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Genetics

Genomic sequencing reveals a 5-methylcytosine-free domain in active promoters and the spreading of preimposed methylation patterns

(adenovirus type 2-transformed hamster cells/late E2A promoter/gene inactivation)

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ABSTRACT **Previous work demonstrated an inverse correlation between methylation at the three 5′-CCGG-3′ sequences in positions +24, +6, and −215 relative to the cap site of the late E2A promoter of adenovirus type 2 (Ad2) DNA and its activity. In the study presented here, we used the genomic sequencing method to detect 5-methyl-2′-deoxycytidine (m^5dC) residues in 5′-CG-3′ sequences other than the 5′-CCGG-3′ (*Hpa* II) sites. The patterns of methylation in all 5′-CG-3′ sequences over a region of about 180 base pairs required for gene activity in the late E2A promoter of integrated Ad2 DNA were determined in cell lines that carry this promoter in an active or inactive state. In cell lines HE1 and uc2, the late E2A promoter is active and all thirteen 5′-CG-3′ sequences between positions +24 and −160 are unmethylated. In cell line HE2, the same promoter is permanently shut off and all 5′-CG-3′ sequences are methylated in both strands. Thus, the inverse correlation is perfect in these cell lines over a region of about 180 base pairs in the late E2A promoter. The same promoter segment was analyzed in cell lines mc23 and mc40, in which a late E2A promoter–chloramphenicol acetyltransferase (CAT) gene construct had been genomically fixed after *in vitro* 5′-CCGG-3′ methylation and subsequent transfection. In cell line mc23, the preimposed methylation pattern was stable and the CAT gene was inactive. Genomic sequencing confirmed the presence of m^5dC in the 5′-CCGG-3′ sequences and revealed the spreading of methylation to neighboring 5′-CG-3′ sequences along the entire promoter. Some of these sites were hemimethylated. In cell line mc40, several of the 120 integrated copies became demethylated in positions +24 and +6, but the promoter was methylated in some of the copies upstream of position −50. Cell line mc40 expressed the CAT gene.**

The regulation of promoter activity is governed by the interaction of specific promoter sequence motifs with regulatory proteins whose binding can entail positive or negative regulatory effects (for review, see ref. 1). While this aspect of promoter modulation has been studied in detail, much less is known about the role of the three-dimensional structure of promoter–protein complexes in the regulation of gene activity. The repertoire of modifications on the DNA side of the binary DNA–protein system is very limited. The methylated nucleoside 5-methyl-2′-deoxycytidine (m^5dC) has been recognized as a genetic signal that can lead to promoter inhibition or inactivation, provided it is present in specific promoter sequences (2–4). It is surmised that this long-term genetic signal functions by compromising some, but not all, specific DNA–protein interactions. The methylation-mediated transcriptional block is temporarily reversible by reactivation mechanisms that act either in trans, like the 289-amino acid protein of adenovirus (5, 6), or in cis, like the strong enhancer from human cytomegalovirus (7). It has been suggested that transient demethylations can occur (8, 9). Hence, several mechanisms seem to be operative that can abrogate the inhibitory effect of promoter methylation.

Promoter methylation, though seemingly uncomplicated chemically, represents a complex genetic signal. We have chosen to limit our studies to two adenovirus promoters, the E1A promoter of adenovirus type 12 (Ad12) DNA (10, 11) and the late E2A promoter of Ad2 DNA (5, 12). Promoter inactivation by *in vitro* methylation of three 5′-CCGG-3′ sequences in the late E2A promoter was demonstrated in transient-expression systems (5, 12), in cell-free transcription assays (13), and after genomic integration of a late E2A promoter–reporter gene construct (14).

The establishment of *de novo* methylation in recently fixed foreign genomes has proven to be a gradual process. Many cell generations are required before a specific pattern of methylation is imposed upon the recently inserted DNA sequences (15, 16). When specifically methylated foreign DNA is permanently fixed in the mammalian genome, the preimposed patterns of methylation appear to be maintained in some of the clonal cell lines but are altered in others (14). Integration of nonmethylated viral DNA can lead to local demethylations of cellular DNA in the vicinity of the integration site (17).

Most studies on patterns of DNA methylation have been based on the use of methylation-sensitive restriction endonucleases (18). The method of genomic sequencing (19) provides an alternative approach that permits analysis of the state of methylation, even of deoxycytidine residues outside the recognition sites of restriction endonucleases. It has been our objective to investigate the mode of methylation in active and in inactive eukaryotic promoters and to include in this analysis all deoxycytidine residues. We have thus studied functionally important sequences in the late E2A promoter of Ad2 DNA in several Ad2-transformed hamster cell lines and in cell lines that carry an integrated late E2A promoter–reporter gene construct. The late E2A promoter was active in some cell lines but was inactivated by methylation in other cell lines.

MATERIALS AND METHODS

Propagation of Cell Lines in Culture. The Ad2-transformed hamster cell lines HE1, HE2, and HE3 (20, 21) and the BHK21 hamster cell lines uc2, mc23, and mc40, which were generated by fixing the unmethylated (uc) or the 5′-CCGG-3′-methylated (mc) pAd2E2AL–CAT construct in the cellular genome by cotransfection with and selection for the

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Abbreviations: m^5dC, 5-methyl-2′-deoxycytidine; Ad2, adenovirus type 2; CAT, chloramphenicol acetyltransferase.

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neomycin phosphotransferase gene (14), were grown in Dulbecco medium (22) supplemented with 10% fetal bovine serum. In the pAd2E2AL–CAT gene construct, the *Kpn* I–*Hin*dIII fragment of the late E2A promoter of Ad2 DNA was fused to the gene for chloramphenicol acetyltransferase (CAT) as an indicator for promoter activity (5). The characteristics of the cell lines are summarized in Table 1. Cells were subcultured at a ratio of 1:4; hence each passage corresponds to about two generations.

Isolation and Enrichment of Cellular DNA Sequences. High molecular mass DNA was isolated from exponentially growing cell cultures by standard procedures (23). In brief, nuclei were isolated in buffer containing Nonidet P-40, protein was removed by proteinase K/SDS treatment followed by one phenol and one chloroform extraction, and DNA was isolated by ethanol precipitation. To decrease the molecular mass of the DNA, samples (1–2 mg) were digested for 1–2 hr with 500 units of *Hin*dIII, which has no cleavage site in the region chosen for sequencing. DNA samples were subsequently treated with RNase followed by several cycles of phenol–chloroform extraction.

The late E2A promoter fragment was enriched according to a published procedure (24). One milligram of DNA was cleaved overnight in small portions with *Kpn* I and *Sac* I for cell lines HE1 and HE2 or with *Hin*dIII and *Sac* I for the uc and mc cell lines (Fig. 1). These enzyme pairs excised the late E2A promoter in a small fragment of about 300 base pairs (bp). However, most of the cellular DNA was cleaved into fragments of >1 kbp. The adenovirus late E2A promoter fragment was then enriched 50- to 100-fold by size fractionation of the DNA on linear 5–25% sucrose gradients in 1 M NaCl/0.01 M Tris·HCl, pH 8.0/1 mM EDTA. Gradients were centrifuged at 25,000 rpm for 24 hr in an SW28 rotor at 18°C in a Beckman ultracentrifuge. Fractions containing DNA fragments 200–400 bp in length were pooled, and the samples were used directly for sequencing reactions. For genomic sequencing of the proximal promoter part at higher resolution, the fractionated DNA preparations were further cleaved with *Bst*XI (Fig. 1).

Genomic Sequencing. Size-fractionated DNA (10–20 μg) was subjected to partial chemical cleavage. DNA fragments generated in this way were subsequently resolved by electrophoresis in a 6% polyacrylamide gel (80 × 30 × 0.1 cm) in 7 M urea and were electrotransferred and UV-crosslinked to GeneScreen (NEN) membranes (19, 26). The sequencing "ladder" on the gel was visualized by autoradiography after hybridizing the fragments to DNA probes that had been ^{32}P-labeled by the indirect end-labeling technique (27). Hybridization probes were prepared in the following way (26). The *Kpn* I–*Hin*dIII fragment of the late E2A gene of Ad2 DNA (Fig. 1) was cloned into M13mp8 or M13mp9 vector DNA and used as a template for the synthesis of single-stranded probes that contained either the top strand (transcribed left to right) or the bottom strand (transcribed right to left). As primers for DNA synthesis, four different 20- to 23-bp oligonucleotides (1–4 in Fig. 1) were synthesized (Applied Biosystems 381A DNA synthesizer) that corre-

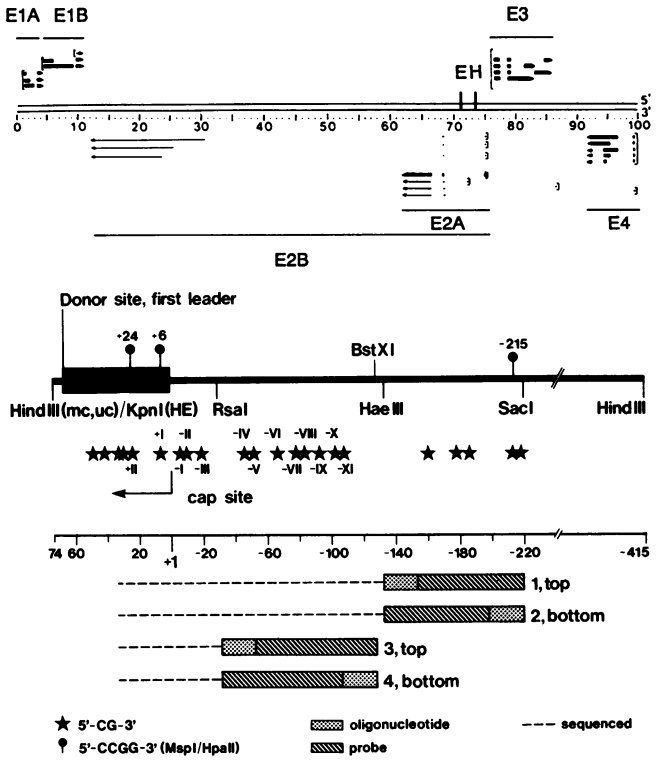

FIG. 1. Map of the late E2A promoter of Ad2 DNA. (*Upper*) Locations of the early regions (E1A, E1B, E2A, E2B, E3, and E4) of Ad2 DNA are shown. For each, the major transcripts are indicated by arrows. Scale numbers represent map units. EH refers to the *Eco*RI–*Hin*dIII fragment containing the late E2A promoter. (*Lower*) Map of the *Kpn* I–*Hin*dIII fragment of the late E2A promoter (nucleotides 25,881–26,369; ref. 25) is presented. Nucleotide scale below the map refers to nucleotides in downstream (+) and upstream (−) positions, relative to the late E2A cap site (nucleotide +1), which is designated by an arrow. Stars and Roman numerals represent 5′-CG-3′ sequences (+II to −XI; see also Figs. 2–4). There are no 5′-CG-3′ sequences between the −XI position and nucleotide −160. Bars at the bottom represent DNA fragments or oligonucleotides that were used as probes in different genomic sequencing experiments.

Table 1. Characteristics of cell lines in which the methylation status of the late E2A promoter (E2AL) of Ad2 DNA was determined

Cell line	Mode of introduction of E2AL	Methylation of 5′-CCGG-3′ in the E2AL*	Gene controlled by E2AL	Activity of E2AL	Number of foreign DNA copies†	Refs.
HE1	Transformation of cultured hamster embryo cells	−	E2A	+	2–4	20, 21
HE2	by infection with UV-irradiated Ad2	+	E2A	−	2–4	20, 21
uc2	Transfection of BHK21 hamster cells with unmethylated pAd2E2AL–CAT	−‡	CAT	+	20	14
mc23	Transfection of BHK21 hamster cells with	+‡	CAT	−	10	14
mc40	5′-CCGG-3′-methylated pAd2E2AL–CAT	±‡	CAT	+	120	14

DNA from these cell lines was extracted and enriched for the Ad2 DNA-containing sequences, and the genomic sequencing technique was applied to the late E2A promoter sequence between nucleotides +24 to −110 relative to the downstream cap site at +1.

*In HE1 and HE2, the integration patterns of Ad2 DNA with internal deletions have been determined (21). In uc2, mc23, and mc40, the unmethylated (uc line) or the 5′-CCGG-3′-methylated (mc lines) pAd2E2AL–CAT construct was integrated in multiple copies (14). In mc40, some of the 5′-CCGG-3′ sequences became demethylated for unknown reasons and the late E2A promoter was reactivated.

†Estimates based on published work (21) and on present study.

‡These cell lines contained only the late E2A promoter of Ad2 DNA and not the entire E2A gene. Consequently, 5′-CCGG-3′ sequences were analyzed only in the promoter segment.

sponded to the 5′ ends of the four different probes. Two of the probes, 1 and 2, facilitated the sequencing of the DNA strands in the late E2A promoter. For improved resolution in the 3′ direction from the *Rsa* I site, probes 3 and 4 were used in some hybridization experiments with size-fractionated, *Bst*XI-cleaved DNA. The same filters were used for the consecutive hybridizations (27) with the strand-specific probes. Kodak XAR5 films were exposed for 1–10 days at −80°C.

Other Methods. RNA transfer and hybridization experiments (28), cell extract preparation and CAT assay (29), and *in vitro* methylation of the late E2A promoter by the *Hpa* II DNA methyltransferase (12) have been described.

RESULTS AND DISCUSSION

Cell Lines. The cell lines described in Table 1 were chosen for an analysis of the state of methylation of all the 5′-CG-3′ residues between nucleotides +24 and −160 in the late E2A promoter (map in Fig. 1) of integrated Ad2 DNA because these cell lines presented this promoter in the active or inactive state. It had been shown that sequence-specific methylations of the late E2A promoter can reduce or eliminate its activity (5, 12–14). We determined the methylation status at the 5′-CCGG-3′ sequences in the same promoter in cell lines HE1 and HE2, which had been generated in culture by transformation with human Ad2. Methylation of the 5′-CCGG-3′ sites in this promoter in cell line HE2 was imitated by transfecting pAd2E2AL–CAT that had been methylated *in vitro* at three sites by *Hpa* II DNA methyltransferase. In cell line mc23, the preimposed methylation pattern was stable. In cell line mc40, the preimposed methylation pattern was only partly stable and the 5′-CCGG-3′ sites became demethylated in some of the 120 copies upon passage in culture (14). The *Hpa* II and *Hha* I sites in the genome of Ad2 account for only 22.5% of all 5′-CG-3′ sequences (30). Hence, it was mandatory to determine the state of methylation at all 5′-CG-3′ sequences in the late E2A promoter. The only method available for this project was the genomic sequencing technique (19). The cell lines chosen for investigation facilitated the determination of methylation patterns in all 5′-CG-3′ sequences in a well-studied promoter that was active or inactive in different cell lines (Table 1).

Methylation Patterns in the Ad2-Transformed Hamster Cell Lines HE1 and HE2. The genomic sequencing technique was applied to size-class-selected DNA from cell lines HE1 and HE2 (Fig. 2). The presence of m^5dC in a nucleotide sequence rendered this site refractory to hydrazine cleavage (31, 32) during the standard chemical sequencing reactions (33). Hence, the presence of m^5dC resulted in the loss of signals in the sequencing ladders at positions where the unmethylated, plasmid-cloned control DNA had produced signals for the same nucleotide sequence. In control experiments, both the unmethylated and the *in vitro* 5′-CCGG-3′-methylated pAd2E2AL plasmid DNA were investigated and, as expected, only the unmethylated DNA was cleaved at the internal cytidine [compare control (Con met) lanes in Fig. 2 with control (Con) lanes in Fig. 3]. Cytidine residues outside the 5′-CCGG-3′ sequences were not methylated in the control reactions. The absence of signals in all 5′-CG-3′ dinucleotides in either strand of the late E2A promoter in DNA from HE2 cells (Fig. 2) indicated the presence of m^5dC residues in the +24 to −110 sequence (see map in Fig. 1). Similar findings (not shown) were adduced for DNA from cell line HE3. The results shown in Fig. 2 were reproduced three times with cellular DNAs prepared from cells in different passage levels. Between nucleotides −110 and −160, 5′-CG-3′ dinucleotides did not occur.

The possibility existed that the late E2A promoter in the DNA from cell line HE2 lacked cytidine-specific chemical

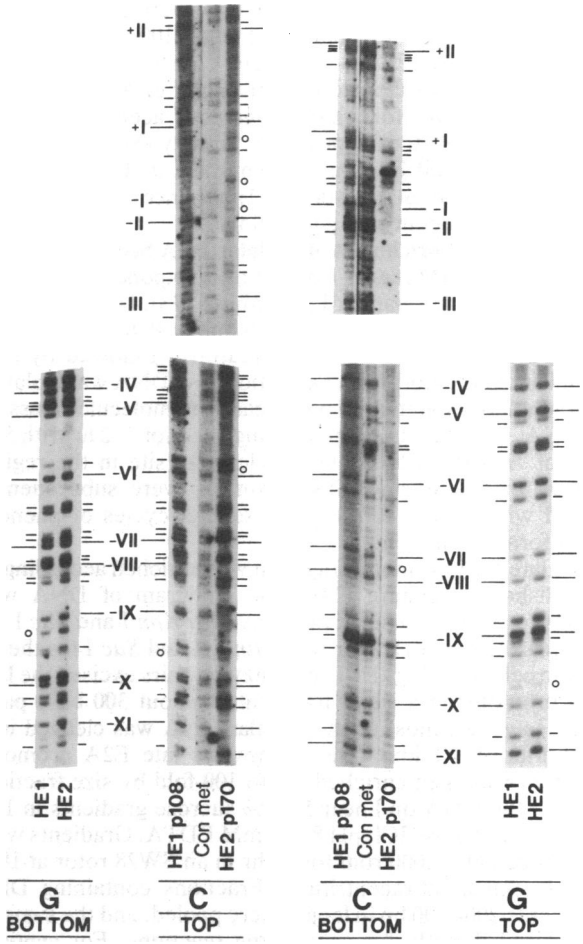

FIG. 2. Autoradiograms of genomic sequencing gels. DNA from cell lines HE1 and HE2 (see Table 1) was analyzed in the late E2A promoter regions of the integrated Ad2 DNA molecules. p, Passage. Details of the genomic sequencing method (19), as adapted for this study, are described in *Materials and Methods*. Roman numerals refer to the 5′-CG-3′ dinucleotide positions of the map (Fig. 1). C and G designate sequence ladders for the cytidine or the guanosine reaction, respectively. Cytidines as well as guanosines in CG dinucleotides are indicated by tick marks. Depending on the hybridization probe, the top- or the bottom-strand signals were visualized. Unexplained bands are indicated by small open circles. In the control (Con met) lanes, the *Hpa* II-methylated late E2A promoter fragment was genomically sequenced as a plasmid clone.

cleavages at the 5′-CG-3′ sequences because of a loss of cytidine residues during cell passages. It has been documented that m^5dC can be eliminated by deamination, which would eventually lead to a G·T mismatch in duplex DNA. The subsequent random repair would then abolish the m^5dC residue (34). DNA from cell line HE2 was therefore treated with dimethyl sulfate to generate G ladders (33), and both DNA strands were sequenced in the late E2A promoter region. The guanosine residues were in the expected positions in both strands (Fig. 2). Hence, none of the C·G pairs in the late E2A promoter had been converted to A·T pairs, and the absence of cytidine signals in the genomic sequencing reaction was due to the presence of m^5dC residues. In some positions in the C tracks of sequencing gels (Figs. 2 and 3), bands were apparent that had no correspondence in the G tracks and lacked a cytidine residue found in the published Ad2 sequence (25). The origin of these bands (indicated by ○ in Figs. 2 and 3) was not apparent.

In contrast to the fully methylated late E2A promoter in the DNA from cell line HE2, the same sequence was unmethylated in the DNA from cell line HE1 (Fig. 2). Previous

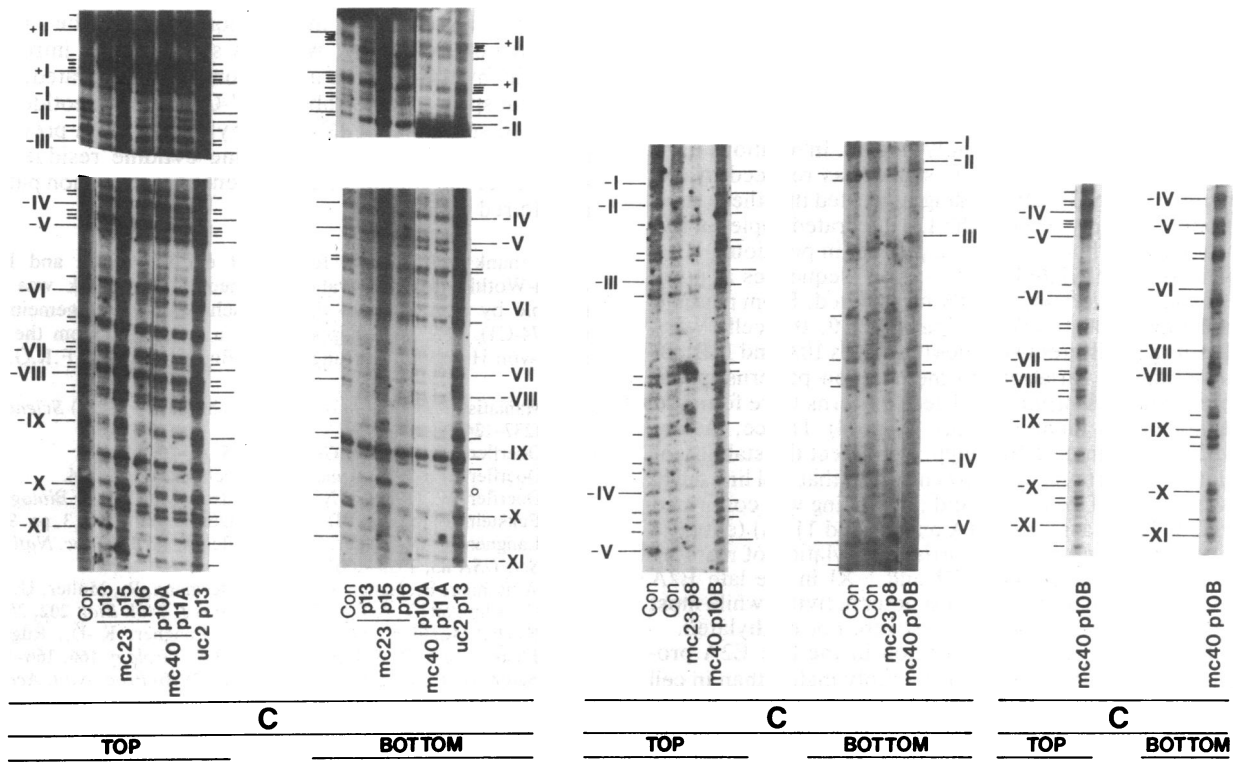

FIG. 3. Autoradiograms of genomic sequencing gels. Cell lines uc2, mc23, and mc40 (see Table 1) were analyzed in different passages (p). In the control (Con) lanes, the unmethylated late E2A promoter fragment was genomically sequenced as a plasmid clone. For further explanations, see legend to Fig. 2.

investigations had established that the E2A gene was inactive in HE2 but active in HE1 (35–37). This result was confirmed for the passage levels of these cell lines currently used by analyzing the cytoplasmic RNA by the RNA transfer-hybridization (Northern) method (data not shown). Hence, straightforward inverse correlations between the methylated and the unmethylated configuration of all 5′-CG-3′ dinucleotides in this sequence and the inactive (HE2, HE3) or the active (HE1) state of the late E2A promoter of integrated Ad2 DNA, respectively, were observed for these cell lines (Figs. 2 and 4). The results obtained with DNA isolated from cell line HE1 (passages 108 and 111) or cell line HE2 (passages 148, 151, and 170) were independent of the different passage levels (data not shown).

Methylation Patterns in the Late E2A Promoter in Recently Established Hamster Cell Lines. In cell line uc2, the unmethylated pAd2E2AL–CAT construct had been genomically fixed in the hamster cell genome by cotransfecting BHK21 cells with that construct and with pSV2neo (refs. 14 and 38; Table 1). The state of methylation in the late E2A promoter in this cell line was determined by genomic sequencing (Fig. 3). All 5′-CG-3′ sequences in this promoter segment were devoid of m^5dC, akin to the DNA from cell line HE1.

A considerably more complex situation was observed in

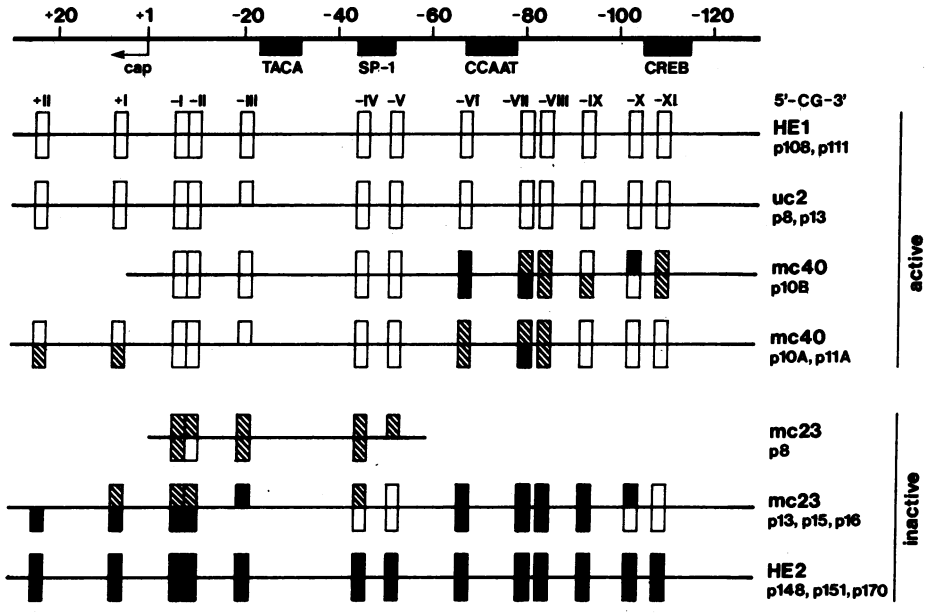

FIG. 4. Methylated and unmethylated 5′-CG-3′ dinucleotides in the late E2A promoter of Ad2 DNA in several transformed hamster cell lines, as shown by genomic sequencing. Scale refers to nucleotide numbers in the late E2A promoter relative to the cap site. 5′-CCGG-3′ (*Hpa* II) sequences are at +6 and +24. Landmark sequences (TACA, CCAAT) or protein binding sites [for transcription factor Sp1 (SP-1) or the cAMP response element-binding protein (CREB)] are indicated. Horizontal lines represent the late E2A promoter segment in cell lines in passages (p) as designated. 5′-CG-3′ sequences in this segment (+II to −XI) are represented by vertical bars: open, unmethylated; solid, completely methylated; hatched, methylated in only some of the integrated promoter copies. Bar above the line designating the promoter sequence represents 5′-CG-3′ in the top strand, bar below the line represents the same dinucleotide in the bottom strand. "Active" and "inactive" refer to the state of the late E2A promoter in the cell lines.

DNA from cell line mc23 (passages 13–16) or mc40 (passages 10 and 11) (Figs. 3 and 4) in which the pAd2E2AL–CAT construct had been introduced after *in vitro* methylation of the 5′-CCGG-3′ (*Hpa* II) sequences (14). In the two passages of cell line mc40, the methylation patterns of the late E2A promoter did not differ significantly (Fig. 3). In positions −VI to −VIII, the intensity of the C signal was reduced in the sequencing gel (Fig. 3). This finding suggested that these sites were modified in only some of the 120 integrated copies of the viral promoter. Other 5′-CG-3′ sequences in positions −I to −XI were not methylated. The 5′-CG-3′ sequences at positions +I and +II were only partly methylated. From passage 7 of cell line mc40 onward to passage 10, the cells were subcultivated as different sublines (passages 10A and 10B), in order to compare variations in methylation patterns in the integrated late E2A promoter. These patterns were found to be similar but not identical (Figs. 3 and 4). Hence, factors other than site of integration might also affect the stability of methylation patterns. It was shown earlier that cell line mc40 expressed the CAT gene (14), and this finding was confirmed for mc40 cells in passages 10 (A and B) and 11 (A) (data not shown). Interestingly, full or partial methylation of many of the sites between positions −VI and −XI in the late E2A promoter was compatible with promoter activity, while most of the other 5′-CG-3′ dinucleotides were not methylated.

The levels of 5′-CG-3′ methylation in the late E2A promoter in cell line mc23 were considerably higher than in cell line mc40. In passages 13–16 of mc23, a definite spreading of the previously imposed methylation pattern was observed. In position −X, the 5′-CG-3′ sequence was hemimethylated (Figs. 3 and 4), similarly to cell line mc40 in passage 10B. Hemimethylated sequences were identified by hybridizing the DNA to the top- or the bottom-strand probes. Apparent hemimethylation could not be accounted for by signal loss during rehybridization, since the DNA on the membrane was first hybridized with the top-strand probe. In this experiment, the cytidine in the −X position did not react. Subsequently, the same filter was washed and probed with the opposite strand, and the C signal became apparent. Hemimethylation in position −X in cell line mc23 was observed in passages 13, 15, and 16 and thus appeared not to be a transient phenomenon. The 5′-CG-3′ sequence in position −IV was partly methylated in the top strand in cell line mc23, and the 5′-CG-3′ sequences in positions −V and −XI were not methylated. The other 5′-CG-3′ sites were methylated. As shown previously, mc23 cells did not express the CAT gene (14), and this finding was confirmed for passages 13–16 of this cell line (data not shown). Interestingly, the patterns of methylation in passage 8 were similar to, but in general lower than, those in passages 13–16. We conclude that there is spreading of the preimposed pattern of methylation to additional sites in the late E2A promoter after genomic fixation, and the spreading may increase with continuous passage.

Conclusions. General conclusions about the importance of the state of methylation at individual 5′-CG-3′ sequences for promoter activity are still difficult to present. A limited number of cell lines and a restricted region of the late E2A promoter have been investigated. The most clear-cut correlations between promoter inactivity and the methylation of all 5′-CG-3′ sites or vice versa over a 180-bp segment in the late E2A promoter have been observed in cells maintained in culture for a long time, like cell lines HE1 and HE2 (Fig. 4), and this consistency may be due to continuous passage in cell culture. In cell lines uc2 and HE1, which carry an active late E2A promoter, all 5′-CG-3′ sequences analyzed have proved to be unmethylated; in cell line HE2, with an inactive late E2A promoter, the same sequences were all methylated (Fig. 4). In cell lines mc23 and mc40, a final pattern apparently has not yet been stabilized. However, the relatively large number of integrated copies in these lines renders simple correlations to promoter activity difficult. The data are consistent with the notion that a preimposed methylation pattern in the late E2A promoter of Ad2 DNA, which is subsequently introduced into cells by transfection and genomically integrated, is not static but spreads to neighboring 5′-CG-3′ dinucleotides. The observed spreading of DNA methylation from a previously methylated cytidine to neighboring cytidine residues may account for the gradual establishment of methylation patterns in cultured cells (16).

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