DNA methylation represses FMR-1 transcription in fragile X syndrome

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ABSTRACT

Fragile X syndrome is the most frequent form of inherited mental retardation and segregates as an X-linked dominant with reduced penetrance. Recently, we have identified the FMR-1 gene at the fragile X locus. Two molecular differences of the FMR-1 gene have been found in fragile X patients: a size increase of an FMR-1 exon containing a CCG repeat and abnormal methylation of a CpG island 250 bp proximal to this repeat. Penetrant fragile X males who exhibit these defects typically show repression of FMR-1 transcription and the presumptive absence of FMR-1 protein is believed to contribute to the fragile X phenotype. It is unclear, however, if either or both molecular differences in FMR-1 gene is responsible for transcriptional silencing. We report here the prenatal diagnosis of a male fetus with fragile X syndrome by utilizing these molecular differences and show that while the expanded CGG-repeat mutation is observed in both the chorion villi and fetus, the methylation of the CpG island is limited to the fetal DNA (as assessed by BstIII digestion). We further demonstrate that FMR-1 gene expression is repressed in the fetal tissue, as is characteristic of penetrant males, while the unmethylated chorion villi expressed FMR-1. Since the genetic background of the tissues studied is identical, including the fragile X chromosome, these data indicate that the abnormal methylation of the FMR-1 CpG-island is responsible for the absence of FMR-1 transcription and suggests that the methylation may be acquired early in embryogenesis.

INTRODUCTION

Fragile X syndrome is the most frequent familial form of mental retardation in humans with an incidence of approximately 1 per 1000 individuals (1). Mental deficiency is the major phenotypic consequence of inheriting the fragile X locus and both sexes may be affected, although males are typically more severe (2). Penetrance is reduced in both sexes being 80% in males and 30% in females (3). Clinical diagnosis is often difficult, particularly in females, and has in the past relied heavily upon the cytogenetic demonstration of the fragile site at Xq27.3 which is associated with the syndrome. The fragile X site is observed as an isochromatic gap of nonstaining material and is induced in vitro by deoxynucleotidyl pool perturbation. Fragile site induction is rarely observed in nonpenetrant males or in most carrier females and is sometimes difficult to demonstrate in either amniotic fluid cells or chorionic villi for prenatal diagnostic purposes.

We have recently identified and cloned the FMR-1 gene at the fragile X locus (4). This gene is normally expressed in brain, tests and other tissues, and is transcriptionally silent in most males with fragile X syndrome (5). Two differences have been noted at the FMR-1 locus between normal individuals and other penetrant or nonpenetrant carriers of the fragile X. The first is an expansion of the DNA sequence within an FMR-1 exon containing numerous repeats of the trinucleotide CGG (no mean repeat number is 29) (4,6,7). DNA of normal individuals, when cleaved with the restriction endonuclease EcoRI, shows a 5.2 kb band on Southern blot analysis following hybridization with the probe pE5.1 (4, 8). Nonpenetrant males (also referred to as transmitting males) and most nonpenetrant carrier females show a variable EcoRI band of approximately 5.4 kb due to an expansion within the CGG repeat of approximately 200 bp and is referred to as a premutation. When this premutation is passed through female meiosis, it may dramatically expand in size in fully penetrant males with EcoRI bands sometimes exceeding 100 kb, called a full mutation (7, 9). Often, this size increase is accompanied by a marked heterogeneity in the bands of penetrant males such that multiple bands or even diffuse smears, a in excess of 6 kb, are observed (7, 10). We have surveyed over 100 penetrant males and EcoRI bands in excess of 6 kb are consistently observed and may be considered pathognomonic of penetrant fragile X syndrome in males.

The second major difference between the DNA of fragile X males and normal males is that a CpG island 250 bp proximal to the FMR-1 CGG repeat is abnormally methylated (5,7,11,12). A BstIII site within this CpG-island has been found to be methylated in affected (or penetrant) males such that digestion with both EcoRI and BstIII shows a pattern identical to EcoRI alone while normal male DNA, when similarly digested with both enzymes, shows 2.4 and 2.8 kb bands. In normal
females, this BssHII site appears methylated on the inactive X chromosome thereby exhibiting all three bands by Southern analysis (2.4, 2.8 and 5.2 kb). It remains unclear whether this abnormal methylation occurs as a consequence of the expansion of the CGG-repeat and if this methylation is responsible for the transcriptional inactivation of the FMR-1 gene in most affected males. We report here, a prospective prenatal diagnosis of fragile X syndrome by molecular examination of the FMR-1 locus that provides data that help clarify these questions.

RESULTS

Family ATL 05 was referred for prenatal studies to evaluate an 11 week pregnancy for fragile X syndrome. The pregnant mother was a nonpenetrant obligate carrier having had a previous male child who is fully penetrant for fragile X syndrome as well as affected maternal relatives. Blood was obtained on the mother and affected son and chorionic villi sampling was performed for prenatal studies. Extracted DNA was cleaved with EcoRI and BssHII and examined by Southern blot analysis following hybridization with probe pE5.1 which contains both the FMR-1 exon with the CGG repeat and the BssHII site of the FMR-1 CpG-island (4). As shown in figure 1a, the affected son (lane 4) shows a typically increased band (6.9 kb) that is resistant to BssHII digestion similar to an unrelated male fragile X patient (7.1 kb; lane 2) and clearly distinct from normal (lane 1). The absence of digestion with BssHII reflects the methylation of this site and the surrounding CpG-island and is typical of pre- and post fragile X males (12). The mother (lane 3) shows a banding pattern consistent with the fragile X mutation in a non-prenatal carrier female (size increase or Δ of 200 bp corresponding to a total of 100 CGG repeats determined by PCR because of the diffuse doubling at ~2.8 kb observed by Southern analysis; data not shown). DNA extracted from the chorionic villi (lane 5) revealed a much different pattern from either normal (lane 6) or fragile X males (lanes 2 and 4) with a 2.4 kb band and a diffuse band centering at 4.6 kb. Since cytogenetic studies on the chorionic villi revealed a male karyotype, the 4.6 kb band represents a fragile X mutation with a Δ of ~1,800 bp or a total of ~600 CGG repeats that is unmethylated. Thus the fetus had a fragile X mutation of similar size as the affected male sib although hypomethylated in the tissue examined.

A diagnosis of fragile X syndrome was made with the opinion that the size increase was consistent with a penetrant phenotype (6–9). An elected termination was performed (at week 13 of gestation) and fetal tissue was examined. In contrast to the chorionic villi, the fetal tissue shows almost complete methylation of the BssHII site. As shown in figure 1b, digestion with both EcoRI and BssHII (lane 4) produces a pattern very similar to that obtained with EcoRI alone (lane 2). Only minor digestion with BssHII is evident from the light 2.4 kb band with the other predicted bands being extremely light due to the heterogeneity of this fragment. Therefore, unlike the DNA of the chorionic villi which was totally unmethylated, fetal DNA was greater than 95% methylated at the BssHII site flanking the FMR-1 gene.

Figure 1. Southern blot analysis. A. DNA cleaved with both EcoRI and BssHII and hybridized with the probe pE5.1 as described showing cleavage of chorionic villi DNA with the methylation-sensitive enzyme BssHII. Lanes 1 (open square) and 2 (closed square) are controls consisting of a normal male and a normally related fragile X male, respectively. Lane 3 (partially filled circle) is an obligate carrier female having had a previous son (lane 4, closed square) with fragile X syndrome. Lane 5 (cvs) is DNA obtained from chorionic villi sampling at 11 weeks gestation. B. DNA cleaved with both EcoRI (left) and both EcoRI and BssHII (right) hybridized with the probe pE5.1 as described showing fetal DNA resistant to BssHII cleavage. Lane 1 and 3 (open squares) is DNA from a control normal male (lymphocyte). Lane 2 and 4 is DNA extracted from fetal tissue obtained following the termination of the pregnancy shown in fig. 1a at 13 weeks gestation.
Since the chorionic villi and fetal tissue represent the identical mutation, in terms of repeat expansion (i.e., ~600 CGG triplets), upon the same genetic background, the methylation differences allowed us to investigate the role of methylation on FMR-1 gene expression. Transcription of FMR-1 was determined by reverse transcription-polymerase chain reaction as previously described (5) using primers downstream of the CGG-repeat and the PCR products analyzed for fluorescence intensity relative to hypoxanthine phosphoribosyl transferase (HPRT) transcripts, as a control. The results, shown in figure 2, reveal that FMR-1 transcription in the methylated fetal tissue is approximately 3% of the transcription of the hypomethylated chorionic villi, relative to HPRT transcription. Although insufficient material was available to perform truly quantitative PCR, the ratio of FMR-1 message relative to that of HPRT was consistent with that observed in lymphocytes of normal individuals (5). Further evidence that the RT-PCR products accurately reflect message abundance is that the slight transcriptional activity seen in the fetal tissue agrees with the Southern analysis (figure 1b) which shows minor hypomethylation of the BshHII site.

**DISCUSSION**

Fully penetrant male patients with fragile X syndrome exhibit a striking expansion of the CGG-repeat associated with the FMR-1 gene as well as methylation of the nearby CpG-island which is normally hypomethylated. In such penetrant males, transcription of the FMR-1 gene is repressed and presumably the absence of the protein product partially or wholly contributes to the clinical phenotype associated with the fragile X syndrome. It is unclear whether the CGG expansion, abnormal methylation, or both, result in the inactivation of the FMR-1 gene. Also underlined is the relationship between the repeat expansion and the DNA methylation. It has been proposed, in essence, that the penetrant fragile X chromosome is transcriptionally silent in the vicinity of the FMR-1 gene due to the failure to erase the imprint of the maternal inactive X chromosome (13). Alternatively, the methylation may be in response to the tremendous density of CpG dinucleotides present in the fully expanded CGG-repeat; the latter being the obligulatory mutational change.

The prenatal diagnosis of an at-risk male pregnancy, reported above, provides some insight into these questions. Analysis of DNA extracted from chorionic villi demonstrated that the fetus inherited the fragile X chromosome, with a trinucleotide expansion estimated to be approximately 600 repeats, close to the size observed in an affected son of the mother. Unlike the son, who exhibited methylation of the associated CpG-island, similar to most penetrant males, the chorionic villi sample showed evidence of impaired digestion with BshHII, an indicator of DNA methylation. Following the diagnosis of fragile X syndrome in the fetus, based upon repeat expansion, and the termination of the pregnancy, fetal tissue was examined. The fetal tissue displayed near total methylation of the CpG-island, as is characteristic for fragile X syndrome. This observation parallels that made by Sutherland et al. (14) who reported the absence of methylation in the chorionic villi DNA of a male fragile X fetus and partial or complete methylation of all other tissue examined. The hypomethylated state in the chorion appears to be ephemeral as some CVS studies of penetrant males have shown partial methylation (15) in both fetal tissues. Therefore, the abnormal DNA methylation associated with the expanded CGG-repeat either is acquired sometime following fertilization, probably in response to the extraordinary density of CpG dinucleotides, or methylation is present in the oocyte of a carrier female, perhaps by failure of imprint erasure as suggested (13), with demethylation occurring in the chorionic villi and partial demethylation in certain other tissues (14).

It has been demonstrated in normal females, that the DNA methylation observed on the inactive X chromosome is frequently absent or partial in chorionic villi and this has been widely interpreted as evidence for the acquisition of methylation in fetal tissue during normal development (16, 17). Rousseau et al. (15) noted that in the chorionic villi of normal females, the FMR-1 associated CpG-island is undermethylated as compared to fetal or adult female tissue, similar to most X-linked loci that undergo X-inactivation. Thus, hypomethylation of chorionic villi is the usual situation for the FMR-1 gene. Second, the failure of reactivation of the inactive fragile X chromosome would suggest a maximum risk of the maternal fragile X chromosome leading to penetrance in offspring to be 50% (if inactivation is random). This is in contrast with the findings of Fu et al. (9) who demonstrated that large premutations (greater than 50 repeats) always undergo transition to full mutations when transmitted while smaller premutations undergo this transition less frequently, in accordance to the repeat length. Thus, a more congruent view may be that methylation occurs in response to the expanded CGG-repeat early in development. However, the actual sequence of events is less certain.

![Figure 2. Chromatogram of reverse transcriptase polymerase chain reaction amplification of FMR-1 and HPRT mRNA from chorionic villi and fetal cells from the pregnancy shown in fig. 1.](image-url)
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... events await analysis of oocytes of carrier females since it remains formally possible that the repeat expansion itself may be transgenerational.

The finding that FMRI transcription is present in the unmethylated chorionic villus sample, despite an expanded full mutation, suggests that (i) the lack of transcription observed in the fetal tissue as well as in most penetrant patients is a consequence of the DNA methylation and (ii) the transcriptional machinery is able, at least to some degree, to proceed through the length of CGG-repeat. These data therefore have important implications. If methylation is responsible for the transcriptional silencing of the FMRI gene, as the data suggest, then induced demethylation, as achieved by 5-aza-cytidine (17), may restore mRNA production. Since it remains uncertain if the CGG-repeat is in fact translated (because a translational start site has yet to be identified 5′ to the repeat), it is possible that the downstream AUG (at position 197 of reference 4) is the authentic translational start. If so, induced hypomethylation with subsequent induction of FMRI transcript could lead to fully functional FMRI protein.

This scenario, although in obvious need of more investigation, does suggest potential therapeutic intervention in fragile X syndrome.

MATERIALS AND METHODS

Southern blot analysis

DNA was extracted from blood leukocytes obtained from family members, from the chorionic villi sample and from fetal tissues obtained after termination. Control DNAs of a normal and affected fragile X syndrome were extracted from established lymphoblastoid lines. 4 μg of DNA from the carrier mother, her affected son and another chorionic villus or the fetus were digested with EcoRI and BamHI following manufacturer’s conditions (Boehringer Mannheim) along with control DNAs from a normal and affected male. Fragments were separated through a 0.8% agarose gel in 1×TAE (40 mM Tris-acetate, 1 mM EDTA) and transferred to Zetaprobe (BioRad) membrane by conventional procedures (18). The probe pES1 (4) (which contains the fragile X CpG island and the FMRI exon subjected to the insertion) was labelled to high specific activity with [3P]dCTP using the random primer method (19) and hybridized to filters at 65°C for 16-24 h in a solution of 0.25 M sodium chloride, 0.25 M sodium phosphate pH 7.0, 7% sodium dodecylsulfate (SDS), 10% polyethyleneglycol 4000 and 1% bovine serum albumin. Prior to autoradiography, filters were washed as a final stringency of 0.1×SSC (1× is 150 mM NaCl, 15 mM sodium citrate), 0.1% SDS at 65°C.

Reverse transcription PCR (RT-PCR) analysis

mRNA was isolated from chorionic villi and fetal tissue using a poly A+ isolation kit from Invitrogen. The RT-PCR technique and primers used to assay for FMRI transcription in chorionic villus sample were described in detail previously (4). PCR reactions were carried out on cDNA synthesized from isolated RNA using FMRI primers at positions 297-320 and 420-442 (designated 4924 and 4925, respectively) designed from the published sequence (4) in addition to HPRT primers 243 and 244 (20). PCR products from FMRI or 3′ internal control HPRT were quantitated by a model 342 Gene Scannner (Applied Biosystems) and displayed as chromatographic peaks.

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