Transcriptional silencing of human Alu sequences and inhibition of protein binding in the box B regulatory elements by 5′-CG-3′ methylation

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Abstract In earlier work, we demonstrated that 5′-CG-3′ methylation inhibits the transcriptional activity of human Alu elements associated with the α_1-globin and the angiogenin genes in a cell-free transcription system from HeLa nuclear extracts. These studies have been extended to different Alu sequences and to investigations on the mechanism involved in transcriptional silencing by methylation. By comparing the results of DNase I and dimethyl sulfate (DMS) in vitro footprinting on a consensus sequence in the RNA polymerase III promoter control B region between the unmethylated and the 5′-CG-3′ methylated B box, evidence has been adduced for effects of 5′-CG-3′ methylation on the interaction of specific nuclear proteins with DNA sequences in the B control region of the Alu elements. These results are consistent with the interpretation that the 5′-CG-3′ methylation interferes with the binding of proteins that are essential for the function of the B control region in these RNA polymerase III-transcribed elements, and that promoter methylation thus inhibits transcription.

Key words: Human Alu element; Regulation of expression; Methylation of regulatory region; DNA–protein binding; Transcription (in vitro)

1. Introduction

The Alu family of repetitive elements comprises about 5% of the human genome (for recent reviews see [1–4]). In cell-free nuclear extracts, Alu elements can be efficiently transcribed in vitro by RNA polymerase III [3,5,6], while most Alu sequences are transcriptionally silent in living cells. In spermatozoa, but not in primary diploid cells, RNA transcripts can be detected which have been initiated at the left border of the box A internal control region ([7], and unpublished results). These transcripts have probably been generated by RNA polymerase III. Interestingly, Alu sequences in general have been found to be undermethylated in sperm [7,8].

The DNA methylation of all 5′-CG-3′ dinucleotides in the Alu elements associated with the α_1-globin and the angiogenin genes leads to the inhibition of transcription in vitro by HeLa cell- or lymphocyte-derived cell-free nuclear extracts [7]. Similar results for several Alu elements have been described recently [6,9]. Methylation of two 5′-CG-3′ sequences in the A box control region of the Alu sequences has been shown to be sufficient to inhibit the in vitro transcription in HeLa nuclear extracts. This effect is thought to be caused indirectly by a protein binding to methylated DNA [6]. The methylation of cytosines has been reported to inhibit the transcription of a tRNA gene but not of 5 S RNA genes [10].

In this report we demonstrate that, by the criteria of in vitro DNase I and DMS footprinting, a 5′-CG-3′ dinucleotide located in the B box regulatory region of the Alu elements may be responsible for the observed differential effect of DNA methylation on the in vitro transcription of several Alu elements.

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2. Materials and methods

2.1. Templates

The plasmid pAngio2 contained a 1686 bp *Pvu*II–*Bgl*II fragment carrying a 5′-CG-3′-rich Alu element upstream of the angiogenin gene cloned in the vector Bluescript KS$^+$ [11]. The plasmid pM14 BglII contained a 2.6 kb *Bgl*II fragment with a 5′-CG-3′-rich Alu element located in intron 8 of the tissue plasminogen activator (tPA) gene as a pBR322 clone [12]. The plasmid pAlu ori contained a 1943 bp *Hin*dIII–*Eco*RI fragment carrying an Alu element located downstream of the α_1-globin gene as a pSV0 clone [5]. The plasmid pACTH contained a 919 bp *Bam*HI–*Bcl*I fragment with a 5′-CG-3′-rich Alu element in intron A of the adrenocorticotropin hormone (ACTH) gene cloned into the Bluescript KS$^+$ vector [13].

2.2. In vitro transcription

The in vitro transcription reactions in HeLa nuclear extracts were performed as described elsewhere [7].

2.3. End-labeling of DNA templates with ^{32}P

For the in vitro footprinting reactions, DNA templates were ^{32}P-labeled at their 5′ termini by using [γ-^{32}P]ATP and T4 polynucleotide kinase [14], or at their 3′ termini by using ^{32}P-labeled dNTPs in fill-in reactions with the Klenow fragment [15] of the *E. coli* DNA polymerase [16]. The labeled DNA preparations were then cleaved with a suitable restriction endonuclease to generate DNA fragments labeled on one terminus. The pAngio2 construct was labeled at a *Nco*I site and then cleaved with *Sac*I in the polylinker of the vector (Fig. 1). The M14BglII construct was labeled at a *Nco*I site and then cleaved with *Stu*I [17]. The pAluori plasmid was cleaved at a *Bcl*I site and then cleaved with *Eco*RI or was labeled at an *Eco*RI site and subsequently cleaved with *Hin*dIII. Lastly, the pACTH construct was labeled at an *Ava*II site and then cleaved with *Sac*I.

2.4. In vitro methylation of DNA

The methylation and mock-methylation reaction using the 5′-CG-3′-specific DNA methyltransferase from *Spiroplasma* spp. (M.SssI) [18] was described elsewhere [19]. In mock-methylation experiments, *S*-adenosylmethionine as the methyl group donor was omitted from the reaction. The completeness of the methylation reaction or the absence of methylation upon mock-methylation was ascertained by chemically sequencing the templates [20].

2.5. In vitro footprinting with DNase I

Under in vitro transcription conditions, 20,000 to 100,000 cpm of end-labeled template DNA were incubated in a total volume of 10 μl

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in 10 mM HEPES (*N*-2-hydroxy-ethylpiperazine-*N'*-2-ethane-sulfonate), pH 7.9, 40 mM KCl, 5 mM $MgCl_2$, 5 mM creatine phosphate, 400 ng competitor pUC18 DNA, 25 μg of protein from crude nuclear extracts [21] which were prepared from exponentially growing HeLa cells. Upon incubation for 15 min at 30°C, samples were placed on ice and 10 μl of ice-cold 5 mM $MgCl_2$, 5 mM $CaCl_2$, 20 ng of DNase I (SERVA; freshly diluted from 1 μg/μl in 40 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.9), were immediately added. The reaction was stopped after 2 min incubation on ice by adding 100 μl of 1% SDS, 20 mM EDTA, 250 mM NaCl, 2 μg of proteinase K, 30 μg of tRNA. Incubation was continued at 37°C for 30 min followed by phenol/chloroform extraction and ethanol precipitation. In control reactions, the end-labeled fragments were incubated under identical conditions, except for the addition of HeLa nuclear extract. The optimal DNase I concentration had been predetermined at 2 ng. DNA fragments were resolved by electrophoresis in 5% polyacrylamide sequencing gels containing 7 M urea.

2.6. In vitro footprinting with dimethylsulfate (DMS)

Incubation with HeLa nuclear extracts was as described above for DNase I in in vitro footprinting experiments. After incubation at 30°C for 15 min, samples were placed on ice, dimethylsulfate (DMS) was added to a final concentration of 0.25%, and incubation was continued at 20°C for 4 min. The optimal DMS concentration had been determined previously. Reactions were terminated by adding 500 μl of 1% SDS, 20 mM EDTA, 100 mM β-mercaptoethanol, 5 μg of sonicated salmon sperm DNA, 2 μg proteinase K. Samples were subsequently incubated at 37°C for 5 min, followed by phenol/chloroform extraction and two cycles of ethanol precipitation. In control reactions, the end-labeled fragments were incubated under the same conditions, except for the addition of HeLa nuclear extracts. After the DMS reaction, samples were treated with 1 M piperidine at 90°C for 30 min in a total volume of 100 μl followed by ethanol precipitation and two rounds of lyophilization.

3. Results

3.1. The in vitro transcription by polymerase III is inhibited by 5'-CG-3' methylation of Alu elements associated with the angiogenin gene, the tPA gene-, and the α_1-globin gene-, but not in the ACTH gene-associated Alu element

We previously documented inhibitory effects of DNA methylation by the 5'-CG-3'-specific DNA methyltransferase (M.SssI) on the in vitro transcription of the α_1-globin gene- and the angiogenin gene-associated Alu elements [7]. In the present communication, a similar inhibition, apparent only at low template concentrations, was observed for the tPA gene-associated Alu element (data not shown), but not for the ACTH gene-associated Alu element (Fig. 1).

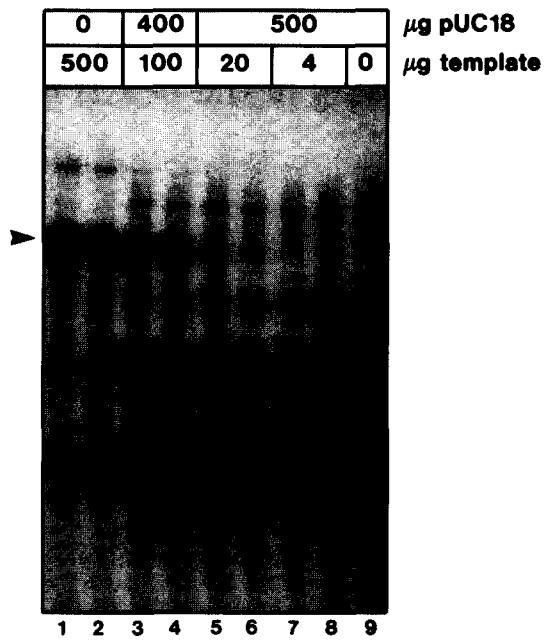

Fig. 1. Autoradiogram of the products from an in vitro transcription experiment using the ACTH gene-associated Alu element as template. Plasmid pACTH was transcribed in vitro in HeLa nuclear protein extracts as described earlier [19]. In this experiment, different amounts of 5'-CG-3' methylated (lanes 1, 3, 5, 7) or mock-methylated template DNA (lanes 2, 4, 6, 8) were used, as indicated. The total amount of DNA in each reaction was balanced with pUC18 DNA to 500 ng. Lane 9, plasmid pUC18 incubated without pACTH as a control. The arrowhead designates the position of the transcription product specific for the ACTH gene-associated Alu element.

3.2. The methylation of 5'-CG-3' sequences results in decreased binding of cellular proteins to the B box regulatory region of the Alu element upstream of the angiogenin gene

The possibility was investigated that the inhibition of the cell-free transcription of Alu elements by 5'-CG-3' methylation documented earlier [7] was due to the interference by template methylation with sequence-specific protein binding. In vitro DNase I and DMS footprinting experiments were performed to study the effect of 5'-CG-3' methylation on protein binding in the B box regulatory region of the Alu element associated with the human angiogenin gene.

The autoradiogram in Fig. 2A presents the results of a DNase I in vitro footprinting experiment. Methylation of the 5'-CG-3' sequences by the DNA methyltransferase from *Spiroplasma* spp. in the pAngio2 construct markedly reduced the binding of proteins from HeLa nuclear extracts to the top strand (Fig. 2a, lane 4) and the bottom strand (lane 11) of the B box region in the Alu element, when compared to the mock-methylated template (lanes 5, top and 12, bottom). In these footprinting experiments, incubation conditions of the ^{32}P-labeled template DNA with HeLa nuclear extracts were identical to those used in cell-free transcription reactions with 5'-CG-3' methylated or unmethylated templates, except that the rNTPs were omitted for footprinting. Under these conditions, footprints in the A box region with unmethylated or methylated templates were not observed (Fig. 2a). In vitro transcription of the pAngio2 plasmid was inhibited by 5'-CG-3' methylation [7].

In Fig. 2b, the autoradiogram of the results of an in vitro DMS footprinting experiment of the same Alu element was shown. In these experiments, the labeled DNA templates were again incubated with HeLa nuclear proteins under in vitro transcription conditions, except that the rNTPs were omitted. Subsequently, DMS was directly added to the reaction mixture. The most pronounced effect of CpG methylation on DMS reactivity could be seen on the bottom strand (lanes 10–18) of the construct with a slight protection of the guanosine residue in the 5'-CG-3' dinucleotide in the mock-methylated template against the attack of DMS and a strong enhancement of the next guanosine residue at a distance of two nucleotides (lanes 13 and 16). Although a minor effect of the DMS reactivity could be observed with the 5'-CG-3' methylated template, the effect was much more pronounced with the mock-methylated template. There was no difference in DMS reactivity detectable

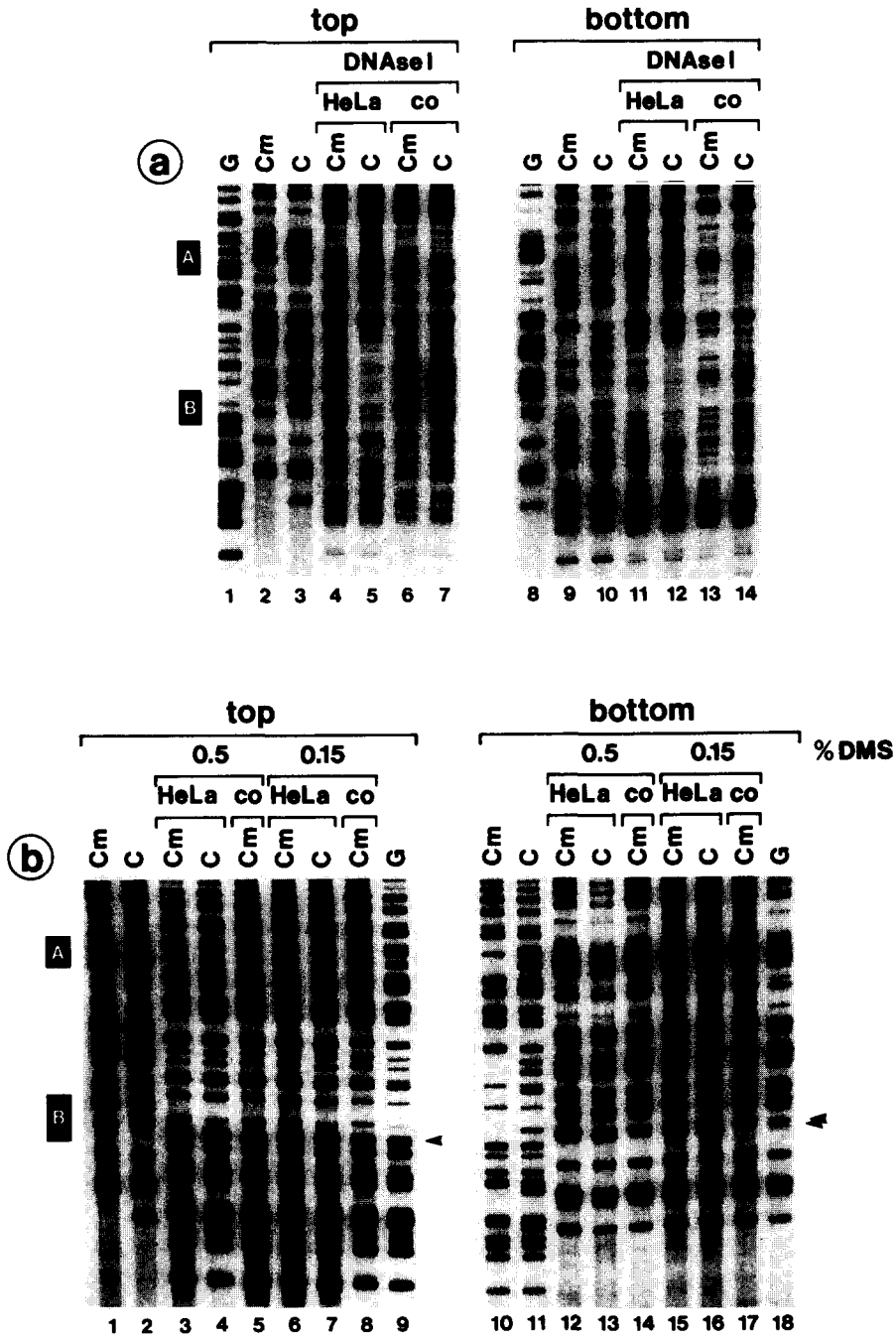

Fig. 2. DNase I and DMS in vitro footprinting of the Alu element located upstream of the human angiogenin gene. (a) Autoradiogram of the results of a DNase I in vitro footprinting experiment. The top strand (lanes 1–7) or the bottom strand (lanes 8–14) of the plasmid pAngio2 construct was ^{32}P-labeled at a *Nco*I site (see section 2). Lanes 1 and 8, G (DMS) reaction; lanes 2 and 9, C (hydrazine) reactions of the 5′-CG-3′ methylated templates; lanes 3 and 10, C reactions of the mock-methylated templates; lanes 4 and 11, DNase I in vitro footprint of the 5′-CG-3′ methylated templates; lanes 5 and 12, DNase I in vitro footprint of the mock-methylated templates; lanes 6 and 13, DNase I control reactions of the 5′-CG-3′ methylated templates; lanes 7 and 14, DNase I control reactions of the mock-methylated templates. In the control reactions, HeLa nuclear extract was omitted. The locations of the A and B box regulatory regions are indicated. The differential accessibility of the 5′-CG-3′ methylated and the mock-methylated control templates to DNase I was described previously [17]. (b) Autoradiogram of a DMS in vitro footprinting experiment. The top strand (lanes 1–9) or the bottom strand (lanes 10–18) of the plasmid pAngio2 construct was ^{32}P-labeled at a *Nco*I site (see a). Lanes 1 and 10, C (hydrazine) reaction of the 5′-CG-3′ methylated templates; lanes 2 and 11, C reaction of the mock-methylated templates; lanes 3, 6, 12, 15, DMS in vitro footprinting reactions for the 5′-CG-3′ methylated templates; lanes 4, 7, 13, 16, DMS in vitro footprinting reactions for the mock-methylated templates; lanes 5, 8, 14, 17, DMS control reactions of the 5′-CG-3′ methylated templates; lanes 9 and 18, G (DMS) reaction under standard conditions [20]. Differences in the reactivity between 5′-CG-3′ methylated and mock-methylated templates in the absence of cellular extracts were not observed (data not shown). The arrowheads indicate differential reactivity to DMS of the unmethylated as compared to the methylated templates (cf. Fig. 4i).

in the A box regulatory segment between the methylated as compared to the unmethylated Alu element. In Fig. 4, the results of the DMS in vitro footprinting experiments are summarized.

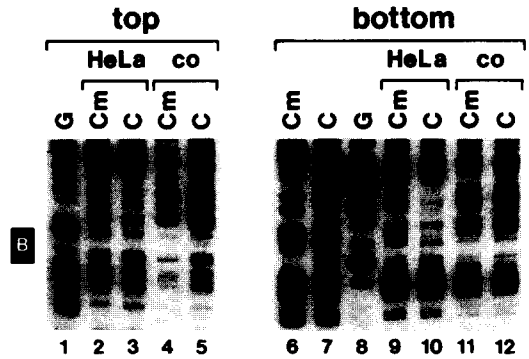

Fig. 3. DNase I in vitro footprinting experiment of the Alu element located in intron 8 of the human tPA gene. Autoradiogram of the results of a DNase I in vitro footprinting experiment. Reactions of the top strand (lanes 1–5) and the bottom strand (lanes 6–12). Lanes 1 and 8, G (DMS) reactions; lanes 2 and 9, DNase I in vitro footprint of the 5′-CG-3′ methylated templates; lanes 3 and 10, DNase I in vitro footprint of the mock-methylated templates; lanes 4 and 11, DNase I control of the 5′-CG-3′ methylated templates; lanes 5 and 12, DNase I control of the mock-methylated templates; lanes 6 and 7, C (hydrazine) reaction on the 5′-CG-3′ methylated and mock-methylated templates, respectively. The location of the B box is indicated.

3.3. *The methylation of 5′-CG-3′ dinucleotides in the B box regulatory region of human Alu elements associated with the α_1-globin and the tissue plasminogen activator (tPA) genes, but not of those linked to the ACTH gene, affects binding of cellular proteins*

The binding of cellular proteins to the B box regulatory regions of the human Alu elements associated with the three specific genes was assessed by DNase I and DMS in vitro footprinting experiments. Protein binding was compared between unmethylated and the 5′-CG-3′ methylated DNA constructs. The results of DNase I in vitro footprinting in the Alu elements associated with the tPA gene (Fig. 3) and the α_1-globin gene (data not shown) demonstrated a marked decrease in protein binding to the B box regulatory region. The data of DMS in vitro footprinting experiments yielded hyper-reactivity of the unmethylated vs. the methylated DNA templates for the two Alu elements (Fig. 4ii and iii), similar to the results described for the angiogenin gene-associated Alu element and its regulatory region.

In contrast to the findings with the angiogenin gene-, the α_1-globin gene-, and the tPA gene-associated Alu elements, in which protein binding was affected by 5′-CG-3′ methylation, the ACTH gene-associated Alu element (data not shown) exhibited a protein binding capacity that was unaffected by 5′-CG-3′ methylation, as determined by DNase I and DMS in vitro footprinting experiments. The results of all DMS in vitro footprinting assays performed with the four human gene-associated Alu elements are summarized in Fig. 4.

It is concluded that the results on methylation effects on the in vitro transcription of the four gene-associated Alu elements in HeLa cell nuclear extracts and on protein binding to the B box regulatory regions of these Alu elements are consistent, in that 5′-CG-3′ methylation inhibits transcription and protein binding in the angiogenin gene-, α_1-globin gene-, and tPA gene-associated Alu elements, but not in the ACTH gene-associated Alu element (Table 1).

4. Discussion

DNA methylation plays a role in the long-term silencing of eukaryotic genes (for reviews see [23,24]). We have investigated the consequences of in vitro 5′-CG-3′ DNA methylation by the M.SssI DNA methyltransferase on the transcriptional activity in a cell-free system from HeLa cell nuclear extracts of four specific human gene-associated Alu elements. The α_1-globin gene-, and the angiogenin gene-associated cloned Alu elements have previously been shown to be inactivated by 5′-CG-3′ methylation [7]. Similar results have now been adduced for the tissue plasminogen activator (tPA) gene-associated Alu element. In contrast, in vitro transcription of the ACTH gene-associated Alu element remains unaffected by 5′-CG-3′ methylation (Fig. 1).

Based on the notion that specific cellular proteins have to bind to the B box control region of the Alu elements to effect their transcription by RNA polymerase III, we have performed DNase I and DMS in vitro footprinting analyses on these control regions of the four gene-associated Alu elements. Protein binding, as shown by protection against enzymatic and chemical modifications, respectively, has been compared between the unmethylated and the 5′-CG-3′ methylated constructs. The results of both types of analyses document that methylation of a 5′-CG-3′ sequence, that is centrally located in the B box regulatory element (Fig. 4), leads to a decrease in the binding of protein(s) in this region among three of the Alu elements. For the ACTH gene-associated Alu element and its B box regulatory region, methylation has not shown this effect. This observation is consistent with the absence of an inhibitory effect on cell-free transcription of this Alu element by DNA methylation. It is worth noting that the B box region of the ACTH gene-associated Alu element does not contain a central 5′-CG-3′ dinucleotide (Fig. 4).

For the following reasons it is likely that transcription factor TFIIIC is among the proteins the binding and functionality of which is compromised by methylation in the Alu B box regulatory region. (i) The Alu sequences belong to the tRNA-type genes in which TFIIIC factor binding has been recognized as a primary step in assembling the transcription complex [25,26]. (ii) In in vitro transcription experiments, the α_1 globin gene-associated Alu element and the adenovirus type 2 VAI gene compete for the same transcription factors [5]. The TFIIIC

Table 1
Summary of the results of 5′-CG-3′ methylation in the regulatory B box regions of four specific, human gene-associated Alu elements

Alu element associated with gene for	Differential protein binding caused by 5′-CG-3′ methylation		Inhibitory effect of CpG methylation on in vitro transcription
	DNase I footprinting	DMS footprinting	
pAngio2	+	+	+
tPA	+	+	+
α_1-globin	+	+	+
pACTH	(−)	(−)	(−)

Summary of the data on the effect of 5′-CG-3′ methylation in DNase I in vitro footprinting (B box and abutting sequence), DMS in vitro footprinting (B box), and in vitro transcription for the four human gene-specific Alu elements.

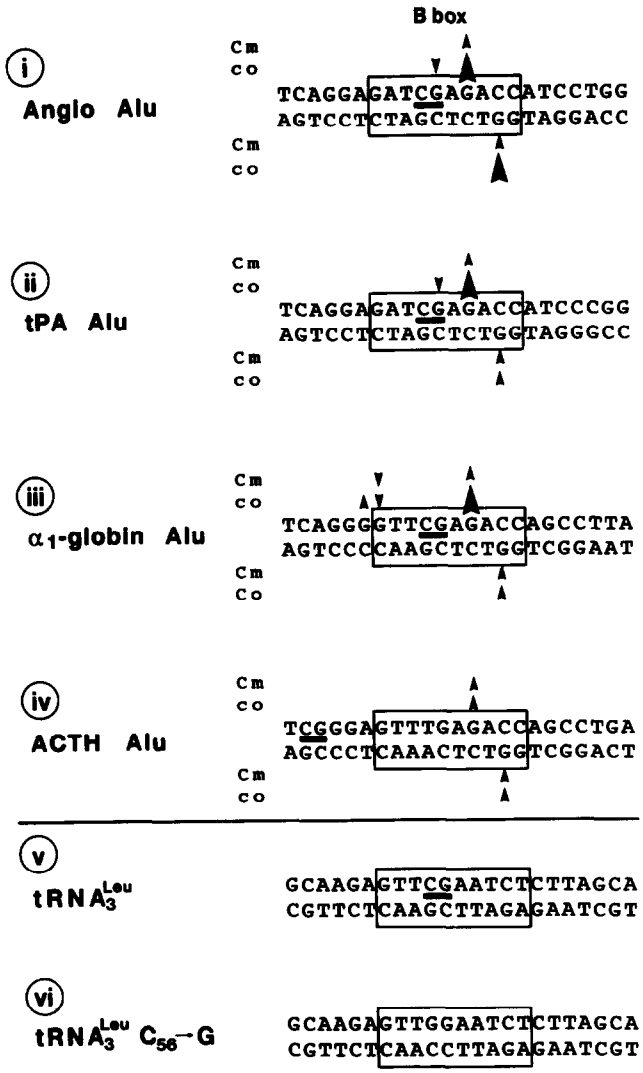

Fig. 4. Summary of the results of the DMS in vitro footprinting experiments on the four investigated Alu elements. The different B box regions are aligned. The bottom strand of the angiogenin (angio)-associated Alu sequence (Fig. 2a) is presented here as the top strand. Only in the boxed region is differential reactivity to DMS induced by 5′-CG-3′ methylation observed. Panels v and vi represent homologous sequences of a yeast tRNA B box and the mutated version [22] of this sequence (see section 4).

factor interacts with the B box of the VAI gene of Ad2 DNA [27], which has a high degree of homology to the B box in the Alu consensus sequence [5]. (iii) Our in vitro transcription and footprinting experiments have been carried out under very similar conditions.

Very similar findings on the inhibitory effect of in vitro 5′-CG-3′ methylation in several human Alu elements on their transcriptional activity in cell-free transcription experiments have recently been reported [6], however, only when low DNA concentrations have been used in these experiments. In experiments with the VAI region of Ad2 DNA we have made similar observations [19].

The results of experiments with methylated APO (apolipoprotein gene) and EPL [28] Alu elements have suggested that a repressor-like function, possibly related to MeCPI (methyl C binding protein I) [29,30] might be responsible for the inactivation of these Alu elements [6].

The findings presented in this report correlate transcriptional inhibition in an in vitro system and interference with protein binding in the B box regulatory region of at least three specific gene-associated Alu elements in human DNA due to 5′-CG-3′ methylation. In a fourth, the ACTH gene-associated Alu element, such inhibitory effects have not been observed, probably because of the absence of 5′-CG-3′ dinucleotides in its B box regulatory region. While the data suggest that 5′-CG-3′ methylation abrogates Alu element transcription in vitro by interfering with the binding of essential proteins, we are aware of the complexity of silencing mechanisms in most Alu elements in the human genome.

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