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The unmethylated state of the promoter/leader and 5'-regions of integrated adenovirus genes correlates with gene expression

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An inverse correlation has been established between the levels of DNA methylation at 5'-CCGG-3' (*Msp*I/*Hpa*II) sites in specific genes of integrated viral DNA in adenovirus type 12 (Ad12)-transformed hamster cell lines and the extent to which these genes are expressed (Sutter and Doerfler, 1979, 1980). In general, early genes are transcribed into mRNA, while late genes are permanently switched off in these cell lines. Adenovirus type 2 genes methylated *in vitro* at 5'-CCGG-3' sites are not transcribed upon microinjection into nuclei of *Xenopus laevis* oocytes – unmethylated genes are expressed (Vardimon *et al.*, 1982a). The *Msp*I sites in the early and in some of the late Ad12 genes in cell lines HA12/7, T637, and A2497-3 have now been precisely mapped. The data presented here reveal that the promoter/leader and 5'-regions of the early genes are unmethylated both at *Msp*I sites and at 5'-GCGC-3' (*Hha*I) sites. In some instances, e.g., in the E2a regions in all three lines, the main parts of the early genes are partly methylated, even though the genes are expressed. In cell line HA12/7, the early region E3 is not expressed, and the promoter/leader and 5'-regions of this segment are fully methylated. All late regions are completely methylated. The results suggest that the state of methylation in the promoter/leader and 5'-regions of integrated adenovirus genes is important in the control of gene expression.

Key words: adenovirus-transformed cells/DNA methylation/gene expression/promoter/regulation

Introduction

Evidence is now accumulating that DNA methylation at highly specific sites in eukaryotic genes constitutes an important signal in the regulation of gene expression (for reviews, see Razin and Riggs, 1980; Doerfler, 1981; Ehrlich and Wang, 1981; Hattmann, 1981; Wigler, 1981). This signal is probably functional in the long-term shut-off of eukaryotic genes. We do not yet understand in what way DNA methylation, i.e., the presence of 5-methylcytosine residues at specific sites, may act in gene regulation. The role DNA methylation plays may be exerted *via* fundamental structural changes in certain stretches of DNA. New concepts about the structure of DNA have been introduced by the discovery of its Z-form (Wang *et al.*, 1979). Recently, a poly(dG-^{5}mdC).poly(dG-^{5}mdC) polymer was shown to have a much higher tendency to assume the Z configuration than the unmethylated polynucleotide (Behe and Felsenfeld, 1981). Similar investigations have not yet been performed for naturally occurring DNAs. It is conceivable that DNA methylation may stabilize DNA in, or facilitate its transition to, an altered configuration. Alternatively, 5-methylcytosine with the methyl group protruding into the major groove might constitute a signal by itself which could direct the binding of regulatory proteins.

Our laboratory has established an inverse correlation between the levels of DNA methylation in specific segments of the integrated adenovirus type 12 (Ad12) or type 2 (Ad2) genome in transformed cells and the extents to which these segments are expressed as mRNA (Sutter and Doerfler, 1979, 1980; Vardimon *et al.*, 1980). Viral genes, in particular the early adenovirus genes that are expressed in transformed cells, are undermethylated at the 5'-CCGG-3' (*Hpa*II/*Msp*I) sites, while the late viral genes that are permanently silenced are completely methylated at these sites. Similar inverse correlations have been observed in many other viral and nonviral eukaryotic systems (for reviews, see Razin and Riggs, 1980; Doerfler, 1981; Ehrlich and Wang, 1981; Hattmann, 1981; Wigler, 1981). It should be emphasized that for those few genes in which such correlations did not seem to hold, methylations at sites other than 5'-CCGG-3' sequences might be important. For the activation of a set of (viral) genes, absence of DNA methylation apparently is a necessary but not a sufficient precondition, since Ad12 genomes integrated into the DNA of Ad12-induced tumor cells show very little methylation at the 5'-CCGG-3' sites, although very small amounts of Ad12-specific RNA are synthesized in these cells (Kuhlmann and Doerfler, 1982).

Direct evidence has been adduced for the involvement of viral DNA methylation at the 5'-CCGG-3' sites in gene regulation (Vardimon *et al.*, 1981, 1982a, 1982b). The cloned E2a gene of Ad2 DNA was methylated *in vitro* using the *Hpa*II DNA methyltransferase from *Haemophilus parainfluenzae* (Mann and Smith, 1977; Quint and Cedar, 1981). Methylated or unmethylated DNA was subsequently microinjected into the nuclei of oocytes from *Xenopus laevis*. Methylated viral genes were not transcribed, whereas unmethylated genes were faithfully expressed using the same late E2a promoter that was used in Ad2-infected human cells. In contrast, when the *Bsu*RI DNA methyltransferase from *Bacillus subtilis* (Günthert *et al.*, 1981) was used, the E2a gene of Ad2 DNA was transcribed in *X. laevis* oocytes (Vardimon *et al.*, 1981, 1982a). Thus, methylated bases had to be positioned in highly specific locations to be recognized as regulatory signals.

We have now precisely mapped many of the methylated and the unmethylated 5'-CCGG-3' (*Hpa*II/*Msp*I) sites in the Ad12 genomes integrated in the three Ad12-transformed hamster cell lines T637, HA12/7, and A2497-3 (Sutter *et al.*, 1978; Sutter and Doerfler, 1979, 1980; Stabel *et al.*, 1980). With few exceptions, the early viral genes are expressed in these lines and the late genes are inactive (Ortin *et al.*, 1976; Schirm and Doerfler, 1981; Esche and Siegmann, 1982). We find that the promoter/leader and 5'-regions of early Ad12 genes carry unmethylated *Hpa*II sites when these genes are expressed. Conversely, when early genes are unexpressed they are methylated in the promoter/leader and 5'-regions. Does DNA methylation exert a crucial function in the promoter/leader and 5'-regions of specific genes?

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Results and Discussion

Principle of experimental approach

The precise localization of methylated 5′-CCGG-3′ sites on the integrated Ad12 DNA in transformed cell lines depended on the availability of maps of Ad12 DNA for frequently cutting restriction enzymes (Figures 2, 5) and of cloned Ad12 DNA fragments which were sufficiently small to probe for specific regions of the viral genome. A large number of clones containing specific Ad12 DNA fragments have been isolated (Vogel *et al.*, 1981). DNA from the three transformed cell lines mentioned was cleaved with the *Hpa*II or the *Msp*I restriction endonuclease in order to distinguish between methylated and unmethylated 5′-CCGG-3′ sites (Waalwijk and Flavell, 1978). Subsequently, the DNA fragments were separated by electrophoresis on agarose slab gels, transferred to nitrocellulose filters (Southern, 1975), and Ad12-specific fragments were visualized by DNA-DNA hybridization (Wahl *et al.*, 1979) followed by autoradiography. Intact Ad12 DNA or specific cloned Ad12 DNA fragments were used as hybridization probes after having been ^{32}P-labeled by nick-translation (Rigby *et al.*, 1977). In some experiments the DNA from transformed cells was cleaved with the restriction endonuclease *Msp*I and subsequently with *Hha*I to detect methylated *Hha*I sites (recognition sequence 5′-GCGC-3′) within the *Msp*I fragments. As a control, Ad12 virion DNA, which was essentially unmethylated (Günthert *et al.*, 1976), was treated in the same way and was co-electrophoresed in many of the experiments. Finally, the results on methylation (Figure 5) were correlated with the expression of specific viral DNA fragments in the transformed cell lines investigated (Ortin *et al.*, 1976; Schirm and Doerfler, 1981).

Mapping of methylated HpaII sites in integrated viral DNA in Ad12-transformed lines T637, HA12/7, and A2497-3

The data presented in Figure 1 provide an overview of the extent of methylation at the 5′-CCGG-3′ sites in four cell lines investigated. The results confirm findings reported earlier (Sutter and Doerfler, 1979, 1980) and show that some of the *Hpa*II sites in the integrated Ad12 genomes in lines T637, HA12/7 and A2497-3 are not methylated, i.e., specific fragments are apparent in the autoradiogram after cleavage with both *Hpa*II and *Msp*I. There is, however, a high level of methylated sites as evidenced by the amount of Ad12-specific sequences not cleaved by *Hpa*II. As shown previously (Sutter and Doerfler, 1980), the Ad12 DNA sequences in the Ad12-induced rat brain tumor line RBT12/15 are not methylated extensively at the 5′-CCGG-3′ sites.

The extent of DNA methylation in the integrated Ad12 genomes in cell lines T637, HA12/7, and A2497-3 has been determined in detail. The cellular DNA from these cell lines was cleaved with restriction endonuclease *Hpa*II or *Msp*I, fragments were separated by electrophoresis and blotted. Cloned Ad12 DNA fragments (Vogel *et al.*, 1981) were ^{32}P-labeled by nick-translation. The map locations of the cloned fragment probes used are indicated in Figure 2 by the hatched or cross-hatched areas. With these probes the state of methylation at all 5′-CCGG sites in the integrated viral genomes could be determined. We have chosen not to present all the data obtained but have summarized the results of many hybridization experiments in Figures 2, and 5. Selected examples of some of the results are shown in Figure 3. It is readily apparent from a comparison of the *Hpa*II and *Msp*I

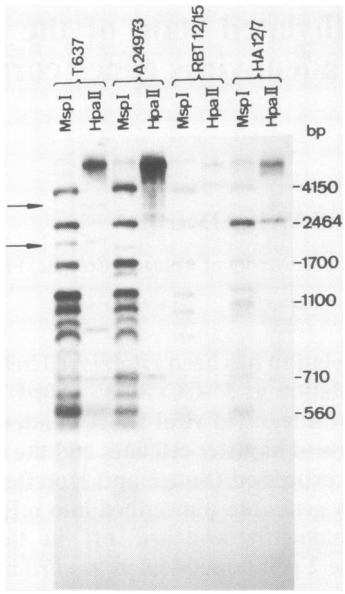

Fig. 1. The levels of methylation at 5′-CCGG-3′ sites in integrated Ad12 DNA in cell lines T637, A2497-3, RBT-12/15, and HA12/7. The DNA from cell lines as indicated was extracted as described elsewhere (Sutter *et al.*, 1978). The DNA was subsequently cleaved with restriction endonuclease *Msp*I or *Hpa*II, the fragments were separated by electrophoresis on a horizontal 2.0% agarose slab gel and transferred to a nitrocellulose filter by the Southern blotting technique. Ad12-specific DNA fragments were visualized as described in the text. As a size marker the *Msp*I fragments of Ad12 DNA were used (not shown); the sizes in base pairs (bp) of some of these fragments were indicated at the right margin. The arrows at the left margin designate some of the off-size bands of viral DNA (Stabel *et al.*, 1980) constituting junction fragments of viral and cellular DNAs. It had been demonstrated that the *Hpa*II restriction endonuclease could not cleave at the 5′-CCGG-3′ site when the internal C residue was methylated, whereas *Msp*I could cleave (Waalwijk and Flavell, 1978). This restriction enzyme pair thus provided a diagnostic tool to determine levels of methylation at the designated sequences.

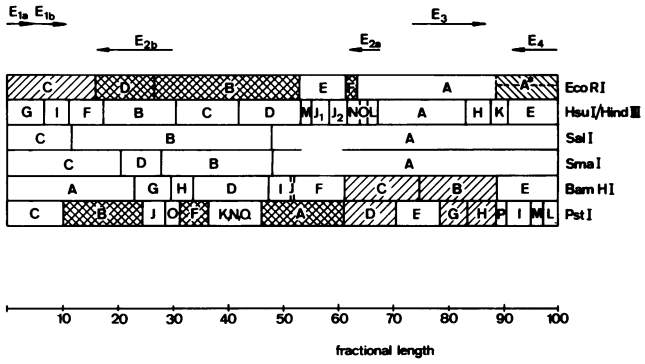

Fig. 2. Methylation map of integrated Ad12 DNA in Ad12-transformed hamster cell lines T637, HA12/7, and A2497-3. The schematic presentation summarizes the data described in this report. The hatched areas indicate regions of the viral genome that are unmethylated, the cross-hatched areas sections that are completely methylated at the 5′-CCGG-3′ sites. For exceptions and a more detailed map see Figure 5. Except for the *Pst*I map of Ad12 DNA, the restriction maps were derived from previously published data (Ortin *et al.*, 1976; Tooze, 1980). We established the *Pst*I map by conventional methods: partial digestion, cleavage of cloned Ad12 DNA fragments (Vogel *et al.*, 1981), double digestion experiments using *Pst*I and another suitable restriction enzyme. The symbol A* in the *Eco*RI map refers to a ~5-kb cloned fragment from the right terminus of Ad12 DNA. The early regions of Ad12 DNA and the directions of their transcription are presented as well as a fractional length scale of Ad12 DNA. In cell line HA12/7, the E3 region is not expressed (Figure 6b, Ortin *et al.*, 1976). In this cell line, the promoter and 5′-regions of the E3 region in the integrated Ad12 genomes are methylated (not shown here, c.f., Figure 5).

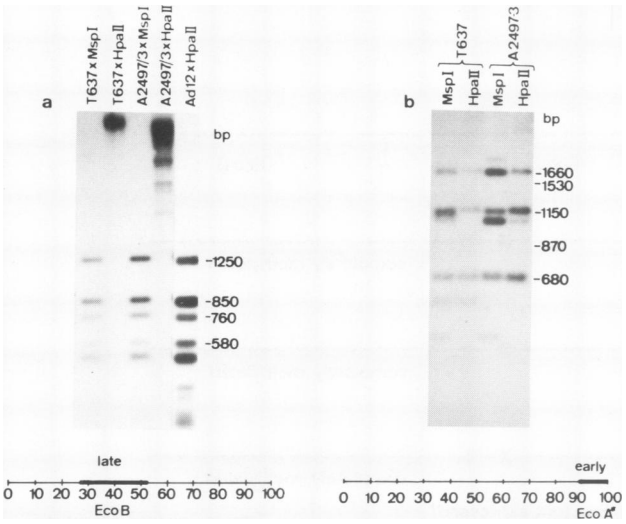

Fig. 3. Analysis of the levels of DNA methylation in specific segments of the integrated Ad12 DNA in cell lines T637, A2497-3, and HA12/7. The experimental approach and details were similar to the ones described in the legend to Figure 1. By using specific cloned fragments (Vogel *et al.*, 1981) of Ad12 DNA as hybridization probes ^{32}P-labeled by nick-translation, the levels of DNA methylation at 5′-CCGG-3′ sequences could be visualized in specific segments of the integrated Ad12 genome. The probes used in individual hybridization experiments were: (**a**) *Eco*RI-B fragment; (**b**) *Eco*RI-A* fragment (Vogel *et al.*, 1981). The map locations of these fragments and their assignments to the functional map of Ad12 DNA are readily apparent from each figure and from Figures 2 and 5. The sizes of Ad12 DNA fragments are also indicated.

cleavage patterns observed that all 5′-CCGG-3′ sites in the *Eco*RI-B fragment of integrated Ad12 DNA in lines T637, HA12/7 (data not shown), and A2497-3 are completely methylated (Figure 3a). In contrast, the 5′-CCGG-3′ sites in the *Eco*RI-A* fragment of integrated Ad12 DNA in the same lines are unmethylated (Figure 3b).

By using appropriate probes as indicated in Figure 2, the entire lengths of the integrated Ad12 genomes in cell lines T637, HA12/7, and A2497-3 have been screened in this way for the state of methylation at 5′-CCGG-3′ sites. The cross-hatched areas designate completely methylated DNA segments, the hatched areas predominantly unmethylated DNA segments. Exceptions will be discussed below (Figure 5). In cell lines T637, HA12/7, and A2497-3 the early regions E1, E2a, and E4, and in lines T637 and A2497-3 the early region E3 as well, are expressed as mRNA. Region E2b and the late regions of Ad12 DNA are not expressed (Ortin *et al.*, 1976; Schirm and Doerfler, 1981; Esche and Siegmann, 1982). Thus, the inverse correlation between gene expression and DNA methylation described previously (Sutter and Doerfler, 1980) is upheld by the present, much more refined, mapping results.

Although cell lines T637, HA12/7, and A2497-3 contained ~22, 3, and 17 copies of Ad12 DNA, respectively (Stabel *et al.*, 1980), it was surprising to find that with few exceptions the *Msp*I sites of all the copies of integrated Ad12 DNA in a given location were either methylated or unmethylated. The exceptions were found in the E2a, E3, and E4 regions of Ad12 DNA in line A2497-3. About 50% of the *Msp*I sites in these regions in line A2497-3 were methylated, indicating that perhaps only part of the persisting early regions could be expressed. In the three lines investigated, all *Msp*I sites located within the *Eco*RI-B and the *Eco*RI-E fragments that contain unexpressed late regions, were completely methylated (Figure 3a).

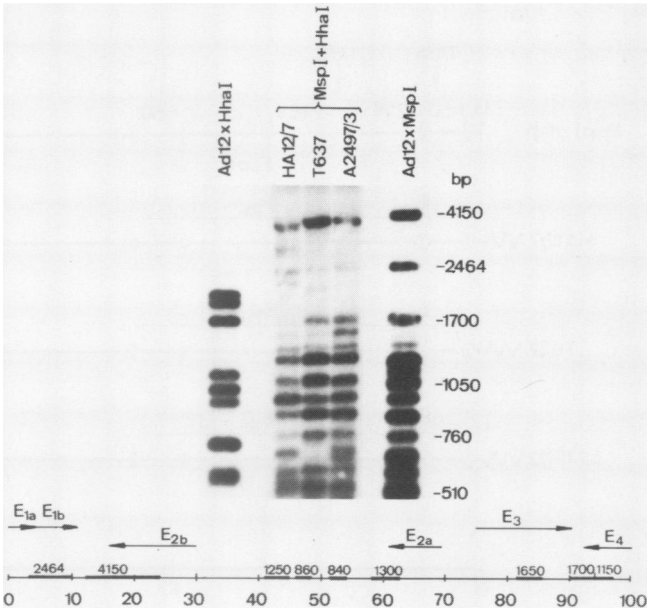

Fig. 4. Analysis of the levels of DNA methylation at the 5′-GCGC-3′ (*Hha*I) sites in lines HA12/7, T637, and A2497-3. The DNA from cell lines as indicated was cleaved with restriction endonucleases *Msp*I and *Hha*I in succession. Ad12 DNA as a marker was cleaved with either *Msp*I or *Hha*I. Subsequently, fragments were separated and transferred as described in the legend to Figure 1. The whole Ad12 DNA molecule ^{32}P-labeled by nick-translation was used as hybridization probe. The objective of cleaving the cellular DNA with both restriction endonucleases was to investigate whether fragments that could be excised by the *Msp*I restriction endonuclease and contained *Hha*I = 5′-GCGC-3′ sites (see Ad12 controls) were methylated or unmethylated at these *Hha*I sites. It is not possible to evaluate the levels of methylation in DNA fragments < 800 bp by this method. The sizes (in bp) of some of the fragments observed and their locations on the functional map of Ad12 DNA are also indicated.

Methylation at 5′-GCGC-3′ (HhaI) sites

So far, the analyses of DNA methylation in the three Ad12-transformed hamster lines were restricted to the 5′-CCGG-3′ sites. Since it was possible that specific and rather complicated patterns of DNA methylation constituted signals of biological significance, we also investigated the levels of methylation at the 5′-GCGC-3′ (*Hha*I) sites by cleaving the DNA from Ad12-transformed cells and from Ad12 virions as internal controls with both the *Msp*I and *Hha*I restriction endonucleases. *Hha*I cannot cleave the sequence 5′-G^{m}CGC-3′. The Ad12-specific cleavage patterns obtained were compared with those adduced when the DNA was cleaved with the restriction endonuclease *Msp*I or *Hha*I alone. The data in Figure 4 show that some of the larger Ad12 DNA fragments, e.g., the 2464-bp fragment, located in early regions of the viral genome that were not methylated at 5′-CCGG-3′ sites, were not methylated at 5′-GCGC-3′ sites and could be further cleaved by the *Hha*I endonuclease. Since a minor portion of the 2464-bp fragment from the E1 region appears to be resistant to cleavage by *Hha*I (Figure 4), it is possible that in a few copies of Ad12 DNA these sites might be methylated. Similar qualifications hold for the interpretation of the *Hha*I cleavage of the 1700-bp fragment in region E4.

Conversely, late fragments methylated at *Msp*I sites, e.g., the 4050-bp or the 1250-bp fragment (Figure 4), were frequently also methylated at *Hha*I sites and hence refractory to this enzyme. Thus, in general, congruent data with respect to the levels of methylation in specific regions of the integrated Ad12 genomes were obtained for two different 5′-CG-3′

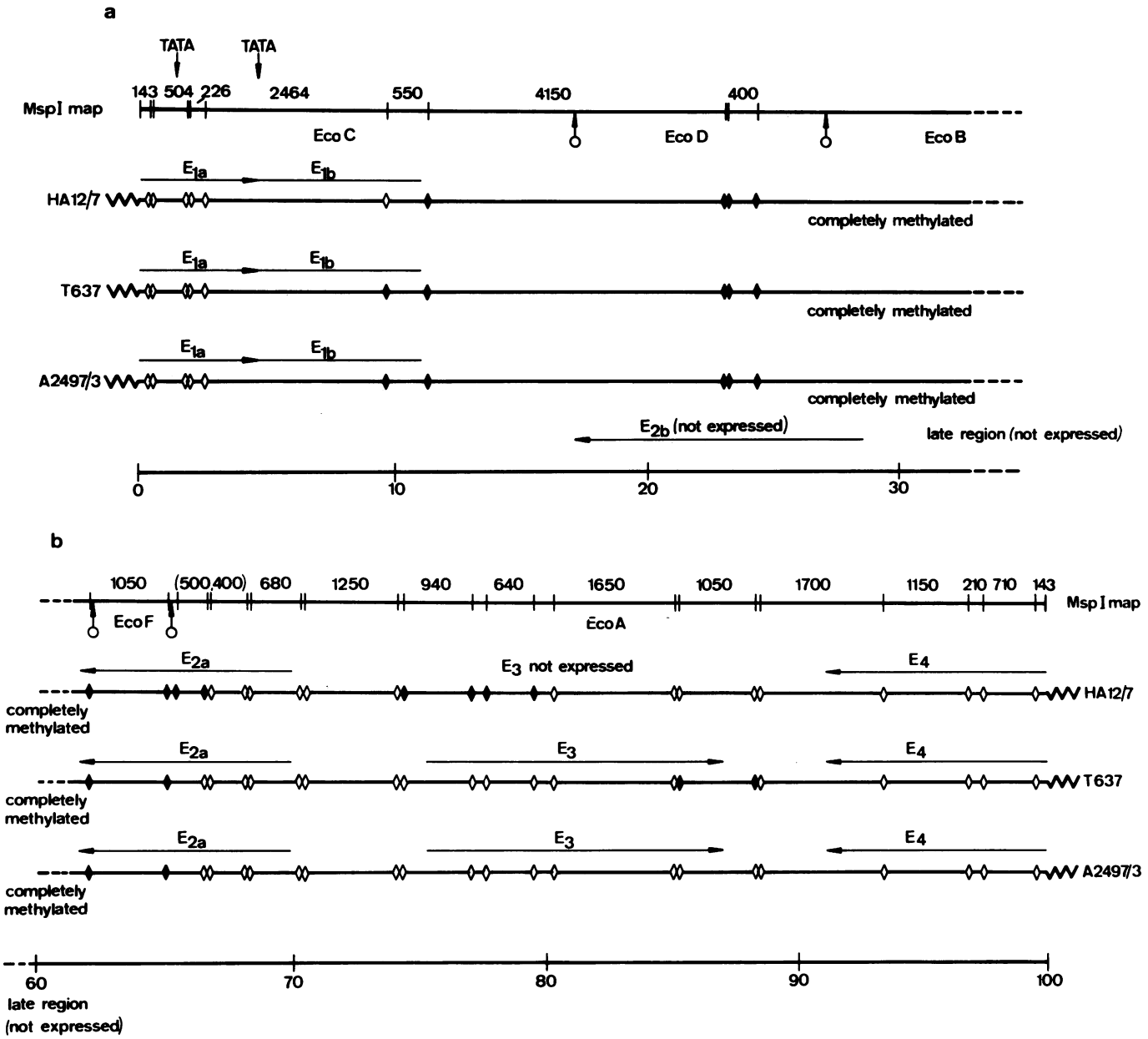

Fig. 5. Functional maps of the left (a) and the right (b) halves of the Ad12 DNA molecules integrated in cell lines HA12/7, T637, and A2497-3. The horizontal lines represent the Ad12 genomes (———) integrated into the genomes of cell lines (ΛΛΛ) as indicated. The *Msp*I maps of the left (a) and right (b) ends of Ad12 DNA are presented in the top line. Vertical bars on and figures above the horizontal lines indicate the locations of the *Msp*I sites and the sizes of some of the *Msp*I fragments, respectively. The arrows (♁) designate *Eco*RI sites. TATA marks the locations of presumptive Goldberg-Hogness signals in the E1a region (Sugisaki *et al.*, 1980; Bos *et al.*, 1981). The unmethylated (◇) and methylated (◆) 5′-CCGG-3′ sites in the integrated Ad12 genomes in the lines HA12/7, T637, and A2497-3 are designated by open or closed symbols as indicated. The horizontal arrows indicate map positions and direction of transcription of the individual early regions in each of the cell lines. Presence of an arrow indicates expression of corresponding regions of the Ad12 genome. *Msp*I sites to the right of the *Eco*RI-D/B and to the left of the *Eco*RI-A/F junctions have not been mapped. The early regions of Ad12 DNA (Ortin *et al.*, 1976; Esche and Siegmann, 1982) and a fractional length scale have also been indicated. The map of the E1 region of Ad12 DNA is as described by Bos *et al.* (1981).

containing tetranucleotide restriction sequences. These findings suggest that within a given region of integrated viral DNA the levels of methylation at different 5′-CG-3′ sites are similar – either the DNA is methylated or unmethylated at most of these sites.

Correlations between methylation at promoter/leader and 5′-regions and expression of integrated viral genes

The data collected from a series of experiments with the early regions of Ad12 DNA integrated in cell lines HA12/7, T637, and A2497-3 are schematically summarized in Figure 2 and in more detail in Figure 5. The bulk of the late regions are not included in the scheme in Figure 5; these regions containing numerous *Msp*I sites are completely methylated. In particular, the region of the major late promoter/leader of Ad12 DNA (16–30 map units) is strongly methylated. It is apparent that with few exceptions the early regions of Ad12 DNA are unmethylated in the three cell lines. In particular the promoter/leader and 5′-regions are completely unmethylated (Figure 5a, b). It is striking to note that in the E1b region, which is expressed in all three cell lines investigated, one 5′-CCGG-3′ site in the main part of the E1b region is methylated in lines T637 and A2497-3 (Figure 5a). Similarly,

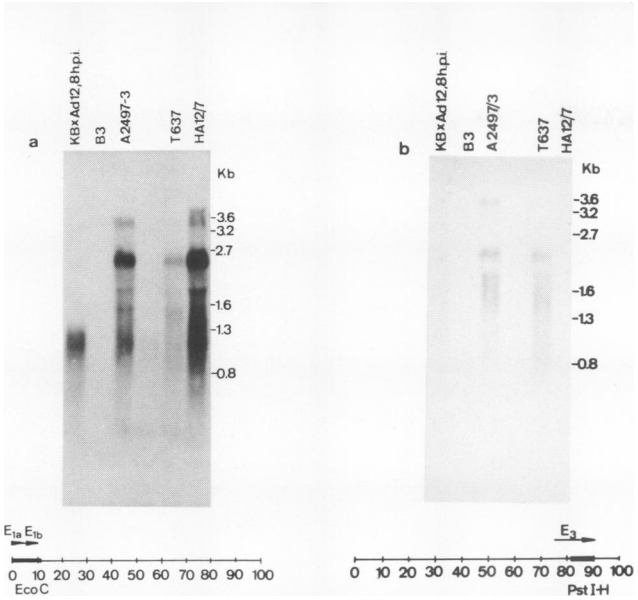

Fig. 6. Expression of Ad12-specific RNA from early regions E1 and E3 in cell lines A2497-3, T637, and HA12/7. Cytoplasmic RNA from the Ad12-transformed cell lines as indicated, from B3 hamster cells and from KB cells productively infected with Ad12 (8 h post-infection) was isolated, fractionated by electrophoresis on 1% formamide agarose slab gels and transferred to nitrocellulose filters as described earlier (Schirm and Doerfler, 1981). Ad12-specific RNA sequences were visualized by hybridization to the cloned *Eco*RI-C (**a**) or the *Pst*I-H (**b**) fragments derived from the E1 or E3 regions of Ad12 DNA, respectively (c.f. Figure 2). The viral DNA fragments were ^{32}P-labeled by nick-translation. The map locations of the cloned DNA probes are schematically indicated. The mol. wt. markers (in bp) correspond to the *Hind*III fragments of Ad12 DNA. Note the absence of Ad12-specific sequences in HA12/7 RNA when probed with the E3 DNA fragment.

5'-CCGG-3' sites towards the 3'-ends of the E2a region in all three lines examined are methylated, whereas the promoter/leader and 5'-regions of the E2a region are completely unmethylated.

There is one additional interesting observation (Figure 5b). The promoter/leader and the 5'-regions of the E3 region of the integrated Ad12 DNA in cell line HA12/7 are strongly methylated. The E3 region is not expressed as mRNA in cell line HA12/7 (Ortin *et al.*, 1976; Schirm and Doerfler, 1981) and *in vitro* translation experiments using RNA selected on Ad12 DNA fragments corresponding to the E3 region have not revealed Ad12-specific products (Esche and Siegmann, 1982). By using the cloned *Pst*I-H or *Pst*I-G fragment of Ad12 DNA (data obtained with the latter fragment not shown) as hybridization probe with cytoplasmic RNA from HA12/7 cells which was electrophoresed and transferred to nitrocellulose filters (Alwine *et al.*, 1977; Schirm and Doerfler, 1981), we have confirmed the absence of E3-specific cytoplasmic RNA in extracts of HA12/7 cells (Figure 6b), whereas the E1 region is expressed in all three transformed cell lines (Figure 6a). We thus conclude that an inverse correlation exists between the levels of DNA methylation at the promoter/leader and the 5'-regions of specific groups of integrated Ad12 genes and the extent to which the regions regulated by these promoters are expressed as mRNA. The state of methylation in the 3'-regions of the gene appears not to be that decisive.

Conclusions

The experiments reported here support the following conclusions:

1. By using highly specific hybridization probes, detailed maps of the methylated and unmethylated 5'-CCGG-3' sites in integrated Ad12 DNA in cell lines T637, HA12/7, and A2497-3 have been obtained. Previous conclusions about an inverse correlation between the extent of DNA methylation of specific genes in integrated viral genomes and the levels of expression of these genes have been confirmed and refined.
2. The mapping data now suggest that the levels of methylation in the promoter/leader and 5'-regions of specific viral genes determine gene expression.
3. The data are consistent with the notion that, in two of the three cell lines investigated, the multiple copies of integrated Ad12 DNA have the same methylation pattern. This finding may suggest that any one of these multiple viral DNA copies is available for expression in the early regions. Which copies are eventually expressed may be determined by factors not exclusively related to DNA methylation.
4. The levels of methylation at 5'-CCGG-3' and at 5'-GCGC-3' sites seem to be the same or very similar.

Materials and methods

All techniques used in this report have been described before (Doerfler *et al.*, 1979; Stabel *et al.*, 1980; Sutter and Doerfler, 1980; Vardimon *et al.*, 1982a). The origin of the cell lines investigated has been described elsewhere (Sutter *et al.*, 1978). The cloned restriction endonuclease fragments of Ad12 DNA were prepared in our laboratory (Vogel *et al.*, 1981).

The *Pst*I map of Ad12 DNA was determined by conventional methods as described in the legend of Figure 2.

The description of all other methods used can be found elsewhere: Southern blot transfer of DNA fragments (Southern, 1975), nick-translation of Ad12 DNA or Ad12 DNA fragments (Rigby *et al.*, 1977; Stabel *et al.*, 1980), DNA-DNA hybridization techniques (Wahl *et al.*, 1979), and blot transfer of RNA fragments and DNA-RNA hybridization techniques (Alwine *et al.*, 1977; Schirm and Doerfler, 1981).

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