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The Structure of Adenovirus Type 12 DNA Integration Sites in the Hamster Cell Genome

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Foreign DNA can integrate into the genomes of mammalian cells, and this process plays major roles in viral oncogenesis and in the generation of transgenic organisms and will be important in evolving regimens for human somatic gene therapy. In the present study, the insertion sites of adenovirus type 12 (Ad12) DNA genomes have been analyzed in detail in the Ad12-transformed hamster cell line T637, its revertants, which have lost most of the >20 Ad12 genome equivalents integrated chromosomally in cell line T637, and in the Ad12-induced tumor T191. Some of these junction sites have been molecularly cloned, and the nucleotide sequences at the sites of transition between viral and cellular DNAs have been determined. The sites of linkage between the hamster cellular and the foreign (viral) DNA are characterized by the frequent occurrence of patch homologies between the recombination partners. The cellular junction sites investigated here are not transcriptionally active. One of the cellular DNA sequences abutting the right Ad12 DNA terminus in cell line T637 (os2) is represented only once in the hamster genome and has a strikingly low abundance of 5′-CG-3′ dinucleotide sequences. One 5′-GCGC-3′ sequence close to the Ad12 DNA integration site is heavily methylated in normal cells, Ad12-transformed cells, and Ad12-induced tumor cells. The second such sequence is more remote from the junction site, is partly methylated in BHK21 hamster cells, and shows differences in methylation in different Ad12-transformed cell lines. This site is unmethylated in liver DNA. The cellular DNA sequence at the site of Ad12 linkage in the tumor T191 exhibits homologies to highly repetitive sequences of the *Alu* family and to an origin of hamster DNA replication containing an *Alu* element. A number of junction sites between Ad12 DNA and hamster or mouse DNA in Ad12-transformed cell lines or Ad12-induced tumor cell lines, investigated here and previously, are characterized by stem-loop structures encompassing the junction sites.

We have been interested in the insertion of foreign DNA into established mammalian genomes and have examined this complex problem by looking at structural details at sites of viral DNA integration in adenovirus type 12 (Ad12)-transformed and Ad12-induced hamster tumor cells (for recent reviews, see references 6 and 7). In addition, we have developed a cell-free system from extracts of hamster cell nuclei for the study of the biochemical mechanisms of foreign DNA-host DNA integrative recombination (11, 20, 49, 50). Integrative recombination of Ad12 DNA proceeds via a mechanism akin to nonhomologous recombination in which patchy homologies between the recombination partners are preferentially exploited (8, 12, 13, 21, 49, 50). Frequently, the cellular sequences at or near the integration sites have been found to exhibit transcriptional activity (14, 40). In one instance, the patterns of cellular DNA methylation in the sequences immediately abutting the integrated Ad12 DNA have been found to be profoundly altered (29). In transformed or hamster tumor cell lines carrying integrated Ad12 genomes at different locations, patterns of DNA methylation in several cellular genes or DNA segments located far remote from the sites of foreign DNA insertion are strikingly altered in comparison to their methylation status in the hamster cell line BHK21 or in primary hamster cells. Similar increases have been observed in several cloned BHK21 cell lines with bacteriophage λ DNA integrated in different loci of the cellular genome (17). It will be interesting to investigate whether and how such changes in DNA methylation of cellular DNA segments can contribute to the process of oncogenic transformation.

Here we describe the nucleotide sequences of several sites of junction between viral and cellular DNA and their possible structures as deduced from computer-aided analyses of these nucleotide sequences. Changes in the patterns of DNA methylation had occurred in one instance but were not the rule in the examples investigated. Application of the fluorescent in situ hybridization (FISH) technique revealed the chromosomal locations of the integrated Ad12 DNA molecules in cell line T637, in the TR12 revertant, in cell line HA12/7, and in the tumor cell line H191.

MATERIALS AND METHODS

Ad12-transformed cells and Ad12-induced tumor cells. Origins and propagation of the Ad12-transformed hamster BHK21 cell line T637 (47), its morphological revertants TR1, TR2, TR3, TR4, TR7, TR11, TR12, TR14, and TR16 (9, 15), cell line HA12/7 (51), the Ad12-induced tumor T191, and the cell line H191 (33, 34) were described elsewhere.

Standard methods in molecular biology. The standard methods used in this study, Southern blot hybridization (22, 42), RNA transfer hybridization (2, 25), determination of nucleotide sequences in DNA by using the chain termination method (37) and an Applied Biosystems A373 DNA sequencer, restriction enzyme analyses of genomic DNA sequences for their status of methylation by using methylation-sensitive enzymes coupled with Southern blot hybridization (48), or the FISH method (30), as applied in our laboratory (17), were described previously.

Molecular cloning of DNA encompassing the Ad12 DNA junction sites and interviral junctions. This procedure was described elsewhere (33). The vector λ GEM-12 (Promega, Madison, Wis.) and the bacterial strain SRB(P_2) (Stratagene, La Jolla, Calif.) were used. The inserts from positive clones were subcloned into the Bluescript vector pBlueIIKS(−) (Stratagene).

Analyses of Ad12 DNA junction sites in the revertant cell lines TR11 and TR12 by using PCR. In a total volume of 50 μl, 1 μg of cellular DNA and 100 ng of each primer were incubated in reaction buffer–10 mM KCl–6 mM $(NH_4)_2SO_4$–20 mM Tris-HCl (pH 8.0)–0.1% Triton X-100–200 μM each deoxynucleoside triphosphate (dNTP)–2 mM $MgCl_2$–1 U of the *Pfu* DNA poly-

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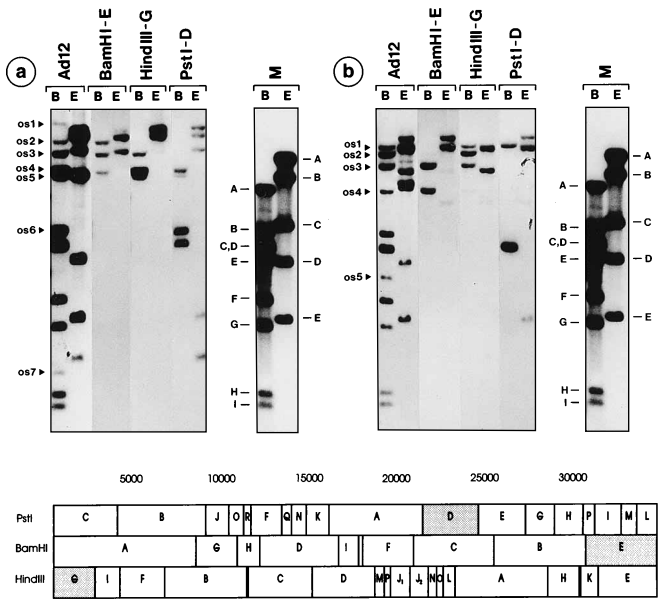

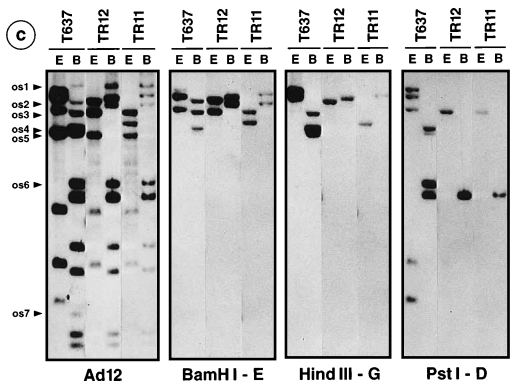

FIG. 1. Patterns of Ad12 DNA integration in the Ad12-transformed hamster cell line T637 (a and c), in the Ad12-induced hamster tumor T191 (b), and in the revertants TR12 and TR11 of cell line T637 (c). From these cell lines, 30 μg of cellular DNA was cleaved with 300 U of *Bam*HI (lanes B) or EcoRI (lanes E) and the fragments were analyzed by standard Southern blot hybridization, using Ad12 DNA or the cloned *Bam*HI E, *Hin*dIII G, or *Pst*I D fragment of Ad12 DNA as hybridization probe as indicated. These fragments are shaded in the Ad12 restriction maps derived from the nucleotide sequence of Ad12 DNA (43) (below panels a and b). Off-size fragments os1 to os7 are designated by arrowheads. As size markers (M), the *Bam*HI (lanes B) or *Eco*RI (lanes E) fragments of Ad12 DNA were coelectrophoresed.

merase (Stratagene) for 5 min at 94°C. This was followed by 30 cycles of 1 min at 94°C, 1 min at 68°C, and 2 min at 72°C in a thermal cycler (Perkin-Elmer). The PCR products were purified by using the Qiaquick-spin PCR purification system (Qiagen, Hilden, Germany). About 1 μg of this DNA, approximately one half of the purified product, was used to determine the nucleotide sequence.

Computer analyses of DNA sequences. For nucleotide sequence analyses, several computer programs of the Genetics Computer Group program package (5) were used on a VAX station 3200. Searches for homologies to known nucleotide sequences in the EMBL sequence library (release 41) (46) were performed with the BLAST program (1).

Nucleotide sequence accession numbers. Nucleotide sequences reported are accessible under EMBL numbers X87239 (T191) and X87240 (T637).

RESULTS AND DISCUSSION

Restriction analyses of integrated Ad12 DNA. Off-size fragments of Ad12 DNA. The cell lines investigated in the present study, T637, the revertants TR12, TR11, the tumor T191, and the Ad12-transformed cell line HA12/7, contained >20, 1 to 2, about 1, about 3, and about 3 copies of Ad12 DNA equivalents per cell, respectively, as previously determined (9, 21, 33, 45). Earlier results had shown that the viral DNA was covalently linked to cellular DNA and did not reside free in the cell nuclei.

Figure 1a and b present the Ad12 cleavage patterns of some of the restriction endonucleases used in this study. These patterns are based on the total DNA sequence of Ad12 DNA determined previously (43). With integrated Ad12 genomes, cleavage with any of these enzymes generated the internal viral DNA fragments which comigrated with the internal DNA fragments of virion DNA. Integrated viral DNA segments covalently linked to cellular DNA or to rearranged viral DNA gave rise to off-size fragments whose sizes did not coincide with any of the virion DNA fragments excised by the same restriction endonucleases. The nature of the off-size fragments was identified in more detail by using cloned Ad12 DNA fragments, in particular the two terminal and the internal *Pst*I D viral DNA fragments. These fragments were used as cloned hybridization probes in Southern blot experiments after cleaving total cellular DNA with several restriction endonucleases.

With the DNA from cell line T637, which carried about 20 genome equivalents of Ad12 DNA, seven different off-size fragments (os1 to os7) were generated (Fig. 1a and c). From the results of the hybridization experiments shown in Fig. 1a, the likely composition of these off-size fragments was deduced. Similar experiments were performed with genomic DNA from the Ad12-induced tumor T191 (Fig. 1b). In the investigations described here, our interest focused on off-size fragments os2 and os3 from cell line T637 and on os1 from the tumor T191. In previously published work, off-size fragments os4 and os5 from cell line T637 and os1 from the tumor T191 were identified as intraviral junctions (33).

Integration patterns of Ad12 DNA segments remaining integrated in the DNAs of the revertants TR11 and TR12 were analyzed in comparison to those of cell line T637 (Fig. 1c). The off-size fragments os2 and probably os1 were still present in the DNAs of the two revertants, whereas fragments os4, os5, os6, and os7 were lost. Fragment os3 was rearranged in revertant line TR12 and probably lost in TR11.

Chromosomal locations of integrated Ad12 DNA in different hamster cell lines and tumor cell lines. Application of the FISH technique revealed that, in the cell lines analyzed, the Ad12 genomes were integrated on one single site. The integration sites were apparently on different chromosomes in cell lines T637 and HA12/7 and in the cell line H191 derived from tumor T191. The site of integration was closer to the centromer in the cell lines T637 and HA12/7 and the revertants of cell line T637 and closer to the telomer in the tumor cell line H191 (Fig. 2). The integration sites in cell line T637 and its revertants TR11 and TR12 seemed to be identical (33).

Molecular cloning and nucleotide sequence analyses of junction sites os2 and os3 from cell line T637 and its revertants TR11 and TR12. (i) The os2 junction sequence. The *Bam*HI-excised off-size fragment os2 from the DNA of cell line T637 was cloned as an approximately 20-kbp fragment into the vector λ GEM-12. Restriction analyses revealed this insert to contain most of the 4-kbp right-terminal *Bam*HI E fragment of Ad12 DNA plus about 16 kbp of cellular DNA. The map in Fig. 3a outlines the structure of this insert and indicates the segments for which part of the nucleotide sequence was determined (Fig. 3b). The nucleotide sequence revealed that at the site of the junction between the viral and cellular DNAs 2 nucleotides of viral DNA were deleted. The remainder of the

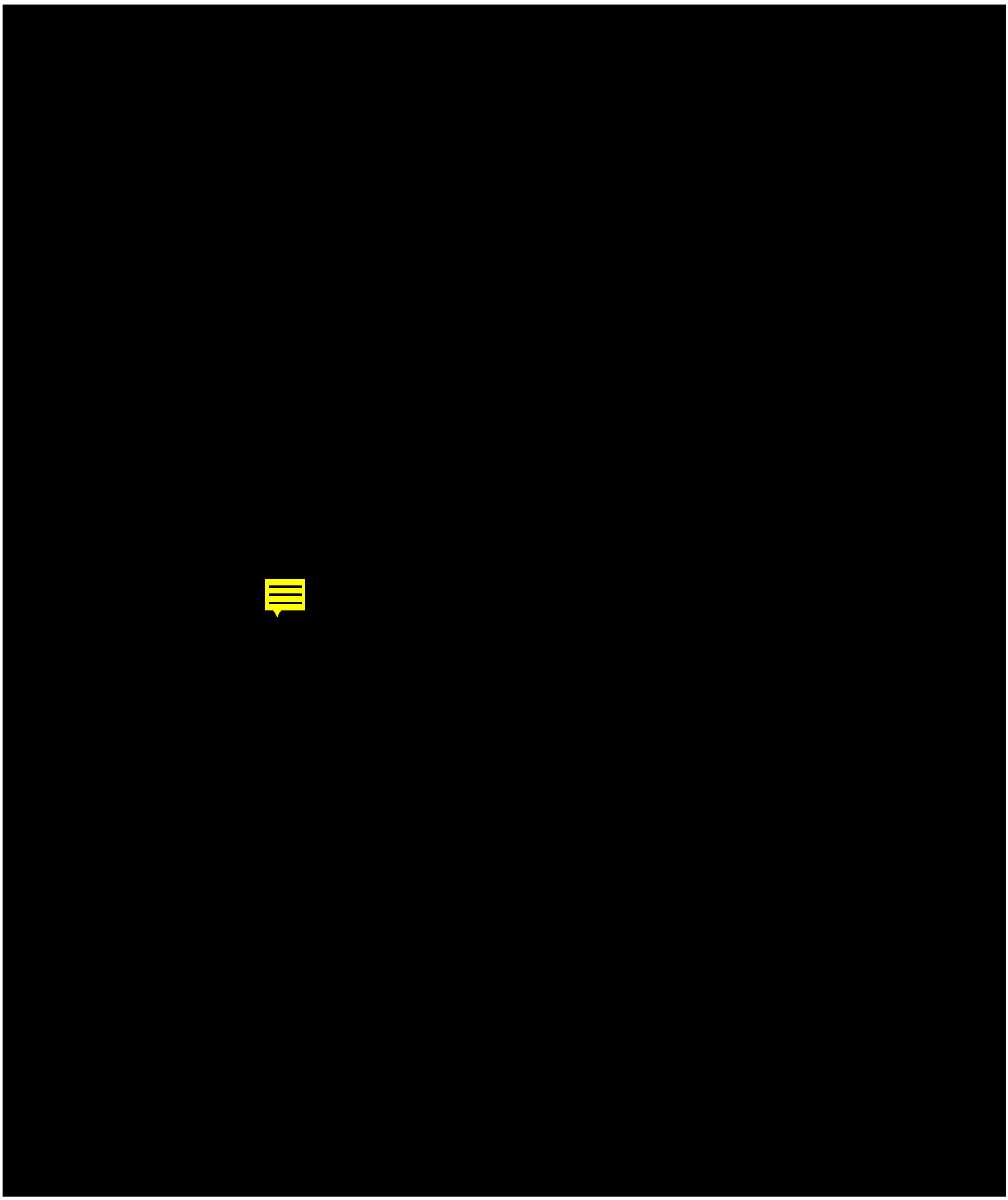

FIG. 2. Chromosomal locations of integrated Ad12 genomes in cell lines H191 (a), HA12/7 (b), T637 (c), and TR12 (d), as determined by the FISH method. Cell line BHK21 (e) served as a control devoid of Ad12 genomes. Ad12 DNA was labeled with biotin-16-2′ dUTP by nick translation (35) and used as hybridization probe.

right-terminal *Pst*I fragments L, M, I, and P of Ad12 DNA (map in Fig. 1) were intact. Patchy homologies between 5 to 10 nucleotides in length (horizontal arrows A to D) were apparent between the right-terminal Ad12 DNA fragment and the cellular DNA in the cloned os2 sequence of T637 hamster DNA (Fig. 3b and c). Nucleotide sequence homologies between the newly determined hamster DNA sequence and previously published sequences were not found, except for a $(GA)_n$ repeat. As indicated in the map in Fig. 3a, the cellular nucleotide sequence between nucleotides 50 and 900 as counted from the T7 end of the cellular DNA sequence contained three repetitive elements of the LINE1 type (41). The entire determined cellular nucleotide sequence of 5,251 nucleotides contained only 13 5′-CG-3′ sequences.

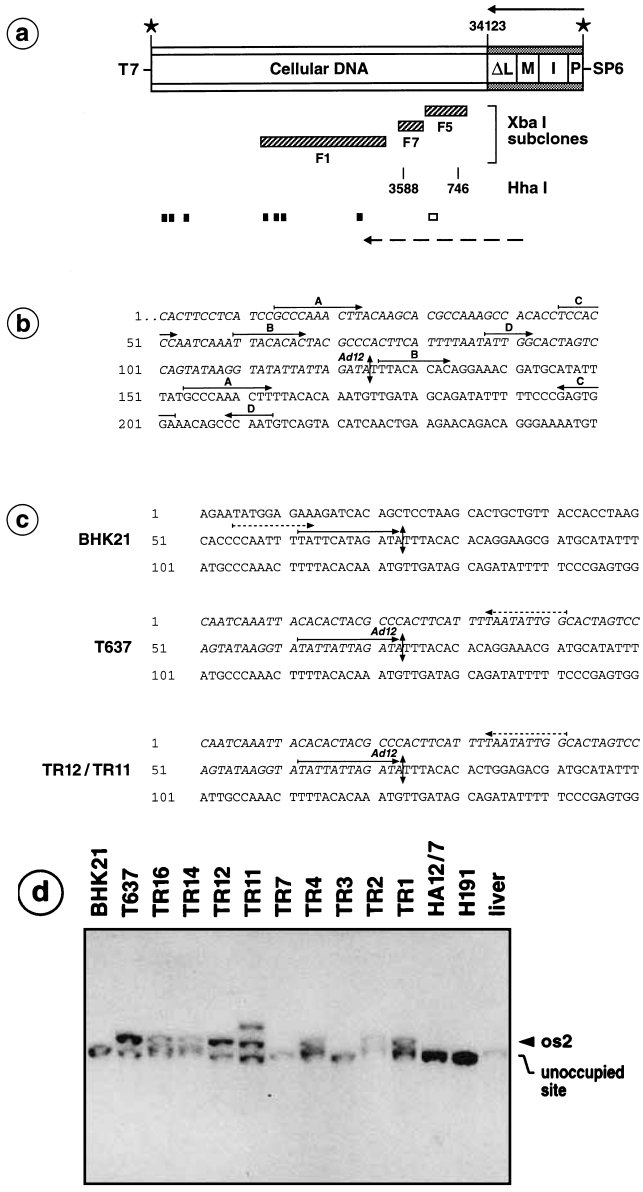

FIG. 3. Structure of the os2 junction site from cell line T637. (a) Map of the junction site between the right terminus of Ad12 DNA and hamster cellular DNA. This fragment was cloned into the λ GEM-12 vector between the T7 and SP6 sites (asterisks). Capital letters refer to the *Pst*I fragments of Ad12 DNA (map in Fig. 1). The *Xba*I subclones F5, F7, and F1, a GAGA motif (□), and repetitive sequences (■) are indicated. The dashed arrow spans the region of the DNA sequence determined and partly presented in panel b. There are two 5′-GCGC-3′ (*Hha*I) sequences at nucleotide positions 746 and 3588, counting the first cellular nucleotide adjacent to the Ad12 DNA sequence as position 1. The sequence contains no 5′-CCGG-3′ (*Hpa*II) sites. The states of methylation of the *Hha*I sites in the os2 sequence in cell line T637 and some of its revertants, in cell lines BHK21, HA12/7, and H191, and in hamster liver were determined (see Fig. 5a). (b) Nucleotide sequence at the os2 junction site (vertical double-headed arrow). The Ad12 DNA sequence (italic characters) starts with nucleotide 34123, i.e., two right-terminal Ad12 nucleotides were deleted (43). Extensive patchy homologies between viral and cellular DNAs are designated by horizontal arrows (A to D). Only part of the determined cellular nucleotide sequence of 5375 nucleotides is presented. The sequence from nucleotides 251 to 5375 is not shown here but is deposited under EMBL accession number X87240. (c) Nucleotide sequence of the preinsertion site in the DNA of BHK21 cells. Junction or insertion sites are indicated by vertical double-headed arrows. Ad12 DNA sequences are in italic type. For comparison, the junction sites in cell lines T637, TR12, and TR11 are also presented. Horizontal arrows indicate patchy homologies between viral and cellular DNA sequences. (d) Presence or deletion of the os2 preinsertion site in the DNA of various hamster cell lines and of hamster liver. The DNAs of the cell lines indicated were cleaved with *Bam*HI, and the fragments were analyzed by Southern blot hybridization using the ^{32}P-labeled subclone F5 (map in panel a) from the junction sequence os2 as hybridization probe.

By using the F5 subclone of the cloned os2 junction sequence (Fig. 3a) as hybridization probe, the preinsertion sequence at this site was also cloned from the DNA of BHK21 cells into the vector λ GEM-12. The nucleotide sequence at the immediate preinsertion site was determined (Fig. 3c) and compared with the sequence at the right terminus of Ad12 DNA. Patchy sequence homologies between these recombination partners were observed in Fig. 3c. The first 6 nucleotides directly at the junction site were identical between the deleted cellular DNA and the integrated Ad12 DNA (TAGATA). Insertion sites in the os2 regions from cell lines T637, TR12, and TR11 were found to be identical. These comparisons were based on nucleotide sequence determinations in PCR amplification products from DNAs of these cell lines (see Materials and Methods).

The map in Fig. 3a also indicates the sizes and positions of three *Xba*I subclones of 2,057 bp (F5), 1,165 bp (F7), and about 7,000 bp (F1) in length. These subclones were derived from the cellular sequences abutting the integrated Ad12 DNA. When subclones F5 and F7 were hybridized after ^{32}P labeling to restricted hamster cell DNA on a Southern blot, they proved to be of the unique sequence type (data not shown). A cellular DNA sequence of about 5 kbp immediately adjacent to the integrated viral DNA was thus of the unique sequence type. Subclone F1 contained repetitive elements of the LINE1 and SINE types of hamster DNA. The repetitive element of the LINE1 type exhibited homologies to a chromosome-specific centromer sequence in Chinese hamster DNA (10).

Previously analyzed cellular DNA sequences at the sites of linkage to viral DNA were transcriptionally active (for a review, see reference 12). This parameter was also assessed for the entire cellular DNA segment in the cloned os2 sequence of T637 DNA. Total cytoplasmic or polyadenylated cytoplasmic RNA was isolated from cell lines BHK21, T637, TR3, TR12, HA12/7, or H191 and analyzed in RNA transfer hybridization (Northern [RNA] blotting) experiments. No hybridization signals specific for any of these clones could be detected, hence the cellular DNA adjacent to the integrated Ad12 DNA in cell line T637 was not detectably transcribed in the cell lines studied.

To a limited extent, we also investigated nucleotide sequence arrangements in the revertant cell lines TR1, TR2, TR3, TR4, TR7, TR11, TR12, TR14, and TR16. The DNAs from these revertants, from cell lines BHK21, T637, HA12/7, and H191, and from hamster liver were cut with *Bam*HI, and the fragments were analyzed by Southern blot hybridization using the ^{32}P-labeled *Xba*I subfragment F5 as hybridization probe (Fig. 3d). The cellular sequence F5 from os2 was present as a single unoccupied fragment from both chromosomal complements in the DNAs from cell lines BHK21, HA12/7, and H191, as well as from hamster liver. Cell lines HA12/7 and H191 obviously carried Ad12 DNA in a location unrelated to the os2 segment of cellular DNA. In cell line T637 and its revertants TR1, TR2, TR4, TR12, TR14, and TR16, two F5-specific fragments were apparent, one from the unoccupied chromosome and the other longer one from the Ad12 DNA insertion site on the other chromosome. Revertant cell lines TR3 and TR7 had lost all Ad12 DNA sequences. Therefore, only the smaller F5 complement from the unoccupied chro-

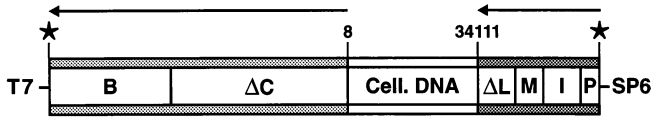

FIG. 4. Structure of the os3 junction site from cell line T637. Symbols and abbreviations are explained in the legend to Fig. 3a.

mosomes was present in these revertant lines (Fig. 3d). In the DNA from revertant cell line TR11, a third, even larger F5-specific fragment was detected, which might be due to an amplification of the cellular and viral sequences in the os2 DNA segment (Fig. 1c).

(ii) The os3 junction sequence. In a similar way, the os3 sequence from cell line T637 was also cloned into the vector λ GEM-12. The left terminus of Ad12 DNA, with a deletion of 7 nucleotides, was coupled via a stretch of cellular DNA of several hundred base pairs to the right terminus of an adjacent Ad12 DNA molecule with a deletion of 14 bp at the right terminus. Thus, nucleotide 8 of the left end of one Ad12 DNA molecule was connected via a bridge of cellular DNA to nucleotide 34111 of the right end of the adjacent Ad12 molecule in an array of >20 Ad12 genome equivalents (Fig. 4). The structure of this clone confirms that the multiple copies of the Ad12 genome in cell line T637 were not arranged in a true tandem array of one Ad12 genome followed immediately by another but that individual Ad12 DNA molecules were separated by cellular DNA (45).

Comparisons of the cellular DNA sequences in the os2 and os3 segments with cellular DNA segments in other Ad12-cellular DNA junctions. The *Xba*I subclones F5 of the os2 (Fig. 3a) and F4 of the os1 (T191) (Fig. 6a) junction sequences were ^{32}P labeled and hybridized to the previously described junction clone T1111(2) (28), CLAC1 (44), or HA12/7 (21), which had been cleaved by *Eco*RI, *Pst*I, or *Sau*3A, respectively, and then electrophoresed and transferred to a nylon membrane by Southern blotting. The autoradiograms revealed no homologies between the described junction segments (data not shown).

TABLE 1. Patchy homologies and A-rich sequences in Ad12 DNA integration sites in hamster and mouse DNA[a]

Nucleotide sequence	Cell line	Reference
Patchy homology		
^{6}ACACAGGAAACGATGCATA^{24}	os2 from T637	This study
^{28}AAGATGAAGCCAGA^{15}	T1111(2)	28
^{49}ACAGGACAC^{41}	CLAC3	4
^{16}GAAGGAGAC^{8}	CLAC1	44
^{25}CAAAGGGAGAAGAC^{38}	CBA-12-1-T (mouse)	39
^{12}TAATATTAAGAAG^{25}	HA12/7	21
^{8}AAAACAATACAGAGAAA^{23}	os1 from T191	This study
^{45}AATAAA^{50}	os1 from T191	This study
A-rich sequence		
^{31}CAAAA^{27}	T1111(2)	28
^{12}GAAA^{15}	os2 from T637	This study
^{42}TAAAA^{38}	os2 from T637	This study
^{33}CAAA^{36}	os2 from T637	This study
^{22}CAAA^{25}	CLAC3	4
^{15}GAAAAA^{20}	CLAC3	4
^{5}CAAA^{8}	CBA-12-1-T (mouse)	39
^{25}CAAA^{28}	CBA-12-1-T (mouse)	39
^{4}GAAA^{7}	HA12/7	21
^{7}CAAAA^{11}	os1 from T191	This study
^{21}GAAA^{24}	os1 from T191	This study
^{35}TAAAA^{39}	os1 from T191	This study
^{41}CAAAAA^{46}	os1 from T191	This study

[a] The nucleotide sequences (numbers for individual sequences noted in superscripts) were compared by using the program Bestfit.

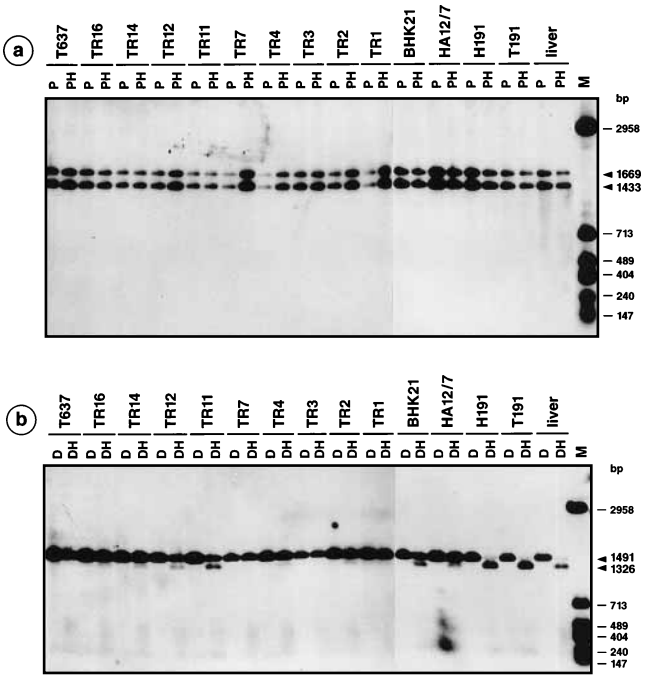

FIG. 5. States of methylation of 5′-GCGC-3′ (*Hha*I) sequences at cellular nucleotide positions 746 (a) and 3588 (b) in the os2 sequence in the DNA from the indicated sources. (a) The DNAs were cleaved with *Pvu*II (lanes P) or with *Pvu*II and *Hha*I (lanes PH), electrophoresed, Southern blotted, and hybridized to ^{32}P-labeled fragment F5. An unmethylated *Hha*I site would yield fragments of 1,102 and 331 bp, instead of a 1,433-bp fragment. *Bam*HI- and *Msp*I-cut pBluescript was used as a size marker (lane M). (b) Experimental procedures were as described in the legend to panel a, except that the different DNAs were cleaved with *Dpn*II (lanes D) or *Dpn*II and *Hha*I (lanes DH). The hybridization probe was the ^{32}P-labeled fragment F7. Appearance of the 1,326-bp fragment was diagnostic for the absence of DNA methylation at least in some of the *Hha*I sites at nucleotide position 3588 (Fig. 3a).

In an extensive computer-aided search, the first 50 nucleotide pairs of cellular DNA from the Ad12 DNA-cell DNA junctions in os2 and os3 from cell line T637 (this study) were compared with the equivalent sequences in previously cloned junction sites from hamster cell lines CLAC3 (4), CLAC1 (44), T1111(2) (28), and HA12/7 (21) or with CBA-12-1-T from mouse DNA (39). Real sequence identities between cellular DNA sequences from different sites of integration did not exist (38). However, patchy homologies of up to 7 nucleotide pairs were abundant. These homologies often comprised motifs of the GAAA, CAAA, TAAA (Table 1), and GAGGACA types. An array of patchy homologies might serve as recognition signals for the recombination machinery.

Status of DNA methylation at the cellular sites of Ad12 DNA integration. The first 5,251 bp of cellular DNA immediately adjacent to the Ad12 DNA sequence in the os2 segment were nonrepetitive DNA, and this sequence was exceptionally poor in 5′-CG-3′ dinucleotide sequences. There were only 13 5′-CG-3′ combinations, among them two 5′-GCGC-3′ (*Hha*I), but no 5′-CCGG-3′ (*Hpa*II), sequences which were amenable to restriction enzyme analyses with the methylation-sensitive enzyme *Hha*I.

As shown in Fig. 3a, the 5′-GCGC-3′ (*Hha*I) sites were at positions 746 and 3588 in the cellular DNA sequence of os2. The sequence at nucleotide position 746 was methylated in the DNA from hamster liver, from BHK21 cells, and from all

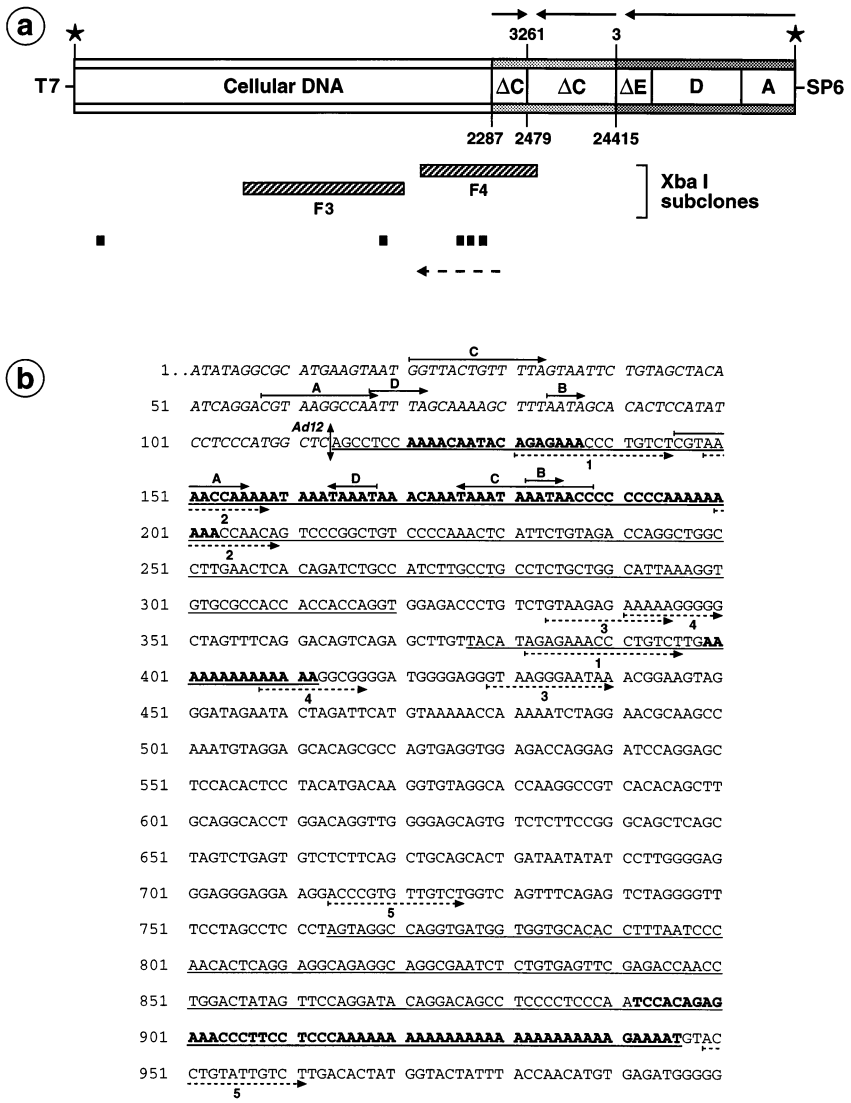

FIG. 6. Structure (a) and nucleotide sequence (b) at the os1 junction site between Ad12 DNA and hamster cell DNA in the tumor T191. (a) The capital letters refer to the *Pst*I fragments of Ad12 DNA (map in Fig. 1). The Δ indicates that parts of the sequence were deleted. The nucleotide segments of Ad12 DNA represented in the os1 clone are identified by nucleotide numbers from the authentic Ad12 DNA sequence (43). *Xba*I subclones are included in the map as well as repetitive sequence motifs (■). The nucleotide sequence was determined (see panel b) for the segment spanned by the dashed arrow. (b) Nucleotide sequence at and adjacent to the site of linkage between viral and cellular DNAs (double-headed vertical arrow). The Ad12 sequence is in italics. This nucleotide sequence comprises *Xba*I subclone F4. Homologies to known hamster DNA sequences and patchy homologies (4 to 13 nucleotides long) between viral and cellular DNAs (horizontal arrows A to D) are designated. A-rich sequences are in bold type. Cellular sequences with homologies to hamster *Alu* sequences are underlined. Dashed arrows indicate direct repeats of *Alu* elements. Only 1,000 nucleotides of the total sequence of 2,266 nucleotides determined are reproduced. The sequence from nucleotide 1001 to 2266 was transferred to the EMBL data bank under accession number X87239.

Ad12-transformed and revertant cell lines (Fig. 5a, lanes PH, cleavage by *Pvu*II and *Hha*I). The *Hha*I sequence at position 3588 was methylated in the DNA from T637 cells and in the DNA from revertants TR1, TR2, TR3, TR4, and TR7. Upon cleavage with *Dpn*II and *Hha*I, Southern blotting, and hybridization to the ^{32}P-labeled probe F7, only one fragment was apparent (Fig. 5b). The same restriction analyses revealed partial cleavage of the DNA from cell lines BHK21 and HA12/7 and from the revertants TR11, TR12, TR14, and TR16 (Fig. 5b). Hence the site was partially methylated. Complete *Hha*I cleavage of the site at position 3588 was observed with the DNA from hamster liver, from H191 cells, and from the tumor T191 (Fig. 5b). The site was therefore unmethylated.

These data indicated that insertion of Ad12 DNA at this site was associated with only minor alterations in the methylation of the two 5′-GCGC-3′ sequences in the adjacent cellular DNA. These changes affected the cellular site more distant (position 3588) from the locus of foreign DNA insertion.

Hybridization of the ^{32}P-labeled right-terminal *Bam*HI E fragment of Ad12 DNA to *Hpa*II- or *Hha*I-cleaved cellular DNA demonstrated that the right terminus of Ad12 DNA in cell lines T637, TR11, and TR12 was strongly, but not completely, methylated (data not shown). Prior to integration, the Ad12 genome is of course completely unmethylated (16, 48).

Increase in DNA methylation in repetitive sequences in the Ad12-transformed hamster cell lines. The subclones F1 of os2 from cell line T637 and F4 of os1 from T191, which contained repetitive DNA of the LINE1 and *Alu*-like types, respectively,

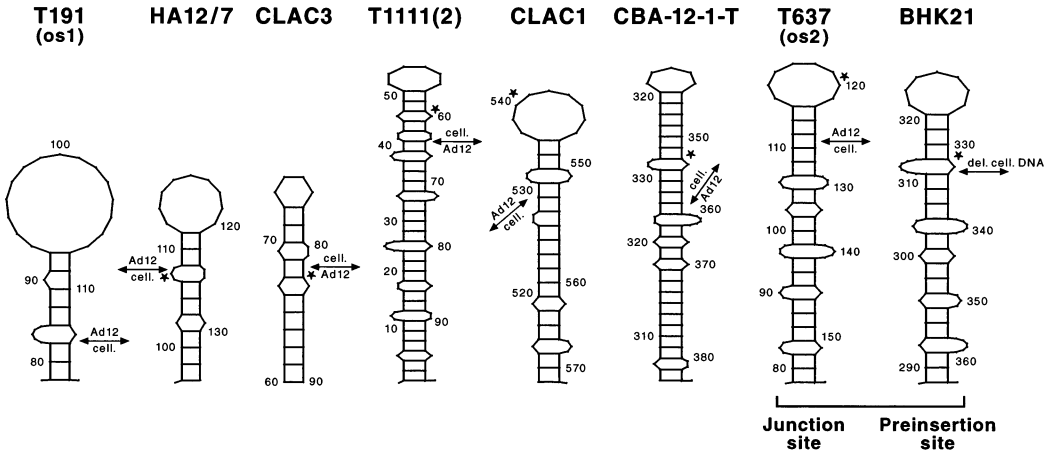

FIG. 7. Stem-loop structures at the sites of linkage between Ad12 DNA and cellular DNA from several Ad12-transformed hamster cell lines and Ad12-induced hamster or mouse tumors or tumor cell lines. A stem-loop structure at the preinsertion site in BHK21 cells was also shown. Details are described in the text. Double-headed arrows, Ad12 DNA-cell DNA junction sites; asterisks, topoisomerase I recognition sites.

were used as hybridization probes with *Hpa*II- or *Msp*I-restricted DNA from cell lines BHK21, T637, TR12, TR7, HA12/7, and H191, from the tumor T191, and from hamster liver. The distribution profiles of the generated DNA fragments demonstrated that the DNA methylation of the analyzed segments of repetitive DNA in cell line T637 and in its revertants TR12 and TR7 was markedly increased in comparison to the methylation in the same segments of hamster liver or the cell line BHK21. In cell lines HA12/7 and H191 and in tumor T191, only moderate increases in repetitive DNA methylation were observed in the os2 and os4 segments (data not shown).

Molecular cloning and nucleotide sequence analyses of junction site os1 from the Ad12-induced tumor T191. The *Bam*HI-generated Ad12-specific off-size fragment os1 from the DNA of tumor T191 (cf. Fig. 1b) was also cloned into the vector λ GEM-12. This insert had a length of approximately 16 kbp and consisted of about 7.4 kbp of rearranged Ad12 DNA sequences, partly derived from the left viral DNA terminus and partly from internal parts of the Ad12 genome and with about 8 kbp of cellular DNA from the hamster cell genome (Fig. 6). The precise map locations of the Ad12 DNA sequences in the junction clone were derived from the nucleotide sequence of parts of the cloned os1 segment (Fig. 6). The nucleotide sequence at the left terminus of one Ad12 DNA molecule had recombined with the left terminus possibly from another Ad12 DNA molecule, with internal Ad12 DNA segments, and with cellular DNA (Fig. 6a). Between the truncated Ad12 DNA sequence from the left terminus (Fig. 6a, ΔC) and the cellular DNA sequence, patchy homologies were observed (Fig. 6b). Such homologies were also found at all sites of intraviral DNA recombination. The cellular DNA sequence adjacent to the site of junction was rich in A and T nucleotides and in A/T-containing repetitions. These sequences were also related to *Alu* elements, as described for hamster DNA sequences involved in DNA amplifications (18).

As described for the os2 segment from cell line T637, the cellular DNA segment in os1 from the tumor T191 was also analyzed for transcriptional activity in cell lines BHK21, T637, TR3, TR12, HA12/7, or H191. No transcriptional activities were detectable by the RNA transfer hybridization (Northern blot) analyses performed with total cytoplasmic or polyadenylated RNA (data not shown). The same RNAs were also hybridized to ^{32}P-labeled actin gene cDNA probes. Actin signals were observed in all hybridization experiments. Hence the cellular os1 and os2 sequences are not transcribed in any of the cell lines tested.

Computer searches in the EMBL DNA sequence library (release 41) for possible sequence similarities of the cellular DNA in os1 to known nucleotide sequences found homologies to sequence elements of the *Alu* family and to a hamster origin of DNA replication containing an *Alu* element (27).

Stem-loop structures at Ad12 integration sites in hamster or mouse DNA. We analyzed the nucleotide sequences at several of the here and previously determined insertion sites of Ad12 DNA into the hamster or the mouse genome for the occurrence of stem-loop structures by using the computer program FOLD (19). For the insertion sites of Ad12 DNA in the hamster tumor cell line CLAC3 or in the mouse cell line CBA-12-1-T, hairpin configurations were described earlier (4, 39). The computer graphs in Fig. 7 present stem-loop designs for the Ad12 insertion sites in the transformed or tumor cell lines T191 (os1) (this study), HA12/7 (21), T1111 (2) (28), CLAC1 (44), CLAC3 (4), CBA-12-1-T (39), and T637 (os2) and the Ad12 preinsertion site in the parental BHK21 cells (this study). In all structures, the virus-cell junctions were designated by double-headed arrows. Possible topoisomerase I recognition sites (asterisks) were indicated as well as nucleotide numbers for the individual nucleotide sequences.

Stem-loop configurations were also reported for a number of nonhomologous recombination events, e.g., the insertion site of unique and repetitive DNA fragments into the aprt locus in the hamster genome (31) or a hotspot for novel amplification joints in *Alu*-like repeats (18) and other structures (23, 26, 32), or for virus-cell DNA integration sites (3, 24, 36).

Conclusions. The mechanisms of nonhomologous recombination in mammalian cells seem to be characterized by a high degree of flexibility and pliability. The major findings in the present report are the following.

(i) In contrast to previously reported observations, the cellular DNA sequences at the sites of junction to foreign (Ad12) DNA analyzed in the present report are not transcriptionally active.

(ii) Junction sites described here abound with patchy homologies between the nucleotide sequences of the recombination partners (Fig. 3b and 6b).

(iii) The cellular DNA segments at the sites of linkage from cell line T637 have a low content of 5′-CG-3′ sequences, and a cellular DNA segment of about 5 kbp is of a unique type and is only followed by repetitive cellular DNA.

(iv) In the cellular sequence vicinity of the Ad12 DNA integration sites, no significant alterations in the patterns of DNA methylation can be found. Subpopulations in some of the revertant T637 cell lines may offer an exception.

(v) In the cellular sequences in junction sites isolated from cell line T637, similarities to known cellular DNA sequences have not been recognized. In contrast, sequences in the cellular DNA segments in os1 from the Ad12-induced tumor T191 carry homologies to nucleotide sequences of the *Alu* family and to a sequence related to a presumptive origin of hamster DNA replication with an *Alu* element. An origin of cellular DNA replication may have an advantage in recombination with foreign DNA. The presumptive origin of DNA replication is extremely rich in AT repetitions.

(vi) The present analyses using the fluorescent in situ hybridization technique prove that in all cell lines investigated the foreign (Ad12) DNA is located at a single chromosomal site even when >20 copies of Ad12 DNA have been integrated. These single insertion sites differ among the different cell lines investigated. The sites of insertion can reside in more telomeric or centromeric positioned locations.

(vii) In the revertants of cell line T637, TR11 and TR12, which have lost most of the Ad12 DNA copies, the chromosomal location has most likely not changed. In cell lines TR3 and TR7, a segment of the adjacent cellular DNA at the junction site seems to have been lost together with Ad12 DNA.

(viii) The data below present computer-aided comparisons of cellular nucleotide sequences of the junction sites to Ad12 DNA studied.

os2 from cell line T637	**os1 from tumor T191**
Right terminal Ad12 fragment	Left terminal Ad12 fragment
No rearrangements at viral terminus	Several rearrangements of viral terminus
Integration adjacent to centromer	Integration close to telomer
Cellular sequence is a single copy sequence	Cellular sequence is highly repetitive
Underrepresentation of 5′-CG-3′ sequences	High 5′-CG-3′ content (25 5′-CG-3′ sequences in 2,153 bp)
No A+T stretches, but CAAA, GAAA, and TAAA sequences	A+T stretches in cellular sequence

The salient features of these analyses confirm earlier conclusions derived from in vivo (13) or in vitro (50) recombination studies, i.e., patchy homologies appear to aid the integrative recombination mechanism. Long stretches of perfect sequence homology have not been observed, and the nucleotide sequences at all junction sites investigated are different from one another.

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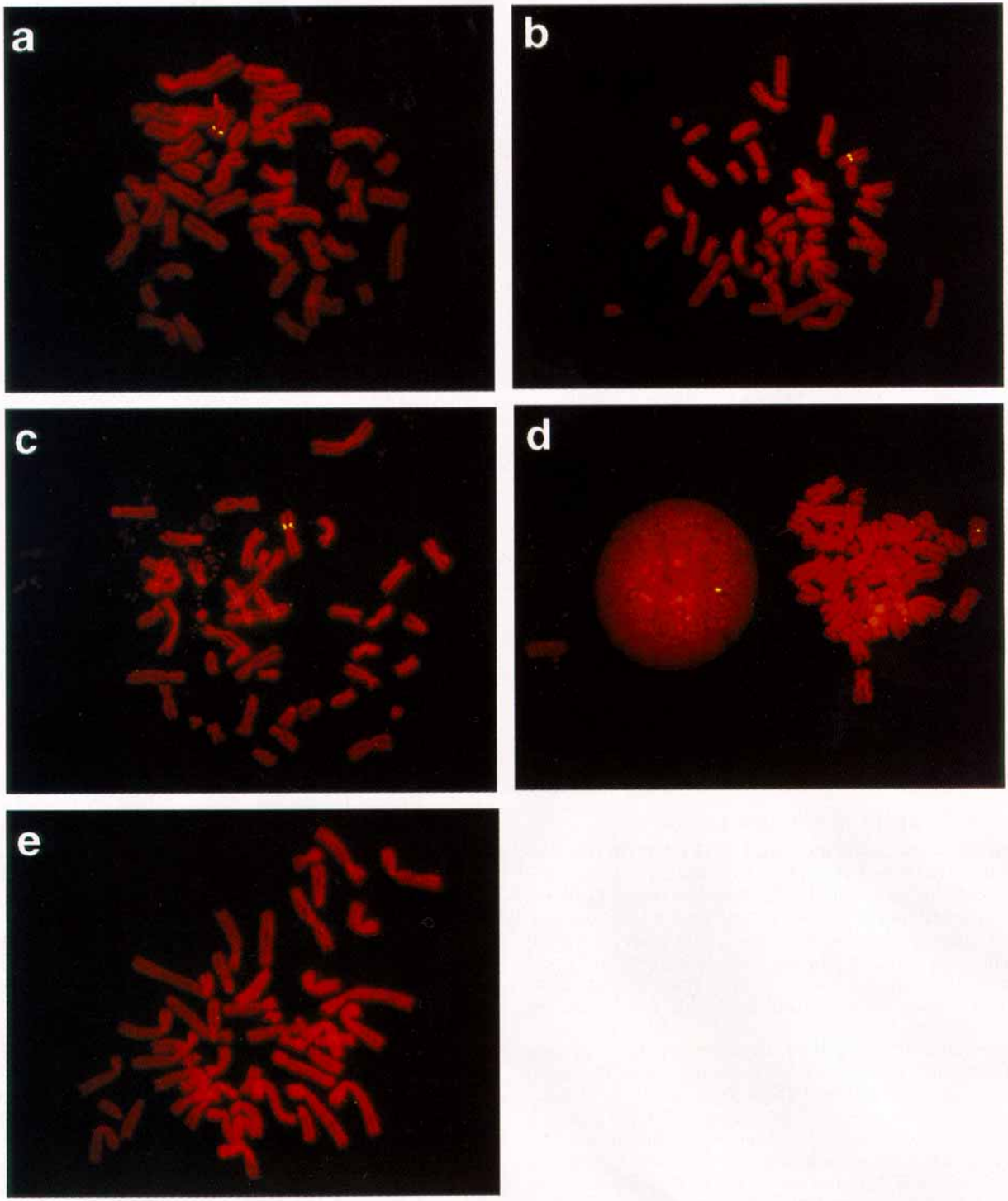
REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Alwine, J. C., D. J. Kemp, and G. R. Stark.** 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc. Natl. Acad. Sci. USA **74:**5350–5354.
3. **Bullock, P., W. Forrester, and M. Botchan.** 1984. DNA sequence studies of simian virus 40 chromosomal excision and integration in rat cells. J. Mol. Biol. **174:**55–84.
4. **Deuring, R., U. Winterhoff, F. Tamanoi, S. Stabel, and W. Doerfler.** 1981. Site of linkage between adenovirus type 12 and cell DNAs in hamster tumour line CLAC3. Nature (London) **293:**81–84.
5. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
6. **Doerfler, W.** 1993. Adenoviral DNA integration and changes in DNA methylation patterns: a different view of insertional mutagenesis. Prog. Nucleic Acid Res. Mol. Biol. **46:**1–36.
7. **Doerfler, W.** 1995. The insertion of foreign DNA into mammalian genomes and its consequences: a concept for oncogenesis. Adv. Cancer Res. **66:**313–344.
8. **Doerfler, W., R. Gahlmann, S. Stabel, R. Deuring, U. Lichtenberg, M. Schulz, D. Eick, and R. Leisten.** 1983. On the mechanism of recombination between adenoviral and cellular DNAs: the structure of junction sites. Curr. Top. Microbiol. Immunol. **109:**193–228.
9. **Eick, D., S. Stabel, and W. Doerfler.** 1980. Revertants of adenovirus type 12-transformed hamster cell line T637 as tools in the analysis of integration patterns. J. Virol. **36:**41–49.
10. **Fatyol, K., I. Cserpan, T. Praznovszky, J. Kereso, and G. Hadlaczky.** 1994. Cloning and molecular characterization of a novel chromosome specific centromere sequence of Chinese hamster. Nucleic Acids Res. **22:**3728–3736.
11. **Fechteler, K., G. Multhaup, B. Schmitz, K. Beyreuther, and W. Doerfler.** Integrative recombination between adenovirus type 12 DNA and mammalian DNA: purification of a multi-protein complex from hamster cells and identification of Rad51 as one of its components. Submitted for publication.
12. **Fechteler, K., J. Tatzelt, S. Huppertz, P. Wilgenbus, and W. Doerfler.** 1995. The mechanism of adenovirus DNA integration: studies in a cell-free system. Curr. Top. Microbiol. Immunol. **199II:**109–137.
13. **Gahlmann, R., R. Leisten, L. Vardimon, and W. Doerfler.** 1982. Patch homologies and the integration of adenovirus DNA in mammalian cells. EMBO J. **1:**1101–1104.
14. **Gahlmann, R., M. Schulz, and W. Doerfler.** 1984. Low molecular weight RNAs with homologies to cellular DNA at sites of adenovirus DNA insertion in hamster or mouse cells. EMBO J. **3:**3263–3269.
15. **Groneberg, J., D. Sutter, H. Soboll, and W. Doerfler.** 1978. Morphological revertants of adenovirus type 12-transformed hamster cells. J. Gen. Virol. **40:**635–645.
16. **Günthert, U., M. Schweiger, M. Stupp, and W. Doerfler.** 1976. DNA methylation in adenovirus, adenovirus-transformed cells, and host cells. Proc. Natl. Acad. Sci. USA **73:**3923–3927.
17. **Heller, H., C. Kämmer, P. Wilgenbus, and W. Doerfler.** 1995. The chromosomal insertion of foreign (adenovirus type 12, plasmid, or bacteriophage lambda) DNA is associated with enhanced methylation of cellular DNA segments. Proc. Natl. Acad. Sci. USA **92:**5515–5519.
18. **Hyrien, O., M. Debatisse, G. Buttin, and B. R. de Saint Vincent.** 1987. A hotspot for novel amplification joints in a mosaic of Alu-like repeats and palindromic A+T rich DNA. EMBO J. **6:**2401–2408.
19. **Jaeger, J. A., D. H. Turner, and M. Zuker.** 1989. Improved predictions of secondary structures for RNA. Proc. Natl. Acad. Sci. USA **86:**7706–7710.
20. **Jessberger, R., D. Heuss, and W. Doerfler.** 1989. Recombination in hamster cell nuclear extracts between adenovirus type 12 DNA and two hamster preinsertion sequences. EMBO J. **8:**869–878.
21. **Jessberger, R., B. Weisshaar, S. Stabel, and W. Doerfler.** 1989. Arrangement and expression of integrated adenovirus type 12 DNA in the transformed hamster cell line HA12/7: amplification of Ad12 and c-myc DNAs and evidence for hybrid viral-cellular transcripts. Virus Res. **13:**113–128.
22. **Koetsier, P. A., J. Schorr, and W. Doerfler.** 1993. A rapid optimized protocol for downward alkaline Southern blotting of DNA. BioTechniques **15:**260–262.
23. **Krawinkel, U., G. Zoebelein, and A. L. M. Bothwell.** 1986. Palindromic sequences are associated with sites of DNA breakage during gene conversion. Nucleic Acids Res. **14:**3871–3882.
24. **Kudo, S., H. Sugisaki, M. Takanami, T. Yamashita, M. Ishino, Y. Shimizu, Y. Iida, and K. Fujinaga.** 1986. Structure of viral DNA in a rat cell line, GY1, transformed by Ad12 HindIII fragment-G. Virology **148:**133–145.
25. **Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker.** 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16:**4743–4751.
26. **Lehrmann, M. A., W. J. Schneider, T. C. Südhof, M. S. Brown, J. L. Goldstein, and D. W. Russell.** 1985. Mutation in LDL receptor: Alu-Alu recombination deletes exons encoding transmembrane and cytoplasmatic domains. Science **227:**140–146.
27. **Leu, T., B. Anachkova, and J. L. Hamlin.** 1990. Repetitive sequence elements in an initiation locus of the amplified dihydrofolate reductase domain in CHO cells. Genomics **7:**428–433.

28. **Lichtenberg, U., C. Zock, and W. Doerfler.** 1987. Insertion of adenovirus type 12 DNA in the vicinity of an intracisternal A particle genome in Syrian hamster tumor cells. J. Virol. **61:**2719–2726.
29. **Lichtenberg, U., C. Zock, and W. Doerfler.** 1988. Integration of foreign DNA into mammalian genome can be associated with hypomethylation at site of insertion. Virus Res. **11:**335–342.
30. **Lichter, P., C.-J. C. Tang, K. Call, G. Hermanson, G. A. Evans, D. Housman, and D. C. Ward.** 1990. High-resolution mapping of human chromosome 11 by *in situ* hybridization with cosmid clones. Science **247:**64–69.
31. **Nablbantoglu, J., C. Miles, and M. Meuth.** 1988. Insertion of unique and repetitive DNA fragments into the aprt locus of hamster cells. J. Mol. Biol. **200:**449–459.
32. **Nicholls, R. D., N. Fishel-Ghodsian, and D. R. Higgs.** 1987. Recombination at the human alpha-globin gene cluster: sequence features and topological constraints. Cell **49:**369–378.
33. **Orend, G., M. Knoblauch, C. Kämmer, S. T. Tjia, B. Schmitz, A. Linkwitz, G. Meyer zu Altenschildesche, J. Maas, and W. Doerfler.** 1995. The initiation of de novo methylation of foreign DNA integrated into a mammalian genome is not exclusively targeted by nucleotide sequence. J. Virol. **69:**1226–1242.
34. **Orend, G., I. Kuhlmann, and W. Doerfler.** 1991. Spreading of DNA methylation across integrated foreign (adenovirus type 12) genomes in mammalian cells. J. Virol. **65:**4301–4308.
35. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113:**237–251.
36. **Romani, M., A. De Ambrosis, B. Alhadeff, M. Purrello, Y. Gluzman, and M. Siniscalco.** 1990. Preferential integration of the Ad5/SV40 hybrid virus at the highly recombingenic human chromosomal site 1p36. Gene **95:**231–241.
37. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
38. **Schellner, J., K. Stüber, and W. Doerfler.** 1986. Computer analyses on the structure of junction sites between adenovirus DNA and cellular DNA. Biochim. Biophys. Acta **867:**114–123.
39. **Schulz, M., and W. Doerfler.** 1984. Deletion of cellular DNA at site of viral DNA insertion in the adenovirus type 12-induced mouse tumor CBA-12-1-T. Nucleic Acids Res. **12:**4959–4976.
40. **Schulz, M., U. Freisem-Rabien, R. Jessberger, and W. Doerfler.** 1987. Transcriptional activities of mammalian genomes at sites of recombination with foreign DNA. J. Virol. **61:**344–353.
41. **Skowronski, J., and M. F. Singer.** 1986. The abundant LINE-1 family of repeated DNA sequences in mammals: genes and pseudogenes. Cold Spring Harbor Symp. Quant. Biol. **51:**457–464.
42. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
43. **Sprengel, J., B. Schmitz, D. Heuss-Neitzel, C. Zock, and W. Doerfler.** 1994. Nucleotide sequence of human adenovirus type 12 DNA: comparative functional analysis. J. Virol. **68:**379–389.
44. **Stabel, S., and W. Doerfler.** 1982. Nucleotide sequence at the site of junction between adenovirus type 12 DNA and repetitive hamster cell DNA in transformed cell line CLAC1. Nucleic Acids Res. **10:**8007–8023.
45. **Stabel, S., W. Doerfler, and R. R. Friis.** 1980. Integration sites of adenovirus type 12 DNA in transformed hamster cells and hamster tumor cells. J. Virol. **36:**22–40.
46. **Stoehr, P. J., and G. N. Cameron.** 1991. The EMBL data library. Nucleic Acids Res. **19:**2227–2230.
47. **Strohl, W. A., H. Rouse, K. Teets, and R. W. Schlesinger.** 1970. The response of BHK21 cells to infection with type 12 adenovirus. III. Transformation and restricted replication of superinfecting type 2 adenovirus. Arch. Gesamte Virusforsch. **31:**93–112.
48. **Sutter, D., M. Westphal, and W. Doerfler.** 1978. Patterns of integration of viral DNA sequences in the genomes of adenovirus type 12-transformed hamster cells. Cell **14:**569–585.
49. **Tatzelt, J., K. Fechteler, P. Langenbach, and W. Doerfler.** 1993. Fractionated nuclear extracts from hamster cells catalyze cell-free recombination at selective sequences between adenovirus DNA and a hamster preinsertion site. Proc. Natl. Acad. Sci. USA **90:**7356–7360.
50. **Tatzelt, J., B. Scholz, K. Fechteler, R. Jessberger, and W. Doerfler.** 1992. Recombination between adenovirus type 12 DNA and a hamster preinsertion sequence in a cell-free system. Patch homologies and fractionation of nuclear extracts. J. Mol. Biol. **226:**117–126.
51. **zur Hausen, H.** 1973. Interactions of adenovirus type 12 with host cell chromosomes. Prog. Exp. Tumor Res. **18:**240–259.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