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Integration of the Deoxyribonucleic Acid of Adenovirus Type 12 into the Deoxyribonucleic Acid of Baby Hamster Kidney Cells

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In a previous report, evidence was presented that the deoxyribonucleic acid (DNA) of adenovirus type 12 (Ad12) is integrated by covalent linkage into the DNA of baby hamster kidney cells (BHK-21 cells). These studies have been extended. The DNA of Ad12 and that of BHK-21 cells grown in medium containing 5-bromodeoxyuridine could be separated by equilibrium centrifugation in alkaline CsCl density gradients. BHK-21 cells were infected with ^{3}H-labeled Ad12, and the total intracellular DNA was analyzed at various times after infection in alkaline CsCl density gradients. The ^{3}H label in the position of cellular DNA hybridized predominantly with viral DNA and to a lesser extent also with cellular DNA. Replication of viral DNA could not be detected in BHK-21 cells. The appearance of viral ^{3}H label in the density stratum of cellular DNA was not significantly affected when DNA synthesis in Ad12-infected BHK-21 cells was inhibited >96% by cytosine arabinoside. These findings provided additional evidence for integration of Ad12 DNA into the DNA of BHK-21 cells. It could be calculated that 5 to 55 Ad12 DNA equivalents per cell are integrated. Replication of viral or cellular DNA was not required for integration. Inhibition of protein or ribonucleic acid synthesis interfered with integration only slightly.

It has been shown that human adenovirus type 12 (Ad12) infects BHK-21 cells abortively (6, 18) and that viral deoxyribonucleic acid (DNA) does not replicate in these cells (6, 7).

In a previous communication (5), it was demonstrated that in Ad12-infected BHK-21 cells ^{3}H-thymidine label from viral DNA becomes associated by covalent linkage with cellular DNA. In these experiments, cellular DNA was made heavy by 5-bromodeoxyuridine (5-BUdR) so that viral and cellular DNA could be separated in CsCl density gradients. Three possible explanations for this finding have been discussed: (i) viral DNA replicates and thus assumes a higher buoyant density; (ii) viral DNA is degraded to mononucleotides which are reutilized in cellular DNA synthesis; (iii) viral genes are integrated into cellular DNA. When the DNA from Ad12-infected BHK-21 cells is fragmented by ultrasonic treatment, the ^{3}H label in the density position of cellular DNA shifts to an intermediate density. This finding supports the conclusion that viral DNA is integrated.

This interpretation has been confirmed by the results of zur Hausen and Sokol (24), although in the cell system (Nil-2 cells) used by these authors degradation of viral DNA and reutilization of mononucleotides appear to have been extensive.

In the present paper, the investigations previously described (5) have been extended. It will be demonstrated that ^{3}H-thymidine label from viral DNA becomes covalently linked to cellular DNA and that this ^{3}H label does hybridize predominantly to viral DNA in DNA-DNA hybridization experiments. Replication of Ad12 DNA in BHK-21 cells cannot be detected by the DNA ribonucleic acid (RNA) hybridization technique, by using Ad12 complementary RNA (cRNA) synthesized in vitro. From the amount of ^{3}H label hybridizing to viral DNA, it can be calculated that 5 to 55 Ad12 genome equivalents per cell are covalently linked to cellular DNA. Free viral DNA also persists in infected cells. Inhibition of DNA synthesis does not interfere with the integration event, and inhibition of protein and RNA synthesis in infected cells appears to affect it only slightly.

MATERIALS AND METHODS

Cell culture media. The composition of the media used has been described previously (5). ETC is rein-

forced Eagle's medium (1) supplemented with 10% calf serum and 10% Tryptose phosphate broth (Difco Manual). Calf serum was purchased from the Grand Island Biological Co., Grand Island, N.Y.

Solutions. TE is 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 7.2 to 7.5), 0.001 M ethylenediaminetetraacetate (EDTA); STE is 0.5% sodium dodecyl sulfate (SDS) in 0.1 M Tris-hydrochloride (*p*H 7.2), 0.02 M EDTA; SSC is 0.15 M NaCl, 0.015 M sodium citrate. PBS is phosphate-buffered saline as described by Dulbecco and Vogt (9). TV is 0.25% trypsin in 0.05% EDTA. Neutral CsCl solution consists of 15 g of CsCl and 10 ml of TE. The alkaline CsCl solution is made up of 16 g of CsCl and 9 ml of 0.05 M sodium phosphate buffer (*p*H 11.75), 0.01 M EDTA, and 0.01 M NaOH. The final *p*H of the solution is 12.3.

Cells and viruses. The origin of the cells and virus preparations used in this study has been described earlier (5, 6). Frozen cells of *Escherichia coli* B, grown to mid-log phase, were bought from the Grain Processing Corp., Muscatine, Iowa.

Chemicals and radioisotopes. The source of most of the chemicals used has been given (6). Diphenylamine, special indicator grade, was purchased from Fisher Scientific Co., Fair Lawn, N.J.; diethylaminoethylcellulose (Cellex-D) was purchased from Bio-Rad Laboratories, Richmond, Calif.; actinomycin D, 5-BUdR, and Acti-Dione (cycloheximide) were obtained from Calbiochem, Los Angeles, Calif.; 1-β-D-arabinofuranosylcytosine (cytosine arabinoside) was a gift of the Upjohn Co., Kalamazoo, Mich. Phenol was redistilled twice, and immediately before use it was saturated with either 1 M Tris-hydrochloride or with 0.1× SSC.

Thymidine-*6*-^3H (20 Ci/mmole) was obtained from the Amersham/Searle Corp. Reconstituted ^{3}H-protein hydrolysate (1 mCi/ml), ^{14}C-sodium formate (53.5 Ci/mmole), the tetralithium salts of the 5′-triphosphates of ^{3}H-guanosine (1.09 Ci/mmole), cytidine-*5*-^3H (12.6 Ci/mmole), uridine-*5*-^3H (12.1 Ci/mmole), and adenosine-*8*-^3H (18 Ci/mmole) were purchased from Schwarz BioResearch, Inc., Orangeburg, N.Y.

Enzymes. Pronase (B grade) was purchased from Calbiochem. Pronase was dissolved in 0.01 M Tris-hydrochloride (*p*H 7.2) at a concentration of 5 mg/ml and was preincubated at 37 C for 2 hr. Deoxyribonuclease (ribonuclease-free) and ribonuclease A (phosphate-free) were obtained from the Worthington Biochemical Corp. Deoxyribonuclease was dissolved at a concentration of 500 μg/ml in 0.01 M Tris-hydrochloride (*p*H 7.2), 0.01 M $CaCl_2$, 0.01 M $MnCl_2$ (13).

DNA-dependent RNA polymerase from *E. coli* B was purified by the procedure of Zillig, Fuchs, and Millette (22). The final purification steps included zonal sedimentation in 10 to 30% sucrose gradients in TMA buffer [0.01 M Tris-hydrochloride (*p*H 7.5), 0.022 M NH_4Cl, 0.01 M $MgCl_2$, and 0.001 M 2-mercaptoethanol] at low salt concentration, followed by sedimentation in 10 to 30% sucrose gradients in 1.0 M KCl in TMA buffer. These final steps were adapted from the procedure described by Burgess (2). The enzyme was stored in 50% glycerol in TMA buffer at −36 C.

Growth and purification of Ad2 and Ad12. Growth and purification of adenovirus type 2 (Ad2) and Ad12 were described earlier (6). Plaque assays of adenovirus were carried out on human embryonic kidney cells (Microbiological Associates, Bethesda, Md.) by the procedure of Strohl, Rabson, and Rouse (17).

Ad2 and Ad12 were radioactively labeled by adding ^{3}H-thymidine (1 μCi/ml) or ^{14}C-sodium formate (0.5 μCi/ml) to the cultures, 10 to 12 hr or 2 hr after infection, respectively. Ad12 labeled with ^{3}H-thymidine will be designated ^{3}H-Ad12. The specific radioactivity of ^{3}H-Ad12 preparations was between 1.28×10^6 and 4.18×10^6 counts per min per optical density (OD_{260}) unit.

Viral DNA was extracted from highly purified virus as described earlier (6).

Analysis of virus and DNA preparations in the analytical ultracentrifuge. Purified Ad12 preparations used for the inoculation of BHK-21 cells were examined for purity by equilibrium sedimentation in CsCl density gradients in the analytical ultracentrifuge as outlined previously (6). In most preparations of Ad12, two distinct bands can be observed in CsCl density gradients. The buoyant density of these two bands differs by $\Delta\rho = 0.002$ g × cm^{-3}, and the two bands have been designated 3A and 3 (B. T. Burlingham and W. Doerfler, *in preparation*). In most experiments, a mixture of both bands has been used.

Viral DNA preparations were examined for purity and homogeneity by equilibrium sedimentation in CsCl density gradients and by zone velocity sedimentation in the analytical ultracentrifuge as described earlier (6). The viral DNA was found to sediment homogeneously as a single, sharp band both in equilibrium and in zone velocity sedimentation experiments.

Growth of BHK-21 cells in 5-BUdR. ETC medium was supplemented with 5 μg of 5-BUdR per ml, and BHK-21 cells were seeded in 60-mm petri dishes at 3×10^5 to 5×10^5 cells per dish and allowed to grow in this medium for 96 hr. Exposure of these cells to light was avoided. These cells will be referred to as 5BU-BHK-21 cells. Cells grown in normal ETC medium without 5-BUdR will be designated N-BHK-21 cells.

Infection of BHK-21 cells with Ad2 or Ad12. Ad2 or Ad12 preparations which had been purified in CsCl density gradients at least three times were dialyzed for 3 to 4 hr against two to four changes of 1,000 ml of PBS each immediately before they were used as inocula. Monolayers of BHK-21 cells (cell numbers per dish are indicated in each experiment) were washed once or twice with 5 ml of PBS. A 1-ml amount of purified Ad2 or Ad12 ($OD_{260} = 2.0$ to 7.0) was added to each monolayer of BHK-21 cells. At the end of a 2-hr adsorption period, the cells were washed at least five times with 5 ml of PBS and then 5 ml of ETC was added. In the experiments in which 5BU-BHK-21 cells were used, the maintenance medium was supplemented with 5 μg of 5-BUdR per ml. BHK-21 cells infected with adenovirus will be designated N-BHK·Ad12 and 5BU-BHK·Ad12 or N-

BHK·^{3}H-Ad12 and 5BU-BHK·^{3}H-Ad12 when ^{3}H-Ad12 was used.

Extraction and purification of intracellular DNA. This method has been described in detail (5).

Analysis of the intracellular DNA by equilibrium sedimentation in neutral and alkaline CsCl density gradients. A 1-ml amount of a solution of DNA that was isolated from the cells by treatment with phenol and ether was layered on top of 4 ml of the neutral or alkaline CsCl solution. In many of the experiments, ^{14}C-labeled Ad12 DNA was added as a marker. In experiments with alkaline CsCl solutions, the DNA in the cell extracts was denatured by adding, per milliliter of extract, 0.05 ml of 0.1 M EDTA and 0.12 ml of 6 N NaOH. Nitrocellulose tubes were used in experiments with neutral CsCl solution, and polyallomer tubes were used in experiments with alkaline CsCl solution. The solutions were centrifuged to equilibrium in the SW39 or the SW50.1 rotor in a Spinco model L2-65B ultracentrifuge at 30,000 rev/min for 60 hr at 20 C. At the end of the run, six- to seven-drop fractions were collected from the bottom of the tube. In every tenth fraction, the refractive index was determined. The OD_{260} in each fraction was measured, and all fractions were precipitated at 0 C with trichloroacetic acid. To each fraction were added 1.0 ml of a 0.01% solution of bovine serum albumin as carrier and 0.2 ml of a solution of trichloroacetic acid containing 100 g of trichloroacetic acid in 100 ml of water. The precipitates were collected on filters, dried, and prepared for liquid scintillation counting.

Fragmentation of DNA by ultrasonic treatment. This procedure has been described in an earlier paper (5). The ^{14}C-labeled Ad12 marker DNA was added after sonic treatment and hence was not fragmented (Fig. 3).

Determination of the DNA content of BHK-21 cells. BHK-21 cells in passages 19, 22, 81, and 84 were analyzed. BHK-21 cells growing in monolayers were washed with PBS and resuspended with TV. The number of cells in suspension in PBS was determined in a hemocytometer. The cells were then lysed by adding SDS to a final concentration of 0.4%. The DNA content in the lysate was determined by the Burton reaction (3). A solution of 0.4% SDS in PBS was used as the control, and calf thymus DNA was used as the standard. All determinations were done at least in duplicate. The DNA content of BHK-21 cells was found to be $1.37 \times 10^{-5} \pm 0.19 \times 10^{-5}$ μg/cell.

In vitro synthesis of Ad12 cRNA. The reaction conditions were similar to those described by So et al. (16). In a total volume of 0.98 ml, the reaction mixture contained 4.3 μg of Ad12 DNA, 169 μg of DNA-dependent RNA polymerase from *E. coli*, 300 nmoles of each of the ribonucleoside triphosphates, 1.9 nmoles of each of the ^{3}H-labeled ribonucleoside triphosphates (specific activities as described above), 240 μmoles of KCl, 75 μmoles of Tris-hydrochloride (*p*H 7.9), 7.5 μmoles of $MgCl_2$, 1.9 μmoles of $MnCl_2$, and 90 μmoles of 2-mercaptoethanol. The reaction was carried out at 37 C for 3 to 4 hr. At the end of the incubation period, 1 volume of 0.01 M Tris (*p*H 7.2), 0.01 M $MnCl_2$, 0.01 M $CaCl_2$, and 50 μg of deoxyribonuclease per ml of solution were added. The mixture was incubated at 37 C for 30 min, then chilled, and extracted twice with equal volumes of phenol saturated with 0.1× SSC. The high-molecular-weight RNA was separated from soluble ribonucleoside triphosphates by gel filtration on Sephadex G-200 (Pharmacia, Uppsala) in 0.1× SSC and was kept frozen until used. It was found that 40 to 50% of the Ad12 cRNA annealed with Ad12 DNA in DNA-RNA hybridization experiments, when 5 μg of Ad12 DNA was fixed to each filter.

DNA-RNA hybridization. DNA-RNA hybridization was carried out by the procedure of Gillespie and Spiegelman (10). In all hybridization experiments, 6× SSC was used and 200 μg of yeast RNA was added with Ad12 cRNA (10^5 to 3×10^5 counts/min) to a total volume of 1 ml. The reaction was carried out in glass scintillation vials at 65 C for 16 to 24 hr. After the incubation period the filters were washed in 0.1× SSC with 20 μg of ribonuclease per ml and washed again with 0.1× SSC. A calibration curve was established which allowed the estimation of an unknown amount of Ad12 DNA in the presence of BHK-21 DNA. Details of the calibration procedure are described in the legend of Fig. 1.

DNA-DNA hybridization. DNA-DNA hybridization was performed by the technique of Denhardt (4), as described in detail previously (6).

Inhibition of macromolecular syntheses. DNA, protein, and RNA syntheses in BHK-21 cells were inhibited by the addition of cytosine arabinoside, cycloheximide, and actinomycin D to the ETC medium at the concentrations indicated in Table 7.

Physical methods. Refractive indexes of the CsCl solutions were determined at 25 C in a thermostated Bausch & Lomb refractometer. The refractive indexes were used to calculate the density of CsCl solutions by the formula of Vinograd and Hearst (21). For experiments in the analytical ultracentrifuge, a Spinco model E was used equipped with ultraviolet optics, monochromator, and a high intensity light source. Ultraviolet absorbency photographs were traced with a Joyce-Loebl microdensitometer.

Radioactive samples were usually counted on glass-fiber papers (Whatman, GF/C), after trichloroacetic acid precipitation, in a Toluene/Liquifluor (New England Nuclear Corp.) system. Aqueous samples were counted in a 1:1 mixture of toluene and methanol containing 42 ml of Liquifluor per liter. A Packard model 3375 Tri-Carb scintillation spectrometer or a Beckman LS 133 liquid scintillation system was used. It could be shown that ^{3}H- or ^{14}C-activities in the samples were unquenched by small amounts of bovine serum albumin or of cellular proteins in the trichloroacetic acid precipitates.

RESULTS

Uptake of Ad2 and Ad12 by BHK-21 cells. Ad2 infects BHK-21 cells productively, whereas Ad12 cannot replicate in these cells (6). The uptake and retention of Ad2 and Ad12 by BHK-21 cells were compared. BHK-21 cells were inoculated with

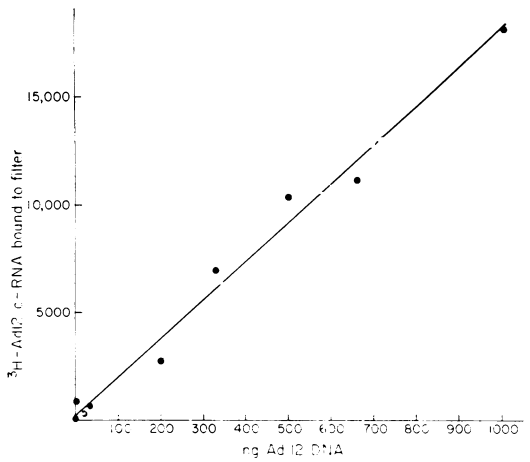

FIG. 1. *Calibration curve for DNA-RNA hybridization. Various amounts of highly purified Ad12 DNA were mixed with approximately 20 μg of BHK-21 DNA, and the DNA was fixed to Schleicher & Schuell membrane filters. A mixture of Ad12 DNA and BHK-21 DNA was used to reproduce the conditions found with DNA from Ad12-infected BHK-21 cells (Fig. 5). The amount of DNA was calculated from the OD_{260} reading of the solution, assuming that 1 ml of a DNA solution of OD_{260} = 1 corresponds to 50 μg of DNA. Ad12 cRNA was synthesized on an Ad12 DNA template. The DNA-RNA hybridization procedure of Gillespie and Spiegelman (10) was used. In 6× SSC, 139,160 counts/min of Ad12 cRNA were added to each filter. Filters with BHK-21 DNA only were used as controls. The amount of Ad12 cRNA hybridizing to the control filters was subtracted from all experimental values. The data of the DNA-RNA hybridization experiments have been fitted to a straight line by the least square method. After the filters had been counted, they were dried and their DNA content was determined by the Burton reaction to ascertain that all filters carried the same amount of DNA.*

different concentrations of ^{3}H-labeled Ad2 and Ad12, and, at various times after inoculation, the cell-associated radioactivity that remained acid-precipitable was determined (Table 1).

Only a small fraction of the Ad2 and Ad12 added was adsorbed to BHK-21 cells. The adsorption of Ad2 was 5 to 15 times more efficient than that of Ad12. Ad2 has a lower particle to plaque-forming unit (PFU) ratio than Ad12. The adsorption at the lower concentrations of virus was relatively better, since at high concentrations the receptors on the cell surface are probably saturated (12). The data in Table 1 also indicate that an increasing amount of radioactive label was lost from the cells into the medium.

The release of ^{3}H-Ad12 from infected BHK-21 cells into the medium was measured at various times after infection (Table 2). Within 24 hr after

TABLE 1. *Uptake of Ad2 and Ad12 by BHK-21 cells*[a]

Virus	Dilution	Percentage ^{3}H activity that is cell-associated and acid-precipitable at		
		2.5 hr PI[b]	22 hr PI	46 hr PI
Ad2	None	0.051	0.009	0.02
	1:10	0.10	0.014	0.028
	1:50	0.49	0.035	0.027
Ad12	None	0.011	0.003	0.006
	1:10	0.02	0.003	0.002
	1:50	0.032	0.006	0.005

[a] BHK-21 cells (1.08×10^7 cells per petri dish) were incubated with 1 ml of undiluted ^{3}H-labeled Ad2 (OD_{260} = 2.4; 4.81×10^6 counts per min per ml) or Ad12 (OD_{260} = 2.64; 7.89×10^6 counts per min per ml), or with dilutions of these viruses in PBS as indicated. After a 2.5-hr adsorption period, the cells were washed five times with 5 ml of PBS each time. The cells were then either lysed with 1 ml of STE or 5 ml of ETC was added. At time intervals as indicated, the cells were again washed five times with 5 ml of PBS and then lysed with 1 ml of STE. The total cell-associated radioactivity that was acid-precipitable was determined. A 1-ml amount of a virus suspension of OD_{260} = 1.0 corresponds to approximately 10^9 to 10^{10} PFU for Ad12.

[b] Postinfection.

infection, >75% and, at 42 hr >82% of the ^{3}H activity that was cell-associated at the end of the 2-hr adsorption period was recovered in the medium. The mechanism by which this shedding occurs has not been investigated. Most of the radioactivity in the medium was found to remain acid-precipitable.

The ^{3}H activity released into the medium was further analyzed by equilibrium sedimentation in CsCl density gradients (Fig. 2). Up to 68 hr post-infection, 70 to 80% of the ^{3}H activity in the medium exhibited a buoyant density identical to or higher than that of Ad12. The radioactivity in the regions denser than Ad12 is probably due to virus particles which have lost part of their capsomers. Therefore, it appears that >80% of the ^{3}H activity associated with the cells after the adsorption period is later again released into the medium. The bulk of the released ^{3}H activity remained in apparently intact or partly uncoated virions which persist in the medium, at least until 68 hr after infection. The released particles have lost their infectivity (5).

Strikingly different results have been reported with Ad12-infected Nil-2 cells, a Syrian hamster cell line (24). In this system, 15.8 and 22.5% of the total radioactivity added with the virus preparation could not be removed by washing of Nil-2

TABLE 2. *Release of Ad12 from infected BHK-21 cells into the medium*[a]

Expt	Time after infection (hr)	Total ^{3}H activity			
		In medium		Cell-associated	
		Counts/min	Per cent	Counts/min	Per cent
1	4.5	1,870	25.5		
	7	820	11.2		
	9	710	9.7		
	11	540	7.4		
	19	490	6.7		
	23.5	130	1.8	2,760	37.7
2	4.5	2,498	33.3		
	6.5	1,116	14.8		
	9	839	11.1		
	11	451	6.0		
	19	702	9.4	1,905	24.9
	24	269	3.6	1,776	23.1
	42	301	4.0	1,330	17.7

[a] N-BHK-21 cells (5.4×10^6 cells per dish in experiment 1; 8.8×10^6 cells per dish in experiment 2) were inoculated with 1 ml of ^{3}H-Ad12 (OD_{260} = 1.4, 2.67×10^6 counts per min per ml in experiment 1; OD_{260} = 4.7, 2.7×10^6 counts per min per ml in experiment 2). After a 2-hr adsorption period, the cell sheets were washed and 5 ml of ETC was added. At time intervals as indicated, the medium was changed. In samples of the used medium, the released ^{3}H activity was determined in duplicate or triplicate. At the end of the experiment, the cell sheets were washed again and lysed in 1 ml of STE, and the total cell-associated radioactivity was measured. The per cent values give the proportion of the total ^{3}H activity that was cell-associated after the adsorption period and could not be removed by washing.

cells which were infected at multiplicities comparable to the highest multiplicities used in the experiments described in Table 1. In the Ad12-infected Nil-2 cells, more than 70% of the radioactivity excreted into the medium became acid-soluble.

Persistence of Ad12 DNA in BHK-21 cells. The nature of the cell-associated radioactivity was further analyzed by the DNA-DNA hybridization procedure. The percentage of the acid-precipitable ^{3}H label at various times after infection of BHK-21 cells with Ad12 was also determined (Table 3). The bulk of the cell-associated viral label remained acid-precipitable up to 88 hr after infection. The parental viral label hybridized predominantly to viral DNA, and, at later times after infection, a significant amount also annealed to cellular DNA. The ratios of ^{3}H label homologous to Ad12 DNA to that homologous to cellular DNA are 25- to 1,380-fold higher in DNA from Ad12-infected cells as compared to DNA from the uninfected control cells (Table 3, experiment 2). The results of the hybridization experiments indicate that a large fraction of the parental viral label remains in viral DNA up to 88 hr after infection and that this DNA hybridizes to both cellular and viral DNA. The question arises whether the ^{3}H label hybridizing to cellular DNA is due to viral genes having become linked to cellular DNA or to the reincorporation into cellular DNA of ^{3}H-thymidine monophosphate, generated by degradation of viral DNA. It has been demonstrated previously (6) that cellular DNA synthesis continues, at least until 114 hr after infection of BHK-21 cells with Ad12.

Parental viral label in cellular DNA. As described earlier (5), free intracellular viral DNA and cellular DNA from BHK-21 cells grown in the presence of 5-BUdR can be separated in neutral and alkaline CsCl density gradients. The DNA from ^{3}H-Ad12-infected BHK-21 cells (designated 5BU-BHK-21·^{3}H-Ad12) was analyzed by equilibrium sedimentation in alkaline CsCl density gradients. In extracts of cells prepared 42 hr after infection, 30% of the total cell-associated label had the buoyant density of cellular DNA, 49% cosedimented with the ^{14}C-labeled Ad12 marker DNA, and 21% exhibited a density slightly less than that of viral DNA (Fig. 3a).

None of the ^{14}C-labeled Ad12 marker DNA, added to the gradient before centrifugation was started, appeared in the position of cellular DNA (5). This finding indicates that the ^{3}H label in the position of cellular DNA is not due to the presence of mechanically included, free viral DNA (Fig. 3a). To rule out this possibility even more rigorously, ^{3}H-Ad12 DNA (0.42 μg) was added directly to a monolayer of 2.4×10^6 5BU-BHK-21 cells which had been washed several times with PBS. The cells were then lysed and the total DNA was extracted and analyzed by equilibrium sedimentation in an alkaline CsCl density gradient (Fig. 4). All of the ^{3}H activity appeared in the density position of Ad12 DNA. Viral DNA did not contaminate the peak of cellular DNA. It can be concluded that the label appearing in the position of cellular DNA in alkaline CsCl density gradients is not due to physically enclosed, free viral DNA.

The equilibrium sedimentation patterns of the DNA from ^{3}H-Ad12-infected 5BU-BHK-21 cells in alkaline CsCl density gradients were compared before and after fragmentation of the DNA by ultrasonic treatment (Fig. 3a, b). The shift of the ^{3}H label from the heavy density stratum in which the mass of the cellular DNA remains, as indicated by the OD_{260} maximum, to an intermediate

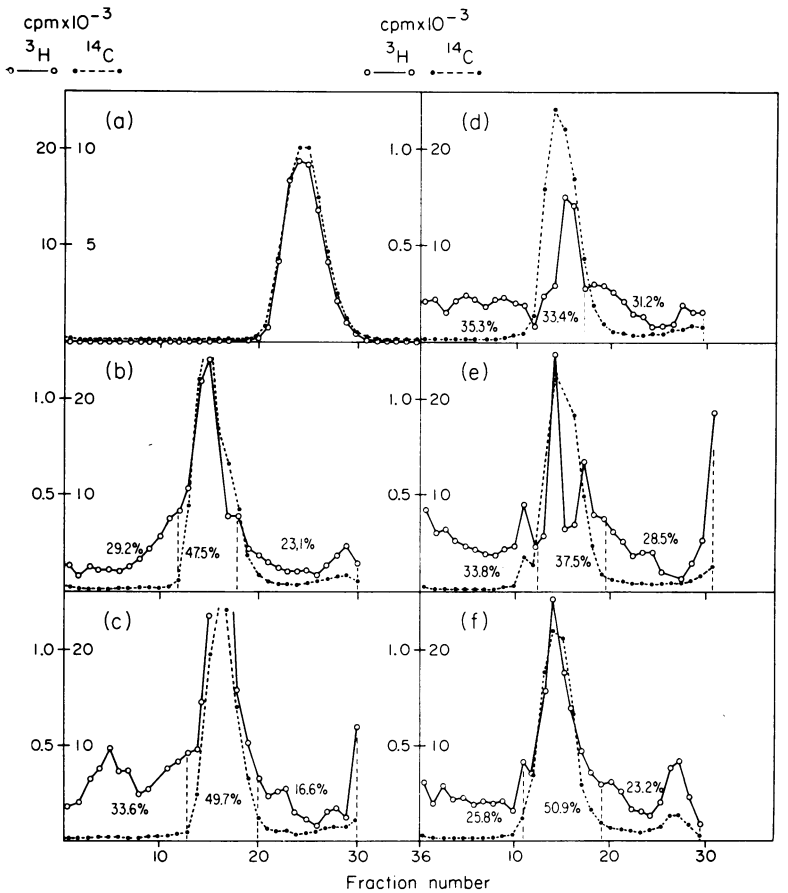

FIG. 2. *Characterization of the ³H activity released from BHK-21 cells infected with ³H-Ad12. BHK-21 cells (2.8 × 10⁶ cells per dish) were infected with 1 ml of ³H-Ad12 (OD_{260} = 4.8, 11.5 × 10⁶ counts per min per ml). After a 2-hr adsorption period, the cells were washed and 5 ml of ETC was added. At various times after infection, the medium was removed and the distribution of ³H activity was determined in CsCl density gradients. To 4.6 ml of medium were added 2.3 g of CsCl and 0.33 OD_{260} unit of ¹⁴C-labeled Ad12 which was used as density marker. The mixture was overlaid with mineral oil and centrifuged to equilibrium in an S50 rotor in a Spinco model L2-65B ultracentrifuge at 4 C for at least 15 hr at 37,000 rev/min. At the end of the run, eight-drop fractions were collected directly into scintillation vials and counted in the toluene-methanol-Liquifluor system. (a) A sample of the ³H-Ad12 inoculum (before or after adsorption) was mixed with 9 ml of ETC, 4.5 g of CsCl, and 0.33 OD_{260} unit of ¹⁴C-labeled Ad12); (b) medium 6 hr postinfection; (c) medium 11.5 hr postinfection; (d) medium 19 hr postinfection; (e) medium 43 hr postinfection; (f) medium 68 hr postinfection. The figures in the graphs give the per cent of ³H activity found in the regions of the gradients as indicated by the vertical broken lines.*

density position can clearly be recognized in Fig. 3b. The percentage of the total intracellular ³H label in the positions of heavy and intermediate density is 22.5% after sonic treatment, as compared to 30% before sonic treatment. In the experiment shown in Fig. 3b, the ¹⁴C-labeled marker DNA was added immediately before centrifugation was started and after the intracellular DNA had been fragmented by ultrasonic treatment. The observation that the peak of free ³H viral DNA appears in a position slightly lighter than that of the unbroken marker DNA may indicate that, in randomly broken Ad12 DNA, relatively lighter fragments of DNA which are rich in adenine-thymine base pairs (8) and which contain the ³H-thymidine are separated from relatively heavier fragments rich in guanine-cytosine base pairs.

The shift in density of the ³H label after sonic treatment from the heavy to an intermediate density position and the separation of a large proportion of the ³H label from the bulk of the cellular

TABLE 3. *Persistence of Ad12 DNA in BHK-21 cells*[a]

Expt	DNA from	Time PI[b] (hr)	Per cent counts/min acid-precipitable	Per cent counts/min bound to filters with		
				Ad12 DNA	BHK-21 DNA	Ratio[c]
1[d]	N-BHK·^{3}H-Ad12	3.0	84.3	89.1	1.2	74.3
		18.5	100	59.1	1.9	31.1
		52.5	77.2	17.2	0.5	34.4
	Ad12			100	0.7	142.8
	BHK-21 cells				9.5	
2[e]	N-BHK·^{3}H-Ad12	10.5	100	55.2	2.0	27.6
		25.0	77.4	56.3	7.5	7.5
		27.5	78.4	11.8	8.3	1.4
		42.0	73.5	7.8	15.4	0.5
		67.5	85.4	6.7	8.1	0.83
		88.5	100	11.6	9.8	1.2
	Ad12			88.6	0.7	126.8
	BHK-21 cells			0.3	15.1	0.02
3[f]	N-BHK·^{3}H-Ad12	2		100	0.3	330
		19		74.9	4.9	15.3
		25		49.9	7.9	6.2

[a] At time intervals after infection as indicated, the infected cells were washed five times with 5 ml of PBS each time and lysed with 1 ml of STE. In portions of 0.15 ml each of all samples, the total and acid-precipitable ^{3}H activity was determined. The remaining sample of 0.7 ml was diluted with TE to 1 ml and layered on 4 ml of the neutral CsCl solution in a nitrocellulose tube, and the DNA was centrifuged to equilibrium. The CsCl was removed by dialysis against TE, and the DNA was hybridized to Schleicher & Schuell filters loaded with Ad12 DNA or BHK-21 cellular DNA.

[b] Postinfection.

[c] Figures in this column indicate the ratio of ^{3}H label annealing to Ad12 DNA to that hybridizing with BHK-21 DNA.

[d] A total of 1.07×10^7 cells per dish were inoculated with 1 ml of ^{3}H-Ad12 (OD_{260} = 4.45; multiplicity of infection, 504 PFU/cell).

[e] A total of 6.4×10^6 cells per dish were inoculated with 1 ml of ^{3}H-Ad12 (OD_{260} = 3.02; multiplicity of infection, 782 PFU/cell).

[f] A total of 7.0×10^6 cells per dish were inoculated with 1 ml of ^{3}H-Ad12 (OD_{260} = 2.70; multiplicity of infection, 3,720 PFU/cell).

DNA (OD_{260} peak) in alkaline CsCl gradients indicate that Ad12 DNA is linked covalently to cellular DNA. Sonic treatment under the conditions employed in this investigation generates DNA fragments of approximately 2×10^6 daltons (5) in the native state which are 10^6 daltons after denaturation. It is likely that only a fragment of viral DNA is integrated, since, if the entire viral genome were integrated, the bulk of the ^{3}H label in the heavy density position would have shifted after sonic treatment to the density position of free viral DNA. This is not observed. The percentage of total ^{3}H label in the position of heavy and intermediate densities was 30% before and 22.5% after sonic treatment.

The graphs in Fig. 3c and d represent the results of control experiments in which the DNA of uninfected 5BU-BHK-21 cells was sedimented to equilibrium in alkaline CsCl density gradients together with ^{14}C-labeled Ad12 DNA, both before (Fig. 3c) and after (Fig. 3d) the cellular DNA had been fragmented by ultrasonic treatment. It is apparent that, on ultrasonic treatment, the maximum of the OD_{260} reading and that of the ^{3}H activity are not separated, in contrast to the experiment shown in Fig. 3b.

These findings are not compatible with the assumption that the ^{3}H label found in the heavy density position is due to reutilization of solubilized mononucleotides derived from the parental viral DNA, nor do the results support the possibility that viral DNA has replicated in BHK-21 cells. The data indicate that viral DNA or fragments of it become integrated into cellular DNA.

Characterization of the ^{3}H label in cellular DNA. The nature of the ^{3}H label found associated with cellular DNA in ^{3}H-Ad12-infected BHK-21 cells was characterized further. The fractions in the cellular (C) and the viral (V) DNA peaks from alkaline CsCl density gradients were pooled as

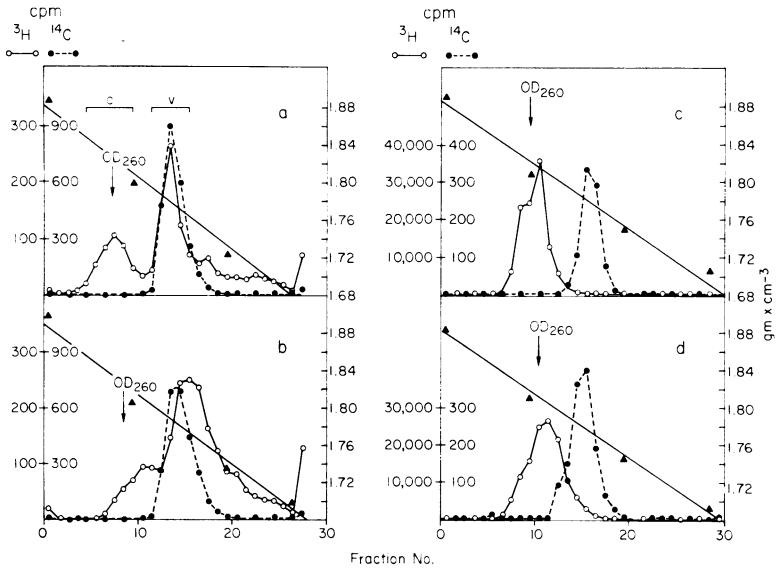

FIG. 3. *Equilibrium sedimentation patterns in alkaline CsCl of DNA from ^3H-Ad12-infected 5BU-BHK-21 cells (a, b) and from mock-infected 5BU-BHK-21 cells (c, d) before and after fragmentation. 5BU-BHK-21 cells were infected with ^3H-Ad12 (OD_{260} = 3.71). At 42 hr after infection, the cells were washed five times with 5 ml of PBS each time and lysed in 1 ml of STE. Mock-infected 5BU-BHK-21 cells were grown in medium containing 2 μCi of ^3H-thymidine per ml. The DNA was extracted and analyzed by equilibrium sedimentation in alkaline CsCl density gradients. To each gradient 5.1 μg of ^{14}C-labeled Ad12 DNA was added as density marker. (a) DNA from Ad12-infected 5BU-BHK-21 cells was sedimented to equilibrium without prior fragmentation. The horizontal bars indicate the fractions of cellular (C) and viral (V) DNA which were pooled in similar experiments and analyzed by the DNA-DNA hybridization procedure (see Table 4). The arrows indicate the position of the peak of the OD_{260} in each of the graphs. (b) DNA extracted from the Ad12-infected cells was first fragmented by ultrasonic treatment; then ^{14}C-labeled Ad12 DNA was added and the mixture was sedimented to equilibrium. (c) DNA from mock-infected 5BU-BHK-21 cells which had been labeled with ^3H-thymidine was centrifuged to equilibrium in an alkaline CsCl density gradient. (d) DNA from mock-infected 5BU-BHK-21 cells was fragmented by ultrasonic treatment; then ^{14}C-labeled Ad12 DNA was added and the mixture was centrifuged to equilibrium.*

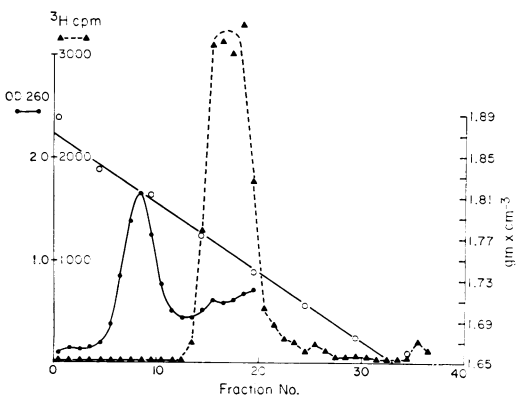

FIG. 4. *Complete separation of Ad12 DNA and 5BU-BHK-21 DNA by equilibrium sedimentation in alkaline CsCl density gradients. The ^3H-labeled Ad12 DNA was added to the cells before they were lysed with STE. The position of the unlabeled cellular DNA in the gradient was monitored by absorbency measurements at 260 nm. All fractions were then precipitated with trichloroacetic acid and counted.*

indicated in Fig. 3a and were hybridized to both Ad12 DNA and BHK-21 DNA. Four independent experiments were performed (Table 4). The intracellular DNA was extracted at various times after infection of 5BU-BHK-21 cells with ^3H-Ad12. In experiments 2 and 4, controls were included in which 4.2 μg of ^3H-labeled Ad12 DNA was mixed with 5BU-BHK-21 DNA, and the viral DNA and cellular DNA were separated in alkaline CsCl density gradients as described in Fig. 3a.

The data in Table 4 demonstrate that between 7.5 and 100% of the ^3H label associated with the cellular DNA peak in alkaline CsCl density gradients is due to viral DNA and that between 0.8 and 58.2% hybridizes to cellular DNA. The ratios listed in Table 4 provide a comparison of the relative amounts of ^3H label hybridizing with viral to that annealing with cellular DNA. In almost all of the experiments in which the ^3H label from the cellular DNA position has been analyzed, these ratios were considerably higher than 1.0. In each experiment, the ratio of the amount

TABLE 4. *Integration of Ad12 DNA into BHK-21 DNA*[a]

Expt	DNA from	DNA extracted (hr PI[b])	Per cent counts/min bound to filters with		
			Ad12 DNA	BHK-21 DNA	Ratio[c]
1[d]	5BU-BHK-21·^{3}H-Ad12				
	Cellular DNA peak	30	34.9		
	Viral DNA peak	30	98.7	1.5	64.6
	Cellular DNA peak	54	7.5	32.3	0.28
	Viral DNA peak	54	100	0.3	298
	Ad12		93.5	0.14	682
	BHK-21 cells		0.3	12.9	0.023
2[e]	5BU-BHK-21·^{3}H-Ad12				
	Cellular DNA peak	28	24.3	11.8	2.1
	Viral DNA peak	28	89.7	0.6	144
	Cellular DNA peak	46	22.1	58.2	0.38
	Viral DNA peak	46	88.6	0.85	105
	Control (5BU-BHK DNA + ^{3}H-Ad12 DNA)				
	Cellular DNA peak		0	0	
	Viral DNA peak		58.4	0.45	130
	Ad12		88.6	0.84	105.4
	BHK-21 cells		0.29	17.6	0.016
3[f]	5BU-BHK-21·^{3}H-Ad12				
	Cellular DNA peak	28	83.2	7.7	10.8
	Viral DNA peak	28	79.2	0.32	247.5
	Cellular DNA peak	37	50.7	1.8	27.6
	Viral DNA peak	37	41.1	0.66	62.2
	Cellular DNA peak	47	28.4	6.6	4.3
	Viral DNA peak	47	57.7	0.88	65.7
	Ad12		33.3	0.37	89.2
	BHK-21 cells		1.0	12.2	0.08
4[g]	5BU-BHK-21·^{3}H-Ad12				
	Cellular DNA peak	28	58.8	1.8	33
	Viral DNA peak	28	26.3		
	Cellular DNA peak	45	63.3	0.8	76
	Viral DNA peak	45	57.8		
	Cellular DNA peak	53	>100	10.0	23.5
	Viral DNA peak	53	87.3		
	Control (5BU-BHK DNA + ^{3}H-Ad12 DNA)				
	Cellular DNA peak		0	0	
	Viral DNA peak		86.7	0.27	241
	Ad12		97.4	0.46	212
	BHK-21 cells		1.2	20.2	0.06

[a] For each hybridization experiment, the total amount of ^{3}H-labeled DNA added to the reaction mixture was determined by counting an identical sample or a portion on a nitrocellulose filter under the same conditions. The per cent figures in the table were calculated as the fraction of the ^{3}H-labeled DNA bound to respective filters. These figures were not further corrected for the lower homologous hybridization of BHK-21 DNA as compared to Ad12 DNA, since the extent of homologous hybridization of cellular DNA fluctuates. At various times after infection as indicated, the ^{3}H-Ad12-infected cells were washed and lysed, and the DNA was extracted. The cellular DNA and free viral DNA were separated in alkaline CsCl density gradients. No ^{14}C-labeled marker DNA was added. The cellular (C) and viral (V) DNA peaks were located by counting 20-μliter samples of each fraction on GF/C filters. Fractions in each peak were pooled as indicated by the bars in Fig. 3a, dialyzed, and hybridized to Ad12 DNA and DNA from BHK-21 cells. DNA extracted from purified Ad12 and from uninfected BHK-21 cells was used as a control. In experiments 2 and 4, controls were included in which ^{3}H-Ad12 DNA and 5BU-BHK-21 DNA were mixed (5BU-BHK DNA plus ^{3}H-Ad12 DNA) and separated as described in Fig. 4. The DNA from the ^{3}H-Ad12 preparations used as inocula in experiments 2, 3, and 4 was extracted as de-

of ^3H label hybridizing with Ad12 DNA to that hybridizing with BHK-21 DNA was compared for the cellular DNA peak from Ad12-infected BHK-21 cells and the DNA from uninfected BHK-21 cells. This ratio was found to be 12 to 1,267 times higher for the cellular DNA from Ad12-infected BHK-21 cells. As expected, the ^3H label in the viral DNA peaks hybridized exclusively with viral DNA. Cellular DNA from uninfected BHK-21 cells hybridized only with cellular DNA, although the extent of hybridization was considerably lower than that of viral DNA to viral DNA.

The results of the control experiments (cf. experiments 2 and 4) confirm the findings presented in Fig. 4. Free viral DNA was not unspecifically enclosed in the heavy cellular DNA peak in alkaline CsCl density gradients.

It can be concluded that the bulk of the ^3H label in the heavy density position constitutes viral DNA, which is either covalently linked to cellular DNA (integration) or, less likely, is present in the heavy position because viral DNA has replicated in the presence of 5-BUdR in the medium. Evidence has been presented earlier (6, 7) that replication of Ad12 DNA in BHK-21 cells cannot be detected. Further evidence against viral DNA replication will be presented in the next section.

A part of the ^3H label in the cellular DNA peak hybridized to cellular DNA. This finding can be explained by the presence of fragments of DNA which consist of both ^3H-labeled viral and unlabeled cellular DNA. Such fragments would be expected to exist in case viral DNA had become integrated, particularly since the ^3H-labeled DNA used in DNA-DNA hybridization experiments is fragmented by ultrasonic treatment before hybridization (4).

The number of viral genome equivalents per cell both in the free state and in association with the cellular DNA can be calculated from the data in Table 4. The DNA from the ^3H-Ad12 preparations used as inocula in experiments 2, 3, and 4 (Table 4) was extracted and its specific radioactivity was determined. The figures presented in Table 5 are estimates and indicate that, 28 to 53 hr after infection of BHK-21 cells with Ad12, 20 to 444 genome equivalents of Ad12 per cell persisted, of which as many as 67% are integrated into cellular DNA. At later times after infection, the fraction of viral DNA linked to cellular DNA increased.

TABLE 5. *Estimate of the number of Ad12 genomes in Ad12-infected BHK-21 cells*[a]

Expt	Time DNA extracted (hr PI[b])	Ad12 genomes per cell	
		Integrated	As free viral DNA
2	28	6	438
	46	5	
3	28	24	140
	37	218	92
	47	55	33
4	28	17	40
	45	13	7
	53	33	16

[a] Based on the data in Table 4, the number of Ad12 genomes per cell in the integrated and the free states was calculated. The assumption was made that during the purification of the intracellular DNA and its fixation to nitrocellulose filters no DNA was lost. Since in most cases close to 90% of the control Ad12 DNA hybridized to viral DNA, no further corrections were made.

[b] Postinfection.

Absence of viral DNA replication. The methods employed in previous investigations (6, 7) did not reveal any Ad12 DNA replication in BHK-21 cells. In the presence of a large amount of highly labeled cellular DNA, the limit of detectability of newly synthesized Ad12 DNA was $\geq 0.1\%$. At high multiplicities of infection, it could not be

scribed previously (6). The specific radioactivity was calculated and found to be 3.31×10^6 counts per min per OD_{260} unit (2), 6.57×10^6 counts per min per OD_{260} unit (3) and 4.53×10^6 counts per min per OD_{260} unit (4), respectively. All of the numbers presented are the means of at least two determinations.

[b] Postinfection.

[c] Figures in this column indicate the ratio of ^3H label hybridizing with Ad12 DNA to that annealing to BHK-21 DNA.

[d] A total of 1.7×10^6 5BU-BHK-21 cells per dish were infected with 1 ml of ^3H-Ad12 (OD_{260} = 4.16; 7.41×10^6 counts per min per ml) at 1,203 PFU/cell.

[e] A total of 1.8×10^6 5BU-BHK-21 cells per dish were infected with 1 ml of ^3H-Ad12 (OD_{260} = 3.36; 1.41×10^7 counts per min per ml).

[f] A total of 2.7×10^6 5BU-BHK-21 cells per dish were infected with 1 ml of ^3H-Ad12 (OD_{260} = 7.0; 1.3×10^7 counts per min per ml).

[g] A total of 1.9×10^6 5BU-BHK-21 cells per dish were infected with 1 ml of ^3H-Ad12 (OD_{260} = 4.92; 1.0×10^7 counts per min per ml).

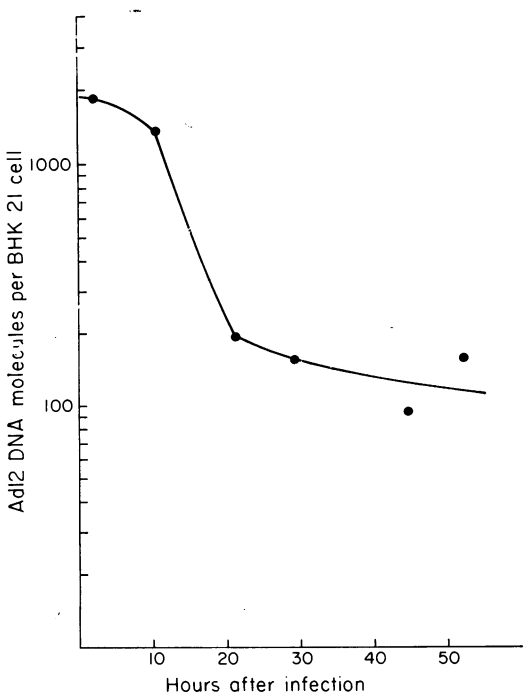

FIG. 5. *Absence of replication of Ad12 DNA in BHK-21 cells. N-BH-K21 cells (6.8 × 10⁶ cells per dish) were infected with unlabeled Ad12. At various times after infection, the cells were washed and lysed, and the total intracellular DNA was purified by equilibrium sedimentation in neutral CsCl gradients. Samples of the purified DNA were denatured by heating and fixed to Schleicher & Schuell membrane filters. Ad12 cRNA was synthesized in vitro and hybridized to the DNA from Ad12-infected BHK-21 cells in 6× SSC by the method of Gillespie and Spiegelman (10). Per filter, 139,160 counts/min of cRNA were added; all determinations were performed in duplicate. Unspecific hybridization was corrected for by subtracting from all results background values determined with filters carrying DNA from uninfected BHK-21 cells. After the filters had been washed and counted, they were dried and the amount of DNA on each filter was determined by the Burton reaction. The amount of DNA per filter was the same as in the calibration experiment (Fig. 1). From the amount of DNA per filter, the equivalent number of BHK-21 cells can be calculated, since it is known that one cell contains 1.37 × 10⁻⁵ μg of DNA. The number of counts/min of cRNA retained per filter can be converted into the number of Ad12 DNA molecules per filter by using the calibration curve in Fig. 1 and can be correlated to the number of infected BHK-21 cells. At 2 hr after infection, there were 54.8 ng of Ad12 DNA per filter, 0.88 × 10⁶ BHK-21 DNA equivalents per filter, and 1,868 Ad12 DNA molecules per cell (control value subtracted). At 10.5 hr after infection, the corresponding values were: 67.9; 1.47 × 10⁶; 1,386. At 21 hr after infection, the corresponding values were: 9.3; 1.43 × 10⁶; 195. At 29 hr after infection, the corresponding values were: 6.8; 1.31 × 10⁶; 156. At 44.5 hr after infection, the corresponding values were: 4.2; 1.31 × 10⁶; 96. At 52 hr after infection, the corresponding values were: 8.1; 1.52 × 10⁶; 160.*

ruled out that 1 to 10% of the input viral genomes might have replicated once (7).

A more sensitive technique was devised to detect an increase of Ad12 genomes in BHK-21 cells should it occur. The DNA of BHK-21 cells infected with unlabeled Ad12 was extracted at various times after infection and fixed to membrane filters. The amount of ^{3}H-labeled Ad12 cRNA synthesized in vitro which bound to the filters was determined (Fig. 5). By using the calibration curve determined for DNA-RNA hybridization (Fig. 1), the number of viral genomes per BHK-21 cell can be calculated. Details of this calculation are described in the legend of Fig. 5. The number of viral genomes per BHK-21 cell declined rapidly; an increase was not observed at any time after infection. This result makes it very unlikely that Ad12 DNA replicates in BHK-21 cells. The loss of viral DNA from the infected cells can be accounted for by shedding of virions (Table 2).

The number of molecules of Ad12 DNA per cell determined by this method is in the same order of magnitude as that calculated from the results of the DNA-DNA hybridization experiments (Tables 4 and 5).

It might be argued that Ad12 DNA replication could be blocked in N-BHK-21 cells (Fig. 5) but might proceed in 5BU-BHK-21 cells. 5BU-BHK-21 cells were infected with unlabeled Ad12 or mock-infected with PBS. After infection, the cells were maintained in medium without 5-BUdR, and ^{3}H-thymidine was added. At various times after infection, the total intracellular DNA was purified and analyzed by DNA-DNA hybridization to Ad12 DNA, as described earlier (7). The results of this experiment likewise do not give any evidence for replication of Ad12 DNA in 5BU-BHK-21 cells (Table 6).

Since the replication of Ad12 DNA in BHK-21 cells can be essentially ruled out, it may be concluded that the viral ^{3}H label found in the position of cellular DNA represents Ad12 genes covalently linked to cellular DNA.

Inhibition of integration. It would be of considerable interest to determine the mechanism by which integration takes place. Is cellular DNA replication a precondition for integration? Are enzymes involved that are coded for by the cellular or the viral genome or both? Studies with inhibitors of macromolecular synthesis may at least partly answer these questions.

The results presented in Table 7 show the extent of inhibition of macromolecular syntheses in BHK-21 cells by chemical inhibitors. 5BU-BHK-21 cells were infected with ^{3}H-Ad12 and, for various periods after infection, DNA, protein, and RNA syntheses were inhibited by cytosine arabinoside, cycloheximide, and actinomycin D, respectively (Table 8). The results indicate that inhibition of DNA synthesis at any time after infection does not affect integration. The same result is obtained when cytosine arabinoside (30 μg/ml) is added 2 hr before infection and is present throughout the 42-hr period after infection. Inhibition of protein or RNA synthesis during the first 24 hr after infection interferes with integration but does not completely suppress it. It can also be noted that the presence of chemical inhibitors in the medium leads to a loss of cell-associated ^{3}H label. The cause of this loss of ^{3}H label and the mechanism by which it occurs have not been investigated further.

It is concluded that inhibition of DNA synthesis to the extent of more than 96% does not markedly affect the integration of viral genes in Ad12-infected BHK-21 cells and that inhibition of protein and RNA synthesis interferes with it only slightly.

TABLE 6. *DNA-DNA hybridization on DNA from Ad12-infected and mock-infected 5BU-BHK-21 cells*[a]

DNA from	Time 20 μCi of ^{3}H-thymidine per ml added (hr)[b]	Per cent counts/min hybridized to Ad12 DNA[c]
5BU-BHK·Ad12	2–19	1.42
5BU-BHK·PBS	2–19	2.85
5BU-BHK·Ad12	2–27.5	1.43
5BU-BHK·PBS	2–27.5	1.28
5BU-BHK·Ad12	2–44.5	0.87
5BU-BHK·PBS	2–44.5	1.99
Ad12		25.6

[a] To each filter 5 μg of unlabeled Ad12 DNA was fixed. More than 80% of the Ad12 DNA was adsorbed to the filters.

[b] Postinfection for 5BU-BHK·Ad12 cells; post-mock infection for 5BU-BHK·PBS cells.

[c] Values are the means of two determinations. 5BU-BHK-21 cells (1.42×10^6 cells per dish) were infected with unlabeled Ad12 ($OD_{260} = 1.25$) or mock-infected with PBS. The cells were maintained in ETC medium without 5-BUdR, and 20 μCi of ^{3}H-thymidine per ml was added. At times as indicated, the intracellular DNA was extracted and purified. Samples of the DNA were analyzed by the DNA-DNA hybridization method by using filters with Ad12 DNA.

DISCUSSION

It is not known at what point the replication cycle of Ad12 in BHK-21 cells is blocked. The results presented in this report demonstrate that Ad12 adsorbs to BHK-21 cells, although the adsorption is markedly less efficient than that of Ad2 which productively infects BHK-21 cells. In Nil-2 cells infected with Ad12, the uptake of Ad12 appears to be greater by two to three orders of magnitude (24). In electron micrographs, Ad12 particles can be observed in cytoplasmic vesicles and free in the cytoplasm of BHK-21 cells (Compans and Doerfler, *unpublished data*). It has been shown that after infection at high multiplicities nearly 100% of the hamster cells synthesize Ad12-specific T antigen (18, 23), which is considered to be one of the early virus-specific functions. Infection of hamster cells with Ad12 leads to a stimulation of cellular DNA synthesis (6, 15, 19, 20). As shown in this paper and in two previous communications, viral DNA replication cannot be detected by a number of techniques (6, 7).

It has been reasoned (5) that the fate of the DNA of Ad12 in nonproductively infected BHK-

TABLE 7. *Inhibition of macromolecular syntheses in BHK-21 cells*[a]

Inhibitor	Per cent inhibition of					
	DNA synthesis		RNA synthesis		Protein synthesis	
	12.5 hr	23 hr	12.5 hr	23 hr	12.5 hr	23 hr
Cytosine arabinoside (30 μg/ml)	92.2	91.0	0	20.9	3.4	38.6
Cycloheximide (75 μg/ml)	74.6	79.2	72.0	82.2	97.2	97.0
Actinomycin D (20 μg/ml)	92.5	97.6	99.6	99.7	0	52.4

[a] N-BHK-21 cells growing in ETC were exposed to inhibitors as indicated. The incorporation into acid-insoluble material of ^{3}H-thymidine (2 μCi/ml), ^{3}H-protein hydrolysate (2 μCi/ml), and ^{3}H-uridine (2 μCi/ml) in the presence and absence of the inhibitors was determined at 12.5 and 23 hr after addition of the chemicals.

TABLE 8. *Inhibition of integration of Ad12 DNA*[a]

Inhibitor[b]	Time after infection inhibitor was present (hr)	Total ^3H counts/min in gradient	Per cent ^3H label in the position of		
			Cellular DNA	Viral DNA	Light fraction[c]
None		917	27	43.7	29.3
Cytosine arabinoside	2–9	446	29.4	50.7	19.9
	9–24	92	21.8	78.2	
	24–42	522	21.9	66.3	11.8
Cycloheximide	2–9	576	11.3	78.3	10.4
	9–24	344	11.6	78.5	9.9
	24–42	465	20.0	61.3	18.7
Actinomycin D	2–24	362	4.4	68.5	21.1
	24–42	244	30.7	50.4	18.9

[a] 5BU-BHK-21 cells (4.0×10^6 cells/dish) were infected with ^3H-Ad12 (3.14×10^6 counts per min per ml). After adsorption, the cells were washed six times with 5 ml of PBS and 5 ml of ETC was added with 5 μg of 5-BUdR per ml. For the time periods after infection as indicated, inhibitors were present in the maintenance medium; at the end of the inhibition period, the medium was removed, the cells were washed once with PBS, and fresh medium containing 5-BUdR was added. At 42 hr after infection, the total intracellular DNA was extracted and analyzed by equilibrium sedimentation in neutral CsCl density gradients as described previously (5). The fractions of the total ^3H activity found in the gradients in the cellular and the viral DNA positions and in a position lighter than that of viral DNA were calculated. The extent of inhibition of macromolecular syntheses was determined in a separate control experiment by using the same periods of inhibition and was found to be as follows: DNA synthesis, 96.1% inhibited; protein synthesis, 98.7% inhibited; RNA synthesis, >99.7% inhibited. These figures represent means of the values obtained for each time interval.

[b] Per milliliter of medium, 30 μg of 1βD-arabinofuranosylcytosine (cytosine arabinoside), 75 μg of cycloheximide, or 30 μg of actinomycin D was added.

[c] As "light fraction" are designated five to six fractions adjacent to the lighter side of the peak of ^{14}C-labeled viral marker DNA (*see* Fig. 3).

21 cells may give a clue to the early events in this virus-cell interaction which eventually leads to the transformation of a small number of BHK-21 cells (17).

Previously, it has been shown that ^3H-thymidine from ^3H-labeled Ad12-infected BHK-21 cells becomes covalently linked to cellular DNA. Three alternative interpretations of this finding have been discussed: (i) degradation of viral DNA to mononucleotides and reutilization of ^3H-thymidine monophosphate; (ii) replication of viral DNA in 5-BUdR-containing medium; and (iii) integration of viral genes into cellular DNA. Evidence in favor of the latter possibility has been presented previously (5, 24).

The purpose of the present paper has been to support further this interpretation, and, if possible, to rule out the first and second possibilities. The results of the DNA-DNA hybridization experiments (Table 4) clearly demonstrate that viral genes shift to the buoyant density of heavy cellular DNA. The finding that hybridization also occurs to cellular DNA is predicted by the integration model. In DNA-DNA annealing experiments, the ^3H-labeled DNA samples are broken by ultrasonic treatment (4); hence DNA fragments will be generated that contain both viral and cellular DNA. Hybridization to cellular DNA could also be explained by reincorporation of mononucleotides into cellular DNA. A small part of the viral ^3H label in cellular DNA may indeed be due to reutilization, as some of the cell-associated ^3H label has been found to be acid-soluble (Table 3). However, the bulk of viral ^3H label in cellular DNA must be due to the presence of viral genes. These genes could be detected by the DNA-DNA hybridization method, which was not possible in the experiments described earlier (7), in which a large excess of highly labeled cellular DNA was present.

If viral DNA replication could be completely ruled out, there would be a strong case for integration. It has been shown that all of the DNA synthesized in Ad12-infected BHK-21 cells sediments with the rate of cellular DNA in zonal sedimentation analysis (5). Moreover, the analysis of the DNA from Ad12-infected BHK-21 cells by zonal sedimentation in alkaline sucrose density gradients, by DNA-DNA hybridization (6, 7), and by DNA-RNA hybridization (Fig. 5) has not

permitted detection of any newly synthesized viral DNA. All of these results argue against viral DNA replication. An analysis of the appearance of viral genes in the heavy density position of cellular DNA also has helped to rule out the possibility of viral DNA replication. The ^3H label shifts directly to the heavy density position, approximately 20 to 24 hr after infection, and cannot be found transiently in an intermediate density stratum (5). The position of the ^3H label exactly coincides with the ultraviolet absorbency peak due to cellular DNA. The ^3H label from the cellular density position sediments with the rate of cellular DNA in band velocity sedimentation experiments (5). The ^3H label shifts, however, to an intermediate density position upon fragmentation of the DNA by sonic treatment to pieces of approximately 2×10^6 daltons, which would not be expected if the viral DNA had become heavy because of replication. Furthermore, when DNA synthesis is inhibited chemically by more than 96% in Ad12-infected BHK-21 cells, the relative amount of ^3H label in the density position of cellular DNA remains constant (Table 8).

Considering all these observations, the likeliest explanation for the appearance of viral ^3H-thymidine label in cellular DNA is that Ad12 DNA or fragments of it are integrated by covalent linkage into cellular DNA. The data in Table 5 suggest that between 5 and 55 equivalents of Ad12 DNA per cell become integrated. In one experiment, >200 equivalents of Ad12 DNA became linked to cellular DNA. The reason for the variation in the number of Ad12 genomes that become integrated is not known.

The objection could be raised that the results presented in this paper were obtained with cellular DNA in which 50% of the thymidine residues were substituted by 5-BUdR (5). However, when the DNA of N-BHK-21 cells infected with ^3H-Ad12 is analyzed by zonal sedimentation in alkaline sucrose density gradients and DNA-DNA hybridization, it is found that the ^3H label in the fast-sedimenting cellular DNA hybridizes with both viral and cellular DNA (Burlingham and Doerfler, *in preparation*).

The finding that the integrated viral DNA shows a shift in buoyant density towards an intermediate density position when the DNA is fragmented implies that the size of the integrated Ad12 DNA is rather small and that the integrated viral genes have replicated with the cellular DNA and contain 5-BUdR in one strand (5).

The mechanism by which integration occurs is not known. It can be speculated that the adenovirus endonuclease which has been found in extracts of infected cells (B. T. Burlingham and W. Doerfler, Fed. Proc. **28:** 434; *in preparation*) and which recently has been localized in the adenovirus pentons (B. T. Burlingham et al., submitted to J. Mol. Biol.) may play a role in integration. The endonuclease is known to cleave the viral genome preferentially at specific sites, probably in the regions relatively rich in guanine-cytosine base pairs (8). The fragments may then be integrated. The assumption that fragments of viral DNA become integrated is consistent with the finding that infectious Ad12 cannot be recovered from Ad12-infected (W. Doerfler, *unpublished data*) or transformed hamster cells (24).

Experiments are in progress in which extracts of ^3H-Ad12-infected N-BHK-21 cells are analyzed in CsCl density gradients in the presence of ethidium bromide (14) or propidium iodide (11) to determine whether the parental Ad12 DNA becomes circularized.

Transformation of BHK-21 cells by Ad12 is an infrequent event (17). The role that integration plays in transformation is not known. Is it a conditio sine qua non? An attractive hypothesis is that integration of a specific viral DNA fragment has to occur at a specific site or a specific combination of sites of the cellular genome to trigger the events that eventually will lead to transformation. It is conceivable that integration at a specific site alters cellular genes which control regulatory functions.

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