In Vitro Methylation of the *Bsu*RI (5'-GGCC-3') Sites in the E2a Region of Adenovirus Type 2 DNA Does Not Affect Expression in *Xenopus laevis* Oocytes

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The early region 2a (E2a) of adenovirus type 2 (Ad2) DNA codes for a 72,000-dalton DNA-binding protein and is expressed in the Ad2-transformed hamster cell line HE1 but not in cell lines HE2 and HE3 (H. Esche, J. Virol. **41**:1076–1082, 1982; K. Johansson et al., J. Virol. **27**:628–639, 1978). An inverse correlation between DNA methylation at the 5'-CCGG-3' sites of the E2a region and of gene expression in these cell lines has been observed (L. Vardimon et al., Nucleic Acids Res. **8**:2461–2473, 1980). When the cloned E2a region of Ad2 DNA is methylated in vitro at the 5'-CCGG-3' sites, the gene is not transcribed after being injected into the nuclei of *Xenopus laevis* oocytes, whereas unmethylated DNA is expressed (L. Vardimon et al., Eur. J. Cell Biol. **25**:13–15, 1981; L. Vardimon et al., Proc. Natl. Acad. Sci. U.S.A. **79**:1073–1077, 1982). These data demonstrate that DNA methylation is directly involved in the shut-off of transcription. In the present communication we investigated in detail the control region of the gene for the DNA-binding protein in Ad2-transformed cell lines and showed that the first late control region (map coordinate 72 on the viral DNA) of the E2a region is present in its entirety in cell lines HE1, HE2, and HE3. The *Hae*III sites (5'-GGCC-3') in the E2a region in all three cell lines were not methylated. When the DNA methyltransferase *Bsu*RI was used, all 5'-GGCC-3' sites in the cloned E2a region of Ad2 DNA were methylated in vitro. It was shown that methylation of these sites did not inhibit the expression of this viral gene in *X. laevis* oocytes. Thus, for methylation to affect gene expression in the E2a region it has to occur at specific sites (e.g., 5'-CCGG-3') which may be different for other genes.

Methylation of the cytosine residues in DNA of eucaryotic cells occurs predominantly at the sequence 5'-CpG-3' (6, 11). One plausible explanation for this finding may be the recognition specificity of the DNA methyltransferases in eucaryotic cells. Recently, considerable evidence has been adduced for the notion that DNA methylation is directly involved in the regulation of gene expression (for reviews see references 5, 7, 15, 19, 20, 33; also W. Doerfler, Annu. Rev. Biochem., in press). Thus, the question arises whether the site 5'-CCGG-3' has structural features that render it particularly well suited for the regulation of gene expression.

In the present study we investigated whether methylation of the sequence 5'-GG^mCC-3' could be found in vivo, i.e., in transformed cells, and whether in vitro methylation of this site would lead to inhibition of gene expression in the same way as the methylation of the 5'-C^mCGG-3' sites inactivated this gene (28, 29). We chose the early region 2a (E2a) of adenovirus type 2 (Ad2) DNA

for these experiments. We have previously demonstrated that in the Ad2-transformed hamster cell lines HE1, HE2, and HE3 (4, 16) multiple copies of the major part of the Ad2 genome persist in an integrated state (27; R. Gahlmann, R. Leisten, L. Vardimon, and W. Doerfler, EMBO J., in press). The structural gene for the viral DNA-binding protein (DBP), located in the E2a region of Ad2 DNA, is present in all three cell lines but is expressed only in HE1 cells. With the isoschizomeric restriction enzymes *Hpa*II and *Msp*I it has been shown that all 5'-CCGG-3' sites in the E2a region are methylated in cell lines HE2 and HE3, which do not express the DBP. The same sites are not methylated at all in cell line HE1, which expresses the DBP (30).

We methylated in vitro the cloned E2a region of Ad2 DNA with the *Hpa*II DNA methyltransferase. In vitro methylation of the *Hpa*II sites inhibits the expression of this viral gene after its microinjection into nuclei of *Xenopus laevis*

oocytes (28, 29). Similar results have been reported for the adenine phosphoribosyltransferase gene in mouse L cells (24) and for the herpes simplex virus thymidine kinase gene (31).

In this communication we show that the *Hae*III sites (5'-GGCC-3') in the E2a region of integrated Ad2 DNA are not methylated in the three Ad2-transformed hamster cell lines HE1, HE2, and HE3. The same 5'-GGCC-3' sites in the cloned E2a region were methylated *in vitro* by the *Bsu*RI DNA methyltransferase. Both the methylated and unmethylated cloned DNAs were found to be expressed as Ad2-specific RNA after microinjection into the nuclei of *X. laevis* oocytes.

How DNA methylation interferes with eucaryotic gene expression is not yet understood. The restriction of the inhibitory effect of DNA methylation on the expression of the E2a region to the sequence 5'-CCGG-3' (and not 5'-GGCC-3') might reflect either the importance of the former sequence or the locations of these two sequences relative to the positions of regulatory signals in this particular gene.

MATERIALS AND METHODS

Most of the methods used in the research described in this report, in particular the methods for microinjection and analyzing DNA and RNA, have been published previously (17, 21, 23, 25-30, 32).

Cleavage of cellular or viral DNA with the *Hae*III restriction endonuclease (New England Biolabs, Beverly, Mass.) was performed according to standard protocols.

The cloning and purification of the *Kpn*I-*Hind*III fragment comprising map units 71.4 to 72.8 on the Ad2 genome (see Fig. 1) was detailed elsewhere (28).

The *Bsu*RI DNA methyltransferase was purified from *Bacillus subtilis* as described earlier (12, 13). For the *in vitro* methylation reaction, DNA at 50 µg/ml was incubated with a saturating amount of enzyme in 300 mM Tris-hydrochloride (pH 8.5)-0.77 mM dithiothreitol-15% glycerol-0.004% Brij 58-1.5 µM S-adenosyl-L-methionine at 37°C for 1 h. The solution was then adjusted to 0.4 M NaCl, 1 mM EDTA, and 0.2% sodium dodecyl sulfate and extracted once with Tris-hydrochloride-saturated phenol and once with chloroform-isoamyl alcohol (24:1, vol/vol). The DNA was precipitated from the aqueous phase with ethanol. The *in vitro*-methylated DNA was redissolved in injection buffer (10 mM Tris-hydrochloride [pH 7.5], 80 mM NaCl) at a concentration of 250 µg/ml. Unmethylated control DNA was treated identically, except that DNA methyltransferase was not added to the reaction mixture. DNA preparations were tested for completeness of the reaction by cleavage with *Hae*III (18). DNA fragments were analyzed by electrophoresis on horizontal 1.5% agarose slab gels.

Details of the procedure for analyzing the Ad2-specific RNA from *X. laevis* oocytes with the single-strand-specific nuclease S1 have been described previously (28). The leftward-transcribed strand of the 71.4 (*Kpn*I site) to 72.8 (*Hind*III site) map unit fragment of Ad2 DNA was used for the hybridization to Ad2-

specific RNA. This fragment contains one of the late promoters of the E2a region of Ad2 DNA.

RESULTS

Late control region of E2a region present in Ad2-transformed cell lines HE2 and HE3. The E2a region of Ad2 DNA encoding the DBP can be controlled by four different promoter/leader regions. One of the control regions, located at about map position 75 on the viral DNA, is used early after productive infection; the other control regions are used late after infection (2, 3, 10). We have shown that in cell lines HE2 and HE3 the early control region as well as two of the late control regions are missing, but at least part of the late control region (map coordinate 72) seems to be present; the entire E2a region persists in cell line HE1 (L. Vardimon, D. Renz, and W. Doerfler, Recent Results Cancer Res., in press). This region is expressed as mRNA, and the 5'-CCGG-3' sites in this region are not methylated. Conversely, in cell lines HE2 and HE3 the E2a region is not expressed (8, 16), and the 5'-CCGG-3' sites in this region are heavily methylated (30).

For an interpretation of the functional meaning of this inverse correlation, it is necessary to determine whether the initiation site of the late control region (map coordinate 72) is present in all three cell lines. A detailed analysis of this control region was therefore performed with restriction enzyme *Msp*I or *Hae*III.

DNA from cell line HE3 or from Ad2 virions was cleaved with one of these restriction enzymes, and the fragments were analyzed by gel electrophoresis and Southern blotting (23). Ad2-specific DNA fragments were detected by hybridization (32) with a ³²P-labeled fragment comprising the map coordinates 71.4 (*Kpn*I site) to 72.8 (*Hind*III site) (28) of Ad2 DNA. This fragment contains the late control region.

Based on the published sequence of the late control region (9), the different viral DNA fragments expected from cleavage with *Msp*I or *Hae*III were drawn schematically (Fig. 1B). The hybridization data (Fig. 1A) showed that in cell line HE3 the 221-base-pair (bp) and >298-bp *Msp*I fragments (the published sequence extends only to the *Eco*RI site at position 70.7) persisted, and that the 285-bp *Hae*III fragment was present in its entire length.

These results prove that the sequence at the site of initiation in the late control region as well as at least 212 nucleotides upstream persisted in the HE3 cell line. Since cell line HE2 exhibits identical patterns of integration (27), it is likely that the same conclusions hold for that line. Evidence that this control region is used in cell line HE1 for transcription of the E2a region has

FIG. 1. Mapping of a late promoter/leader region of the E2a region of Ad2 DNA. (A) DNA from cell line HE3 (20 μ g; lanes e and f) from Ad2 (1.2×10^{-3} μ g; lanes c and d), or from pBR322 (3×10^{-3} μ g; lanes a and b) was cleaved with *MspI* (lanes d and f), *HaeIII* (lanes a, c, and e), or *HinfI* (lane b). The fragments were analyzed as described in the text. The *KpnI* (71.4 map units) to *HindIII* (72.8 map units) fragment of Ad2 DNA (see panel B) was labeled with 32 P by nick translation (21) and used as hybridization probe (lanes c through f). Similarly, pBR322 DNA was also labeled (lanes a and b). The fragment lengths in base pairs are indicated. (B) Restriction map of the late promoter/leader region as derived from sequence data (9). *MspI* (●) and *HaeIII* (○) sites are indicated. The sites of initiation of transcription of the E2a region are shown by arrows (2).

been published elsewhere (Vardimon et al., in press).

5'-GGCC-3' (*HaeIII*) sites in the E2a region of Ad2 DNA not methylated. The importance of the sequence 5'-CCGG-3' has been demonstrated previously (17, 25, 26, 28–30). We next analyzed to what extent the inverse sequence 5'-GGCC-3' is methylated in the E2a region of cell lines HE1, HE2, and HE3. DNAs extracted from these cell lines and from Ad2 virions were cleaved with the restriction enzyme *HaeIII*. This enzyme cannot cleave the methylated sequence 5'-GG^mCC-3' (18). The DNA fragments were separated by electrophoresis on a 1.5% agarose gel, blotted onto nitrocellulose filters, and hybridized with the cloned *HindIII* fragment A of Ad2 DNA labeled with 32 P by nick translation (21). In all three cell lines, cleavage with *HaeIII* yielded the same fragment pattern as did virion DNA (Fig. 2). Ad2 virion DNA has been shown to be unmethylated (14, 30). The *HindIII*-A fragment

of Ad2 DNA comprises the structural gene and the first late control region of the E2a region. These results unequivocally demonstrate that the 5'-GGCC-3' sites in the E2a region of Ad2 DNA are completely unmethylated and that the inverse correlation between DNA methylation at the 5'-CCGG-3' sites and gene expression (17, 25, 26, 28–30; Vardimon et al., in press) cannot be extended to the 5'-GGCC-3' sites in the E2a region as well.

Expression of in vitro-methylated E2a region of Ad2 DNA. In vitro methylation of the cloned E2a region of Ad2 DNA by *HpaII* DNA methyltransferase at all 5'-CCGG-3' sites inhibits the specific transcription of the cloned viral DNA fragment in *X. laevis* oocytes (28, 29). This observation is compatible with the notion that DNA methylation at specific sites is causally related to the shut-off of specific genes.

An obvious next question pertains to the specificity of the sites whose methylation could

FIG. 2. 5'-GGCC-3' (*Hae*III) sites of Ad2 DNA are unmethylated in cell lines HE1, HE2, and HE3. DNA (20 μ g) from cell lines (lanes) HE1 (f), HE2 (e), and HE3 (d), or 1.2×10^{-3} μ g of Ad2 virion DNA (c) was cleaved with the *Hae*III restriction endonuclease and analyzed as described in the text. pBR322 DNA cut with *Hae*III (b) or *Hinf*I (a) was used as a size marker. Marker fragment sizes are indicated.

affect gene expression. As a first approach to this problem, we methylated the reverse sequences in vitro, the 5'-GGCC-3' sites of the cloned E2a (*Hind*III-A) fragment of Ad2 DNA, with the *Bsu*RI DNA methyltransferase. Methylated or unmethylated DNA was microinjected into the nuclei of *X. laevis* oocytes as described previously (28, 29). Viral gene expression was then monitored by testing for the presence of Ad2-specific RNA in oocytes.

We first examined whether the in vitro-methylated DNA stayed in the methylated state in oocyte nuclei during the 24-h period of the experiment. DNA was extracted from the oocytes 24 h after microinjection and cleaved with *Hae*III or *Hpa*II. The DNA fragments were separated on a 1.5% agarose slab gel, blotted to nitrocellulose filters, and hybridized with 32 P-labeled Ad2 DNA. The data showed that the methylated DNA in oocytes remained methylated at the 5'-GGCC-3' sites and therefore was not cleaved by the *Hae*III restriction enzyme (Fig. 3). Most of the 5'-CCGG-3' restriction sites in the methylated DNA remained sensitive to the

*Hpa*II restriction enzyme. It was not clear why a few of these sites had become resistant to *Hpa*II after methylation at the 5'-GGCC-3' sites and microinjection. The unmethylated DNA that had been microinjected into oocytes remained sensitive to the *Hae*III and *Hpa*II restriction endonucleases (Fig. 3) over the 24-h period of the experiment.

The expression of the microinjected viral genes in *Xenopus* oocytes was tested by extracting the total intracellular RNA from the oocytes

FIG. 3. Cloned E2a region of Ad2 DNA methylated in vitro at the 5'-GGCC-3' sites remained methylated for at least 24 h after microinjection. Experimental details are described in the text. At 24 h after microinjection of *X. laevis* oocytes with the methylated E2a region (lanes a and c) or with the unmethylated DNA (lanes b and d), DNA was extracted from the oocyte and analyzed by cleavage with the restriction enzymes *Hae*III (lanes a and b) or *Hpa*II (lanes c and d).

FIG. 4. Methylation of the cloned *Hind*III-A fragment of Ad2 DNA by the *Bsu*RI DNA methyltransferase at 5'-GGCC-3' sites does not inhibit transcription after microinjection into *X. laevis* oocytes. Experimental details are described in the text. (a) RNA from oocytes injected with methylated DNA (5'-GG^mCC-3'). (b) RNA from oocytes injected with unmethylated DNA (5'-GGCC-3'). (c) Ad2 DNA cleaved with the *Hind*III restriction endonuclease used as a marker. Arrows indicate the Ad2-specific DNA.

at 24 h after microinjection. The RNA extracted was analyzed for the presence of Ad2-specific transcripts by gel electrophoresis, blotting (1, 22), and DNA-RNA hybridization. Total Ad2 DNA, labeled with ³²P by nick translation, was used as probe. After the microinjection of the methylated or unmethylated cloned Ad2 DNA fragment, virus-specific RNA could be detected (Fig. 4). No difference in the pattern of Ad2 DNA transcription was found between the oocytes injected with the methylated or the unmethylated DNA fragments (Fig. 4). The complexity of the RNA pattern was probably due to the fact that a pBR322 clone of the *Hind*III-A fragment of Ad2 DNA was directly microinjected-

ed and that the viral gene had not been excised from the plasmid before its injection. Thus, transcription could have started in the vector or could have proceeded into the vector. The Ad2-specific RNA bands (arrows, Fig. 4) did not hybridize with a ³²P-labeled pBR322 probe, whereas most of the other areas of hybridization did (data not shown).

FIG. 5. S1 nuclease analysis of Ad2-specific RNA from *X. laevis* oocytes injected with unmethylated or 5'-GGCC-3' methylated *Hind*III-A DNA from Ad2. Many of the experimental details are described in the text. Oocytes were injected with the unmethylated, cloned E2a region of Ad2 DNA in pBR322 or with the same DNA which was completely methylated at the 5'-GGCC-3' sites with *Bsu*RI DNA methyltransferase. RNA was extracted 24 h after injection and hybridized to the leftward-transcribed strand of the 71.4 to 72.8 map unit fragment of Ad2 DNA (28). Cytoplasmic RNA from human KB cells productively infected with Ad2 was isolated 12 h postinfection and used as a positive control. Lanes: (a and b) Size markers from a sequence analysis experiment. The position of 70 bases is marked by an arrow. (c) Total RNA from oocytes microinjected with *Bsu*RI-methylated DNA. (d) Total RNA from oocytes microinjected with unmethylated DNA. (e) Total RNA from oocytes that were not microinjected. (f) Cytoplasmic RNA from KB cells 12 h after infection with Ad2. (g) Cytoplasmic RNA as in lane f but not treated with S1 nuclease. (h) No RNA added.

We also analyzed the RNA extracted from *Xenopus* oocytes injected with the unmethylated or the 5'-GGCC-3' methylated E2a fragment by S1 mapping as described previously (28). The data showed that in both experiments Ad2-specific RNA was initiated at the same site and to about the same extent (Fig. 5). Identical data were obtained when RNA from human KB cells productively infected with Ad2 was used (Fig. 5). In all these experiments, the leftward-transcribed strand of the 71.4 (*Kpn*I site) to 72.8 (*Hind*III site) map unit fragment of Ad2 DNA was used for hybridization with RNA before S1 digestion, gel electrophoresis, and autoradiography. The DNA sequence protected in the *Kpn*I-*Hind*III fragment by RNA from microinjected oocytes was 70 ± 2 nucleotides long (Fig. 5) (28), as expected from the known sequence of this late E2a promoter. These data clearly demonstrate that 5'-GGCC-3' methylated and unmethylated DNAs are equally well transcribed in *Xenopus* oocytes and furthermore are transcribed in the same way as they are in homologous KB cells. We thus conclude that in vitro methylation of the *Hind*III-A fragment of Ad2 DNA at the 5'-GGCC-3' sites does not inhibit the specific transcription of viral DNA in *X. laevis* oocytes.

DISCUSSION

We showed that the late promoter/leader of the E2a region at map position 72 on the Ad2 genome is present in its entirety in cell line HE3 (Fig. 1) and probably also in cell line HE2. Failure to express the E2a region in cell lines HE2 and HE3 (8, 16) thus cannot be due simply to lack of a functional promoter. Moreover, we showed previously that the late promoter/leader at 72 map units of Ad2 DNA is expressed in cell line HE1 (Vardimon et al., in press) and thus represents a functional promoter. In previous work, a perfect inverse correlation was established between the E2a region of integrated Ad2 DNA in cell lines HE1, HE2, and HE3 and the extent to which this region is expressed in these cell lines (30). In cell line HE1, which expressed the DBP, all 5'-CCGG-3' sites of the E2a region were unmethylated; in cell lines HE2 and HE3, which failed to express the DBP, all of the 5'-CCGG-3' sites were methylated. Since DNA methylation at 5'-CCGG-3' sites in the E2a region of Ad2 DNA served as a regulatory signal, we chose these sites in the E2a region for in vitro investigation. Two of these *Hpa*II sites were located 4 or 20 nucleotides downstream from the site of initiation of RNA synthesis (Fig. 1b) (2). The results of a series of recently published experiments (28, 29) corroborated the interpretation assigned to the in vivo results. In vitro methylation of the cloned E2a region of

Ad2 DNA at 5'-CCGG-3' sites leads to the transcriptional inactivation of this region in oocytes from *X. laevis* (28, 29). Similar results have been reported for the adenine phosphoribosyltransferase gene in mouse L cells (24) and for the herpes simplex virus thymidine kinase gene (31). Although for other genes methylation at the same or at different sites might have regulatory significance, expression of the E2a region of Ad2 DNA is highly sensitive to methylation at 5'-CCGG-3' sites. In fact, evidence from many different higher eucaryotic systems points to that particular sequence having signal value in regulatory functions (5, 7, 15, 19, 20, 33; Doerfler, in press).

The data summarized here show that the converse nucleotide sequence, i.e., 5'-GGCC-3', is unmethylated in the E2a region in integrated Ad2 DNA in cell lines HE1, HE2, and HE3 (Fig. 2), and consequently in vitro methylation of the cloned E2a region at these sites did not cause transcriptional inactivation after its microinjection into *X. laevis* oocytes (Fig. 4 and 5). Transcription was initiated from the same sites as it was in KB cells productively infected with Ad2 (Fig. 5).

These results together support the notion that methylation at highly specific sites in specific genes can affect gene expression, perhaps by influencing the binding of regulatory proteins. It cannot be decided from the results of these experiments whether the methylated sequence by itself is important for the ability to inhibit transcription or whether its location relative to regulatory signals on the gene is decisive.

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