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The impact of 5′-CG-3′ methylation on the activity of different eukaryotic promoters: a comparative study

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Abstract

The inhibiting or inactivating effects of position-specific promoter methylation in different viral or human cellular promoters Ad2 E2AL, SV40, LTR-MMTV, HSV-tk, TNFα) have been compared by in vitro 5′-CCGG-3′ methylation by M-*Hpa*II or the M-*Sss*I DNA-methyltransferase, respectively. In most promoters, 5′-CG-3′ methylation reduces activity to a few percent of that of mock-methylated controls. The number of 5′-CG-3′ dinucleotides in a promoter does not strictly correlate with the extent of methylation inhibition. The LTR-MMTV promoter, which lacks 5′-CG-3′ dinucleotides, is not affected by methylation. The late E2A promoter of Ad2 DNA cannot be inactivated by 5′-CCGG-3′ methylation when the construct carries the strong cytomegalovirus enhancer devoid of this sequence. In contrast, 5′-CG-3′ methylation shuts this promoter off even in the presence of this enhancer.

Key words: Sequence-specific DNA methylation; DNA-methyltransferase; Viral promoter; Human TNFα gene promoter; Chloramphenicol acetyltransferase or luciferase reporter gene; DNA transfection

1. Introduction

Through studies on many different eukaryotic promoters the concept that sequence-specific DNA methylation can lead to the inactivation of promoters [1] has gained acceptance (see references in [2]). The methylation-sensitive site(s) in a given promoter cannot be predicted but must be experimentally determined. It is likely that promoter methylation interferes with transcription factor binding [3–6] or promotes the binding of methyl group-dependent factors to promoter motifs [7]. This interpretation could explain why the methylation of different promoter motifs is associated with gene inactivation.

We have shown previously that sequence-specific promoter methylation inactivates the RNA polymerase II-transcribed late E2A promoter of adenovirus type 2 (Ad2) DNA [8–11], the E1A promoter of Ad12 DNA [12], the major late promoter of Ad2 DNA [13] or even the p10 gene promoter of the insect virus, *Autographa californica*, nuclear polyhedrosis virus DNA in insect cells [14]. The RNA polymerase III-transcribed elements for VA (virus associated) RNA of Ad2 DNA [15] and Alu elements from the human genome [16] are also transcriptionally inhibited by the sequence-specific methylation of control sequences in these elements.

Here, we report on the results of a comparative study testing the effects of 5′-CG-3′ methylation on the activities of the late E2A promoter of Ad2 DNA, the early SV40 promoter, the LTR (long terminal repeat)-located promoter of mouse mammary tumor virus (MMTV), the thymidine kinase promoter from herpes simplex virus (HSV), and of the promoter of the human tumor necrosis factor (TNF) α gene. With the exception of the early SV40 promoter, which is strongly inhibited, 5′-CG-3′ methylation practically inactivates all the other promoters tested.

2. Materials and methods

2.1. Cell lines used in transfection experiments

Human HeLa cells (ATCC CCL 2), a cervical carcinoma cell line, the human PA-1 cell line derived from an ovarian teratocarcinoma (ATCC CRL 1572) [17], and the Ad5-transformed human cell line, 293 [18], were propagated in Dulbecco's medium enriched with 10% fetal calf serum.

2.2. Plasmids employed in this study

Table 1 presents an overview of all plasmid constructs used, the promoters and the reporter genes, and lists the derivation of these constructs.

2.3. Newly made plasmid constructs

2.3.1. pAd2-E2AL-LUX. The 490 bp *Hin*dIII fragment carrying the late E2A promoter of Ad2 DNA was re-cloned into the *Hin*dIII site of the plasmid, pGB-Basic (Promega).

2.3.2. pTNFαEN-CAT(-LUX). The 632 bp *Sma*I–*Eco*NI (−615 to + 17) fragment from the human TNFα gene promoter [26] was

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treated with Klenow polymerase [27] and cloned into the *Sma*I site of pBS$^+$ (Stratagene, LaJolla, CA, USA). From this construct, the TNFα promoter was re-cloned into the pSV0-CAT plasmid as a *Hin*dIII–*Nde*I fragment or into the pGL-Basic plasmid as a *Hin*dIII–*Kpn*I fragment.

2.3.3. pTNFα HP-CAT(-LUX). The 795 bp *Hpa*II fragment (−592 to + 203) of the human TNFα gene promoter was cloned first into the *Acc*I site of the pBS$^+$ vector and then re-cloned into the pSV0-CAT plasmid as a *Hin*dIII–*Nde*I fragment or into the pGL-Basic plasmid as a *Hin*dIII–*Kpn*I fragment.

Plasmid constructs were prepared using standard cloning techniques. Plasmid DNAs were purified by using Qiagen (Diagen, Hilden, Germany) Midi- and Maxiprep systems.

2.4. The in vitro methylation of DNA

Plasmid DNAs were methylated in vitro by the bacterial M-*Hpa*II (MBI-Fermentas, Vilnius, Lithuania) or by the M-*Sss*I (CpG) [28] DNA-methyltransferase (New England Biolabs, Beverly, MA, USA, or Amersham-USB, Cleveland, OH, USA). NEB buffer No. 2 was made 160 μM *S*-adenosylmethionine (SAM) and used in all methylation reactions which proceeded for 2–4 h at 37°C using 2 U of enzyme per μg of DNA in a volume of 10 μl. In mock-methylation reactions, SAM was omitted. At the end of the methylation or mock-methylation reaction, DNA was re-extracted by phenol–chloroform and ether, ethanol precipitated, washed with 70% ethanol and resuspended at a final DNA concentration of 1 μg in 10 μl of 0.01 M Tris-HCl, pH 7.5, 1 mM EDTA (TE). Complete methylation was assessed by restricting DNA with *Hpa*II or by determining the nucleotide sequence. In addition, changes in plasmid topoisomer distributions were tested by electrophoresis of uncut DNA in 0.8% agarose gels. All enzyme preparations used produced remarkable relaxation of the supercoiled form of plasmid DNA both in mock- and methylation reactions. Plasmid preparations with an excess of relaxed plasmid topoisomers were not used in transfection experiments.

2.5. Transfection and reporter gene activity assays

Human cell lines were transfected 24 h after re-plating with promoter–reporter gene constructs by the calcium phosphate precipitation technique [29]. Amounts of 2–5 μg of plasmid DNA were used per 5 cm diameter dish of HeLa or 293 cells, and 0.5–1.0 μg for PA-1 cells. The published protocol was applied with the following modifications: the HEPES buffer solution was pH 6.96; the glycerol shock step was omitted; medium was first changed 24 h after the addition of the DNA-Ca^{2+} precipitates; the cells were harvested 48 h after transfection.

CAT activity was determined according to standard procedures [12,19] and expressed as percent conversion of chloramphenicol (CAM) to acetylated CAM forms during a 1 h incubation. Activity of β-galactosidase was determined according to Hall et al. [21] and expressed in arbitrary units. LUX activity was determined using the Promega assay system and a Lumat LB9501 luminometer (Berthold, Bad Wildbad, Germany). LUX activity was expressed in relative light units. Reporter gene activities were normalized relative to the amounts of cellular protein in culture lysates. Protein concentration was determined according to [30]. The data presented in the tables were typical values derived from at least 3, but in most cases from 10–12 independent transfection experiments.

3. Results and discussion

3.1. Aim of project

We have previously shown that the late E2A promoter of Ad2 DNA can be inhibited or inactivated by in vitro methylation at the 5′-CCGG-3′ sequences [9,11]. We have now compared this promoter activity upon methylation at the three 5′-CCGG-3′ sequences at + 6, + 24, and −215 relative to the cap site at + 1 with its activity when all 5′-CG-3′ sequences were methylated by different DNA-methyltransferases and upon transfection of these methylated constructs into different human cell lines. We have also demonstrated that the 13S E1A function of Ad2 or of Ad5 DNA can overcome, at least partly, the inhibitory effect of methylation in this promoter [11,23]. Similarly, presence of the strong early enhancer from human cytomegalovirus (HCMV) counteracts the inactivating function of 5′-CCGG-3′ methylation in the late E2A promoter [24].

It has been the second aim of this study to compare the effects of 5′-CCGG-3′ with those of 5′-CG-3′ methylation on a series of viral and mammalian cellular promoter constructs to underline the fact that results derived

Table 1
Plasmid constructs used in transcription experiments[a]

Plasmid	Promoter	Reporter gene	Reference
pSV2-CAT	SV40 early	CAT	[19]
pSV0-CAT	none	CAT	[19]
pBLCAT2	HSV tk	CAT	[20]
pCH110	SV40 early	β-Gal	[21]
pMMTV	MMTV-LTR	β-Gal	[22]
pAd2E2AL-CAT	E2AL	CAT	[23]
pAd2E2AL-HCMV-CAT	E2AL+HCMV	CAT	[24]
pAd2E2AL-HCMV (Bam-CAT)	E2AL−HCMV	CAT	[24]
pGL-Control	SV40 early	LUX	Promega, Madison, WI, USA
pGB-Basic	none	LUX	Promega
pAd2E2AL-LUX	E2AL	LUX	This work
pTNFα-EN-CAT	P-TNFα	CAT	This work
pTNFα-HP-CAT	P-TNFα	CAT	This work
pTNFα-EN-LUX	P-TNFα	LUX	This work
pTNFα-HP-LUX	P-TNFα	LUX	This work
pE2AL-Δ46-CAT	E2A-Δ46	CAT	[25]

Promoters: SV40 early, Simian virus 40 early region of transcription; HSV tk, Herpes simplex virus thymidylate kinase; E2AL, adenovirus type 2 late E2A gene promoter; pTNFα, human tumor necrosis factor α gene promoter; MMTV-LTR, mouse mammary tumor virus long terminal repeat; HCMV, human cytomegalovirus immediate early enhancer; Reporter genes: CAT, chloramphenicol acetyltransferase; LUX, firefly luciferase; β-Gal, bacterial β-galactosidase.

Table 2
5′-CpG-3′ methylated E2A late gene promoter is inactivated

Plasmid construct	Methylation by DNA-methyltransferase from	Cell line used for transfection (% activity)[a]		
		HeLa	PA-1	293
pAd2-E2AL-CAT	M-*Hpa*II	20	25	100
	M-*Sss*I	< 1	2	1
pAd2-E2AL-HCMV-CAT	M-*Hpa*II	100	100	ND[b]
	M-*Sss*I	< 1	3	ND
PAd2-E2AL-HCMV(Bam)-CAT	M-*Hpa*II	100	100	ND
	M-*Sss*I	2	3	ND

[a]Activities of promoter constructs were expressed as percent activity of mock-methylated promoter, which was arbitrarily set at 100%.
[b]ND = not determined.

from viral promoter systems are valid for human cell promoters as well.

3.2. The methylation of 5′-CG-3′ sequences inactivates the late E2A promoter of Ad2 DNA

The data in Table 2 document that 5′-CG-3′ methylation of the late E2A promoter of Ad2 DNA by the M-*Sss*I DNA-methyltransferase from *Spiroplasma* spp. inactivates this promoter, even in the presence of the HCMV enhancer. As described earlier [24] and reconfirmed by some of the results in Table 2, 5′-CCGG-3′ methylation by the M-*Hpa*II DNA-methyltransferase does not even slightly affect the activity of late E2A promoter–CAT gene constructs which contain the strong HCMV enhancer. These data can probably be explained by the fact that the HCMV enhancer [31] does not carry a single *Hpa*II site, but seventeen 5′-CG-3′ dinucleotides which render it susceptible to M-*Sss*I methylation. The silencing of the late E2A promoter of Ad2 DNA by complete 5′-CG-3′ methylation cannot be overcome either by the E1A adenovirus functions which are constitutively expressed in the human cell line, 293 (Table 2). In contrast, the inhibitory effect of 5′-CCGG-3′ methylation of the late E2A promoter by the M-*Hpa*II DNA-methyltransferase is counteracted by the E1A functions present in 293 cells. The transactivating effect of the E1A 13S gene product on the late E2A promoter [11] is obviously compromised when all 5′-CG-3′ dinucleotides are methylated.

It should be mentioned that the absolute strength of the three promoter constructs presented in Table 2 differ and is highest in the human PA-1 teratocarcinoma cell line. The presence of the HCMV enhancer in two of the unmethylated constructs raises their absolute activities by a factor of about ten (data not shown).

3.3. Different promoters differ in activity upon transfection into human HeLa cells

Prior to determining the effect of 5′-CG-3′ methylation on different viral and human cell promoters, the relative activities of these promoters were assessed in combination with three different indicator genes upon transfection into human HeLa cells. The relative values are juxtaposed in Table 3 and demonstrate the following: (i) With the exception of the early SV40 and possibly the HSV-tk promoters, all other promoter–reporter gene constructs have about comparable relative activities independent of the indicator genes used (CAT, β-Gal, or LUX). The early SV40 and the HSV tk promoters have higher activities with the CAT reporter gene. (ii) Two slightly differently designed human TNFα gene promoter constructs exhibit appreciable activities, similar in conjunction both with the CAT and LUX gene reporters.

3.4. The human TNFα gene promoter is expressed in human epithelial cell lines and is completely inactivated by 5′-CG-3′ methylation

In both human HeLa (Table 3) and PA-1 cells, the TNFα gene promoter activates the CAT and LUX reporter genes. This activity is unaffected by TNFα inducers, such as phorbol ester or bacterial lipopolysaccharides (data not shown). As shown by the results of 5′-CG-3′ methylation experiments in Table 4, the human TNFα promoter is almost completely inactivated by this modification.

3.5. Promoter inactivation by 5′-CG-3′ methylation: a comparative study

The presentation of data in Table 4 correlates the

Table 3
Relative activities in HeLa cells of different promoters with the same reporter gene

Promoter[a]	Number of 5′-CG-3′ dinucleotides[b]	Relative strength of promoters in HeLa cells with different reporter genes		
		CAT	β-Gal	LUX
E2AL	11	1	ND[d]	1
E2AL-Δ46[c]	11	0.5	ND	ND
TNFα-EN	4	2	ND	3
TNFα-HP	4	0.5	ND	5
SV40 early	7	300	3	10
MMTV	0	ND	1	ND
HSV tk	16	10	ND	ND

[a]Relative strength of promoters was compared in constructs carrying different promoters but the same reporter gene.
[b]Number of 5′-CG-3′ inucleotides in 120 nucleotides upstream from the cap site.
[c]In this promoter construct, the nucleotide + 7 to + 52 sequence relative to the + 1 cap site in the late E2A promoter encompassing the + 6 and + 24 5′-CCGG-3′ site was deleted [25].
[d]ND = not determined.

Table 4
A comparison of the sensitivities of different viral and human cellular promoters to inactivation by 5′-CG-3′ methylation

Promoter[a]	Number of 5′-CG-3′ dinucleotides[b]	Reporter gene		
		CAT	β-Gal	LUX
E2AL	11	< 1	ND[c]	< 1
E2AL-Δ46[d]	11	< 1	ND	ND
TNFα-EN	4	< 1	ND	< 1
TNFα-HP	4	< 1	ND	5
SV40 early	7	40	50	20
MMTV	0	ND	100	ND
HSV tk	16	2	ND	ND

[a]Activity of CpG methylated promoters was expressed as percent of the mock-methylated counterparts.
[b]Number of 5′-CG-3′ dinucleotides in 120 nucleotides upstream from the cap site.
[c]ND = not determined.
[d]In this promoter construct, the + 7 to + 52 nucleotide sequence in the late E2A promoter was deleted [25].

number of 5′-CG-3′ dinucleotides in the different promoters and their sensitivities to inactivation by 5′-CG-3′ methylation in test constructs. Even low numbers of 5′-CG-3′ dinucleotides, e.g. four in the human TNFα promoter, suffice to elicit complete inactivation. In contrast, the activity of the early SV40 promoter with seven 5′-CG-3′ dinucleotides is only moderately sensitive to 5′-CG-3′ methylation. Lastly, the LTR-MMTV promoter, which is devoid of 5′-CG-3′ dinucleotides, does not respond to 5′-CG-3′ methylation; its activity remains unabated.

It is plausible to suggest that the sensitivity of a promoter to 5′-CG-3′ methylation depends crucially on the position of these sequences relative to essential protein-binding sites and less on the absolute number of these sites. It would be premature to speculate on the possible sites affected by 5′-CG-3′ methylation in each of the promoter constructs investigated. This comparative study demonstrates that viral and cellular promoters are similarly affected in their activities by sequence position-specific DNA methylation.

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