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# Methylation of integrated adenovirus type 12 DNA sequences in transformed cells is inversely correlated with viral gene expression

(differential DNA methylation/*Hpa* II/*Msp* I isoschizomers/Southern blot technique)

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ABSTRACT **The adenovirus type 12 (Ad12) DNA sequences integrated into the DNA of four lines of Ad12-transformed hamster cells are extensively methylated. Methylation in mammalian cell DNA is believed to occur predominantly at 5′-C-G-3′ sequences. The majority, although not all, of the 5′-C-C-G-G-3′ sequences present in integrated Ad12 DNA are methylated. Ad12 DNA isolated from purified virions, on the other hand, is not methylated to any significant extent. The segments of the integrated viral DNA comprising early genes, which are expressed as mRNA in two lines of Ad12-transformed hamster cells, are undermethylated in comparison to late viral segments, which are not expressed and are extensively methylated. In contrast, in two lines of Ad12-induced rat brain tumor cells, some of the late viral genes have been shown to be expressed as mRNA. The segment of the integrated Ad12 DNA that comprises these late genes, the *Eco*RI B fragment, is undermethylated in comparison to the extensive methylation of the same fragment in Ad12-transformed hamster cells. Thus, there appears to exist a striking inverse correlation between the levels of methylation of specific DNA segments and the extent to which these segments are expressed as mRNA. The functional significance of this correlation remains to be determined. It may provide a clue to understanding the regulation of gene expression in transformed cells and perhaps in eukaryotic cells in general.**

The DNAs of adenovirus type 2 (Ad2) and type 12 (Ad12) are strongly undermethylated in comparison to the DNA of their host. Previously it was shown (1) that Ad2 and Ad12 DNAs contain 0.01 and 0.02% $N^6$-methyladenine per adenine and 0.04 and 0.06% 5-methylcytosine (MeCyt) per cytosine, respectively. The DNA of human KB cells, the host for adenovirus propagation, however, contains 3.57% MeCyt (1). The DNAs of two lines of Ad12-transformed hamster cells (T637 and HA12/7) contain 3.11 and 3.14% MeCyt, respectively; the DNA of BHK21 cells contains only 2.22% (1).

In eukaryotic DNA, about 3–4% of the cytosine bases are methylated (2, 3). The main recognition sequence of DNA methyltransferases is presumably 5′-C-G-3′ (4, 5), although it is likely that a more complex signal is required for recognition because not all C-G doublets are methylated. In some cases, C residues in 5′-C-T-3′ sequences may also be methylated (6). Recently, Waalwijk and Flavell (7) demonstrated that the restriction endonucleases *Hpa* II and *Msp* I are isoschizomers, both cleaving at 5′-C-C-G-G-3′ sequences. *Hpa* II cannot cleave the sequence 5′-C-$^m$C-G-G-3′, whereas the sequence 5′-$^m$C-C-G-G-3′ seems to be cut (8). *Msp* I, however, is able to cleave at both of these methylated sequences (7). Thus, a comparison of the cleavage patterns obtained with the isoschizomer enzyme pair will reveal the 5′-C-G-3′ sites modified by methylation in a particular DNA molecule.

The availability of this isoschizomer pair has made it possible to investigate the methylation of integrated Ad12 DNA sequences in Ad12-transformed hamster cells. The patterns of integration of Ad12 DNA sequences in four lines of Ad12-transformed hamster cells (9, 10) and four lines of Ad12-induced rat brain tumor cells (10, 11) have been analyzed in detail. During the analysis of the DNA from Ad12-transformed hamster cells it was noted that *Hpa* II (5′-C-C-G-G-3′) and *Sma* I (5′-C-C-C-G-G-G-3′) did not effectively cleave integrated Ad12 DNA sequences, whereas *Eco*RI, *Bam*HI, and *Bgl* II cleaved integrated viral DNA as efficiently as control virion DNA. These data suggested that integrated Ad12 DNA sequences were extensively methylated (9).

Recently, Desrosiers *et al.* (12) reported that the DNA of herpesvirus saimiri, which purportedly persists in a nonintegrated state in lymphoid tumor cells, is also extensively methylated, whereas viral sequences in virus-producing lymphoid lines are not methylated.

In the present report we confirm that the major portion of the integrated Ad12 DNA sequences is inserted into the host genome as the entire DNA molecule (9, 10) and demonstrate that the integrated Ad12 DNA sequences in the HA12/7, T637, A2497-2, and A2497-3 lines are extensively methylated. However, not all of the 5′-C-C-G-G-3′ sites are in the 5′-C-$^m$C-G-G-3′ configuration. Upon cleavage with either *Hpa* II or *Msp* I, Ad12-specific DNA fragments that do not match any of the virion DNA bands are apparent. Presumably, these fragments represent virus–cell DNA junctions. Evidence will be adduced that, in the Ad12-transformed hamster cell lines T637 and HA12/7, the early regions of the Ad12 genome located in the *Eco*RI A and C fragments are strikingly less methylated than one of the major late regions located in the *Eco*RI B fragment. In these two transformed hamster lines, only early regions of the viral genome are expressed as mRNA.

Thus, there appears to exist an inverse correlation between the levels of methylation of specific viral DNA segments and the extent of their expression. In the Ad12-induced rat brain tumor cell lines RBT12/3 and RBT12/6, the late region located in the *Bam*HI D fragment (contained in the *Eco*RI B fragment) is at least partly expressed (11), and this region is undermethylated in rat cells in comparison to the hamster lines T637 and HA12/7. The possibility exists that the overall level of methylation of integrated Ad12 DNA in rat cells is lower than in hamster cells.

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Abbreviations: Ad2, adenovirus type 2; Ad12, adenovirus type 12; MeCyt, 5-methylcytosine; NaCl/Cit, 0.15 M NaCl/0.015 M Na citrate, pH 7.

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## MATERIALS AND METHODS

Most of the methods used and the origins of the Ad12-transformed cell lines HA12/7, T637, A2497-2, and A2497-3 as well as of the Ad12-induced rat brain tumor line RBT12/6 have been described (9–11). Ad2 and Ad12 were propagated and purified and the viral DNAs were extracted as outlined (13). *Hpa* II was purchased from Bethesda Research Laboratories (Bethesda, MD) and *Msp* I was from BioLabs (Beverly, MA); they were used as recommended by the manufacturers.

**Analysis of Intracellular DNA for the Presence of Viral DNA Sequences by the Technique of Southern.** DNA from transformed cells was isolated as described (9) and was cleaved with restriction endonucleases as indicated. The fragments were separated by electrophoresis in 1.5% agarose (Sigma) horizontal slab gels in 0.1 M Tris·HCl, pH 8.3/77 mM $H_3BO_3$/2.5 mM EDTA (TEB buffer) at 50 V for 16 hr. After electrophoresis, the DNA was denatured by submerging the gels in 0.5 M NaOH/1.5 M NaCl at room temperature for 4–8 hr. Subsequently, the gels were neutralized in 0.5 M HCl/0.1 M Tris·HCl, pH 7.5/1.5 M NaCl for 1.5–4 hr. The denatured DNA fragments were then transferred to nitrocellulose filter sheets (14). Transfer was effected with 20× standard saline citrate (NaCl/Cit; 0.15 M NaCl/0.015 M Na citrate, pH 7) for 3 days at room temperature. The filters were then briefly rinsed in 2× NaCl/Cit, dried at 80°C, sealed into plastic bags (Dazey Products Co., Industrial Airport, KS), and preincubated for 24 hr at 68°C in 50–100 ml of Denhardt's mixture (15) in 3× NaCl/Cit containing 300 μg of heat-denatured calf thymus DNA per ml. Subsequently, the filters were incubated for 24 hr at 68°C with Ad12 DNA or the *Eco*RI fragments of Ad12 DNA that had been $^{32}$P-labeled by nick translation (16). Hybridization was performed in the same solution freshly prepared. At the end of the hybridization reaction, the filters were washed at 68°C in 6× NaCl/Cit/0.5% sodium dodecyl sulfate (five times, each with 500 ml, overnight) and for at least 4 hr in two 500-ml portions of 2× NaCl/Cit. Finally, the filters were dried and autoradiographed as described (9).

**Purification of the *Eco*RI Fragments of Ad12 DNA.** In some of the hybridization experiments described (Figs. 3–5), the *Eco*RI fragments of Ad12 DNA were $^{32}$P-labeled by nick translation. The *Eco*RI fragments of Ad12 DNA were purified by three cycles of electrophoresis in cylindrical 0.8% agarose gels (2.4 × 20 cm) in TEB buffer or in 0.04 M Tris·HCl, pH 7.8/5 mM sodium acetate/1 mM EDTA as described elsewhere (10).

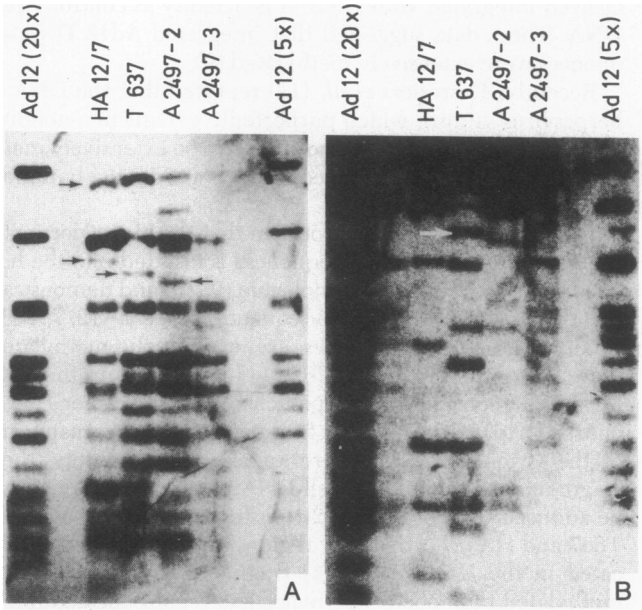


FIG. 1. Methylation patterns of the DNA from four Ad12-transformed hamster cell lines after digestion with *Msp* I and *Hpa* II. The DNA from the Ad12-transformed lines HA12/7, T637, A2497-2, and A2497-3 was isolated, and 10 μg of each DNA or 0.64 ng (Ad12 20×) or 0.15 ng (Ad12 5×) of Ad12 DNA isolated from purified virions was cleaved with *Msp* I (*A*) or *Hpa* II (*B*). These amounts of Ad12 virion DNA correspond to 20 genome equivalents of Ad12 DNA per cell (based on the 10 μg of cellular DNA used) or to 5 genome equivalents per cell, respectively. The fragments of cellular or viral DNA, as indicated, were separated by electrophoresis on 1.5% agarose slab gels and transferred to nitrocellulose filters (14). Ad12-specific sequences were detected by hybridization with $^{32}$P-labeled intact Ad12 DNA and autoradiography as described. Kodak XR-5 film was used for all autoradiographs. The autoradiogram in *B* was overexposed in comparison to the one in *A*. →, Ad12-specific DNA fragments in the bulk of cellular DNA which do not correspond in size to any of the Ad12 virion DNA fragments and presumably are due to virus–cell DNA junctions. The sequences cleaved by *Msp* I or *Hpa* II are: *Msp* I, 5′-C-C-G-G-3′, 5′-$^{m}$C-C-G-G-3′, 5′-C-$^{m}$C-G-G-3′; *Hpa* II, 5′-C-C-G-G-3′, 5′-$^{m}$C-C-G-G-3′.

## RESULTS AND DISCUSSION

**Analysis of the DNA of Ad12-Transformed Hamster Cells with *Hpa* II and *Msp* I.** The DNA isolated from four Ad12-transformed hamster cell lines (HA12/7, T637, A2497-2, and A2497-3) was cleaved with *Hpa* II or *Msp* I. In these lines a major portion of the Ad12 genome (probably the entire genome) is integrated into cellular DNA (9, 10). The fragments generated were separated by electrophoresis in horizontal 1.5% agarose slab gels, and the fragments were subsequently transferred to nitrocellulose filters by the Southern technique. The distribution of Ad12 DNA sequences in the bulk of cellular DNA was determined by DNA·DNA hybridization with intact Ad12 DNA $^{32}$P-labeled by nick translation followed by autoradiography.

The data presented in Fig. 1*B* confirm findings reported previously (9). *Hpa* II cleaved integrated Ad12 sequences incompletely, even when a 4-fold excess of *Hpa* II over the amount required for complete cleavage of Ad12 virion DNA was used (data not shown). Virion DNA was cleaved by *Hpa* II into the same number of fragments, regardless of whether or not transformed cell DNA was present, indicating that no enzyme inhibitor was present in the DNA preparations investigated. Some of the 5′-C-C-G-G-3′ sites in integrated Ad12 DNA, presumably those not modified by methylation and possibly also those containing the rare 5′-$^{m}$C-C-G-G-3′ configuration, are recognized by *Hpa* II and the DNA was cleaved at these sequences (Fig. 1*B*). (The autoradiograph in Fig. 1*B*

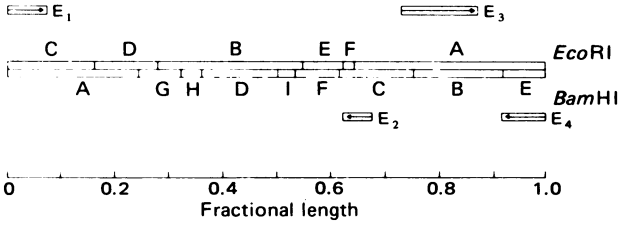


FIG. 2. Transcriptional map of the early genes of Ad12 DNA. The positions of the *Eco*RI and *Bam*HI fragments on the Ad12 genome have been indicated as well as those regions of the viral DNA that are transcribed early (8 hr after infection) as mRNA in permissively infected cells (17, 18) (i.e., regions $E_1$–$E_4$. Regions $E_1$–$E_4$ are expressed as mRNA in T637 cells; regions $E_1$, $E_2$, and $E_4$ are partly expressed in HA12/7 cells (17). In the Ad12-induced rat brain tumor cell lines RBT12/3 and RBT12/6, the early viral regions are expressed as mRNA, as well as part of the late viral region localized in the *Eco*RI B fragment.

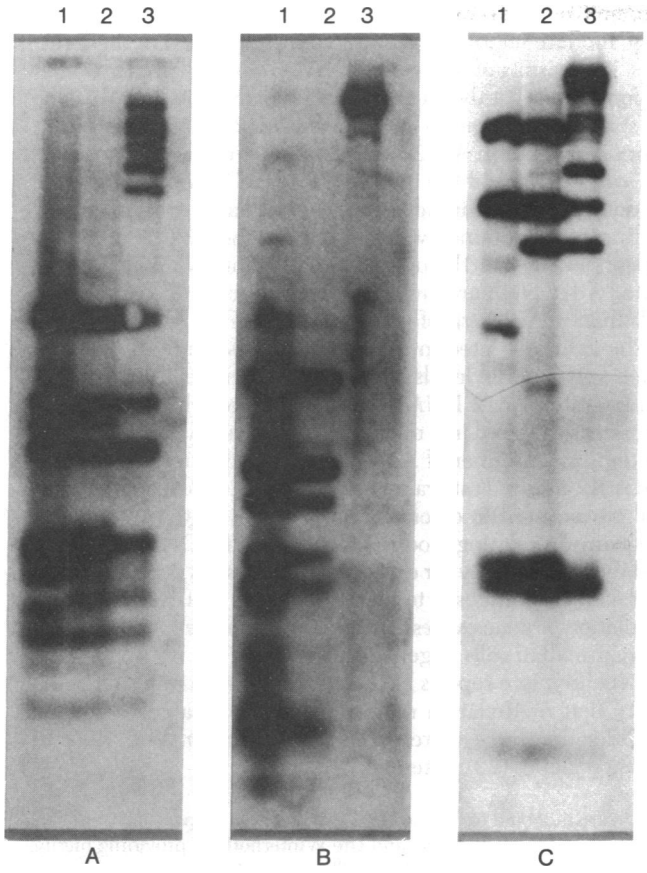


FIG. 3. Autoradiograms of Southern blots of T637 hamster cell DNA digested with *Msp* I (lane 2) or *Hpa* II (lane 3). Lane 1 is Ad12 marker DNA cleaved with *Msp* I; identical cleavage patterns were obtained when Ad12 DNA was cut with *Hpa* II (see Fig. 1). The fragments were separated by electrophoresis on 1.5% agarose slab gels and blotted onto nitrocellulose filters (14). Three such filters were prepared for hybridization with $^{32}$P-labeled *Eco*RI fragment A (*A*), fragment B (*B*), or fragment C (*C*) of Ad12 DNA. Autoradiograms on Kodak XR-5 film were produced as described (9). The amounts of cellular DNA and of Ad12 DNA used were the same as in Fig. 1, except that the marker Ad12 DNA was mixed with B 3 (BHK) carrier DNA.

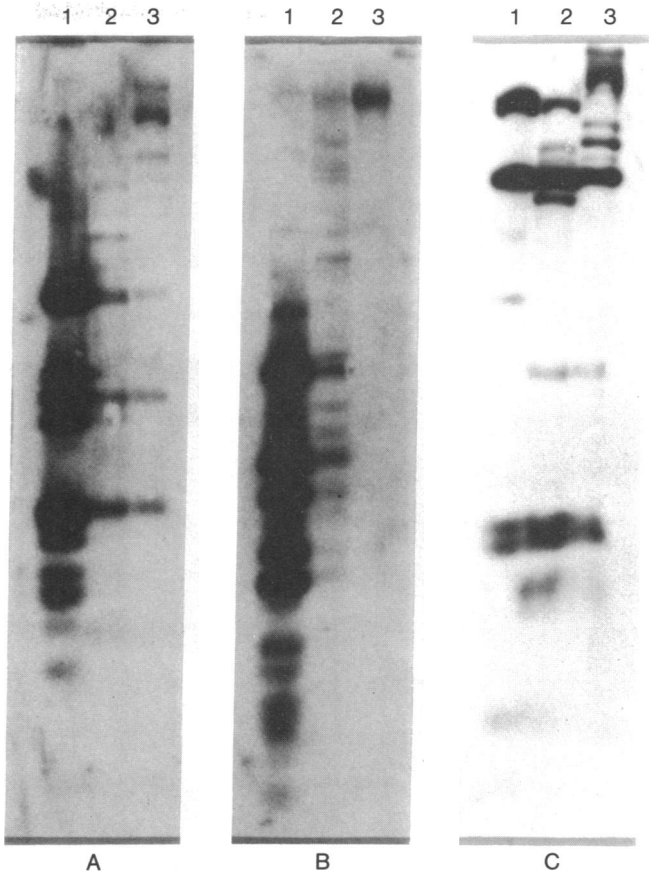


FIG. 4. Autoradiograms of Southern blots of HA12/7 hamster cell DNA digested with *Msp* I or *Hpa* II. Experimental conditions and the arrangements of the autoradiograms are as in Fig. 3.

was considerably overexposed in order to document unequivocally that there are only few sites available for *Hpa* II cleavage). In the four different transformed lines the distributions of the 5′-C-C-G-G-3′ sites accessible to *Hpa* II were not identical.

Different patterns of distribution of Ad12 DNA sequences were observed when the DNAs from Ad12-transformed hamster cells were cleaved with *Msp* I (Fig. 1*A*). This endonuclease cleaves at all 5′-C-C-G-G-3′ sites, methylated or not. There was good correspondence between the patterns of distribution of Ad12-specific fragments derived from Ad12 virion DNA and of integrated Ad12 sequences. Thus, a detailed comparison of the restriction patterns of specific DNA molecules after cleavage with *Hpa* II and *Msp* I is diagnostic for the extent and for the sites of methylation at 5′-$^{m}$C-G-3′ sequences.

In the DNA of all four lines of Ad12-transformed hamster cells, there were at least two Ad12-specific DNA fragments that did not correspond to any of the fragments generated by cleavage of the virion marker DNA with *Hpa* II or *Msp* I. These fragments (indicated by arrows in Fig. 1) probably contain the sites of junction between viral and cellular DNAs. Similar fragments have been found after cleavage with *Eco*RI, *Bam*HI, and *Hin*fI (9, 10). It is striking, however, that these virus–cell DNA fragments generated by *Hpa* II or *Msp* I appear to have similar, but not identical, sizes in all four transformed lines. Moreover, these "off-size" fragments appear to be the same ones, regardless of whether *Hpa* II or *Msp* I was used in the analysis.

We conclude that the Ad12 DNA sequences integrated in the HA12/7, T637, A2497-2, and A2497-3 lines are extensively methylated, in contrast to Ad12 DNA isolated from purified virions propagated on human KB cells (1). Not all of the numerous 5′-C-C-G-G-3′ sequences in integrated Ad12 DNA contain the modified 5′-$^{m}$C-G-3′ doublet, although many of them do. In general, the role of DNA methylation in eukaryotes has not been elucidated. It is conceivable that the very striking differences in the levels of MeCyt between Ad12 virion DNA and integrated Ad12 DNA in transformed cells simply reflect differences in the activity of the methyltransferase systems in permissively infected human and transformed hamster cells. It has been suggested in a previous report from our laboratories (1) that the methyltransferase system in permissively infected cells is inactivated as a consequence of virus infection.

**Differential Methylation of Integrated Ad12 Sequences.** We investigated the possibility that correlations may exist between the levels of methylation in specific sequences of eukaryotic DNA and the extent to which these sequences are expressed. Adenovirus-transformed cells are a suitable system for such studies because the entire viral genome persists in the transformed cells, although not all of the viral sequences are expressed as mRNA. In Ad12-transformed hamster cells, only the early regions $E_1$–$E_4$, or parts of them (Fig. 2), are expressed as mRNA, as has been shown for lines HA12/7 and T637 (17). It is apparent from the transcriptional map of Ad12 DNA (18)

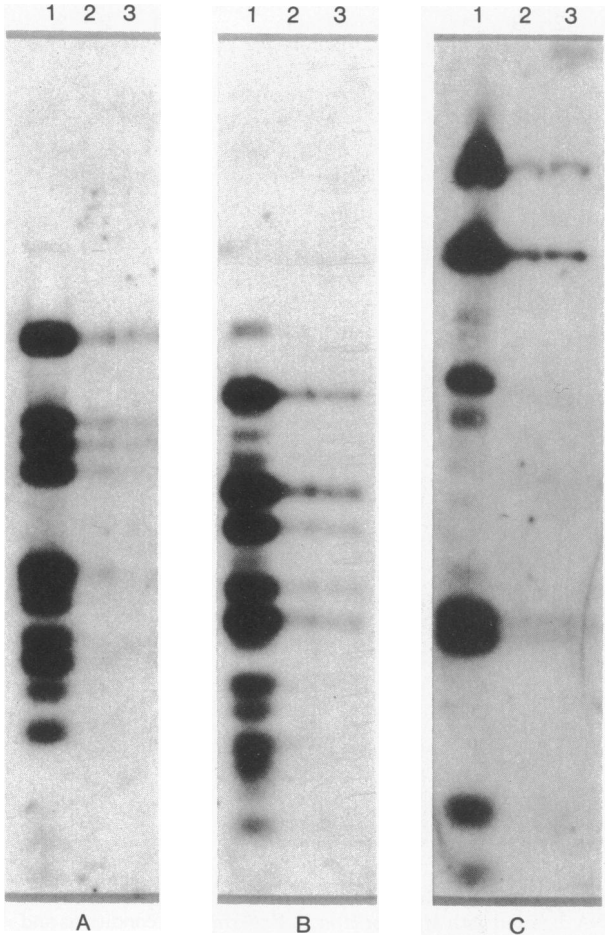


FIG. 5. Autoradiograms of Southern blots of RBT12/6 rat cell DNA digested with *Msp* I or *Hpa* II. Experimental conditions and the arrangements of the autoradiograms are the same as in Fig. 3.

(Fig. 2) that the early regions of the Ad12 genome are located mainly on the *Eco*RI A and *Eco*RI C fragments. The *Eco*RI B fragment, on the other hand, constitutes exclusively late viral DNA sequences which are not expressed in the Ad12-transformed hamster lines HA12/7 and T637 (17).

We therefore analyzed the levels of methylation in early and in late Ad12 DNA segments persisting in the integrated state in transformed hamster cells. The DNAs of the transformed cell lines HA12/7 and T637 were cleaved with the isoschizomer enzyme pair *Msp* I and *Hpa* II as before (Fig. 1). The DNA was blotted (14) and then hybridized to $^{32}$P-labeled *Eco*RI fragment A, B, or C. In the hamster lines T637 (Fig. 3) and HA12/7 (Fig. 4), practically all of the 5′-C-C-G-G-3′ sequences located in *Eco*RI fragment B, which comprises late genes of Ad12 DNA, are methylated. The integrated viral DNA sequences located in the *Eco*RI B fragment were not cleaved by *Hpa* II whereas *Msp* I cut at all 5′-C-C-G-G-3′ sequences in this fragment. In the *Eco*RI A and C fragments, which contain early genes of Ad12 DNA, most of the 5′-C-C-G-G-3′ sites are not methylated in the two hamster lines studied, as revealed by a comparison of the *Hpa* II and *Msp* I cleavage patterns. It should be noted that only 60–70% of the *Eco*RI C fragment constitute early sequences (17), a fact that may explain the finding that some of the 5′-C-C-G-G-3′ sequences in this fragment are methylated.

It has recently been shown that, in the Ad12-induced rat brain tumor lines RBT12/3 and RBT12/6, late sequences located in the *Eco*RI B fragment (Fig. 2) of the integrated Ad12 DNA are at least partly expressed as mRNA (11). It was therefore interesting to determine the levels of methylation in the early and late segments of Ad12 DNA integrated in these rat lines. The results of analysis of DNA from the RBT12/6 line demonstrated that the late regions of the integrated viral DNA are strikingly less methylated in the Ad12-induced rat brain tumor cells than in Ad12-transformed hamster cells (Fig. 5). The early regions of the integrated Ad12 DNA in the rat line are unmethylated as in Ad12-transformed hamster cells. Similar findings were obtained with DNA from the RBT12/3 line. It appears likely that the overall levels of methylation of integrated Ad12 DNA are lower in Ad12-induced rat brain tumor cells than in Ad12-transformed hamster cells.

The data presented in this report suggest an inverse correlation between the levels of methylation in specific segments of integrated Ad12 DNA and the extent to which these segments are expressed into mRNA and presumably translated into proteins in a number of Ad12-transformed hamster cell lines and Ad12-induced rat brain tumor cell lines. Much further work will be required to elucidate the possibly quite complicated mechanisms relating modification of specific DNA sequences and their availability for expression. The results presented raise tantalizing questions as to the role of DNA methylation in the regulation of gene expression in transformed cells and perhaps in mammalian cells in general.

Recently, two reports have appeared which raise the possibility that methylation may somehow be related to genetic function in the sea urchin (*Echinus esculentus*) (19) and chicken globin (20) systems.

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