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Integration Sites of Adenovirus Type 12 DNA in Transformed Hamster Cells and Hamster Tumor Cells

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The patterns and sites of integration of adenovirus type 12 (Ad12) DNA were determined in three lines of Ad12-transformed hamster cells and in two lines of Ad12-induced hamster tumor cells. The results of a detailed analysis can be summarized as follows. (i) All cell lines investigated contained multiple copies (3 to 22 genome equivalents per cell in different lines) of the entire Ad12 genome. In addition, fragments of Ad12 DNA also persisted separately in non-stoichiometric amounts. (ii) All Ad12 DNA copies were integrated into cellular DNA. Free viral DNA molecules did not occur. The terminal regions of Ad12 DNA were linked to cellular DNA. The internal parts of the integrated viral genomes, and perhaps the entire viral genome, remained colinear with virion DNA. (iii) Except for line HA12/7, there were fewer sites of integration than Ad12 DNA molecules persisting. This finding suggested either that viral DNA was integrated at identical sites in repetitive DNA or, more likely, that one or a few viral DNA molecules were amplified upon integration together with the adjacent cellular DNA sequences, leading to a serial arrangement of viral DNA molecules separated by cellular DNA sequences. Likewise, in the Ad12-induced hamster tumor lines (CLAC1 and CLAC3), viral DNA was linked to repetitive cellular sequences. Serial arrangement of Ad12 DNA molecules in these lines was not likely. (iv) In general, true tandem integration with integrated viral DNA molecules directly abutting each other was not found. Instead, the data suggested that the integrated viral DNA molecules were separated by cellular or rearranged viral DNA sequences. (v) The results of hybridization experiments, in which a highly specific probe (143-base pair DNA fragment) derived from the termini of Ad12 DNA was used, were not consistent with models of integration involving true tandem integration of Ad12 DNA or covalent circularization of Ad12 DNA before insertion into the cellular genome. (vi) Evidence was presented that a small segment at the termini of the integrated Ad12 DNA in cell lines HA12/7, T637, and A2497-3 was repeated several times. The exact structures of these repeat units remained to be determined. The occurrence of these units might reflect the mechanism of amplification of viral and cellular sequences in transformed cell lines.

Since the first demonstration of adenovirus DNA integration (4, 6), a great deal of work has been done to elucidate the state of viral DNA persisting in adenovirus-transformed cells. There is now very good evidence for the integrated state of adenovirus DNA in a number of different systems (for review, see reference 6). In most adenovirus type 12 (Ad12)-transformed cell lines or Ad12-induced tumor cell lines, the entire viral genome persists in multiple copies (8, 10, 14, 20) in a very stably integrated form (8, 15, 16, 19, 33). However, in many of the Ad2- and Ad5-transformed rat cell lines investigated, only fractions of the viral genome are present (26) in an integrated form (12). It has been shown that transfection of rat cells with fragments from the molecular left ends of Ad5, Ad7, or Ad12 DNA can lead to the phenotypic transformation of cells (13, 21, 27, 28, 37). The apparent difference in persistence patterns between Ad12-transformed hamster cells and Ad2- and Ad5-transformed rat cells may best be explained by the fact that rat cells are semipermissive for Ad2 and Ad5 (11), whereas hamster cells are totally nonpermissive for Ad12 (5, 10). Since semipermissive infections will eventually result in cell killing, there must be a very strong selective force directed against persistence of the intact Ad2 and Ad5 genomes in rat cells. Such restrictions would not apply in the Ad12-hamster cell system. Intact genomes allow transformation of cells if integrated at certain sites or expressed in an appropriate way.

The integrated state of viral genomes in trans-

formed cells is associated with an altered control of viral gene expression. In some of the Ad12-transformed hamster lines, only early genes are transcribed into mRNA (22). Early and late genes are expressed in Ad12-induced rat brain tumor cell lines (19). However, expression of late genes in adenovirus-transformed cells appears to be the exception rather than the rule. Recently, it has been demonstrated that methylation of integrated Ad12 and Ad2 DNA sequences is nonrandom, and that its extent is inversely correlated to the level of expression of specific segments of the viral genomes (31, 32, 38). DNA methylation may constitute a signal for long-term inactivation of genes.

From the Ad12-transformed hamster cell line T637, morphological revertants have been isolated under nonselective conditions (17, 18). These revertants have retained some of the biological properties of transformed cells, but have lost considerable portions of the Ad12 genome (9). The occurrence of these revertants indicates that integrated viral sequences, although generally very stable in the transformed cells (33), can be lost in rare instances by a mechanism which is totally unknown.

In the present study, we have started to characterize the sites of viral DNA integration in Ad12-transformed hamster cells and in Ad12-induced hamster tumor cells.

MATERIALS AND METHODS

Cells and virus. The origin and the method of propagation of the Ad12-transformed hamster cell lines T637, HA12/7, and A2497-3, as well as of the B3 subline of BHK-21 cells, were described previously (10). Lines CLAC1 and CLAC3 were derived from tumors which were induced in inbred hamsters, strain CLAC (apricot), by injection of CsCl-purified Ad12. Lines CLAC1 and CLAC3 were maintained in Dulbecco's modification of Eagle medium (1) supplemented with 10% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.). DNA for analysis was extracted from the CLAC lines in passages 10 to 15.

The propagation of Ad2 and Ad12 on human KB cells in suspension cultures and the purification of virus by equilibrium centrifugation in CsCl density gradients were reported elsewhere (5).

Purification of viral DNA and of Ad12 DNA fragments: cloned fragments of Ad12 DNA. Viral DNA was extracted from CsCl-purified virions as described earlier (5). Ad12 DNA was cleaved with the *Eco*RI restriction endonuclease (Boehringer, Mannheim, Germany) according to published protocols. *Eco*RI fragments A through E of Ad12 DNA were purified by three cycles of electrophoresis on 0.8% agarose gels in TEB buffer (0.1 M Tris-hydrochloride, pH 8.6, 0.077 M H_3BO_3, and 0.0025 M EDTA) at 30 to 50 V for 24 to 36 h. After the gels had been stained in a solution of 0.5 to 1.0 μg of ethidium bromide per ml, fragments A through E were excised with a razor blade under the control of UV light. Each fragment was subsequently electroeluted from the gel slices at 200 mA and 0°C overnight into 0.01 M Tris-hydrochloride, pH 7.5, and 0.001 M EDTA. Elution was controlled under a UV lamp. Eluates were concentrated approximately fivefold under a stream of N_2, and each fragment was reelectrophoresed twice under the conditions described above, except that the time of electrophoresis varied between 24 h (E fragment) and 64 h (A fragment), depending on the size of the fragment. Purity of the fragments was ascertained by hybridizing each fragment, which was ^{32}P labeled by nick translation, to Southern blots of *Eco*RI-cleaved Ad12 DNA (see Fig. 2). In some of the experiments, cloned fragments of Ad12 DNA were used in the analysis. We have cloned the *Eco*RI fragments D, E, and F and parts of fragments A and C in the plasmid pBR325 (2), and *Eco*RI fragment B in bacteriophage λgt WES·λB DNA (34) (S. Vogel, M. Brötz, U. Winterhoff, and W. Doerfler, manuscript in preparation).

^{32}P labeling of Ad12 DNA, of specific viral DNA fragments, and of cloned DNA by nick translation. The nick translation procedure of Rigby et al. (24) was used with minor modifications as described elsewhere (35). Cloned DNA fragments were labeled together with vector DNA. It has been shown that the DNA of λ WES or of pBR325 does not exhibit homology to Ad12 DNA or to hamster cell DNA (D. Eick and W. Doerfler, unpublished data).

Labeling the terminal *Msp*I fragments of Ad12 DNA to be used as probes in hybridization experiments. A 10-μg amount of Ad12 DNA was treated for 10 min at 13°C with exonuclease III from *Escherichia coli* (23) under conditions as described earlier (8) to remove terminal nucleotides from the 3′ termini. The depleted sequences were filled in with the four α-^{32}P-labeled deoxyribonucleoside triphosphates, using the Klenow fragment of DNA polymerase I (Boehringer, Mannheim) from *E. coli* as outlined elsewhere (8). Subsequently, the DNA was cleaved with the restriction endonuclease *Msp*I. This procedure generated a probe which carried ^{32}P label predominantly in the terminal *Msp*I fragments of Ad12 DNA (see Fig. 10), as shown by gel electrophoresis and autoradiography. A 143-base pair (bp) band contained the bulk of the label. It has been shown previously that both terminal *Msp*I fragments (143 bp) lie within the inverted terminal repetition of Ad12 DNA and hence are equal in size (8). Before being used as probe, the ^{32}P-labeled terminal 143 bp fragment of Ad12 DNA was purified by electrophoresis on 2% agarose gels. The DNA was electroeluted from the gel slice and used directly in hybridization experiments.

Preparation of the *E. coli* plasmid pBR322 (30). The plasmid pBR322 was prepared as described earlier (8).

Extraction and purification of cellular DNA. The sodium dodecyl sulfate (SDS)-pronase B-phenol procedure was outlined in detail elsewhere (33). The DNA was also treated with RNase and proteinase K and was purified by equilibrium centrifugation in CsCl density gradients (33).

Analysis of cellular DNA by the Southern blotting technique (29). The total intracellular DNA from transformed cells was cleaved with various re-

striction endonucleases (*Eco*RI, *Bam*HI, *Bgl*II, *Hin*fI, *Msp*I, or *Pst*I), using published protocols (Biolabs and Boehringer specifications). Restriction endonucleases were purchased from Boehringer, Mannheim (*Eco*RI, *Bam*HI, *Bgl*II, and *Pst*I), or from Biolabs, Beverly, Mass. (*Hin*fI, *Msp*I, and *Pst*I). Fragments of cellular DNA, 10 μg per slot, were separated by electrophoresis in horizontal slab gels consisting of 0.5 to 2% agarose (Sigma Chemical Co., St. Louis, Mo.) in TEB buffer. As controls, Ad12 DNA in amounts ranging from 1 to 20 genome equivalents per cell (0.026 to 0.52 ng of Ad12 DNA) were mixed with 10 μg of carrier B3 DNA, cleaved with the same restriction endonuclease, and coelectrophoresed in the same gel. Carrier DNA was used to compensate for the dependence of migration rates on DNA concentration for the larger Ad12 DNA fragments (33). Viral genome equivalents per cell were calculated on the basis of 10 μg of cellular DNA per slot, a DNA content of 1.3×10^{-5} μg per hamster cell (10), and a molecular weight of 20×10^6 for Ad12 DNA (5). B3 DNA was shown previously to be devoid of homologies to Ad12 DNA (33).

At the end of electrophoresis, the DNA was transferred to sheets of BA85 nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) after denaturation of the DNA by submersing the gels in 0.5 N NaOH–1.5 M NaCl at room temperature for 4 h. Subsequently, the gels were neutralized in 0.5 N HCl–0.1 M Tris-hydrochloride (pH 7.5)–1.5 M NaCl for 1 to 2 h. During denaturation and neutralization, the gels were rotated gently on a shaker. The denatured DNA fragments were then transferred to nitrocellulose filter sheets by the Southern technique (29). Transfer was effected with 20× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) for 2 to 3 days at room temperature. The filters were then briefly rinsed in 2× SSC, dried at 80°C, and sealed in plastic bags (Dazey Products Co., Industrial Airport, Kansas).

DNA-DNA hybridization and autoradiography. The DNA-DNA hybridization method of Wahl et al. (39) was applied. Depending on size, filters were preincubated in plastic bags in total volumes of 30 to 50 ml containing 50% formamide, fivefold-concentrated Denhardt reagent (1× reagent contains 20 mg each of bovine serum albumin, Ficoll-400, and polyvinylpyrrolidone in 100 ml), and 300 μg of heat-denatured salmon sperm or calf thymus DNA per ml in 0.05 M sodium phosphate, pH 6.5, and 5× SSC. It was necessary that the preincubation volume be abundant or else background binding of ^{32}P-labeled DNA to the filters was considerable. The filters were preincubated at 42°C for 12 to 24 h. All of the preincubation solution was then removed from the plastic bags and replaced by 20 ml of a solution containing the heat-denatured ^{32}P-labeled Ad12 DNA probe (150×10^6 to 200×10^6 cpm) in 50% formamide, 1× Denhardt reagent, and 100 μg of heat-denatured calf thymus DNA per ml in 5× SSC, 0.02 M sodium phosphate, pH 6.5, and 10% sodium dextran sulfate 500 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). Sodium dextran sulfate was added as a 50% solution. The solution was thoroughly mixed in the bag and incubated at 42°C for 12 to 14 h. Subsequently, the filters were washed three times with 250 ml of 2× SSC and 0.1% SDS for 5 min each at room temperature and twice with 250 ml each of prewarmed 0.1× SSC and 0.1% SDS at 50°C for a total of 30 min; if background radiation was found to be low, filters were dried and autoradiographed on Kodak XR5 film at −80°C for various lengths of time. If the background was not found to be satisfactory, washes in 0.1× SSC and 0.1% SDS were continued for another 30 min at 50°C.

In some experiments (see, e.g., Fig. 4), in which *Eco*RI-cleaved Ad12 DNA was used as molecular weight marker, the tracks carrying these marker DNA fragments were cut off from the main part of the filter before hybridization. The filter strips containing Ad12 DNA were then hybridized separately with ^{32}P-labeled intact Ad12 virion DNA. The DNA on the main part of the filter was hybridized to isolated *Eco*RI fragments. After washing and drying, the marker DNA strips were precisely fitted back on the main filter and then the entire filter was autoradiographed.

Scanning and quantitation of autoradiographic intensities. Autoradiograms were scanned in a Vitatron Electronic photometer which was capable of integrating intensities of individual bands. Different autoradiograms of varying exposure times were tested for linearity of autoradiographic intensities, using a reconstitution series of viral DNA fragments in which 1, 5, 10, and 20 genome equivalents of Ad12 DNA per cell were electrophoresed and were hybridized to *Eco*RI fragments A, B, C, D, or E of Ad12 DNA (see Fig. 2). The intensities measured over each of these fragments in the reconstitution series were then used to quantitate the amount of each corresponding Ad12 DNA fragment in the DNA extracted from transformed cells (see Fig. 2 and Table 1).

RESULTS

Patterns of integration: survey of different hamster cell lines. The arrangements of integrated Ad12 DNA molecules in the genomes of three different Ad12-transformed hamster cell lines, HA12/7, T637, A2497-3, and two Ad12-induced hamster tumor lines, CLAC1 and CLAC3, were determined by cleavage of the entire intracellular DNA with restriction endonuclease *Eco*RI (Fig. 1a), *Bam*HI (Fig. 1b), or *Bgl*II (Fig. 1c). The fragments were separated by electrophoresis on 0.5% agarose gels and transferred to nitrocellulose filters by the Southern blotting method, and the DNA on the blots was hybridized with intact ^{32}P-labeled Ad12 DNA. This experimental approach was based on a size comparison of specific viral DNA fragments in virion DNA and in cellular DNA containing integrated viral DNA sequences. Comigration of specific viral DNA fragments from virion DNA and from transformed cell DNA indicated that a specific viral DNA fragment persisted intact and in a colinear arrangement with virion DNA. Viral DNA segments linked to cellular DNA would assume a higher molecular weight than the corresponding virion DNA fragments. In Southern blots, these virus-cell DNA fragments gave rise to viral specific bands in off-

size positions, i.e., in positions not corresponding in size to any of the virion DNA fragments. Frequently, off-size bands exceeded in size the largest virion DNA fragments. The identity of specific viral DNA bands in Southern blots was established by hybridization experiments using isolated or cloned viral DNA fragments as ^{32}P-labeled probes (see below). Covalent linkage of viral and cellular DNAs in cell lines T637, HA12/7, A2497-2, and A2497-3 was demonstrated previously by two-step filter hybridization experiments (15, 16).

Irrespective of whether the cells were transformed in vitro or derived from tumors in animals, the autoradiograms in Fig. 1 revealed certain similarities in the patterns of viral DNA fragments for all five cell lines investigated. The results presented were compatible with the following interpretations.

(i) In all lines, the majority of the Ad12 genomes were integrated as complete or nearly complete molecules. The centrally located fragments of Ad12 DNA, i.e., the *Eco*RI fragments D, B, E, and possibly F (Fig. 1a), the *Bam*HI fragments G, D, F, C, B, H, and I (Fig. 1b), and the *Bgl*II fragments A, D, E, and F (Fig. 1c) comigrated with the corresponding marker DNA fragments in those tracks in which virion DNA cleaved with the same enzymes was electrophoresed.

(ii) The displacement of the terminal Ad12 DNA fragments, *Eco*RI-C and -A (Fig. 1a), *Bam*HI-A and -E (Fig. 1b), and *Bgl*II-B and -C (Fig. 1c), indicated that free viral DNA did not persist in these lines (see Fig. 2) and that the sites of linkage between viral and cellular DNA were at or close to the termini of viral DNA.

(iii) It has been shown previously that multiple copies of Ad12 DNA persist in the in vitro-transformed hamster lines T637, HA12/7, and A2497-3 (10). This quantitation was based on the results of reassociation kinetics. In the present study, we tried to estimate the amount of Ad12 DNA present in the hamster cell lines from the autoradiographic intensities of viral DNA bands. The autoradiographic intensities of Ad12-specific bands in the DNA from transformed hamster cells were compared photometrically with the intensities of the authentic virion DNA bands in the reconstitution series representing 1, 5, 10, and 20 Ad12 genome equivalents per cell (Fig. 1a). From this comparison and from the data presented in Fig. 2, it was deduced that line HA12/7 contained 3, line T637 contained 22, line A2497-3 contained 17, line CLAC1 contained 12, and line CLAC3 contained about 5 genome equivalents per cell (Table 1).

(iv) Although in all cell lines multiple copies of viral DNA were persisting in the integrated state, only a limited number of off-size bands was detected by using the *Eco*RI restriction endonuclease in the analysis: three in line HA12/7, three (perhaps four) in line T637, six in line A2497-3, two in line CLAC1, and three in line CLAC3 (Fig. 1a). An even smaller number of off-size bands was observed upon cleavage of cellular DNA with the *Bam*HI restriction endonuclease (Fig. 1b): two in lines CLAC1 and -3, HA12/7, and A2497-3 (plus two minor ones), and three to four in line T637. Upon cutting cellular DNA with the restriction endonuclease *Bgl*II, two off-size bands were observed in line HA12/7, four in line T637, two to three in line A2497-3, two in line CLAC1, and two to three in line CLAC3 (Fig. 1c). The sizes of these off-size bands were different for each cell line investigated and for each restriction endonuclease used. This latter finding suggests that at least the macroenvironments at the sites of integration were different in each cell line. Judging from the intensities of the off-size bands, most of them contained more than one genome equivalent of viral DNA fragment per cell.

(v) The patterns of viral DNA in each of the lines examined were specific for one characteristic line with respect to the number and sizes of off-size bands.

A trivial explanation for the occurrence of off-size bands would be incomplete digestion of DNA by restriction endonucleases. This possibility was ruled out by demonstrating that the DNAs of T637 and HA12/7 cells gave rise to the same integration patterns and off-size bands, irrespective of whether 15, 30, or 50 U of the *Eco*RI restriction endonuclease was used per μg of cellular DNA (data not shown). An amount of 15 U of enzyme cleaved 1 μg of Ad12 DNA to completion.

We conclude that multiple copies of the entire Ad12 DNA were integrated at a limited number of sites. Different cell lines exhibited different sizes of off-size bands. Free viral DNA could not be detected. The general patterns of integration were similar for viral DNA from cell lines which were obtained by transformation in cell culture and for DNA from cell lines established from Ad12 tumors in hamsters.

Specific fragments of Ad12 DNA as hybridization probes. Specific segments of Ad12 DNA could be identified in the DNA of the five transformed lines by using *Eco*RI fragments A, B, C, D, or E of Ad12 DNA, all of which were ^{32}P labeled by nick translation, as hybridization probes in Southern blots of *Eco*RI-cleaved cellular DNA (Fig. 2). In each blot, increasing amounts of Ad12 virion DNA were mixed with

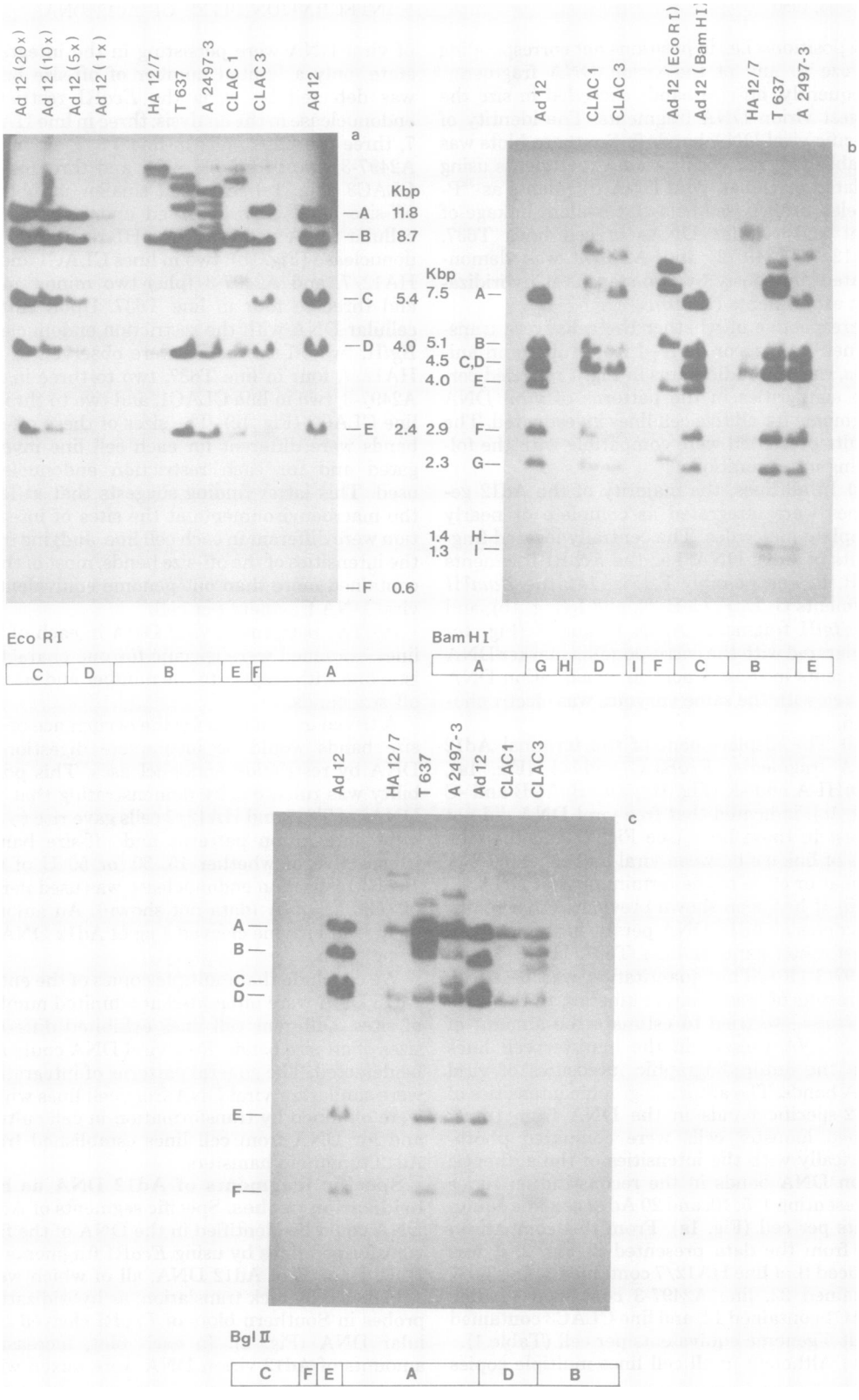
Ad 12 (20x)
Ad 12 (10x)
Ad 12 (5x)
Ad 12 (1x)
HA 12/7
T 637
A 2497-3
CLAC 1
CLAC 3
Ad12
a
Kbp
A 11.8
B 8.7
C 5.4
D 4.0
E 2.4
F 0.6
Ad 12
CLAC 1
CLAC 3
Ad 12 (Eco RI)
Ad 12 (Bam HI)
HA 12/7
T 637
A 2497-3
b
Kbp
7.5 A
5.1 B
4.5 C,D
4.0 E
2.9 F
2.3 G
1.4 H
1.3 I
Eco RI
C D B E F A
Bam HI
A G H D I F C B E
Ad 12
HA 12/7
T 637
A 2497-3
Ad 12
CLAC1
CLAC3
c
A
B
C
D
E
F
Bgl II
C F E A D B

TABLE 1. *Quantitation of integrated viral DNA molecules in three lines of Ad12-transformed hamster cells and two lines of Ad12-induced hamster tumor cells*[a]

Cell line	No. of copies[b]			No. of Ad12 DNA molecules/ cell	Range
	*Eco*RI fragment A	*Eco*RI fragment B	Total Ad12 DNA		
HA12/7	3	4	3	3	3-4
T637	15	21	26	22	21-26
A2497-3	16	18	12	17	12-20
CLAC1		13	10	12	10-13
CLAC3	5	5	5	5	4-5

[a] Experimental details are described in the text. All calculations were based on an amount of 1.3×10^{-5} μg of total cellular DNA per Ad12-transformed hamster cell (10).

[b] Based on photometric scans of the fragment bands as indicated or on the sum of all the *Eco*RI bands of Ad12 DNA (total Ad12 DNA). Estimates of copy numbers were derived from a comparison with the intensities of marker DNA bands in the reconstitution series (Fig. 1 and 2).

10 μg of B3 DNA, cleaved with the *Eco*RI restriction endonuclease, and coelectrophoresed on the same gel with the DNA from transformed cells. Ad12 DNA was used in amounts corresponding to 1, 5, 10, and 20 genome equivalents of viral DNA per cell (0.026, 0.13, 0.26, and 0.52 ng of Ad12 DNA). This reconstitution series of Ad12 DNA served as an internal reference in the quantitation of Ad12 DNA sequences in transformed cell DNA. Each DNA probe (fragments A to E) hybridized exclusively to the corresponding fragment in the reconstitution series, attesting to the purity of each probe used. There was a low degree of cross-hybridization between the terminal *Eco*RI fragments A and C (Fig. 2a, c) due to the inverted terminal repetition of Ad12 DNA (36). Such cross-hybridization was also found with cloned DNA fragments.

The results of the hybridization experiments using specific probes are presented in Fig. 2 and can be summarized as follows.

The terminal *Eco*RI fragments A and C (see map, Fig. 1) were displaced to off-size positions due to the attachment of cellular DNA sequences (Fig. 2a, c). Covalent linkage of Ad12 and cellular DNAs in the lines investigated was shown earlier (15, 16). With few exceptions, the internal fragments B, D, and E comigrated precisely with the marker DNA fragments (Fig. 2b, d, e). This pattern of distribution of specific viral DNA segments was consistent with a model in which multiple copies of the entire Ad12 DNA were integrated at a limited number of cellular DNA sites. There were, however, some bands homologous to the terminal *Eco*RI fragments (see below) which indicated that some copies of Ad12 DNA had become fragmented during integration (33). In particular, *Eco*RI-D sequences in line A2497-3 and *Eco*RI-B sequences in line CLAC3 were found in off-size positions. These data imply fragmentation of some viral genomes.

The positions of all Ad12-specific bands and the sequences detected in these bands are indicated in the scheme in Fig. 3. Bands not specifically designated were identical with the Ad12 marker bands they comigrated with. All off-size bands in the DNA of each line are referred to by numbers as designated (Fig. 3).

In preliminary experiments, we demonstrated that the *Eco*RI-B fragment of virion DNA was identical to the *Eco*RI-B fragments isolated from lines HA12/7 and T637. The B fragment from each of these sources was cloned in λ WES DNA and further analyzed by cleavage with the *Bam*HI, *Hin*fI, *Hpa*II, or *Msp*I restriction endonuclease. These restriction patterns were identical for the B fragments of virion or cellular origin (R. Deuring, U. Winterhoff, S. Stabel, and W. Doerfler, unpublished data). These results demonstrate that a major internal part of the Ad12 genome remained unaltered in the integrated state.

In the DNAs of cell lines HA12/7, T637, and A2497-3, off-size bands existed that hybridized equally intensely with both *Eco*RI fragments A and C, i.e., both termini of Ad12 DNA. The

FIG. 1. *Integration patterns of Ad12 DNA in Ad12-transformed hamster cells and Ad12-induced hamster tumor cell lines. Ad12 virion DNA or DNA from cell lines as indicated was cleaved with the EcoRI (a), the BamHI (b), or the BglII (c) restriction endonuclease. In each track, 10 μg of cellular DNA was used. Ad12 DNA was mixed with 10 μg of B3 hamster cell DNA. Fragments were separated by electrophoresis in 0.5% agarose gels and transferred to nitrocellulose filters by Southern blotting (29). Virus-specific sequences were detected by hybridization with Ad12 DNA ^{32}P-labeled by nick translation followed by autoradiography. Experimental details are described in the text. In (a), increasing amounts of Ad12 virion DNA [1, 5, 10, and 20 genome equivalents per cell, designated Ad12 (1×), Ad12 (5×), etc.] were mixed with each 10 μg of B3 DNA and were cleaved with the EcoRI restriction endonuclease as indicated. The intensity of bands in the marker DNA series served as an approximate measure for the quantities of viral DNA persisting in transformed cells (see text). In (b), EcoRI- and BamHI-cleaved Ad12 DNA were used as size markers. The sizes of these viral DNA fragments in kilobase pairs (kbp) were also indicated.*

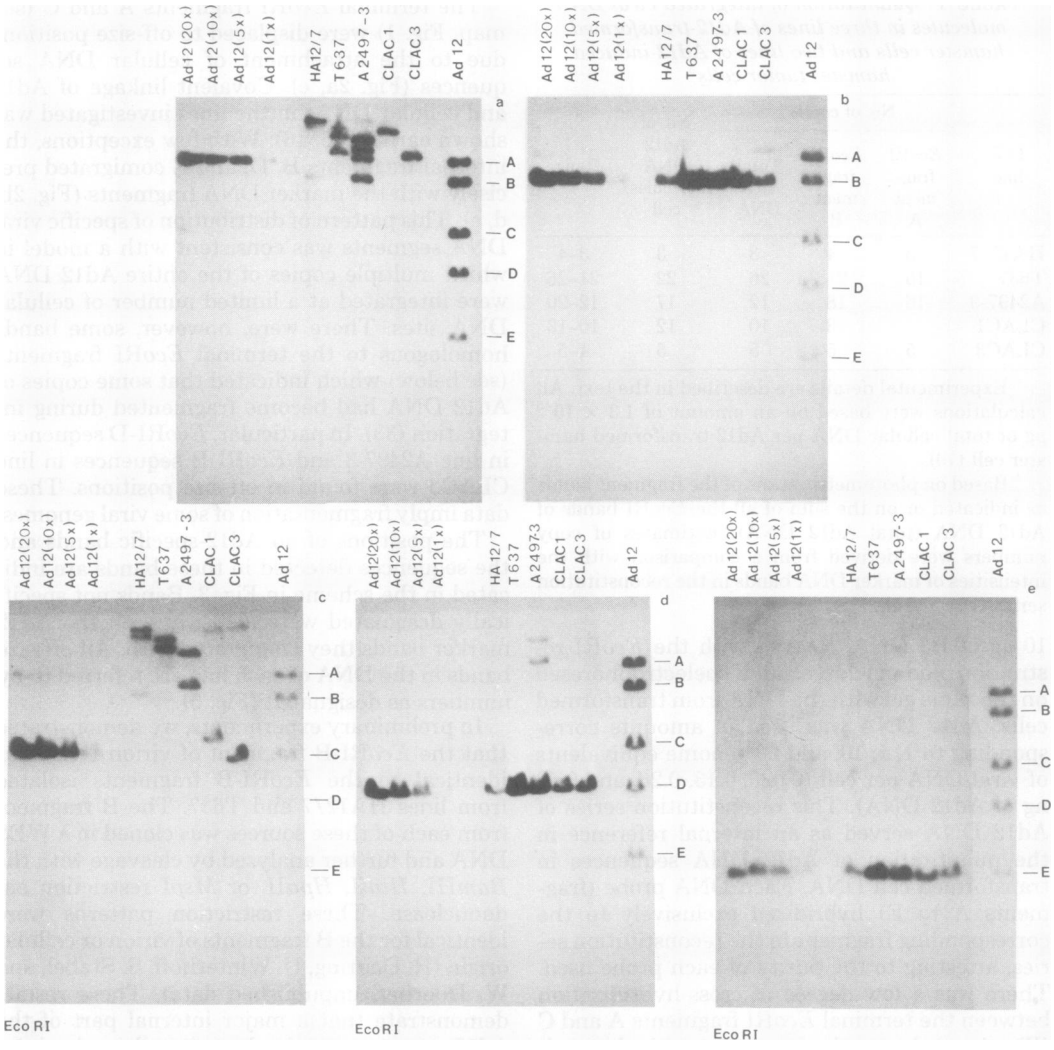

FIG. 2. *Analysis of Southern blots of DNA from three Ad12-transformed hamster lines and two Ad12-induced hamster tumor lines, using the EcoRI fragments of Ad12 DNA as hybridization probes. The DNA from cell lines as indicated (10 μg each) was cleaved with the EcoRI restriction endonuclease. Amounts of 10 μg of B3 DNA were mixed with 0.026 ng (1×), 0.13 ng (5×), 0.26 ng (10×), and 0.52 ng (20×) of Ad12 virion DNA corresponding to 1, 5, 10, and 20 genome equivalents per cell, and the mixtures were also cleaved with the EcoRI restriction endonuclease. Electrophoresis, Southern blotting, hybridization with the purified EcoRI fragments A (a), B (b), C (c), D (d), and E (e), and autoradiography were carried out as described in the text. The reconstitution series using increasing amounts of Ad12 DNA served to quantitate viral DNA in transformed cell DNA (see Table 1). B3 DNA was added to virion DNA to ensure identical electrophoresis conditions in all tracks.*

lengths of the off-size bands 1 and 2 in the DNA from line HA12/7, which were homologous to *Eco*RI fragments A and C, were directly compared (Fig. 4). A similar comparison was done for off-size bands 1 and 2 in the DNA from line T637 and for the off-size bands in the DNA from line A2497-3 (Fig. 4). In separate tracks, the DNAs of T637, HA12/7, and A2497-3 cells, as well as the marker Ad12 DNA, were run in duplicate after *Eco*RI cleavage. After transfer, the filter was cut into three parts. Part a was hybridized with Ad12 DNA, part b was hybridized with *Eco*RI fragment C, and part c was hybridized with *Eco*RI fragment A (Fig. 4). All probes were ^{32}P labeled. The off-size bands 1 and 2 in line HA12/7 and in line T637 were homologous to both the A and C fragments. In line A2497-3, off-size band 2 was complementary to

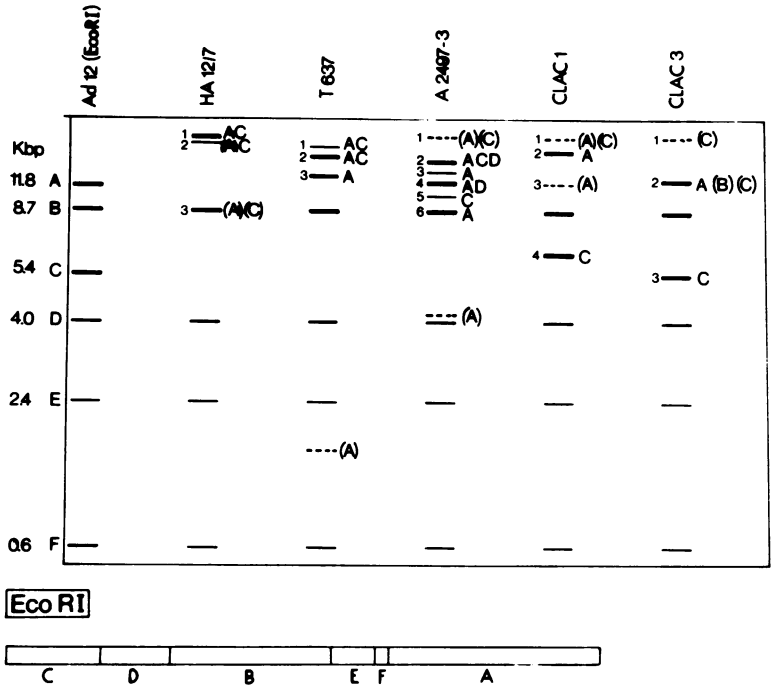

FIG. 3. *Schematic representation of Ad12-specific sequences in EcoRI-cleaved DNA from three transformed hamster cell lines and two Ad12-induced hamster tumor cell lines. This scheme summarizes the data shown in Fig. 1a and Fig. 2. The letters A to E designate homology to the EcoRI fragments A to E of Ad12 DNA, respectively. The locations of these fragments on the Ad12 virion DNA map are indicated on the bottom of the scheme. The figures on the extreme left present the sizes of the virion DNA fragments in kilobase pairs (kbp). The numerals to the left of some of the Ad12-specific bands designate off-size Ad12-specific bands for easy reference in the text. Dotted lines represent weak bands.*

both terminal fragments. Other bands appeared to be uniquely complementary to the A or C fragment. The finding that certain off-size bands appeared to contain sequences from either terminus of Ad12 DNA could be coincidence. The DNA of the five cell lines was, therefore, cleaved with the *Bam*HI (Fig. 5) or *Bgl*II (Fig. 6) restriction endonuclease. The DNA fragments were separated by electrophoresis, transferred to nitrocellulose filters by Southern blotting, and hybridized to the ^{32}P-labeled *Eco*RI fragment A (Fig. 5a and 6a) or C (Fig. 5b and 6b). The data are summarized schematically in Fig. 7 and 8 and demonstrate again that off-size bands were generated that seemed to contain sequences from the left and right termini. Thus, any model describing the integrated state of Ad12 DNA will have to take into consideration the finding that, upon cleavage of the DNA from line HA12/7, T637, or A2497-3 with three different restriction endonucleases, fragments were generated which contained cellular DNA and sequences from both termini of the Ad12 DNA molecule. Except for one weak band in the DNA from line CLAC1, bands with double homology to either terminus of Ad12 DNA were not apparent in the DNAs from lines CLAC1 and CLAC3 (Fig. 3 and 8).

Recently, many of the experiments described in this section were repeated by using cloned fragments of Ad12 DNA in order to confirm the data presented. Identical results were obtained, attesting again to the high degree of purity of the fragment probes used.

Quantitation of viral DNA persisting in three Ad12-transformed hamster lines and two Ad12-induced hamster tumor lines. The data presented in Fig. 2a–d were used to estimate the amounts of each of the *Eco*RI fragments A to D persisting in the five hamster lines investigated. The quantitation of viral genome copies per cell was based on photometric scans of these blots comparing intensities of individual Ad12 DNA bands with the corresponding bands in the reconstitution series. These series were used for quantitation only if the increase in DNA concentration and the increase in photometric intensities were linearly related. All tracks in such autoradiograms were scanned as described, and the amount of each of

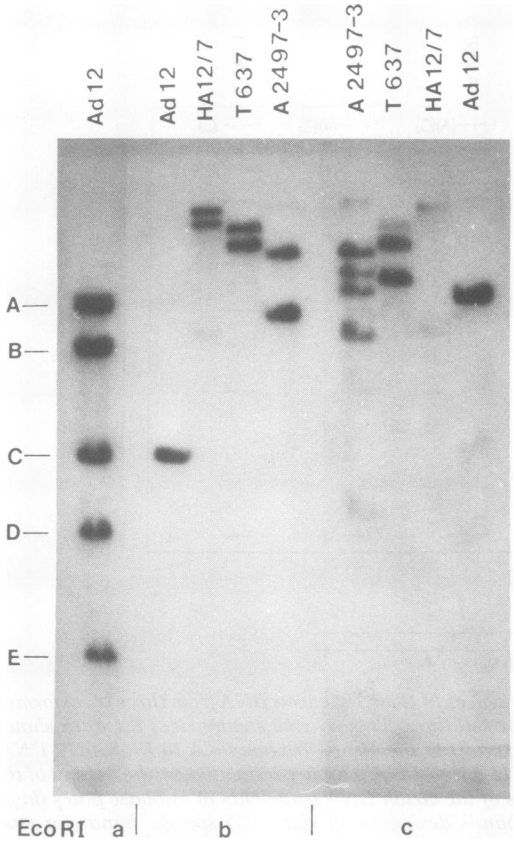

FIG. 4. *Identification of off-size bands in the HA12/7, T637, and A2497-3 cell lines. Amounts of 10 μg of DNA from the HA12/7, T637, and A2497-3 lines and from B3 cells were cleaved with the EcoRI restriction endonuclease. Before cleavage, B3 DNA was mixed with 0.52 ng (20 genome equivalents) of Ad12 DNA. Electrophoresis and Southern blotting were performed as described in the text. Subsequently, the nitrocellulose filter was cut (|) into three parts, a, b, c, as indicated on the bottom of the autoradiogram. Part a was incubated with Ad12 DNA, part b was incubated with purified EcoRI fragment C, and part c was incubated with purified EcoRI fragment A as probes. Probes were ^{32}P labeled by nick translation, and hybridization was carried out as described in the text. After the filter pieces were washed, they were precisely fitted together again and autoradiographed. In part c, the two Ad12-specific bands in track HA12/7 are weak; they do, however, comigrate with the corresponding bands in part of this figure as shown in overexposures of the same autoradiogram.*

the *Eco*RI fragments A to D persisting in each line was determined, using the intensities of bands of Ad12 DNA in the reconstitution series as internal references (0.026 to 0.52 ng, corresponding to 1 to 20 genome equivalents per cell) (Table 1). The most reliable estimates of the number of Ad12 genomes persisting could be derived from the internal *Eco*RI fragments B and D, since they comigrated with the corresponding virion DNA fragments. These fragments supposedly transferred with the same efficiencies in the tracks carrying cellular DNA and Ad12 virion DNA, respectively. The terminal fragments in the DNA from transformed cells, on the other hand, were displaced to high-molecular-weight off-size bands which might transfer less efficiently. Hence, the amount of *Eco*RI fragments A and C in cellular DNA might have been underestimated by this method. Even a quantitation based on the off-size bands A gave results compatible with those derived from internal fragments. Quantitation of minor off-size bands revealed <1 genome equivalent per cell of a particular Ad12 DNA fragment. This result confirmed the conclusion that fragmented genomes were also persisting in nonstoichiometric amounts.

Absence of free viral DNA from Ad12-transformed cell lines and from Ad12-induced tumor cell lines. The data presented in Fig. 1 and Fig. 2a,c demonstrated that the terminal fragments of Ad12 DNA in the DNA of the hamster lines, i.e., the *Eco*RI fragments A and C, the *Bam*HI fragments E and A, or the *Bgl*II fragments B and C, did not comigrate with the terminal fragments in virion marker DNA (see Fig. 3, 7, and 8). These findings demonstrated that the terminal segments of Ad12 DNA were linked to cellular DNA and that free viral DNA did not occur in the Ad12-transformed hamster cell lines or the Ad12-induced tumor cell lines investigated.

This correlation was corroborated by the results of experiments presented in Fig. 5 and 6. The DNA from cell lines as indicated was cleaved with the *Bam*HI (Fig. 5) or *Bgl*II (Fig. 6) restriction endonuclease, and upon transfer to nitrocellulose filters by Southern blotting, these DNA fragments were annealed with the ^{32}P-labeled *Eco*RI fragment A (Fig. 5a and 6a) or fragment C (Fig. 5b and 6b). As was apparent from the restriction maps of Ad12 DNA (Fig. 1a–c), the *Eco*RI fragment A hybridized to *Bam*HI fragments B, C, and E (Fig. 5a, lane 4 from the left), whereas the *Eco*RI fragment C hybridized exclusively to *Bam*HI fragment A (Fig. 5b, lane 4 from the left). Upon hybridization with the *Eco*RI fragment A, no homology was found in the position of *Bam*HI fragment E in any of the five hamster lines investigated (Fig. 5a). Similarly, no homology to *Eco*RI fragment C was found in the position of *Bam*HI fragment A in any of the five lines studied (Fig. 5b), even when the autoradiograms were heavily overexposed. The experiments carried out with *Bgl*II-

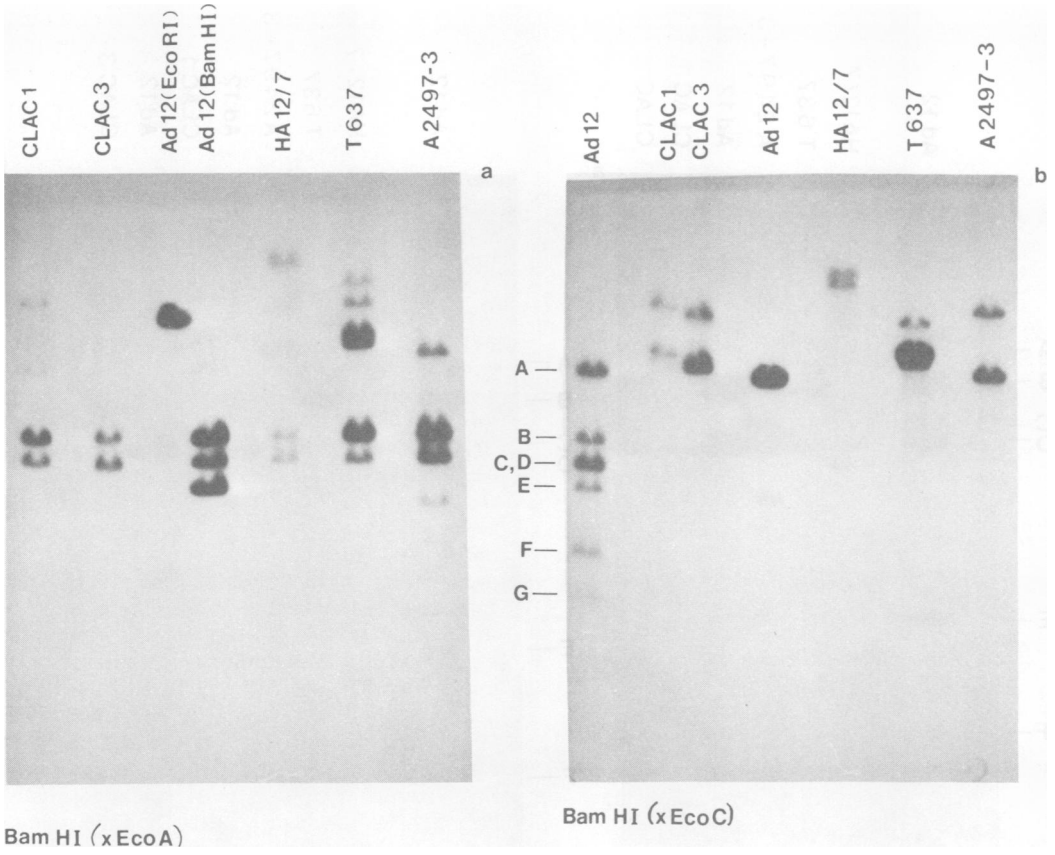

FIG. 5. *Analysis of Ad12 DNA sequences in three Ad12-transformed hamster lines and two Ad12-induced hamster tumor lines, using the BamHI restriction endonuclease and hybridization with the terminal EcoRI fragments of Ad12 DNA. DNA from cell lines (10 μg/slot) as indicated or a mixture of 10 μg of B3 DNA mixed with 0.52 ng of Ad12 DNA was cleaved with the BamHI endonuclease, except where indicated differently (EcoRI). The DNA fragments were electrophoresed and blotted, and the DNA on the filters was hybridized to highly purified, ^{32}P-labeled terminal EcoRI fragment A (a) or C (b). Hybridization to EcoRI-cut Ad12 DNA (third track from the left, panel a) or to BamHI-cut Ad12 DNA (fourth track from the left, panel b) demonstrated the purity of these probes. The DNA in the left-most track of panel b was hybridized to ^{32}P-labeled Ad12 DNA, and the corresponding filter strip was cut off for hybridization and fitted back on for autoradiography.*

cleaved cellular DNAs yielded similar results (Fig. 6). Upon hybridization with the *Eco*RI fragment A (Fig. 6a), no hybridization was found in the position of virion DNA *Bgl*II fragment B; upon hybridization with *Eco*RI fragment C (Fig. 6b), no hybridization was observed in the position of virion DNA *Bgl*II fragment C. It was concluded that intact free Ad12 genomes were absent from lines HA12/7, T637, A2497-3, CLAC1, and CLAC3.

Persistence of the entire viral genome. The restriction endonucleases *Hin*fI, *Msp*I, and *Pst*I have been shown to cut Ad12 DNA into a large number of fragments (8). We analyzed the viral sequences in the transformed hamster and hamster tumor cell lines after cleavage with each of these endonucleases in order to determine whether the entire viral genome was persisting. In the DNA from the five hamster lines tested with the restriction endonuclease *Pst*I, all of the Ad12 DNA fragments located in *Eco*RI fragment B coelectrophoresed exactly with the corresponding fragments in the B fragment of virion marker DNA (Fig. 9b). With the exception of a few bands, this was also true for the *Pst*I fragments in the right terminal *Eco*RI fragment A (Fig. 9a) and the left terminal *Eco*RI fragment C (Fig. 9c). The off-size fragments in these blots were due to the sites of junction between cellular and viral DNA. The high degree of correspondence between Ad12 DNA fragments in the DNA from the transformed hamster lines and the virion marker DNA indicated that the entire viral genome persisted in the integrated state.

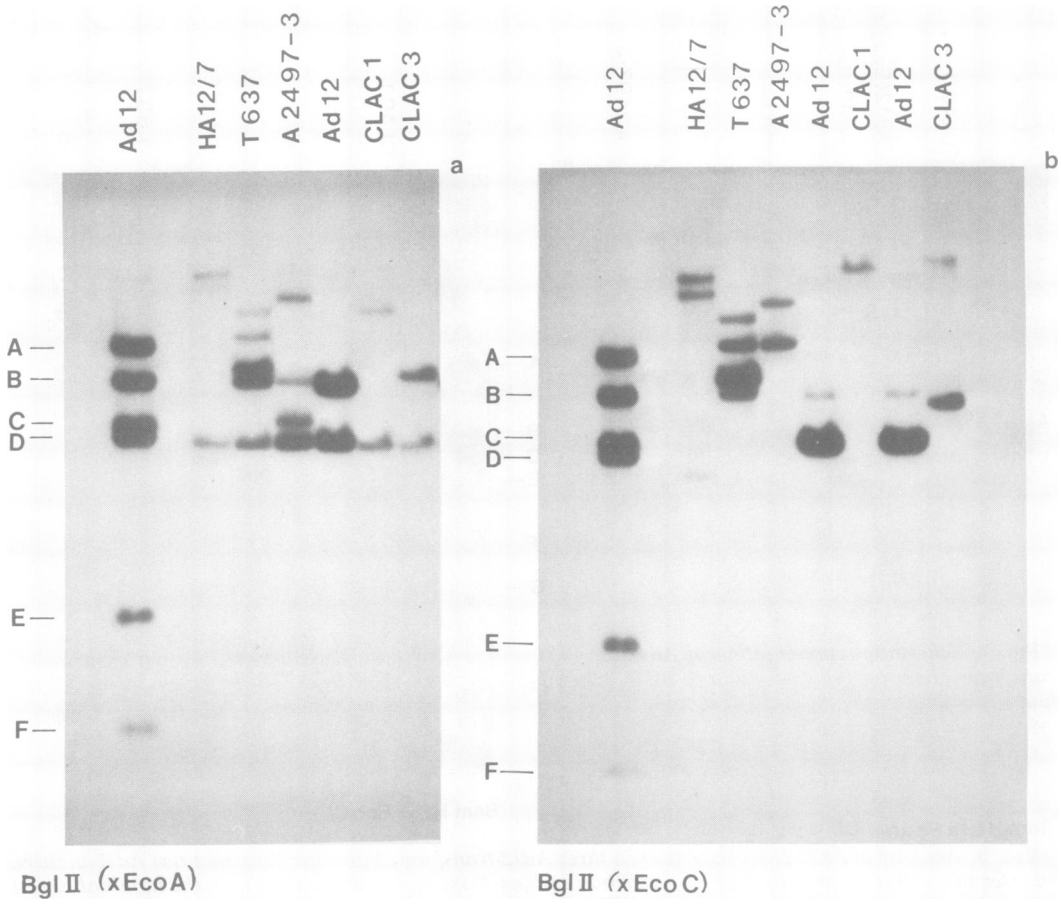

FIG. 6. *Analysis of Ad12 DNA sequences in transformed cells and in tumor cells, using the BglII restriction endonuclease. Experimental conditions were similar to those described in the legend to Fig. 5, except that the BglII restriction endonuclease was used in the analyses.* ^{32}*P-labeled EcoRI fragment A (a) or C (b) was used as hybridization probe.*

Was Ad12 DNA integrated via a circular intermediate? Upon cleavage of the DNA from lines HA12/7, T637, and A2497-3 with three different restriction endonucleases, *Eco*RI, *Bam*HI, and *Bgl*II, off-size bands were generated that hybridized to both terminal *Eco*RI fragments A and C (Fig. 3, 7, and 8). This striking result would be consistent with model (b) in Fig. 10. Model (a) predicted that multiple copies of Ad12 DNA were colinearly integrated into repetitive sequences (H_1) of host DNA, and that the host sequences H_1 did not contain the recognition sequences of restriction endonucleases *Eco*RI and *Bam*HI. Thus, off-size fragments would arise containing both termini of viral DNA and cellular DNA. Model (b) assumed a circular intermediate of Ad12 DNA in the recombination process. If recombination had occurred inside the *Eco*RI fragment A of Ad12 DNA, a part of the *Eco*RI fragment A and the entire fragment C would be included in an off-size band [Fig. 10, model (b)].

An experiment was devised to distinguish between these two possibilities. Transformed cell DNA was cleaved with the restriction endonuclease *Msp*I. If model (a) is correct (Fig. 10), the terminal *Msp*I fragments of Ad12 DNA, which have been shown to be identical and 143 bp long (8), would be contained in fragments of varying off-size lengths (>143 bp). If model (b) applies, the two terminal *Msp*I fragments would be covalently linked and would give rise to a fragment of 286 to 290 bp in length. (The length of 290 bp included the two unmatched bases at the site of the staggered *Msp*I cut.) The same result would be expected in the case of a tandem arrangement (concatenates) of Ad12 DNA molecules [Fig. 12, model (c)]. Model (b) in Fig. 10 further implied that extensive deletions had not occurred during integration at the sites of linkage of the A and C fragments.

We prepared a highly specific ^{32}P-labeled

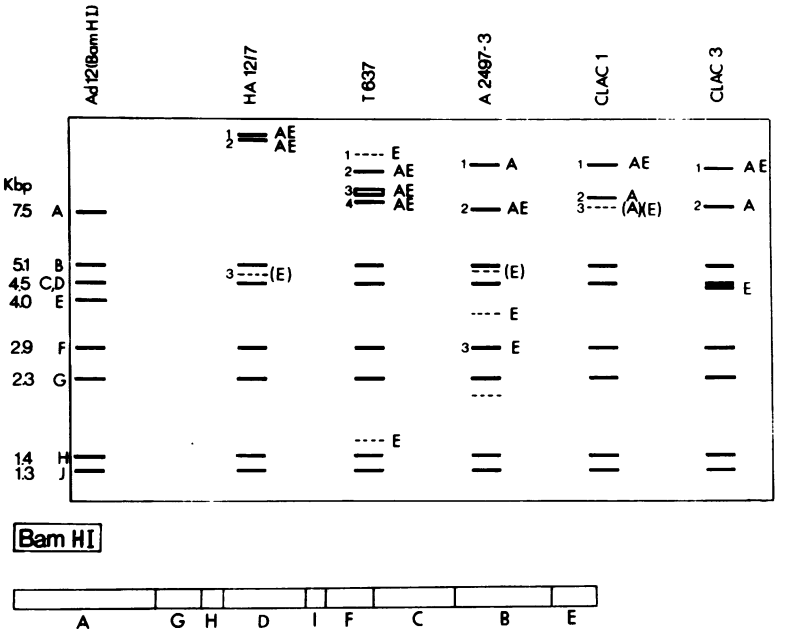

FIG. 7. *Schematic representation of Ad12-specific sequences in BamHI-cleaved DNA from three Ad12-transformed hamster cell lines and two Ad12-induced hamster tumor cell lines. The scheme summarizes the data shown in Fig. 1b and Fig. 5. Symbols are as in Fig. 3. The BamHI restriction map of Ad12 DNA is given on the bottom of the scheme.*

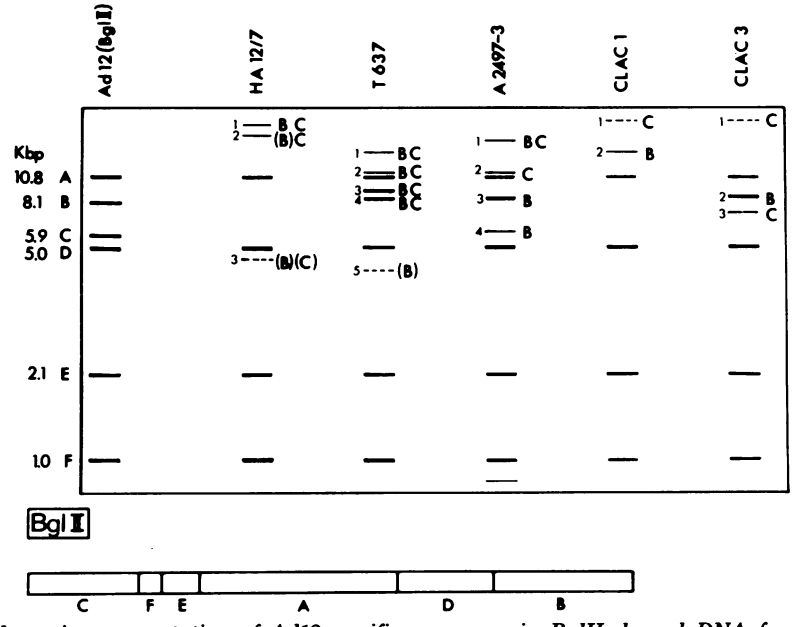

FIG. 8. *Schematic representation of Ad12-specific sequences in BglII-cleaved DNA from three Ad12-transformed hamster cell lines and two Ad12-induced hamster tumor cell lines. The scheme summarizes the data shown in Fig. 1c and Fig. 6. Symbols are as in Fig. 3. The BglII restriction map of Ad12 DNA is given on the bottom of the scheme.*

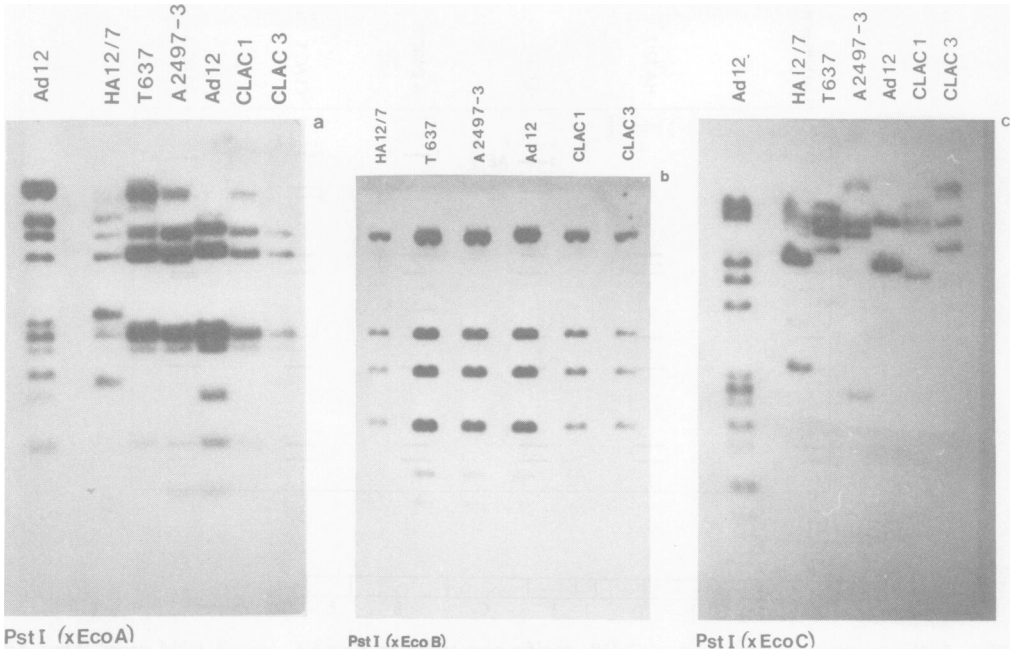

FIG. 9. *Analysis of Ad12 DNA sequences in transformed cells and in tumor cells, using the PstI restriction endonuclease. Experimental conditions were similar to those described in the legend to Fig. 5, except that the PstI restriction endonuclease was used. ^{32}P-labeled EcoRI fragment A (a), B (b), or C (c) was used as hybridization probe. In (a) and (b), 1.3% agarose gels were used for the analysis; in (c), 0.8% gels were used.*

probe consisting of the terminal *Msp*I fragment only (see Materials and Methods). Ad12 DNA was digested to a very limited extent with exonuclease III from *E. coli* (23) under reaction conditions that were shown not to allow digestion to transgress the limits of the terminal *Msp*I fragment (8). Subsequently, the depleted regions of Ad12 DNA were repolymerized with polymerase I from *E. coli*, using α-^{32}P-labeled deoxyribonucleoside triphosphates. Finally, this DNA was cleaved with the *Msp*I restriction endonuclease; then the terminal *Msp*I fragment was purified and isolated by gel electrophoresis and used as hybridization probe (Fig. 10 and 11).

The *Msp*I-cleaved DNA from transformed cells was subjected to gel electrophoresis on horizontal 2% agarose slab gels and transferred to nitrocellulose filters by Southern blotting. The DNA on these filters was hybridized with the ^{32}P-labeled terminal *Msp*I fragment of Ad12 DNA (see above). DNA from the *E. coli* plasmid pBR322 was cleaved with restriction endonuclease *Hin*fI, and the *Hin*fI fragments were used as precise molecular weight markers whose sequence had been determined (30). The sections of the filter carrying the pBR322 marker fragments were cut off the main part of the filter and hybridized separately to ^{32}P-labeled pBR322 DNA. Similarly, Ad12 DNA cleaved with *Msp*I was coelectrophoresed and transferred to the filter, and the filter strip carrying Ad12 DNA was incubated with ^{32}P-labeled Ad12 DNA (Fig. 11).

The results of this analysis demonstrated that DNA fragments with homologies to the terminal *Msp*I fragments of Ad12 DNA were not generated in the size range below 380 bp, with the exception of a weak band of about 228 bp in the DNA from cell line T637 (not visible in Fig. 11). In the control experiment, B3 DNA was shown to be devoid of homologies to the terminal Ad12 DNA fragment (Fig. 11). Off-size fragments complementary to the terminal *Msp*I fragments of Ad12 DNA were observed in all five lines investigated: 410 and 380 bp in length in the DNA of cell line HA12/7; 1,150, 550, and 228 bp in length in the DNA of line T637; 680 bp in length in the DNA of line A2497-3; 540 and 435 bp in length in line CLAC1; and 850 and 720 bp in length in line CLAC3 (Fig. 11). These fragments corresponded to the sites of linkage between viral and cellular DNA. As was concluded before, there were far fewer sites of linkage than there were copies of viral DNA persisting in cell lines T637, A2497-3, CLAC1, and CLAC3. Furthermore, a comparison of the total intensities of the bands with homologies to the terminal segments of Ad12 DNA relative to the intensities of the 143-bp fragment in the tracks containing 50 or 10 genome equivalents of Ad12 marker DNA re-

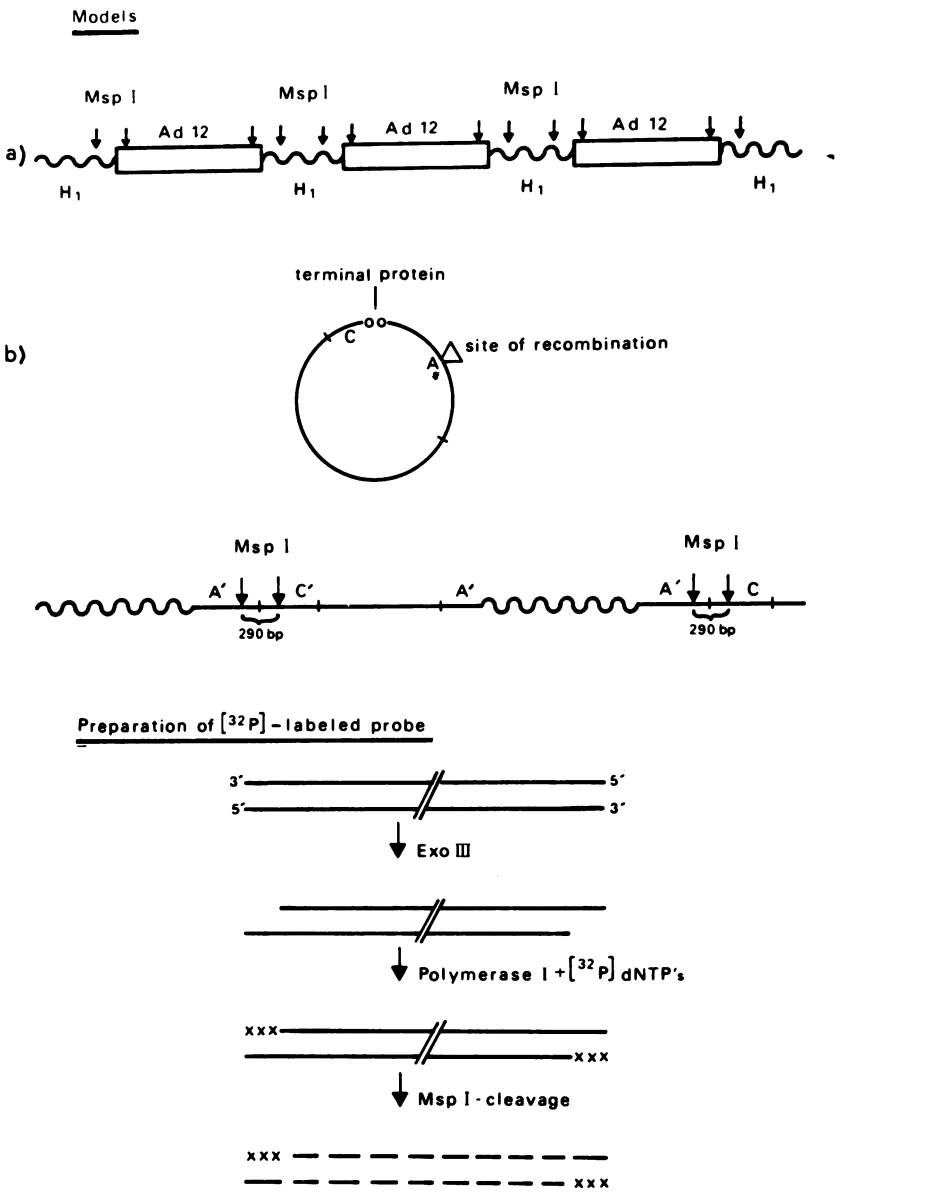

FIG. 10. *Design of an experiment to determine the mode of integration of Ad12 DNA in Ad12-transformed hamster cells. Details of this experiment were described in the text. Arrows indicate sites of cleavage of the MspI restriction endonuclease (only a few of these sites are indicated). Crosses designate label in [^{32}P]-deoxyribonucleotides incorporated into DNA.*

vealed that cell lines HA12/7, T637, and A2497-3 contained far more copies of the terminal *Msp*I fragments of Ad12 DNA than copies of Ad12 DNA. Cell lines CLAC1 and CLAC3 apparently did not carry more copies of the terminal *Msp*I fragments of Ad12 DNA than of the viral genome (Fig. 11). This apparent discrepancy in copy numbers could best be explained by an amplification of the termini of the integrated Ad12 DNA molecules. Thus, a model of integration of Ad12 DNA in the cell lines investigated emerged in which the termini of integrated Ad12 DNA comprising sequences shorter or slightly longer than the terminal 143 bp and, possibly, adjacent cellular DNA sequences were amplified several times. The exact structure of these repeat sequences is currently under investigation.

The data presented in this section were not consistent with model (b) in Fig. 10, which suggests circularization of the Ad12 genome and

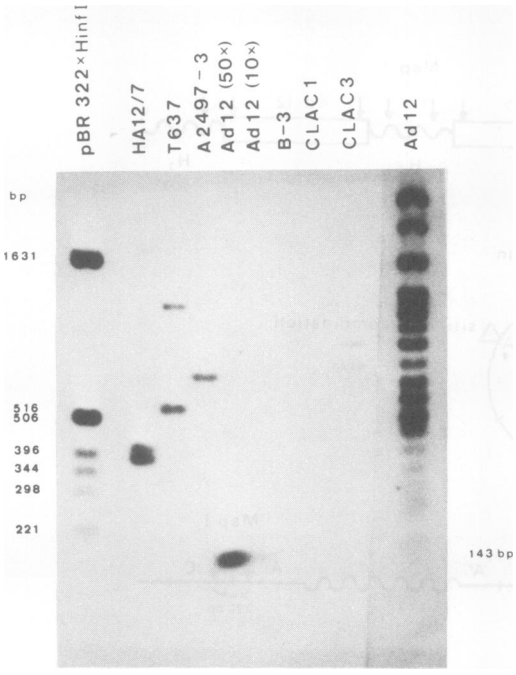

FIG. 11. *Terminal MspI fragment of Ad12 DNA as hybridization probe: amplification of termini of integrated Ad12 DNA molecules in lines HA12/7, T637, and A2497-3. Ad12 DNA (Ad12) and the DNAs of cell lines as indicated (10 μg/slot) were cleaved with the MspI restriction endonuclease; the DNA of the bacterial plasmid pBR322 was cleaved with the HinfI restriction endonuclease (pBR322 × HinfI). The fragments were separated by electrophoresis on horizontal 2.0% agarose slab gels and, after denaturation, were transferred to nitrocellulose filters. The leftmost track of the filter was cut off and incubated with ^{32}P-labeled pBR322 DNA; similarly the right-most track was incubated with ^{32}P-labeled Ad12 DNA. The DNA fragments in the central major portion of the filter were hybridized to the ^{32}P-labeled terminal MspI fragment of Ad12 DNA (see Fig. 10 and Materials and Methods). After hybridization and washing, the different parts of the filters were very carefully reassembled and autoradiographed. The figures indicate the sizes of the pBR322 marker fragments (30) in base pairs; the size of the terminal MspI fragment of Ad12 DNA is also given. In the tracks designated Ad12 (10×) or Ad12 (50×), 10 or 50 genome equivalents of Ad12 DNA mixed with 10 μg of B3 DNA each were cleaved with the MspI restriction endonuclease and coelectrophoresed as internal controls. The sizes of the Ad12-specific off-size DNA fragments were calculated in comparison with the pBR322 × HinfI fragments.*

subsequent recombination with host DNA in one of the terminal fragments of Ad12 DNA (see also Fig. 12d). The results also tended to rule against the model of true tandem integration of Ad12 DNA and the formation of head-to-tail, head-to-head, or tail-to-tail concatenates (Fig. 12c). In the DNA of line T637, one very weak band 228 bp in length was found with homology to the terminal *Msp*I fragment of Ad12 DNA. It is conceivable that this band was derived from two directly adjacent Ad12 DNA molecules in which some of the 290 bp of the terminal concatenate had been deleted. This structure, however, was exceptional. In line T637, the majority of the 20 to 22 persisting copies of Ad12 DNA were serially arranged with interspersed cellular and/or rearranged viral DNA sequences. The termini of the integrated Ad12 DNA were amplified several times.

DISCUSSION

General properties of integration patterns. We have investigated integration patterns both of Ad12-transformed hamster cells and of Ad12-induced hamster tumor cells. The patterns in both types of cells are characterized by the persistence of multiple copies of the entire Ad12 genome. The internal parts and probably the entire lengths of most of these genomes are colinearly integrated; i.e., the restriction maps of Ad12 virion DNA and of integrated Ad12 DNA are identical. The structure of the termini of the integrated Ad12 genomes is not entirely clear yet; in lines HA12/7, T637, and A2497-3, sequences close to the ends of Ad12 DNA are amplified several times (see Fig. 10 and 11). Table 1 lists the approximate numbers of Ad12 genome copies persisting per cell. These numbers range from 3 in the HA12/7 line to 22 in the T637 line. The data presented in Fig. 9 and similar results published elsewhere (8) support the notion that most of the persisting Ad12 DNA molecules represent the entire viral genome. We cannot rule out the possibility that a few nucleotides, e.g., at the termini of Ad12 DNA, might have been deleted during integration. In addition to the entire Ad12 genomes, fragments of Ad12 DNA persist (Fig. 3, 7, and 8). Free viral DNA does not occur in any of the cell lines investigated (Fig. 1, 2a, c, 3, 7, and 8). There are clearly fewer sites of recombination between viral and host DNAs detectable in any of the cell lines than there are Ad12 DNA copies persisting. This finding suggests a number of possible models of integration patterns which will be discussed below in the light of all the results presented here.

It is striking that the modes of persistence of viral genomes are similar in certain respects between in vitro- and in vivo-transformed hamster cells. It is conceivable that transformations in vivo and in vitro occur by similar mechanisms, although the mechanisms of selecting for these cells in culture and in the animals could be quite different. It was shown earlier by two-step filter hybridization experiments (15, 16) that Ad12

DNA and cellular DNA were covalently linked in Ad12-transformed hamster cells. It is not clear in general whether the cellular DNA into which viral DNA is integrated in transformed cells is part of the chromosome proper or of a very large extrachromosomal element. This distinction might be difficult to make.

Quantitation of viral genomes. The quantitation of viral genomes persisting in the five cell lines analyzed is based upon a comparison of the amounts of ^{32}P-labeled Ad12 DNA sequences bound to Ad12 DNA sequences from transformed cells with that bound to known amounts of virion DNA in a reconstitution series on Southern blots (Table 1; Fig. 1a and 2). In this calculation, the assumption is implicit that virion DNA fragments are transferred from agarose gels to nitrocellulose filters with the same efficiency as Ad12 DNA fragments in cellular DNA. This assumption is justified at least for the internally located *Eco*RI fragments B, D, and E of Ad12 virion DNA, which exactly comigrate with the corresponding fragments from integrated Ad12 genomes (see scheme in Fig. 3). Transfers in the gel regions of very high molecular weight fragments are known to be sometimes less complete, as can be shown by staining the slab gels with ethidium bromide before and after transfers. We have checked for the efficiency of transfer by restaining the remaining gels with ethidium bromide after Southern transfers and in general have found very little DNA left in the gels, except sometimes in the high-molecular-weight regions. No DNA was left in size classes equal to or smaller than the *Eco*RI fragment B. The results of this quantitation also suggest that some of the weaker off-size bands, particularly those of lower molecular weights (see Fig. 3, band 4 in line T637, band 6 in line A2497-3, and perhaps band 3 in line CLAC3) might contain only fractions of the terminal *Eco*RI fragments of Ad12 DNA. It is also interesting to note that off-size bands 2 and 4 in the integration pattern of line A2497-3 contained sequences from the internal *Eco*RI fragment D, even when cloned fragment D was used as probe. These observations confirm earlier findings that parts of the viral genomes become fragmented before or during integration (10). It is not understood exactly how these fragments arise.

In an earlier report, we tried to estimate the amount of viral DNA persisting in lines HA12/7, T637, and A2497-3 by using measurements of reassociation kinetics of labeled Ad12 DNA in the absence and presence of transformed cell DNA (10). The numbers obtained are not identical to those presented in Table 1, although they come close considering that completely different methods of quantitation were used. In previous work we may have somewhat overestimated the amount of some of the scattered fragments of viral DNA. In general, the method of quantitation based on a comparison of autoradiographic intensities seems to yield data comparable or perhaps preferable in reliability to those obtained by reassociation kinetic measurements.

Absence of free viral genomes. The cell lines investigated do not carry intact free viral DNA. All viral genomes are integrated into cellular DNA. Using three different restriction endonucleases, *Eco*RI, *Bam*HI, and *Bgl*II, the terminally located fragments of Ad12 DNA were found exclusively in off-size positions, even when autoradiograms were heavily overexposed (see Fig. 1, 3, 7, and 8). The same results were obtained when the terminal *Eco*RI fragments A and C were used as hybridization probes (Fig. 2, 5, and 6). In Ad12-induced rat brain tumor cell lines, free, unintegrated viral genomes have not been found either (19).

Sites of integration. It has not yet been proven that one off-size band represents only one site of recombination between viral and host DNA. It should be remembered, however, that upon cleavage of the DNA from transformed cells with restriction endonuclease *Hin*fI (8), *Pst*I (Fig. 9), or *Msp*I (Fig. 11), each of which cuts Ad12 DNA many times, only a few Ad12-specific off-size bands are generated. The analyses of Ad12-specific off-size bands obtained by cleavage with the *Msp*I restriction endonuclease by using a terminal 143-bp probe of Ad12 DNA reveal that there appear to be multiple copies of both terminal fragments of Ad12 DNA at some of these sites. This finding is also consistent with the observation that some off-size fragments generated from HA12/7, T637, or A2497-3 DNA exhibit double homology to both termini of Ad12 DNA (Fig. 3, 7, and 8). In the CLAC lines this apparent linkage of terminal fragments of Ad12 DNA in off-size bands is only rarely found (Fig. 3 and 7) or not found at all (Fig. 8). The data also imply that multiple viral DNA molecules have probably become linked to cellular DNA at the same sites of repetitive sequences, since only a few off-size bands were detected. Alternatively, a single copy of Ad12 DNA has been amplified together with the adjacent cellular sequences during or after the integration event. It is impossible to distinguish between these two possibilities, although the latter mechanism appears to be more likely. The process of amplification may have involved the termini of the integrated Ad12 DNA molecules which appear to have been amplified disproportionately (see below).

Off-size band 2 in T637 DNA has equal homologies to the *Eco*RI fragments A and C. Judging from the estimated size of this band (18

kilobase pairs [kbp]), it could represent a head-to-tail concatenate consisting of the terminal A and C fragments (17.2 kbp). If off-size band 2 in T637 DNA does indeed represent concatenates of the terminal fragments of Ad12 DNA, none of the remaining off-size bands with homologies to both A and C fragments from any of the cell lines would fit in size to a simple concatenate with the terminal Ad12 DNA fragments abutting each other. Off-size bands of higher molecular weights, e.g., band 1 in the DNA from HA12/7, also contain DNA which is complementary to *Eco*RI fragments A and C. If these sequences were in the same molecule, they would have to be separated by (repetitive) cellular DNA or by amplified viral DNA termini lacking *Eco*RI sites. However, when the DNA from line T637 was cut with the *Bgl*II restriction endonuclease, off-size bands 3 and 4 (Fig. 8) with strong homologies to both termini of Ad12 DNA appeared in molecular weight regions between 8 and 10 kbp, i.e., a size class region which is incompatible with the sizes of head-to-tail concatenates of Ad12 DNA consisting of *Bgl*II fragments B plus C with a size of 14 kbp. There is only one weak band in T637 DNA that is homologous to both terminal *Bgl*II fragments B and C and comes close in size to a head-to-tail concatenate. In line T637, the bulk of the integrated viral DNA is not organized in concatenates, and two viral genomes at most might abut each other directly. In none of the other cell lines investigated were fragments found which constituted true concatenates of Ad12 DNA. We conclude that true concatenates of Ad12 DNA do not occur in the hamster lines investigated. There is one minor band in the T637 pattern which might be an exception.

Models. The fact that multiple copies of Ad12 DNA persist in the cell lines investigated (Table 1) precludes simple models describing the patterns of integration. The schemes presented in Fig. 12 summarize four of the models discussed in the following. In model (a), intact Ad12 genomes are colinearly integrated into repetitive host DNA sequences (H_1). Alternatively, a single copy of Ad12 DNA and the flanking host sequences have been amplified during or after integration. This model would be consistent with the data obtained for lines HA12/7, T637, and A2497-3, at least for most of the viral DNA molecules persisting. It is also possible that some of the DNA sequences between the intact viral genomes represent amplified termini of Ad12 DNA.

For other molecules and for viral DNA copies integrated in lines CLAC1 and CLAC3, a simpler model (b) might apply. Again viral DNA is colinearly integrated into host DNA sequences (H_1, H_2), perhaps of the repetitive type, which contain sites for the restriction endonucleases *Eco*RI, *Bam*HI and *Bgl*II. Thus, fragments are produced which contain one of the terminal viral DNA fragments and the flanking host sequences. The host-virus DNA units depicted could be arranged serially or independently in the genome. The former possibility is the more likely one, since the repetition might be the result of amplification events. Except for maximally two copies of viral DNA in line T637, the

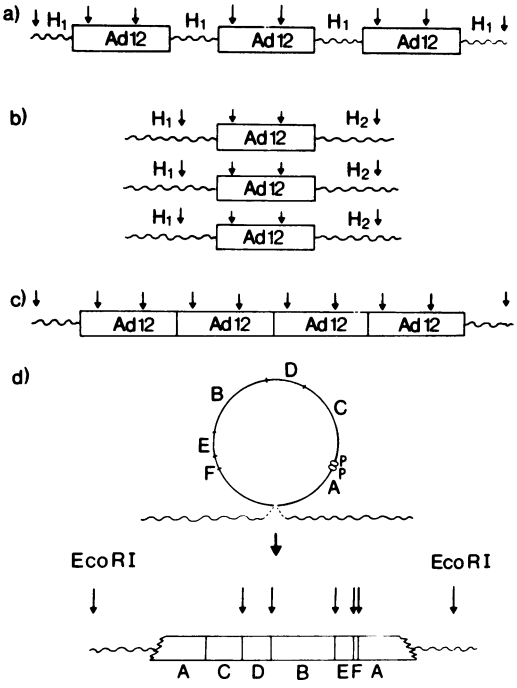

FIG. 12. *Possible models for the integration of Ad12 genomes in the DNA of transformed hamster cells. Details are explained in the text. A and C refer to the terminal EcoRI fragments A and C of Ad12 DNA. H_1, H_2, and H represent specific hamster sequences of the repetitive type. H_1 could also contain rearranged viral DNA sequences. Arrows indicate cleavage sites of the EcoRI restriction endonuclease. (a) Integration into repetitive DNA, or postintegrational amplification. The cellular sequences separate viral genomes in a regular spacer array. (b) Integration into repetitive hamster DNA. (c) True tandems of Ad12 DNA integrated into cellular DNA. (d) This model suggests that Ad12 DNA is integrated via a circular intermediate. The circular molecule of Ad12 DNA is stabilized by the terminal adenovirus protein (P). Recombination with the host DNA (∿∿) is assumed to occur inside the right terminal EcoRI fragment A of Ad12 DNA. This model was not confirmed by the results of the experiment described in Fig. 10 and 11.*

model of a true tandem arrangement of Ad12 genomes directly abutting each other (Fig. 12c) is incompatible with the experimental evidence (see "Sites of integration" above), since simple concatenates consisting exclusively of the viral termini have not been found (see Fig. 8, 10, and 11).

The finding of high-molecular-weight off-size bands in lines HA12/7, T637, and A2497-3 with homology to both termini of Ad12 DNA is also consistent with model (d) (Fig. 12d), which suggests that Ad12 DNA is circularized through the action of the terminal protein (3, 25). The circular intermediate could also be a covalently closed molecule. The circular molecule could then recombine inside the right terminal *Eco*RI fragment A with host DNA. Thus, one off-size fragment would arise that contained host DNA and a portion of the right and the entire left terminal *Eco*RI fragment of Ad12 DNA; another off-size fragment would contain the remainder of fragment A and host DNA (Fig. 12d).

In previous studies (7), evidence for the occurrence of covalently closed circles of Ad12 DNA in hamster cells could not be adduced. Preliminary recent results obtained with highly sensitive hybridization techniques also have not provided any evidence for the existence of such structures (I. Kuhlmann and W. Doerfler, unpublished experiments). The results of the experiments described in Fig. 10 are not consistent with model (d) in Fig. 12. We therefore favor model (a) and/or (b) to describe the state of integrated Ad12 genomes in the cell lines investigated. Further refinement of these proposed models will have to await the results of an analysis of the adjacent cellular DNA sequences.

Amplification of terminal sequences of integrated Ad12 genomes. The finding that off-size fragments generated by the restriction endonucleases *Eco*RI, *Bam*HI, and *Bgl*II with the DNA from lines HA12/7, T637, and A2497-3 exhibited homologies to both termini of Ad12 virion DNA suggested a particular arrangement of viral DNA sequences at the sites of junction with cellular DNA. This double homology can be explained by postulating that the inverted terminal repeats occurring in Ad12 virion DNA (36) have been amplified several times at the sites where colinearly inserted Ad12 DNA is linked to cellular DNA. These segments comprising repeats of the terminal nucleotide sequences would be hybridizing with DNA probes from either terminus of Ad12 DNA. The results of the experiment outlined in Fig. 10 and 11 lend further support to this notion. We do not know at present the extent and the exact structure of these repeat units. Attempts to clone the terminal Ad12 DNA fragments and the adjacent cellular DNA sequences in a number of different vectors have proved difficult so far, whereas internal parts of the integrated Ad12 genomes could be readily recloned from the DNA of lines T637, HA12/7, and CLAC3.

The results of the experiment described in Fig. 10 and 11 are incompatible with models (c) and (d) in Fig. 12. We therefore favor the model of colinear integration of Ad12 DNA molecules, perhaps in serial arrangements in the cell lines investigated (Fig. 12a,b).

Morphological revertants of line T637. We have previously described the isolation and characterization of morphological revertants of line T637 (17, 18). The analysis of the patterns of integration of Ad12 DNA in these revertants has helped in the elucidation of the arrangement of viral DNA in line T637. These data are presented and discussed in the accompanying paper (9).

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