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Prenatal Diagnosis and Carrier Screening for Fragile X by PCR

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During the past three years, we have conducted fragile X DNA studies for carrier screening and prenatal diagnosis using a previously described PCR protocol that accurately resolves normal FMR1 alleles and premutations and detects most full mutations [Brown et al., JAMA 270:1569–1575, 1996]. A total of 344 pregnant women with a family history of mental retardation of unknown cause were screened and 6 fragile X carriers were identified: two had full mutations, and four had premutations. The mentally retarded relatives of two other women were found to be fragile X positive although the women themselves were not carriers. In all, 6 carriers and 8 fragile X families were identified by this screening. We have also screened 40 pregnant women who were members of previously identified fragile X families, but whose carrier status was unknown. Ten were found to be carriers and were offered prenatal diagnosis. Prospective prenatal testing of 84 carrier women correctly detected 31 fetal samples (19 females, 12 males) with full mutations and 6 with premutations (2 females, 4 males). No false positives but one false negative occurred early on due to undetected maternal cell contamination. In addition, screening of 806 males with developmental delays of unknown cause gave positive results in 33 (4.1%). Potential problems and pitfalls of direct DNA testing are discussed. Because of the proven success of fragile X screening with direct molecular analysis, screening of all undiagnosed individuals with mental retardation and at risk pregnant women should now be considered. The identification of fragile X carriers and prenatal diagnosis of their pregnancies should significantly reduce the prevalence of this syndrome.

KEY WORDS: fragile X syndrome, prenatal diagnosis, genetic screening, mental retardation

INTRODUCTION

The fragile X syndrome is the most common inherited form of mental retardation [Hagerman and Silverman, 1991; Brown and Jenkins, 1992] and is second only to Down syndrome as a specific genetic cause of mental retardation. The molecular basis for the syndrome is usually an expansion of a repetitive CGG triplet sequence located in the 5′ untranslated region of the fragile X gene, FMR1 [Verkerk et al., 1991; Fu et al., 1991; Eichler et al., 1993; Warren and Nelson, 1994]. In rare cases the syndrome is due either to a deletion of part or all of the FMR1 gene [Gedeon et al., 1992; De Graaff et al., 1995; Qan et al., 1995] or to a point mutation within an RNA binding domain [DeBoulle et al., 1993; Siomi et al., 1994]. Triplet expansion above a threshold of approximately 200 repeats results in hypermethylation of the FMR1 promoter region and a lack of gene expression [Pieretti et al., 1991]. Because of its prevalence and medical importance, efficient means for accurate diagnostic screening and prenatal testing are needed [Oostra et al., 1993].

Polymerase chain reaction (PCR) is rapid and requires little DNA for analysis. Initial attempts to PCR amplify the CGG repeat region in the fragile X locus by PCR were unsuccessful [Kremer et al., 1991]. Because the region is high in CG content, special methods were needed for successful PCR amplification. Fu et al. [1991] developed a PCR method which amplified normal sized alleles and most premutation alleles, but failed to amplify full mutations with >200 CGGs. We developed a PCR protocol which allowed resolution of normal alleles and amplification of premutations and most full mutations [Brown et al., 1993, 1994]. The method replaced dGTP with 7-deaza-2-dGTP and employs a non-radioactive detection system. This PCR protocol permits rapid, cost-effective screening for fragile X carrier status and diagnosis of prenatal samples.

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Here we report an update of our experience with this protocol and discuss potential problems in fragile X testing.

MATERIALS AND METHODS

Control samples were primarily drawn from a New York based population and included 2500 unrelated X chromosomes that were approximately 95% Caucasian with 35% of Ashkenazi Jewish background. Prenatal specimens were referred from local, national and international obstetrical settings.

The previously described PCR protocol was employed with minor modifications [Brown et al., 1993, 1994]. In general, 20–200 nanograms of DNA were amplified in 10 μl reactions. Primers 1 (5′-GAC GGA GGC GCC GCT GCC AGG-3′) and 3 (5′-GTG GGC TGC GGG CGC TCG AGG-3′), yielding a 152 bp product for a normal 30 repeat allele, were used for most samples. The chemiluminescent detecting probe used, a $(CGG)_{7-11}$ oligonucleotide labeled with a chemiluminescent detection motif and supplied as a kit (FMC), provides rapid results and avoids the use of radioisotopes [Brown et al., 1993, 1994]. The use of 100% 7-deaza-2′-dGTP does not allow visualization of the PCR product with ethidium bromide. Lowering the ratio of 7-deaza-2′-dGTP to dGTP from 100% to 50%:50% or to 25%:75% allows for direct visualization with ethidium bromide [Chong et al., 1994; Wang et al., 1995]. However, we have found that many premutation and most full mutation alleles fail to amplify with less than 100% 7-deaza-2′-dGTP. Amplification of all premutation sized alleles and approximately 90% of full mutations are successful with this primer set and conditions. Full mutations generally appear as a high molecular weight band or a series of fragments. Some increase in secondary background banding is occasionally seen which can be reduced by optimizing the magnesium concentration to approximately 0.75 mmol/L. In addition, all prenatal PCR results were confirmed by Southern analysis and hybridization with the probe StB12.3 following the method of Rousseau et al. [1991].

RESULTS

The FMR1 repeat distribution of 2500 unrelated X chromosomes with fewer than 60 repeats is illustrated in Figure 1. The individual allele frequencies, given in Table I, yielded a calculated heterozygosity $(1 - \Sigma q^2)$ of 79.9%. Thus, for the screening of women and female fetal samples, approximately 80% of those that are normal will have two differently sized alleles. The protocol allows accurate resolution of alleles within the normal repeat range of ~5–55, and thus such normal allele sizes can be readily resolved.

We have screened 344 pregnant women with a family history of mental retardation of unknown cause over a 4-year period, 1992 through 1995. Two women were identified that had full mutations of >200 repeats and 4 had premutations with 70, 59, 59, and 56 repeats. The woman with 70 came from a large family with mentally retarded individuals that were subsequently found to

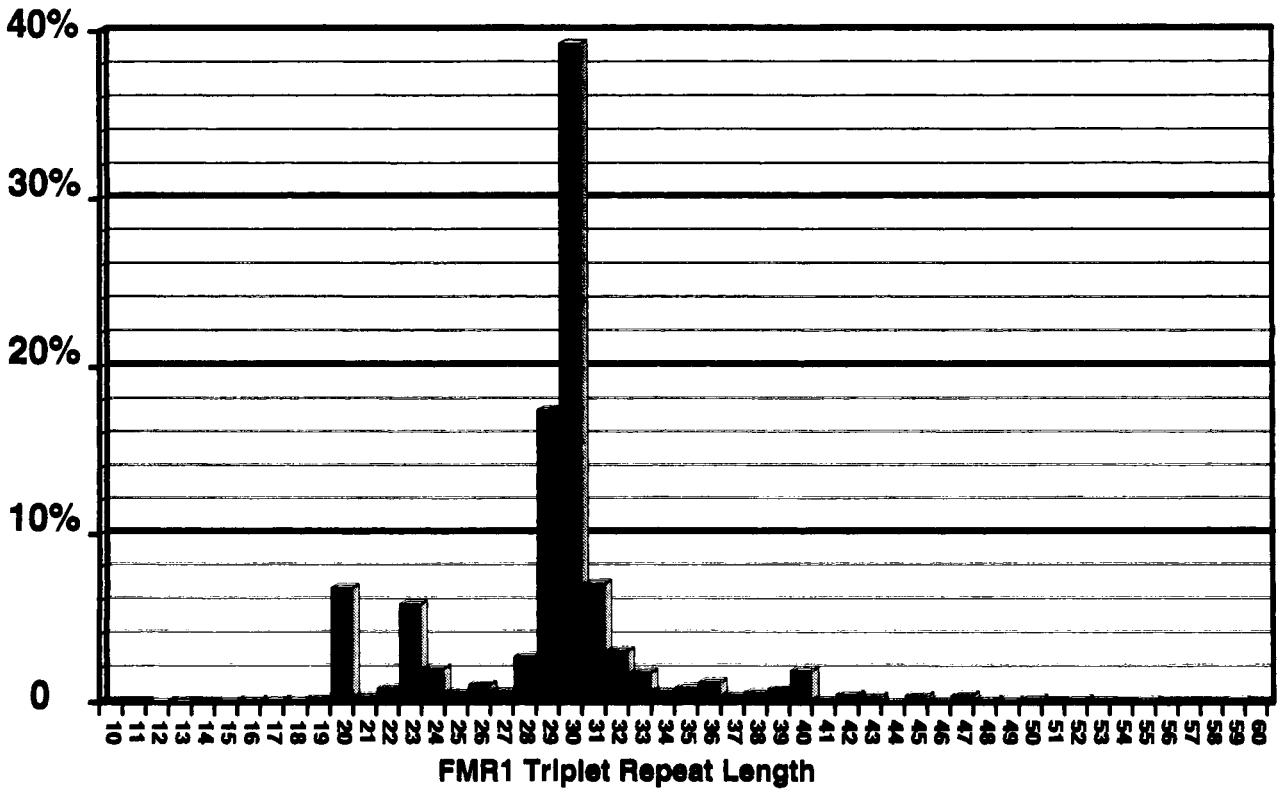

Fig. 1. Distribution of FMR1 repeats in 2,500 New York controls.

TABLE I. Distribution of FMR1 Repeats in 2,500 Controls

Repeats	No.	Percent#
12	2	0.08
13	1	0.04
14	—	—
15	3	0.12
16	4	0.16
17	4	0.16
18	4	0.16
19	7	0.28
20	170	6.80
21	9	0.36
22	21	0.84
23	146	5.84
24	50	2.00
25	15	0.60
26	26	1.04
27	17	0.68
28	68	2.72
29	437	17.48
30	982	39.28
31	176	7.04
32	76	3.04
33	44	1.76
34	17	0.68
35	22	0.88
36	30	1.20
37	11	0.44
38	14	0.56
39	20	0.80
40	47	1.88
41	5	0.20
42	12	0.48
43	9	0.36
44	2	0.08
45	10	0.40
46	3	0.12
47	11	0.44
48	4	0.16
49	3	0.12
50	6	0.24
51	—	—
52	5	0.20
53	1	0.04
54	—	—
55	3	0.12
56	—	—
57	—	—
58	—	—
59	3	0.12
60	—	—

have full mutations. Upon obtaining the information that she was a carrier, she elected not to have a prenatal diagnosis. The first woman with a 59 repeat allele had a mentally retarded brother (unavailable for testing) and she transmitted her normal 29 repeat allele. The second woman with a 59 repeat allele passed the 59 repeat unchanged to her male fetus. Analysis for AGG interspersion of this 59 repeat allele showed one AGG at position 9 indicating she carried a pure CGG repeat of 50 [Zhong et al., 1996]. Prenatal diagnosis in the woman with the 56 repeat allele showed this allele was stably transmitted to a female fetus. The woman had a mother and both an emotionally disabled and normal brother, all of whom had a 53 repeat allele that was 3 repeats less than hers. AGG analysis of a brother showed one AGG at position 12 indicating the woman's unstable 56 repeat allele had 44 pure CGG repeats. Although no full mutation individual had been documented in these latter three families, the size of the pure repeats and the instabilities present leads us to classify them as probable fragile X families.

We also screened 40 pregnant women who were members of previously identified fragile X families, but whose carrier status was unknown. Ten were found to be carriers. Eight had their pregnancies monitored. Normal alleles were transmitted in 6 pregnancies and full mutations were transmitted in the remaining 2. One woman with 23 and 33 repeats was included in this series because the 33 repeat was a reversion from her mother's premutation allele of 82 repeats [Brown et al., 1996]. Two of her pregnancies were monitored to examine the stability of the revertant 33 repeat allele. In each instance, the fetus inherited the other chromosome with 23 repeats.

Prospective prenatal testing with PCR and direct genomic Southern analysis was carried out on 84 pregnancies of fragile X carrier women. Of these, 44 were chorionic villus samples (CVS) and 40 were amniotic fluid samples (AFS). Full mutations were detected in 31 fetal samples and premutations in 6. Among the full mutations, 19 were female (9 CVS and 10 AFS) while 12 were male (11 CVS and 1 AFS). The premutations included 4 males (1 CVS and 3 AFS) with sizes of 59, 65, 70, and 80 and 2 females (1 CVS and 1 AFS) with sizes of 56 and 100 repeats. Follow-up of the male with 70 repeats at 2 years of age showed no developmental delay. The male fetus with a 59 repeat spontaneously aborted at approximately 26 weeks gestation. The 2 male fetuses with 65 and 80 were electively terminated based on the detected instability of the alleles and parental anxiety, even though, at present there is no information to counsel that such males would have any increased risk of mental impairment. The female fetuses with 56 and 100 repeats were continued to term and are apparently normal infants.

The preliminary PCR results were generally available within 3 to 5 working days and Southern blot results generally 2–5 weeks later. When the mother's normal allele was transmitted to a male fetus this information could be given with confidence. Similarly for a female fetus, when the normal alleles of the mother and father were of different repeat numbers, this information could also be given with confidence. In 3 pregnancies, a full mutation allele of a female fetus was not identified by PCR and required a Southern analysis for diagnosis. In one, the maternal and paternal alleles were of different sizes and the absence of the mother's normal allele gave an early indication that the fetus carried the full mutation. In the other 2 cases, the parental normal alleles were the same size. The preliminary negative results were revised when the direct genomic analysis was completed. In one of these two pregnancies, one spontaneously occurring fragile X [fra(X)(q27.3)] chromosome was observed out of 20 cells examined following culture in CDM medium (Sigma). Among the 9 full mutation male fetuses, one failed to

amplify by PCR but was positive by genomic Southern analysis.

Although methylation was incomplete or absent in most chorionic villus samples, CVS analysis was effective in identifying potentially affected fetuses. In the pregnancies monitored by Southern blot analysis of CVS where the affected chromosome was inherited, incomplete methylation made it difficult to distinguish between full mutation and mosaic cases, but this distinction was not critical since both are generally affected. However, it is potentially difficult to distinguish between mosaic and premutation carrier cases because of the difficulty in excluding the presence of a full mutation allele when methylation is incomplete. In the cases where CVS was used to analyze fetuses carrying an affected allele, this situation was not encountered. In this relatively rare situation, where CVS analysis may be ambiguous, a follow-up amniocentesis sample can be obtained for clarification of methylation status.

Using these molecular approaches, we have had no false positives. One false negative occurred in 1991 when cytogenetics was still the primary technique used to identify the fragile X chromosome [Jenkins et al., 1995]. Retrospective analysis of this case indicated that this negative was due to undetected maternal cell contamination with maternal cell overgrowth in a few of the flasks grown for a prolonged period of time for genomic Southern analysis. With the ability to size alleles accurately from directly isolated samples, such a false negative we regard now as very unlikely.

We have also screened by PCR 806 males with a clinical history of mental retardation or developmental delay of unknown cause. Among these, 33 (4.1%) gave a positive result. This positive yield is comparable to the numbers of fragile X males we have detected by cytogenetic screening of similar populations [Jenkins et al., 1992]. Among the 806 males, 15 (1.9%) were found to have an allele size of 45 to 60. In addition, one male with mental retardation was identified with a repeat size of 75 and was not mosaic by Southern blot analysis. His family history was otherwise negative and his mother carried a 70 repeat allele. His clinical history indicated microcephaly and seizures. Thus, his transmitting male status was likely to be a coincidence.

DISCUSSION

Our experience has shown that fragile X screening and prenatal diagnosis employing PCR as the primary technique can be conducted in a rapid and efficient manner. PCR screening has several distinct advantages over direct Southern blot analysis because it is faster, less expensive and requires only minimal DNA. After DNA isolation and PCR amplification, the analysis can be completed in as little as 8 hrs. Because nanogram amounts of DNA are sufficient for PCR analysis, prenatal samples can be studied directly. In addition, the PCR product length of a normal 30 repeat allele using our primers is 151 bps, whereas analysis of Southern blots of restriction enzyme digested DNA identifies fragments several kb long. The small size of the PCR product allows resolution and diagnosis of small premutations which are very difficult to differentiate on a genomic Southern blot. Individual family studies examining allele stability in the high normal range can also be conducted with PCR whereas the same is not possible with Southern analysis. However, PCR analysis does have the limitation that amplification of a full mutation cannot always be accomplished. In our prenatal experience approximately 13% of full mutations overall failed to amplify successfully by PCR; 3 of 19 female fetuses and 1 of 12 male fetuses. This is comparable to our experience of approximately 10% of negative PCR results on blood specimens from fragile X affected individuals. PCR also does not give information about the methylation status of the CGG repeat region. Because of the limited ability to resolve alleles with more than 150 repeats by PCR, and the inability to provide methylation information by PCR, both PCR and Southern analysis are important to have available for routine diagnostic and for prenatal testing purposes.

The number of CGG repeats in the normal population is highly variable (Fig. 1). As a consequence, approximately 80% of normal women are heterozygous with two distinguishable alleles. This means that carrier screening with our protocol is highly efficient because most normal females show two alleles. Of the 20% who are homozygous, 75% will have a 30 repeat and 15% a 29 repeat. For this latter group, Southern analysis is required to make certain the diagnosis of a full mutation female was not missed.

For identification of mosaic individuals, Southern blot analysis is the preferred technique. We have not experienced a significant problem with detection of prenatal mosaicism in either CVS or AFS. Review of our prenatal results showed that approximately 40% of samples with a full sized mutation were judged to be mosaic. Most full mutation CVS samples analyzed have shown a partially or completely unmethylated pattern which is consistent with the concept that the FMR1 locus in CVS generally undergoes methylation at a time somewhat later than 10 weeks gestation.

The detection of one positive cell on routine cytogenetic analysis in the case described above was the second such instance of positive fragile X cells we have observed during the standard prenatal cytogenetic analysis of AFS uninduced for fragile sites [Jenkins et al., 1995]. Another such spontaneously occurring true fragile X was also observed in the laboratory of Dr. Teshima [Jenkins et al., 1995]. These results indicate that spontaneously occurring fragile X sites should be kept in mind as a possible marker of true fragile X mutations. However, Shapiro et al. [1994] has reported follow-up studies on 33 cases of apparent spontaneously occurring fragile X chromosomes. Although molecular confirmations were not reported, all were cytogenetically negative. More recently, another case was reported to be positive by direct DNA testing [Shapiro et al., 1995]. When such spontaneously occurring fragile sites are observed in the future, we recommend molecular studies to confirm carrier status of such pregnant women and prenatal molecular analysis in those found to be carriers.

Because PCR analysis is relatively fast and inexpensive, all pregnancies at risk for fragile X could be monitored similarly to maternal α-fetoprotein analysis. Specifically, all pregnant women could be screened to determine their carrier status. In a survey of known fragile X families, the smallest premutations we observed to expand to full mutations in one generation were from two women with 59 repeats (Nolin et al., personal communication). Thus, women with a premutation allele of ≥59 should consider having prenatal diagnosis. However, the stability of gray zone alleles in the range of 45–58 repeats is unclear and these are expected to be found in approximately 2% of alleles (Table I). We have performed analysis of the underlying AGG patterns on male samples for alleles in this size range [Zhong et al., 1996] and have found that about 50% had zero or one AGG and thus have ≥35 pure CGG repeat alleles which are potentially unstable. With presently available techniques only male alleles can be analyzed for AGG patterns. Thus, family studies on samples within this size range to determine stability and AGG patterns are recommended. Preconceptual screening is recommended to allow time for adequate genetic counseling of identified carriers. In addition, all developmentally delayed children can be screened by this approach. When positive, they can benefit from early intervention while their relatives benefit from genetic counseling.

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