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Intracellular Forms of Adenovirus DNA

V. Viral DNA Sequences in Hamster Cells Abortively Infected and Transformed with Human Adenovirus Type 12

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The persistence of viral DNA in BHK-21 cells abortively infected with human adenovirus type 12 has been investigated using reassociation kinetics. No indication of an increase in the amount of viral DNA per cell has been found. On the contrary, the amount of intracellular viral DNA sequences decreases rapidly after infection. Thus, free adenovirus type 12 DNA does not replicate in BHK-21 cells. The influence of the multiplicity of infection on the amount of persisting adenovirus type 12 DNA has also been explored. The viral DNA sequences persisting in four lines of hamster cells transformed in vitro by adenovirus type 12 at various multiplicities of infection have been quantitated and mapped by reassociation kinetics experiments using restriction endonuclease fragments of ^{3}H-labeled adenovirus type 12 DNA. All the *Eco*RI restriction nuclease fragments of the adenovirus type 12 genome are represented in each of the four cell lines. Individual fragments of the viral genome are represented in multiple copies in non-equimolar amounts.

Human adenovirus type 12 (Ad12) induces tumors in newborn hamsters (21, 36) and transforms hamster cells in tissue culture (22, 27, 34). Ad12 infects BHK-21 cells abortively (6, 7, 28, 32, 33), and viral DNA replication is not detectable in this system (7, 8, 10, 28). Even many hours after infection, viral DNA sequences can be detected in the cell, associated with the host chromosomes (39), covalently linked to the cellular DNA (6, 8), and free in the cell (6, 8).

Ad12-transformed hamster cells contain viral DNA (18, 19), although the physical state of this DNA has not been determined. With hamster cells transformed by CELO virus, an avian adenovirus, evidence based on the network technique indicates an integrated state of the viral DNA (1). In 10 different rat cell lines transformed by adenovirus type 2 (Ad2), the molecular left 14% of the Ad2 genome was found to persist in all of the transformed lines (14, 29, 30). The same fraction of the Ad2 genome may be sufficient to induce phenotypic transformation (16, 17).

The present study describes the influence of multiplicity of infection (MOI) on the persistence of Ad12 DNA in abortively infected BHK-21 cells and confirms the absence of viral DNA replication in this virus-host cell system. In addition, we report the results of experiments in which restriction fragments of Ad12 DNA were used to map viral DNA sequences in four different Ad12-transformed hamster cell lines.

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MATERIALS AND METHODS

Cells and virus. The A2497-2 and A2497-3 cell lines were derived from LSH inbred hamster embryo cells infected with Ad12 in vitro at an MOI of 5 PFU/cell and were a gift of A. M. Lewis, Jr., NIH, Bethesda, Md. They were grown in monolayers in Eagle minimal essential medium (13) with 10% fetal calf serum (Flow Laboratories or Laborservice, Munich). The T637 line was derived by W. A. Strohl from BHK-21 cells by transformation in vitro with Ad12 at an MOI of 350 PFU/cell (35). HA12/7 was isolated by H. zur Hausen from primary Syrian hamster cells infected at an MOI of 10 PFU/cell (40). The origin of the BHK-21 cells (31), KB cells (12), human Ad12 (strain Huie), as well as the media and methods of propagation, have been described elsewhere (24, 25). Some of the properties of the Ad12-transformed cell lines used in this study are summarized in Table 1.

Radioisotopes and chemicals. The [6-^{3}H]thymidine (specific activity, 24 to 26 Ci/mmol) was purchased from Amersham-Buchler, Braunschweig, Germany. Agarose (Indubiose A37) was obtained from L'Industrie Biologique, Genevilliers, France, and α-amylase was from Sigma Chemical Co., St. Louis, Mo. The sources of other chemicals have been described previously (9).

Enzymes. The single-strand-specific nuclease S1 was purified from α-amylase by the procedure of Vogt (37). Restriction endonuclease *Eco*RI was iso-

TABLE 1. *Properties of Ad12-transformed hamster cell lines*

Cell line	Cells used for transformation	Multiplicity of Ad12 used in transformation	T antigen	Oncogenicity in animals	Reference
T637	BHK-21	350	+	+	36
HA12/7	Primary Syrian hamster	10	−/+[a]	+	40
A2497-2	LSH inbred hamster embryo	5	+	+	A. M. Lewis, Jr., personal communication
A2497-3	LSH inbred hamster embryo	5	+	+	

[a] These cells are T antigen negative by immunofluorescence (H. zur Hausen, personal communication), but T antigen positive by complement fixation (K. Raška, Jr., personal communication).

lated according to the method of Yoshimori (Ph.D. thesis, Univ. of California, San Francisco, Calif., 1971) as described previously (9).

Restriction endonuclease *Bam*HI was prepared by R. Greenberg and J. Ortin in our laboratory as described elsewhere (25). Pronase B, endonuclease free, was obtained from Calbiochem and was preincubated at 37°C for 2 h before use.

Preparation of ^{3}H-labeled Ad12 DNA. Preparation of ^{3}H-labeled Ad12 DNA of high specific activity (10^6 to 2×10^6 cpm/μg) has been described elsewhere (25).

Isolation of Ad12 DNA fragments generated by *Eco*RI and *Bam*HI restriction endonucleases. The ^{3}H-labeled Ad12 DNA was cleaved with *Eco*RI as described earlier (9) or with *Bam*HI, as reported elsewhere (25). The resulting fragments were separated electrophoretically on 1.5% polyacrylamide-0.8% agarose gels (26). The gels were sliced, and the DNA was eluted from each slice by incubation at 37°C in 0.1% sodium dodecyl sulfate.

Reassociation kinetics. Reassociation kinetics (2, 38) was performed essentially as described previously (9). The reassociation mixtures were composed of ^{3}H-labeled Ad12 DNA (whole Ad12 genome or one of the restriction fragments) as the probe and either salmon sperm DNA (Worthington Biochemicals) as a viscosity control or a test DNA prepared from Ad12-infected or -transformed cells. The DNA was sheared with a Branson Sonifier B-12 to fragments of about 500 nucleotides in length, as shown by sedimentation in neutral and alkaline sucrose density gradients. After denaturation by boiling for 15 min, the mixtures were adjusted to 1 M NaCl, 0.01 M Tris-hydrochloride, pH 7.5, and 0.001 M EDTA and incubated at 68°C. At various times after starting the reaction, samples were withdrawn, usually 0.1 ml containing 400 to 500 cpm, and diluted in S1 buffer to 0.3 M NaCl, 0.024 M sodium acetate, pH 4.6, 0.005 M $ZnSO_4$, and the samples were frozen at −20°C. At the end of the experiment, the samples were thawed and digested for 2 h at 45°C with an amount of S1 nuclease (diethylaminoethyl-cellulose fraction) sufficient to acid solubilize >98% of single-stranded DNA and <5% of double-stranded DNA under the same conditions. Double-stranded DNA was precipitated with 1 M HCl containing 1% NaH_2PO_4 and 1% sodium pyrophosphate; the precipitates were collected on glass-fiber filters (Whatman GF/C), dried, and counted in a toluene-based scintillation fluid. Tri-Carb liquid scintillation spectrometers, Packard models 3380 and 3330, were used in these experiments. Results were calculated according to methods published elsewhere (15, 30).

Reconstruction experiments performed with ^{3}H-labeled Ad12 DNA in the presence of different amounts of unlabeled Ad12 DNA demonstrated the reliability of the technique (Table 2).

Infection of BHK-21 cells with Ad12. Infection of BHK-21 cells with Ad12 was carried out as described earlier (8). Fresh Ad12, purified by two to three cycles of equilibrium centrifugation in CsCl, was dialyzed for 2 h against three changes of 1 liter of phosphate-buffered saline (PBS) (11) before inoculating the cells. BHK-21 cells, about half-confluent monolayers in 10-cm dishes, were washed one to two times with 5 ml of PBS. Freshly dialyzed virus was added to each dish of cells at the desired MOI. One optical density unit at 260 nm corresponded to 10^{10} PFU/ml (7). After a 2-h adsorption period, the cells were washed five to six times with 5 ml of PBS, and then 20 ml of fresh medium was added.

Extraction and purification of intracellular DNA. With minor modifications, intracellular DNA was isolated according to procedures published elsewhere (6, 8). The medium was removed, and the cells were washed five to six times with 5 ml of cold PBS. To a 10-cm-diameter dish, Pronase B (0.15 ml of a 5-mg/ml solution) and 1.2 ml of TE (0.01 M Tris-hydrochloride, pH 7.5, 0.001 M EDTA) were added first; then, 0.15 ml of 10% sodium dodecyl sulfate was added and the cells were incubated at 37°C for 30 to 60 min. The DNA was extracted three times with Tris-saturated phenol and three times with ether. Ether was evaporated with N_2. DNA from BHK-21 cells was further purified in most cases by equilibrium centrifugation in neutral CsCl, as described elsewhere (6). DNA from transformed cells was incubated in 0.1 N NaOH at 37°C overnight to digest RNA, neutralized, concentrated by vacuum dialysis or ethanol precipitation, if necessary, and dialyzed against TE. Under these conditions, >99.5% of ^{3}H-labeled rRNA added to the DNA was made acid-soluble. DNA concentrations were measured in a Zeiss spectrophotometer or by the Burton reaction using diphenylamine (3).

RESULTS

Infection of BHK-21 cells with Ad12. The state of the cells at the time of infection, the MOI, and the conditions of maintenance used for the infected cells influence the outcome of the infection of BHK-21 cells with Ad12. Infection of BHK-21 cells may result in cell death or the cells may survive infection, and some of the cells may become transformed (35). The survival of BHK-21 cells infected with different MOIs was examined (Fig. 1). When growing cells were infected at an MOI of 10^3 PFU/cell, cells continued to grow, with a certain lag period, at least up to 48 h postinfection (p.i.). At an even higher multiplicity (10^4 PFU/cell), the number of cells per dish remained constant (Fig. 1). In these cultures the medium contained very few floating cells that had detached from the dish. Under these conditions, then, "cell killing" was not one of the more likely fates of the infected cells, although an MOI of 10^4 PFU/cell was sufficient to stop further growth of the culture, at least for the first 48 h p.i.

TABLE 2. *Reconstruction experiment*[a]

Concn of ^{3}H-labeled Ad12 probe DNA (OD_{260}/ml)	Concn of unlabeled Ad12 DNA added (OD_{260}/ml)	$C_0t_{1/2}$ (mol/liter × s)	Concn of Ad12 DNA calculated (OD_{260}/ml)
1.27×10^{-4}	0	7.34×10^{-3}	
1.32×10^{-4}	9.95×10^{-5}	1.13×10^{-4}	9.48×10^{-5}
1.33×10^{-4}	1.99×10^{-4}	1.47×10^{-4}	1.62×10^{-4}
1.22×10^{-4}	2.99×10^{-4}	1.98×10^{-4}	2.74×10^{-4}

[a] ^{3}H-labeled Ad12 DNA (specific activity, 3.0×10^7 cpm/optical density unit at 260 nm [OD_{260}]) was reassociated in the presence of 9.05 OD_{260} of salmon sperm DNA per ml and different known amounts of unlabeled Ad12 DNA, as described in the text. The amount of unlabeled Ad12 DNA was calculated from the acceleration of reassociation by published methods (30).

MOI and the persistence of viral DNA in Ad12-infected BHK-21 cells. When BHK-21 cells were infected at a high MOI (10^3 to 10^4 PFU/cell), Ad12 DNA was found to persist in the cells up to 88 h p.i., and a portion of the viral DNA became covalently linked to the host genome (6, 8). However, the influence of MOI on the persistence of Ad12 DNA in the cell had not been examined in those experiments.

BHK-21 cells growing in monolayers were infected with freshly CsCl-purified Ad12 at MOIs ranging from 29 to 10^4 PFU/cell. At 24 h p.i., the total intracellular DNA was extracted, purified, and used in reassociation kinetics ex-

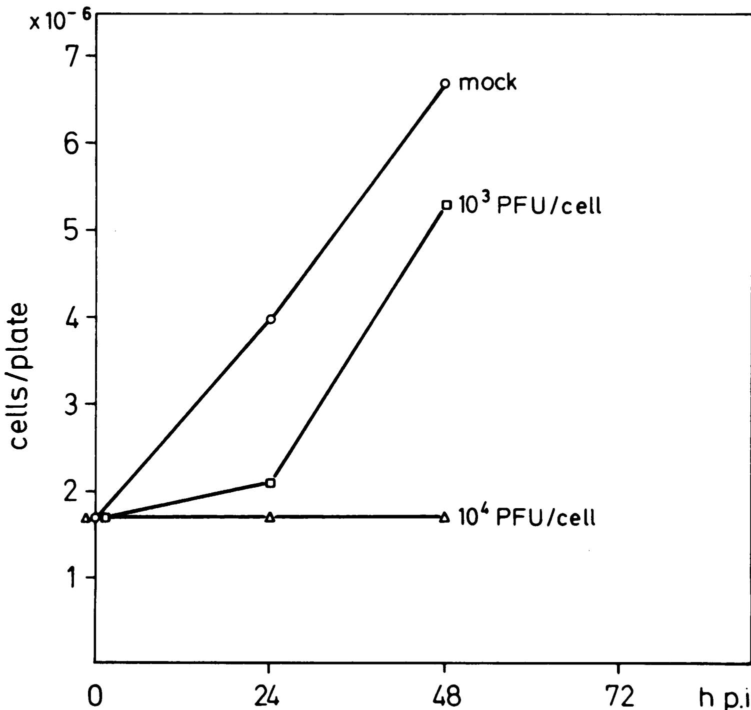

FIG. 1. *Survival of BHK-21 cells abortively infected by Ad12. BHK-21 cells were seeded in monolayers (3 × 10^4 cells/35-mm-diameter dish). Later at a titer of 2.3 × 10^5 cells/dish, cells were either mock-infected with PBS or infected with freshly CsCl-purified Ad12 in PBS at an MOI of 10^3 or 10^4 PFU/cell. At various times after infection, the cells were trypsinized and counted in a hemocytometer.*

periments with ^{3}H-labeled Ad12 DNA as the probe (Table 3). The number of viral genome equivalents per diploid cell was calculated according to Gelb et al. (15). The amount of intracellular Ad12 DNA per cell hardly increases from an MOI of 29 up to 500 to 600 PFU/cell, but even higher multiplicities result in a corresponding rise in the amount of viral DNA persisting in the cell. There are several possible explanations for these results. First, the adsorption of Ad12 to the cell is very inefficient; less than 0.1% of the inoculum is taken up by the cell even at low multiplicities (8). Furthermore, within 24 h p.i., more than 75% of the adsorbed virus is shed as noninfectious particles into the medium (8). Moreover, the cell cultures continue to grow during the 24-h period after infection. As a consequence, the infecting viral genomes may be diluted, since Ad12 DNA does not replicate in BHK-21 cells. Thus, several factors could combine to minimize the persistence of intracellular Ad12 DNA at lower MOIs.

Persistence of viral DNA with time after infection. In previous investigations, hybridization and sedimentation methods have been used (7, 8, 10, 28) to search for the replication of Ad12 DNA in BHK-21 cells. None has been observed. On the contrary, the amount of intracellular viral DNA has been found to decrease after infection.

This problem has been reinvestigated, using reassociation kinetics as a more sensitive tool to determine persisting viral DNA sequences. BHK-21 cells growing in monolayers were infected with CsCl-purified Ad12 at multiplicities of 1,000 to 3,000 PFU/cell. At various times after infection, the DNA was extracted, purified, denatured, and reassociated in the presence of ^{3}H-labeled Ad12 DNA as the probe. The rate of reassociation of the ^{3}H-labeled Ad12 DNA was enhanced by the addition of the DNA from Ad12-infected BHK-21 cells (Fig. 2a).

The results from a series of such experiments indicate that the amount of intracellular viral DNA decreases rapidly with time after infection (Table 4; Fig. 2b). By 48 to 72 h p.i., the amount of Ad12 DNA per cell appears to stabilize at a value of <5 genome equivalents/cell. In agreement with earlier results, there is no evidence for the replication of the Ad12 genome. The measurements presented here would have been sensitive enough to detect an increase of 0.5 genome equivalents of Ad12 DNA per diploid cell. The fact that the number of viral genomes per cell reaches a plateau value (Fig. 2b) may indicate that the viral DNA replicates with the cellular genome, probably in an integrated form (8).

Persistence of viral DNA in Ad12-transformed hamster cells. The persistence of viral DNA was investigated in four different Ad12-transformed hamster cell lines, T637, HA12/7, A2497-2, and A2497-3, whose origin has been described in Materials and Methods. DNA from these cell lines was prepared and used in reassociation kinetics experiments with ^{3}H-labeled Ad12 DNA as a probe (Fig. 3). The results of these experiments have been presented as reciprocal plots (38).

Second-order kinetics of reassociation should yield a straight line in the plot shown in Fig. 3, as indeed is the case when ^{3}H-labeled Ad12 DNA reassociates in the presence of salmon sperm DNA (Fig. 3, control). The failure of the probe to reassociate in a true second-order reaction in the presence of DNA from the T637 and HA12/7 cell lines and, to a lesser extent, the other two transformed lines, indicates that some of the sequences in the probe are present at frequencies quite different from those of others or may be totally absent from the transformed cell DNA. Thus, meaningful reassocia-

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

MOI[b] (PFU/cell)	Concn of test DNA[c] (OD_{260}/ml)	Concn of probe DNA (OD_{260}/ml)	Control $C_0t_{1/2}$[d]/experimental $C_0t_{1/2}$[e]	Ad12 equivalents/diploid cell[f]
29	26.4	2.79×10^{-4}	1.01	ND[g]
84	18.3	3.00×10^{-4}	1.36	1.1
120	17.0	4.44×10^{-4}	1.37	1.2
120	15.2	2.71×10^{-4}	1.41	1.3
500	16.8	4.00×10^{-4}	3.13	9.2
900	7.9	4.52×10^{-4}	1.43	4.5
1,400	16.2	3.48×10^{-4}	1.22	43.8
3,000	21.6	2.74×10^{-4}	8.20	16.6
10,000	6.3	4.72×10^{-4}	6.28	72.3

[a] BHK-21 cells were inoculated with CsCl-purified Ad12 at multiplicities as indicated. At 24 h p.i., the total intracellular DNA was extracted and analyzed for the presence of viral DNA as described in the text.

[b] Multiplicities are based on the previously determined (7) relation that 1 optical density unit at 260 nm (OD_{260}) is equivalent to 10^{10} PFU.

[c] DNA extracted from BHK-21 cells infected 24 h prior to extraction with varying multiplicities of Ad12.

[d] $C_0t_{1/2}$ value of the ^{3}H-labeled Ad12 DNA used as a probe, in the presence of salmon sperm DNA ($5.62 \pm 0.37 \times 10^{-3}$ mol/liter × s).

[e] $C_0t_{1/2}$ value of the ^{3}H-labeled Ad12 DNA in the presence of DNA from Ad12-infected BHK-21 cells.

[f] Viral genome equivalents per diploid cell were calculated from the enhancement of the $C_0t_{1/2}$ value as described by Gelb et al. (15).

[g] ND, None detected.

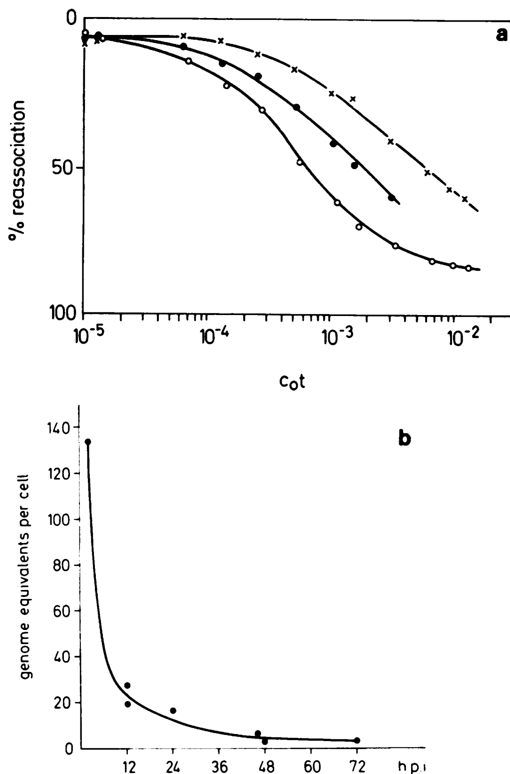

FIG. 2. *Persistence of Ad12 DNA in BHK-21 cells with time after infection. BHK-21 cells growing in monolayers (20 × 10^6 to 25 × 10^6 cells/100-mm-diameter dish) were infected with freshly CsCl-purified Ad12 in PBS at an MOI of 10^3 to 3 × 10^3 PFU/cell. The cells were harvested at different times after infection, and the DNA was isolated for use in reassociation experiments. (a) Sixteen 100-mm-diameter dishes (17 × 10^6 cells/dish) were infected with Ad12 at an MOI of 3,000 PFU/cell. The DNA was prepared from eight dishes at 24 h p.i. and from the other dishes at 72 h p.i. by the sodium dodecyl sulfate-Pronase-phenol method and sedimented to equilibrium in neutral CsCl density gradients. The control reassociation mixture included 24.1 optical density units at 260 nm (OD_{260}) of salmon sperm DNA and 0.012 μg of ^{3}H-labeled Ad12 DNA per ml. The experimental mixtures contained 21.6 OD_{260} (24 h p.i.) or 26.4 OD_{260} (72 h p.i.) of unlabeled DNA from infected cells per ml, instead of salmon sperm DNA. The reassociation kinetics of ^{3}H-labeled Ad12 DNA in the presence of salmon sperm DNA is indistinguishable from that of viral DNA in the presence of DNA from uninfected BHK-21 cells or of calf thymus DNA (data not shown). Symbols: (×) control, reassociation of ^{3}H-labeled Ad12 DNA probe by itself; (○) reassociation of ^{3}H-labeled Ad12 DNA in presence of DNA from Ad12-infected BHK-21 cells isolated 14 h p.i. and (●) at 72 h p.i. (b) Ad12-infected BHK-21 cells were harvested at various times after infection, and the DNA was prepared for use in reassociation kinetics experiments similar to the ones shown in (a). The number of Ad12 genome equivalents per cell was calculated according to Wetmur and Davidson (38). The DNA prepared at 24 and 72 h p.i. was derived from the same infection, whereas DNA prepared at 2, 12, 46, and 48 h p.i. came from separate experiments. The data from the individual reaction mixtures are presented in Table 4.*

tion analysis of the viral DNA sequences present in these cells requires the use of less complex probes, i.e., restriction endonuclease fragments of the Ad12 genome.

Analysis of the viral DNA sequences in four Ad12-transformed hamster cell lines using restriction endonuclease fragments of Ad12 DNA. Restriction endonuclease fragments of Ad12 DNA were produced using the *Eco*RI and *Bam*HI nucleases and were isolated by gel electrophoresis as described in Materials and Methods (25). The *Eco*RI fragments of Ad12 DNA were reassociated in the absence and presence of DNA from the T637 cell line (Fig. 4). The initial rate of reassociation of the ^{3}H-labeled Ad12 DNA probe both in the absence and presence of T637 DNA proceeded according to apparent second-order kinetics. All of the *Eco*RI fragments are represented in the T637 line (Fig. 4 and 5). It is interesting to note that the *Eco*RI fragments D and E (cf. Fig. 5) occur most frequently of all parts of the Ad12 genome in the T637 line, although they are not transcribed in these cells (25).

Similar analyses were performed with the DNA from the other three Ad12-transformed hamster cell lines (Table 5; Fig. 5). To define more precisely which portions of the *Eco*RI A fragment of Ad12 DNA were present in the transformed cells, *Bam*HI fragments from the right end of the Ad12 genome (Fig. 5) were used

TABLE 4. *Persistence of Ad12 DNA at various times postinfection*[a]

DNA from Ad12-infected BHK-21 cells isolated at (h p.i.):	Concn of test DNA (OD_{260}/ml)	Concn of ^{3}H-labeled Ad12 probe DNA (OD_{260}/ml)	Control $C_0t_{1/2}$/experimental $C_0t_{1/2}$	Viral genome equivalents/diploid cell
2	8.1	2.71 × 10^{-3}	3.21	134.0
12	3.9	2.06 × 10^{-4}	3.86	27.5
12	8.0	2.91 × 10^{-4}	3.93	19.4
24	21.6	2.74 × 10^{-4}	8.20	16.6
46	9.2	2.84 × 10^{-4}	2.14	6.4
48	11.4	3.20 × 10^{-4}	1.59	3.0
72	26.4	2.54 × 10^{-4}	3.09	3.65

[a] BHK-21 cells were infected with Ad12 as described in the text. The total intracellular DNA was extracted and analyzed at various times after infection. All other explanations are given in Table 3.

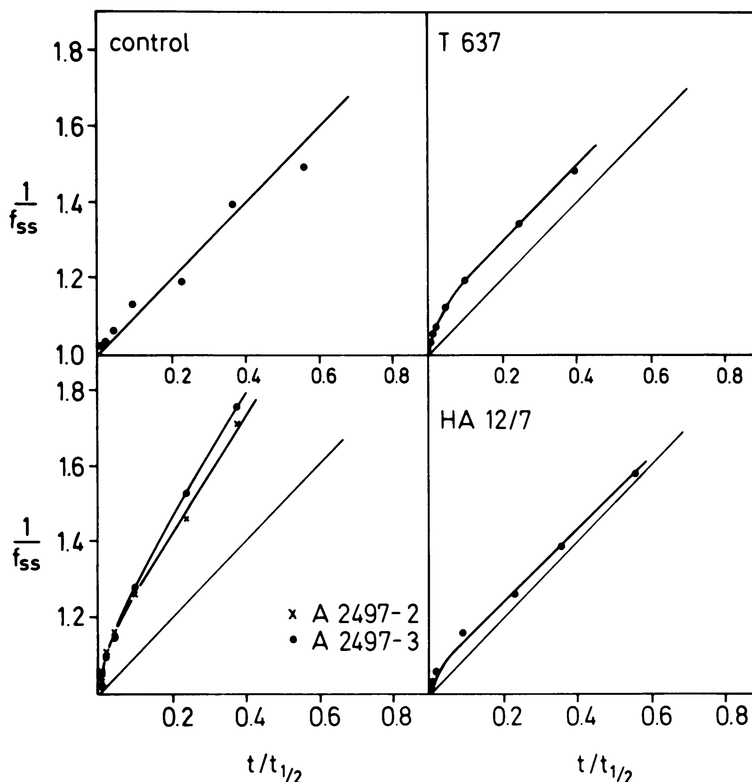

FIG. 3. *Persistence of viral DNA sequences in four Ad12-transformed hamster cell lines. DNA was extracted by the sodium dodecyl sulfate-Pronase-phenol method from the four Ad12-transformed hamster cell lines T637, HA12/7, A2497-2, and A2497-3. Prior to use in reassociation experiments with ^{3}H-labeled Ad12 DNA (specific activity, 3.0×10^{7} cpm/optical density unit at 260 nm [OD_{260}]) as the probe, the DNA was incubated in 0.1 N NaOH overnight at 37°C to hydrolyze the RNA. The reassociation mixtures contained per milliliter: 1.9×10^{-4} to 2.1×10^{-4} OD_{260} units of ^{3}H-labeled Ad12 DNA and 4.7 OD_{260} units of T637 DNA, or 4.5 OD_{260} units of HA12/7 DNA, or 5.4 OD_{260} units of A2497-2 DNA, or 4.1 OD_{260} units of A2497-3 DNA, or 4.5 OD_{260} units of salmon sperm DNA in the control experiment. The reassociation reaction was performed at 68°C under conditions outlined in Materials and Methods. The results were calculated and plotted as reciprocal plots (38).*

in the analysis. The results of these experiments are summarized in Fig. 5 and suggest that in the cell lines investigated, all segments of the Ad12 genome are represented in multiple copies. The number of copies is unequal for different fragments of the Ad12 genome. However, we cannot rule out the possibility that some small fraction of the viral genome may be absent from the transformed cells.

DISCUSSION

Persistence of viral DNA in abortively infected cells. After the infection of BHK-21 cells with Ad12, viral DNA is lost rather rapidly from the infected cells, possibly due to shedding of virions or subviral particles into the medium (8). We do not understand the mechanism of the disappearance of viral genetic material from the cells. It is, however, conceivable, and perhaps even likely, that BHK-21 cells containing integrated viral genetic material have a selective disadvantage in comparison to cells that have been cured or have lost viral genes. The validity of this hypothesis could be assessed by cloning Ad12-infected BHK-21 cells.

An increase in the number of viral genomes per cell cannot be detected at any time postinfection with the technique of reassociation kinetics or any of the other procedures used in earlier work (8). Thus, the block in Ad12 multiplication in BHK-21 cells must lie before the step of DNA replication (8), although the pattern of Ad12 transcription in BHK-21 cells is indistinguishable from that found early in productively infected cells (24). Perhaps there is a factor missing in BHK-21 cells that is required to initiate late transcription and/or Ad12 DNA replication. It will also be interesting to investi-

gate whether the viral inhibitory factor described by Champe et al. (4, 5) plays a role in the abortive infection cycle.

It has been shown previously (8) that a portion of the intracellular Ad12 DNA becomes covalently linked to the DNA of the BHK-21 cells. The integrated viral sequences probably replicate together with the host genome. The data reported here show that the amount of viral DNA persisting in abortively infected cells is lower than previously estimated.

Persistence of Ad12 DNA in Ad12-transformed hamster cells. The pattern of viral genetic material persisting in Ad12-transformed hamster cells is summarized in the scheme presented in Fig. 5, which also shows the regions of the Ad12 genome transcribed into nuclear RNA in the T637 and HA12/7 hamster lines (25). Although all segments of the Ad12 genome appear to be represented in multiple copies in

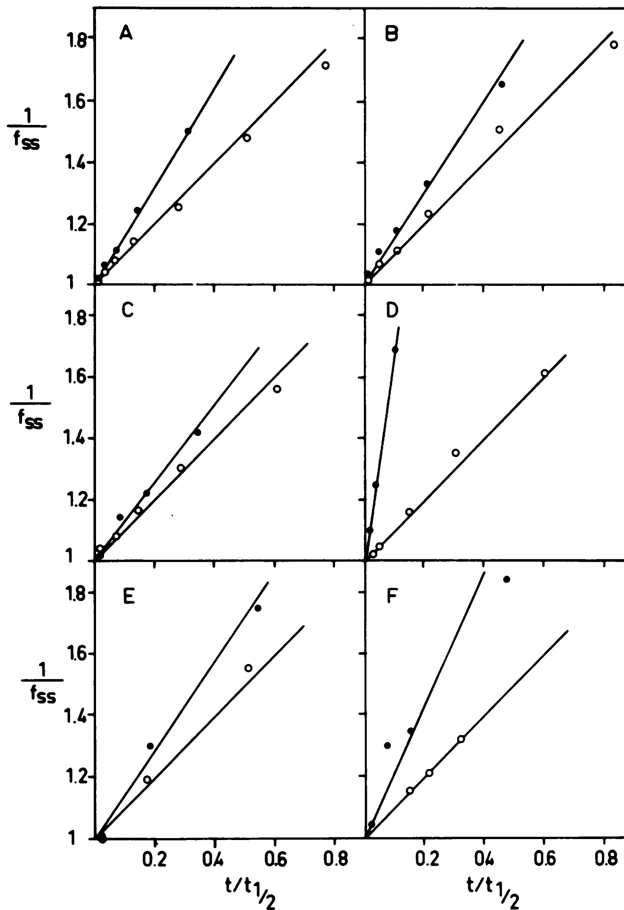

FIG. 4. *Reassociation kinetics experiments using DNA from Ad12-transformed T637 cells and the EcoRI restriction endonuclease fragments of ³H-labeled Ad12 DNA. The DNA was extracted from T637 cells by the sodium dodecyl sulfate-Pronase-phenol method and incubated with 0.1 N NaOH as described in the text to hydrolyze the RNA. The EcoRI restriction endonuclease fragments of ³H-labeled Ad12 DNA (specific activity, 4 × 10⁵ cpm/μg) were prepared as described elsewhere (25) and were used as probes in reassociation experiments with T637 DNA. The reassociation mixtures contained (per milliliter): 0.014 μg of fragment A, 0.019 μg of fragment B, 0.014 μg of fragment C, 0.005 μg of fragment D, 0.012 μg of fragment E, and 0.0005 μg of fragment F. In reassociation experiments involving the EcoRI fragments A, B, C and E, 630 μg of T637 DNA or salmon sperm DNA in the control experiments was added; for EcoRI fragment D 1,640 μg of cell DNA was added and for EcoRI fragment F 1,480 μg of cell DNA was used. The reassociation experiments were carried out as described in the text. The results were calculated as detailed elsewhere (29, 30). (A-F) Reassociation experiments using the EcoRI fragments A through F, respectively. Symbols: (○) reassociation of the ³H-labeled fragments A through F in the presence of salmon sperm DNA; (●) reassociation in the presence of T637 DNA.*

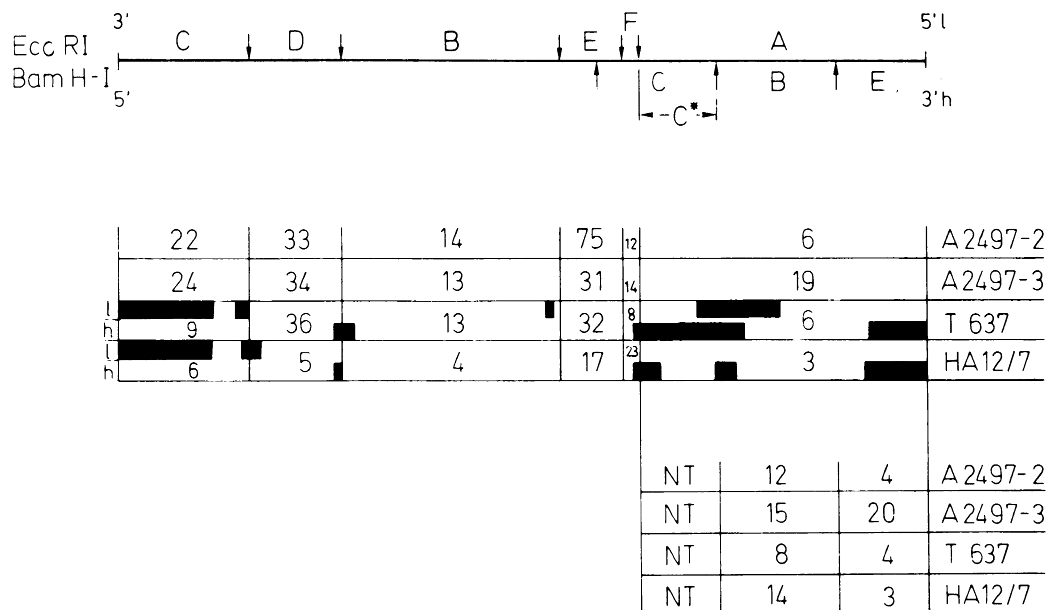

FIG. 5. *Persistence of viral DNA fragments in Ad12-transformed hamster cell lines. Restriction endonuclease fragments of ^{3}H-labeled Ad12 DNA generated by the EcoRI and BamHI restriction endonucleases were reassociated in the presence of salmon sperm DNA as a control or in the presence of DNA from four lines of Ad12-transformed hamster cells, T637, HA12/7, A2497-2, and A2497-3. The composition of the reaction mixtures is described in detail in Table 5. The number of equivalents persisting per diploid cell for each fragment of the Ad12 genome appears in the column underneath the corresponding fragment shown on the physical map of Ad12 DNA. These numbers have been calculated (15) from the increase in the rate of reassociation of individual Ad12 DNA fragments in the presence of DNA from transformed hamster cells, as compared to salmon sperm DNA, and present mean values of the data shown in Table 5. The physical map of Ad12 DNA has been determined by Mulder et al. (23; Mulder, personal communication), and the polarity has been established by Ortin et al. (25). The shaded blocks indicate the regions of the Ad12 genome that are transcribed into nuclear RNA as determined by mapping of nuclear RNA on the separated strands of each of the restriction endonuclease fragments (25). NT, Not tested.*

the transformed hamster cells, at least in two of the lines only those fractions of the Ad12 genome that are also expressed early in productive infection or in abortively infected cells are transcribed into mRNA (25). These findings suggest a complex control mechanism which regulates the transcription of the persisting viral genes. The expression of some of these viral genes may be under host control in transformed cells.

The Ad12-transformed lines A2497-2 and A2497-3 represent different clones derived from the same transformation experiment. It is interesting to note that the frequencies of occurrence of viral DNA segments from different parts of the Ad12 genome are very similar in the two cell lines, with the exception of the right terminus (*Eco*RI fragment E and *Bam*HI fragment E) of the Ad12 DNA molecule (Fig. 5).

The physical state of the persisting Ad12 DNA in the transformed hamster cell lines described here has also been investigated in our laboratory. The data obtained so far suggest that the viral genes persist in an integrated form in covalent linkage with the cellular DNA (Groneberg, Chardonnet, and Doerfler, submitted for publication). Hamster cells transformed by infection with Ad12 at multiplicities ranging from 5 to 350 PFU/cell carry most segments of the viral genome in multiple copies, probably in an integrated form. There is apparently no correlation between the MOI used to transform the cells and the amount of viral DNA persisting in them after transformation. This conclusion is limited by the fact that the four Ad12-transformed cell lines studied have been developed in three different laboratories whose titration systems for measuring the multiplicity of infection are obviously difficult to compare.

These results contrast with those obtained by examining 10 lines of Ad2-transformed rat cells; nine of the cell lines were found to contain only certain parts of the Ad2 DNA, the left end of the viral genome being common to all these

TABLE 5. *Persistence of viral DNA fragments in Ad12-transformed hamster cell lines*

Cell line	Probe[a]	Probe DNA concn (OD_{260}/ml)	Cell DNA concn[b] (OD_{260}/ml)	Control[c] $C_0t_{1/2}$/experimental $C_0t_{1/2}$	Viral genome equivalents/diploid cell[d]
T637	*Bam*HI E	1.41×10^{-4}	31.4	1.53 ± 0.38[e]	3.7
	B	1.17×10^{-4}	4.65	1.25 ± 0.15	7.6
	*Eco*RI A	3.09×10^{-4}	8.99	1.35 ± 0.23	6.4
	B	4.14×10^{-4}	8.94	1.40 ± 0.08	12.6
	C	2.92×10^{-4}	8.94	1.24 ± 0.17	9.1
	D	3.60×10^{-4}	8.66	1.66 ± 0.33	44.7
	D	9.94×10^{-5}	34.88	6.93 ± 0.78	27.7
	E	2.61×10^{-4}	8.66	1.40 ± 0.12	31.9
	F	1.07×10^{-4}	31.4	1.26 ± 0.14	8.4
HA12/7	*Bam*HI E	1.78×10^{-4}	39.4	1.44 ± 0.23	3.1
	B	9.95×10^{-5}	40.6	5.76 ± 2.77	14.1
	*Eco*RI A	7.27×10^{-5}	16.7	2.37 ± 0.57	3.2
	B	3.08×10^{-4}	24.31	1.48 ± 0.32	4.2
	C	4.31×10^{-4}	24.2	1.12 ± 0.08	4.8
	C	1.59×10^{-4}	33.12	2.26 ± 0.16	7.0
	D	1.46×10^{-4}	33.12	1.68 ± 0.23	4.9
	E	1.98×10^{-5}	29.92	1.96 ± 0.40	16.7
	F	6.46×10^{-5}	32.5	2.20 ± 0.76	22.7
A2497-2	*Bam*HI E	1.43×10^{-4}	5.44	1.10 ± 0.05	4.2
	B	1.90×10^{-4}	18.45	1.99 ± 0.53	12.4
	*Eco*RI A	5.38×10^{-5}	38.24	8.97 ± 2.15	5.9
	B	4.46×10^{-4}	5.31	1.26 ± 0.13	15.3
	B	3.49×10^{-4}	9.99	1.53 ± 0.16	12.8
	C	9.63×10^{-5}	38.24	11.56 ± 3.83	30.6
	C	2.38×10^{-4}	9.99	1.46 ± 0.28	12.6
	D	1.06×10^{-4}	38.24	8.33 ± 2.04	33.3
	E	2.26×10^{-4}	26.64	4.37 ± 1.45	75.2
	F	8.81×10^{-5}	31.8	1.44 ± 0.18	11.6
A2497-3	*Bam*HI E	1.47×10^{-4}	3.97	1.37 ± 0.11	20.0
	B	1.27×10^{-4}	4.02	1.40 ± 0.26	15.3
	*Eco*RI A	5.89×10^{-5}	25.92	20.21 ± 2.45	23.1
	A	4.27×10^{-4}	3.94	1.26 ± 0.23	14.2
	B	1.42×10^{-4}	26.2	3.45 ± 1.14	9.1
	B	4.46×10^{-4}	3.94	1.23 ± 0.09	17.5
	C	1.09×10^{-4}	25.92	5.90 ± 0.99	23.8
	D	1.31×10^{-4}	25.92	5.15 ± 1.17	34.4
	E	6.75×10^{-5}	26.2	5.55 ± 1.53	30.9
	F	5.50×10^{-5}	25.5	1.67 ± 0.44	13.8

[a] Restriction endonuclease fragments (*Eco*RI and *Bam*HI) of ^{3}H-labeled Ad12 DNA (specific activity, 2×10^5 to 6×10^5 cpm/μg) were prepared as described in the text. OD_{260}, Optical density units at 260 nm.

[b] DNA extracted from transformed cell lines as described in the text or salmon sperm DNA as a control.

[c] Average acceleration of reassociation rate of labeled Ad12 fragment in the presence of transformed DNA (experimental) relative to the rate in salmon sperm DNA (control), calculated using matched controls according to Sharp et al. (30). $C_0t_{1/2}$ of the control reactions for *Eco*RI A-F and *Bam*HI B and E, respectively, were $2.77 \pm 0.66 \times 10^{-3}$, $2.60 \pm 0.72 \times 10^{-4}$, $1.34 \pm 0.21 \times 10^{-3}$, $1.54 \pm 0.11 \times 10^{-3}$, $8.33 \pm 2.29 \times 10^{-4}$, $2.56 \pm 1.39 \times 10^{-4}$, $1.39 \pm 0.36 \times 10^{-4}$, and $9.16 \pm 3.74 \times 10^{-4}$ mol/liter × s.

[d] The number of copies of labeled DNA per diploid cell multiplied by the average acceleration of reassociation rate minus the number of copies of labeled DNA per diploid cell. It was assumed that diploid cell DNA has a molecular weight of 4×10^{12} (15). The actual amount of DNA per cell, determined colorimetrically as described in the text, was 1.62×10^{-5} μg for BHK-21, 1.33×10^{-5} μg for T637, 1.28×10^{-5} μg for HA12/7, and 1.12×10^{-5} μg for A2497-2 cells.

[e] Standard deviation of the mean.

lines (14, 29, 30). Thus, the persistence of only part of the viral genome in adenovirus-transformed cells is not a general phenomenon. Factors such as MOI, conditions of infection, and, in particular, the method of selection for transformed clones may play a decisive part in determining which parts of the viral genome will eventually persist. The fact that rat cells are semipermissive for Ad2 and hamster cells are nonpermissive for Ad12 may also affect selection for the loss of viral sequences. Moreover, it may be important to know whether some of the transformed hamster cells may have arisen by transformation with incomplete particles of Ad12. A systematic study of these problems has not yet been carried out.

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ADDENDUM

After submission of this paper, a report by M. R. Green et al. (20) came to our attention. Their paper demonstrates that two lines of Ad 12-transformed hamster cells harbor sequences representing most of the viral genome.

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