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Biochemistry

DNA methylation of three 5′ C-C-G-G 3′ sites in the promoter and 5′ region inactivate the *E2a* gene of adenovirus type 2

(*in vitro* DNA methylations/microinjection of *Xenopus laevis* oocytes/RNA blots/S1 nuclease analysis)

KLAUS-DIETER LANGNER, LILY VARDIMON*, DORIS RENZ, AND WALTER DOERFLER

Institute of Genetics, University of Cologne, Cologne, Federal Republic of Germany

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ABSTRACT **The *E2a* gene of human adenovirus type 2 (Ad2) encodes the 72-kilodalton DNA-binding protein. We previously described perfect inverse correlations between the methylation of all 5′ C-C-G-G 3′ sequences in the Ad2 *E2a* gene in virus-transformed hamster cells containing viral DNA sequences in an integrated state and the extent to which this gene is expressed. We subsequently showed that *in vitro* methylation of all 14 5′ C-C-G-G 3′ sequences in the cloned *E2a* gene by prokaryotic *Hpa* II DNA methyltransferase leads to transcriptional inactivation after microinjection into *Xenopus laevis* oocytes. The unmethylated cloned *E2a* gene is expressed in these cells. We report here the construction of partly methylated clones of the *E2a* gene. In the promoter (5′)-methylated construct, three 5′ C-C-G-G 3′ sequences at the 5′ end of the subclone were methylated. One of these sites is located 215 base pairs (bp) upstream (bp 26,169 of Ad2 DNA), and two sites are located 5 and 23 bp downstream from the cap site (bp 25,931 and 25,949 of Ad2 DNA) of the *E2a* gene. This construct was transcriptionally inactive upon microinjection into nuclei of *X. laevis* oocytes. In the gene (3′)-methylated construct, 11 5′ C-C-G-G 3′ sequences in the main part of the transcribed gene region were methylated *in vitro*. This construct was transcribed in *X. laevis* oocytes, and at least some of the Ad2-specific RNA synthesized was initiated at the same sites as in Ad2-infected human KB cells. Both mock-methylated constructs were transcribed into Ad2-specific RNA in *X. laevis* oocytes. These results demonstrate that DNA methylations at or close to the promoter and 5′ end of the *E2a* gene cause transcriptional inactivation. Perhaps only one methyl group would be adequate for inactivation; *in vivo* methylation of more than one cytosine may be a form of safeguard or redundancy.**

It has been demonstrated clearly in several eukaryotic systems that DNA methylations at specific sites can affect regulation of gene expression. While there are examples that do not seem to support such correlations, in many instances methylations at critical sites of genes have been shown to lead to their inactivation (for reviews, see refs. 1–8). In many genes, 5′ C-C-G-G 3′ sequences are such critical sites; in other genes, these sites have not been pinpointed. This failure may explain apparent discrepancies. In adenovirus-transformed cells, striking inverse correlations between the extent of DNA methylation in integrated viral genes and their expression have been reported (9–11). A causal role of DNA methylation in gene inactivation has been documented by the *in vitro* methylation of several eukaryotic genes (12–17). More recently, the critical sites for DNA methylation leading to inactivation of gene expression have been localized at the 5′ end and in the promoter region of several genes (18–24).

In an even more detailed study, we have been able to demonstrate that methylation of two 5′ C-C-G-G 3′ sites or of 5′ G-C-G-C 3′ sites upstream from the "TATA" signal in the promoter of the *E1a* gene of adenovirus type 12 (Ad12) DNA inactivated this promoter, whereas methylation of one 5′ C-C-G-G 3′ or of one 5′ G-C-G-C 3′ site downstream from the TATA signal in the promoter of protein IX gene of Ad12 DNA left this promoter unaffected (25). The activity of methylated or unmethylated promoters was tested (25) after linkage to the chloramphenicol acetyltransferase gene (*CAT*) and transfection into mouse cells (26). For the globin gene, methylations of all cytidine residues between nucleotides −760 to +100 inactivate the gene, whereas methylations between nucleotides +100 and +1950 do not influence gene expression (27). Methylations of highly specific sites in the promoter and 5′ regions cause long-term shut-off of eukaryotic genes (4). The biochemical mechanisms by which site-specific DNA methylations lead to transcriptional inactivation are less certain. It is conceivable that specific DNA methylations can cause or stabilize structural alterations of DNA (28, 29).

It will be demonstrated that methylations of three 5′ C-C-G-G 3′ (*Hpa* II) sites at the 5′ end of the *E2a* gene [at nucleotides 25,931, 25,949, and 26,169 of adenovirus type 2 (Ad2) DNA] inactivate this gene upon microinjection into nuclei of *Xenopus laevis* oocytes. In contrast, the complete methylation of all 11 5′ C-C-G-G 3′ sites in the 3′ body of the same gene, including introns, does not inactivate it in the same cells. The results presented directly support the notion that critical promoter methylations cause transcriptional inactivation of certain genes.

MATERIALS AND METHODS

Subclone of the *Hin*dIII A Fragment Containing the *E2a* Region of Ad2 DNA. The cloned *Hin*dIII A fragment of Ad2 DNA was excised with *Hin*dIII and *Bam*HI, and this fragment was subcloned in pBR322.

Construction of Partly Methylated *E2a* DNA. Standard techniques were used in construction experiments. The *Bam*HI–*Hin*dIII subclone in pBR322 was cleaved with *Bam*HI and *Eco*RI or with *Eco*RI and *Hin*dIII. DNA fragments were separated by electrophoresis on horizontal agarose gels and electroeluted from the gel at 100 V for 0.5–1 hr. DNA fragments were religated with T4 ligase (New England Biolabs) in 140 mM Tris·HCl, pH 7.8/28 mM $MgCl_2$/55 mM dithiothreitol/2.6 mM ATP at 15°C for 2 hr and at 4°C for 16 hr. The DNA was reextracted with Tris·HCl-saturated phenol and isoamyl alcohol and was precipitated with ethanol. Supercoiled circular DNA was subsequently purified by equilibrium centrifugation in CsCl/ethidium bromide density gradients (30).

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Abbreviations: Ad2 and Ad12, adenovirus types 2 and 12; bp, base pair(s).

*Present address: Developmental Biology Laboratory, Cumming Life Science Center, University of Chicago, Chicago, IL 60637.

***In Vitro* Methylation of DNA Fragments with *Hpa* II DNA Methyltransferase.** DNA fragments were methylated at 37°C for 18 hr in 167 mM Tris·HCl, pH 7.5/33.3 mM EDTA/267 μM *S*-adenosylmethionine/16.7 mM 2-mercaptoethanol with 3 units of *Hpa* II DNA methyltransferase (ref. 31; Biolabs) per μg of DNA. Mock-methylation reactions were performed under similar conditions, except that *S*-adenosylmethionine was omitted from the reaction mixture. Subsequent to the methylation or mock-methylation reaction, the DNA was purified by treatment with phenol and isoamyl alcohol, followed by precipitation with ethanol.

Microinjection of the Nuclei of *X. laevis* Oocytes. *X. laevis* females were induced by the injection of 100 units of human chorionic gonadotropin (32). Oocytes were removed from the ovaries 7–10 days later and were kept in Barth's medium (33) at 18°C. Suitable oocytes were selected under the microscope and fixed onto a nylon grid by low-speed centrifugation at 18°C for 10 min. The nuclei of these oriented oocytes were injected by using glass capillaries (Clark Electromedical Instruments, Pangbourne, England) with 5–7.5 ng of DNA in 20–30 nl of injection buffer (88 mM NaCl/10 mM Tris·HCl, pH 7.5). Subsequently, the oocytes were incubated at 18°C for 24 hr.

Extraction of RNA or DNA from Microinjected Oocytes. Oocytes were dissolved in a solution of 2% $NaDodSO_4$ and 1 mg of proteinase K per ml in 10 mM Tris·HCl, pH 7.5/10 mM NaCl/1.5 mM $MgCl_2$. The solution was adjusted to a pH value of 5.2 or 7.5 by the addition of Na acetate (final concentration, 0.3 M) depending on whether RNA or DNA, respectively, was extracted. The total intracellular nucleic acid was further purified by repeated extractions with Tris·HCl-saturated phenol/chloroform/isoamyl alcohol, 25:24:1 (vol/vol), and with a mixture of chloroform and isoamyl alcohol. In some experiments the DNA or RNA were destroyed by incubation with DNase or RNase (13).

Other Methods. Southern blotting (34), RNA blotting (35, 36), nick translation of DNA (37), DNA·DNA hybridization (38), S1 mapping of RNA (39, 40), and DNA transfection (41) have been detailed (10, 13, 17, 24, 42).

RESULTS

Design of Selective *in Vitro* Methylation Experiments. The *E2a* gene of Ad2 DNA was chosen to selectively methylate the promoter and 5′ region or the body of this gene including the introns and to test the effects of these critical site methylations on gene expression after microinjection into nuclei of *X. laevis* oocytes. There are 3 5′ C-C-G-G 3′ (*Hpa* II) sites in the promoter and 5′ region and 11 such sites in the body of the *E2a* gene (Fig. 1). One of the 5′ C-C-G-G 3′ sites in the promoter is located 215 base pairs (bp) upstream from the site of initiation of RNA transcription, two sites are located 5 bp and 23 bp downstream from that landmark (Fig. 1). The 5′ C-C-G-G 3′ sites were selected for *in vitro* methylation because it had been shown previously that the *E2a* gene can be inactivated transcriptionally by methylations at these sites (12, 13). Moreover, an excellent inverse correlation has been observed between methylation of these sites in the *E2a* gene and gene activity in the Ad2-transformed hamster cell lines HE1, HE2, and HE3 (11).

The construction of partly methylated *E2a* DNA molecules is described schematically in Fig. 2. All construction experiments utilized the *Bam*HI–*Hin*dIII subclone of the

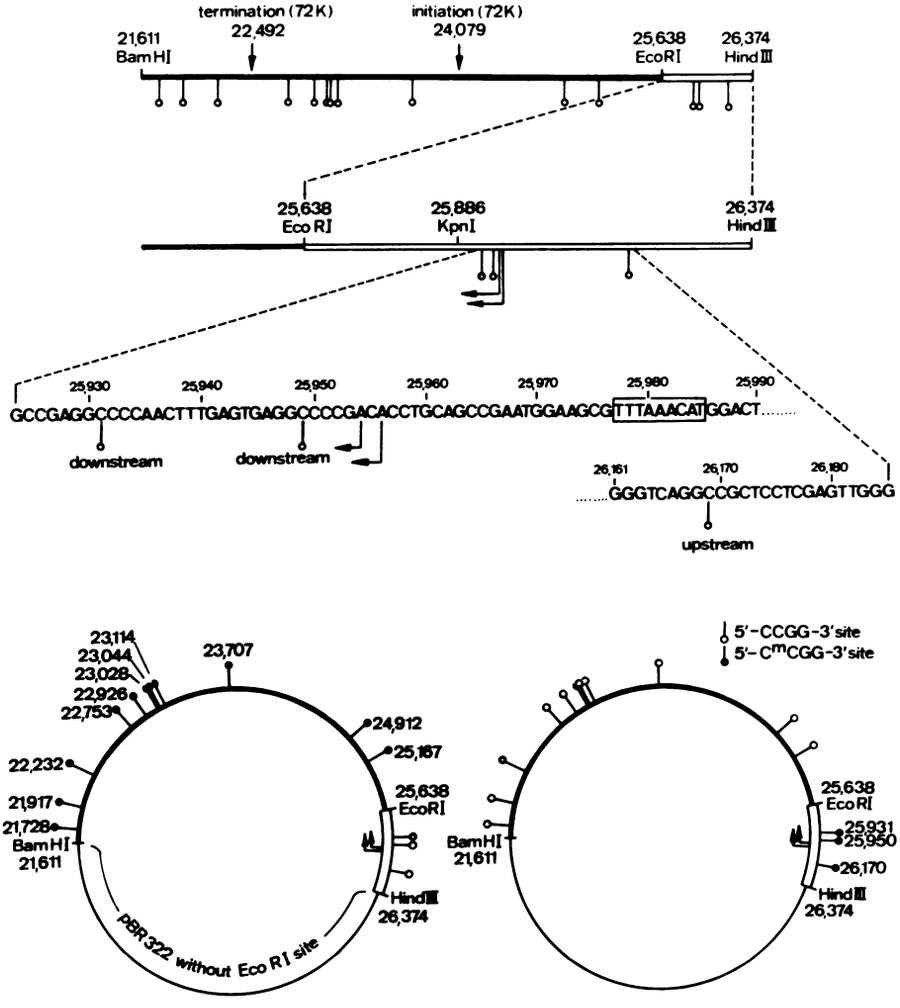

FIG. 1. Detailed map of the *E2a* subclone (*Bam*HI–*Hin*dIII fragment) and maps of the promoter (5′)-methylated and gene (3′)-methylated constructs. The DNA-binding protein (DBP = 72-kilodalton protein) is read from right to left. ↓, Initiation and termination sites of translation of the 72-kilodalton protein; ○, 5′ C-C-G-G 3′ site; ●, 5′ C-mC-G-G 3′ site. The nucleotide sequence of part of the promoter and cap regions (↩) are shown. The *E2a* region lacks a canonical TATA (Hogness–Goldberg) signal (43). The possible equivalent of such a signal has been indicated in a box.

E2a gene in pBR322. The plasmid clone was cut either with *Bam*HI and *Eco*RI to isolate the body of the gene (experiment 1) or with *Hin*dIII and *Eco*RI to isolate its promoter and 5′ region (experiment 2). The DNA fragments thus generated in step a (Fig. 2) of the construction experiment were separated and isolated by electrophoresis on horizontal agarose slab gels. In experiment 1 of Fig. 2, the body of the *E2a* gene including its introns was excised from the plasmid and subsequently methylated by the *Hpa* II methyltransferase in step b of the reaction. Similarly, in experiment 2 (Fig. 2), the 5′-end fragment of *E2a* including the promoter was methylated (step b). In the same step, the methylated and the unmethylated fragments in each experiment were religated. In order to select for the correctly rejoined combination of fragments, the ligation products were cleaved with *Eco*RI, and the proper fragment was reisolated by agarose gel electrophoresis (Fig. 2, step c). The correct reisolated fragment was designated by a dotted box. In step d, the selected fragment was recircularized by ligation and isolated by equilibrium sedimentation in CsCl/ethidium bromide density gradients (30).

The constructs thus generated will subsequently be designated the gene (3′)-methylated construct and the promoter (5′)-methylated construct (Fig. 1). These abbreviated designations do not directly relate the fact that the gene (3′)-methylated construct contains also intron segments of the *E2a* region and that the promoter (5′)-methylated construct carries 5′-terminal regions of the transcribed region in addition to the promoter. In these experiments the vector was not methylated. The transcriptional activities of either construct were tested upon microinjection into nuclei of *X. laevis* oocytes.

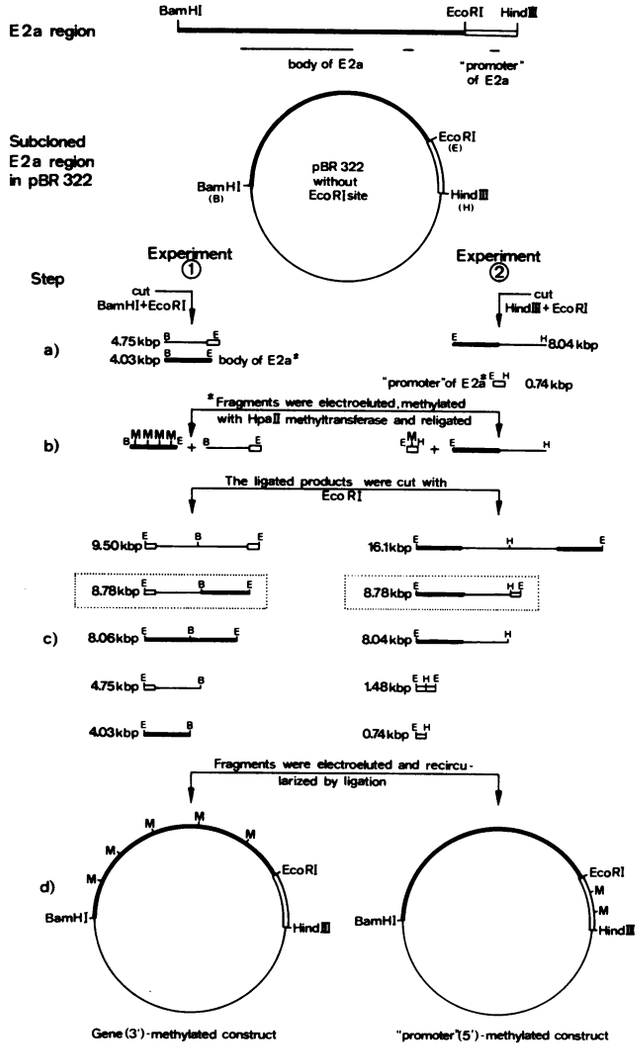

FIG. 2. Scheme for the design of the promoter (5′)-methylated and gene (3′)-methylated constructs. Most details have been described in the text. The number of methylated 5′ C-C-G-G 3′ sites (M) indicated does not correspond to the actual number (see Fig. 1) but schematically shows that all 5′ C-C-G-G 3′ sites in a region have been methylated. In the religation experiment depicted in step b, the two fragments were mixed at a larger-to-smaller fragment ratio of 1:1 in experiment 1 and at a ratio of 10:1 in experiment 2.

***In Vitro* Methylations of the Promoter (5′)- and Gene (3′)-Methylated Constructs Were Complete.** Since relatively large amounts of construct DNA had to be microinjected into *Xenopus* oocytes, it was essential to prove that the promoter (5′)- or the gene (3′)-methylated construct was completely methylated in the *in vitro* reaction (Fig. 2, step b). After this reaction and prior to religation, the *in vitro* methylated DNA fragments (see step b in Fig. 2) were cleaved with the diagnostic restriction endonuclease pair *Msp* I or *Hpa* II (44), and the fragments were electrophoretically separated, blotted (34), and hybridized (38) to ^{32}P-labeled (37) Ad2 DNA. The data demonstrate that both the *Eco*RI–*Hin*dIII promoter (5′) fragment and the *Bam*HI–*Eco*RI (3′) fragment of the *E2a* gene are totally refractory to *Hpa* II cleavage (Fig. 3)—i.e., completely methylated at all 5′ C-C-G-G 3′ sites. This control also ruled out minor contaminations by other possible constructs because no unexpected bands were apparent. It was essential to analyze the methylated DNA fragments by Southern blotting and hybridization to Ad2 DNA because ethidium bromide-stained gel analyses proved to be insufficiently sensitive.

Specific Promoter Methylations Inactivate the *E2a* Gene of Ad2 DNA. The unmethylated plasmid clone containing the *Bam*HI–*Hin*dIII fragment of the *E2a* gene was transcribed after microinjection into nuclei of *X. laevis* oocytes, whereas the clone methylated at all 14 5′ C-C-G-G 3′ sites was not (Fig. 4). This result has previously been published (12, 13). More importantly, it was also shown that 24 hr after microinjection, the promoter (5′)-methylated construct was not expressed in *X. laevis* oocytes, whereas the gene (3′)-methylated construct was readily transcribed. In this latter experiment (Fig. 4*a*), the same size classes of Ad2-specific RNA were synthesized as in the oocytes injected with the completely unmethylated plasmid clone.

Two control experiments were performed. The total DNA was extracted from the oocytes 24 hr after microinjection with the promoter (5′)-methylated or gene (3′)-methylated

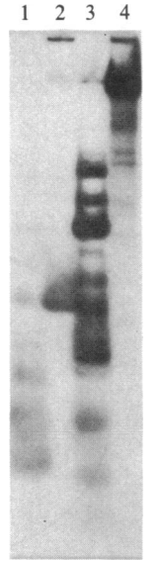

FIG. 3. Southern blot of the *Eco*RI–*Hin*dIII promoter (5′)-methylated fragment (lanes 1 and 2) and *Bam*HI–*Eco*RI gene (3′)-methylated fragment (lanes 3 and 4) of region *E2a* upon cleavage with *Hpa* II (lanes 2 and 4) or *Msp* I (lanes 1 and 3). The experiment was performed to demonstrate that either segment had been completely methylated at all 5′ C-C-G-G 3′ sites by treatment with the *Hpa* II DNA methyltransferase. After methylation, the segments were cut with *Msp* I or *Hpa* II as indicated, the fragments were separated by gel electrophoresis on a 1.5% agarose slab gel, transferred to a nitrocellulose filter (34) and hybridized (38) to ^{32}P-labeled (37) Ad2 DNA. After washing and drying of the filter, it was autoradiographed on Kodak XAR-5 film.

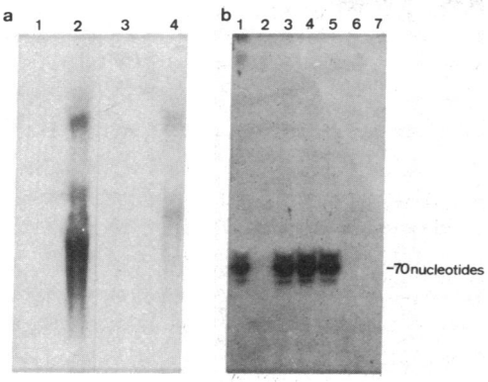

FIG. 4. Methylation of the three 5′ C-C-G-G 3′ sites in the *Hin*dIII–*Eco*RI fragment of gene *E2a* causes transcriptional inactivation. *X. laevis* oocytes were microinjected with the *Hpa* II methylated (lane 1) or the unmethylated (lane 2) plasmid clone containing the entire *Bam*HI–*Hin*dIII fragment of gene *E2a* or with the *Eco*RI–*Hin*dIII promoter (5′)-methylated construct (lane 3) or the *Bam*HI–*Eco*RI gene (3′)-methylated construct (lane 4) as indicated. In each experiment, 5 ng of DNA per oocyte was injected. At 24 hr after microinjection, the total intracellular RNA from 15 oocytes was extracted and fractionated on a 0.8% agarose slab gel containing 2.2 M formaldehyde (36). (*a*) The RNA was subsequently transferred to a nitrocellulose filter, and Ad2-specific RNA sequences were detected by hybridization to ^{32}P-labeled Ad2 DNA followed by autoradiography. (*b*) RNAs as indicated below were hybridized to the *Kpn* I–*Hin*dIII fragment of the *E2a* region (Fig. 1). This fragment was previously 5′-labeled with [γ-^{32}P]ATP and polynucleotide kinase. The hybrids were treated with S1 nuclease (200 units), separated on a sequencing gel, and identified by autoradiography. Sources of RNA in each lane: 1, Ad2-infected KB cells, 12 hr after inoculation; 2, oocytes injected with the promoter (5′)-methylated construct; 3, oocytes injected with the gene (3′)-methylated construct; 4, oocytes injected with the cloned unmethylated *E2a* gene; 5, same as lane 4 except that the *E2a* gene was grown on a *dam*$^-$ *E. coli* strain; 6, RNA from *Zea mays*, negative control; 7, same as lane 5 except that *dam*$^-$ plasmid was completely methylated at 5′ C-C-G-G 3′ sites.

construct. The DNA preparations were electrophoresed on agarose gels, blotted, and hybridized to ^{32}P-labeled Ad2 DNA. In this way, it could be ascertained that the microinjection experiments had been successful and that both construct DNAs persisted in the oocytes for at least 24 hr.

In a second type of control experiment, the constructions were performed similarly to the ones shown in Fig. 2 (step b) except that the appropriate DNA fragments were mock-methylated by omission of the methyl donor, *S*-adenosylmethionine, from the reaction mixture. Both constructs were then completed as described and separately microinjected into the nuclei of *X. laevis* oocytes. The total oocyte RNA was assayed 24 hr later for Ad2-specific RNA sequences. Both the promoter (5′)-methylated and the gene (3′)-methylated constructs were transcribed (data not shown). Thus, transcriptional inactivation of DNA fragments is not an artifact due to *in vitro* methylation. The same conclusions had been documented earlier (12, 13).

The data described here demonstrate that methylations of 3 5′ C-C-G-G 3′ sites in the promoter and 5′ region but not of 11 such sites in the gene itself lead to transcriptional inactivation of the *E2a* gene of Ad2 DNA.

Mapping of the RNA That Was Synthesized in Oocytes Injected with the *E2a* Gene of Ad2 DNA. We previously have mapped the Ad2-specific RNAs synthesized in *Xenopus* oocytes injected with the cloned *E2a* gene and have compared the RNAs to those synthesized in human KB cells productively infected with Ad2 virions (13). The method of Berk and Sharp (39) was used as described in detail (13). The Ad2-specific RNAs in oocytes injected with gene (3′)-methylated construct (Fig. 4*b*, lane 3) or injected with unmethylated construct (Fig. 4*b*, lanes 4 and 5) were initiated at the same cap site in the *Kpn* I–*Hin*dIII fragment (Fig. 1) as in Ad2-infected KB cells (Fig. 4*b*, lane 1). In oocytes injected with promoter (5′) or totally methylated constructs (Fig. 4*b*, lanes 2 or 7, respectively), viral RNA was not detectable. Constructs propagated in *dam*$^-$ *Escherichia coli* strains, which were deficient in adenine methylation, expressed Ad2-specific RNA upon microinjection when unmethylated at 5′ C-C-G-G 3′ sites (lane 5). Methylation at 5′ C-C-G-G 3′ sequences also inactivated *dam*$^-$ grown constructs (lane 7). Thus, adenine methylation did not seem to affect gene expression in this system.

DISCUSSION

Eukaryotic genes can be permanently inactivated by the methylation of cytidine residues in highly specific sequences. It has been shown for a number of genes that the sequences decisive in this long-term transcriptional shutoff are located in the promoter and 5′ regions of these genes (18–25, 27). It is a problem of prime importance to understand the regulation of *de novo* DNA methylations and to elucidate the factors involved in regulating the maintenance of DNA methylations. By using the specific inhibitor of DNA methyltransferases, 5-azacytidine, previously inactivated genes or sets of genes could be rendered transcriptionally active (45–49). The biochemical mechanisms for transcriptional inactivation by 5-methylcytidine may be sought in structural alterations of DNA and/or effects on highly specific DNA–protein interactions at or at sites close to the promoter region of a gene.

We have shown here that the *in vitro Hpa* II-methylation of one 5′ C-C-G-G 3′ site 215 bp upstream and of two such sites 5 bp and 23 bp downstream from the left-most initiation site (25,954 of Ad2 DNA) of transcription of the *E2a* gene (Fig. 1) inactivates this gene in *X. laevis* oocytes. The *in vitro* methylation of 11 5′ C-C-G-G 3′ sites in the 3′ main part of the *E2a* gene affects transcription in *X. laevis* oocytes only slightly, if at all (Fig. 4).

The design of the present experiments has been based on the outcome of previous work with the *E2a* gene of Ad2 DNA. This gene is not only faithfully transcribed in *X. laevis* oocytes (12, 13), but the mRNA is also translated into the 72-kilodalton DNA-binding protein (50). *In vitro* methylation of all 14 5′ C-C-G-G 3′ sites in the subcloned *E2a* gene (bp 21,611 to 26,374 of Ad2 DNA) inactivated transcription of this gene in *X. laevis* oocytes (12, 13). Preliminary results indicate that the Ad2 *E2a* gene is inactivated by methylation also in mammalian cells (unpublished data). Evidence for the functional significance of DNA methylations at 5′ C-C-G-G 3′ sites in the *E2a* region of Ad2 DNA (11) and in other adenoviral genes (9, 10, 18) had been previously adduced from the observation of inverse correlations between methylations at 5′ C-C-G-G 3′ sites in integrated viral genes in transformed cell lines and gene expression. We also have shown that methylations of the 5′ G-G-C-C 3′ sites in the *E2a* gene of Ad2 DNA do not inhibit its expression (51).

The main conclusion derived from the data presented here relates *in vitro* DNA methylations at specific sequences at, or at sites close to, the promoter and 5′ region of the *E2a* gene to its transcriptional inactivation. The promoter methylated is the late promoter of the *E2a* region. There are additional early promoters that are not included in the DNA fragment used here (13, 43). Methylations at 5′ C-C-G-G 3′ sites in the main part of the *E2a* gene appear to have no, or a strikingly lesser, effect. Very similar conclusions have recently been documented for the *E1a* promoter of Ad12 DNA after transfer into mammalian cells (25) and of the promoter of the globin gene in mammalian cells as well (27). The critical sites in the *E1a* promoter of Ad12 DNA could be located

at two 5′ C-C-G-G 3′ or a few 5′ G-C-G-C 3′ sequences upstream from the TATA signal of the *E1a* gene. Methylations of these sites downstream from the TATA signal in the polypeptide IX gene of Ad12 DNA did not decrease transcription. Due to the design of the *in vitro* methylation experiment of the globin gene (27), the critical promoter sites could not be determined precisely. It appears likely that the methylation of very few (perhaps even of one) highly specific sites in the promoter region is sufficient to inactivate the gene. In the late promoter of simian virus 40 DNA, methylation of a single 5′ C-C-G-G 3′ site causes transcriptional inactivation (16). The methylations of many cytidines found *in vivo* may be a safeguard or serve additional functions.

It was emphasized (3, 4, 6–8) that DNA methylation may be recognized as an inactivation signal for eukaryotic genes that have to be silenced permanently or for a long time. Thus, specific DNA methylations may assume prime importance in understanding the changes in transcriptional programs occurring during differentiation or in malignant growth. In contrast, genes or sets of genes that have to be occasionally reactivated are probably not shut off by DNA methylations but by other mechanisms. It is still unknown how frequently and by what mechanisms established patterns of methylation are subject to alterations. Specific *in vitro* DNA methylations of the ribosomal genes of *X. laevis* did not affect their expression (52). Is the expression of genes transcribed by the DNA-dependent RNA polymerase I less sensitive to DNA methylation?

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