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ORIGINAL INVESTIGATION

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Evaluation of the fragile X (FRAXA) syndrome with methylation-sensitive PCR

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Abstract The fragile X (FRAXA) syndrome is the most common form of inherited mental retardation in males. Its peculiar pattern of inheritance results from the parent of origin-specific expansion of a CGG-repeat within the FMR1 gene on the X chromosome. In patients, gene function is abolished by hypermethylation of the promoter and the massively expanded repeat. We have developed a methylation-sensitive polymerase chain reaction (MS-PCR) strategy that combines repeat-length and methylation analysis of the CGG-repeat and the promoters of the FMR1 and XIST genes. The allelic methylation of the latter opposes that of the FMR promoter and serves as an internal control and standard for semiquantitative analyses. This system enables the delineation of 11 distinct patterns encountered in nonaffected, carrier, and affected males and females. We have evaluated our system on well-defined samples with different FMR1 mutations and have used it for the diagnostic evaluation of 253 male and 80 female probands. In the male group, we have identified five full mutations, and three gray-zone and premutation alleles with 54, 55, and 62 repeats, respectively. The female group consists of 33 normal homozygote and 41 heterozygote individuals, two of whom harbor a gray-zone allele with 47 repeats, none with a premutation, and six with a full mutation. Our MS-PCR approach allows the currently most comprehensive diagnostic evaluation of the FRAXA syndrome in a cost- and time-efficient fashion. In addition, it is a valuable tool for the analysis of clonality and skewing phenomena in females.

Electronic database information: accession numbers and URLs for the data in this article are as follows:
GenBank, http://www.ncbi.nlm.nih.gov/Genbank/, for human FMR1 and XIST (accession numbers L29074, L38501, U80460)
Online Mendelian Inheritance in Man (OMIM), http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/, for FMR1 (MIM 309550)
PE Biosystems, Foster City, Calif., USA (Product Bulletin), http://www.appliedbiosystems.com/ab/md/fraxb.html

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Introduction

The fragile X (FRAXA) syndrome [MIM 3009550] is the most prevalent cause of inherited mental retardation in males (Turner et al. 1996). Its perplexing molecular genetic pathomechanism and its unusual pattern of inheritance pose an extraordinary challenge for its diagnostic evaluation in the laboratory and for the genetic counseling of affected families (de Vries et al. 1998a; Bardoni et al. 2000; Jin and Warren 2000; Kooy et al. 2000). The clinical phenotype of the FRAXA syndrome consists of moderate to severe intellectual impairment, macroorchidism, large ears, a prominent jaw, and high-pitched jocular speech (de Vries et al. 1998; Bardoni et al. 2000). It results from the functional abolishment or, less often, deprived expression of the FMR1 gene, which is located in the telomeric region of the long arm of the X chromosome, X(q27) (de Vries et al. 1998; Bardoni et al. 2000; Jin and Warren 2000; Kooy et al. 2000). In the vast majority of cases, gene transcription is impaired by an unphysiological expansion of a polymorphic CGG-triplet repeat within the untranslated exon 1 of the FMR1 gene (de Vries et al. 1998; Bardoni et al. 2000; Jin and Warren 2000; Kooy et al. 2000). In normal unaffected individuals, the number of repeats ranges from 6 to 52 copies, with an average of approximately 30. Individuals with alleles in the so-called premutation range between 52 and 200 repeats are clinically also unaffected (de Vries et al. 1998; Bardoni et al. 2000; Jin and Warren 2000; Kooy et al. 2000). However, they have a variable risk for producing diseased offspring with full mutations that are characterized by an expansion with a triplet repeat number of more than 200. This risk depends on the size of the premutation and the sex of the transmitting parent (Heitz et al. 1992; Turner et al. 1994). Longer premutation alleles are more

likely to expand to full mutations than shorter ones (Heitz et al. 1992; Turner et al. 1994). In addition to the above-described expansion mechanism, other rare mutations such as small deletions of the FMR1 gene region or point mutations within the coding sequence can also impair the normal production of the FMR1 protein (for a review, see de Vries et al. 1998).

In individuals with repeat numbers in the normal and premutation range, the FMR1 gene is unmethylated, whereas in affected individuals with a full mutation, the expanded repeat in the 5' untranslated region and the 5'-region surrounding the promoter of the FMR1 gene become de novo methylated (Kirchgessner et al. 1995; Carrel and Willard 1996; Wöhrle et al. 1996; Schwemmle et al. 1997; Stöger et al. 1997). However, even FMR1 alleles with very repeat numbers (300–800) can be reactivated in vitro by treatment with demethylating agents such as 5-azadeoxycytidine (Chiurazzi et al. 1998). This observation, together with reports of males with normal intelligence, an unmethylated full mutation, and near-normal levels of protein, has led to the conclusion that de novo methylation of the FMR1 promoter rather than that of the expanded repeat impairs gene function (Kirchgessner et al. 1995; Wang et al. 1996; Sandberg and Schalling 1997). Varying degrees of methylation or methylation mosaicism may also influence the severity of the phenotype (Rousseau et al. 1991b; Kolehmainen and Karant 1994; Kirchgessner et al. 1995; Allingham-Hawkins et al. 1996; Cohen et al. 1996; Dobkin et al. 1996; Maddalena et al. 1996; Stöger et al. 1997; Wöhrle et al. 1998). Such situations can be encountered in females with skewed inactivation of either the normal or the abnormal X chromosome or in mosaic males with a combination of full and premutation cell lines (Rousseau et al. 1991b; Kolehmainen and Karant 1994; Kirchgessner et al. 1995; Allingham-Hawkins et al. 1996; Cohen et al. 1996; Dobkin et al. 1996; Maddalena et al. 1996; Stöger et al. 1997; Wöhrle et al. 1998).

Currently available procedures for the diagnostic evaluation of the FRAXA syndrome include cytogenetic, Southern-blot, polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR), and immunohistochemical analyses (Rousseau et al. 1991a; Brown et al. 1993; Cao et al. 1994; Pai et al. 1994; El-Aleem et al. 1995; Wang et al. 1995; Carrel and Willard 1996; Haddad et al. 1996; Holden et al. 1996; Spence et al. 1996; Willemsen and Oostra 2000). The most commonly applied molecular genetic-based techniques depend on either the detection of the expanded repeat, the de novo methylation of the affected gene regions, or both (Brown et al. 1993; Cao et al. 1994; Pai et al. 1994; El-Aleem et al. 1995; Wang et al. 1995; Carrel and Willard 1996; Haddad et al. 1996; Holden et al. 1996; Spence et al. 1996; Hecimovic et al. 1997; Panagopoulos et al. 1999; Strelnikov et al. 1999). Only radioactive or non-radioactive Southern-blotting of DNA double-digested with methyl-sensitive and methyl-insensitive restriction enzymes is able reliably to cover the whole spectrum of premutations, full mutations, and mosaicism that may be encountered in the context of this syndrome (Brown et al. 1993; Kolehmainen and Karant 1994; Kirchgessner et al. 1995; Allingham-Hawkins et al. 1996; Carrel and Willard 1996; de Vries et al. 1998). PCR is mainly applied to determine the length of the triplet repeat in the normal and premutation range. It therefore helps to refine the determination of the repeat lengths of normal and premutation alleles and to detect repeat-length and methylation mosaicism. The high CG content of the repeat region renders a direct and reliable amplification of full mutations difficult. It is only reliable within the lower repeat unit range, particularly of male patients, either following 7-deaza-2'-dGTP incorporation (Brown et al. 1993) or by applying a commercial test system (PE Biosystems).

To circumvent the above-described problems, PCR-based alternatives have been developed that are based on the identification of the methylated FMR1 promoter or the absence of gene expression (Pai et al. 1994; Das et al. 1997–1998; Dawson et al. 1995; Kirchgessner et al. 1995; Carrel and Willard 1996; Hmadcha et al. 1998; Strelnikov et al. 1999). The absence of the FMR1 mRNA in male individuals with full mutations can be demonstrated by RT-PCR (Pai et al. 1994; Dawson et al. 1995; Hmadcha et al. 1998), whereas the absence of the respective protein can also be identified immunohistochemically with a FMR1-directed antibody (Willemsen and Oostra 2000). Nevertheless, at best, only male individuals can be reliably evaluated with these tests. The various combinations of normal, premutation, and full mutation alleles together with the possibility of skewed X-chromosome inactivation and size or methylation mosaicism still pose a diagnostic challenge that has so far been impossible to cover with one simple diagnostic procedure (Brown et al. 1993; Rousseau et al. 1991b; Kolehmainen and Karant 1994; Kirchgessner et al. 1995; Allingham-Hawkins et al. 1996; Cohen et al. 1996; Dobkin et al. 1996; Maddalena et al. 1996; Stöger et al. 1997; Panagopoulos et al. 1999). We therefore present a simple PCR strategy that combines repeat-length and methylation analysis in a cost- and time-efficient fashion. It requires only small amounts of DNA and reliably detects virtually all normal and pathological FRAXA variants in males and, in particular, females.

Material and methods

Conceptual design of the PCR assay

Our test system takes advantage of the varying repeat lengths that define normal, premutation, and full mutation FMR1 alleles. In addition, it takes into account the different methylation patterns of the CGG repeat and the promoters of the FMR1 and XIST gene that are present in nonaffected and different types of affected males and females (Fig. 1). Since the XIST gene promoter is methylated on the active X chromosome, its allelic methylation pattern opposes that of the FMR1 promoter (Carrel and Willard 1996). Therefore, the XIST gene promoter provides an internal standard for the semiquantitative comparison of the XIST/FMR1 methylation ratio, that at the same time also serves as an optimal internal control (Kubota et al. 1999). Thus, the combined determination of the repeat size and the methylation patterns of the FMR1 gene within the promoter and repeat reactions, respectively, resolve all male and female FRAXA patients. This approach can advance investigations that assess the amount and distribution of skewing and mosaicism in various tissues of affected females (Rousseau et al. 1991b; Carrel

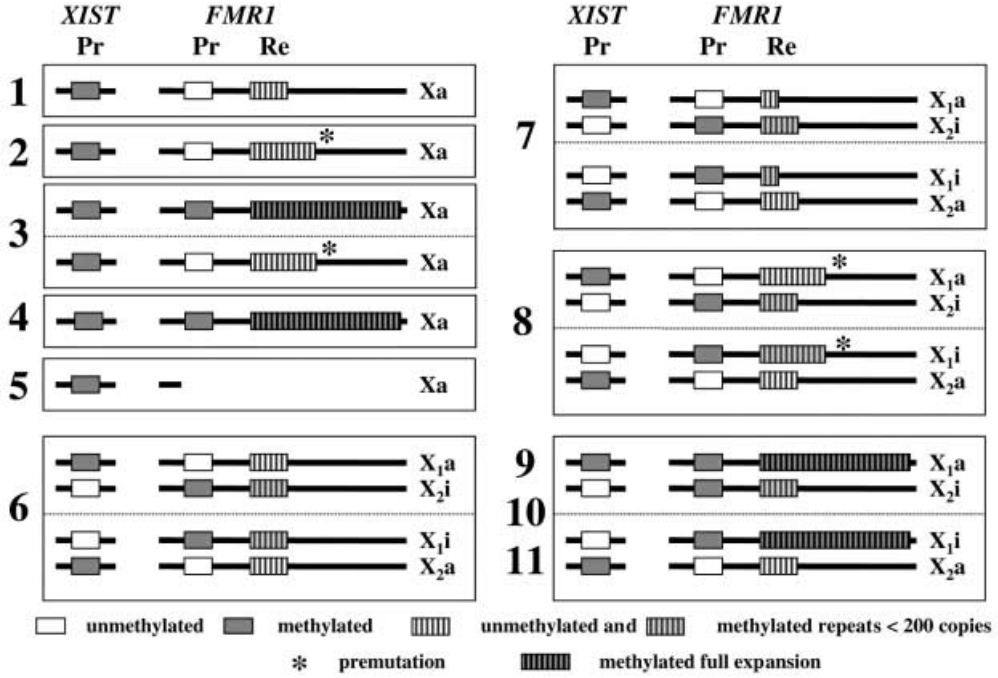

Fig. 1 Schematic presentation of the size polymorphisms and respective methylation status of the triplet repeat within the first untranslated exon of the FMR1 gene and the respective methylation patterns of the FMR1 and XIST gene promoters encountered in male (*1–5*) and female individuals (*6–11*) with repeat units in the normal (*1, 6–11*) premutation (*2, 3, 8*), and full mutation (*3, 4, 9–11*) range or a deletion (*5*). These heterogeneous patterns provide the rationale for our assay. Males have only one active X chromosome, and females one active and one inactive X chromosome. The XIST promoter is always methylated on the active X chromosome, whereas in females, the FMR1 allele (promoter and repeat) that is located on the inactive X chromosome is physiologically methylated (Carrel and Willard 1996; Willard 1996; Kubota et al. 1999). Therefore, female samples always contain a mixture of unmethylated and methylated XIST and FMR1 gene promoters. In normal males with a repeat size up to 52 (*1*) and those with a premutation (between 52 and 200 repeats; *2*), both the promoter and the repeat of the FMR1 gene are unmethylated, whereas in the case of a full mutation (>200 repeats), the FMR1 promoter and repeat become de novo methylated (*3, 4*). The fully expanded methylated repeat cannot be amplified with our MS-PCR. In males with a premutation/full mutation mosaicism, a combination of FMR1 promoter methylation and an unmethylated repeat size in the premutation range can be observed (*3*). FRAXA cases that are attributable to deletions within this region lack the FMR1 gene promoter and the repeat (*5*). However, they are identified by the methylated XIST product. In all other instances, XIST has no diagnostic value in males and solely serves as an internal control. Normal females with a homozygous repeat length and random X inactivation have a mixture of one unmethylated and one methylated XIST and FMR1 promoter product with an approximate ratio of 1:1 together with one unmethylated and one methylated FMR1 repeat product, respectively (*6*). Normal heterozygous females possess the same FMR1 and XIST promoter pattern, but with two unmethylated and two methylated XIST and FMR1 promoter products that correspond to the two alleles with unequal repeat lengths (*7*). Females with one premutation allele reveal a similar pattern as normal heterozygous females, except that one repeat is more than 52 repeat units long (*8*). Irrespective of its location on the active or the inactive X chromosome, the FMR1 gene promoter is always methylated on the fully expanded allele in females with a full mutation. In the case of random X inactivation, the methylation ratio of the FMR1 gene promoter is 3:1 in favor of the methylated one, whereas the methylation ratio of the XIST gene promoter remains 1:1. Because the fully expanded methylated repeat cannot be detected with MS-PCR, the FMR1 repeat sequence in these patients appears homozygous (*9*). In females with a full mutation and either skewed X inactivation or methylation mosaicism (*10, 11*), the methylation pattern is identical to that of affected females with random X inactivation. However, skewing and mosaicism can be identified by virtue of a semiquantitative comparison of the XIST promoter methylation ratio with that of the FMR1 gene promoter and the FMR1 repeat. The methylation ratio of the XIST promoter serves as a standard, because it remains 1:1 in all instances. In contrast, the methylation ratio of the FMR1 promoter and that of the methylated repeat shifts either toward the unmethylated or toward the methylated allele depending on whether the normal chromosome or that containing the expanded allele is skewed. An identical pattern is observed in mosaic females with a normal cell population and one that harbors a full mutation. The numbering system corresponds to that used in Tables 2, 3, Fig. 3

and Willard 1996). Such investigation can help to evaluate to which extent the clinical phenotype in affected women is influenced by such epigenetic modifications (Rousseau et al. 1991b; Kolehmainen and Karant 1994; Allingham-Hawkins et al. 1996; Cohen et al. 1996; Dobkin et al. 1996).

To distinguish between unmethylated and methylated homologous sequences, we take advantage of the fact that unmethylated, but not methylated, cytosine is converted into uracil by chemically deaminating DNA with sodium bisulfite (Herman et al. 1996; Das et al. 1997–1998; Zeschnigk et al. 1997; Kubota et al. 1999). When this modified DNA replicates, uracil is replaced by thymidine. Therefore, this procedure generates derivative DNA sequences from originally homologous, but differentially methylated alleles, and also sense and antisense strands. Based on the assumption that in the unmethylated gene regions, all CpGs are unmethylated, whereas in the methylated regions all CpGs are methylated, we simply deduced the sequence of the deaminated unmethylated DNA by converting all cytosines to thymidines (Hornstra et al. 1993). In the case of a deaminated methylated sequence, we only changed cytosines that were not contained in CpGs.

Based on this sequence data, we have designed primers that are specific for the deaminated unmethylated and methylated adjacent

Table 1 Characteristics of 13 primers used for our PCR analyses (*pmol* amount in pmol of each primer used per 25-µl reaction mix): seven primers in the promoter reaction (PUF, PMF, P-R, XUF, XMF, XUR, XMR), four in a repeat reaction (RUF, RMF, RUR, RMR), and two in control amplifications (FMF, FMR). The repeat number "*n*" can be calculated according to the formula provided for the product size (bp)

Target sequence	Primer	Primer designation	Sequence	Position (bp) [1]Acc#L29074, L38501 [2]Acc#U80460	Concentration per reaction (pmol)	Designation of product	Product length (bp)
Promoter PCR							
FMR1							
Unmethylated promoter	Forward	PUF	5'-GTGTTTGATTGAGGTTGAATTTTTG-3'	[1]13712–13688	5	PU	318
	Reverse	P-R	5'-ATTTAATTTCCCAC(A/G)CCACTAAATACAC-3'	[1]13395–13422	10		
FMR1							
Methylated promoter	Forward	PMF	5'-GTTGCGGGTGTAAATATTGAAATTACG-3'	[1]13683–13657	5	PM	288
	Reverse	P-R	5'-ATTTAATTTCCCAC(A/G)CCACTAAATACAC-3'	[1]13395–13422	–		
XIST							
Methylated promoter	Forward	XMF	5'-AATTAAAGTAGGTATTCGCGGTTTCG-3'	[2]19049–19024	8	XM	241
	Reverse	XMR	5'-TTTTTCCTTAACCCATCGAAATATCG-3'	[2]18834–18809	8		
XIST							
Unmethylated promoter	Forward	XUF	5'-AAAAGTGGTTGTTATTTTAGATTTGTT-3'	[2]19238–19260	8	XU	198
	Reverse	XUR	5'-CTACCTCCCAATACAACAATCACAC-3'	[2]19435–19411	8		
Repeat PCR and alternative primers for the deletion of the methylated FMR promoter in a single-step PCR							
				Acc#L29074, L38501			
FMR1							
Unmethylated repeat	Forward	RUF	5'-TTTGAGAGGTGGGTTGTGGGTGTTTGAGG-3'	13925–13897	10	RU	64+3×$(CGG)_n$
	Reverse	RUR	5'-AACACCACTACCAAAAAACATACAACAACACAAC-3'	13802–13835	10		
FMR1							
Methylated repeat	Forward	RMF	5'-TTTCGAGAGGTGGGTTGCGGGCGTTCGAG-3'	13926–13898	10	RM	120+3×$(CGG)_n$
	Reverse	RMR	5'-CCGCCTCTAAACGAACGACGAACCGACGAC-3'	13747–13776	10		
FMR1							
Methylated promoter	Forward	FMF	5'-CGTCGTCGGTTCGTCGTTCGTTTAGAGGC-3'	13777–13749	10	FP	231
	Reverse	FMR	5'-CCGACCGATTCCCAACAACGCGCATACG-3'	13546–13574	10		

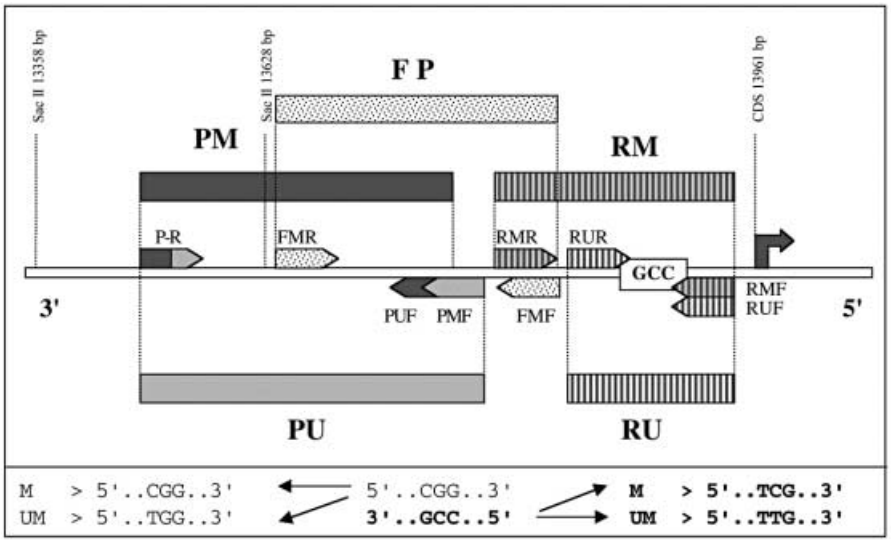

Fig. 2 Location of primers that are specific for the deaminated methylated and unmethylated antisense strand of the 5'-FMR-1 region. The initial second strand synthesis from the reverse primers generates the double-stranded templates for the further amplification steps in a strand-specific manner. The primers are specific for the deaminated antisense strand and cannot bind to the deaminated sense or the native FMR1 DNA sequences. The basepair numbers of *Sac*II restriction sites and of the coding region startsite (*CDS*) are shown for reference (Gene Bank accession numbers L29074, L38501)

regions of the triplet repeat and the FMR1 and XIST gene promoters (Table 1, Fig. 2). By determining the presence or absence of one or the other of the respective PCR products, we distinguish between nonaffected and affected males. In addition, we are also able to identify premutations, full mutations, mosaicism, and the rare cases with deletions in the respective regions (Figs. 1, 3). Comparison of the ratio of the methylated and unmethylated FMR1 promoter with that of the reciprocally methylated XIST gene promoter and with the ratio of the product of the unmethylated and methylated FMR1 repeat facilitates the distinction of nonaffected and the vast majority of affected females. This system also takes advantage of the fact that we are unable directly to amplify full expansions with PCR.

Samples and DNA preparation

EDTA-anticoagulated peripheral blood samples from normal controls and from affected individuals were collected and aliquots of 500 µl were stored frozen at –20°C until DNA extraction. In some instances, we used methanol/acetic-acid-fixed cell samples from cytogenetic analysis. Lymphoblast cell lines GM06891 (male) and GM06896 (female) with a premutation range repeat length of 100 and 95–120+23 CGG repeat units, respectively, were obtained from the Coriell Institute (Camden, N.J.). DNA from fibroblast cell line TC43–97 with 220 unmethylated FMR-1 repeat units was kindly provided by Robert Burman (Portland, Ore.; Burman et al. 1999) and DNA samples with different types of mutations by Peter Steinbach (Ulm, Germany) and Jean Louis Mandel (Strasbourg, France).

DNA for deamination and subsequent MS-PCR analysis was extracted from 80 µl blood samples (stored frozen) with DNAzol (Vienna Lab, Vienna, Austria) and resuspended in 30 µl sterile water. For Southern blot analysis, DNA was extracted in the same way from cell pellets (10^6–10^7 cells) and prepared by lysing red blood cells with two successive washes in 1–3 vol lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM NaEDTA). Pellets from cell lines were treated in an identical fashion.

DNA deamination

DNA (0.5 µg) was deaminated according to a previously published protocol with some modifications (Herman et al. 1996; Zeschnigk et al. 1997)**.** The first and most critical step in MS-PCR is a complete denaturation of the DNA before bisulfite treatment. In case of a high CG content, the DNA has to be treated for 3 min at 95°C in 300 mM NaOH, otherwise denaturing will not be complete, and the GC-rich sequences will remain partially resistant to bisulfite conversion. The time for deamination at 55°C was reduced from 16 to 2 h and addition of 8 µl polyacryl carrier (MRC, Cincinnati,

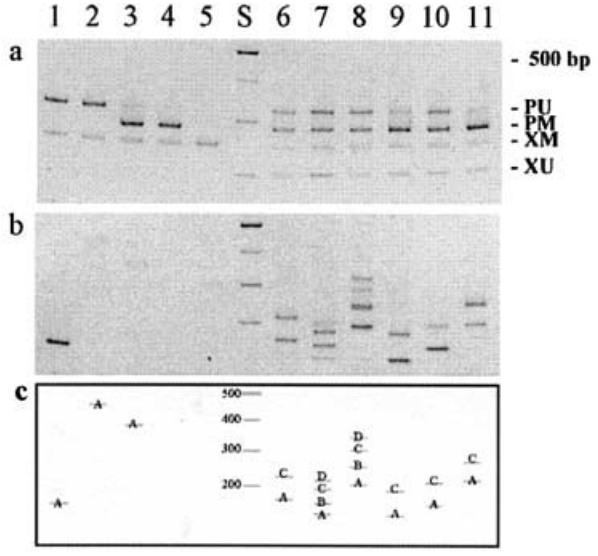

Fig. 3 Representative examples of the results obtained with the promoter (**a**) and the repeat (**b**) MS-PCR assay. The promoter PCR (**a**) detects the unmethylated (*PU*) and methylated (*PM*) promoter sequences of the FMR1 and the XIST (*XU*, *XM*) gene, whereas the repeat PCR (**b**) detects the unmethylated (*RU*) and methylated (*RM*) FMR1 triplet repeat up to at least 160 copies. The unmethylated (*A, B*) and methylated repeat alleles (*C, D*) are depicted in the scheme for easy reference (**c**). The numbering system corresponds to that in Fig. 1, Table 2, 3 (*n* repeat number). The sizes of the respective promoter PCR products and the 500 bp band of the 100 bp ladder are indicated. *Lane 1* Normal male [*n*=34], *lane 2* male with a premutation [*n*=130], *lane 3* mosaic male [*n*>200+*n*=100], *lane 4* male with a full mutation [*n*>200], *lane 5* male with a deletion of the FMR1 gene promoter and the repeat, *lane 6* normal homozygous female [*n*=33], *lane 7* normal heterozygous female [*n*=23+30], *lane 8* female with a premutation [*n*=33+66], *lane 9* female with a full mutation [*n*>200+*n*=23], *lane 10* female with a full mutation and an increased proportion of cells with an active normal allele due to skewed X-inactivation [*n*>200+*n*=30; increased amount of RU-product], *lane 11* female with an increased proportion of cells with a full mutation due to skewed X-inactivation [*n*>200+*n*=46; increased amount of RM-product], *S* size standard. The mosaicism in the male individual shown in *lane 4* is defined by the presence of the methylated promoter product (*PM*) in the promoter reaction and of the expanded (*n*=100) unmethylated repeat product (*RU*) in the repeat reaction

Ohio) was added to speed up the final DNA precipitation to 10 min at –20°C. The deaminated DNA redissolved in 20 µl sterile water was stored at 4°C for several weeks; for long-term storage, DNA was stored at –20°C. Methanol/acetic-acid-fixed material was resuspended in 70 µl water after removal of the fixative by centrifugation, and the DNA was deaminated as described without further need of DNA extraction.

Primers

We designed 13 primers whose sequences were deduced from the deaminated DNA sequence of the respective unmethylated or methylated gene regions (Fig. 2, Table 1). The promoter PCR consists of seven primers and contains two forward primers (PUF, PMF) and one common reverse primer (P-R) that are specific for the unmethylated and methylated FMR1 promoter, and two forward (XUF, XMF) and two reverse primers (XUR, XMR) that are specific for the unmethylated and methylated XIST promoter (Table 1). The repeat PCR consists of four primers and contains two forward primers (RUF, RMF) and two reverse primers (RUR, RMR) that are specific for the unmethylated and methylated triplet repeat region (Table 1). An additional primer pair (FMF, FMR) detects the methylated FMR1 promoter (FP) but cannot be combined with the other primers. To reduce the melting temperature from Tm≈95°C to Tm≈75°C (calculated value of deaminated dsDNA), we based the construction of the XUF and XUR primers on the sense strand and all other primers on the sequence of the antisense strand. Primers that are specific for the methylated triplet repeat region are important for the detection and semiquantitative evaluation of the differentially methylated alleles of the two X chromosomes in females. With this approach, we can amplify triplet repeats within the normal and premutation range up to at least 160 repeat units, but no full mutations. The normal and premutation repeat number can be easily calculated from the length of the PCR products (Table 1).

MS-PCR analysis

The PCR was set up in 25-µl reaction volumes under oil, with 1 µl being taken from 20 µl deaminated DNA from patients and normal controls in the same deamination lot. For the promoter PCR, we used amplification buffer F-511 (10 mM TRIS pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100; Finnzymes, Espoo, Finland); optimized buffer EXT (50 mM TRIS, 15 mM NH_4Cl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, pH 9,0) was used for the repeat PCR with 4% DMSO and 60 mM TMAC, and for amplification of FP without any enhancer. The concentration of each deoxynucleotide was 200 µM; optimized primer-concentrations are listed in Table 1. Amplifications were run on a Biometra TrioBlock (Biometra, Göttingen, Germany) and started with 1 U Dynazyme 501L (Finnzymes) during an initial denaturation step at 95°C for 5 min; the promoter PCR profile was 35 cycles at 95°C for 30 s, 60°C for 20 s, and 72°C for 40 s; the repeat and the FP profile consisted of 35 cycles at 95°C for 45 s, 63°C for 1 min, and 72°C for 1 min; final extension was performed in all cases at 72°C for 7 min.

PCR products (10 µl) were separated under nondenaturating conditions on NOVEX pre-cast TBE gels (Novex, San Diego, Calif.); bands were visualized by ethidium-bromide staining, and densito-

Table 2 Schematic presentation of the expected product patterns and XIST/FMR1 methylation ratios of normal, premutation, and full mutation individuals that are detected with our MS-PCR assay. The numbering system corresponds to that used in Figs. 1 and 3 (– no PCR product, + PCR product, *2+* two differently-sized products, ▲ relative increase, ▼ relative decrease of PCR product)

		Promoter PCR				Repeat PCR	
	Target gene sequences	*FMR1* promoter		*XIST* promoter		*FMR1* repeat	
No.	PCR product	PU	PM	XU	XM	RU	RM
1	Normal males	+	–	–	+	+	–
2	Males with premutation	+	–	–	+	+	–
3	Male mosaics with pre- and full mutation	+	+	–	+	+	–
4	Males with full mutation	–	+	–	+	–	–
5	Males with deletion	–	–	–	+	–	–
6	Normal females with random X-inactivation and equal repeat lengths on both alleles	1:1		1:1		+	+
7	Normal females with random X-inactivation and unequal repeat lengths on both alleles	1:1		1:1		2+	2+
8	Females with a premutation and random X-inactivation	1:1		1:1		2+	2+
9	Females with a full mutation and random X-inactivation	1:3		1:1		+	+
10	Females with a full mutation and an increase of cells with the normal allele on the active X chromosome	(>) 1:3		1:1		▲	▼
11	Females with a full mutation and an increase of cells with the expanded allele on the active X chromosome	(<) 1:3		1:1		▼	▲
	Mosaic females with a premutation	(>) 1:3		1:1		▲	▼
		or				or	
		(<) 1:3				▼	▲
	Mosaic females with a full mutation	(>) 1:3		1:1		▲	▼
		or				or	
		(<) 1:3				▼	▲

metric image analysis was performed by using the KODAK-1D 2.0.2 software package (Kodak, New Haven, Conn.).

Deamination of non-methylated cytosines generates a significant amount of uracil in the DNA that is only subsequently substituted by thymidine during the first rounds of amplification. Therefore, amplification of deaminated DNA is much less efficient at the beginning than that of untreated native DNA. The original amount of 500 ng DNA is significantly degraded by the bisulfite treatment, so that only approximately 25 ng (12.5 ng unmethylated and 12.5 ng methylated) single-stranded, deaminated, and non-degraded DNA may remain amplifiable. Amplification with fluorescence-labelled primers and quantification of the products on an ABI 310 sequencer revealed that even with this more sensitive approach products were only detectable after at least 25 PCR cycles. The multiplex reaction was linear up to 31 cycles (R^2=0.99) and the duplex reaction up to 33 cycles (R^2=0.99; data not shown). In ethidium-bromide-stained gels, on the other hand, hardly any product is visible after 30 cycles. A reproducible densitometric quantification and reliable interpretation is therefore only feasible after 33 and 35 PCR cycles, respectively.

Results

The simultaneous analysis of two intragenic and one intergenic parameter together with the concurrent evaluation of their methylation status allows the definition of virtually all FRAXA variations encountered in male and female patients, including full mutations, repeat-length and methylation mosaicism, and skewing of X inactivation.

Table 3 Results of the densitometric analysis of the promoter PCR products obtained from females. Net intensities, calculated ratios of unmethylated and methylated products, and the standardized ratio based on the intergenic XIST standard ratio (XM/XU) are shown (columns are numbered according to Figs. 1, 3). The ratio of methylated and unmethylated XIST amplicons that are specific for the active and inactive X chromosome, respectively, serve as internal standard ($mean_{[XM/XU]}$=0.935±0.14). Under the selected conditions the PU/PM ratio is not exactly as theoretically expected, viz., 1, but ranges from 0.7 to 1.0. The standardized intergenic comparison of the two intragenic ratios derived from the methylated and unmethylated FMR1 promoter and XIST alleles, respectively, show a clear difference that approach the 95% confidence interval in females with a full mutation and random X-inactivation (sample 9) or an increase of cells that carry the expanded allele on the active X chromosome (sample 11; $mean_{[PU/PM]/[XM/XU]}$=1.02± 0.14). In females with a full mutation and an increase of cells that carry the normal allele on the active X chromosome (sample 10), the ratio is as expected between normal females and full mutation females with random X-chromosome inactivation. In this instance, the unequal band intensities of the RU and RM PCR products also indicate the presence of the full mutation (sample 10 and Fig. 3b)

Product (bp)	Sample [net intensity]					
	6	7	8	9	10	11
PU (318)	7070	13075	11232	6664	12864	4233
PM (288)	10122	13019	12460	22203	20389	26111
XM (241)	1579	4514	2607	3821	3931	3261
XU (198)	2143	5329	2702	4320	3520	3072
Ratio	Intragenic ratios					
PU/PM	0.70	1.00	0.90	0.30	0.63	0.16
XM/XU	0.74	0.85	0.96	0.88	1.12	1.06
	Standardized intergenic ratios					
(PU/PM)/(XM/XU)	0.95	1.19	0.93	0.34	0.57	0.15

With only two PCRs and one ethidium-bromide-stained gel, we have thus been able to distinguish 11 different patterns (Figs. 1, 2, Tables 1, 2, 3). In contrast to most of the other systems, this information is achieved without any further hybridization or digestion with restriction enzymes.

First, we evaluated our system in a blinded fashion on well-defined samples with various extraordinary forms of FMR1 mutations. We identified the correct repeat numbers and associated methylation pattern in the 30 samples. This panel consisted of six female and five male samples with full mutations, one male sample each with a premutation mosaicism and a deletion of the promoter- and repeat-primer-binding sites, three females and four males with premutations between 61–160 repeats, and seven female and five male normal controls. Representative examples of the different patterns are shown in Fig. 3.

Next, we used this MS-PCR system for the diagnostic evaluation of 253 male and 80 female probands. In the male group, we identified five full mutations, one premutation with 62 repeats and two "gray-zone" alleles with 54 and 55 repeats, respectively. The female group consisted of 33 normal homozygote and 41 heterozygote individuals, two of whom harbored a "gray-zone" allele with 47 repeats, none with a premutation, and six with a full mutation.

Discussion

Our MS-PCR approach is the most comprehensive system currently available for the diagnostic evaluation of FRAXA syndrome. Its key components are the DNA deamination, the combined assessment of the repeat length and methylation pattern, and the introduction of the XIST gene promoter as an internal standard and control. DNA deamination serves two purposes. It converts exclusively unmethylated cytosine into uracil and subsequently thymidine and, thus, enables the distinction between homologous, but differentially methylated, DNA sequences by PCR (Herman et al. 1996; Zeschnigk et al. 1997; Kubota et al. 1999; Panagopoulos et al. 1999). Moreover, the generation of AT-rich sequences from the originally GC-rich sequences of the triplet repeat and the FMR1 and XIST promoters significantly improves the PCR conditions for the amplification of both the unmethylated and methylated counterparts. Under these circumstances, two alleles differing in methylation pattern and repeat number can be reliably resolved. This increased sensitivity facilitates the determination of the exact size of polymorphic alleles and, consequently, the recognition of heterozygous females with normal and premutation alleles.

The introduction of the reciprocally methylated XIST promoter not only facilitates the discrimination of various types of mutations in females, but also between male and female samples (Carrel and Willard 1996; Stöger et al. 1997). In addition, the ability of the promoter PCR assays to detect cell populations with methylation differences is far more sensitive than that of Southern blot analysis. Various degrees of skewing of X chromosome inactivation

have previously been found in a high proportion of normal females and, in addition, may change with increasing age in FRAXA cases, preferentially toward the active healthy allele (Rousseau et al. 1991b).

Although we can delineate females with premutations and full mutations from females with normal alleles, it sometimes proves difficult to distinguish those with a premutation from those with a full mutation. This difficulty arises from primer competition that results in amplification of only the allele of normal repeat size, but not the expanded allele, when the repeat unit differs by more than 100 repeats. In the peripheral blood of females, we were able to amplify a combination of 25 and 132 repeat alleles, but not that of 27 and 158 repeats, whereas in male fibroblasts, our longest amplified repeat was 220 triplets long (Burman et al. 1999). However, whether and how well expanded repeats in females are amplifiable is also strongly influenced by the presence and extent of methylation mosaicism and skewing. In both our promoter and repeat PCR, premutation cases will be overdiagnosed, because they mimic the pattern that derives from affected females with a full mutation and skewed X-inactivation toward the expanded allele. Thus, in rare situations, it will be necessary either to amplify the methylated or unmethylated triplet repeat with the RU and RM primer pairs in separate PCRs or to try to determine the length of the triplet repeat with a different PCR system or Southern blot analysis.

The small amount of DNA needed for our MS-PCR test is advantageous for many purposes, such as for prenatal diagnosis, for the analysis of DNA extracted from Guthrie cards, and for studies from small tissue samples obtainable from fine needle biopsies (Pai et al. 1994; Dawson et al. 1995; Brown et al. 1996; Abrams et al. 1999; Spence et al. 1996; Larsen et al. 1997). Moreover, it may facilitate non-invasive diagnosis from hair bulbs and buccal smears. Finally, it can also be easily adapted for fluorescence-based automated fragment analysis and is thus suitable for large-scale screening purposes (Spence et al. 1996; Larsen et al. 1997; Strelnikov et al. 1999).

References

Abrams MT, Kaufmann WE, Rousseau F, Oostra BA, Wolozin B, Taylor CV, Lishaa N, Morel ML, Hoogeveen A, Reiss AL (1999) *FMR1* gene expression in olfactory neuroblasts from two males with fragile X syndrome. Am J Med Genet 82:25–30

Allingham-Hawkins DJ, Brown CA, Babul R, Chitayat D, Krekewich K, Humphries T, Ray PN, Teshima IE (1996) Tissue-specific methylation differences and cognitive function in fragile X premutation females. Am J Med Genet 64:329–333

Bardoni B, Mandel JL, Fisch GS (2000) *FMR1* gene and fragile X. Am J Med Genet 97:153–163

Brown WT, Houck GEJ, Jeziorowska A, Levinson FN, Ding X, Dobkin C, Zhong N, Henderson J, Brooks SS, Jenkins EC (1993) Rapid fragile X carrier screening and prenatal diagnosis using a nonradioactive PCR test. JAMA 270:1569–75

Brown WT, Nolin S, Houck GJr, Ding X, Glicksman A, Li SY, Stark-Houck S, Brophy P, Duncan C, Dobkin C, Jenkins E (1996) Prenatal diagnosis and carrier screening for fragile X by PCR. Am J Med Genet 64:191–195

Burman RW, Yates PA, Green LD, Jacky PB, Turker MS, Popovich BW (1999) Hypomethylation of an expanded FMR1 allele is not associated with a global DNA methylation defect. Am J Hum Genet 65:1375–1386

Cao J, Tarleton J, Barberio D, Davidow LS (1994) A simple fragile X PCR assay with 7-deazaguanine-substituted DNA visualized by ethidium bromide. Mol Cell Probes 8:177–180

Carrel L,Willard HF (1996) An assay for X inactivation based on differential methylation at the fragile X locus, FMR1. Am J Med Genet 64:27–30

Chiurazzi P, Pomponi MG, Willemsen R, Oostra BA, Neri G (1998) In vitro reactivation of the FMR1 gene involved in fragile X syndrome. Hum Mol Genet 7:109–113

Cohen IL, Nolin SL, Sudhalter V, Ding XH, Dobkin CS, Brown WT (1996) Mosaicism for the FMR1 gene influences adaptive skills development in fragile X-affected males. Am J Med Genet 64:365–369

Das S, Kubota T, Song M, Daniel R, Berry-Kravis EM, Prior TW, Popovich B, Rosser L, Arinami T, Ledbetter DH (1997–1998) Methylation analysis of the fragile X syndrome by PCR. Genet Test 1:151–155

Dawson AJ, Chodirker BN, Chudley AE (1995) Frequency of FMR1 premutations in a consecutive newborn population by PCR screening of Guthrie blood spots. Biochem Mol Med 56:63–69

Dobkin CS, Nolin SL, Cohen I, Sudhalter V, Bialer MG, Ding XH, Jenkins EC, Zhong N, Brown WT (1996) Tissue differences in fragile X mosaics: mosaicism in blood cells may differ greatly from skin. Am J Med Genet 64:296–301

El-Aleem AA, Bohm I, Temtamy S, El-Awady M, Awadalla M, Schmidtke J, Stuhrmann M (1995) Direct molecular analysis of the fragile X syndrome in a sample of Egyptian and German patients using non-radioactive PCR and Southern blot followed by chemiluminescent detection. Hum Genet 96:577–584

Haddad LA, Mingroni-Netto RC, Vianna-Morgante AM, Pena SD (1996) A PCR-based test suitable for screening for fragile X syndrome among mentally retarded males. Hum Genet 97:808–812

Hecimovic S, Barisic I, Muller A, Petkovic I, Baric I, Ligutic I, Pavelic K (1997) Expand long PCR for fragile X mutation detection. Clin Genet 52:147–154

Heitz D, Devys D, Imbert G, Kretz C, Mandel JL (1992) Inheritance of the fragile X syndrome: size of the fragile X premutation is a major determinant of the transition to full mutation. J Med Genet 29:794–801

Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93:9821–9826

Hmadcha A, De Diego Y,Pintado E (1998) Assessment of FMR1 expression by reverse transcriptase-polymerase chain reaction of KH domains. J Lab Clin Med 131:170–173

Holden JJ, Chalifoux M, Wing M, Julien-Inalsingh C, White BN (1996) A rapid, reliable, and inexpensive method for detection of di- and trinucleotide repeat markers and disease loci from dried blood spots. Am J Med Genet 64:313–318

Hornstra IK, Nelson DL, Warren ST, Yang TP (1993) High resolution methylation analysis of the FMR1 gene trinucleotide repeat region in fragile X syndrome. Hum Mol Genet 2:1659–1665

Jin P, Warren ST (2000) Understanding the molecular basis of fragile X syndrome. Hum Mol Genet 9:901–8

Kirchgessner CU, Warren ST, Willard HF (1995) X inactivation of the FMR1 fragile X mental retardation gene. J Med Genet 32:925–929

Kolehmainen K, Karant Y (1994) Modeling methylation and IQ scores in fragile X females and mosaic males. Am J Med Genet 51:328–338

Kooy RF, Willemsen R, Oostra BA (2000) Fragile X syndrome at the turn of the century. Mol Med Today 6:193–198

Kubota T, Nonoyama S, Tonoki H, Masuno M, Imaizumi K, Kojima M, Wakui K, Shimadzu M, Fukushima Y (1999) A new assay for the analysis of X-chromosome inactivation based on methylation-specific PCR. Hum Genet 104:49–55

Larsen LA, Gronskov K, Norgaard-Pedersen B, Brondum-Nielsen K, Hasholt L, Vuust J (1997) High-throughput analysis of fragile X $(CGG)_n$ alleles in the normal and premutation range by PCR amplification and automated capillary electrophoresis. Hum Genet 100:564–568

Maddalena A, Yadvish KN, Spence WC, Howard-Peebles PN (1996) A fragile X mosaic male with a cryptic full mutation detected in epithelium but not in blood. Am J Med Genet 64: 309–312

Pai JT, Tsai SF, Horng CJ, Chiu PC, Cheng MY, Hsiao KJ, Wuu KD (1994) Absence of FMR-1 gene expression can be detected with RNA extracted from dried blood specimens. Hum Genet 93:488–493

Panagopoulos I, Lassen C, Kristoffersson U, Aman P (1999) A methylation PCR approach for detection of fragile X syndrome. Hum Mutat 14:71–79

Rousseau F, Heitz D, Biancalana V, Blumenfeld S, Kretz C, Boue J, Tommerup N, Van Der Hagen C, De Lozier-Blanchet C, Croquette MF, et al (1991a) Direct diagnosis by DNA analysis of the fragile X syndrome of mental retardation. N Engl J Med 325:1673–1681

Rousseau F, Heitz D, Oberle I, Mandel JL (1991b) Selection in blood cells from female carriers of the fragile X syndrome: inverse correlation between age and proportion of active X chromosomes carrying the full mutation. J Med Genet 28:830–836

Sandberg G, Schalling M (1997) Effect of in vitro promoter methylation and CGG repeat expansion on FMR-1 expression. Nucleic Acids Res 25:2883–2887

Schwemmle S, Graaff E de, Deissler H, Glaser D, Wohrle D, Kennerknecht I, Just W, Oostra BA, Dorfler W, Vogel W, Steinbach P (1997) Characterization of FMR1 promoter elements by in vivo-footprinting analysis. Am J Hum Genet 60:1354–1362

Spence WC, Black SH, Fallon L, Maddalena A, Cummings E, Menapace-Drew G, Bick DP, Levinson G, Schulman JD, Howard-Peebles PN (1996) Molecular fragile X screening in normal populations. Am J Med Genet 64:181–183

Stöger R, Kajimura TM, Brown WT, Laird CD (1997) Epigenetic variation illustrated by DNA methylation patterns of the fragile-X gene FMR1. Hum Mol Genet 6:1791–1801

Strelnikov V, Nemtsova M, Chesnokova G, Kuleshov N, Zaletayev D (1999) A simple multiplex FRAXA, FRAXE, and FRAXF PCR assay convenient for wide screening programs. Hum Mutat. 13:166–169

Turner AM, Robinson H, Wake S, Laing SJ, Leigh D, Turner G (1994) Counselling risk figures for fragile X carrier females of varying band sizes for use in predicting the likelihood of retardation in their offspring. Am J Med Genet 51:458–462

Turner G, Webb T, Wake S, Robinson H (1996) Prevalence of fragile X syndrome. Am J Med Genet 64:196–197

Vries BB de, Halley DJ, Oostra BA, Niermeijer MF (1998) The fragile X syndrome. J Med Genet 35:579–589

Wang Q, Green E, Bobrow M, Mathew CG (1995) A rapid, nonradioactive screening test for fragile X mutations at the FRAXA and FRAXE loci. J Med Genet 32:170–173

Wang Z, Taylor AK, Bridge JA (1996) FMR1 fully expanded mutation with minimal methylation in a high functioning fragile X male. J Med Genet 33:376–378

Willard HF (1996) X chromosome inactivation, *XIST*, and pursuit of the X-inactivation center. Cell 86:5–7

Willemsen R, Oostra BA (2000) FMRP detection assay for the diagnosis of the fragile X syndrome. Am J Med Genet 97:183–188

Wöhrle D, Schwemmle S, Steinbach P (1996) DNA methylation and triplet repeat stability: new proposals addressing actual questions on the CGG repeat of fragile X syndrome. Am J Med Genet 64:266–267

Wöhrle D, Salat U, Glaser D, Mucke J, Meisel-Stosiek M, Schindler D, Vogel W, Steinbach P (1998) Unusual mutations in high functioning fragile X males: apparent instability of expanded unmethylated CGG repeats. J Med Genet 35:103–111

Zeschnigk M, Lich C, Buiting K, Doerfler W, Horsthemke B (1997a) A single-tube PCR test for the diagnosis of Angelman and Prader-Willi syndrome based on allelic methylation differences at the SNRPN locus. Eur J Hum Genet 5:94–98