JOURNAL OF VIROLOGY, Sept. 1969, p. 256-263
Vol. 4, No. 3

Fate of Adenovirus Type 12 Genomes in Nonpermissive Cells

HARALD ZUR HAUSEN[1] AND FRANTISEK SOKOL

Virus Laboratories, The Children's Hospital of Philadelphia, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, 19146, and The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

Received for publication 22 April 1969

The fate of ^{3}H-thymidine-labeled adenovirus type 12 deoxyribonucleic acid (DNA) was studied in Nil-2 cells of Syrian hamster origin. It was found that a substantial fraction of ^{3}H-adenovirus type 12 DNA became degraded within 24 hr after infection and was released into the culture fluid. After infection of 5-bromodeoxyuridine (BUdR)-prelabeled cells with ^{3}H-adenovirus type 12, viral DNA became readily separable from cellular DNA by equilibrium centrifugation in CsCl. Part of the viral radioactivity was found to shift gradually to the position of cellular DNA as time progressed after infection. When exponentially growing cells were exposed simultaneously to BUdR, 5-fluorodeoxyuridine, and ^{3}H-adenovirus type 12, up to 50% of the viral radioactivity shifted within 24 hr from the density of viral DNA to that of cellular DNA after equilibrium centrifugation in CsCl. Upon denaturation of the cellular DNA, the isotope was preferentially found to be associated with the "heavy" strand which was synthesized after infection. Upon hybridization of the "heavy" and the "light" strands with sonically treated, denatured ^{3}H-adenovirus type 12 DNA, small and nearly equal amounts of counts hybridized with both strands. The number of counts annealed was in a range similar to that of those annealed with the same amount of DNA derived from adenovirus type 12-transformed hamster cells. These results demonstrate that (i) a substantial proportion of the adsorbed virus becomes degraded within 24 hr; (ii) part of the degradation products is reutilized for cellular DNA synthesis; (iii) only a small fraction, mainly fragments, of viral DNA becomes integrated into both the newly synthesized and the parental strands of cellular DNA.

Syrian hamster cells are nonpermissive for adenovirus type 12. Neither production of infectious virus (8, 12, 16) nor replication of viral deoxyribonucleic acid [DNA (5)] have been observed after infection of such cells, although early viral functions, such as induction of T-antigen (8, 12, 16) synthesis and of chromosomal aberrations, are expressed (18).

Recently, observations by Doerfler suggested integration of viral genomes into host cell DNA, which increased as time progressed (5). Yet, in his study, the possibility of incorporation of degradation products of viral DNA into cellular DNA has not been rigidly excluded. Therefore, we designed experiments to determine (i) the extent of degradation of infecting adenovirus type 12 genomes in growing cultures of Syrian hamster cells, (ii) possible reutilization of degradation products for cellular DNA synthesis, and (iii) the extent of integration of viral DNA, if any, into host cell DNA and the mechanisms by which it might be achieved. The results of these studies are described in the present report.

MATERIALS AND METHODS

Cells and medium. Origin and maintenance of the Nil-2 line of Syrian hamster cells (4), as well as of human KB cells, have been described (17, 18). The adenovirus type 12-transformed hamster cell lines H-A12-4 and H-A12-7 were established in this laboratory and will be described elsewhere (zur Hausen, *in preparation*). All cells were maintained on medium RPMI-1640 (Grand Island Biological Co., Grand Island, N.Y.) containing 10% fetal calf serum (FCS), 100 units of penicillin per ml, and 100 μg of streptomycin per ml.

Virus. Adenovirus type 12 was propagated in KB cells (17) and assayed by the plaque method described by Ledinko (10). The virus was purified by the method of Green and Piña (6). After three consecutive equilibrium centriguations in RbCl, the virus was dialyzed against three changes of 0.01 M tris(hydroxymethyl)-

[1] Present address: Virologisches Institut der Universität Würzburg, 8700 Würzburg, Germany.

aminomethane(Tris)-chloride at *p*H 8.1 for 24 hr and then used immediately for infection.

Labeling of virus with ^{3}H-thymidine. The virus-labeling procedure has been described previously (17). Approximately 5×10^8 KB cells were infected at input multiplicities between 10 and 100 plaque-forming units (PFU)/cell. ^{3}H-thymidine was added 18 to 20 hr postinfection at a concentration of 2.5 μc/ml. These cells were harvested 3 days after infection, and the virus was extracted and purified.

Infection of cells Cultures of Nil-2 cells in 6-cm plastic petri dishes (10^6 cells unless stated otherwise) were used for infection. The medium was removed, and the cell sheet was washed three times with Hanks balanced salt solution (HSS) before 0.4 ml of the viral suspension was added per petri dish. After 2 hr at 37 C, unadsorbed virus was removed, the cell sheet washed again three times with HSS, and fresh medium was added.

Labeling of cellular DNA with 5-bromodeoxyuridine (BUdR). Two different cellular DNA-labeling procedures were employed. (i) Nil-2 cells were grown for 96 hr in 6-cm plastic petri dishes in the presence of 5 μg of 5-bromodeoxyuridine (BUdR) as described by Doerfler (5). Thereafter, the cells were infected with purified ^{3}H-adenovirus type 12. At this time every petri dish contained approximately 3×10^6 cells. After the adsorption period fresh medium, again containing 5 μg/ml BUdR and 10% dialyzed FCS, was added. (ii) Cells were seeded at a density of approximately 5×10^5 cells per petri dish. After 24 hr the cultures, containing approximately 10^6 cells per petri dish, were infected with purified ^{3}H-labeled adenovirus type 12 diluted in HSS containing 5 μg of BUdR and 15 μg of 5-fluorodeoxyuridine (FUdR) per ml. After the adsorption period, the cell sheets were washed, and medium was added which contained 10% dialyzed FCS, 5 μg of BUdR per ml, and 15 μg of FUdR per ml.

Isolation of viral DNA. Viral DNA was isolated from purified virus as described by Levine and Ginsberg (11).

Isolation of cellular DNA. The cells were washed three times with phosphate-buffered saline solution (PBS) and then were lysed with 2 ml of a 1% sodium lauryl sulfate (SDS) solution at *p*H 7.8, containing 0.05 M Tris-chloride and 0.01 M ethylendiaminetetraacetate (EDTA). Pronase B (500 μg/ml) was added 20 min later. The mixture was incubated for 30 min at 37 C, and the lysate was extracted three times with two volumes of phenol saturated with 0.01 M Tris-chloride at *p*H 7.8. The phenol was removed by dialysis against three changes of 1,000 ml each of SSC (0.15 M NaCl plus 0.015 M citrate) for 24 hr.

Equilibrium centrifugation of DNA in CsCl solutions. DNA in SSC was brought to a density of 1.73 g/cm^3 by addition of solid CsCl and centrifuged in the SW 50 rotor of a Spinco centrifuge at $74,000 \times g$ at 25 C for 48 hr. Fractions of 0.1 ml were collected from the bottom of the tube, and their refractive indexes at 25 C were determined. The density was calculated from the refractive index as described by Schildkraut et al. (14). The fractions were then diluted with 0.3 ml of distilled water and the optical densities at 260 and 280 nm were determined.

Denaturation of DNA. DNA in 0.01 × SSC (SSC diluted 100-fold with distilled water) was diluted with the same buffer solution to a concentration of less than 35 μg/ml and heated for 10 min at 100 C. The sample was then chilled immediately in an ice bath.

Rate zonal centrifugations. These were carried out in linear 5 to 25% sucrose density gradients in 0.15 M NaCl, 0.05 M Tris-chloride, and 0.001 M EDTA at *p*H 7.8. The labeled viral DNA was mixed with 0.8 mg of Nil-2 cell ribosomal ribonucleic acid (RNA), layered on top of the sucrose gradient, and centrifuged at 5 C in the SW 25.1 rotor of a Spinco centrifuge at $51,000 \times g$ for 16 hr. Fractions of 1.5 ml were collected after piercing the bottom of the tube. Sedimentation coefficients were determined according to Martin and Ames (13) by using the cold ribosomal RNA as markers.

Determination of acid-soluble radioactive material. Precooled samples of acid-soluble radioactive material were diluted with equal volumes of cold 10% trichloroacetic acid and kept in an ice bath for 30 min. A few drops of 0.5% bovine serum albumin (BSA) solution were added as carrier when necessary, and samples, were filtered through Bac-T-Flex, B-6, 25-mm membrane filters (Schleicher & Schuell, Keene, N.H.). The radioactivity of the filtrate was then determined in Bray's scintillation fluid (2). After the filters were washed two times with cold 5% trichloroacetic acid, they were dried and assayed for radioactivity in Liquifluor.

DNA-DNA homology studies. DNA-DNA hybridizations were performed by the technique described by Aloni et al. (1). Heat-denatured nonlabeled DNA in 5 ml of 2 × SSC was filtered through Schleicher & Schuell membrane filters, presoaked in 2 × SSC. On filtration of radioactive denatured adenovirus type 12 DNA, about 80% of the DNA was retained by the membranes. The membranes were washed with 100 ml of 2 × SSC and dried at room temperature and then at 80 C for 4 hr. They were placed into scintillation vials and incubated with 1.5 ml of 0.04% BSA solution in 3 × SSC for 6 hr at 60 C. Purified ^{3}H-labeled adenovirus type 12 DNA (specific activity 24,000 counts per min per μg of DNA) was sonically treated in a Raytheon sonicator at maximum output for 15 min. It was subsequently denatured by heating and added in 0.25-ml volumes to the membranes. After incubation at 60 C for an additional 23 hr, the membranes were removed from the vials and rinsed three times with 0.003 M Tris (*p*H 9.4); both sides were washed with 100 ml of the same solution. After drying, the radioactivity was determined in Liquifluor. Empty membranes, as well as membranes containing comparable amounts of DNA from uninfected cells, were included as controls in every experiment. The extent of nonspecific binding to these control membranes was similar and showed an increase from 4 counts/min at an input of 2,000 counts/min to 106 at an input of 100,000.

Radioisotopes and chemicals. ^{3}H-Thymidine (specific activity 12 c/mmole), ^{14}C-thymidine (specific activity 12 mc/mmole), ^{3}H-BUdR (specific activity 12.3 c/mmole), as well as nonradioactive BUdR, and CsCl (optical grade) were purchased from Schwartz Bio-Research, Orangeburg, N.Y. Pronase B was obtained

from Calbiochem, Los Angeles, Calif. FUdR was a generous gift of Hoffman La Roche, Inc., Nutley, N.J.

Autoradiography. The method of autoradiography used has been described previously (19).

RESULTS

Degradation of adenovirus type 12 DNA during interaction with hamster cells. To study the fate of adenovirus type 12 DNA in nonpermissive cells, cultures of 10^6 Nil-2 cells were infected with purified ^{3}H-labeled adenovirus type 12 at an input multiplicity of 6×10^3 PFU/cell. The total and acid-soluble radioactivities in various fractions of the cultures were then determined at intervals (Table 1).

After an adsorption period of 2 hr, a substantial fraction of "adsorbed" radioactivity was removed by three consecutive washings of the cell sheets with 2 ml of HSS each. Further washing (not shown in the table) released only negligible amounts of radioactivity. After addition of medium and incubation of the infected cultures at 37 C for 24 hr, 40 to 49% of the adsorbed radioactivity was released into the medium. More than 50% of this radioactivity was acid-soluble, demonstrating that degradation of viral DNA had occurred. The cell-bound radioactivity recovered in the DNA extracted from these cells at 24 hr postinfection by SDS and phenol did not contain significant amounts of dialysable radioactivity. Indeed, rate zonal centrifugation of viral DNA isolated 24 hr postinfection from BUdR- and FUdR-treated cells and separated from cellular DNA by banding in CsCl solution revealed that the vast majority of the viral DNA molecules were not degraded. Additional experiments showed that the proportion of total radioactivity adsorbed to the cells and released into the medium within 24 hr postinfection as acid-soluble material was essentially independent of an input multiplicity within the range of 10^2 to 10^4 PFU/cell.

TABLE 1. *Degradation of adenovirus type 12 DNA within 24 hr after infection of Nil-2 cells with purified ^{3}H-thymidine-labeled virus*[a]

No.	Prepn[a]	Radioactivity[b]	
		Total	Acid-soluble
		%	%
1	Original virus	100	0.02; 0.06
2	Nonadsorbed radioactivity 2 hr PI	68.2; 78.2	0.14; 0.2
3	Adsorbed radioactivity	21.8; 31.8	
4	First wash fluid (2 hr PI)	4.6; 8.0	0.05; 0.12
5	Second wash fluid (2 hr PI)	0.5; 1.2	ND[c]
6	Third wash fluid (2 hr PI)	0.1; 0.1	ND
7	Adsorbed radioactivity not removable by washing	15.8; 22.5	
8	Radioactivity released into medium within 24 hr PI	6.3; 10.9	3.5; 6.1
9	Radioactivity recovered in cells lysed 24 hr PI by SDS	4.2; 6.6	
10	Recovered counts after phenol extraction and dialysis of sample no. 9	4.7; 8.5	

[a] Nil-2 cells (10^6) were infected with 6×10^3 PFU of purified ^{3}H-labeled adenovirus type 12 (corresponding to 200,000 counts/min) per cell. PI = postinfection.

[b] Data from two independent experiments.

[c] Not done.

The following two experiments were carried out to determine whether the degradation of viral DNA during the 24-hr incubation period at 37 C was due to the interaction of the virus and the host cell or whether it occurred upon release of virus from the cells.

(i) The 25,000 counts/min of ^{3}H-labeled adenovirus type 12 (specific activity 25,000 counts/min per μg of DNA) was exposed at 37 C for 24 hr to culture medium containing 10% FCS, and the amount of radioactive material which became acid-soluble was determined.

(ii) Nil-2 cells (10^6) were infected with 200,000 counts/min of the same viral preparation, and the amount of acid-soluble material released into the medium within 24 hr postinfection was determined. Only 1.4% of the radioactivity was rendered acid-soluble by exposure of the virus to the medium. In contrast, the infected cells released 14,000 counts/min into the medium, of which 70.5% was acid-soluble. The media from both experiments were analyzed by equilibrium centrifugation in CsCl solution. The virus exposed to medium banded as a sharp peak at 1.34 g/cm^3, whereas the radioactivity released from the infected cells was distributed throughout the gradient.

Interaction of BUdR-labeled host cells with ^{3}H-labeled adenovirus type 12. Cultures of Nil-2 cells were exposed to BUdR for 96 hr prior to infection with 10^4 PFU/cell of ^{3}H-adenovirus type 12. At the time of infection, each petri dish contained approximately 3×10^6 cells. The DNA

was extracted from some of the infected cultures after the 2-hr adsorption period and from others after 24 and 48 hr of further incubation. In addition, the DNA was extracted from uninfected cultures which had been exposed to both unlabeled BUdR and ^{3}H-BUdR (0.1 μc/ml). The various DNA preparations were banded in CsCl. Normal cellular DNA labeled with ^{14}C-thymidine was added as a marker. The buoyant density of BUdR-DNA (1.760 to 1.770 g/cm^3) isolated from noninfected cells was considerably higher than that of the marker ^{14}C-DNA (1.700 g/cm^3) isolated from cells not exposed to BUdR (Fig. 1A). When cultures which had been treated with BUdR for 96 hr were exposed to 1 μc of ^{3}H-thymidine per ml for 24 hr and then examined by autoradiography, only 10 to 20% of the nuclei were found to be labeled. The labeled DNA extracted immediately after the 2-hr adsorption period from ^{3}H-adenovirus infected, BUdR-treated cultures banded exclusively in the position of normal viral DNA (Fig. 1B). In DNA preparations obtained 24 and 48 hr postinfection, however, an increasing proportion of the label banded with the cellular DNA (Fig. 1C, 1D). The shift of the label from the position of viral DNA to that of cellular DNA in the course of incubation was previously observed by Doerfler, who used a similar experimental approach (5).

To further explain this phenomenon, a different technique for the labeling of cellular DNA with BUdR was employed. Cultures containing 10^6 Nil-2 cells were infected with 10^4 PFU of ^{3}H-adenovirus type 12 per cell. After the adsorption period, BUdR and FUdR were added simultaneously. The DNA was isolated 24 hr later and banded in CsCl. In the presence of the two analogues, a "hybrid" DNA molecule was formed. This was composed of a "heavy" strand, in which thymidine was almost completely replaced by BUdR, and a "light" strand containing thymidine instead of BUdR. A minor fraction of DNA started a second cycle of replication as evidenced by the presence in some preparations of a small amount of DNA which banded at 1.805 g/cm^3. Under these experimental conditions, both the infected and the noninfected cultures still revealed growth. Autoradiography showed that after cultures treated with nonlabeled BUdR and FUdR were exposed for 24 hr to 0.1 μc of ^{3}H-BUdR per ml, 92% of the nuclei were labeled. Up to 50% of the radioactivity recovered from the infected cells 24 hr postinfection was found in the "hybrid" cellular DNA (Fig. 2). To decide whether this radioactivity was associated with one or both of the strands of cellular DNA, the "hybrid" DNA was rebanded and freed of CsCl by dialysis. After being denatured by heating, it was banded again in CsCl (3). The position of the bands was determined by measuring the absorbance of the fractions collected at 260 nm. The buoyant density of the "heavy" strand was 1.825 g/cm^3, whereas that of the

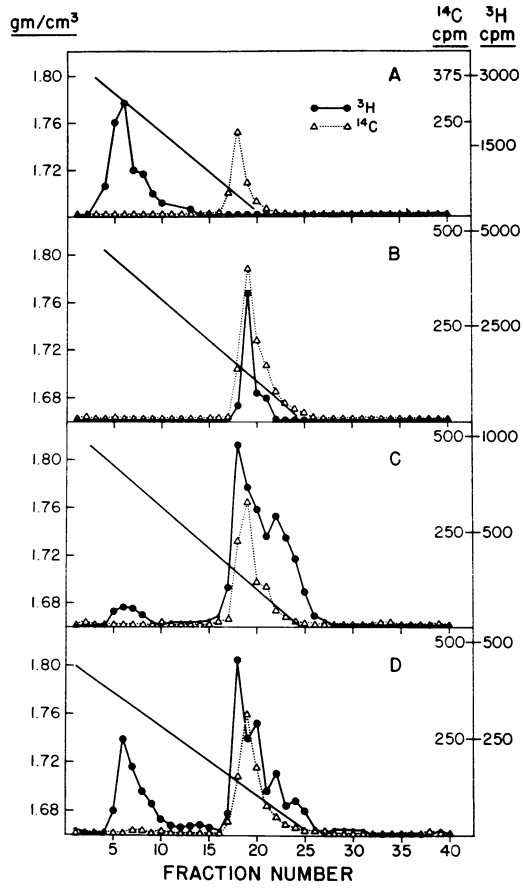

FIG. 1. *Shift of originally virus-associated radioactivity to the buoyant density of BUdR-substituted cellular DNA. Cells were grown for 96 hr in the presence of 5 μg of BUdR per ml. Thereafter, they were infected with 10^4 PFU of purified ^{3}H-labeled adenovirus type 12 per cell. The DNA was extracted at various intervals intervals and banded in CsCl density gradients. Cellular ^{14}C-labeled DNA was added as a marker. (A) Banding of DNA derived from noninfected cells at 96 hr after addition of nonlabeled BUdR and 0.1 μc ^{3}H-BUdR per ml. The ^{3}H-radioactivity bands at much higher density than the marker DNA. (B) Banding of DNA isolated from BUdR-treated cells at 2 hr after infection with ^{3}H-adenovirus type 12. Marker DNA and the radioactivity of viral DNA band at the same density. (C) The same preparation 24 hr postinfection. Part of the originally virus-associated radioactivity shifted to the buoyant density of BUdR-substituted cellular DNA. (D) Increasing shift of radioactivity to cellular DNA at 48 hr postinfection.*

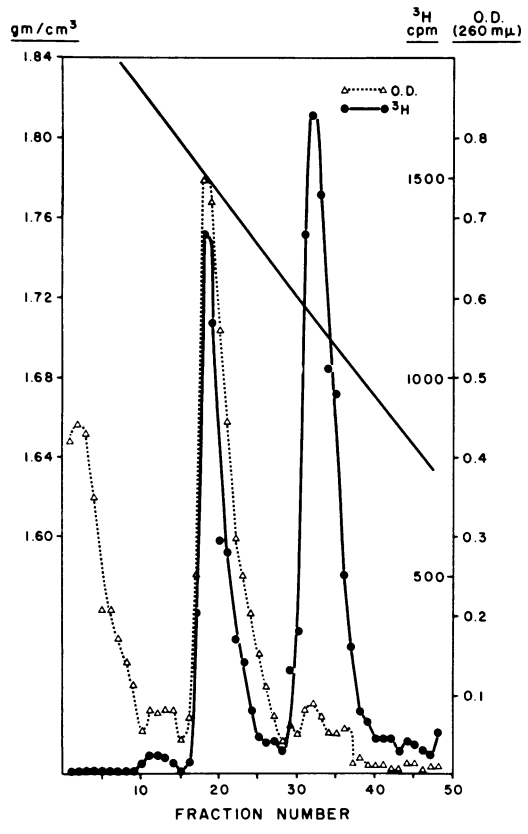

FIG. 2. *Shift of originally virus-associated radioactivity to the buoyant density of BUdR-substituted cellular DNA in growing cells. Exponentially growing cells were treated simultaneously with BUdR, FUdR, and 10^4 PFU of ^{3}H-adenovirus type 12 per cell. The DNA was extracted 24 hr later and banded in CsCl. The optical adsorbance of the bottom fractions represents RNA. A large proportion of originally virus-associated radioactivity banded at the buoyant density of BUdR-substituted cellular DNA.*

"light" strand was 1.717 g/cm³ (Fig. 3). In six separate experiments, the radioactivity was found to be predominantly associated with the "heavy" strand; i.e., the DNA strand synthesized after infection. However, a small amount of radioactivity was also found in the band at the position of the "light" strand.

Hybridization of cellular DNA isolated from infected cells with ^{3}H-adenovirus type 12 DNA. Nil-2 cells were infected with nonlabeled adenovirus type 12 at an input multiplicity of 100 PFU/cell and treated with BUdR and FUdR as described. The DNA was isolated 24 hr postinfection, and the "hybrid" cellular DNA was separated from viral DNA by banding in CsCl solution. The "heavy" and the "light" strands of host cell DNA were separated and isolated as described above. Of each strand, 40 μg was then filtered through a membrane filter. The denatured DNA was annealed with various amounts of fragmented, denatured ^{3}H-labeled adenovirus type 12 DNA. The saturation level was reached at an input of about 5×10^4 counts/min of viral DNA and corresponded to hybridization of 80 to 100 counts/min. In all four experiments, a slightly higher degree of hybridization was observed in the "light" strand in than the "heavy" strand (Fig. 4). Since the specific activity of the labeled viral DNA was 25,000 counts/min/μg, the 40 μg of cellular

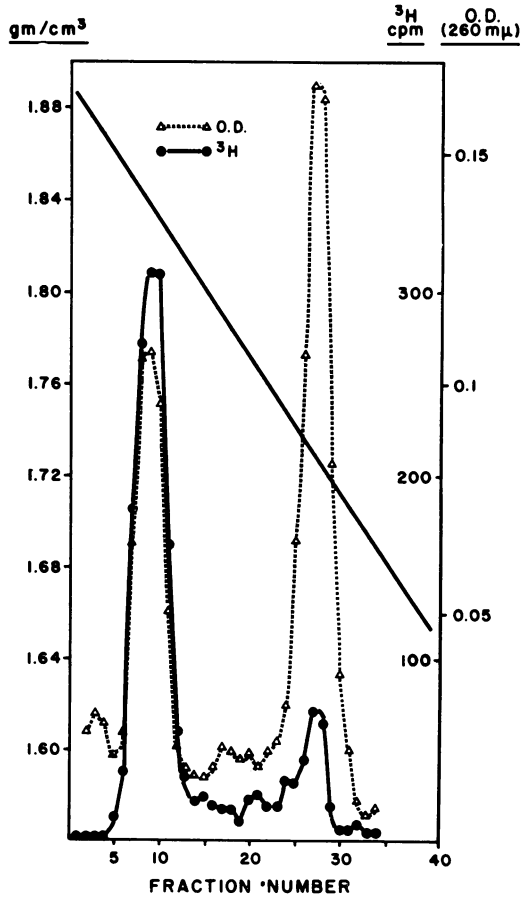

FIG. 3. *Separation of the "heavy" and "light" strands of "hybrid" cellular DNA isolated 24 hr postinfection with ^{3}H-labeled adenovirus type 12 by denaturation and banding in CsCl. The "hybrid" cellular DNA recovered from the gradient shown in Fig. 2 was rebanded in CsCl, freed of CsCl by dialysis, denatured by heating, and banded again in a CsCl density gradient. Note that most of the radioactivity which shifted into the cellular DNA bands with the "heavy" strand which was synthesized after infection.*

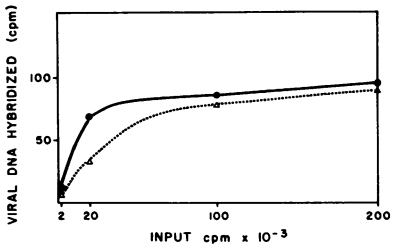

FIG. 4. *Hybridization of "heavy" and "light" strands which were derived from "hybrid" cellular DNA isolated at 24 hr postinfection from adenovirus type 12-infected Nil-2 cells with ^{3}H-adenovirus type 12 DNA. Of the "heavy" and "light" strands, 40 μg each was hybridized with increasing inputs of fragmented and denatured ^{3}H-adenovirus type 12 DNA. Figure 4 reveals a slightly higher extent of hybridization with the "light" strand. A saturation level is reached at 80 to 100 counts hybridized per min.*

DNA contained about 0.004 μg, or 0.01%, of nonlabeled viral DNA.

Data presented in Table 2 show that the extent of hybridization was similar in DNA isolated 24 and 48 hr postinfection, respectively, and that it was not appreciably affected by the input multiplicities which ranged from 100 to 2,000 PFU/cell. DNA isolated from adenovirus type 12-transformed cells hybridized to about the same extent with a saturating amount of ^{3}H-adenovirus type 12 DNA as DNA extracted from adenovirus type 12-infected Nil-2 cells.

To determine whether cellular DNA of intermediate density is associated with viral DNA, fractions 15–22 (Fig. 3) were pooled, freed of CsCl, filtered through membranes, and hybridized with 5×10^4 counts/min of ^{3}H-adenovirus type 12 DNA. The amount of nonlabeled cellular DNA bound to the membrane could not be determined because of the low concentration. Approximately four times less counts were hybridized to the DNA of intermediate density than to the "light" and "heavy" strands recovered from the gradients.

The following experiment was carried out to exclude the possibility that the small extent of apparent hybridization between viral and cellular DNA from infected cells was not due to contamination by nonintegrated viral DNA. Cellular DNA from BUdR- and FUdR-treated cells (100 μg), extracted 24 hr after addition of the analogues, was mixed with 25,000 counts/min (1 μg) of ^{3}H-labeled adenovirus type 12 DNA. The cellular DNA was separated from the viral DNA by two cycles of banding in CsCl and found to be free of contamination (i.e., of detectable radioactivity).

DISCUSSION

Evidence has been presented that a substantial proportion of adenovirus type 12 genomes is degraded within 24 hr after adsorption of ^{3}H-labeled virus to Syrian hamster cells. The extent of degradation was independent of the input multiplicity of infection within the range of 10^2 to 10^4 PFU/cell. Most of the degradation products were released into the culture medium. When labeled virus was exposed merely to culture medium for 24 hr at 37 C, only insignificant amounts of DNA were degraded. These experiments were performed in growing cultures, since in nongrowing cultures degradation of viral DNA is delayed (zur Hausen, *unpublished data*). The results parallel, to some extent, observations made with respect to the RNA of poliomyelitis virus (7), but no evidence of viral DNA degradation has been found in lytic infections by adenovirus type 5 (9).

In view of the degradation of adenovirus type 12 DNA in hamster cells, data obtained in studies on the apparent incorporation of radioactive viral DNA into cellular DNA have become open to different interpretations. In cultures prelabeled for 96 hr with BUdR, cellular DNA synthesis is markedly inhibited (15). In these slowly growing cultures, a portion of originally virus-associated radioactivity shifted within 24 hr, and

TABLE 2. *Hybridization of nonlabeled DNA isolated from noninfected cells, adenovirus type 12-infected cells, and adenovirus type 12-transformed Syrian hamster cells with ^{3}H-adenovirus type 12 DNA*

DNA on membrane[a]	Radioactivity bound (counts/min)
None	33
Nil-2, noninfected "light" strand	32
Nil-2, noninfected "heavy" strand	33
Nil-2, 100 PFU/cell, 24 hr PI, "light" strand	122
Nil-2,100 PFU/cell, 24 hr PI, "heavy" strand	118
Nil-2, 100 PFU/cell, 48 hr PI, "light" strand	134
Nil-2, 100 PFU/cell, 48 hr PI "heavy" strand	121
Nil-2, 2,000 PFU/cell, 24 hr PI, "light" strand	163
Nil-2, 2,000 PFU/cell, 24 hr PI, "heavy" strand	142
H-A12-4 (adenovirus 12 transformed cell line)	207
H-A12-7 (adenovirus 12 transformed cell line)	148

[a] Nonlabeled denatured DNA (40 μg) was hybridized with 5×10^4 counts/min (2 μg) of fragmented and denatured ^{3}H-adenovirus type 12 DNA. PI = postinfection.

more clearly within 48 hr, to the cellular DNA as shown by banding in CsCl. No radioactivity was detected at the density of cellular DNA 2 hr after infection, which indicated the absence of radioactive contaminants from the virus preparation. These results were similar to those reported by Doerfler (5). His interpretation that the radioactivity transferred to the cellular DNA represented viral DNA covalently linked with host cell DNA was tested in the present experiments with cells exposed simultaneously to BUdR, FUdR, and ^{3}H-labeled adenovirus type 12. Under these conditions, the majority of the cells undergo only one cycle of DNA replication. Up to 50% of the label recovered from the gradient shifted within one generation cycle from the position of the viral DNA to that of the cellular DNA. When the "heavy" and "light" strands of the "hybrid" DNA molecules were separated by heat denaturation and equilibrium centrifugation, most of the radioactivity was found to be associated with the "heavy" strand. Since this strand was synthesized after infection, this finding indicated that the shifted radioactivity represented either nucleotides from degraded viral DNA which were reutilized for synthesis of cellular DNA or viral DNA integrated into replicating host cell DNA.

To differentiate between these alternatives, DNA-DNA hybridization experiments were performed. If integration of parts of adenovirus type 12 DNA into the "heavy" strand of cellular DNA caused the shift of radioactivity, a preferential hybridization of ^{3}H-adenovirus type 12 DNA with the "heavy" strand should be expected. This did not turn out to be the case, however, since both strands of cellular DNA hybridized with small amounts of labeled viral DNA. It is concluded therefore that the vast majority of label found in the cellular DNA 24 hr after infection with ^{3}H-labeled adenovirus type 12 represents nucleotides derived from degraded viral DNA and reutilized for cellular DNA synthesis.

The hybridization of viral DNA with both strands of cellular DNA may denote either that viral DNA replication was not completely inhibited in the FUdR- and BUdR-treated hamster cells or that a small proportion of viral DNA became integrated into the host cell genome. Thus far, no evidence has been obtained to indicate that adenovirus type 12 DNA can replicate even in the absence of the analogues in Syrian hamster cells. Fusion of Nil-2 cells at various times after infection by adenovirus type 12 with human KB cells failed to provide evidence for viral DNA replication (H. Zur Hausen, B. Knowles, and H. Koprowski, *in preparation*). In addition, DNA-DNA hybridization and sedimentation experiments with DNA from infected BHK or Nil-2 cells failed to provide evidence of viral DNA replication (Doerfler, *personal communication*; zur Hausen, *unpublished data*). The hybridization between viral DNA and the two strands of cellular DNA derived from infected Nil-2 cells was, therefore, most likely due to integration of viral genomes into host cell DNA; this integration seemed to be essentially completed within 24 hr after infection. The fact that the extent of hybridization was independent of the input multiplicity of the infection in the range of 100 to 2,000 PFU/cell indicated that the infected cells were saturated with viral genomes already at the lowest virus-to-cell ratio used.

The similar extent of hybridization observed with the "heavy" and the "light" strands raised another problem. Assuming that the cellular DNA is fragmented during the extraction, rebanding, and denaturation procedures and that the average molecular weight of the fragments is 10^7 daltons, or less, the integrated viral genome should be preferentially recovered in fragments banding between the "heavy" and the "light" strands of cellular DNA or within the "light" strand. This is expected since the molecular weight of single strands of adenovirus type 12 DNA approximates 10^7 daltons and most of the denatured BUdR-substituted cellular strands containing longer sequences of "light" viral DNA should band at intermediate or "light" densities. Fractions of intermediate density contained about 20% of the total DNA which could be hybridized with adenovirus type 12 DNA. On the other hand, the "light" strand of cellular DNA revealed only a slightly higher ability to hybridize than the "heavy" strand. These results are compatible with the assumption that fragments of viral DNA had become integrated into both strands of host cell DNA. Although contamination of preparations of the "light" strand with some nonintegrated viral DNA cannot be excluded, this possibility seems remote since fractionation of artificial mixtures of ^{3}H-labeled viral DNA and BUdR-labeled Nil-2 DNA yielded cellular DNA preparations devoid of detectable radioactivity. Further experiments are required, however, to elucidate the sequence of events leading to the integration of the viral DNA both into the newly synthesized strand of cellular DNA and its template.

In conclusion, it has been shown that a substantial fraction of adenovirus type 12, adsorbed to Nil-2 cells, becomes degraded within 24 hr after infection. Part of the degradation products are reutilized for cellular DNA synthesis. A small proportion of viral DNA is inserted into both the newly synthesized and parental strands

of cellular DNA probably during the repair of cellular DNA. Most likely, the majority of this viral DNA becomes integrated as small fragments.

ACKNOWLEDGMENTS

We thank W. Henle for helpful discussion and valuable suggestions during the preparation of this manuscript. The excellent technical assistance of Rosemary Makarczyk and Evelyn Walter is gratefully acknowledged.

This investigation was supported by Public Health Service Research grants CA 04568, and RO1-CA 10594 from the National Cancer Institute.

LITERATURE CITED

1. Aloni, Y., E. Winocour, and L. Sachs. 1968. Characterization of the simian virus 40-specific RNA in virus-yielding and transformed cells. J. Mol. Biol. 31:415–429.
2. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. 1:279–285.
3. Chun, E. H. L., and J. W. Littlefield. 1961. The separation of the light and the heavy strands of bromouracil-substituted DNA. J. Mol. Biol. 3:668–673.
4. Diamond, L. 1967. Two spontaneously transformed cell lines derived from the same hamster embryo culture. Intern. J. Cancer 2:143–152.
5. Doerfler, W. 1968. The fate of the DNA of adenovirus type 12 in baby hamster kidney cells. Proc. Nat. Acad. Sci. U.S.A. 60:636–643.
6. Green, M., and M. Piña. 1963. Biochemical studies on adenovirus multiplication. IV. Isolation, purification, and chemical analysis of adenovirus. Virology 20:199–207.
7. Joklik, W. K., and J. E. Darnell, Jr. 1961. The adsorption and early fate of purified poliovirus in HeLa cells. Virology 13:439–447.
8. Kitamura, I., G. Van Hoosier, Jr., L. Samper, G. Taylor, and J. J. Trentin. 1964. Characteristics of human adenovirus type 12 induced hamster tumor cells in tissue culture. Proc. Soc. Exp. Biol. Med. 116:563–568.
9. Lawrence, W. C., and H. S. Ginsberg. 1967. Intracellular uncoating of type 5 adenovirus deoxyribonucleic acid. J. Virol. 1:851–867.
10. Ledinko, N. 1967. Stimulation of DNA synthesis and thymidine kinase activity in human embryonic kidney cells infected by adenovirus 2 or 12. Cancer Res. 27:1459–1469.
11. Levine, A. J., and H. S. Ginsberg. 1968. Role of adenovirus structural proteins in the cessation of host-cell biosynthetic functions. J. Virol. 2:430–439.
12. McBride, W. D., and A. Wiener. 1964. *In vitro* transformation of hamster kidney cells by human adenovirus type 12. Proc. Soc. Exp. Biol. Med. 115:870–874.
13. Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. 236:1372–1379.
14. Schildkraut, C. L., J. Marmur, and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. 4:430–443.
15. Simon, E. H. 1963. Effects of 5-bromodeoxyuridine on cell division and DNA replication in HeLa cells. Exp. Cell Res. Suppl. 9:263–269.
16. Strohl, W. A., H. C. Rouse, and R. W. Schlesinger. 1963. *In vitro* cultivation of malignant cells derived from adenovirus induced hamster tumors. Virology 21:513–516.
17. zur Hausen, H. 1967. Induction of specific chromosomal aberrations by adenovirus type 12 in human embryonic kidney cells. J. Virol. 1:1174–1185.
18. zur Hausen, H. 1968. Assocation of adenovirus type 12 deoxyribonucleic acid with host cell chromosomes. J. Virol. 2:218–223.
19. zur Hausen, H., W. Henle, K. Hummeler, V. Diehl, and G. Henle. 1967. Comparative study of cultured Burkitt tumor cells by immunofluorescence, autoradiography, and electron microscopy. J. Virol. 1:830–837.