Revertants of Adenovirus Type 12-Transformed Hamster Cell Line T637 as Tools in the Analysis of Integration Patterns

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Spontaneously arising morphological revertants of the adenovirus type 12 (Ad12)-transformed hamster cell line T637 had been previously isolated, and it had been demonstrated that in these revertants varying amounts of the integrated Ad12 genome were eliminated from the host genome. In this report, the patterns of persistence of the viral genome in the revertants were analyzed in detail. In some of the revertant cell lines, F10, TR3, and TR7, all copies of Ad12 DNA integrated in line T637 were lost. In lines TR1, -2, -4 to -6, -8 to -10, and -13 to -16, only the right-hand portion of one Ad12 genome was preserved; it consisted of the intact right segment of Ad12 DNA and was integrated at the same site as in line T637. In revertant lines G12, TR11, and TR12, one Ad12 DNA and varying parts of a second viral DNA molecule persisted in the host genome. These patterns of persistence of Ad12 DNA molecules in different revertants supported a model for a mode of integration of Ad12 DNA in T637 hamster cells in which multiple (20 to 22) copies of the entire Ad12 DNA were serially arranged, separated from each other by stretches of cellular DNA. The occurrence of such revertants demonstrated that foreign DNA sequences could not only be acquired but could also be lost from eucaryotic genomes. There was very little, if any, expression of Ad12-specific DNA sequences in the revertant lines TR7 and TR12. Moreover, Ad12 DNA sequences which were found to be undermethylated in line T637 were completely methylated in the revertant cell lines G12, TR11, TR12, and TR2. These findings were consistent with the absence of T antigen from the revertant lines reported earlier. Hence it was conceivable that the expression of integrated viral DNA sequences was somehow dependent on their positions in the cellular genome. In cell line TR637, the early segments of Ad12 DNA were expressed and undermethylated; conversely, in the revertant lines G12, TR11, TR12, and TR2, the same segments appeared to be expressed to a limited extent and were strongly methylated.

Mammalian cells have a mechanism that enables them to incorporate foreign DNA into their genomes. The uptake and integration of viral DNA have so far been studied most thoroughly, in particular in virus-transformed cells (for review, see reference 3). The integration of specific genes and of bacteriophage DNA into the genomes of mammalian cells has also been reported (9, 19). On the other hand, different clones of morphological revertants of adenovirus type 12 (Ad12)-transformed hamster cells have been shown to have lost a portion or all of the integrated viral DNA sequences (7, 8). Similarly, revertants of simian virus 40-transformed (14) and polyoma-transformed cells (2) have also been described. Thus, foreign DNA sequences can also be eliminated from the genomes of mammalian cells. The question arises whether in the process of excision of foreign (viral) DNA, cellular sequences adjacent to viral sequences are removed at the same time. Since these cel-

lular DNA sequences are possibly of the repetitive type (5, 13), their loss may not affect cell survival.

The T637 line of Ad12-transformed hamster cells has been studied extensively in our laboratory (5, 10, 13, 16). Approximately 20 to 22 copies of Ad12 DNA per cell are integrated into the genome of these cells at two or three different sites. Most, perhaps all, of the Ad12 genomes are separated by cellular DNA sequences. A true tandem arrangement of Ad12 DNA has not been observed (5, 13).

The four early regions of Ad12 DNA are expressed as mRNA in T637 cells (10). T637 cells synthesize the Ad12-specific T antigens. Spontaneously arising morphological revertants of T637 cells have been isolated and characterized (7, 8). The revertants exhibit a morphology strikingly different from that of T637 cells and are T-antigen negative according to the immunofluorescence test (7). Among 18 revertant clones

analyzed, 3 seem to have lost most, if not all, Ad12 DNA sequences. Fourteen clones have lost considerable amounts of viral DNA. Several of the 18 clones have not been independent isolates. The revertants are not useful in the analysis of Ad12 genes essential for the maintenance of the transformed state, because all revertant clones are about as oncogenic as the T637 line or the BHK-21 parent line from which the T637 cells originated (7).

In the present report, we have used the revertants as tools in the analysis of integration patterns of Ad12 DNA in the T637 line. According to the patterns of persisting Ad12 DNA sequences, the 18 clones fall into five classes. We have analyzed in detail which parts of the viral genome have been eliminated from each of the revertant lines. The data obtained further support the model presented in the accompanying paper (13) for the integration of Ad12 DNA in T637 cells, i.e., multiple viral genomes being integrated possibly into repetitive DNA. Intact viral DNA molecules are separated by stretches of cellular DNA, and multiple cell-virus DNA combinations are serially arranged. There is little, if any, expression of the remaining Ad12 genomes in the revertant lines, and the pattern of methylation has become strikingly altered in the revertants in comparison with the T637 line.

MATERIALS AND METHODS

Cells and viruses. The origin and the methods of propagation of the T637 cells (6) and of the morphological revertants (7) were previously described. Among the 18 revertant clones, G12 and F10 were obtained first and independently from lines TR1 through TR16. The clones of the series TR1 through TR16 were isolated from a single culture of T637 cells which had been derived from a single independently reisolated clone of T637 cells. It was demonstrated that many of the TR clones (TR1, -2, -4, -5, -6, -8, -9, -10, -13, -14, -15, and -16) exhibited identical integration patterns of Ad12 DNA and, therefore, were likely to represent reisolates of the same original revertant clone.

Ad12 was propagated on human KB cells growing in suspension culture or monolayer culture. The methods of Ad12 propagation, virus purification, and isolation of Ad12 DNA were described elsewhere (10).

Isolation of the *EcoRI* fragments of Ad12 DNA.

Isolation procedures are outlined in detail elsewhere (5, 13). The same methods were used to purify the *Bam*HI or *Hind*III fragments of Ad12 DNA. In later experiments, *EcoRI* fragments of Ad12 DNA were used which had been cloned in the bacterial plasmid pBR325 (S. Vogel, M. Brötz, U. Winterhoff, and W. Doerfler, manuscript in preparation).

Other methods. The methods used in the isolation of cellular DNA (16), in nick translation of Ad12 DNA or viral DNA fragments (11), and in the analysis of viral DNA sequences in cellular DNA by blotting (12), by DNA-DNA hybridization (18), and autoradiography are all described in detail elsewhere (5, 13, 16).

Methylation of integrated Ad12 DNA sequences. The methods of cleavage of DNA from T637 cells and from the revertants with the *Msp*I and *Hpa*II restriction endonucleases are described elsewhere (15). Intact, 32 P-labeled Ad12 DNA was used as hybridization probe.

RESULTS

Survey of integration patterns of Ad12 DNA in all revertant lines and in T637 cells.

The DNAs from all revertant lines (F10, G12, and TR1 through TR16) and from the parent line, T637, were isolated and analyzed by Southern blotting upon cleavage with the *EcoRI* restriction endonuclease. The DNA on the blots was subsequently hybridized with Ad12 DNA which was 32 P labeled by nick translation. The data presented in Fig. 1a demonstrate five distinct patterns of integration in revertant lines F10, G12, TR12, TR11, and TR2. Moreover, it was established that the revertant lines TR1, -2, -4, -5, -6, -8, -9, -10, -13, -14, -15, and -16 exhibited identical patterns (Fig. 1). Thus, these latter isolates probably represented reisolates of the same revertant clone. As previously reported (7), in revertant lines F10, TR3, and TR7, viral DNA sequences could not be detected (Fig. 1a,b), even though a much more sensitive method for DNA-DNA hybridization (18) was applied in this study than in earlier studies (7, 8). In Fig. 1a, the five principal integration patterns of Ad12 DNA in revertant lines F10, G12, TR12, TR11, and TR2 are presented. In the DNA from line F10, viral DNA sequences were not apparent. In the DNAs from lines G12, TR11, and TR12, the

FIG. 1. Analysis of the DNA from 18 morphological revertants and from line T637 for the patterns of integration of Ad12 DNA. DNAs from revertant cell lines or from Ad12 as indicated were cleaved with the restriction endonuclease *EcoRI*, and the fragments were separated by electrophoresis in horizontal 0.5% agarose slab gels. Each gel slot contained 10 μ g of DNA. The DNA fragments were then transferred to nitrocellulose filters by the Southern technique and hybridized with Ad12 DNA which was 32 P labeled by nick translation. Ad12-specific DNA fragments were visualized by autoradiography. In the gel slots designated 5 $\times$ Ad12, five genome equivalents of Ad12 DNA mixed with 10 μ g of B3 DNA used as substrate were electrophoresed after *EcoRI* digestion. The calculation of genome equivalents was based on a molecular weight of 20×10^6 for Ad12 DNA (4) and a DNA content of 1.3×10^{-5} μ g per T637 cell (6). All methods used are outlined in detail elsewhere (5, 13, 16). In (a), the *EcoRI* map of Ad12 DNA and the sizes of the *EcoRI* fragments A to E are given in kilobase pairs (kbp).

internal *EcoRI* fragments B, D, and E of Ad12 DNA comigrated with the corresponding fragments in the virion marker DNA. The intensities of these bands in the lines mentioned were, however, considerably reduced in comparison with the T637 parent line. The DNA from all three revertant lines also contained off-size band 3 of T637 DNA (13). In addition, the DNA from line TR12 carried a weak off-size fragment (band 2), and the DNA from line TR11 contained an Ad12-specific fragment intermediate in size between the *EcoRI* fragments A and B of Ad12 DNA (Fig. 1a). The DNAs from line TR2 and all its subclones contained off-size band 3, a band comigrating with the *EcoRI* fragment E, and a band of slightly higher molecular weight than the *EcoRI* fragment C of virion marker DNA. The identities of most of these bands were determined by using specific viral DNA fragments as hybridization probes.

The amounts of Ad12 DNA persisting in the revertant lines were quantitated by photometrically comparing intensities of Ad12-specific bands in the autoradiograms of Fig. 1. This method is described in the accompanying report (13). The results of this quantitation indicated that the DNA from the revertant lines TR1, -2, -4 to -6, -8 to -10, and -13 to -16 contained about one copy of a fragment of Ad12 DNA per cell (see below). The DNAs from the revertant lines G12, TR12, TR11, and TR2 contained considerably less than five copies of Ad12 DNA and more than one copy, probably about two copies, at least of some parts of the Ad12 genome.

Analysis of integration patterns in revertants, using specific Ad12 DNA fragments as hybridization probes. We repeated the Southern blotting experiments with the DNAs from line T637 and from the revertants F10, G12, TR12, TR11, and TR2, using ³²P-labeled *EcoRI* fragment A, B, C, or D as hybridization probe. In some experiments, the ³²P-labeled *Bam*HI fragments or some of the cloned *EcoRI* or *Bam*HI fragments of Ad12 DNA were used as probes also. The data presented in Fig. 2 can be summarized as follows.

(i) Off-size band 3 (13; Fig. 3) in revertant lines G12, TR12, TR11, and TR2 contained sequences that were homologous to *EcoRI* fragment A of Ad12 DNA. Homology to fragment A was also found in the DNA from line G12 in a band comigrating with *EcoRI* fragment B, and in DNA from line TR11 in the Ad12-specific band electrophoresing in a position between *EcoRI* fragments A and B (Fig. 2a). In the DNA from line TR12, off-size band 2 hybridized to *EcoRI* fragments A and C.

(ii) In the DNA from lines G12, TR12, and

TR11, the Ad12-specific band comigrating with the authentic *EcoRI* fragment B represented in fact fragment B. In the DNA from line TR2, the Ad12-specific band electrophoresing in a position slightly above *EcoRI* fragment C represented part of the *EcoRI* fragment B of Ad12 DNA (Fig. 2b). This fragment showed weak homology to fragment A also, suggesting that the truncated B fragment might still be linked to the same cellular DNA site via a short fragment from the original left terminus of Ad12 DNA (Fig. 2a). The weak homology to *EcoRI* fragment B in off-size band 3 (Fig. 2b and 3) might have been due to a contaminant in the probe.

(iii) *EcoRI* fragment C was missing altogether from the DNA of line TR2. Homology to *EcoRI* fragment C of Ad12 DNA was found in the DNA from line TR12 in off-size band 2 and in the DNA from line TR11 in the position between *EcoRI* fragments A and B (Fig. 2c). In the DNA from line G12, a band comigrating with *EcoRI* fragment B also displayed homology to *EcoRI* fragment C. The weak apparent homology of off-size band 3 in the revertant cell lines to *EcoRI* fragment C could possibly be explained by hybridization of the C fragment due to the inverted terminal repeat.

(iv) The DNA from lines G12, TR12, and TR11 contained the authentic D fragment. This fragment was absent from the DNA of line TR2 (Fig. 2d).

(v) None of the *EcoRI* fragments was detectable in the DNA of line F10.

All of the data presented are summarized schematically in Fig. 3. All DNA bands with homologies to *EcoRI* fragments A, B, C, and D were identified by hybridization to the isolated *EcoRI* fragments of Ad12 DNA as indicated (Fig. 3). The identities of the *EcoRI* fragments E and F were deduced from the fact that they comigrated with the authentic virion marker DNA fragments.

Right-hand end of Ad12 DNA is preserved intact in revertant cell lines. The data presented in Fig. 4 demonstrate that the right terminal 2.9 to 3 kilobase pairs (kbp) of Ad12 DNA were persisting intact in the T637 cell line and in the revertant cell line TR2. The right terminal *Hind*III fragment E of Ad12 DNA (2.9 to 3 kbp) was ³²P labeled by nick translation and was used in hybridization experiments with the DNAs from cell line T637 and from the revertant line TR2. The DNAs of these cell lines were cleaved with the restriction endonuclease *Mbo*I or *Pst*I; the fragments were electrophoresed, blotted onto nitrocellulose filters, and hybridized to the probe (Fig. 4). The 1.97- and 0.29-

FIG. 2. Analysis of integration patterns of five revertant lines and of T637 cells, using *EcoRI* fragment A, B, C, or D of Ad12 DNA as the hybridization probe. Experimental conditions were similar to those described in the legend to Fig. 1. The DNAs of revertant lines F10, G12, TR12, TR11, and TR2 were analyzed, using *EcoRI* fragment A (a), B (b), C (c), or D (d) as hybridization probe. These fragments were ^{32}P labeled by nick translation.

kbp (not visible) *MboI* fragments as well as the 1.52- and 0.83-kbp *PstI* fragments comigrated with the corresponding Ad12 virion marker DNA fragments, whereas the terminal 1.16-kbp

MboI and the terminal 1.15-kbp *PstI* fragments were displaced to off-size positions due to the linkage of viral to cellular DNA (Fig. 4). We conclude that the right terminal 2.9- to 3-kbp

FIG. 3. Schematic representation of Ad12-specific DNA bands in the DNAs of line T637 and of 18 morphological revertants. The identities of all bands were indicated by the letters A through F, which refer to the *EcoRI* fragments of Ad12 DNA. The *EcoRI* map of Ad12 DNA is also depicted.

segment of Ad12 DNA was preserved intact in the DNAs of the T637 line and of the revertant cell line TR2. There was a very limited number of off-size bands in line T637 and only one off-size band in line TR2. The latter off-size band was identical to one off-size band in line T637, suggesting that the sites of integration in line T637 and in the revertant were the same.

Expression and methylation of the early regions in the revertant cell lines. It has been previously reported that the revertant cells do not express the Ad12-specific T antigen as determined by immunofluorescence (7, 8). Preliminary data (S. Schirm and W. Doerfler, manuscript in preparation) indicated that in the revertant lines TR7 and TR12, very little, if any, Ad12-specific RNA could be detected by hybridization of ^{32}P -labeled Ad12 DNA or of the left terminal *Bam*HI fragment A (see Fig. 1b in reference 13) to total cytoplasmic RNA extracted from revertant cell lines TR7 and TR12 and covalently linked to diazobenzoyloxymethyl paper by the method of Alwine et al. (1). In T637 cells, on the other hand, Ad12-specific sequences were readily found by the same methods. It was concluded that Ad12 DNA did not appear to be expressed extensively as mRNA in revertant lines TR7 and TR12. For line TR7, this finding was expected since Ad12 DNA sequences did not persist in this line (Fig. 1b).

We have reported earlier that in cell line T637 the early regions of Ad12 DNA corresponding to

EcoRI fragments A and C are undermethylated, whereas the late regions in *EcoRI* fragment B are strongly methylated (15). Similar results have been obtained with Ad2 DNA sequences in Ad2-transformed hamster cells (17). It was, therefore, of interest to investigate the extent of methylation of the Ad12 DNA sequences in the revertant cell lines G12, TR12, TR11, and TR2. The DNAs of cell lines T637, G12, TR12, TR11, and TR2 were cleaved with the restriction endonuclease *Msp*I or *Hpa*II, and the fragments were electrophoresed and transferred to nitrocellulose filters as described (15, 17). The DNA on the filters was then hybridized with ^{32}P -labeled Ad12 DNA. The results of these experiments (Fig. 5) demonstrate that the Ad12 DNA sequences were almost completely methylated in the revertants and undermethylated in the T637 line as previously found (15). The restriction endonuclease *Msp*I cleaved at all of the CCGG sites in Ad12 DNA in the revertants, whereas the enzyme *Hpa*II did not (Fig. 5). Thus, the Ad12 DNA sequences persisting in the revertants might have come under completely different control as a consequence of their repositioning in the reversion process. It was also conceivable, though unlikely, that unmethylated sequences of Ad12 DNA might have been lost preferentially.

DISCUSSION

The analysis of the patterns of integration of

FIG. 4. Presence of the right terminal segment of Ad12 DNA in cell lines T637 and TR2. The DNAs from Ad12 or from cell lines T637 and TR2 were cleaved with restriction endonuclease *Mbo*I or *Pst*I as indicated. The fragments were separated by electrophoresis on 2.0% agarose slab gels and transferred to nitrocellulose filters by the Southern procedure. The DNA was then hybridized to the 32 P-labeled HindIII fragment E of Ad12 DNA which contained the right terminal 2.9 to 3 kbp of Ad12 DNA. The numbers on the left margin of each autoradiogram designate the sizes in kilobase pairs of individual DNA fragments. The maps on the bottoms of each graph indicate the relative positions of the right terminal *Mbo*I and *Pst*I fragments and their sizes in kilobase pairs. Maps were taken from reference 5.

Ad12 DNA in morphological revertants of T637 cells has revealed five different modes of persisting Ad12 genomes (Fig. 3 and 6). In lines F10, TR3, and TR7, apparently all viral DNA sequences have been lost. In line TR2 and its identical subclones TR1, -4 to -6, -8 to -10, and -13 to -16, all viral genomes have been lost except a portion of one viral DNA molecule. This interpretation is consistent with and based upon model (a) in the accompanying paper (13), i.e., a serial arrangement of multiple copies of the entire Ad12 genome integrated into cellular DNA separated from one another by repetitive cellular DNA sequences. An episomal location of the integrated Ad12 DNA copies on a very

FIG. 5. Extent of methylation of Ad12 DNA in line T637 and in revertant lines G12, TR12, TR11, and TR2. The DNAs of Ad12 or of the cell lines as indicated were cleaved with restriction endonuclease *Msp*I or *Hpa*II. Fragments were separated by electrophoresis on 1.3% agarose slab gels. All other conditions were described elsewhere (5, 13, 15, 16); 32 P-labeled, intact Ad12 DNA was used as hybridization probe.

large extrachromosomal element would also explain the occurrence of revertants. At present, there is no evidence for such episomal structures. In the process of deletion of viral and cellular DNA, the entire block of DNA comprising all the viral and cellular DNA sequences is excised, leaving only one viral DNA equivalent which has been truncated in the process inside *Eco*RI fragment B (Fig. 3 and 6). This model is in agreement with the persistence of off-size band 3 homologous to *Eco*RI fragment A, of fragments E and F, and of a truncated B fragment (Fig. 3). It has also been demonstrated that the right terminal segment of Ad12 DNA comprising 2.9 to 3 kbp persists intact in cell lines T637 and

FIG. 6. Proposed models for the integration of Ad12 DNA in four revertant lines. The letters A through F refer to the *EcoRI* fragments of Ad12 DNA. The shaded areas represent cellular DNA sequences; the vertical arrows indicate the known viral or the unmapped cellular *EcoRI* sites. TR2, G12, TR11, and TR12 refer to individual revertant lines (see text).

TR2 (Fig. 4). The sites of linkage of Ad12 to cellular DNA in lines T637 and TR2 seem to be identical.

In lines G12, TR11, and TR12, one entire Ad12 genome has been conserved in the process of excision. In addition, the adjacent viral genome, which is separated by cellular sequences, has been truncated at different sites inside the right terminal *EcoRI* fragment A. Truncation might also have occurred inside the cellular DNA sequences separating the two persisting viral DNA molecules. The schemes presented in Fig. 6 (G12, TR11, and TR12) indicate the hypothetical sites of truncation within the *EcoRI* fragment A of the second persisting Ad12 DNA molecule. The nucleotide block consisting of a truncated fragment A, of cellular sequences, and of the left terminal fragment C of the viral DNA molecule persisting intact varies in length in each of the three lines, depending on where the break inside fragment A has occurred (Fig. 3 and 6). In the hypothetical schemes presented in Fig. 6, the exact locations of the *EcoRI* sites in the cellular DNA sequences and the lengths of the cellular DNA separating viral DNA sequences are not known.

The patterns of persistence of the viral DNA sequences remaining in the genomes of the revertants analyzed support the general model (a) proposed for the mode of integration of Ad12 DNA in T637 cells (13). The mechanism of excision of DNA sequences from transformed cells is not known. If the models elaborated here are correct, viral and cellular sequences have been eliminated jointly. The fact that as many as about 20 viral genomes can be excised jointly strengthens the notion that many Ad12 DNA

copies are integrated into cellular sequences en bloc as depicted in model (a) in the accompanying report (13). We have not yet determined how and at what site(s) the remaining truncated viral genomes are reconnected to cellular DNA upon completion of the excision event. It cannot be ruled out at the present time that the remaining viral sequences in revertants have been completely excised and reintegrated at a different site. However, we consider this possibility unlikely, because the sizes of off-size band 3 in the T637 line and the corresponding band in the revertant lines are apparently identical (Fig. 3). Moreover, the sites of integration at the right and perhaps the left ends of Ad12 DNA in lines T637 and TR2 are the same or very similar (Fig. 4).

It cannot be stated how frequently DNA sequences are excised from virus-transformed cells or from mammalian cells in general. Investigations on this fascinating subject may be facilitated by the use of virus-transformed cells.

It is quite interesting to note that the revertants analyzed do not seem to contain the Ad12-specific T antigen that line T637 synthesizes (7, 8). By DNA-RNA hybridization techniques using a very sensitive method (1), we have detected minute amounts, if any, of Ad12-specific RNA in the cytoplasm of the revertant line TR12. Apparently, the Ad12 genome is not expressed in these revertants. In line T637, on the other hand, all four early regions of Ad12 DNA are expressed as mRNA (10), and these transcripts can readily be detected by the methods used in the present study. We have also shown that the extent of DNA methylation of Ad12 DNA sequences in all of the revertant cell lines has been

markedly enhanced in comparison with that in the parent line T637 (Fig. 5). This finding would also imply that the Ad12 genome copies persisting in the revertants have become genetically inactivated, perhaps as a consequence of their repositioning in the cellular genome. We have reported previously (15, 17) on an inverse correlation between the levels of DNA methylation and the extents of expression of the same segments of viral genomes in adenovirus-transformed cells. It is rather less likely that some of the Ad12 sequences in line T637 had been undermethylated to begin with and that these Ad12 molecules had been lost preferentially.

The findings presented on the altered levels of expression and methylation of Ad12 genes in revertants suggest that expression of viral genes in transformed cells may be somehow dependent on their position in the cellular genome.

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ADDENDUM IN PROOF

(i) The results of recent experiments have shown that in the revertant lines G12 and TR11 sequences at the right terminal 2,900 to 3,000 base pairs of the persisting Ad12 DNA have been rearranged and thus are no longer colinear with the virion DNA sequences, as has been found for the parent line T637.

(ii) In the cytoplasmic RNAs of the revertant lines, very few, if any, sequences have been detectable that hybridized with Ad12 DNA.

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