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Loss of Viral Genomes from Hamster Tumor Cells and Nonrandom Alterations in Patterns of Methylation of Integrated Adenovirus Type 12 DNA

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The insertion stability and DNA methylation patterns of integrated adenovirus type 12 (Ad12) genomes were investigated in Ad12-induced tumors and in tumor cell lines established from them as a function of time of passage under culture conditions. Upon subcultivation of cells from some of the tumors, the viral genomes were eliminated, apparently in a stepwise process with segments of the left termini of Ad12 DNAs persisting the longest. Morphological variants of these tumor cells lost all viral DNA and yet retained the oncogenic phenotype. All 13 independently isolated clones from one revertant line were devoid of Ad12 DNA. It could not be ruled out that very short sequence elements of viral DNA, such as promoters or enhancing sequences, could have persisted in these variants. The extent of viral DNA methylation was minimal in Ad12-induced tumors, although the viral genome was not extensively expressed, if at all. Upon passage in culture, the levels of viral DNA methylation increased. It was interesting that establishment of the final methylation pattern of integrated Ad12 DNAs required many cell generations after the fixation of foreign DNA in the host genome. The shift in methylation was nonrandom. The late parts of the inserted viral genomes became methylated more extensively than did the early gene segments.

The results of a detailed study of the integration and methylation patterns of adenovirus type 12 (Ad12) DNA in 39 Ad12-induced tumors and cell lines established from these tumors have been reported previously (9, 10). In over 70 different adenovirus-transformed cell lines and Ad12-induced tumors or tumor lines, the site of viral DNA insertion differs from cell line to cell line (3). In general, the patterns of viral DNA insertion have proven to be quite stable for a given cell line (16). However, morphological variants of Ad12-transformed hamster cells (6) or of Ad12-induced hamster tumor cells (9) have been observed which appeared more fibroblastic than the original tumor cells. Although some of these variants have been characterized by the complete elimination of viral DNA from the host genome (5, 9), these cells retain their oncogenic phenotype upon injection into hamsters (9). From these findings, it has been concluded that the oncogenic phenotype is compatible with the complete loss of Ad12 DNA from cells that had originally been transformed by Ad12. Presumably, in hamster cells, Ad12 infection triggers hereto unknown mechanisms that entail oncogenic transformation, and this transformation is accompanied by fixation of multiple copies of viral DNA. At least in the cells in which the viral genome was completely lost, stability of the oncogenic phenotype is not premised on the persistence of viral genes (3, 9). Conceivably, the decisive transforming events are elicited by the fixation of viral genes, expression of viral genes, or both, but the continued realization of transformation once initiated may be independent of viral functions.

There is now extensive evidence for the notion that DNA methylation in highly specific sequences can play a decisive role in the long-term shutoff of genes in eucaryotes (for recent reviews, see reference 4 and W. Doerfler, Curr. Top. Microbiol. Immunol., in press). It appears that the absence of DNA methylation is a necessary but not sufficient precondition for gene expression to proceed (9, 17). This latter concept had been supported by the striking observation that the levels of Ad12 DNA methylation in Ad12-induced tumors were very low, if DNA methylation could be detected at all, at 5'-CCGG-3' and 5'-GCGC-3' sites (10). However, viral gene expression not at all detectable in these tumors or at an exceedingly low level is not commensurate with the low level of DNA methylation (10). Upon explantation and continued cultivation of these tumor cells, the extent

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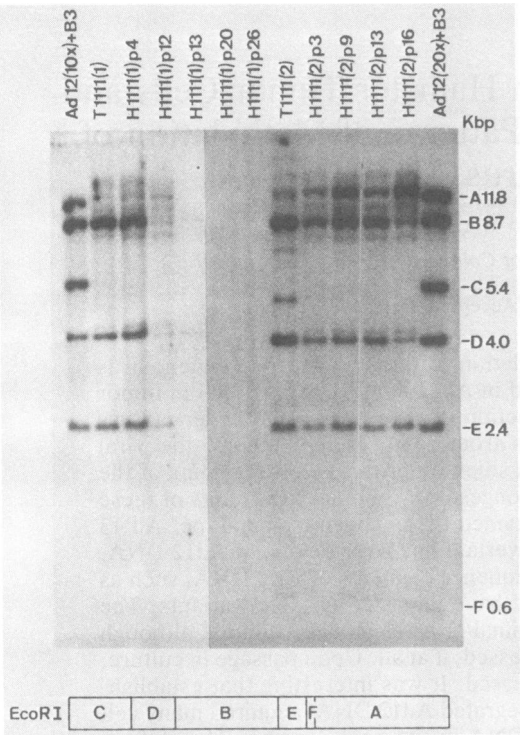

FIG. 1. Integration patterns of viral DNA in the Ad12-induced hamster tumors T1111(1) and T1111(2) and in cell lines H1111(1) and H1111(2) established from these tumors, respectively. DNA was extracted from tumors or from cells at stages of passage (p) after explantation from the tumors as indicated. DNA preparations (10 μg) were cleaved with *Eco*RI, the fragments were separated by electrophoresis on 0.5% agarose gels and blotted (13) to nitrocellulose filters, and Ad12-specific fragments were visualized by hybridization to ^{32}P-labeled (11) Ad12 DNA and autoradiography. Ad12 DNA (0.5 ng is equivalent to 20 genome equivalents [20×] per cell, corresponding to 10 μg of cellular DNA and the equivalent number of cells) was mixed with 10 μg of B3 (BHK-21) DNA and treated in the same manner. The authentic virion DNA fragments (A to F) and their sizes in kilobase pairs (Kbp), as well as the *Eco*RI map of Ad12 DNA, are also shown.

of DNA methylation in the integrated Ad12 sequences increases gradually (10).

In the present communication, we demonstrate that Ad12 DNA can be lost from tumor cells in a stepwise fashion with the left termini of the viral DNAs vanishing last. The increase in the levels of Ad12 DNA methylation in established hamster tumor cell lines was found to be nonrandom. The 5′-CCGG-3′ sites at the right termini of Ad12 DNAs were increasingly methylated. In this way, the Ad12 DNA methylation patterns approached those described earlier for Ad12-transformed cell lines cultured over long periods of time (8, 15). Viral gene expression in Ad12-induced tumors was minimal.

The induction of tumors by Ad12 in newborn hamsters and the establishment of cell lines from these tumors, as well as the methods used to determine patterns of viral DNA integration and methylation, have been described earlier (9, 10). In these experiments, the Southern blotting technique (13) and a DNA-DNA hybridization procedure (20) were used, employing nick-translated (11) ^{32}P-labeled Ad12 DNA or cloned viral DNA fragments (18). DNA methylation of integrated viral DNA sequences was detected by hybridization of ^{32}P-labeled Ad12 DNA to blotted tumor cell DNA that had previously been cleaved with the isoschizomeric restriction endonuclease pair *Hpa*II or *Msp*I (19) or with *Hha*I. In the latter instance, *Hha*I-cleaved Ad12 virion DNA, which is unmethylated (7), was used as a reference.

From 39 Ad12-induced tumors, 13 cell lines were established, and in 8 cases, the cell lines were carried continuously over 10 or more passages. In three lines, the patterns of integration and copy numbers of viral DNA per cell did not change. In three other lines, viral DNA was no longer detectable even after the first passage. One cell line was not analyzed further. In cell line H1111(2), only minor changes were observed (Fig. 1); e.g., some of the off-size bands disappeared. In cell line H1111(1), all 10 to 11 viral genome equivalents were lost in passage 20 (Fig. 1). In passage 12, 3 to 4 genome equivalents remained, exhibiting the same integration pattern as in previous passages. The number of Ad12 genome equivalents persisting in passage 12 of line H1111(1) was estimated from the intensity of the internal viral DNA bands as shown in Fig. 1 by a spectrophotometric scanning procedure as described previously (14). Both the internal Ad12 fragments and the Ad12-specific off-size bands were decreased in intensity. Thus, it was most likely that total Ad12 genomes had been lost during passage of the tumor cells. In passage 13 of cell line H1111(1), one to two copies of the left terminus of Ad12 DNA persisted, the *Eco*RI D fragment and an off-size fragment hybridizing to both termini. In this experiment, the molecularly cloned termini of Ad12 DNA, the *Eco*RI C and A* fragments (18), were used as hybridization probes (reference 9, cf. maps in Fig. 1 and 2b). In DNA from passage 20 onward, viral DNA could not be detected, even though these cells continued to be oncogenic in hamsters (9). The sensitivity of the blot hybridization experiments was such that one copy of the left 10% of the genome per cell would have been detected. In passage 12 of cell line H1111(1), 13 cellular clones were established. In the DNA of these clones, viral DNA

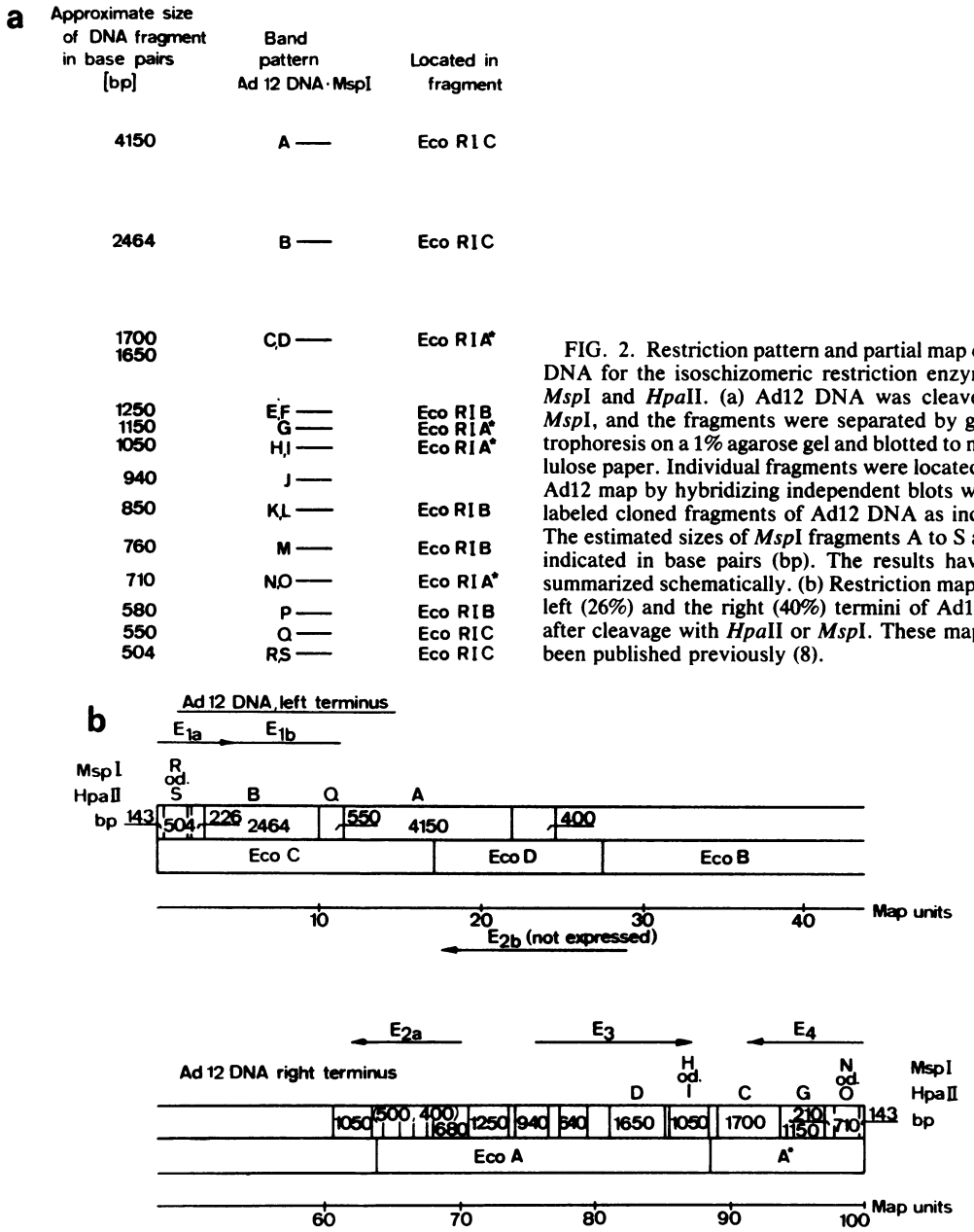

FIG. 2. Restriction pattern and partial map of Ad12 DNA for the isoschizomeric restriction enzyme pair *Msp*I and *Hpa*II. (a) Ad12 DNA was cleaved with *Msp*I, and the fragments were separated by gel electrophoresis on a 1% agarose gel and blotted to nitrocellulose paper. Individual fragments were located on the Ad12 map by hybridizing independent blots with ^{32}P-labeled cloned fragments of Ad12 DNA as indicated. The estimated sizes of *Msp*I fragments A to S are also indicated in base pairs (bp). The results have been summarized schematically. (b) Restriction maps of the left (26%) and the right (40%) termini of Ad12 DNA after cleavage with *Hpa*II or *Msp*I. These maps have been published previously (8).

could not be detected by Southern blotting, even when up to 300 μg of cellular DNA was blotted on nitrocellulose filters. In these hybridization experiments, the ^{32}P-labeled total Ad12 genome as well as cloned fragments, in particular the left terminal *Eco*RI C fragment (comprising the E1 region of Ad12 DNA) and *Eco*RI D fragment, were used as hybridization probes (for maps, see Fig. 2).

We conclude that viral DNA can be deleted apparently in a stepwise manner from Ad12-induced hamster tumor cells. The morphological variants that lost all viral DNA can retain their oncogenic phenotypes, although they do not carry the E1 region of Ad12 DNA. It will be very difficult to rigorously rule out the possibility that small elements of viral DNA, e.g., a promoter or enhancer sequence (2, 21), do persist in the variant cell line. It should be pointed out that the cell lines established from tumors were probably close to being clonal, as most tumors exhibited rather homogeneous integration patterns of

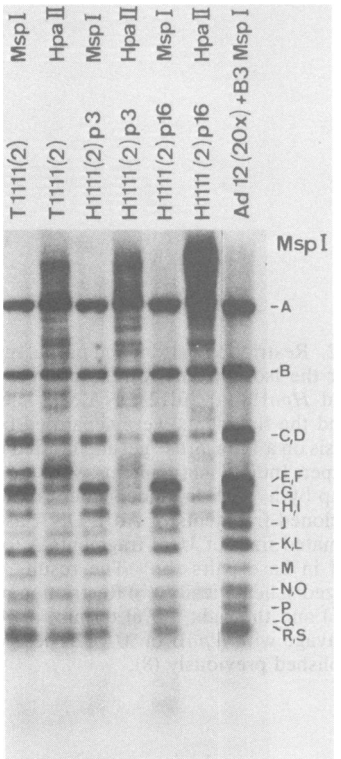

FIG. 3. Nonrandom changes of methylation patterns of Ad12 DNA in an Ad12-induced hamster tumor line. DNA was isolated from the Ad12-induced tumor T1111(2) and from cell line H1111(2) established from this tumor. DNA was purified in passage 3 (p3) or 16 (p16) of this cell line. All procedures used in DNA isolation have been reported elsewhere (10, 15). These DNA preparations (10 μg per slot) or 0.5 ng of Ad12 virion DNA (equivalent to 20 genome equivalents per cell), mixed with 10 μg of B3 hamster DNA as carrier, were cleaved with *Msp*I or *Hpa*II as indicated. Fragments were separated by electrophoresis on a 1% agarose gel, blotted (13), and hybridized to Ad12 DNA that was ^{32}P-labeled by nick translation (11). Letters A to S designate marker DNA fragments.

Ad12 DNA. Moreover, the cell lines described here were derived from cloned cells.

We also investigated in detail the shifts in viral DNA methylation that occurred upon cultivation of Ad12-induced hamster tumor cells. These shifts from low-level DNA methylation to more extensive levels did not occur randomly but followed certain patterns. The increase in Ad12 DNA methylation was surveyed by cleaving the DNA from tumor cells at various passages with *Hpa*II or *Msp*I, by gel electrophoresis and blotting, and by hybridization to ^{32}P-labeled Ad12 DNA. The *Msp*I (recognition sequence 5′-CCGG-3′) map of the left terminal 26% and the right terminal 40% of the Ad12 genome has been established previously (8). Although *Msp*I sites in the remaining 34% of the viral genome have not yet been mapped, the *Msp*I fragments E, F, K, L, M, and P were localized in the *Eco*RI B fragment by blotting *Msp*I-cleaved Ad12 DNA and by hybridization to the cloned *Eco*RI B fragment (18). In addition, fragment B harbored a large number of very small *Msp*I fragments. The schemes in Fig. 2a assigned map locations to *Msp*I fragments A to S (4,150 to about 504 base pairs in length). Figure 2b presents previously published partial *Msp*I maps (8).

The result presented in Fig. 3 actually demonstrated the increase in DNA methylation in the integrated Ad12 genomes by comparing the *Hpa*II and *Msp*I cleavage patterns of viral DNA from the tumor T1111(2) with those from cell line H1111(2) in passage 3 and passage 16. It was apparent that *Hpa*II bands predominantly from the (late) *Eco*RI B fragment and from the right terminal *Eco*RI A fragment started disappearing at low passage levels, whereas the *Hpa*II bands

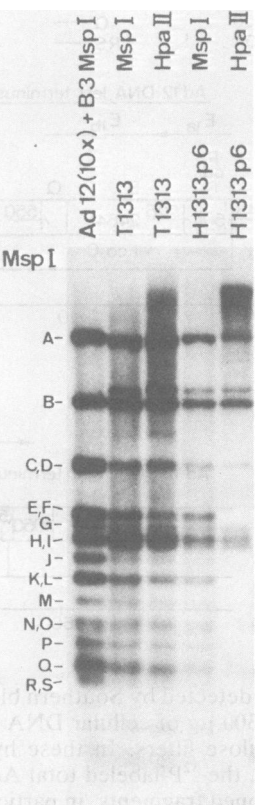

FIG. 4. Changes in methylation patterns in Ad12-induced hamster tumor line H1313. Experimental conditions were similar to those described for Fig. 3. T1313 and H1313 p6 refer to DNA from the Ad12-induced tumor T1313 and the cell line H1313 in passage 6, respectively.

in the early regions of the genome, particularly in region E1, remained unaltered. The data presented in Fig. 3 and 4 demonstrated that with increasing passage of cells in culture the *Hpa*II bands C, D, E, F, H, I, K, L, M, and probably smaller fragments as well started to disappear from passage 3 onward. Disappearance of *Hpa*II bands obviously signified methylation at the corresponding 5'-CCGG-3' sites. At higher passage levels, mainly the *Hpa*II sites in early regions of the integrated viral genome remained unmethylated. Thus, the pattern of DNA methylation approached that observed in Ad12-transformed cell lines (15). Similar results were presented for DNA from tumor T1313 and cell line H1313 (Fig. 4). It was interesting to note that establishment of the final pattern of methylation required many cell generations after fixation of the viral DNA in the host genome. Similar observations were made with other tumors and tumor lines and also after cleavage of the DNA with *Hha*I (10). In several of the Ad12-induced tumors and tumor lines a 1,400-base-pair off-size fragment was apparent that corresponded to the junction between the left terminus of viral DNA and cellular DNA. In some cell lines, the 5'-CCGG-3' sites flanking this fragment became methylated with continued passage; in other lines, the same sites remained unmethylated.

In Table 1, the results of many experiments are summarized. In general, the conclusion held that with increasing passage the late regions of the Ad12 genome were progressively methylated at 5'-CCGG-3' sites.

A total of 17 Ad12-induced tumors and 3 tumor cell lines were screened for the occurrence of Ad12-specific cytoplasmic RNA. By Northern blotting (1, 12), viral RNA was detected in only two tumors in small amounts (data not shown). Thus, the low level of viral DNA methylation did not correspond to full-fledged expression of the inserted viral genome. On the contrary, the viral genomes seemed to be completely shut off; not even the E1 region seemed to be expressed. It will be interesting to investigate what factors influence the levels of methylation of inserted Ad12 DNA and by what mechanisms the expression of the viral genome is controlled at early and late times after the fixation of foreign DNA in the tumor cells. Obviously, the stringency of these control mechanisms could change with time of passage under culture conditions.

TABLE 1. Shifts of DNA methylation at specific sites upon passage of Ad12-induced hamster tumor cells in culture

*MspI-Hpa*II fragment[c]	Increase[a] in the extent of DNA methylation at:					5'-CCGG-3' sites in DNA of Ad12-induced tumor cell lines[b]:						
	T313	H313 (p20)	T314	H314 (p4)	T412	H412 (p4)	T1111(1)	H1111(1) (p4)	T1111(2)	H1111(2) (p16)	T1112(1)	H1112(1) (p10)	T1313	H1313 (p6)
B		+												
C, D	++	++				++	+	+	+	++				+
E, F		+++				+++	+	++	+	+++				+++
G				+										
H, I	++	+++	++	++	+	+++	+	++	++	+++	+	+	+	
J		+++	+	+		+++	+	++		+++			+	+++
K, L		++	+	+		+++	+	+		+++	+	+		+++
M	+	+++	++	++	+	+++	+	+	+	+++	+	+	+	+++
N, O						+++				+				+

[a] The extent of methylation of the 5'-CCGG-3' sites has been graded from + to +++, indicating a gradual (+, ++) or nearly complete (+++) disappearance of individual *MspI-Hpa*II fragments from blots similar to the ones shown in Fig. 3 or 4. Disappearance of individual restriction fragments from the authentic gel position signifies methylation at one or both 5'-CCGG-3' sites bounding this DNA fragment.

[b] T and H followed by a number designate individual Ad12-induced tumors and hamster cell lines established from them, respectively. Number of passages (p) at time of analysis is also indicated.

[c] B to O refer to the *MspI-Hpa*II fragments of Ad12 DNA. Methylation was not detected with fragment A. The *MspI-Hpa*II fragments E, F, K, L, M, and P and many of the very small fragments are located in the *Eco*RI B fragment which contains predominantly late viral genes.

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