, clone 13, were originally isolated by Stoker and Macpherson[2] and were given to us by Dr. Carabazzo of the Rockefeller Foundation Virus Laboratory. Primary human embryonic kidney (HEK) cells were purchased from Microbiological Associates, Inc. Seed virus preparations of human adenovirus type 12, strain Huie, were supplied by the American Type Culture Collection and by Dr. Wallace Rowe of the National Institute of Allergy and Infectious Diseases.

Chemicals: CsCl was obtained from the Harshaw Chemical Co. as optical-grade powder. Pronase, B-grade, and 5-bromodeoxyuridine (5-BUdR) were obtained from Calbiochem., Inc. Dr. A. Kornberg of Stanford University supplied dAT-copolymer. All other chemicals used were of analytical grade.

Radioisotopes: Thymidine-methyl-H^3 (15–17 c/mmole), thymidine-2-C^{14} (30 mc/mmole), and C^{14}-amino acids mixture (uniformly labeled) were purchased from the New England Nuclear Corp.; thymidine-6-H^3 (17–22 c/mmole) was purchased from Nuclear-Chicago, and 5-BUdR-6-H^3 (2.5 c/mmole) from Schwarz BioResearch, Inc.

Plaque assay of ad 12: The procedure of Strohl *et al.*[3] was followed with minor modifications.

Virus growth and purification: Ad 12 was grown in KB cells in suspension cultures in Spinner medium and was purified by a modification[9] of the procedure of Green and Piña.[10] All virus preparations used in this study were purified by three cycles of equilibrium sedimentation in CsCl. They were found to be free of contaminating adeno-associated virus by ultracentrifugal and electron microscopical examinations. The final

virus preparations contained 0.003% cellular DNA and 0.8% cellular protein. The recovery of biological activity in this procedure was >90%. The specific infectivity of virus preparations was 2×10^9 to 2×10^{10} PFU/ODU.

Radioactively labeled virus: Eleven hours after infection, 1 μc/ml of H^3-methyl or 6-H^3-thymidine or 0.2 μc of thymidine-2-C^{14} were added. H^3-labeled virus preparations (H^3-ad 12) had specific activities of $1-2 \times 10^6$ dpm/OD_{260} unit.

The DNA of ad 12 was prepared by pronase digestion of the virus and three phenol extractions as described elsewhere.[9] The DNA of such preparations of ad 12 had an S of 29, and approximately 80% of its single chains were intact.[9]

Growth and infection of BHK21 cells: Plastic dishes, 60 mm in diameter, were seeded with $2-3 \times 10^5$ BHK21 cells in the 10th to the 50th passage and grown in 5 ml ETC containing 5-BUdR (5 μg/ml). After incubation for 96 hr at 37°C, the cell density was $3-5 \times 10^6$ cells/dish. The cell sheets were washed twice with 5-ml vol of PBS, and 1 ml (OD_{260} = 1.7–4.4) of 3 × CsCl-purified ad 12 virus was added per plate. Immediately prior to infection, the virus suspension was dialyzed for at least 4 hr against four 1-liter changes of PBS, and bovine plasma albumin was added to a concentration of 0.2%. For virus adsorption, 2.5 hr at 37°C were allowed. The cell sheets were then washed 7 times with 5-ml vol of PBS to remove all virus that had not become associated with the cells. Subsequently, 5 ml of prewarmed ETC (5% TPB and 5% calf serum) were added containing 5-BUdR (5 μg/ml), unless otherwise stated. In some experiments, H^3-5-BUdR or H^3-thymidine was also added.

Extraction of cellular DNA: At various times after adsorption of radioactive virus to monolayers of BHK21 cells, the maintenance medium was removed, and aliquots were analyzed for radioactivity. The cell sheets were washed at least 6 times with 5-ml vol of PBS. Subsequently, 1 ml STE was added to each plate, and 10–15 min at room temperature were allowed for complete cell lysis to occur. The use of pipettes to handle cell extracts was avoided to minimize shear forces. The lysates were incubated with 250 μg/ml of pronase B (preincubated at 37°C for 120 min) for 15–20 min at 37°C. (In an STE lysate of cells labeled with a C^{14}-amino acid mixture, >80% of the C^{14} counts was rendered acid-soluble by treatment with pronase B.) The cell lysates were extracted twice at room temperature with twice the volume of 2 times redistilled phenol saturated with 1 M Tris, pH 7.5. Phenol was extracted from the aqueous layer with 3×5 ml ether, and the remaining solvent was evaporated with N_2. Subsequently, 1 ml of cell extract was layered on top of 4 ml of a CsCl solution consisting of 15–15.4 gm CsCl and 10 ml TE. Equilibrium sedimentation was carried out in an SW 39 rotor at 33,000 rpm for 60–65 hr at 25°C in a Spinco model L-2 ultracentrifuge. After centrifugation, the bottom of the nitrocellulose tube was punctured with a wide-gauge needle, and fractions of 0.1–0.13 ml were collected. The fractions containing cellular DNA were dialyzed against TE and analyzed by velocity sedimentation in sucrose gradients at pH 7.6 and 12.5.

Preparative zone centrifugation in sucrose density gradients was carried out as described previously.[11] Alkaline sucrose solutions of 5% (pH 12.7) and 20% (pH 12.2) were made up in TE containing 0.7 M NaCl and 0.3 N NaOH. In all experiments, 0.4 μg of C^{14}-labeled ad 12 DNA was added to the sample as a marker. The S values of DNA samples were calculated according to Martin and Ames.[12] Centrifugation was carried out in an SW 39 rotor in a Spinco model L-2 ultracentrifuge at 33,000 rpm for 180 min at 4–6°C. After centrifugation, 0.2-ml fractions were collected, and the trichloroacetic acid-insoluble radioactivity was determined. DNA samples were denatured by alkali as described earlier.[11]

Analysis of cell extracts by CsCl equilibrium centrifugation was performed as described above. In every fifth fraction, the refractive index was determined, and the density was calculated as described earlier.[11]

For equilibrium sedimentation in alkaline CsCl, 1 ml of an alkali-denatured DNA sample was layered on top of 4 ml CsCl solution of the following composition: 6.4 gm CsCl and 3.8 ml of a solution containing 0.5 ml 0.5 M sodium phosphate, pH 11.75, 0.5 ml 0.1 M EDTA, 0.05 ml 1 N NaOH, and 4.0 ml H_2O (pH 12.1). The buoyant density of ad 12 DNA

in CsCl at pH >12 was calculated to be $\rho = 1.762$ gm $\times$ cm^{-3}, relative to dAT ($\rho = 1.732$ gm $\times$ cm^{-3}). These figures were not corrected for pressure effects.

Sonication of cellular DNA: Cell extracts containing 0.4 μg C^{14}-ad 12 marker DNA were sonicated in an ice-water bath with the microtip of a model LS-75 sonifier (Branson Instruments, Inc.) at a setting of 3; 6.8 amp for 30 sec. After fragmentation, the DNA sedimented as a homogeneous peak of 13*S*, equivalent to a molecular weight[13] of 1.9×10^6 (see Fig. 3*c*).

Absorbance was measured in a Zeiss PMQ II spectrophotometer, and pH was measured with a Radiometer with G222B and K130 electrodes. Simultaneous determination of C^{14} and H^3 was carried out in a Packard model 3375 Tri-Carb scintillation spectrometer. All manipulations of DNA or cell extracts were performed under sterile conditions.

Results and Discussion.—The difference in buoyant density of ad 12 DNA and the DNA of BHK21 cells in CsCl equilibrium gradients is $\Delta\rho = 0.006$ gm $\times$ cm^{-3}. The density of cellular DNA can be increased by 0.060 gm $\times$ cm^{-3} to $\rho = 1.759$ gm $\times$ cm^{-3} by growing BHK21 cells for 96 hours in a medium containing 5-BUdR. The increase in density of cellular DNA corresponds to approximately 50 per cent substitution[14] of thymine (T) by 5-BU residues, which are replaced by T in further growth in the absence of 5-BUdR. A reconstitution experiment (Fig. 1) characterizes the system that is used for separating viral and cellular DNA.

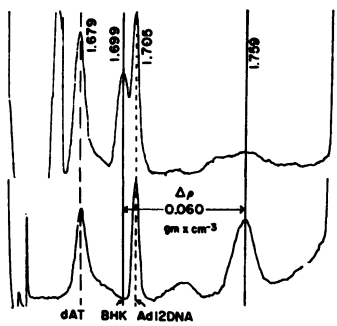

FIG. 1.—Separation of ad 12 and BHK21 cellular DNA by CsCl equilibrium sedimentation in the analytical ultracentrifuge.

Top: 10 μl cell extract in TE, 0.66 ml TE, 5 μl dAT ($OD_{260} = 3.1$); 3 μl ad 12 DNA ($OD_{260} = 2.1$) and CsCl to yield a $\rho_{25°} = 1.736$ gm/cm^3. *Bottom:* 0.1 ml cell extract (cells grown for 96 hr in 5-BUdR), 0.59 ml TE, 5 μl dAT ($OD_{260} = 3.1$); 5 μl ad 12 DNA ($OD_{260} = 3.7$) and CsCl to yield $\rho_{25°} = 1.729$ gm/cm^3.

Sedimentation was carried out at 44,000 rpm at 25°C. Photographs were taken at 265 mμ and traced with a Joyce-Loebl microdensitometer.

For infection of BHK21 cells grown in 5-BUdR-containing medium with H^3-ad 12, multiplicities of infection of 10^3 to 10^4 PFU/cell were used because of the low specific radioactivity of the virus. After infection, a variable amount of radioactivity is released into the maintenance medium. The cell-associated radioactivity decreases concomitantly. Most of the counts in the medium are precipitable by trichloroacetic acid, and 80–90 per cent exhibit a buoyant density identical to or higher than that of ad 12. On the basis of the amount of radioactivity found in cell extracts, 0.1–0.5 per cent of the DNA of input virus remains inside the cells.

Infectious virus disappears rapidly from cells after infection (Table 1); only small amounts can be detected up to 120 hours after infection. Infected BHK21 cells were passed in 5-BUdR-free medium for up to two months, and infectious virus was never found intra- or extracellularly beyond the first passage. It is concluded that infectious ad 12 particles are not produced in BHK21 cells.

TABLE 1. *Disappearance of infectious adenovirus 12 after infection of BHK21 cells.*

Hours after infection	Surviving Fraction of PFU/Plate	
	Intracellular	Extracellular
4	4.4×10^{-5}	6×10^{-5}
6	3.4×10^{-7}	3×10^{-5}
8	3.1×10^{-7}	8×10^{-7}
16	9.4×10^{-9}	2×10^{-6}
28.5	—	1.7×10^{-7}
51.5	3.1×10^{-9}	1.9×10^{-7}

BHK21 cells (6.8×10^6 per plate) grown in ETC without 5-BUdR were inoculated with 1.3×10^{12} PFU/plate of ad 12. After a 2.5-hr adsorption period, the monolayers were washed 6 times with 5-ml vol of PBS, and 5 ml fresh ETC was added. At times, as indicated, plaque-forming virus in the medium and intracellular virus were determined. Sonication was used for the disruption of cells.

Does viral DNA become linked to cellular DNA? At various times after infection of cells with H^3-ad 12, the fate of viral DNA can be followed by determining the distribution of H^3-label in CsCl equilibrium gradients. Early after infection (3–6 hr), almost all the H^3-label appears in the density position of ad 12 DNA, which is marked by C^{14}-ad 12 DNA (Fig. 2*a*, *b*). There is practically no soluble label in the gradients. Later on (16–40 hr), an increasing percentage of the cell-associated radioactivity assumes the density of cellular DNA; 63 hours after infection, almost none of the label remains in the position of viral

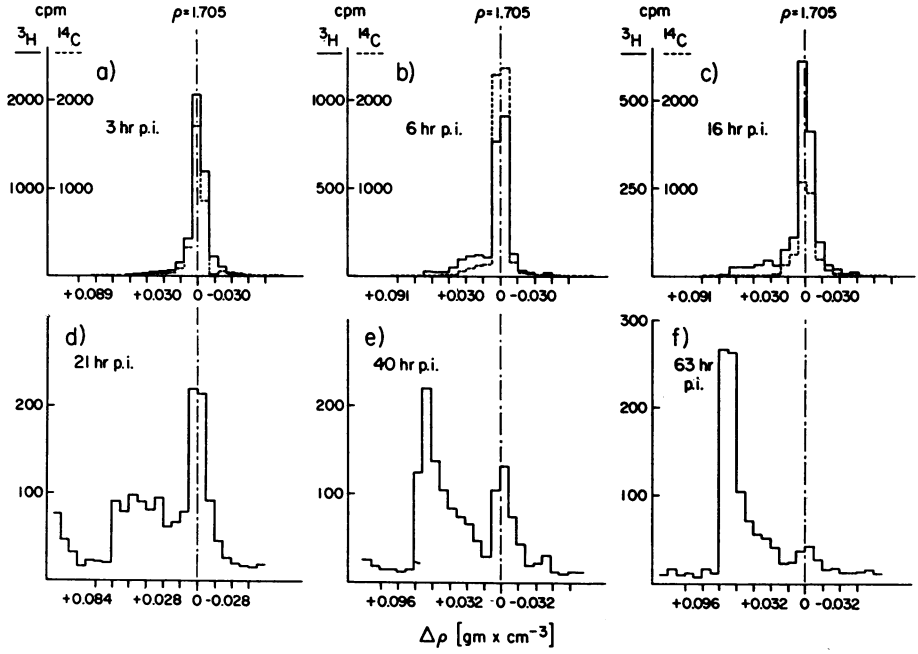

FIG. 2.—Equilibrium sedimentation in CsCl of extracts of BHK21 cells grown in the presence of 5-BUdR and infected with H^3-ad 12. The cells were lysed and extracted at various times after infection (*p.i.*), as indicated. In the experiments presented in parts *a–c*, 0.4 μg of C^{14}-labeled ad 12 DNA was added to each extract. All $\Delta\rho$-values were calculated relative to ρ = 1.705 gm $\times$ cm^{-3} for ad 12 DNA. The fractions from the top of each gradient are free of radioactivity and are not shown in the diagrams.

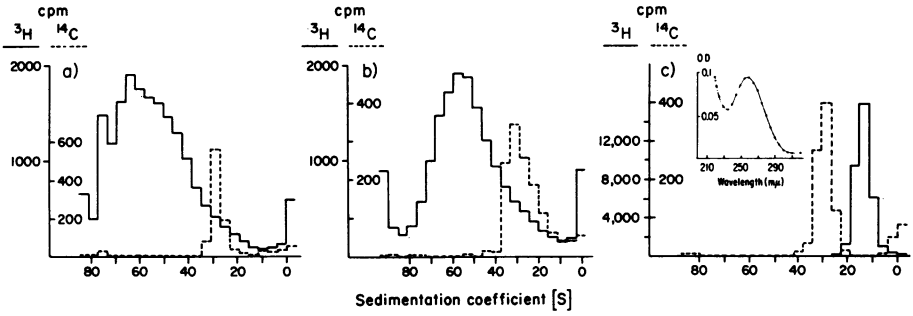

FIG. 3.—Velocity sedimentation of DNA from BHK21 cells infected with unlabeled ad 12. DNA synthesized after infection was labeled with H^3-5-BUdR. Aliquots of cellular DNA (0.2 ml) isolated from a CsCl gradient were sedimented in sucrose gradients at pH 7.6 (*a*), and after alkali denaturation, at pH 12.5 (*b*). C^{14}-labeled ad 12 DNA (0.4 μg) was added as a marker. The value of 63*S* for the peak fraction of cellular DNA is consistent with a molecular weight[13] of 177×10^6. DNA extracted from uninfected cells showed identical profiles. The sedimentation profile of sonicated DNA at pH 7.6 is shown in (*c*). The insert contains the spectrum of such DNA when purified with CsCl from uninfected BHK21 cells grown in a medium free of 5-BUdR.

DNA (Fig. 2*c–f*). The gradual increase in the density of the H^3-label associated with cellular DNA reflects its continued replication. The appearance of H^3-label in the density stratum of heavy (cellular) DNA could be the result of any of the following mechanisms: (1) Viral DNA is degraded to mononucleotides; H^3-thymidine 5′-monophosphate (TMP) is phosphorylated by cellular kinases to H^3-thymidine 5′-triphosphate (TTP) and reincorporated during cellular DNA synthesis which continues after infection, as will be shown below. (2) Viral DNA replicates and, due to the incorporation of 5-BUdR, its buoyant density increases. (3) The viral genome, or part of it, becomes linked to cellular DNA. In the following discussion, evidence will be presented that eliminates possibility (2) and argues in favor of (3), but does not completely rule out (1).

No difference is detectable in extracts of uninfected and infected BHK21

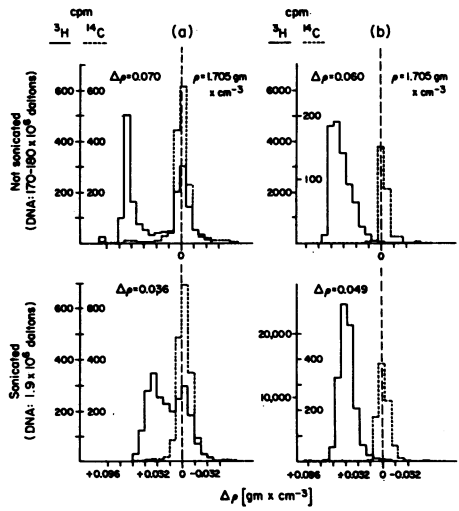

FIG. 4.—CsCl profiles of unsonicated and sonicated extracts from BHK21 cells grown in the presence of 5-BUdR and infected with: (*a*) H^3-ad 12; cells extracted 44 hr after infection; and (*b*) unlabeled ad 12, 2 μc H^3-5-BUdR per ml medium were added after infection, cells were extracted 44 hr later (0.4 μg C^{14}-ad 12 DNA per ml extract was added as marker).

All $\Delta\rho$ values were calculated relative to $\rho = 1.705$ gm $\times$ cm^{-3} for ad 12 DNA.

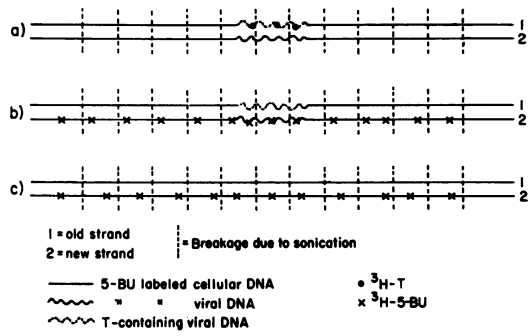

FIG. 5.—Scheme of the experiments described in Fig. 4. BHK21 cells were grown in the presence of 5-BUdR and infected with (*a*) H^3-ad 12, and (*b*) unlabeled ad 12 (H^3-5-BUdR added after infection). (*c*) Uninfected BHK21 cells were labeled with H^3-5-BUdR.

cells in the amounts of H^3-thymidine incorporated into acid-insoluble material. To determine what type of DNA was synthesized after infection of cells, BHK21 cells that had been exposed to 5-BUdR for 96 hours were infected with unlabeled ad 12, and after adsorption, 10 μc of H^3-5-BUdR were added. The DNA was extracted 44 hours after infection and centrifuged to equilibrium in CsCl. All the label appears in the density position of heavy DNA (see Fig. 4*b*, *top*). An aliquot of the combined heavy DNA fractions was dialyzed and sedimented in a sucrose density gradient. All the label sediments with the *S* value of cellular DNA, both at pH 7.6 and 12.5 (Fig. 3*a*, *b*). This finding argues against, but does not rule out, the replication of free ad 12 DNA because ad 12 DNA could replicate and form covalently linked aggregates. This latter possibility is eliminated by the result of an experiment similar to the one described in the top part of Figure 4*b*, except that H^3-thymidine, and not 5-BUdR, was added to the maintenance medium and the cells were extracted 24 hours after infection. In a CsCl gradient, no label appears in the position of viral DNA. If ad 12 DNA had replicated in 5-BUdR-free medium, H^3-label should have been found in the position of ad 12 DNA, whether aggregates were or were not formed. Thus the H^3-label in the position of high density is due to replication of cellular DNA. Under identical conditions, the amounts of H^3-5-BUdR or H^3-thymidine incorporated into cellular DNA of infected and uninfected cells are the same. It is concluded that cellular DNA synthesis in growing BHK21 cells is not affected by infection with ad 12. Replication of free ad 12 DNA cannot be detected in 5-BUdR BHK21 cells. This eliminates possibility (2).

One can distinguish between possibilities (1) and (3) by breaking the DNA from cells infected with H^3-ad 12 through sonication (see *Materials and Methods* and Fig. 3*c*) and by comparing equilibrium sedimentation patterns of the sonicated and unsonicated DNA samples. It is observed that the peak of the H^3-label in the position of cellular DNA ($\Delta\rho$ = 0.070 gm $\times$ cm^{-3}, relative to ad 12 DNA) shifts upon sonication to a position of lower (hybrid) density ($\Delta\rho$ = 0.036 gm $\times$ cm^{-3}) (Fig. 4*a*). Such a shift in density would be expected after

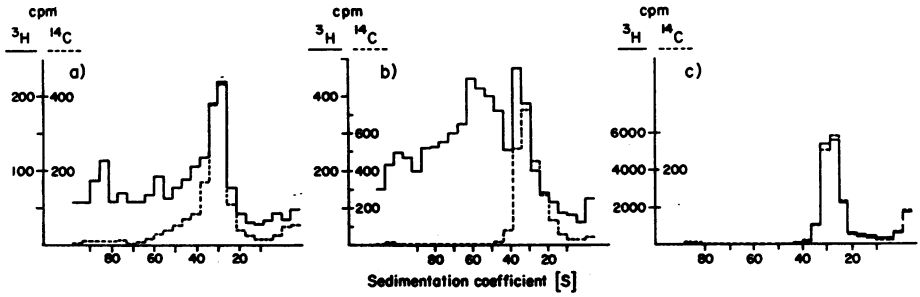

FIG. 6.—Velocity sedimentation of cellular and viral DNA. Extracts were prepared 43 hr after infection of 5-BU-labeled BHK21 cells with H^3-ad 12. Viral and cellular DNA were separated as described in Fig. 2. Fractions from the cellular DNA peak were pooled, dialyzed against TE, and resedimented in a sucrose density gradient (*a*) at pH 7.6, and (*b*) after alkali denaturation of the sample, at pH 12.5. (*c*) Fractions from the viral DNA peak were treated identically and resedimented at pH 7.6.

sonication if viral DNA had become linked to cellular DNA and had replicated with it. This shift is probably due to the breakage of bonds between heavy-heavy cellular and heavy-light viral DNA (Fig. 5*a*). It would not be found if the H^3-label in the position of cellular DNA was exclusively attributable to reincorporation of H^3-TMP (Fig. 5*c*).

The following control experiments were performed: BHK21 cells grown in the presence of 5-BUdR were infected with unlabeled ad 12, and H^3-5-BUdR was added after infection (Fig. 5*b*). The densities of unsonicated and sonicated DNA from an extract prepared 43 hours after infection differ by only 0.011 gm $\times$ cm^{-3} in their peak fractions (Fig. 4*b*). When uninfected cells are used in the same experiment, this difference is 0.007 gm $\times$ cm^{-3}.

These data are interpreted as evidence for the contention that at least part of the H^3-label appearing in the density position of cellular DNA is due to substantial regions of viral DNA linked to cellular DNA. The size of these regions cannot, however, be estimated. It cannot rigorously be excluded that another part of the radioactivity found in cellular DNA comes from reincorporation of low-molecular-weight fragments. Supporting evidence for the linkage of the ad 12 genome to the genome of mammalian cells is provided by autoradiographic studies on chromosomes of HEK cells infected with ad 12.[15]

Alkali stability of H^3-label associated with cellular DNA: Cellular DNA isolated from a CsCl gradient experiment similar to the one presented in Figure 2*e* was analyzed by sucrose gradient velocity sedimentation at pH 7.6 and 12.5 (Fig. 6*a*, *b*). At either pH, there is label sedimenting with the rate of cellular DNA and label cosedimenting with C^{14}-ad 12 DNA. The occurrence of this latter component at alkaline pH is due to the release of intact, noncovalently bound ad 12 DNA molecules. Intact ad 12 DNA is also liberated at neutral pH, which suggests that some of it is only very loosely associated with cellular DNA. It is important to note that >80 per cent of the viral DNA found 43 hours after infection in the density position of free ad 12 DNA still consists of intact molecules sedimenting with the *S* value of C^{14}-ad 12 marker DNA (Fig. 6*c*).

An extract from infected cells was also centrifuged to equilibrium in CsCl at

pH >12 (Fig. 7). The label in the cellular DNA position is stable after chain separation in alkali; its amount is comparable to that found in neutral CsCl gradients. The increased width of the cellular DNA peak and its lower buoyant density are probably due to breakage of 5-BU-labeled DNA in alkali. These results suggest that viral genes may have become covalently linked to cellular DNA. Experiments to further support this conclusion are in progress.

The design of the experiments described in this report precludes a direct correlation of the results to those obtained with transformed cells. The findings discussed provide evidence in a model system that the DNA of a mammalian virus can become integrated into the host genome.

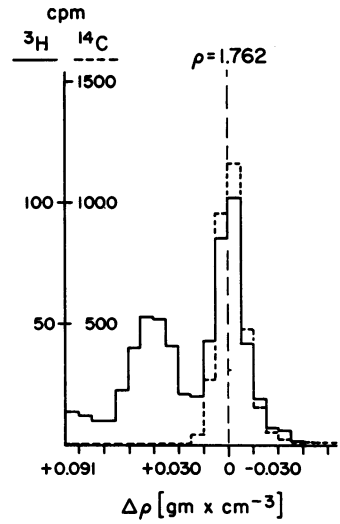

FIG. 7.—Equilibrium sedimentation in alkaline CsCl. Cells were extracted 41 hr after infection with H^3-ad 12. 0.4 μg C^{14} ad 12 DNA was added as marker. $\Delta\rho$ values were calculated relative to ρ = 1.762 gm × cm^{-3} for ad 12 DNA in alkaline CsCl.

Summary.—Adenovirus 12 does not replicate in BHK21 cells, and viral DNA is not synthesized. 5-BUdR BHK21 cells have been infected with H^3-ad 12, and the distribution of label has been followed in CsCl density gradients at various times after infection. At early times, all the label has the density of ad 12 DNA; later on, an increasing amount is found in the position of heavy cellular DNA, both at neutral and alkaline pH. Upon sonication, the label shifts to a position of hybrid density. These results are interpreted as evidence for linkage between the genome of ad 12, or part of it, and cellular DNA.

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