Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 7586–7590, December 1983
Genetics

Expression of the chloramphenicol acetyltransferase gene in mammalian cells under the control of adenovirus type 12 promoters: Effect of promoter methylation on gene expression

(genetic engineering/*in vitro* DNA methylation/early promoters of adenovirus/DNA methylation and expression control/DNA transfection)

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Communicated by Igor Tamm, August 31, 1983

ABSTRACT **The effect of DNA methylation at specific promoter sites on gene expression was tested by using a sensitive and quantitative assay system. The plasmid pSVO CAT contains the prokaryotic gene chloramphenicol acetyltransferase (CAT) and a *Hin*dIII site in front of it for experimental promoter insertion. Upon insertion into pSVO CAT, the E1a and protein IX gene promoters from adenovirus type 12 (Ad12) DNA were capable of mediating CAT expression upon transfection in mouse cells. In many viral and nonviral eukaryotic genes, DNA methylation at highly specific sites in the promoter region can attain a regulatory function in gene expression. One of the important sites is the 5′ C-C-G-G 3′ sequence. The CAT-promoting activity of the early simian virus 40 promoter in plasmid pSV2 CAT is refractory to methylation by the *Hpa* II or *Hha* I DNA methyltransferase at 5′ C-C-G-G 3′ or 5′ G-C-G-C 3′ sequences, respectively, because this promoter lacks such sites. The CAT coding sequence of this plasmid carries four *Hpa* II and no *Hha* I sites. Methylation of the *Hpa* II sites in the coding region does not affect expression. The E1a promoter of Ad12 DNA comprising the leftmost 525 base pairs of the viral genome carries two 5′ C-C-G-G 3′ and three 5′ G-C-G-C 3′ sites upstream from the leftmost "TATA" signal. Methylation of the *Hpa* II or *Hha* I sites incapacitates this promoter. The promoter of protein IX gene of Ad12 DNA contains one 5′ C-C-G-G 3′ and one 5′ G-C-G-C 3′ site downstream and two 5′ G-C-G-C 3′ sites >300 base pairs upstream from the TATA motif and probably outside the promoter. The protein IX promoter is not inactivated by methylation of these sites. These data demonstrate that critical 5′ methylations in the promoter region decrease or eliminate transcription; methylations of sites too far upstream or probably any sites downstream from the TATA site do not affect expression.**

Several lines of evidence support the notion that DNA methylation at highly specific sites can affect the regulation of eukaryotic gene expression. Inverse correlations between the extent of DNA methylation and the level of gene expression in higher eukaryotes have been established (refs. 1 and 2; for reviews, see refs. 3–6). The results of *in vitro* methylation experiments using cloned viral or nonviral genes have demonstrated that genes methylated at specific sites are not expressed; unmethylated genes are expressed upon microinjection into oocytes of *Xenopus laevis* or into mammalian cells (7–12). Moreover, the cytidine analogue 5-azacytidine, an inhibitor of cellular DNA methyltransferase(s), efficiently induces some previously dormant genes (13–18). In several systems absence of DNA methylation has been shown to be a necessary, but not sufficient, precondition for gene activation (19–22).

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We have used the adenovirus system to study the function of DNA methylation (6, 23). Recently, we have shown in adenovirus type 12 (Ad12)-transformed hamster cell lines that methylation at 5′ C-C-G-G 3′ (*Hpa* II) or 5′ G-C-G-C 3′ (*Hha* I) sites at the promoter region or the 5′ end of integrated viral genes (or both) correlates with the inactive state of these genes (24). Similar conclusions have been drawn in other systems (21, 25, 26). We have now employed an experimental system that allows the direct manipulation of promoter activity.

The prokaryotic gene for chloramphenicol acetyltransferase (CAT) had been inserted (27) into the vector pSV2-βG based on pBR322 and simian virus 40 (SV40) DNAs (B. H. Howard, personal communication). The vector pSVO CAT (27) carries a *Hin*dIII site just in front of the CAT gene. At this site any eukaryotic promoter signal can be inserted and tested for activity. We have adopted that plasmid and assessed the efficiency of several Ad12 promoters in short-term transfection experiments, with the plasmid probably in an episomal state. The results demonstrate that the enzymatic methylation of Ad12 promoters at critical 5′ C-C-G-G 3′ or 5′ G-C-G-C 3′ sites upstream from the "TATA" (Hogness–Goldberg) signal causes inactivation of the gene. *In vitro* methylations of the same sites too far upstream or probably of any sites downstream from this signal seem to have no effect on promoter strength. Promoters lacking 5′ C-C-G-G 3′ or 5′ G-C-G-C 3′ sites, as the early SV40 promoter in the plasmid pSV2 CAT, cannot be inactivated by methylation; neither does methylation of the four *Hpa* II sites in the CAT coding sequence inactivate expression.

MATERIALS AND METHODS

Recipient Cells. Recipient cells used in transfection experiments were mouse Ltk$^-$ cells.

Plasmids and Plasmid Constructions. The plasmid pSVO CAT (27) was opened at the *Hin*dIII site (Figs. 3–5). Nucleotide sequences derived from the Ad12 genome, as indicated in Fig. 1*b*—i.e., the E1a promoter or the gene IX promoter—were excised from the cloned *Eco*RI-C fragment of Ad12 DNA. All fragments were adapted to the *Hin*dIII site in the plasmid pSVO CAT by ligating *Hin*dIII linkers (Bethesda Research Laboratories) to either end. These linkers were phosphorylated by using polynucleotide kinase and [γ-^{32}P]ATP. The DNA fragments had to be filled in by using deoxyribonucleoside triphosphates

Abbreviations: Ad12, adenovirus type 12; CAT, chloramphenicol acetyltransferase; CAM, chloramphenicol; SV40, simian virus 40; bp, base pair(s).

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and the Klenow fragment (28) of DNA polymerase I (29). Subsequently, the phosphorylated *Hin*dIII linkers were ligated to the prepared inserts. The *Hin*dIII-cut pSVO CAT plasmid was pretreated with bacterial alkaline or calf intestinal phosphatase. Insert and pretreated plasmid DNAs were ligated and transfected into *Escherichia coli* strain HB101/λ. Positive clones were selected (30) by hybridization to the isolated ^{32}P-labeled *Eco*RI-C fragment. Details of these techniques have been published elsewhere (31, 32). The insert and its orientation in the pSVO CAT plasmid were determined by cleavage with diagnostic restriction endonucleases.

***In Vitro* Methylation Using the *Hpa* II or *Hha* I DNA Methyltransferase.** The DNA methyltransferase *Hpa* II (*Hemophilus parainfluenzae*) methylated *de novo* the internal cytidine in the sequence 5′ C-C-G-G 3′. The enzyme was purified by a published procedure (33). The assay mixture contained in 10 μl of 10 mM EDTA/20 mM 2-mercaptoethanol/50 mM Tris·HCl, pH 7.5/4 μM ^{3}H-labeled or unlabeled *S*-adenosylmethionine (Amersham; 60 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) 1 μg of salmon sperm DNA and 2.5 μl of the enzyme and was incubated at 37°C for 16 hr. Methylated [^{3}H]DNA was measured by thin-layer chromatography on polyethyleneimine plates. The DNA methyltransferase *Hha* I (*Hemophilus hemolyticus*) (New England BioLabs) methylated the internal cytidine in the sequence 5′ G-C-G-C 3′. The reaction proceeded at 37°C for 1 hr with 1 μg of DNA in 10 μl of 50 mM Tris·HCl, pH 7.5/10 mM EDTA/80 μM *S*-adenosylmethionine/5 mM 2-mercaptoethanol.

Because large amounts of methylated (or unmethylated DNA were used in transfection, it was essential to test each preparation of methylated DNA for complete methylation at 5′ C-C-G-G 3′ or 5′ G-C-G-C 3′ sites. The DNA was cut with the diagnostic resistriction endonuclease pair *Hpa* II and *Msp* I (34) or with *Hha* I and was analyzed by Southern (35) blot hybridization. In some experiments *in vitro* methylated DNA was incubated with *Hpa* II to cleave all unmethylated DNA. The supercoiled DNA was then repurified by equilibrium sedimentation in dye buoyant density gradients, eliminating all unmethylated (linearized) DNA fragments.

Transfection Experiments. Published methods were followed (27, 36). About 20 μg of pure construct DNA was used; in some experiments, mixtures of 0.1–1 μg of construct DNA and 20–19 μg of pSVO CAT carrier DNA, respectively, were used. Mouse Ltk$^-$ cells growing in monolayers were used at about 70–80% confluency. About 12 hr after transfection, the cell cultures were divided 1:2 to ensure continued replication and to improve expression.

Preparation of Cell Extracts. Cells transfected with DNA 48 hr previously were scraped off after five washes in phosphate-buffered saline deficient in Ca^{2+} and Mg^{2+} (37). Total cell extracts were prepared by ultrasonic treatment using the microtip of a Branson B12 sonifier and were clarified. Assays for CAT were performed immediately.

Assays for CAT. Published procedures were used (27). Briefly, 150 μl of clarified extract was mixed with 20 μl of 4 mM acetyl-coenzyme A (P-L Biochemicals) and 4 μl of [^{14}C]chloramphenicol (CAM; 400 nCi; specific activity = 43.2 mCi/mmol) and incubated for 1 hr at 37°C. CAM and derivatives were extracted with ethyl acetate, dried, resuspended in ethyl acetate, and then applied to a thin-layer silica gel plate (Merck). The positions of CAM and its acetylation products were identified by autoradiography.

Extraction of Cytoplasmic RNA and Blot Analysis. Published procedures were followed (24, 38, 39).

Southern Blot Analysis of Nuclear DNA. Nuclear DNA from transfected cells was extracted and analyzed as described (24, 35,40).

RESULTS

Experimental Design. We have developed a system that permitted *in vitro* methylation by the *Hpa* II or *Hha* I DNA methyltransferase of specific promoters and facilitated quantification of the effect these modifications exerted on promoter efficiency. A combination of early Ad12 promoters and the pSVO CAT plasmid (27) that carried the gene for CAT but lacked eukaryotic promoters provided such a system. The E1a or polypeptide IX promoter of Ad12 DNA (for the map, see Fig. 1) was inserted at the *Hin*dIII site of the plasmid pSVO CAT. The efficiency of each of these promoters in mediating the expression of the CAT gene after transfection of mouse cells was quantitatively assessed by using the CAT assay.

The promoter segments used in constructing pAd12 CAT plasmids were the following (Fig. 1*b*): (*i*) the *Hin*dIII–*Ava* II fragment comprising 29 bp of the plasmid pAT153 and the left-terminal 525 bp of Ad12 DNA, with two TATA motifs starting at bp 276 and 414 in the E1a region. The pSVO CAT construct containing this promoter was designated pAd12-1a CAT. This promoter carries 2 *Hpa* II sites 133 and 124 bp upstream from the TATA signal starting at bp 276 (Fig. 1*b*). The locations of all *Hpa* II and *Hha* I sites in the E1a and gene IX promoters are listed in Table 1. In the promoter region of the E1a segment, a cluster of G+C-rich sequences is apparent between bp 110 and 154, with several partly interdigitating *Hpa* II and *Hha* I sites. The *Hpa* II site at bp 152 is part of an enhancer (41) (Fig. 1*b*); (*ii*) the *Hin*dIII–*Ava* II fragment between bp 2,320 and 3,549 contains the TATA signal for the gene of polypeptide IX and one *Hpa* II site 137 bp downstream from the TATA motif starting at bp 3,323. There is 1 *Hha* I site downstream from the TATA signal and 2 *Hha* I sites >300 bp upstream from it. This construct was termed pAd12-IX CAT (Fig. 1*b*). The protein IX gene of adenovirus DNA can apparently be expressed independently of the E1a promoter. In tests on the effect of *Hpa* II or *Hha* I methylation on promoter strength, the construct pSV2 CAT (27) has been used as a control. Here, CAT gene expression is mediated by the early SV40 promoter, which lacks 5′ C-C-G-G 3′ and 5′ G-C-G-C 3′ sites. Controls using this construct were essential, because both the CAT gene and the bacterial plasmid contained 4 and 9 *Hpa* II sites, respectively (see Fig. 3). The CAT gene had no *Hha* I site; the plasmid had 14 such sites. Thus, the effect of methylation at these sites had to be controlled by using a construct that lacked *Hpa* II or *Hha* I sites in the promoter region but had such sites in the plasmid and CAT sequences.

The E1a Promoter Mediates Expression of the CAT Gene and Is Inhibited by Methylation. The E1a promoter of Ad12 DNA can promote CAT activity in mouse Ltk$^-$ cells with efficiency about equal to that of the early SV40 promoter (Fig. 2). The pSVO CAT construct (27) lacks eukaryotic promoters and thus cannot express the gene. Positive controls that used commercial CAT were included to mark the chromatographic positions of 1-acetyl- and 3-acetyl-CAM. When the pAd12-1a CAT construct was completely methylated *in vitro* at 5′ C-C-G-G 3′ sites (Fig. 3*a*) by *Hpa* II DNA methyltransferase, CAT activity was abolished or markedly reduced (Fig. 3*b*). When the pAd12-1a CAT DNA was methylated at *Hha* I sites, CAT expression was also abolished (Fig. 3*b*). The results of the pSVO CAT and CAT controls are also shown. It was essential to prove prior to transfection that the pAd12-1a CAT DNA was completely methylated. Southern blot analysis (Fig. 3*c*) demonstrated that the insert in pAd12-1a CAT was completely refractory to cleavage by the restriction endonuclease *Hpa* II—i.e., methylated at all 5′ C-C-G-G 3′ sites—while *Msp* I did cut the DNA as expected. In contrast, when the pSV2 CAT DNA was completely methylated in the same way, expression of the

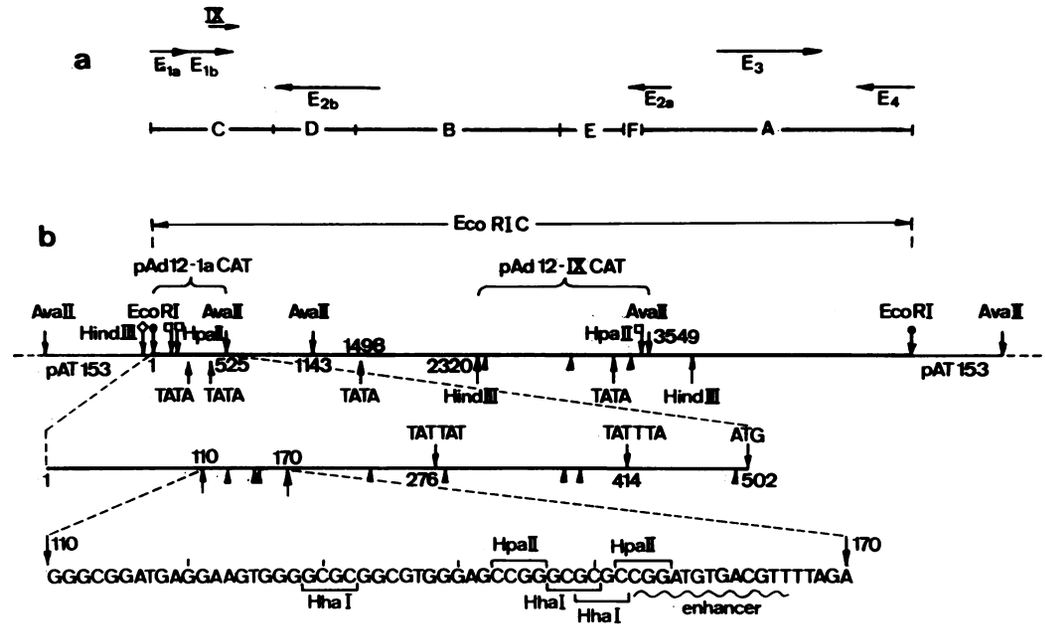

FIG. 1. Map of the Ad12 genome and functional anatomy of its leftmost *Eco*RI-C fragment. (*a*) Functional map of Ad12 DNA (24). E1a, E1b, E2a, E2b, E3, and E4 designate the early regions of the viral genome; IX designates the location of the protein IX gene of Ad12. Letters refer to DNA fragments on the restriction maps. (*b*) Detailed map of the left-terminal *Eco*RI-C fragment with restriction sites as indicated. Numbers refer to base pairs (bp) as counted from the left terminus of viral DNA. TATA (TATTAT, TATTTA) designate the presumptive Hogness–Goldberg boxes. The nucleotide sequence between bp 110 and 170 (42) is also included. *Hpa* II (5′ C-C-G-G 3′) and *Hha* I (5′ G-C-G-C 3′) sites (▲) are indicated.

CAT gene proceeded unabated (Fig. 4). The early SV40 promoter lacks appropriate sites for *Hpa* II or *Hha* I methylation, and, consequently, its activity cannot be modulated by methylation. Methylations of the four *hpa* II sites inside the CAT gene obviously have no effect on expression.

In most experiments, 20 μg of pAd12-1a CAT DNA was used for transfection. The quantity of pAd12-1a CAT DNA could be reduced to 0.1–1 μg by adding pSVO CAT DNA as carrier. The reduced amount still yielded efficient expression of the CAT gene (data not shown). The pSVO CAT DNA is not active by itself (Figs. 2, 3, and 5). It is unknown how the pSVO CAT carrier DNA is able to augment expression of the pAd12-1a CAT construct, possibly by a complicated mechanism.

These data demonstrate that methylations of specific sites upstream from the TATA signal in the E1a promoter eliminate or strongly reduce its activity in mediating expression of the CAT gene in short-term (48 hr) experiments after transfection of mouse cells. When the E1a promoter was mock-methylated with *Hpa* II DNA methyltransferase in the absence of *S*-adenosylmethionine, no effect on its activity was observed (data not shown).

Methylation of the *Hpa* II or *Hpa* I Site Downstream from the TATA Signal in the Protein IX Gene Does Not Affect CAT Expression. The promoter of the protein IX gene (Fig. 1*b*) lo-

Table 1. Coordinates of the TATA signal, *Hpa* II sites, and *Hha* I sites in the E1a and gene IX promoter regions of Ad12 DNA

Promoter region	TATA signal	*Hpa* II sites	Effect of methylation of *Hpa* II sites on expression	*Hha* I sites	Effect of methylation of *Hha* I sites on expression
E1a	276 414	143 152	Decrease or turn off	129 147 231 284 369 380 494 (d)	Decrease or turn off
Gene IX	3,323	3,460 (d)	Weakly stimulating, if any	2,392* 2,975* 3,402 (d)	Weakly stimulating, if any

Coordinates are expressed as bp numbers from the left terminus of Ad12 DNA (42, 43). Sites downstream from the promoter are indicated by (d).

*These sites lie 931 and 348 bp upstream from the TATA box and probably outside the promoter.

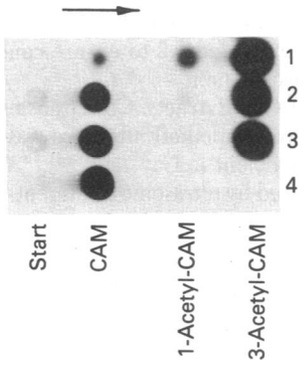

FIG. 2. Expression of the CAT gene under the control of viral promoters. Mouse Ltk⁻ cells were transfected with the plasmid pSV2 CAT (lane 2), pAd12-1a CAT (lane 3), or pSVO CAT (lane 4) as indicated. Extracts were prepared at 48 hr after transfection and incubated with [^{14}C]CAM (27). Acetylation products of CAM were separated by chromatography and visualized by autoradiography as outlined. CAT enzyme control (lane 1) refers to a control experiment in which [^{14}C]CAM was incubated with commercial CAT (P-L Biochemicals) to determine authentic marker positions of CAM and its derivatives (see text). Similar conditions were employed in the experiments described in the legends to Figs. 3–5.

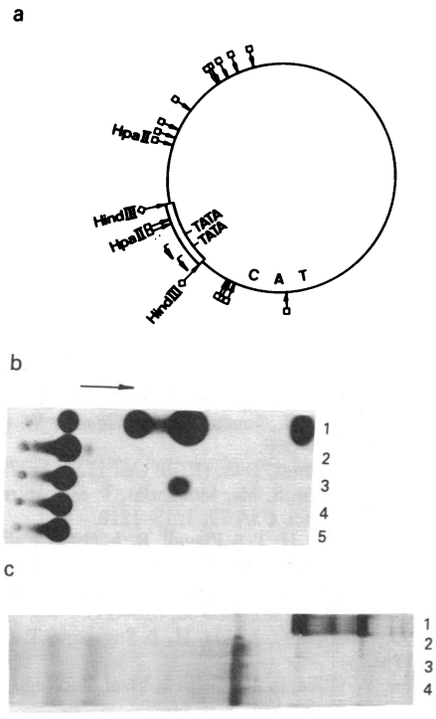

FIG. 3. The E1a promoter of Ad12 DNA mediates CAT expression and is inhibited by methylation. (*a*) Map of the pAd12-1a CAT construct. ⚲ designates 5′ C-C-G-G 3′ (*Hpa* II) sites; ⌣ designates the approximate TATA positions. (*b*) Experimental design and details were similar to those described in the legend to Fig. 2. The pAd12-1a CAT preparation was *in vitro* methylated by the *Hpa* II or *Hha* I DNA methyltransferase. The precise locations of the *Hpa* II and *Hha* I sites relative to the TATA signal are listed in Table 1. Lane 1, CAT enzyme control; lane 2, pSVO CAT; lane 3, pAd12-1a CAT; lane 4, pAd12-1a CAT methylated (*Hpa* II); lane 5, pAd12-1a CAT methylated (*Hha* I). (*c*) Southern blot hybridization analyses of the methylated (lanes 1 and 2) and unmethylated (lanes 3 and 4) pAd12-1a CAT constructs prior to transfection. DNAs as indicated were cleaved with *Hpa* II (lanes 1 and 3) or *Msp* I (lanes 2 and 4) to ascertain complete methylation. Details were described in the text. The ^{32}P-labeled (40) pSVO CAT was used as hybridization probe.

cated inside the E1b region with a TATA signal at bp 3,323 of Ad12 DNA (43) was inserted into the *Hin*dIII site of the plasmid pSVO CAT. Transfection with this construct led to expression of the CAT gene in mouse Ltk$^-$ cells (Fig. 5). Methylation

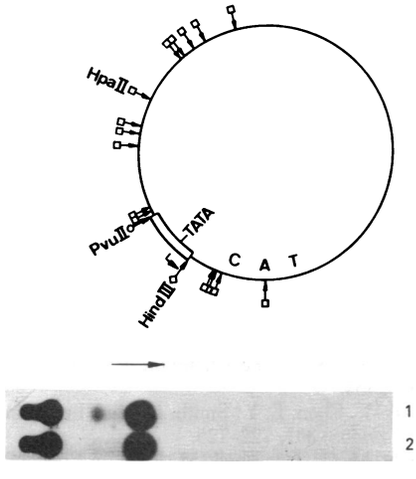

FIG. 4. The early promoter of SV40 DNA in the pSV2 CAT construct is not inactivated by methylation. Lane 1, pSV2 CAT methylated; lane 2, pSV2 CAT.

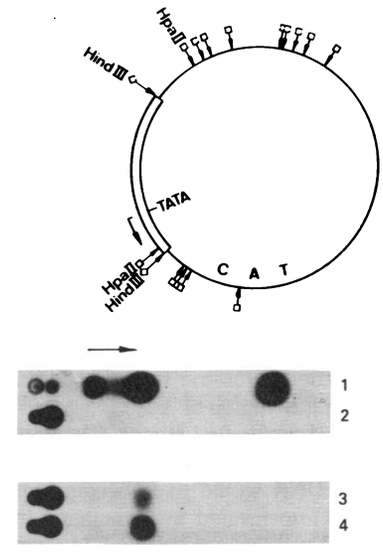

FIG. 5. The protein IX gene promoter mediates CAT expression and is not inactivated by methylation. Lane 1, CAT enzyme control; lane 2, pSVO CAT; lane 3, pAd12-IX CAT; lane 4, pAd12-IX CAT methylated.

of the 5′ C-C-G-G 3′ sites in this construct did not inactivate CAT expression (Fig. 5) nor did methylation of the 5′ G-C-G-C sites (data not shown). The efficiency of the protein IX gene promoter may even be somewhat enhanced by methylation. The only 5′ C-C-G-G 3′ site in this promoter is located 137 bp downstream from the TATA signal (Figs. 1*b* and 5). One *Hha* I site is also located downstream from the TATA signal; two other *Hha* I sites are far upstream from the TATA signal and probably lie outside the promoter (Table 1). These findings strengthen the concept that DNA methylation at highly specific sites inside promoter regions can exert regulatory effects on gene expression. Apparently, methylations downstream from the TATA signal are not effective.

The Effect of Promoter Methylation Apparently Is Exerted at the Transcriptional Level. Methylations of the *Hpa* II sites upstream from the TATA signal in the E1a promoter region of plasmid pAD12-1a CAT inactivated CAT expression (Fig. 3). We have demonstrated that this inactivation was probably due to a transcriptional block. Mouse Ltk$^-$ cells were transfected with methylated or unmethylated pAd12-1a CAT DNA (Fig. 3) or with methylated or unmethylated pAd12-IX CAT DNA (Fig. 5). At 48 hr after transfection, cytoplasmic RNA was extracted and analyzed by gel electrophoresis, blotting (38, 39), and hybridization to the pSVO CAT DNA ^{32}P-labeled by nick-translation (40). CAT-specific RNA sequences can be detected after transfection with unmethylated, but not after transfection with methylated, pAd12-1a CAT DNA. Upon transfection both with methylated and unmethylated pAd12-IX CAT DNA, CAT-spesific RNA was detectable (data not shown). Assuming that transcription starts at the same site in a plasmid not affected and in one affected by methylation, the mRNAs from these two promoters are of equal stability. Thus, methylation at highly specific promoter sites presumably stops transcription.

DISCUSSION

The experimental findings demonstrate that methylations of specific sites in the promoter of certain genes can affect gene expression. It appears that 5′ C-C-G-G 3′ and 5′ G-C-G-C 3′ sites upstream from the TATA signal of the E1a promoter of Ad12 DNA are decisive in this regulatory function. Methylations of sites >300 bp upstream from the TATA signal have no

effect (Table 1). Methylations of the four *Hpa* II sites in the coding sequence of the CAT gene in pSV2 CAT, for example, had no effect on CAT expression (Fig. 4). Of course, the possibility cannot be excluded that specific DNA methylations at 5′ CpG 3′ sites other than *Hpa* II or *Hha* I sites in the body of the CAT gene or the E1a gene may have an effect on gene expression. This problem has not been addressed here, because DNA methyltransferases with appropriate specificities are not available.

It was pointed out previously (6, 23) that *in vitro* DNA methylation experiments with constructions containing methylated sequences would preferably be patterned according to results of analyses on methylated sequences in the same genes in cellular DNA. In all adenovirus-transformed cell lines or Ad12-induced tumors, the E1a region of Ad12 DNA was hypomethylated and expressed (1, 2, 20, 24). Hence, it was not obvious from studies on cellular DNA whether 5′ C-C-G-G 3′ sites in this segment of viral DNA would ever become methylated. Recently, a cell line transformed by the cloned left terminus of Ad5 DNA was analyzed that carried the E1a region of Ad5 DNA in a highly methylated state and consequently expressed viral genes at a reduced rate (44). Thus, the E1 region constituted no exception. In many systems studied, the 5′ C-C-G-G 3′ sequence proved to be of importance (1–6, 23). However, in some genes, methylation of this sequence seems to have no effect on expression.

Mouse cells were chosen in the experiments reported here because they do not allow the replication of adenovirus DNA. The pSVO CAT and pSV2 CAT constructs lack a eukaryotic origin of replication. However, with the pAd12-1a CAT plasmid, an adenoviral origin of replication was introduced into mouse cells. We have demonstrated by Southern blot analyses that in the 48-hr period of the transfection experiment, the pAd12-1a CAT plasmid does not replicate or integrate in mouse Ltk^- cells to any appreciable extent. Thus, problems due to the loss of methyl groups in transfected DNA as a consequence of replication were not to be expected.

In conclusion, it may be of interest to mention that in the E1a promoter region used in the pAd12-1a CAT construct, the pentanucleotide G-G-G-C-G occurred six times. The same or the complementary motif was also found clustered several times in the 21-bp repeats of SV40 DNA (45), in the *myc* oncogene (46), in the immediate early promoter of herpes simplex virus type 1 (47), in the promoter region of the thymidine kinase gene (48), as well as in hamster and human DNA sequences linked to Ad12 DNA in the Ad12-induced tumor line CLAC1 (49) and in the symmetric recombinant (SYREC) between Ad12 and human DNAs (50), respectively.

We are indebted to Cori M. Gorman and Bruce H. Howard (National Institutes of Health, Bethesda, MD) for gifts of the pSV2 CAT and pSVO CAT plasmids. A. van der Eb (Leiden, The Netherlands) kindly supplied the *Eco*RI-C fragment of Ad12 DNA cloned in pAT153. Peter Gruss (Heidelberg) provided mouse Ltk^- cells. We also thank Ute Brüggemann for preparing *Hpa* II DNA methyltransferase, Hanna Mansi-Wothke for media production, and Petra Böhm for editorial assistance. This research was supported by grants from the Deutsche Forschungsgemeinschaft through SFB74-C1 and the Ministry of Science and Research of the State of Nordrhein–Westfalen.

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