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IN VITRO DNA METHYLATION INHIBITS FMR-1 PROMOTER

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SUMMARY: In fragile X syndrome, the FMR-1 gene is changed by a CGG repeat mutation and an abnormal methylation at a CpG-island 5′ to the gene. To elicit if methylation itself inactivates the gene, FMR-1 promoter was defined by deletion mapping and primer extension assay and was analyzed by *in vitro* methylation. Promoter activity was measured by transient expression and chloramphenicol acetyl transferase assay. Although this promoter contains several HpaII sites, it was not affected by methylation with HpaII methylase. However, the promoter was completely repressed by methylation with M. SssI which methylates all cytosines of CpG dinucleotides. This repression could not be overridden by SV-40 enhancer. This study indicates that methylation could be the direct cause of FMR-1 inactivation in fragile X syndrome.

Fragile X syndrome, one of the most important hereditary form of mental retardation, is characterized by the chromosome Xq27.3 fragile site and a peculiar form of transmission [1]. FMR-1 gene at the fragile X locus contains two differences in patients [2]. The first is an expansion of CGG repeat within an FMR-1 exon [3]. The length of expansion ranges from approximate 200bp in transmitting males (premutation) to several kb in penetrant males (full mutation). The risk of expansion during oogenesis increases with the number of repeat, thus accounts for the Sherman paradox that the risk of mental impairment in fragile X pedigree is contingent upon position of individual in the pedigree. The second change in fragile X syndrome is an abnormal methylation at a 5′ CpG-island which is neither methylated in normal males nor in active X chromosomes of normal females [4]. This methylation is correlated with the loss of expression of FMR-1 mRNA. It remains unclear if this abnormal methylation occurs as a consequence of the expansion of CGG repeat, or if this methylation is responsible for the transcriptional inactivation of the FMR-1 gene. It was observed that a mutated FMR-1 gene in chorionic villi, where the gene is hypomethylated, is transcriptionally active [5]. So the CGG repeat mutation itself may not

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Abbreviation: CAT, chloramphenicol acetyl transferase.

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totally prevent the transcription of FMR-1 gene. This paper defines the FMR-1 promoter and shows that *in vitro* DNA methylation completely inhibits FMR-1 promoter. The role of methylation in molecular basis of fragile X syndrome is discussed.

MATERIALS AND METHODS

Plasmids. pBlue-FX and pCAT-FX were constructed by inserting a 466bp PstI/XhoI fragment of pE5.1, which contains the CpG-island and CGG repeat of FMR-1 gene [3], into pBluescript II and pCAT-Basic. All pCAT plasmids are obtained from Promega, USA. pCAT-Basic and pCAT-Enhancer contain the promoterless Chloramphenicol acetyl transferase (CAT) genes. The CAT genes in two control plasmids, pCAT-Promoter and pCAT-Control, are driven by SV-40 promoter. Both pCAT-Enhancer and pCAT-Control contain SV-40 enhancer. 3´ serial deletion was performed by cutting and protecting at DraII site of pBlue-FX, then digesting with ExoIII at XhoI site which is aided by an Erase-A-Base kit (Promega, USA). Deleted promoters, named by their lengths, were cloned into pCAT-Basic and pCAT-Enhancer for functional assays.

DNA transfection, CAT assay and primer extension. HeLa and HepG2 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum. For each 9cm dish, 5μg of DNA (with 10μg of pGEM4 carrier DNA) was transfected by calcium phosphate precipitation technique [6]. 48 hours after transfection, 30μg of total protein was assayed for CAT activity by TLC method and imaged by a phosphoimager (Molecular Dynamics, USA). For primer extension assay, cytoplasmic RNA from transfected cells were annealed in 40mM PIPES pH6.4, 1mM EDTA, 0.4M NaCl and 0.2% SDS with 1pmole of gamma-[^{32}P]ATP labeled CAT primer (5´-TTT,AGC,TTC,CTT,AGC,TCC, TGA,AAA,TCT-3´) at 37^{o}C overnight. Extension was performed with 5U AMV reverse transcriptase (Promega), 1mM each of dNTPs, 10mM DTT and 10U of RNasin. The final product was loaded onto a sequencing gel with sequence ladder.

Methylation. 5μg of plasmid DNA was methylated by either 10U of HpaII methylase (Boehringer Mannheim, USA) or 2U of M. SssI methylase (BioLab, USA) [7] in 10mM EDTA, 50 mM Tris-HCl pH7.5, 5mM 2-mercaptoethanol and 400μM S-adenosyl-methionine overnight. The completeness of methylation was checked by HpaII digestion. Methylated DNA was used for transfection assay.

RESULTS

The FMR-1 promoter. Because the 5´ end of FMR-1 cDNA sequence is not complete [2], a series of 3´ deletion clones were constructed to define the transcription initiation site. Fig.1 shows that the 466bp insert in pCAT-FX contains high promoter activity. This insert contains part of the first exon of FMR-1 gene and the CpG-island which is methylated in fragile X patients [3]. There is a tremendous decrease in CAT activities from pCAT-FXD291 to pCAT-FXD272, so there may be an initiation site or an enhancing element in this region. Position 273 was later proved to be a transcription initiation site by primer extension assay from cells transfected with pCAT-FXD291 (Fig.2). Sequence TTACA was located 26 nucleotides 5´ to this start site. The CAT activity of pCAT-FXD324 is slightly higher than pCAT-FX, and that of pCAT-FXD291 is slightly lower. There may be some transcription regulating elements in these regions.

FMR-1 promoter is sensitive to in vitro methylation. FMR-1 promoter is GC rich with several HpaII sites [3], but methylation with HpaII methylase did not affect its activity

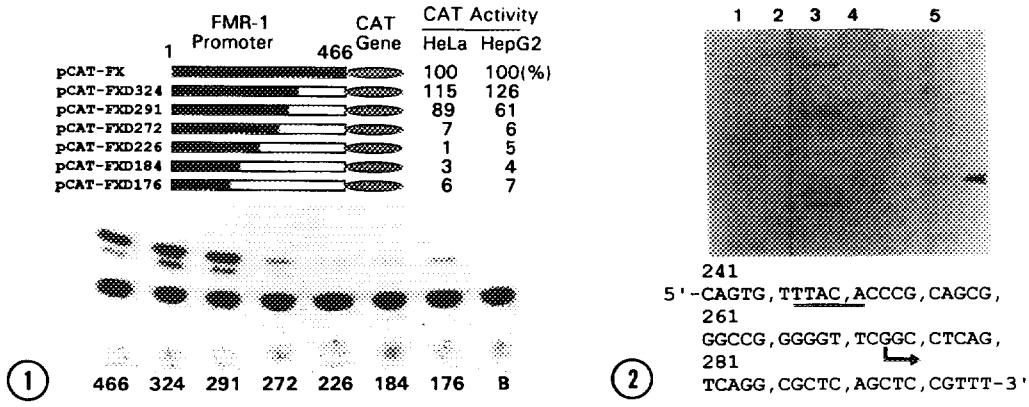

Fig.1. Deletion mapping of FMR-1 promoter. 3′ deletion clones are inserted into pCAT-Basic and named by their individual lengths (Left panel). Open boxes indicate the sequences deleted. CAT activities are shown in the right panel. All data are the average of three independent experiments and expressed relative to the full length clone. The lower panel shows the autoradiography of one representative experiment in HeLa cells. B denotes pCAT-Basic.

Fig.2. Primer extension analysis. Lanes 1 to 4 are sequence ladder. Arrowhead indicates the product of primer extension (lane 5). The lower panel shows part of the FMR-1 promoter sequence including a TATA-like box (underline) and a transcription initiation site at nucleotide 273 (arrow).

(data not shown). However, when M. SssI methylase was used to methylate cytosines of all CpG dinucleotides, FMR-1 promoter activity was completely destroyed (Fig.3). This proves that methylation could inhibit FMR-1 gene at its promoter. Because enhancer element may override methylated promoters if they are lightly repressed [8], the effect of

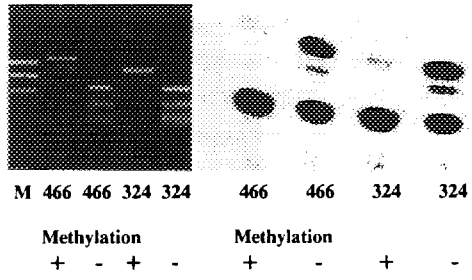

Fig.3. FMR-1 promoter is repressed by *in vitro* methylation. DNA of pCAT-FX(466) and pCAT-FXD324(324) is methylated by M. SssI methylase or mock methylated. After methylation, DNA is either cut by HpaII (left panel) or transiently expressed for CAT activity in HeLa cells (right panel). M denotes DNA molecular weight marker.

SV-40 enhancer was tested. FMR-1 promoters with (pCAT-Enhancer-FXD324) or without (pCAT-FXD324) SV-40 enhancer were methylated with M. SssI. As a control, SV-40 promoters with (pCAT-Control) or without (pCAT-Promoter) SV-40 enhancer were compared. As shown in Fig.4 in HeLa cells, pCAT-Control retains 5 to 10% of promoter activity after methylation. But from the average of three independent experiments, after methylation, the CAT activity of pCAT-Enhancer-FXD324 does not differ from that of pCAT-FXD324 or the pCAT-Basic negative control. Methylation did not decrease the CAT activity of pCAT-Control at all in BHK cells (data not shown), so the CAT structural gene itself is not affected by methylation. We conclude that FMR-1 promoter is strongly repressed by methylation if the density of methylation is high.

DISCUSSION

It is known that methylation is involved in gene regulation and differentiation. McGhee and Ginder first reported that chicken beta-globin genes were less methylated in erythrocytes than in oviduct tissue [9]. More direct evidences are derived from gene transfer experiments. Gamma-globin gene could be inhibited by *in vitro* methylation [10], and the demethylation of alpha-actin gene promoter was observed following transfection into myoblasts where the gene is active [11]. Genes regulated by methylation are usually tissue specific with few CpG dinucleotides in their promoters. The inhibition of these sparsely methylated promoters could be overcome by the presence *in cis* of an SV-40 enhancer [8].

In contrast to tissue-specific genes, constitutively expressed housekeeping genes generally contain CpG islands that are completely unmethylated in both germ line and all somatic cells [12]. Even in cell lines where high levels of *de novo* methylation happens,

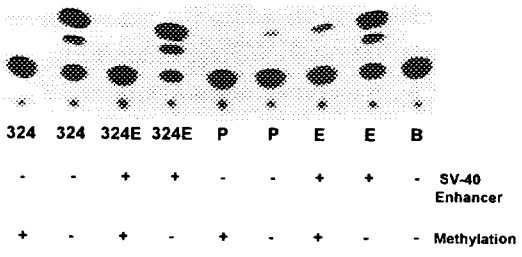

Fig.4. Action of SV-40 enhancer on the repression of FMR-1 promoter by methylation. pCAT-FXD324 (324) is compared with pCAT-Enhancer-FXD324 (324E) which contains SV-40 enhancer. Two controls are included in the study, the pCAT-Promoter (P) which contains only SV-40 promoter, and pCAT-Control (E) which contains both the SV-40 promoter and enhancer. DNA is methylated with M. SssI or mock methylated. Transfections were performed in HeLa cells. B denotes pCAT-Basic.

the islands at housekeeping genes stay unmethylated [13]. The expression of FMR-1 gene is thought to be ubiquitous, and the structure of its promoter is similar to other housekeeping gene promoters. FMR-1 promoter is GC rich with several SP1 sites [3]. There is a TATA like box (TTACA) 26bp 5′ to the transcription initiation site. From the minor bands in primer extension analysis and the residual promoter activities of upstream deletion clones, other initiation sites are also possible. So it is extremely unusual to see that such a promoter is methylated and repressed, especially in a human disease.

Though normally unmethylated, the effect of methylation on housekeeping gene promoters could be studied by *in vitro* methylation. These promoters are usually not affected by methylation with HpaII methylase which attacks only a small portion of the cytosines. But when the density of methylation increases, these promoters are completely suppressed. SV-40 enhancer can not overcome this suppression as they does for sparsely methylated promoters. The FMR-1 gene in fragile X patients is so heavily methylated that it resists the digestion of all known methylation sensitive restriction enzymes. Because an FMR-1 gene with full CGG repeat mutation expresses mRNA when the gene is hypomethylated [5], methylation should be the direct cause of FMR-1 inactivation in fragile X syndrome.

Methylation may interfere with transcription by directly preventing the binding of transcriptional factors [14] or by altering chromatin structures [15], possibly through the action of some methyl-CpG-binding proteins [16,17]. But these do not explain how methylation is induced by the expansion of CGG repeat in fragile X syndrome. There is one possibility that this segment of DNA, composed of solely G and C nucleotides for several kb in length, may alter chromatin structures and induce DNA methylation. The FMR-1 promoter and CGG repeat will be a very good model in the study of mechanisms of DNA methylation.

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