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Integration of viral DNA into the genome of the adenovirus type 2-transformed hamster cell line HE5 without loss or alteration of cellular nucleotides

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ABSTRACT

Hamster cell line HE5 has been established from primary LSH hamster embryo cells by transformation with adenovirus type 2 (Ad2) (1). Each cell contains two to three copies of integrated Ad2 DNA (2, 3). We cloned and sequenced the sites of junction between viral and cellular DNAs. The terminal 10 and 8 nucleotides of Ad2 DNA were deleted at the left and right sites of junction, respectively. The integrated viral DNA had an internal deletion between map units 35 and 82 on the Ad2 genome. At the internal site of deletion, the remaining viral sequences were linked via a GT dinucleotide of unknown origin. From HE5 DNA, the unoccupied sequence corresponding to the site of insertion was also cloned and sequenced. Part of this sequence was shown to be expressed as cytoplasmic RNA in HE5 and primary LSH hamster embryo cells. The viral DNA had been inserted into cellular DNA without deletions, rearrangements or duplications of cellular nucleotides at the site of insertion. Thus, insertion of Ad2 DNA appeared to have been effected by a mechanism different from that of bacteriophage λ in Escherichia coli and from that of retroviral genomes in vertebrates. It was conceivable that the terminal viral protein (4) was somehow involved in integration either on a linear or a circularized viral DNA molecule.

INTRODUCTION

Uptake and fixation of viral DNA into the genome of mammalian cells seem to be among the important steps in the stable transformation or in tumorigenesis by adenoviruses or by adenoviral DNA (for recent reviews, 5, 6). Furthermore, viral DNA insertion provides a suitable model system for studies on the mechanism of recombination between foreign and host DNA in eucaryotic cells.

So far, scarce information is available on recognition signals operative in the integration event. Attachment sites or extensive homologies comparable to those found in the integra-

tion of phage lambda into the E. coli chromosome via homologous recombination (7) have not been observed in the integration of adenoviral DNA. Patchy homologies as well as possible secondary structures at or near sites of junction between adenoviral and cellular DNA have been described (8-10). The functional significance of these structures at the nucleotide level is not clear.

Detailed descriptions of the patterns of integration of viral DNA exist in Ad2-, adenovirus type 5 (Ad5)-, and type 12 (Ad12)-transformed and Ad12-induced tumor cells of hamster, rat or mouse origin (3, 11-19). However, due to possible postintegrational events as rearrangements, amplifications or deletions of integrated viral DNA sequences, integration patterns are often complex and difficult to interpret. It has proved essential to investigate sites of linkage between viral and cellular DNA at the nucleotide level and to compare nucleotide sequences at the sites of insertion with those at the identical unoccupied cellular sites.

In the present communication, we describe a complete analysis of the sites of linkage of integrated Ad2 DNA in hamster cell line HE5. Junction sites between the right and left termini of viral DNA and cellular DNA as well as the internal link between Ad2 DNA at map units 35 and 82 have been molecularly cloned and the nucleotide sequences have been determined. Similarly, the unoccupied cellular site has been cloned and sequenced. Integration models will be discussed.

MATERIALS AND METHODS

Cells, cellular DNA and RNA

The Ad2-transformed hamster cell line HE5 was obtained by transformation in culture of primary LSH hamster embryo cells by ultraviolet light-irradiated Ad2 (1, 2). The cells were propagated as described earlier (3). Primary LSH hamster embryo cells were isolated from 14 day old LSH hamster embryos and were cultured in Dulbecco-modified Eagle medium containing 15-20 % fetal calf serum. Cellular DNA was extracted and purified by a procedure described elsewhere (12). Cytoplasmic RNA from HE5 cells was prepared as outlined (20). RNA from primary hamster embryo cells was isolated by a modification of this procedure.

Molecular cloning and procaryotic vectors

Molecular cloning of the right terminal junction fragment and of the cellular DNA fragment corresponding to the unoccupied site in cell line HE5 DNA in the DNA of bacteriophage lambda [designated here as clones λ24 and λ19 (Fig. 1)], and recloning in the plasmid pUR2 have been described earlier (9). In principle, the left junction fragment was cloned from HE5 DNA in the same way as reported earlier (8, 9), except that the vector λ Charon 4A was used. The exact locations of some of the cloned fragments, which were subcloned (pXba4) in pUR250 (21) or pBR322 (pG1a and pSK1), are indicated in Fig. 1. As described elsewhere (9), the unoccupied cellular DNA site from cell line HE5 was preselected by zone sedimentation on sucrose gradients and cloned in λgtWES·λB DNA. Positive plaques were identified using as hybridization probe the right junction fragment p24 (Fig. 1) which was [^{32}P]-labeled by nick translation.

Most of the HindIII fragments of Ad2 DNA were cloned in pBR322 and were provided by S.-L. Hu and D. Solnick, Cold Spring Harbor, N.Y. The pBE5 plasmid containing in pBR322 the left terminal BglII-E fragment of Ad2 DNA was made available by N. Stow, Glasgow.

DNA and RNA techniques

Restriction mapping, agarose gel electrophoresis of DNA or RNA, Southern blotting and DNA-DNA or DNA-RNA hybridization procedures were performed according to standard protocols which were described in detail elsewhere (3, 9, 11, 12, 20, 22). DNA was nick translated as reported (23) and DNA-DNA and DNA-RNA hybridizations were performed as described (20, 24). Some filters were used for multiple hybridization experiments. DNA from previous hybridization reactions was washed off from the filters by boiling them in distilled water for at least 15 minutes and by several rinses in H_2O.

Nucleotide sequences were determined according to the method of Maxam and Gilbert (25, 26).

Computer analyses of nucleotide sequences were carried out as detailed elsewhere (9).

RESULTS

Mapping, cloning, and subcloning of the junction site between the left end of Ad2 DNA and hamster cell DNA

Restriction analyses of the patterns of Ad2 DNA integration in cell line HE5 had revealed one off-size EcoRI fragment containing Ad2 DNA from the left terminal EcoRI fragment A and additional sequences from the internal EcoRI fragment D. Apparently the internal deletion of Ad2 DNA had generated a fusion of parts of the left terminal EcoRI fragment A and of fragment D of Ad2 DNA accompanied by the loss of the EcoRI site between these fragments (3). From the DNA of cell line HE5 a λ Charon 4A clone (λL1) was isolated which contained an EcoRI fragment comprising the viral regions described and cellular sequences contiguous with the left viral terminus. In order to map the adenoviral sequence of this clone, λL1 DNA and Ad2 DNA were cleaved either with EcoRI and one of the following restriction endonucleases, BamHI, BglII, DdeI, HaeIII, HindIII, HinfI, HpaI, KpnI, PstI, PvuII, RsaI, SacI, SalI, Sau3A, SmaI, TaqI or XbaI, or with only one of the restriction endonucleases specified. The fragments were separated on agarose gels, blotted and hybridized to [^{32}P]-labeled probes of total Ad2 DNA or separately to one of the following Ad2 fragments cloned in pBR322: BglII E (comprising 0-9.1 % of the Ad2 map), or HindIII C, B, I, D, A, H (comprising 7.7 % to 37.3 % and 41.0 % to 79.6 % of the Ad2 map), or EcoRI D (comprising 76.0 % to 83.5 % of the Ad2 map) (data not shown).

The results of these experiments and of additional restriction mapping experiments of subclones pSK1 and pXba4 (Fig. 1) were in accordance with the following conclusions:
1) Less than 268 basepairs of the left terminal Ad2 DNA were deleted. 2) Internal viral DNA sequences from about map unit 35 up to map unit 82 were deleted (Fig. 1). 3) The conserved segments of viral DNA were integrated colinearly with virion DNA. 4) Two point mutations inside the remaining viral regions could be mapped: A HindIII site at map position 17.1 % was missing; a new HindIII site was positioned at 6.4 %.

Inside the cellular part of clone λL1, the BamHI and XbaI sites designated in Fig. 1 and recognition sites of additional

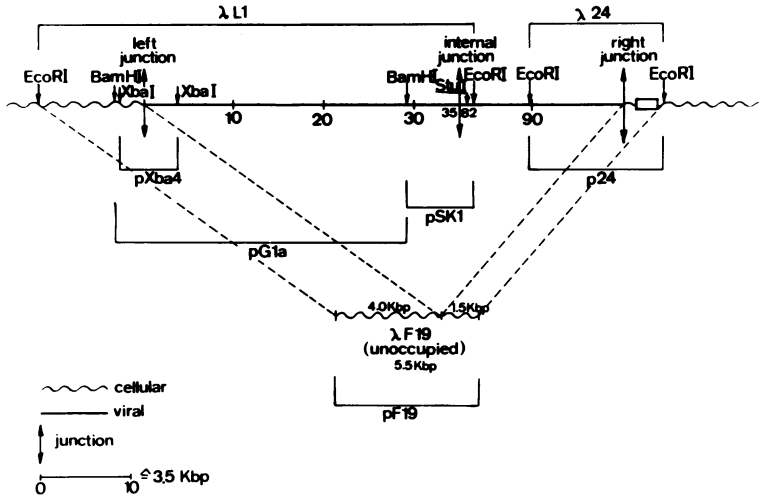

Fig. 1

Map of integrated Ad2 DNA in hamster cell line HE5. Both EcoRI junction fragments comprising viral and cellular DNA and a purely cellular fragment representing the unoccupied site of integration were cloned from HE5 DNA in λ vectors. The clones used for further analyses were λ24 (right junction), λL1 (internal and left junctions), and λF19 (unoccupied site). The EcoRI fragments from clones λ24 and λF19 were recloned in pUR2 to yield clones p24 and pF19, respectively. Parts of the λL1 clone were subcloned in pUR250 to yield clone pXba4 or in pBR322 to yield clones pG1a and pSK1. The subclone pSK1 carries the joint between the remaining Ad2 fragments at the internal deletion which ranges from map unit 35 to 82. DNAs of the clones p24, pXba4, pF19 and pSK1 have been used to sequence the sites of junction between the right and left viral termini and cellular DNA sequences, the cellular sequence at the site of insertion, and the site of the internal viral junction, respectively, as indicated.

restriction endonucleases were determined (data not shown). For further analyses parts of the cloned fragment were subcloned in plasmids. The fragments cloned in pUR250 (pXba4) and in pBR322 (pG1a and pSK1) by conventional methods are indicated in the map of Fig. 1.

Comparison of hamster cell DNA sequences in the λ clones containing the left junction, the right junction and the unoccupied site DNA fragment

The DNAs from the clones containing the left junction site, the right junction site and the unoccupied cellular site of insertion

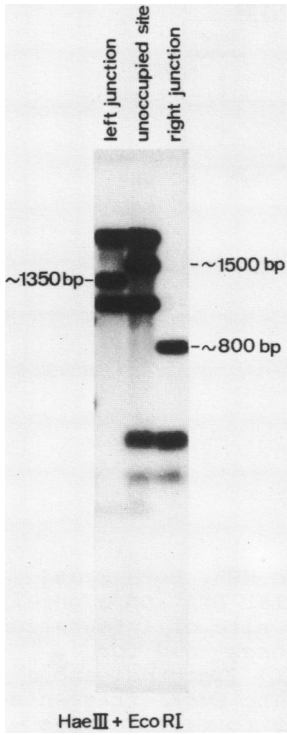

Fig. 2

Comparison of hamster cell DNA sequences present in the left and right junction clones, as well as in the clone containing the purely cellular DNA fragment from the unoccupied site. DNAs of all three λ clones (λL1, λF19, λ24, cf. Fig. 1) were cleaved with EcoRI and HaeIII. The DNA fragments were separated on an agarose gel, transferred to a nitrocellulose filter, hybridized to the purely cellular DNA fragment recloned in pUR2 (pF19, cf. Fig. 1) which was [^{32}P]-labeled by nick translation. The filter was autoradiographed. The lengths of individual fragments were estimated using as markers subfragments of clones λ24 and λF19, which had been sized previously.

were cleaved with restriction endonucleases EcoRI and HaeIII simultaneously. The fragments were separated on an agarose gel, transferred onto a nitrocellulose filter and hybridized to the [^{32}P]-labeled unoccupied site fragment recloned in pUR2 (cf. Fig. 1, and 9). From the autoradiographic data shown in Fig. 2, we concluded that viral DNA had been integrated into a cellular HaeIII fragment of about 1.5 kilo basepairs since this fragment was displaced in the junction fragments to different molecular weight positions. Integration of viral DNA led to the generation of a left- and a right-terminal HaeIII junction fragment comprising viral and cellular DNAs of about 1.35 kbp (kilo basepairs) at the left and 0.8 kbp at the right junction (Fig. 2). The cellular DNA sequences flanking the inserted viral DNA were probably unaltered, as suggested by the finding that the remaining cellular fragments of the unoccupied site clone comigrated with cellular DNA fragments of either the left or the right junction site.

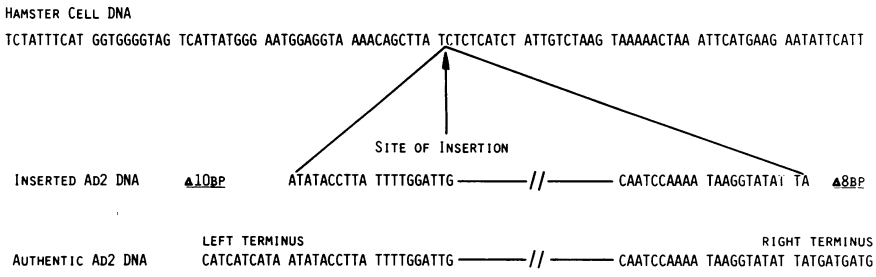

Fig. 3

DNA sequences at the site of insertion of Ad2 DNA into the cellular DNA of hamster cell line HE5. No cellular nucleotides were deleted at the site of integration, but 10 left terminal and 8 right terminal viral nucleotides were missing at the sites of junction (marked by ↑). The viral genome is schematically presented by its terminal sequences and an interrupted line designating the remainder of the genome with an internal deletion ranging from map units 35 to 82.

Sequence analysis of the site of viral DNA integration into hamster cell DNA

The nucleotide sequence at the right terminal site of junction between viral and cellular DNA and the sequence of the corresponding cellular sequence at the unoccupied site have been described earlier (9). In order to determine the sequence at the left terminal viral-cell DNA junction, DNA of subclone pXba4 (Fig. 1) was 5'-labeled at a unique BalI site representing the conserved viral BalI site at a distance of 268 nucleotides from the left terminus of Ad2 DNA. The nucleotide sequence was determined towards the left junction site. This sequence starting at basepair 263 of Ad2 DNA and the published left terminal sequence of Ad2 DNA (27) were colinear up to viral basepair 11. Ten terminal viral nucleotides were deleted. The cellular sequence continuing toward the left from the conserved viral sequence at the left terminal site of junction was identical with the cellular sequence of the unoccupied cellular site (9). By superimposing the nucleotide sequences at the unoccupied cellular site and the cellular sequences at the right (9) and left junction sites (Fig. 3), it became apparent that all cellular nucleotides at the site of viral DNA integration were conserved. Deletions, rearrangements, or repetitions of cellular nucleotides could not be de-

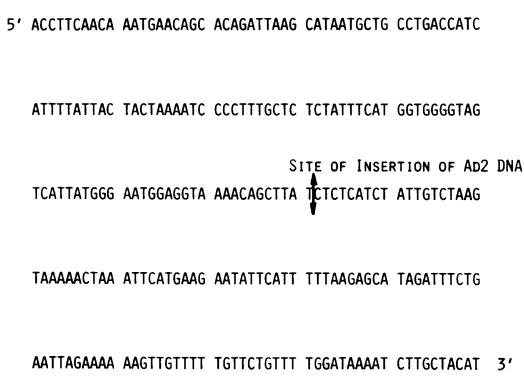

Fig. 4
Unique hamster cell DNA sequence from the site of insertion (↕) of Ad2 DNA. This sequence was determined from subclone pF19 and was found to be identical to the corresponding cellular sequences at the right and left junction sites.

tected. In contrast, 10 left terminal and 8 right terminal (9) viral basepairs had been deleted. The viral sequences continuing from nucleotides 9 (right terminus) and 11 (left terminus), respectively, were colinear with the authentic Ad2 DNA. In a previous publication (9), the viral deletion at the right terminus was inadvertantly stated to comprise only 5 instead of 8 basepairs. We subsequently corrected this error (28). A total of 250 cellular nucleotides at the site of insertion of Ad2 DNA are listed in Fig. 4. This cellular sequence of the unoccupied site was determined using subclone pF19 (Fig. 1) and sequencing both strands of DdeI subfragments after strand separation. The site of viral DNA insertion has been marked by a double-headed arrow and is located between nucleotides 131 and 132. Cellular nucleotide sequences at the left and right junction sites were shown to be identical to the corresponding sequences at the unoccupied cellular site. A computer search for nucleotide homologies between Ad2 sequences and the cellular sequence at the site of integration was performed as detailed previously (9). Except for short patch type homologies at the right and left junction sites (9), extensive homologies between viral and cellular DNAs at the immediate site of junction did not exist. Secondary structures near or at the sites of junction or in the unoccupied site DNA were not found.

Nucleotide sequence analysis of the internal junction of viral DNA sequences

The subclone pSK1 (Fig. 1) spans the internal linkage of Ad2 DNA in cell line HE5. This joint connects the remaining viral frag-

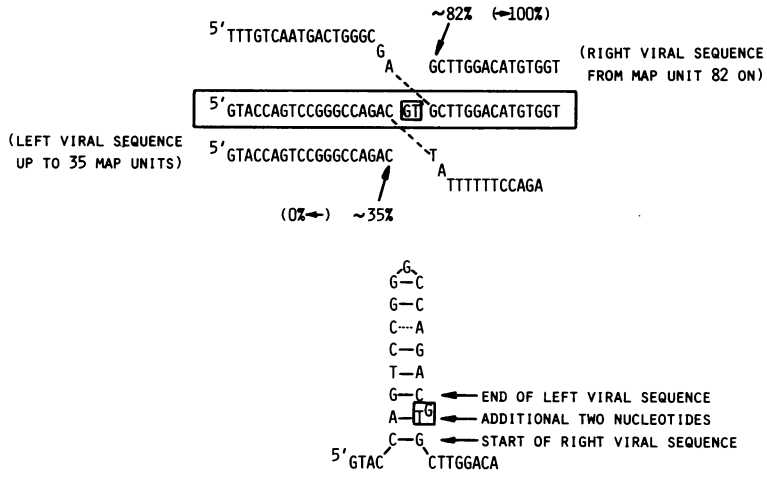

Fig. 5

DNA sequence at the site of linkage between viral DNA fragments at the internal deletion ranging from Ad2 DNA map unit 35 to 82. The sequence at the junction site (box) and an additional dinucleotide (GT) were indicated. Sequences outside the box and connected by dashed lines represented the immediately adjacent authentic viral sequences that had been deleted in the DNA of cell line HE5. A region of dyad symmetry was apparent at the site of linkage (bottom). This structure may not be stable.

ments. An internal deletion has been previously noted (3). Subclone pSK1 was used for the sequence determination at the internal junction. The DNA was [^{32}P]-labeled at the 5' ends of a unique StuI site and sequenced through the internal junction (Fig. 1). A total of 46 nucleotides was determined. This sequence was identical to the known authentic sequences of Ad2 DNA at around map units 35 and 82 (29, 30). As shown in detail in Fig. 5, the truncated viral sequences at the internal site of deletion were linked by the dinucleotide GT of unknown origin. A computer search did not reveal homologies between the deleted and the conserved viral DNA sequences at or near the site of junction. A short region of dyad symmetry was apparent at the site of linkage (Fig. 5).

DISCUSSION

Adenoviral DNA can be integrated at many different sites in the

mammalian genome (5, 6). From previously published results, it has been difficult to design comprehensive models about the viral DNA insertion event, since one could not rule out the possibility that the sites of viral DNA integration might have been post-integrationally altered by rearrangements, deletions or amplifications. A number of adenoviral integration sites have been characterized at the nucleotide level by cloning and sequencing sites of junction between viral and cellular DNAs (8, 9, 10, 15, 31). Similarly, an interviral DNA linkage between integrated adenovirus type 5 (Ad5) termini from an Ad5-transformed rat cell line has also been cloned and sequenced (32). In two instances, in the Ad2-transformed cell line HE5 (9), and in the adenovirus type 12-transformed hamster tumor line CLAC1 (10), the unoccupied cellular nucleotide sequences corresponding to the sites of insertion have also been cloned and sequenced. Comparisons of adenoviral and cellular nucleotide sequences at several sites of linkage have revealed more or less extensive patch-type homologies at or close to the site of junction (8-10). Two types of patch homologies have been noted: One between the deleted terminal viral DNA sequence and the cellular DNA sequence replacing it (10), another one between the persisting viral and the adjacent cellular DNA sequences (8, 9). The biological significance of these patch homologies was not certain.

In the present report a complete sequence analysis of the right (9) and left adenoviral-cellular DNA junction sites, of the unoccupied cellular sequence corresponding to the site of insertion, and of the internal viral junction linking the two remaining Ad2 DNA fragments in the hamster cell line HE5 has been presented. The comparison of cellular sequences at the unoccupied site of insertion and at the sites of viral-cellular junction has revealed a perfect conservation of cellular nucleotides at the site of adenoviral insertion. Not a single cellular nucleotide has been deleted. Based on this information, a detailed search for homologies between viral and cellular sequences at or near the sites of insertion was conducted. Except for short patch homologies at the right and left terminal cell-viral junction sites, extensive homologies or conspicuous secondary structures were not apparent. Since viral DNA integration in

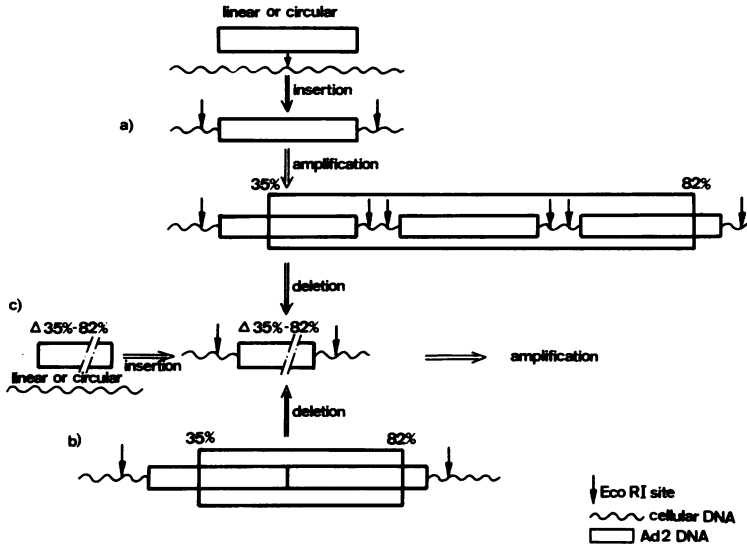

Fig. 6

Possible models describing insertion and amplification of Ad2 DNA in cell line HE5.

a) A linearly inserted, intact viral genome was amplified and the internal part of the amplified viral and cellular DNA sequences were deleted as depicted in the scheme. Finally, the remaining viral and the adjoining cellular sequences were amplified.
b) Tandemly arranged, multiple copies of intact Ad2 DNA were integrated and subsequently partly deleted. Finally, the remaining viral and the adjoining cellular sequences were amplified.
c) Viral DNA internally truncated prior to the integration event was subsequently amplified.

These models are to be considered working hypotheses at the present time.

cell line HE5 led to a clean insert without deletions or rearrangements of cellular nucleotides, the structure at the sites of junction must reflect the situation right after the integration event. There is clearly no evidence for secondary postintegrational alterations in this cell line. Moreover, short duplications of cellular nucleotides at the sites of integration have not been seen. Such duplications have been reported in the integration of retroviral genomes (33). At least for Ad2 DNA integration in cell line HE5, we can therefore conclude that the mechanism of insertion is different from that of bacteriophage λ

in E. coli (7) and different from that of retroviruses (33).

It has been shown earlier that 2 to 3 copies of Ad2 DNA per HE5 cell persist (2, 3). The simplest explanation for the presence of multiple viral genome copies is the postintegrational amplification of the inserted viral DNA together with the adjoining cellular DNA sequences. The internal truncation of viral DNA might be due to one of the following events (Fig. 6). a) Amplification of a single copy of integrated intact Ad2 DNA and subsequent deletion of viral and cellular DNA with break points inside viral DNA followed by subsequent amplification (Fig. 6a). b) Integration of multiple, tandemly arranged, intact Ad2 DNA molecules and subsequent deletion of viral DNA sequences as indicated followed by amplification (Fig. 6b). c). Integration of a viral DNA molecule that carried already the internal deletion and subsequent amplification of viral and cellular sequences (Fig. 6c). The preexisting deletion could have been introduced by UV-irradiation of Ad2 virions used in transformation (1).

The fact that the region of contact between viral and host DNA is limited to the viral termini and two neighboring cellular nucleotide pairs at the site of insertion is consistent with a circular intermediate of viral DNA that is formed prior to or at least during the recombination process, perhaps without covalent linkage of the viral termini. The circular structure could possibly be mediated by the terminal viral protein. It is also possible that both viral termini contact the insertion site in immediate succession without actually ever circularizing the viral DNA molecule.

A common feature of integrated adenoviral DNA is the deletion of terminal viral nucleotides ranging from 2 up to 174 base pairs at different sites of junction examined (6, 8-10, 15, 36). In this context, the results of a recently performed sequence analysis (35) might be of interest. Terminal duplications of left end sequences transposed to the right end of the genome were observed in frequently passaged preparations of adenovirus type 16 (Ad16) (34, 35). The internal junction between the right and left terminal viral nucleotide sequences revealed a deletion of eight right terminal nucleotides in both Ad16 variants examined (35). The same number of nucleotides were deleted in cell

line HE5 at the right terminal junction of Ad2 and cell DNAs. Ten nucleotides were deleted at the left site of junction. In an Ad12-induced mouse tumor, CBA-12-1-T, nine viral nucleotides were deleted at the left site of junction (6, 36). It is tempting to consider the possibility that a hot spot of recombination might exist around nucleotides 8-10 at the viral termini. This spot happens to be located just in front of a sequence comprising ten base pairs (ATAATATACC) which are highly conserved in all human adenoviruses. This particular sequence is considered important as a signal in the initiation of viral DNA replication (37-40).

At the site of junction of the internally truncated viral genome, an insertion of two nucleotides of unknown origin has been observed. Similar insertions have also been noted at junctions between non-homologously recombined SV40 and pBR322 DNA molecules in CV1 monkey kidney cells (41). It is still unknown whether such insertions are haphazard or point to a complex recombination event possibly involving other (cellular?) sequences.

Preliminary results indicated that the cytoplasmic RNA from HE5 and LSH primary hamster embryo cells hybridized to the cloned cellular DNA from the unoccupied site of insertion in cell line HE5. This RNA appeared to be equal in size in transformed HE5 cells and in untransformed primary LSH hamster embryo cells (data not shown).

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