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Methylation Status of CpG Sites and Methyl-CpG Binding Proteins Are Involved in the Promoter Regulation of the Mouse Xist Gene

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The mouse Xist gene is expressed exclusively from the inactive X chromosome and is involved in the initiation of X inactivation. We previously reported that the −1157/+917 region of the Xist promoter was ubiquitously functional in mammalian cells and that experiments in a transient expression system revealed no *trans*-acting element responsible for the inactive X specific expression of Xist. In somatic tissues, the 5′ end of the silent Xist allele on the active X is known to be fully methylated whereas the expressed allele on the inactive X is unmethylated. In the present study we have used a bisulphite genomic sequencing method to evaluate DNA methylation at all cytosines including CpG dinucleotides within the Xist promoter. We report and confirm that methylation of specific sites plays a key role in Xist gene expression. In vitro DNA methylation of the 5′-region drastically reduced transcriptional activity in transiently transfected fibroblasts. Mobility shift assays showed that methylation does not inhibit Xist promoter activity by preventing the binding of transcription factors and that two distinct nuclear proteins bind in a sequence methyl-CpG-specific manner. Therefore, we suggest that Xist repression involves its promoter methylation and two distinct methylated DNA binding proteins.

Xist gene X inactivation Transcriptional activity Methylation Methyl-DNA binding proteins

X chromosome inactivation is the process by which one of the two mammalian X chromosomes in female somatic cells is transcriptionally silenced, resulting in dosage compensation for X-linked gene products between male and female (37). The inactive X chromosome assumes a compact, condensed structure and replicates asynchronously. Most of its genes are not transcribed into messenger RNAs. The active X chromosome has the open structure of functional chromatin and is transcribed normally. In female embryos, X inactivation first occurs in the extraembryonic lineage, where the paternal X chromosome is preferentially silenced (19,33,39,40,52,53,55). The imprint is erased in the embryonic lineage, at around the time of gastrulation, resulting in silencing of either the maternal or paternal X chromosome (41). Progress towards understanding X inactivation has been made through attempts to identify the X inactivation center (Xic), a *cis*-acting locus that regulates this process (48). Studies of rearranged and translocated X chromosomes in human and mouse allowed to narrow down the Xic region to 680–1200 kb within band Xq13 in humans (5,34) and to the distal part of band XD in the mouse (8,12,31,38,47). Xic seems to be involved in the first X inactivation steps, but not in the maintenance (11). Initiation involves a counting mechanism that determines how many X chromosomes and autosomes are contained in the cell, so that only one X is maintained active per two autosome sets. The Xic is thought to take part in counting, in choosing which X chromosome remains (or becomes) active, and in propagating the signal for spreading along the chromosome as demonstrated by X chromosome rearrangements.

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The Xist gene, which lies within the Xic region in human and mouse, has the unique property of being exclusively expressed from the inactive X chromosome (2,6,9). Xist is first transcribed from the paternal allele in the four-cell embryo, prior to X inactivation (29,30,42). This imprint, which seems associated with Xist methylation level, marks the paternal X chromosome for preferential inactivation in the trophectoderm and primary endoderm lineage in the blastocyst (16,29,42). Later, Xist becomes randomly expressed in the embryo prior to gastrulation (29,30).

Xist is transcribed only from the inactive X chromosome and its expression precedes both imprinted and random X chromosome inactivation. The large Xist transcript lacks protein coding potential and is in direct association with the inactive X chromosome in interphase nuclei (7,10,15). Overall, these lines of evidence support a role for Xist in the initiation of X inactivation. Generation of a targeted deletion of the Xist gene has confirmed its importance in effecting the onset of X inactivation, but left open the possibility that other genes might be involved in counting (44). A 450-kb region including the Xist locus was shown to provide the counting and choosing factors essential to X inactivation initiation (35) and, recently, Xist introduced onto an autosome was shown to carry out at least some aspects of chromosome counting and to produce gene inactivation in *cis* (24). Thus, the Xist gene seems to exhibit properties of the Xic.

We have recently characterized by in vitro CAT assays a minimal constitutive promoter in the −81/+1 region of the gene (45). A first site located at −30/−25 contains a TTAAAG sequence that binds a nuclear protein called NP1. Further analyses have identified a second region at −82/−41 containing a TCCTC sequence, binding a different nuclear protein called NP2 (unpublished, Pillet et al.). Neither of these transcription factors is responsible for the inactive X-specific expression characteristic of the Xist gene. The recent study of the human XIST gene promoter also supports its constitutive activity, as shown by transient transfection and analysis of the protein interactions with the minimal promoter (22). These results lead to the proposal that the XIST/Xist minimal promoter is constitutively active on all X chromosomes prior to inactivation. Its transcriptional silencing requires a repression factor acting on the single X chromosome in males and the active X in females. Although the exact nature of this repression agent is not yet known, a likely candidate mechanism is differential DNA methylation. Previous studies indicated that, in somatic tissues, the 5′ end of the silent Xist allele on the active X was fully methylated whereas the expressed allele on the inactive X was characterized by a complete lack of methylation (42). However, in female mouse embryonic stem cells (ES), Xist alleles were shown to be mosaically rather than differentially methylated in the 5′ region (44,49). Thus, the control of Xist regulation in the embryo proper is apparently not due to methylation. However, methylation plays a supportive role in maintenance of Xist silence, as it was recently demonstrated in male ES cells homozygous for a strong mutation in the DNA methyltransferase gene (1). Thus, DNA demethylation in these ES cells induces Xist expression during differentiation.

In this report, we describe experiments which support the conclusion that DNA methylation represses promoter activity, presumably through methyl-CpG binding proteins. To investigate the molecular mechanisms, a reporter gene construct containing the Xist promoter was methylated in vitro and transfected into murine fibroblasts. Methylation resulted in total repression of the Xist promoter. Mobility shift assays indicated that methylation did not inhibit the DNA binding of NP1 and NP2 transcription factors to the Xist promoter. In contrast, two distinct nuclear proteins were able to bind to the −53/−31 and −10/+26 regions in a sequence methyl-CpG-specific manner.

MATERIALS AND METHODS

Bisulphite Genomic Sequencing

Isolation of Xist Promoter Region. The bisulphite reaction was carried out on genomic DNA extracted from male and female mouse tails (129J/C57BL6 F1 mouse). Genomic DNA (10 μg) was digested with *Sty*I and *Ssp*I (Boehringer Mannheim) for 6 h at 37°C in a volume of 30 μl. The DNA fragment of interest, 646 bp long, corresponding to the Xist promoter region (−437 to +209), was selectively isolated using a specific biotinylated primer (XistP1R: 5′-biotin-GCCATAAGGCTTGGTGGTAGGGG-3′) and streptavidin-coated paramagnetic beads. Digested DNA was denatured for 10 min at 95°C, immediately cooled on ice for 2 min, and annealed with 1.5 pmol of biotinylated XistP1R during 40 s at 55°C. The reaction mixture was added to 35.7 μl (357 μg) of prewashed Dynabeads M-280 Streptavidin (Dynal) and incubated at room temperature for 20 min. The solid supports were subsequently washed once with 2× binding and washing buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 2 M NaCl. The immobilized product was recovered from the beads by adding 50 μl of 0.15 M NaOH and incubating for 10

min at 37°C. The supernatant was neutralized with 50 μl of 0.15 M HCl and 10 μl of 1 M Tris-HCl, pH 7.7.

Bisulphite Conversion Reaction. Recovered DNA was denatured by NaOH (final concentration: 0.3 M) for 15 min at 37°C. The denatured template was then treated with 5 M sodium bisulphite (Sigma), pH 5.0, and 10 mM hydroquinone (Fluka), both freshly prepared, to a final concentration of 3.1 M and 0.5 mM, respectively, and incubated at 53°C for 16 h. Modified DNA was desalted using the Wizard DNA clean-up system (Promega) according to the manufacturer's instructions, eluted in 50 μl H_2O, and added to 50 μl of 20 mM Tris, 0.2 mM EDTA, pH 8.0 buffer. Desulphonation step was performed by NaOH treatment (final concentration: 0.3 M) for 15 min at 37°C. The solution was neutralized by addition of NH_4OAc, pH 7.0, to 3 M and the DNA was ethanol precipitated, dried, and resuspended in 20 μl of TE 1×, pH 8.0.

PCR Amplification. Amplifications were performed in 50 μl reaction mixtures containing 1 μl of bisulphite-treated DNA, 200 μM dNTPs, 1 μM primers, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 μl (2.5 units) AmpliTaq DNA polymerase (Perkin Elmer) in a Perkin Elmer Cetus 9600 thermal cycler. PCR conditions were as follows: 94°C/5 min × 1 cycle; 94°C/1 min, 47°C/90 s, 72°C/90 s, × 33 cycles; 72°C/10 min × 1 cycle. Specific primers were used to amplify bisulphite fully converted DNA: forward primer XS-437MF: 5′-TAAGGAGTTATTTT-GTGAGGTATTT-3′; reverse primer XS+77MR: 5′-AACTTAATAATAAAAAAACT-3′. A second round of amplification, with nested forward primer XS-392MF: 5′-TTGAGGTTGTTAATTAATGTAGAAG-3′ and reverse primer XS+77MR was performed under the following conditions: 94°C/5 min × 1 cycle; 94°C/40 s, 47°C/1 min, 72°C/1 min, × 33 cycles; 72°C/10 min × 1 cycle.

Cloning and Sequencing. Amplified DNA was ligated into pGEM-T vector (Promega) and transformed into competent *E. coli* (XL1-Blue). Cloned DNA was sequenced using an Automated Laser Fluorescent ALF DNA sequencer and the AutoRead sequencing kit (Pharmacia Biotech).

Cell Culture

The BALB/3T3 and BLK/CL.4 cell lines were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a 5% CO_2 atmosphere.

For treatment of the BLK/CL.4 cell line with 5-azacytidine, cells were cultured in DMEM containing 10 or 16 μM 5-azacytidine (Sigma) and total RNA was isolated after 48 or 72 h of culture.

RNA Isolation and RT-PCR

RNA was prepared from cultured cells by the guanidium isothiocyanate/phenol/chloroform method as described by Chomczynski and Sacchi (13). Approximately 0.8 μg of RNA was transcribed in a total volume of 20 μl containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 1 mM dNTP, 20 units of RNasin, 50 units of MuLV reverse transcriptase, and 2.5 μM of random hexamers. The reaction was incubated 10 min at room temperature, 15 min at 42°C, 5 min at 99°C, and 5 min at 5°C. First strand synthesis (5 μl) was amplified by PCR in a total volume of 25 μl. PCR reaction contained 50 mM KCl, 10 mM Tris at pH 8.3, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 25 pmol of each primer, 1 μCi of [α-^{32}P]dCTP, and 1.5 units of Taq polymerase. The amplification consisted of a denaturation step at 94°C for 5 min, followed by 30 cycles of PCR amplification at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a 10-min extension at 72°C. Products were analyzed by electrophoresis on an 8% polyacrylamide gel.

Transcription Reporter Constructs and Transfection Studies

The pCAT/4844B and the promoter deletional mutants were previously described (45). The pCAT(B)/4844B and its derivatives were constructed by introducing an A + T-rich sequence and the SV40 enhancer in front of the Xist promoter, which was linked to the CAT reporter gene. BALB/3T3 and BLK/CL.4 cells ($1–2 \times 10^5$) were transiently transfected by liposome-mediated DNA transfection (DOTAP, Boehringer Mannheim) using 5 μg of the construct. Relative transfection efficiency was determined by cotransfection with a pSV/β-galacosidase reporter gene (pSV/β-gal). After transfection, cells were incubated for 48 h, harvested, and then lysed in a lysis buffer (Promega). Extracts were heated for 10 min at 65°C to abolish endogenous deacetylating activity. After removal of cellular debris, protein concentrations were determined using the BCA protein assay (Pierce). CAT assays were carried out using 30–100 μg of cell extracts and butyrylated chloramphenicol was separated on a thin-layer chromatography plate. The results were normal-

ized to the value of galactosidase activity measured from the cotransfected pSV/β-gal and adjusted to the protein concentration of the cell extracts. The CAT experiments were carried out 3 to 10 times depending on the constructs and a mean activity was reported.

DNA Methylation

Plasmid DNA and synthetic oligonucleotides were methylated using the bacterial CpG methylase *Sss*I according to the supplier's instructions (New England Biolabs). Reaction products were phenol extracted and ethanol precipitated. The completeness of methylation was tested by digesting an aliquot with the methylation-sensitive *Hpa*II or *Sac*II and methylation-insensitive *Msp*I restriction enzyme followed by agarose and polyacrylamide gel electrophoresis of plasmid DNA and oligonucleotides, respectively.

Preparation of Nuclear Extracts

BALB/3T3 and BLK/CL.4 cells from 10-cm dishes were used for each preparation. Cell nuclear extracts were prepared as described by Dent (17). Briefly, cells were harvested by scraping, washed in cold phosphate-buffered saline, and incubated in 5 packed cell volume of buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF)] for 10 min at 4°C. Cells were collected by microcentrifugation, resuspended in 3 volumes buffer A, and homogenized after addition of Nonidet P-40 (NP40) to 0.05%. The crude nuclei released were collected by microcentrifugation and resuspended in 1 ml of buffer C [5 mM HEPES (pH 7.9), 26% glycerol (v/v), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF]. NaCl was added to a final concentration of 300 mM. Nuclei were incubated at 4°C for 30 min and clarified by microcentrifugation for 20 min. Nuclear extracts were frozen on dry ice and stored at −70°C.

DNA Mobility Shift Assay

Double-stranded oligonucleotide probes and competitors were prepared by annealing complementary single-stranded oligonucleotides in a heating block. The probes were gel purified and end-labeled with [α-^{32}P]dCTP and the Klenow fragment. Typical mixtures (20 μl) for in vitro binding reactions contained 1 μg of poly(dI-dC), 1× binding buffer [20 mM HEPES (pH 7.8), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, and 0.5 mM β-mercaptoethanol], 2–5 μg of nuclear extract proteins, and 2×10^{-2} pmol of the ^{32}P-labeled probe. Reaction mixtures were incubated for 20 min on ice and analyzed by electrophoresis on a 4–6% native polyacrylamide gel at 4°C and 200 V for 2–3 h in 1× Tris-acetate/EDTA buffer (TAE). Competition reactions were performed by adding 5–20 pmol of competitor oligonucleotides to the binding reaction mixture prior to the addition of labeled probe. Gels were then dried under vacuum at 80°C for 30 min and exposed to X-OMAT AR films.

RESULTS

Methylation Analysis of the Xist Promoter by Bisulphite-Mediated Genomic Sequencing

Norris et al. showed that the 5′ end of the silent Xist allele on the active X is fully methylated in somatic tissues whereas the expressed allele on the inactive X is characterized by a complete lack of methylation (42). The technical limitations of this Southern blotting-based approaches restricted the analysis to those few DNA sequences that were accessible to methylation-sensitive restriction enzymes. Therefore, only 6 CpGs out of 20 could be assessed (Fig. 1A). Bisulphite genomic sequencing allows to determine methylation status of each cytosine residue in a defined sequence for each individual strand of genomic DNA (14,20). This technique relies on the ability of bisulphite to specifically convert unmethylated cytosine residues, but not 5-methylcytosine, to uracil by deamination. Bisulphite-modified DNA is then PCR amplified to yield products in which uracil residues have been amplified as thymine and 5-methylcytosine residues as cytosine. The amplified fragments are then cloned and sequenced, and the methylation status of all CpG is established by comparison to the wild-type sequence. The methylation status of the Xist promoter was determined by bisulphite genomic sequencing from male and female mouse tails genomic DNA. Sequencing of 14 clones from male mice indicates that statistically all cytosines within CpG dinucleotides located from −357 to + 52 are methylated on the single silent Xist allele (Fig. 1A). Figure 1B highlights the sequence pattern of the −60 to +20 region. In contrast, analysis of female genomic DNA demonstrated that, in addition to the methylated sites on the silent Xist (50% of the sequenced clones), there is a fully unmethylated allele corresponding to the expressed Xist (50% of the sequenced clones).

Treatment of BLK/CL.4 Cells With 5-Azacytidine Results in Transcriptional Activation of the Xist Gene

We previously demonstrated that the Xist promoter region −1157/+917 and the different *trans*-act-

A

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                 14 14   14
-357 GTCCAAGACG CGGAGCGATA CATGGTTTGT CCAAGTAGAA GATATATTGA AATTTTGCAT -298

                                                                  13
-297 AGACAGGTGT GTGACCTAAT GTACATTATT TAATGTTTAT GTGGAAGTTC TACATAAACG -238

                                      14 14             14 14
-237 TTTTTAGCTG TAAAATAGGA TAATCCTTCA TTATCGCGCA AAATAATGAA ATTCACGCGT -178

                        14
-177 CATGTCACTG AGCTTACGTA CCTCCATCTT TATTCATTTT AATTTTTTTA TAATATAGTT -118

-117 AGACCTAAAG GTCCAATAAG ATGTCAGAAT TGCAATCTTT GTGGCCACTC CTCTTCTGGT -58

           10         14 14 14             14       13 4
-57  CTCTCCGCCT TCAGCGCCGC GGATCAGTTA AAGGCGTGCA ACGGCTTGCT CCAGCCATGT +3

           10       11          9      14
+4   TTGCTCGTTT CCCGTGGATG TGCGGTTCTT CCGTGGTTTC TCTCCATCTA +52
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B

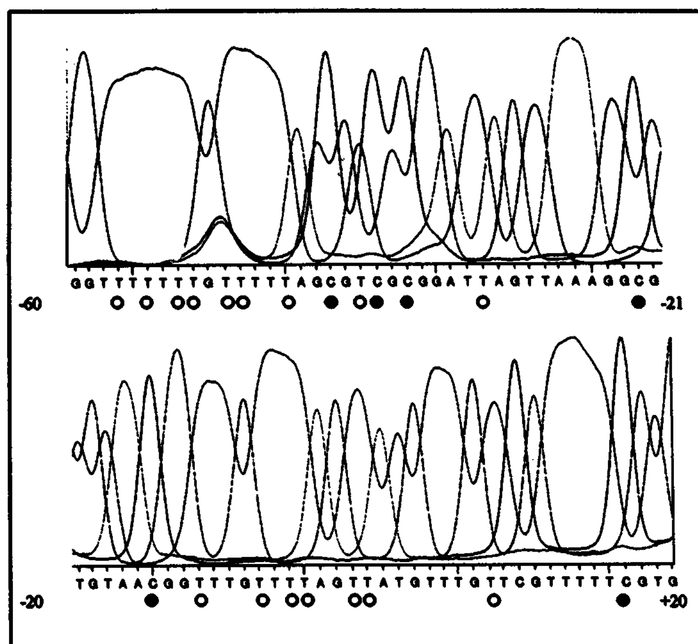

FIG. 1. Methylation status of the Xist promoter studied by bisulphite genomic sequencing. (A) CpG methylation in the –357 to +52 Xist region. Cytosines of underlined CpG remain unmodified after bisulphite reaction in most of the 14 analyzed clones. Number of clones in which corresponding cytosine is not modified by the treatment is indicated above the sequence. The arrow indicates the transcription initiation site and the black circles show the CpG sites accessible to methyl-sensitive restriction enzymes. (B) Genomic sequence profile of a cloned PCR product following sodium bisulphite conversion of Xist promoter (–60 to +20). Methylated CpG cytosines retained as cytosines are indicated by closed circles and unmethylated cytosines converted to thymines are shown by open circles.

ing factors were not responsible for the inactive X-specific expression of Xist, which led to the proposal that methylation is the Xist transcriptional repressor. To investigate the putative repressor function of Xist promoter methylation, the XY murine BLK/CL.4 cell line was treated with 5-azacytidine. Increasing concentrations of this DNA methylation inhibitor (10 and 16 μM) were added to the culture for 48 or 72 h and the presence of Xist transcript was tested by RT-PCR. 5-Azacytidine-treated BLK/CL.4 cells transcribed Xist, whereas no transcript was detected in untreated cells (Fig. 2). Transcription analysis of the housekeeping insulin receptor (Insr) gene served as a positive control. These findings suggest that Xist promoter methylation does play a role, either directly or indirectly, in the transcriptional repression of this gene in BLK/CL.4 cell line.

Methylation of the Promoter Construct Inhibits CAT Activity

Because DNA seems implicated as a modulator of Xist expression, we tested the effect of in vitro methylation of Xist promoter on transcription by transient expression assays. We generated the pCAT(B)/4844B construct, which contains the Xist promoter region from −1157 to +917 driving expression of the CAT gene. This sequence induced efficient transcription when transiently transfected into BALB/3T3 murine cells (Fig. 3). In vitro methylation of pCAT(B)/4844B by *Sss*I methylase, an enzyme that adds a methyl group to the 5′ position of all cytosine residues within CpG dinucleotides, abolished all CAT activity (Fig. 3). By contrast, methylation of the control plasmid pCAT(B)/promoter resulted in only a 10% reduction in CAT activity. This control contained the CAT gene and the simian virus 40 (SV40) promoter, which was previously described to be insensitive to methylation (21). As positive control, the plasmid pCAT(B)/PGK was treated with the *Sss*I methylase, what repressed all transcriptional activity. In fact, PGK-1 promoter is known to be inhibited by methylation. These results showed that Xist promoter was particularly sensitive to CpG methylation and that methylation of the reporter gene itself had little or no effect. The transfection efficiency was monitored by cotransfecting tested constructs with pSV/β-Gal, which contains the β-galactosidase gene linked to the strong early SV40 promoter. To insulate the promoters from adjacent CpG sites in the plasmid sequence, an A + T-rich region was cloned in front of the promoters.

This repression could depend upon methylation of specific CpG sites of the Xist promoter or, alternatively, upon the extent or the density of methylation. To distinguish among these possibilities, deletions in the −1157 to +917 were established and analyzed as before. Methylation of the pCAT (B)/−132/+20 construct containing as few as eight CpGs within the −132/+20 region still abolished CAT activity, suggesting that the extent of repression did not depend on the density of the methylation (Fig. 3).

CpG Methylation Has No Effect on the Binding of NP1 and NP2, But Induces Interaction of a New Methyl-CpG Binding Protein

NP1 and NP2 are two ubiquitous transcription factors previously shown by electrophoretic mobility shift assays (EMSA) to bind to the −182/+20 region

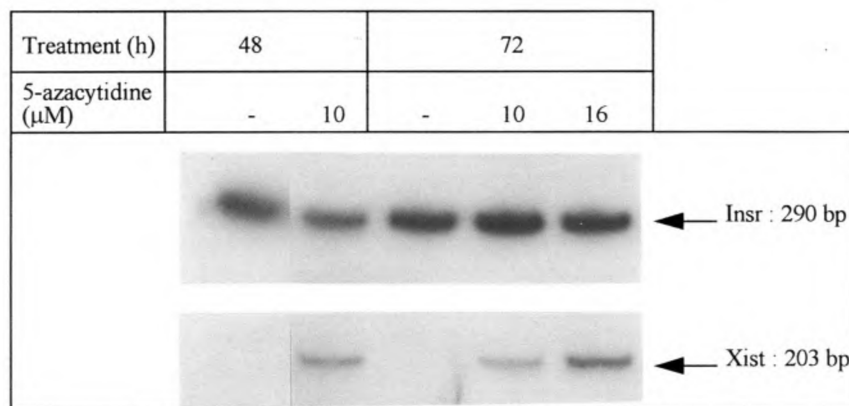

FIG. 2. Reverse transcriptase PCR demonstrates transcriptional activation of the Xist gene after treatment with 5-azacytidine. The BLK/CL.4 cells were treated with increasing concentrations of 5-azacytidine. Forty-eight or 72 h after treatment, RNA was extracted and RT-PCR amplification was performed with primers designed to amplify the Xist transcript (Xist-F: GGGACCTAACTGTTGGCTTTATCAG and Xist-R: GAAGTGAATTGAAGTTTTGGTCTAG), with or without reverse transcriptase to exclude DNA contamination. The products of RT-PCR were electrophoresed on a 8% polyacrylamide gel. The transcription analysis of Insr, a constitutively active gene, serves as a positive control (Insr-F: ACCTGCTTCTCTTCCGTGTCTATGG and Insr-R: CCCACATCCCTCGTTGTCATCTTTG).

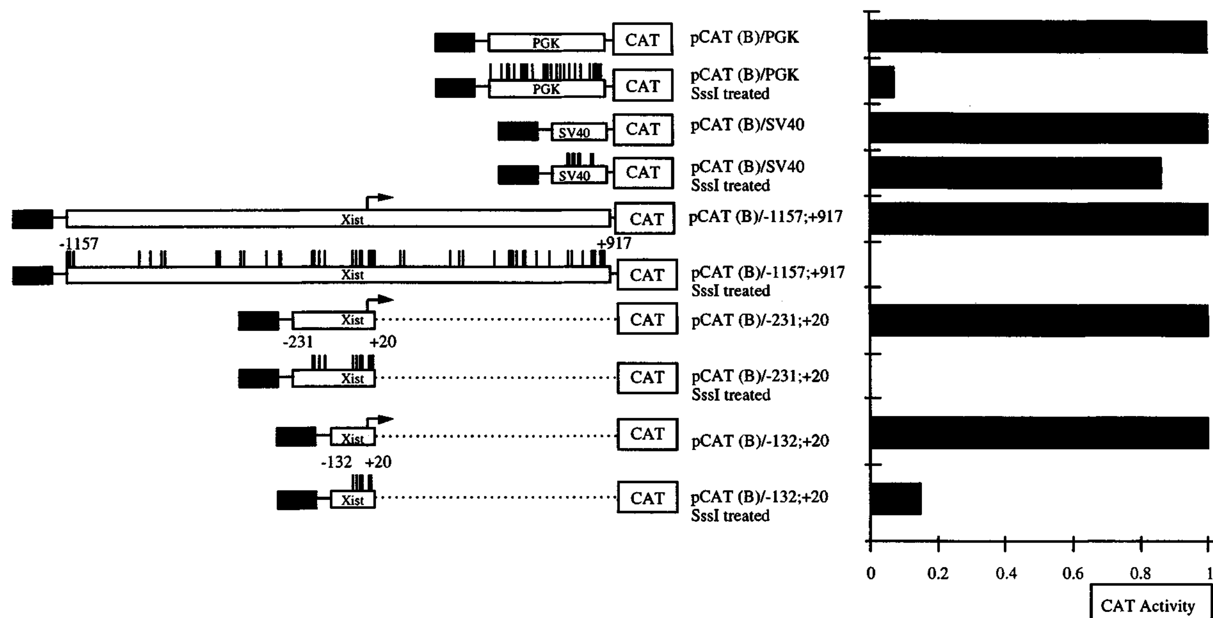

FIG. 3. Xist promoter activity is inhibited by DNA methylation and the extent of this repression is independent on the density of methyl-CpG. BALB/3T3 cells were transfected with either unmethylated or *Sss*I-methylated constructs using DOTAP. Relative CAT activities compared with that of each unmethylated construct are presented. Three different Xist promoter regions were analyzed and compared with both PGK-1 and SV40 promoters, which had been previously shown to be sensitive and insensitive, respectively, to methylation. The arrow indicates the main cap site (+1) and the vertical bars indicate the methylated CpG sites. All the plasmids have in common the CAT sequence and are flanked by an A + T-rich sequence together with the SV40 enhancer (black box).

of the Xist gene (45). The impact of methylation on both NP1 and NP2 binding activities was investigated by EMSA using methylated and nonmethylated oligonucleotides. The synthesized GMSA1B (−53 to −18) and GMSA7B (−87 to −31) oligonucleotides retain the NP1 and NP2 binding elements, respectively. Both oligonucleotides were methylated with *Sss*I methylase. Incubation of radiolabeled and methylated GMSA1B with nuclear extract prepared from BALB/3T3 (Xist active) and BLK/CL.4 (Xist inactive) resulted in the formation of an NP1–methylated DNA complex, suggesting that NP1 binding is not sensitive to CpG methylation (data not shown). In addition, a new complex was observed with the methylated probe. The specificity of this complex was identified by competition reactions with unlabeled homologous methylated and nonmethylated GMSA1B and heterologous methylated and nonmethylated oligonucleotides. EMSA with labeled methylated GMSA7B also indicated that NP2 binding to the TCCTC element was not inhibited by methylation (data not shown). Here again a new complex was formed. Competition reactions and migration patterns suggested that the same methyl-CpG binding protein was interacting with methylated GMSA7B. These data indicate that this nuclear protein, named Met1, is able to bind in a sequence methyl-CpG-specific manner to GMSA1B (−53/−18) and GMSA7B (−87/−31). These two oligonucleotides overlap between positions −53 and −31 where four CpGs are located (CCGCCTTCAGCGCCGCGGATCAG).

To check that the −53/−31 sequence, located between the NP1 and NP2 binding sites, is indeed the Met1-specific recognition sequence, the GMSA17 (−57/−33) oligonucleotide was synthesized. Incubation of labeled and methylated GMSA17 with nuclear extract from female cells resulted in the formation of a Met1–methylated DNA complex. The specificity of this complex was verified by competition reactions with unlabeled methylated and unmethylated oligonucleotides (Fig. 4). The competition reaction with the GMSA1K oligonucleotide (−53/−12), whose CpGs were mutated in TpG, showed that methylated cytosines are necessary to Met1 binding (Fig. 4). However, these mutations do not alter NP1 fixation nor constitute a new DNA–protein interaction site.

Met1 Binding Does Not Seem to be the Only Event Necessary to Promote Xist Repression Through Methylation

To investigate the Met1 functional importance in Xist transcriptional repression through methylation, the CpG mutations previously shown to abolish

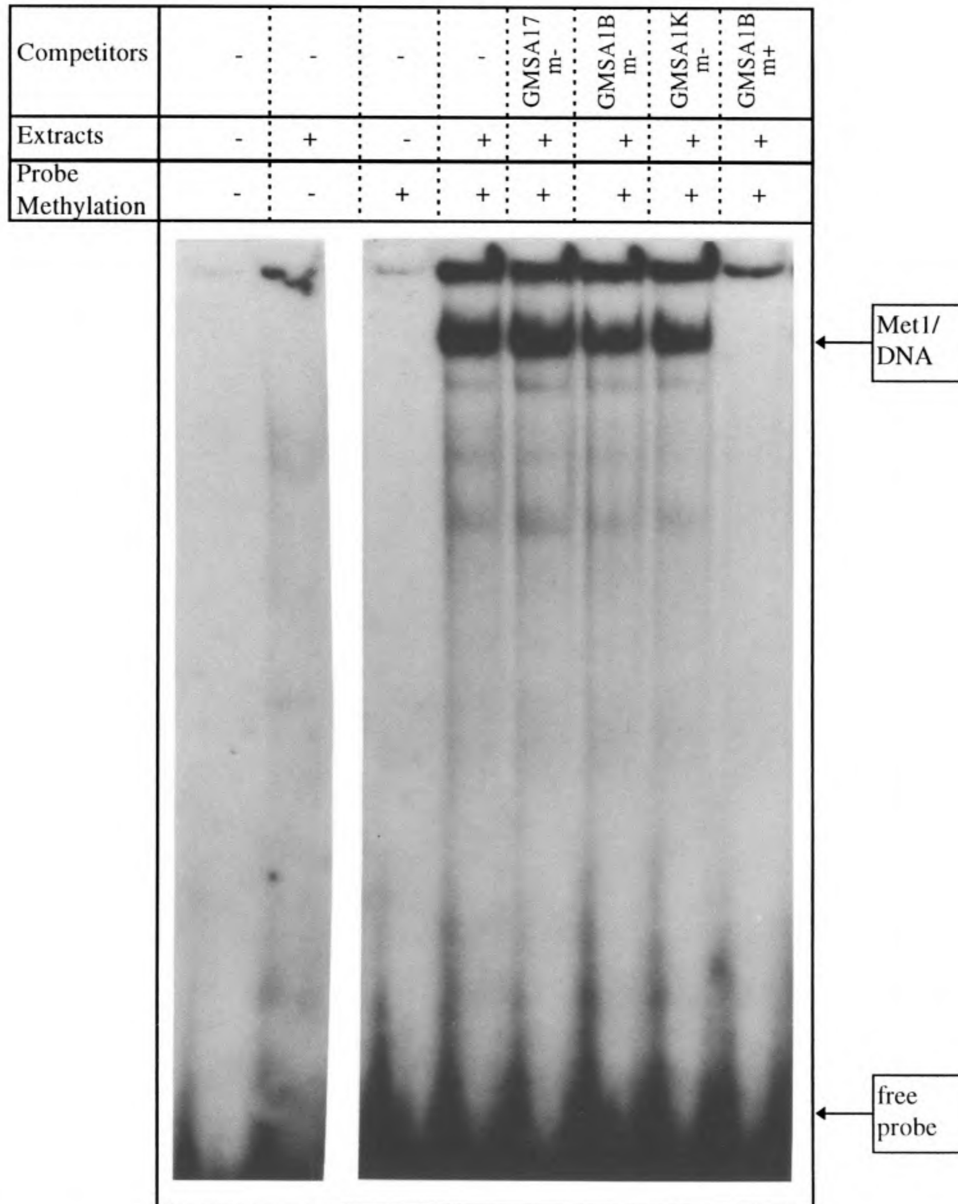

FIG. 4. DNA binding of Met1, a methylated DNA binding protein, to the –57/–33 region. Labeled unmethylated and methylated GMSA17 oligonucleotides (–57/–33: CTCTCCGCCTTCAGCGCCGCGGATC) were used for EMSA without or with BALB/3T3 nuclear extracts. The reaction mixture for competition experiments contained unlabeled nonmethylated GMSA17 (m–), nonmethylated GMSA1B (m–), mutant GMSA1K, and methylated GMSA1B (m+).

DNA–Met1 interaction were introduced into the –132 to +20 promoter fragment by PCR. The mutated region was cloned in front of the CAT reporter gene and female cells were transfected with the resulting pCAT(B)/–132;+20 (MT1) construct containing mutated CpG sites at position –52; –43; –40 and –38 all at once. CAT assays revealed that the methylation of the remaining CpG sites in the pCAT(B)/–132;+20 (MT1) construct abolished all promoter activity (Fig. 5), despite the inability of Met1 to bind to the mutated –53/–31 element. These data suggest that methylation of the CpG sites located at –23; –16; +9 and +16 positions are sufficient to repress Xist, possibly involving Met1 or another methyl-CpG–binding protein interaction.

A Second Methylation-Dependent DNA Binding Activity Recognizes the –10 to +26 Xist Promoter Region

To investigate whether the –23; –16; +9 and +16 CpGs interact with Met1 or another methyl-CpG

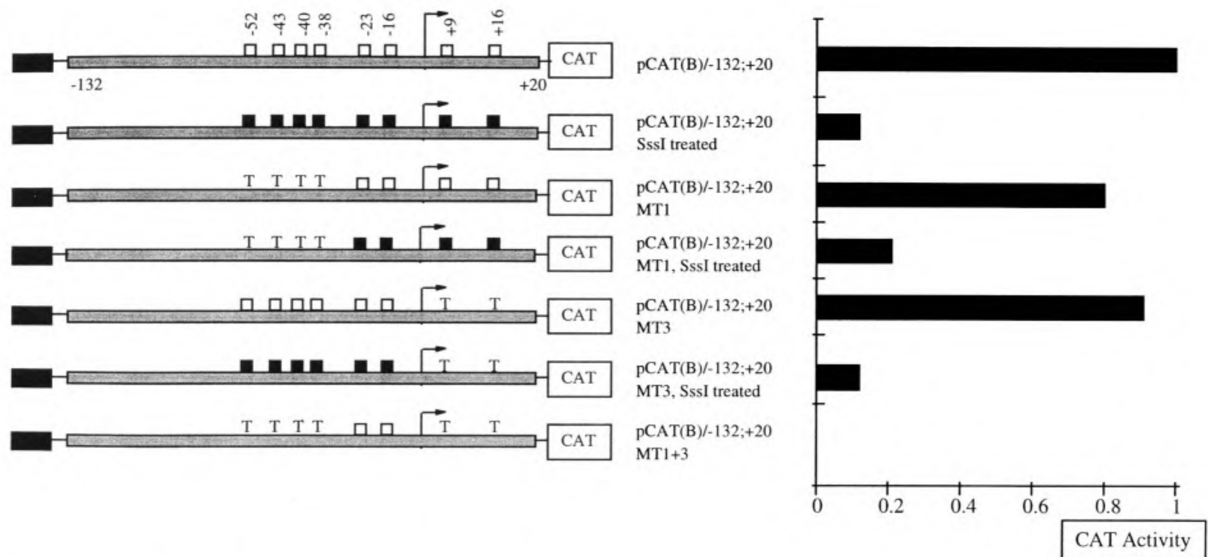

FIG. 5. Mutation of the −52, −43, −40, −38, −23, −16 CpG sites into TpG were introduced into the −132 to +20 CAT construct and promoter activity was tested by CAT assay in female cell line after methylation. The white squares indicate the unmethylated CpG sites, the black squares are the methylated CpG and the T letters correspond to the mutated CpG sites into TpGs.

binding protein, the GMSA18 (−27 to −3) and GMSA19 (−10 to +26) were synthesized. Incubation of labeled and methylated GMSA19 with nuclear extract resulted in the formation of a new protein–methylated DNA complex (Fig. 6). The specificity of this complex was verified by competition reactions with unlabeled homologous methylated and nonmethylated GMSA19 and heterologous methylated and nonmethylated oligonucleotides. Absence of competition with unlabeled methylated GMSA17 and migration patterns suggested that the −10/+26 element interacts with another protein, called Met2, different from Met1 (Fig. 6). EMSA with labeled methylated GMSA18 did not show any protein–methylated DNA complex formation (data not shown).

To determine the Met2 functional importance in the Xist regulation, the cytosines of both CpG sites in the −10 to +26 region were mutated to thymine in the pCAT(B)/−132;+20 construct. CAT assays after transient transfection of female cells with the pCAT(B)/−132;+20 (MT3) revealed that the remaining methylated CpG sites in the construct allows Met1 interaction on the Xist promoter so that the transcriptional activity was completely abolished (Fig. 5), despite the inability of Met2 to bind to the mutated −10/+26 element. By introducing mutations at the −52, −43, −40, −38, +9, and +16 CpG sites in the pCAT(B)/−132;+20 construct, we should be able to prevent Met1 and Met2 interaction on the Xist promoter. Unfortunately, the resulting pCAT(B)/−132;+20 (MT1+3) construct did not show any CAT activity even before methylation (Fig. 5).

DISCUSSION

The lack of *cis* element in the −1157/+917 promoter region responsible for the inactive X specific expression of Xist and the absence of sex-specific *trans*-acting factors suggest that female-specific Xist expression relies on other mechanisms. Several studies have suggested that DNA methylation is involved in the regulation of the Xist gene. In somatic tissues, the expressed Xist allele on the inactive X chromosome was found completely unmethylated at all studied CpG sites, whereas the silent Xist allele was methylated (42). In addition, null mutation of the DNA methyltransferase gene, which induces Xist demethylation in mutant male ES cells, leads to Xist expression after differentiation (1). In sperm and eggs, three CpG sites were differentially methylated in the promoter region (16,29,42,56). This suggests that the gametic methylation imprint marks the paternal X chromosome for preferential inactivation in the trophectoderm and primary endoderm lineage, which express only the paternal hypomethylated Xist allele. However, recent studies of Xist methylation in undifferentiated female ES cells suggested that the methylation pattern in the 5′ region of the Xist is not stable (49), favoring mosaic rather than differential methylation. Therefore, all these data led to the proposal that methylation, if not responsible for the primary Xist regulation in the early embryo, should be involved in Xist repression in differentiated cells, maybe playing a supportive role in maintenance of Xist silence.

In vertebrates, DNA methylation at CpG dinucleo-

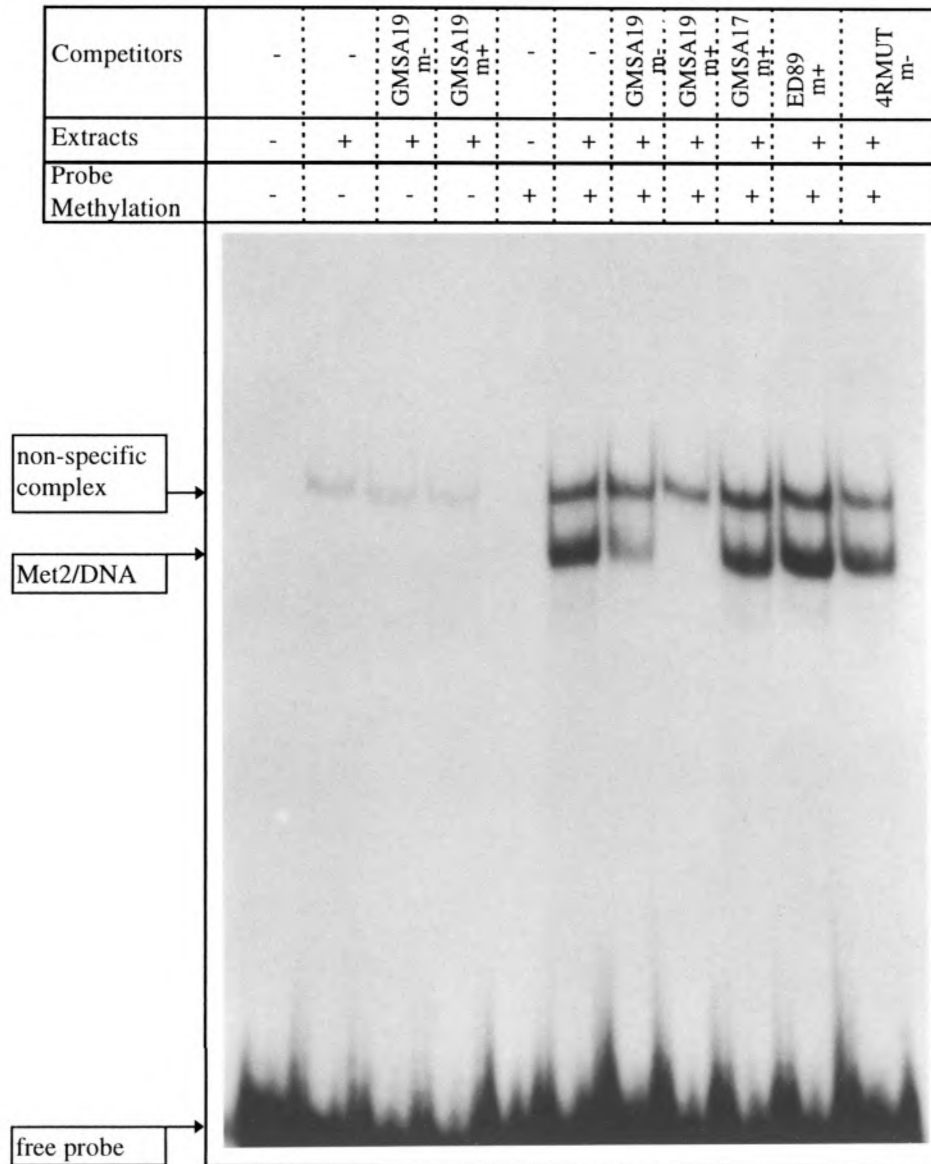

FIG. 6. DNA binding of Met2, a second methylated DNA binding protein distinct of Met1. Labeled unmethylated and methylated GMSA19 oligonucleotide (GCTCCAGCCATGTTTGCTCGTTTCCCGTGGATGTGC) was used for EMSA without or with BALB/3T3 nuclear extracts. The reaction mixture for competition experiments contained unlabeled nonmethylated GMSA19 (m–), methylated GMSA19 (m+), methylated GMSA17 (m+), heterologous methylated competitor ED89 (m+), and heterologous nonmethylated competitor 4RMUT (m–). A nonspecific protein–DNA complex migrates more slowly than the Met2–DNA complex.

tides is involved in fundamental biological processes such as tissue-specific regulation of gene expression, genomic imprinting, and X chromosome inactivation. The precise role of DNA methylation in the regulation of eukaryotic genes is unknown. Numerous studies indicate that there is an inverse correlation between the extent of DNA methylation and the level of gene expression: hypermethylation of promoter regions may lead to gene inactivation whereas hypomethylation of the same regions is associated with transcriptional activity. The molecular mechanisms by which DNA methylation causes this transcriptional silencing are only partly understood. First, CpG methylation may directly block the binding of *trans*-acting factors. Various sequence-specific DNA binding proteins inhibited by DNA methylation have been described (26). However, other transcription factors, such as Sp1, were found to be insensitive to DNA methylation (25). In addition, many transcription factors do not contain CpG dinucleotides within their binding sites and CpG methylation is thus unlikely to have a direct effect on their DNA binding

capacity. Second, CpG methylation may act indirectly, either by affecting the conformation of chromatin or by interacting with methyl-CpG-specific proteins. Several lines of evidence support the assumption that chromatin structure is involved in mediating the inhibitory effect of DNA methylation on transcriptional activity. Transfected methylated DNA preferentially assumes a closed, transcriptionally inactive chromatin structure (32). Furthermore, histone H1 has been shown to bind preferentially to CpG-methylated DNA and to repress in vitro transcription from CpG-methylated templates (36). A number of DNA binding proteins that bind specifically to CpG-methylated DNA have been identified to date. Human placental MDBP1 (methyl-DNA binding protein 1) binds to DNA in a sequence-specific manner dependent on the density of methyl-CpG (51). MECP-1 (methyl-CpG-binding protein 1) binds to DNA that has at least 15 methyl-CpGs in a sequence-independent way and inhibits various methylated promoters. MECP-2 only requires a single methyl-CpG for binding and inhibits transcription of both methylated and unmethylated DNA templates (3,4,23,46,54). Another protein, MEDBP-2, a member of the histone H1 family, represses the avian vitellogenin gene (27,28,43). Two mouse nuclear proteins have been described that bind selectively to a methylated c-Myc binding sequence (50) and a sequence-independent methyl-CpG binding protein (DBPm) has also been described for plants (18). The precise function of most of these proteins remains to be elucidated. However, several studies suggest that binding of these proteins may sterically block the binding of transcription factors and/or bring about changes in chromatin structure.

We observed that treatment of the BLK/CL.4 cell line with 5-azacytidine resulted in transcriptional activation of the Xist gene. This finding suggests that DNA methylation does inhibit Xist expression in the BLK/CL.4 cell line, either directly or indirectly. Using a transient expression system, we showed that in vitro DNA methylation of the 5′ Xist region drastically reduced transcriptional activity, suggesting that specific repression of the Xist gene is achieved by DNA methylation. Experiments with sequential deletions of the promoter showed that methylation of only eight CpG sites within the −132/+20 region is still sufficient to reduce transcriptional activity, indicating that the extent of repression was not dependent on the density of methylation. It is probable that CpG methylation blocks the binding or interaction of transcription factors, by changing the conformation of chromatin and/or facilitating the binding of methyl-CpG-specific repressor proteins.

We have investigated the effects of CpG methylation on the binding of NP1 and NP2 proteins to the Xist promoter. EMSA showed that none of the factors interacting with the promoter is directly inhibited from binding to its recognition site when the sequence is methylated. However, a new nuclear protein was able to bind to the −53/−31 region in a sequence methyl-CpG-specific manner. To elucidate the functional role of Met1 in the transcriptional repression of the Xist promoter, we mutated all the CpG sites located between −53 and −31. These mutations partly blocked methylation of the −53 to +16 region and prevented the binding of Met1 protein, but still failed to protect the Xist promoter from the effect of CpG methylation at other nonmutated positions. These data suggest that methylation of the CpG sites located at −23, −16, + 9, +16 positions are sufficient to repress Xist, possibly involving Met1 or another methyl-CpG–binding protein interaction. Using an oligonucleotide encompassing the −10 to +26 Xist region (GMSA19), we have identified a second nuclear protein, called Met2, which binds to the methylated DNA in a sequence-specific manner. Even though the functional role of Met1 and Met2 could not be determined, as mutations of the −52, −43, −40, −38, +9, and +16 sites abolished all CAT activity before any methylation, our data highly suggest a repression action of Met1 and Met2 on the transcriptional activity of the Xist gene. Met1 and Met2 do not share any common feature with previously described methyl-CpG binding proteins such as MECP-1 or MECP-2 (4,23,46,54). As previously described, MECP-1 (120 kDa) and MECP-2 (81 kDa) bind to methylated DNA in a sequence-independent manner, which is not the case for Met1 and Met2. In addition, both Met1 and Met2, whose approximate molecular weight are, respectively, 75 and more than 120 kDa, interact with DNA containing few methyl-CpG, whereas MECP-1 binding needs at least 15 methylated CpG.

Our results indicate that methylation and two distinct methyl-CpG DNA binding proteins play a role in the regulation of Xist transcription. How could Met1 and Met2 maintain Xist silencing without preventing the binding of *trans*-acting factor NP1 and NP2 on the minimal promoter? A possible explanation could be that Met1 and Met2 inhibit activation by interfering with the critical binding of a transcriptional activator that was not detected with our experimental conditions. Moreover, the loss of promoter activity after the mutation of the −52, −43, −40, −38, +9, and +16 CpG sites suggests a critical role for this sequence in Xist transcription, possibly due to a transcription factor binding at this site. Another explanation could be that the binding of the negative elements Met1 and Met2 close to NP1 and NP2 sites blocks the transmission of NP1 and NP2 activation

signals to the transcriptional initiation complex. We propose to isolate both Met1 and Met2 to study their role on Xist promoter and on imprinting, as the methyl-CpGs necessary to their binding have been shown to be differentially methylated in gametes (56).

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