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Integration of foreign DNA into mammalian genome can be associated with hypomethylation at site of insertion

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Summary

The methylation patterns in the genome of mammalian cells are remarkably stable, although occasional changes are observed. In mammalian cells, the non-methylated DNA of human adenovirions (Günthert et al., 1976) undergoes de novo methylation after integration into the host hamster genome (Sutter et al., 1978). The establishment of these specific patterns of methylation in the integrated adenovirus sequences (Sutter and Doerfler, 1979, 1980) requires a considerable number of cell divisions after integration (Kuhlmann and Doerfler, 1982, 1983). Recently, we have reported the analysis of the site of linkage between the left terminus of adenovirus type 12 (Ad12) DNA and unique hamster DNA in the Ad12-induced tumor T1111(2) (Lichtenberg et al., 1987). In what way, if any, are the methylation patterns of the adjacent cellular DNA affected by the insertion of unmethylated foreign (adenoviral) DNA? In normal hamster kidney and spleen DNA and in several Ad12-transformed hamster cell lines, this preinsertion sequence is completely methylated at the 5′-CCGG-3′ (*Hpa*II) and 5′-GCGC-3′ (*Hha*I) sequences. The same preinsertion sequences in the DNA of cell line BHK21 and on the non-occupied chromosome in the tumor cell line H1111(2) in passage 9 (p9) are almost completely methylated. In contrast, the same sequence on the chromosome, that carries the integrated Ad12 DNA sequence in the tumor T1111(2), is unmethylated at the 5′-CCGG-3′ and 5′-GCGC-3′ sequences, as are the abutting Ad12 DNA sequences. Thus, the insertion of unmethylated foreign DNA can lead to the hypomethylation of the flanking cellular DNA in the target sequences.

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Foreign DNA can be inserted at many different sites in the genome of mammalian DNA. There is evidence that the transcriptional activity of cellular DNA may predispose a certain chromatin structure to recombination with foreign DNA (Gahlmann et al., 1984; Schulz et al., 1987). It has been demonstrated for several eukaryotic genes that specific promoter methylation can lead to the inactivation of genes (for reviews see Doerfler, 1981, 1983; Doerfler et al., 1988). Methylated CpG sequences in the mammalian genome appear to be clustered in the 5′ flanking sequences of genes (Doerfler et al., 1982; Bird, 1986). When foreign DNA is inserted into the mammalian genome, the methylation pattern in the flanking sequences might be changed or remain unaltered, depending on the host site, on the inserted DNA, or on both. For the insertion of Moloney murine leukemia provirus DNA into mouse DNA, the induction of de novo methylation of the flanking host sequences has been domonstrated (Jähner and Jaenisch, 1985). This methylation correlates with gene inactivation. In the present report, we demonstrate that upon insertion of an unmethylated Ad12 genome into hamster DNA in the Ad12-induced tumor T1111(2), the flanking cellular DNA sequences become hypomethylated. The same cellular sequences are much more heavily methylated in normal hamster tissues or in a number of established hamster cell lines, as well as in the allelic, unoccupied chromosome in the tumor T1111(2). Insertion of unmethylated foreign DNA entails demethylation of the abutting cellular sequences. The hamster cell DNA corresponding to the T1111(2) integration site that is immediately adjacent to the integrated Ad12 DNA is not transcribed in several Ad12-transformed hamster cell lines and in cell line BHK21 (Lichtenberg et al., 1987).

The maps in Fig. 1 present details of the linkage site between Ad12 DNA and hamster DNA in the hamster tumor T1111(2) (p17) and of the preinsertion site in hamster DNA (p16) (Lichtenberg et al., 1987). The preinsertion site belongs to the unique type of hamster DNA sequences and represents that segment into which the Ad12 DNA molecule has been inserted. This segment is surrounded by two different repetitive sequence elements which are depicted in Fig. 1. (i) An intracisternal A particle (IAP) genome is located about 600 basepairs to the left of the site of insertion and is represented as a cross-hatched box. (ii) About 600 basepairs to the right, another repetitive DNA element has been partly sequenced (Fig. 1) which shows 80% homology to the Alu-equivalent repetitive sequence in Chinese hamster DNA (Haynes et al., 1981). The maps also show the locations of the cloned DNA fragments that have been used as hybridization probes in the present study. The sites of linkage between viral (filled-in box) and cellular DNA have been explained in the legend to Fig. 1. The hamster preinsertion sequence contains several 5′-CCGG-3′ (*Hpa*II/*Msp*I) and 5′-GCGC-3′ (*Hha*I) sites as designated in Fig. 1. Restriction endonucleases *Hpa*II and *Hha*I do not cut the methylated sequences

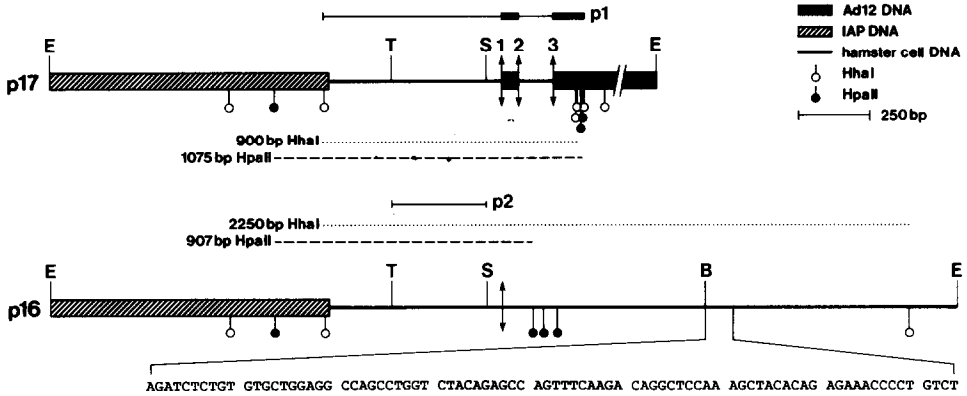

Fig. 1. Maps of the junction site between AD12 DNA and hamster cell DNA from tumor T1111(2) (p17) and of the cellular preinsertion site (p16). Tumor T1111(2) was induced in newborn hamsters by injection of Ad12 virions (Kuhlmann et al., 1982; Kuhlmann and Doerfler, 1983). One of the approximately ten copies of Ad12 DNA that were integrated into cellular DNA proved unstable upon passage of cells derived from this tumor in culture. In a previous report (Lichtenberg et al., 1987), we have described the anatomy of this junction sequence and of the corresponding preinsertion sequence in detail. The 7.25 kbp p17 fragment contains the junction site, the 3.1 kbp p16 fragment the corresponding preinsertion sequence into which the Ad12 genome was inserted. The restriction sites relevant to this study are as designated: B, *Bgl*II; E, *Eco*RI; S, *Sph*I; T, *Taq*I. The double-headed arrows indicate the site of insertion (p16) or the sites of junction (p17). In p17, there are three such sites. From left to right these sites are (1) the junction between hamster DNA and an inverted Ad12 DNA fragment comprising nucleotides 1361 to 1290, (2) the junction between this Ad12 DNA fragment and 127 bp of cellular DNA, and (3) the junction between the latter DNA and nucleotide 65 at the left terminus of the integrated Ad12 genome (interrupted scale). For details see Lichtenberg et al. (1987). The exact locations of the hybridization probes p1 (*Hha*I fragment) and p2 (*Taq*I—*Sph*I fragment) have been indicated. The maps also indicate the sizes of the *Hpa*II (dashed line) or *Hha*I (dotted line) DNA fragments generated upon cleavage with these restriction endonucleases and their precise locations. The nucleotide sequence of part of the hamster Alu-equivalent repeat has been determined in a cloned *Bgl*II fragment. IAP designates the endogenous sequence of the hamster internal A particle genome.

5′C^{m}CGG-3′ and 5′-G^{m}CGC-3′, respectively, whereas *Msp*I cuts the former sequence (Waalwijk and Flavell, 1978). Due to the insertion of viral DNA, fragment lengths of *Hpa*II and *Hha*I cleavage products are different at the occupied and the non-occupied hamster sites. Thus, fragments of the preinsertion site on the unoccupied chromosome and of the integration site on the chromosome, that carries the Ad12 genome, can be distinguished by sizes. Fragment lengths from the DNA of cells, that are devoid of viral genomes, are identical in size to fragments from the unoccupied chromosome in the Ad12-induced tumor T1111(2).

DNA has been analyzed from the tumor T1111(2) and from a cell line H1111(2) established from this tumor, in passages 3 (p3) and 9 (p9), furthermore DNA from hamster kidney and spleen and from the Ad12-transformed cell line T637 which carries viral DNA molecules in cellular sequences different from those in the tumor T1111(2). The DNA was extracted by the standard SDS-proteinase K-phenol method (Sutter et al., 1978). DNAs from hamster organs were extracted after freezing the organs in liquid nitrogen and pulverizing the tissue in a mortar (Blin

and Stafford, 1976). Subsequently, about 40 μg of cellular DNA was first cleaved with *Eco*RI (sites cf. Fig. 1) and ethanol precipitated. The fragments (10 μg) were then further cut with *Hpa*II or *Msp*I, or with *Hha*I. Marker DNAs (p16 and p17 DNA in amounts of about 10 genome equivalents per cell) were cleaved with the same combination of restriction enzymes and mixed with 10 μg of ultrasonically treated salmon sperm DNA as carrier. DNA fragments were separated by electrophoresis on 1% agarose gels, and the DNA fragments were blotted onto nitrocellulose filters (Southern, 1975). The hybridization probe p2 was a ~ 340 bp fragment of cellular DNA adjacent to the site of integration (cf. Fig. 1). This fragment was excised from the M13 vector DNA, purified by agarose gel electrophoresis and ^{32}P-labeled by nick translation (Rigby et al., 1977). Hybridization conditions were similar to those described (Wahl et al., 1979). The hybridization temperature was 42° C, and filters were washed at 50° C.

The autoradiographs in Fig. 2 demonstrate the *Hpa*II/*Msp*I DNA fragments and the *Hha*I fragments generated when DNAs from the different cells were cleaved, and when the fragments were visualized by hybridization to the probe p2 (Fig. 1). The 7.25 kilobase pair (kbp) fragment represents the *Eco*RI junction fragment in the tumor T1111(2) which has been cloned in the plasmid p17 (Fig. 2b, d). The 3.1 kbp *Eco*RI-fragment (Fig. 2b) corresponds to the preinsertion site segment on the unoccupied chromosome which has been cloned in plasmid p16. The same fragment is also apparent in the DNA from hamster kidney and spleen cells or from cell lines BHK21, T637, and H1111(2) (Fig. 2).

Upon cleavage with *Hha*I, the 3.1 kbp *Eco*RI-fragment is not recut in the DNAs of any of the cell lines examined. Thus the expected 2.25 kbp *Hha*I-fragment from the preinsertion site is not detectable in the autoradiographs (Fig. 2a, b, except in lanes p16 × *Hha*I). In contrast to DNA from the preinsertion site, in the tumor T1111(2) the 7.25 kbp *Eco*RI junction fragment disappears upon cleavage with *Hha*I and the expected 900 bp *Hha*I fragment corresponding to the junction site is visualized (Fig. 2b).

Similar findings indicating extensive methylation of the preinsertion site have been obtained after cleavage with *Hpa*II (Fig. 2c, d). Upon cleavage with *Eco*RI and *Hpa*II, the 3.1 kbp preinsertion fragment does not disappear in the DNA of any of the cells examined. *Msp*I-cleavage results in a complete disappearance of the *Eco*RI preinsertion fragment. Instead, a 907 bp fragment is visible (Fig. 2c, d). Again, in contrast to the preinsertion fragment, in the tumor T1111(2) the 7.25 kbp *Eco*RI junction fragment is cleaved by *Hpa*II, and a 1075 bp *Hpa*II-fragment is generated (Fig. 2d) which appears also after *Msp*I treatment.

In the DNA from cell line BHK21 (Fig. 2d) and from the tumor line H1111(2) (Kuhlmann et al., 1982) in passage p9 (Fig. 2c), weak additional *Hpa*II fragments are generated which might indicate that in a subset of the cells the 5′-CCGG-3′ sites are not completely methylated. Similarly, the weak additional *Hha*I bands in hamster kidney DNA, in DNA from BHK21 cells or from the tumor T1111(2) (Fig. 2b) can be explained by some of the *Hha*I sites in a minority of cells in which the DNA is not completely methylated.

The results presented characterize the state of methylation in the hamster DNA

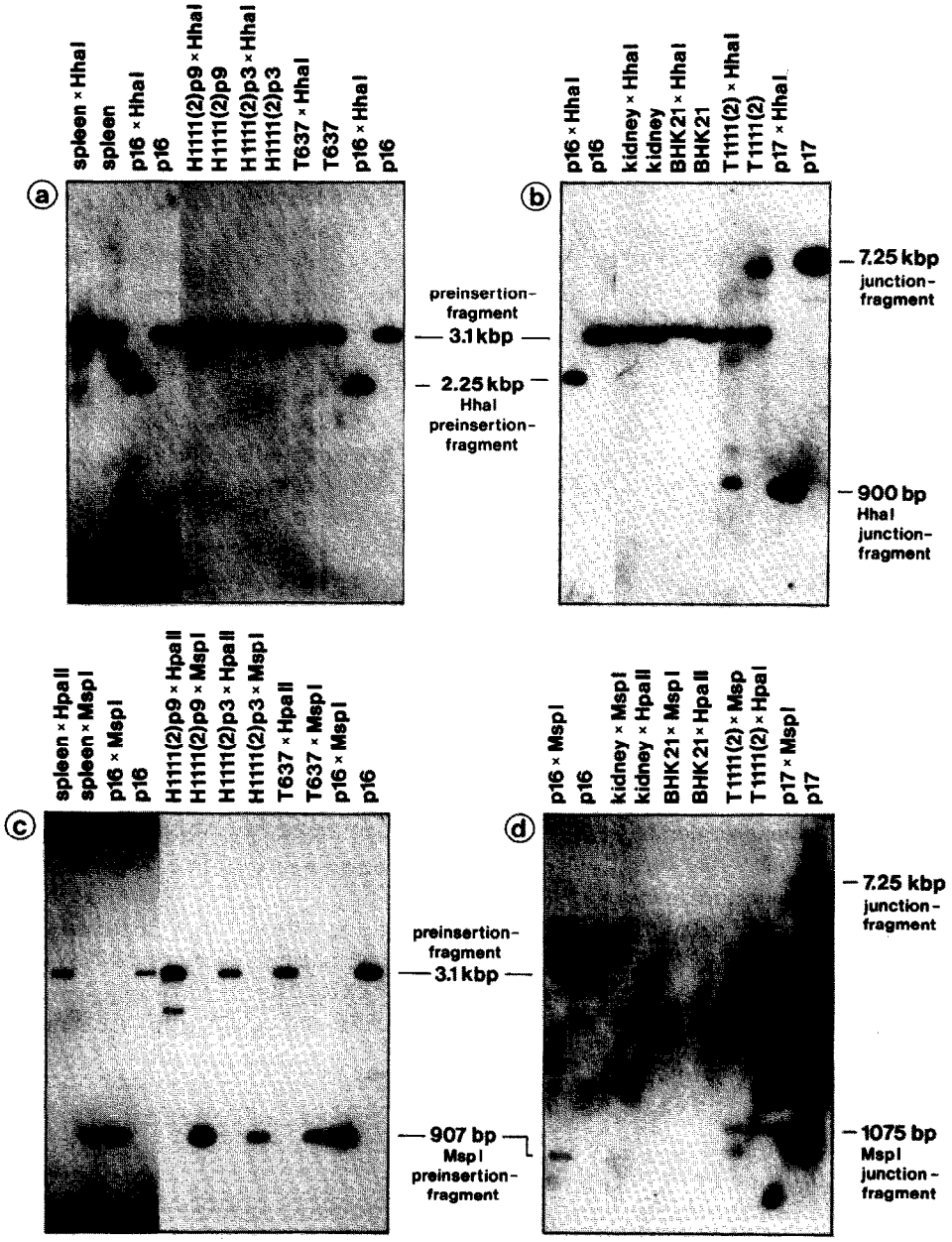

Fig. 2. Cleavage patterns of various hamster DNAs with *Hha*I (a, b) or *Hpa*II/*Msp*I (c, d) and visualization of fragments with the p2 DNA fragment (Fig. 1) as hybridization probe. Experimental details have been described in the text. Sources of DNA and restriction endonucleases used have been indicated for each lane. DNAs in all lanes have been precut with *Eco*RI and subsequently cleaved with restriction endonucleases as indicated or have not been further treated. The hamster kidney and spleen DNAs have been extracted from organs of animals that carried Ad12-induced tumors. The DNAs from these organs have, however, been shown to be devoid of Ad12 DNA sequences (data not shown).

sequence that lies immediately adjacent to the integrated Ad12 DNA sequence in the tumor T1111(2). *Hpa*II/*Msp*I and *Hha*I restriction analyses of the integrated Ad12 DNA molecule have previously shown that the integrated viral DNA is hypomethylated (Kuhlmann and Doerfler, 1983). This finding has been corroborated in the present study by using the Ad12 DNA containing pl insert (Fig. 1) as hybridization probe (data not shown). In the tumor T1111(2) the cellular DNA segment abutting this integrated viral genome has become significantly demethylated. In primary hamster DNA from isolated organs and in the DNA from hamster cell lines devoid of Ad12 DNA or carrying the Ad12 genomes at sites different from those in the tumor T1111(2), the same sequences in the DNA segment corresponding to the T1111(2) junction sequence are strongly or completely methylated at the *Hpa*II and *Hha*I recognition sequences investigated. It is striking that the demethylation in the T1111(2) sequences affects only the DNA on the chromosome that has been occupied by the foreign (Ad12) DNA molecule and not the DNA on the non-occupied chromosome. Experimentally, the allelic cellular sequences can be distinguished due to differences in restriction fragment lengths that are caused by insertion of Ad12 DNA on one of the two chromosomes. Apparently, the hypomethylated state of the inserted viral chromosome can influence the extent of methylation in its immediate vicinity. We do not know how this effect is exerted on the selective activity of the DNA methyltransferase, that is responsible for maintaining a given level of DNA methylation. There is no evidence that the previously transcriptionally inactive cellular sequence at the site of junction was activated concomitantly with demethylation. Obviously, there are no simple, generally applicable rules to predict whether foreign (e.g. viral) DNA sequences, which have been inserted into mammalian DNA, mimick the methylation patterns of their cellular environment or impose their own methylation pattern on that of their environment as demonstrated here. As a third alternative, evidence has been adduced previously that foreign DNA induced de novo methylation of flanking host sequences (Jähner and Jaenisch, 1985).

It has been indicated that in genomic imprinting allelic DNA sequences on different chromosomes can differ significantly in their patterns of methylation (Reik et al., 1987; Sapienza et al., 1987; Swain et al., 1987). Thus cellular mechanisms seem to exist that generate or maintain differences in the extent of DNA methylation in allelic sequences.

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