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Interference with protein binding at AP2 sites by sequence-specific methylation in the late E2A promoter of adenovirus type 2 DNA

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The in vitro methylation of the +6, +24, and −215 located 5'-CCGG-3' sequences in the late E2A promoter of adenovirus type 2 (Ad2) DNA abrogates promoter function. 5-Methyldeoxycytidine (5-mC) at positions +6 and +24 in both or either of the two DNA complements in the late E2A promoter abolishes the formation of a high-molecular-mass DNA–protein complex that is essential for promoter function. The formation of this complex can be competed for by an oligodeoxyribonucleotide with a consensus AP2 sequence, but not by AP1, AP3, or CREB sequences. The AP2 sites comprise the +6 and +24 located 5'-CCGG-3' sequences in the late E2A promoter; the AP1, AP3, and CREB sequences are in their immediate vicinity. Methylation of either the +6 or the +24 5'-CCGG-3' sequence also compromises formation of the DNA–protein complex. A 40 nucleotide pair oligodeoxyribonucleotide encompassing the −215 5'-CCGG-3' site in the late E2A promoter can also form DNA–protein complexes which is not affected by the introduction of a 5-mC residue in the −215 position. The data suggest that the AP2 protein together with other proteins is involved in the generation of a transcription-activating complex with the late E2A promoter of Ad2 DNA, and the formation of this complex is completely abolished when both the +6 and +24 5'-CCGG-3' sequences are methylated.

Promoter methylation; Regulation of gene expression; DNA–protein interaction; Band shift assay; Competition with protein binding

1. INTRODUCTION

The late E2A promoter of Ad2 DNA has been used as a model to study the mechanism by which sequence-specific promoter methylations lead to gene inactivation [1–3]; (for reviews [4–6]). The inactivation of this promoter by sequence-specific methylation is associated with the elimination of a specific DNA–protein complex. This complex is formed when a downstream nucleotide sequence comprising nucleotides +37 to −13 in an oligodeoxyribonucleotide is incubated with nuclear extracts from human HeLa cells. The generation of this complex is abrogated when two cytidine (C) residues in 5'-CCGG-3' sequences in this region are methylated or hemimethylated at positions +6 and +24 [7]. The interaction of proteins with specific promoter motifs is affected by DNA methylation in some instances [7–11] but not in others [12,13]. The locations of the functionally decisive sites in eukaryotic promoters for the methylation that causes inactivation have to be determined experimentally.

It will be shown here that in the downstream +6, and +24 region of the late E2A promoter of Ad2 DNA, the AP2 sites and not sequences equivalent to AP1, AP3, or CREB motifs are affected by DNA methylation in that the binding of proteins from nuclear extracts of HeLa cells at the methylation sensitive sites can be competed for by an oligodeoxyribonucleotide with the AP2 consensus sequence. Methylation of the −215 nucleotide does not cause loss of protein binding.

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2. MATERIALS AND METHODS

Most of the techniques employed in this work have been previously described [7].

2.1. *Nuclear extracts from HeLa cells*

HeLa cell nuclei were liberated by a published method [14]. Nuclear extracts were made in 0.42 M NaCl, the proteins were precipitated by 40% $(NH_4)_2SO_4$, dialyzed against 20 mM HEPES, pH 7.8, 20 mM KCl, 20% glycerol, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonylfluoride, aprotinin (2.5 U/ml), and partly purified on a heparin-sepharose column as described [7]. The protein fractions eluting between 0.6 and 0.8 M KCl from this column were used for further studies on DNA–protein binding.

2.2. *Oligodeoxyribonucleotides employed in competition experiments for DNA–protein binding*

DNA–protein binding studies were performed as described [7]. The following oligodeoxyribonucleotides were synthesized in an Applied Biosystems 381A synthesizer: The oligodeoxyribonucleotides containing the AP1 consensus sequence, 5'-AGCTTGATGAGTCAGCCG-3' [15]; the AP2 consensus sequence, 5'-GATCGAACTGACCGCCCGCGGCCCCTCTAG-3' [16]; the AP3 consensus sequence 5'-GATCGGGTGTGGAAAGTCACTG-3' [16]; or the CREB (ATF) consensus sequence, 5'-CCACCCCATTGACGTCAATGGGAGTT-3' [17] (cf. map in Fig. 1). The oligodeoxyribonucleotides were added to the DNA–protein binding reaction in amounts of 0.5 to 200 ng, i.e. in 10- to 4000-fold excess over the DNA substrate and simultaneously with the ^{32}P-labeled DNA substrate [7].

2.3. *Oligodeoxyribonucleotide methylated in one cytidine residue*

The 50-nucleotide-pair DNA segment comprising nucleotides +37

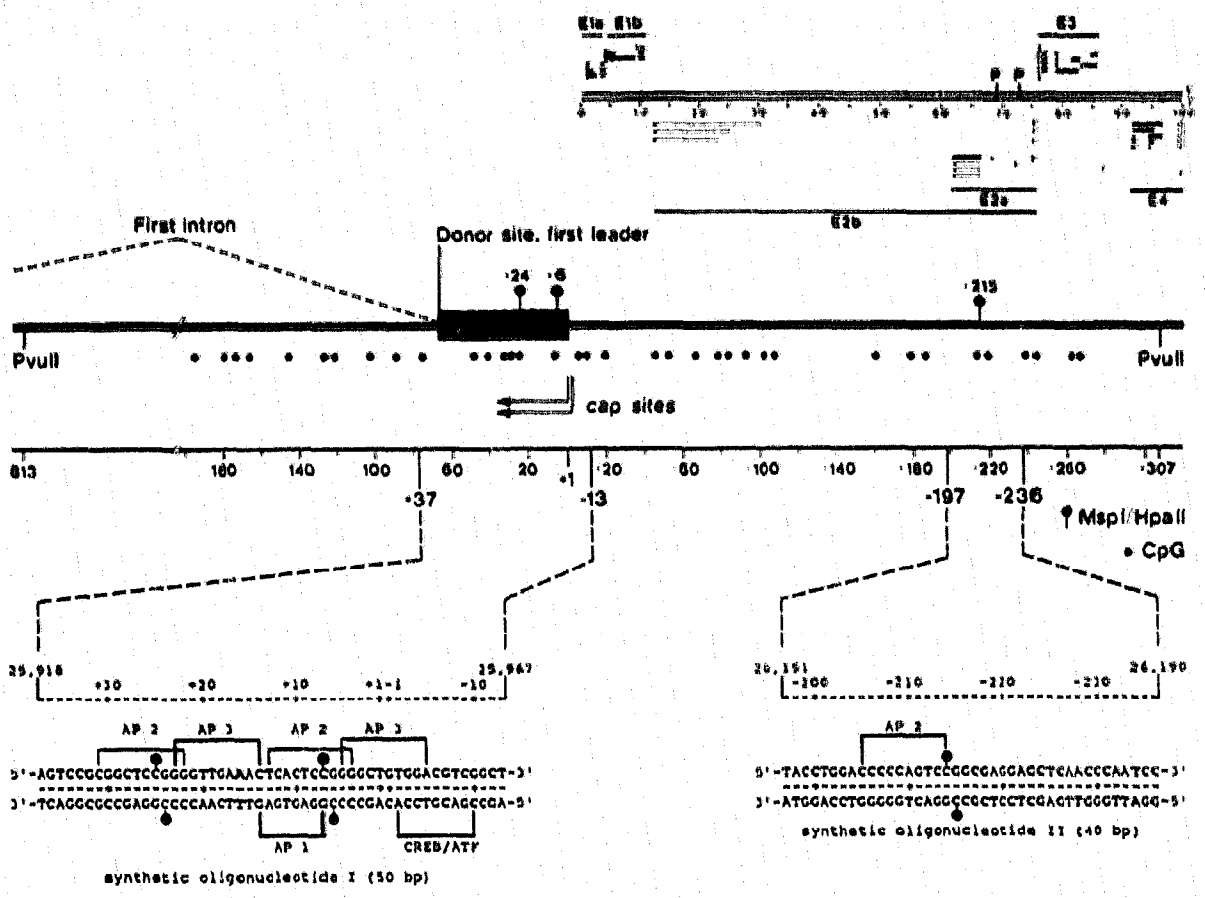

Fig. 1. Map and factor binding sites in the late E2A promoter and its downstream region of Ad2 DNA. The insert (upper right) presents a map of the early regions of the Ad2 genome. The *PvuII-PvuII* fragment whose map is detailed in the central part of the graph is designated P-P in the Ad2 DNA map. In the *PvuII-PvuII* fragment, all 5'-CG-3' dinucleotides (●) and the 5'-CCGG-3' sequences with the internal C at nucleotide positions +6, +24 and −215 relative to the downstream cap site have been indicated. The scale presents nucleotide positions upstream (−) and downstream (+) from this site. Nucleotide sequences akin to known factor binding sites have been bracketed in the bottom part of the scheme.

to −13 was synthesized such that either the C residue in position +6 or in +24 was replaced by 5-mC.

In other experiments an unmethylated or a 5'-CCGG-3' methylated 40-base-pair oligodeoxyribonucleotide was used that represented positions −198 to −237 in the late E2A promoter of Ad2 DNA. The 5-mC residue was introduced in position −215 of this oligodeoxyribonucleotide (cf. Fig. 1).

3. RESULTS AND DISCUSSION

3.1. *Location of the promoter motifs, the methylation sensitive sites and the synthetic oligodeoxyribonucleotides in the late E2A promoter of Ad2 DNA*

The map in Fig. 1 presents the structural details of the late E2A promoter which we have been using in the studies on the mechanism of promoter inactivation by sequence-specific methylation. The two AP2 sites cover the +6 and +24 located 5'-CCGG-3' sequences whose methylation abrogates the formation of a high molecular mass DNA-protein complex [7] (Fig. 2). AP1, AP3, and CREB sequence equivalents have also been indicated in Fig. 1. The methylation-sensitive residues are located at positions +6, +24 and at −215. These three C residues are only a subset of the 36 5'-CG-3' dinucleotide combinations (filled circles in Fig. 1) which are all completely methylated, e.g. in the Ad2-transformed hamster cell line HE2 or HE3 [18,19]. The map also presents the positions of the synthetic oligodeoxyribonucleotides which have been used for studies on DNA-protein binding. For most experiments, the 50-bp fragment comprising nucleotides +37 to −13 (nucleotides 25 918 to 25 967 in the Ad2 DNA sequence [20]) or the 40-bp fragment between nucleotides −198 and −237 (nucleotides 26 151 to 26 190 [20]) of the late E2A promoter has served as DNA substrate. In many DNA-protein binding assays, synthetic oligodeoxyribonucleotides with the consensus AP3, AP2, AP1, or CREB sequence have been employed as competitors in order to identify the DNA motif essential for protein binding. The migration delay (gel shift) procedure [21,22] has been chosen as analytical tool.

3.2. *AP2 sequences are involved in the formation of the methylation-sensitive DNA-protein complex in the late E2A promoter*

Within the 50-nucleotide-pair sequence in the late

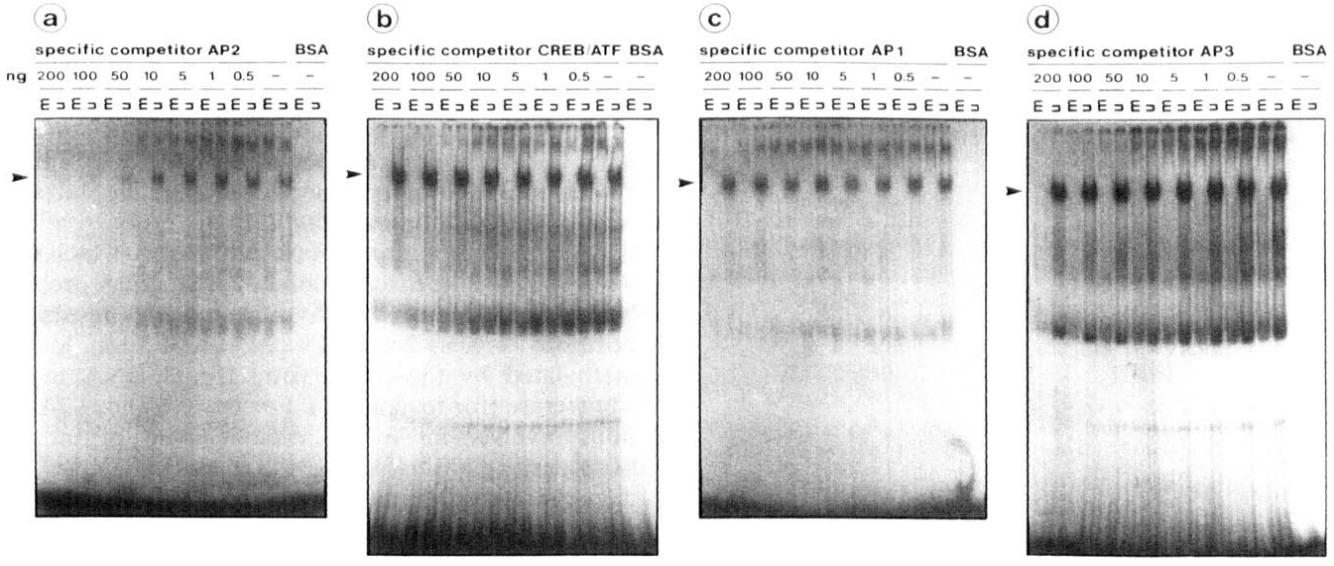

Fig. 2. Formation of the high-molecular-mass complex between the oligodeoxyribonucleotide comprising nucleotides +37 to −13 of the late E2A promoter of Ad2 DNA and partly purified nuclear extracts: Competition by an oligodeoxyribonucleotide with the AP2 consensus sequence. The experimental details have been described in the text. The specific oligodeoxyribonucleotides (nucleotide sequences reproduced in section 2) and their amounts used in each experiment have been indicated above each autoradiogram: consensus sequence AP2 site (a), CREB/ATF site (b), AP1 site (c), or AP3 site (d). The arrowheads locate the positions of the high-molecular-mass DNA-protein complex. BSA, bovine serum albumin was used as a negative control, instead of partly purified nuclear extracts from HeLa cells. The ^{32}P-labeled 50 nucleotide pair oligodeoxyribonucleotide between nucleotides +37 and −13 in the late E2A promoter of Ad2 DNA was used for DNA-protein binding and migration delay experiments in the unmethylated (u) or the nucleotide +24 and +6 methylated (m) form. Autoradiograms of polyacrylamide electrophoresis experiments were shown in which the uncomplexed oligodeoxyribonucleotide and the DNA-protein complexes were separated.

E2A promoter of Ad2 DNA between nucleotides +37 and −13, several sequences with similarities to recognized motifs of transcription factor binding sites have been identified, such as AP1-, AP2-, AP3- and CREB-binding sequences (Fig. 1). We have now performed competition experiments in DNA-protein binding assays by using increasing amounts (10- to 4000-fold excess) of oligodeoxyribonucleotides with the described consensus sequences. The composition of these oligodeoxyribonucleotides has been described in section 2. The data presented as autoradiograms of band shift assays (Fig. 2a–d, lanes without or with low amounts of competitors added) confirm the previously documented finding [7] that a high-molecular-mass DNA-protein complex is formed between the 50-nucleotide-pair oligodeoxyribonucleotide comprising nucleotides +37 to −13 of the late E2A promoter and proteins from partly purified nuclear extracts of HeLa cells. Bovine serum albumin (BSA) used as a negative control is inert in the formation of such a complex. The DNA-protein complex is not established when the oligodeoxyribonucleotide is methylated in the C residues corresponding to positions +6 and +24 of the late E2A promoter of Ad2 DNA (Fig. 2a–d, lanes without or with low amounts of competitors added; for map, see Fig. 1). It is also apparent that the oligodeoxyribonucleotides with the consensus sequence of the CREB/ATF, the AP1, or the AP3 site do not compete for the +6 and +24 binding sites even when added in 4000-fold excess (Fig. 2b, c and d, respectively). On the other hand, the oligodeoxyribonucleotide with the AP2 sequence starts to compete significantly for the site essential for complex formation when the AP2 oligodeoxyribonucleotide is added in 200-fold excess and almost completely eliminates the formation of the complex when it is present in 2000- to 4000-fold excess (Fig. 2a). These data demonstrate that one or both of the AP2-like sites in the +37 to −13 nucleotide sequence of the late E2A promoter are directly involved in, perhaps even responsible for, the formation of the DNA-protein complex which is eliminated by the methylation of the C residues in positions +6 and +24.

It had been shown earlier that the formation of the high-molecular-mass complex can be competed for by the unmethylated nucleotide +37 to −13 oligodeoxyribonucleotide, but not by the methylated form of this oligodeoxyribonucleotide [7].

3.3. *Methylation of either 5'-CCGG-3' sequence in the nucleotide +37 to −13 region affects the formation of the DNA-protein complex*

Do both methyl groups in the +37 to −13 oligodeoxyribonucleotide (Fig. 1) contribute to the destabilization of the DNA-protein complex in the downstream region of the late E2A promoter? Synthetic oligodeoxyribonucleotides were prepared which carried the 5-mC residue either in the +6 or in the +24 position in both strands of the construct. The data reproduced in Fig. 3

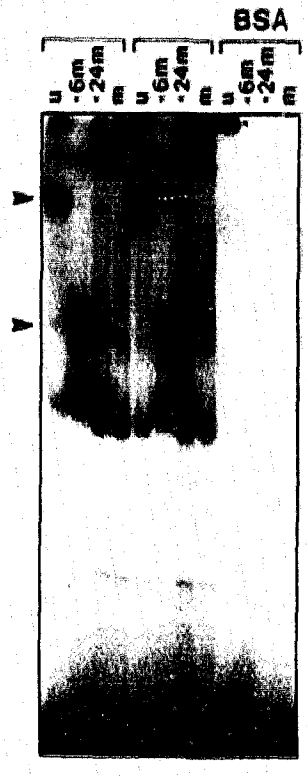

Fig. 3. The methylation of either 5'-CCGG-3' sequence in the nucleotide +37 to −13 oligodeoxyribonucleotide destabilizes the high-molecular-mass DNA-protein complex. Experimental conditions were described in the text or in the legend to Fig. 2 where symbols and abbreviations were explained. The designations +6m, +24m indicate that in the 50-nucleotide-pair oligodeoxyribonucleotide, which corresponds to nucleotide positions +37 to −13 in the late E2A promoter of Ad2 DNA, the C residue in position +6 or +24 was replaced by 5-mC, respectively.

demonstrate that methylation of the C residue in position +6 almost completely abolishes the formation of the high-molecular-mass DNA–protein complex. Apparently, some of the ^{32}P-labeled oligodeoxyribonucleotide is shifted to the position of a lower molecular-weight mass (Fig. 3, lower arrowhead). Some of the proteins in the original complex may have been released from it. Similarly, when the methylated C residue is placed in position +24 of the late E2A promoter, a complex of much lower molecular mass is generated that has not been detectable when the unmethylated oligodeoxyribonucleotide has been used. This finding is consistent with the interpretation that a 5-mC residue in position +6 leads to the release of some of the protein(s) from the methylation-sensitive high-molecular-mass complex. These results suggest that both the +6 and the +24 C residues contribute to the formation of the methylation-sensitive DNA–protein complex in the downstream region of the late E2A promoter in Ad2 DNA. The distance of 18 nucleotides between these two essential sites would argue that the formation of the complex requires both protein–DNA and protein–protein interactions. Methylation of the C residue in position +6 only, still seems to leave a sizable complex intact which is, however, much smaller than the original high-molecular-mass complex.

3.4. *Methylation of the C residue in position −215 of the late E2A promoter fails to alter the formation of a DNA–protein complex*

In all of our previous experiments, which have demonstrated the inactivation of the late E2A promoter by sequence-specific methylation, the C residues in positions +6, +24, and −215 have been in vitro methylated by the prokaryotic HpaII DNA methyltransferase (for review, [5]). For the +6 and +24 position, involvement in the establishment of methylation-sensitive DNA–protein interactions has been demonstrated. Presumably, these interactions are essential for promoter functionality, their obliteration leads to promoter inhibition. The role of the C residue at position −215 has previously not been separately assessed. The data in Fig. 4 show that a 40-nucleotide-pair oligodeoxyribonucleotide (composition and map location, cf. Fig. 1), comprising this −215 site in the late E2A promoter of Ad2 DNA forms one distinct DNA–protein complex with partly purified nuclear ex-

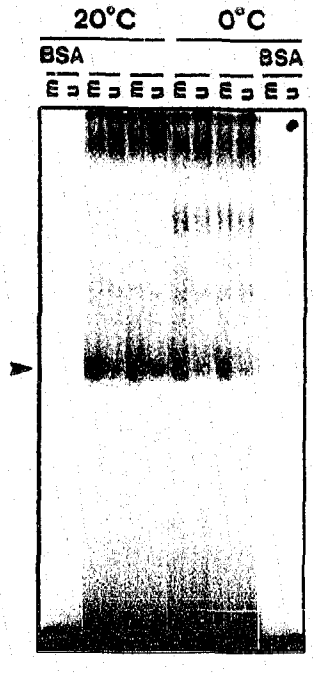

Fig. 4. Protein binding at the upstream 5'-CCGG-3' position at nucleotide −215 in the late E2A promoter of Ad2 DNA. The ^{32}P-labeled 40-nucleotide-pair oligodeoxyribonucleotide around promoter position −215 (nucleotide sequence at lower right in Fig. 1) in the upstream region of the late E2A promoter of Ad2 DNA was incubated with the heparin-sepharose column fractions described in section 2 (also cf. [7]). This oligodeoxyribonucleotide carried a C (u) or a 5-mC (m) residue in position −215. The DNA–protein complex formed irrespective of DNA methyl and was indicated by an arrowhead.

tracts of HeLa cells. Methylation of the C residue at position −215 does not affect the formation of this complex. Perhaps, complex formation in this case is more pronounced with the methylated oligodeoxyribonucleotide. Of course, in a functional sense the complexes with the unmethylated or the −215 methylated oligodeoxyribonucleotide might still differ. On the basis of the results presented in Fig. 4 we consider it unlikely that the methylation of the C residue in position −215 can contribute significantly to the inactivation of the late E2A promoter when the three specific 5'-CCGG-3' sequences are methylated.

4. CONCLUSIONS

1. Methylation of the C residues in positions +6 and +24 in the downstream region of the late E2A promoter of Ad2 DNA probably suffices to inactivate this promoter in the experimental systems used for analysis.
2. The methylation at these sites abrogates completely the formation of a high-molecular-mass DNA-protein complex.
3. The formation of this complex seems to be essential for promoter function. In living cells, a much more complicated DNA-protein complex may be found.
4. For the formation of the high-molecular-mass DNA-protein complex, the AP2 site(s) in the downstream region of the late E2A promoter are important; closely adjacent AP1, AP3, and CREB sites do not have a demonstrable effect.
5. The data suggest that the formation of the +6 and +24 downstream promoter complex involves both DNA-protein and protein-protein interactions, since
   (a) the methylation of the C position +6 only allows the formation of a complex which is much smaller than the high-molecular-mass structure, and since
   (b) positions +6 and +24 appear to be too far apart for complex formation to occur solely on the basis of DNA-protein interactions.

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