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Intragenic Probe Used for Diagnostics in Fragile X Families

Annemieke J.M.H. Verkerk, Bert B.A. deVries, Martinus F. Niermeijer, Ying-Hui Fu, David L. Nelson, Stephen T. Warren, Danielle F. Majoor-Krakauer, Dicky J.J. Halley, and Ben A. Oostra

Department of Clinical Genetics, University Hospital Dijkzigt (A.J.M.H.V., B.B.A.dV., M.F.N., D.F.M-K., D.J.J.H.); Department of Cell Biology (B.A.O.), Erasmus University, Rotterdam, The Netherlands[1]; Institute for Molecular Genetics (Y-H.F., D.L.N.), Baylor College of Medicine, Houston, Texas; Howard Hughes Medical Institute and Department of Biochemistry and Pediatrics (S.T.W.), Emory University School of Medicine, Atlanta, Georgia

The intragenic (FMR-1) probe pE5.1 was used for DNA analysis in fragile X families. With this probe fragments of altered size can be detected in female carriers, affected individuals and transmitting males. The length of the altered fragments was found to vary from one generation to another as well as between sibs. This instability of the DNA detected by pE5.1 was also seen in peripheral blood within single individuals. These phenomena are illustrated by 4 exemplary families segregating the fragile X syndrome. We demonstrate the diagnostic contribution of intragenic analysis to carrier detection as well as the identification of normal transmitting males carrying premutations. One of the families illustrates the passage of a premutation to a male through 2 generations.

KEY WORDS: Fragile X syndrome, carrier detection, repeat sequence, FMR-1, DNA diagnostic, X-linked mental retardation.

INTRODUCTION

We have recently described the isolation of the FMR-1 gene at the fragile X [fra(X)] locus, which may be directly involved in the manifestation of the fra(X) syndrome [Verkerk et al., 1991]. A 5.2 kb EcoRI fragment was isolated, containing the majority of breakpoints in hybrid cell lines at the fra(X) site [Warren et al., 1990], a CpG island that is preferentially methylated in fra(X) patients [Vincent et al., 1991; Bell et al., 1991], and an exon of the FMR-1 gene [Verkerk et al., 1991] including an unusual repetitive CGG sequence. The CGG repeat exhibits polymorphic length variation in the normal population. The number of CGG repeats ranged from 6-46, with an average of 29, in more than 200 individuals [Fu et al., 1991]. In fra(X) patients the 5.2 kb EcoRI fragment is considerably increased in size and shows somatic length variation.No difference was found in sequences flanking the CGG repeat region in carriers and patients, placing the source of the length variation within the CGG repeat itself [Kremer et al., 1991, Fu et al., 1991].

The diagnosis of the fra(X) syndrome has been based on cytogenetic testing of the presence of a fragile site at Xq27.3. Cytogenetics has serious limitations in carrier detection because 50% of obligate carriers fail to express the fragile site. In addition, the fragile site normally is not observed in normal transmitting males [Sherman et al., 1985].

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[1]Address reprint requests to Dr. B.A. Oostra, Dept. of Cell Biology, Erasmus University, PO Box 1738, 3000 DR Rotterdam, The Netherlands

The possibilities for carrier detection and prenatal diagnosis have been extended by DNA analysis, using closely linked polymorphic markers flanking the fra(X) locus [Suthers et al., 1991]. However, a widespread application of polymorphic markers was hampered by limited informativeness. Using the 5.2 kb EcoRI fragment as a probe in diagnosis is a major improvement [Oberlé et al.,1991; Kremer et al., 1991; Pieretti et al., 1991]. Carriers and patients now can be detected by showing the presence of fragments of abnormal size.

In this paper we describe the analysis of four fra(X) families using the 5.2 kb EcoRI fragment (pE5.1) as a probe. We detected female carriers and distinguished between normal, affected and transmitting males. The diagnostic potential of intragenic DNA analysis is discussed.

MATERIALS AND METHODS

The DNA probe used was pE5.1 [Verkerk et al., 1991], which was later determined to be 5222 bp long [Fu et al., 1991].

Total genomic DNA was isolated from leukocytes from individuals from different Fra(X) families as described [Miller et al., 1988]. DNA (8 μg) was digested to completion with the restriction endonuclease EcoRI, separated by gel electrophoresis and subjected to Southern blot analysis. The probe was labeled by the random oligonucleotide priming method [Feinberg and Vogelstein, 1983] and was competed for 2 hours with 120 μg of total human DNA in 5 x SSC at 65°C. After prehybridization and hybridization the filters were washed to 0.1 x SSC at 65°C prior to autoradiography.

RESULTS AND DISCUSSION

A family with 2 affected brothers, A3 and A6 is shown in Fig. 1A. They showed cytogenetic fra(X) expression in 4% and 14% of their cells, respectively. Abnormal fragments appeared in EcoRI digests of both patients. Instead of discrete bands, smears of DNA fragments were observed, indicating somatic mosaicism: different cells may have fragments with different increased sizes in the CGG repeat sequence. A band of normal size was observed in their sister A5 as well as a band with an increased fragment length, although its size is smaller than is seen in her brothers. Besides a normal band, a band that is increased in size, but that is smaller than in A5, was found in the mother A1.

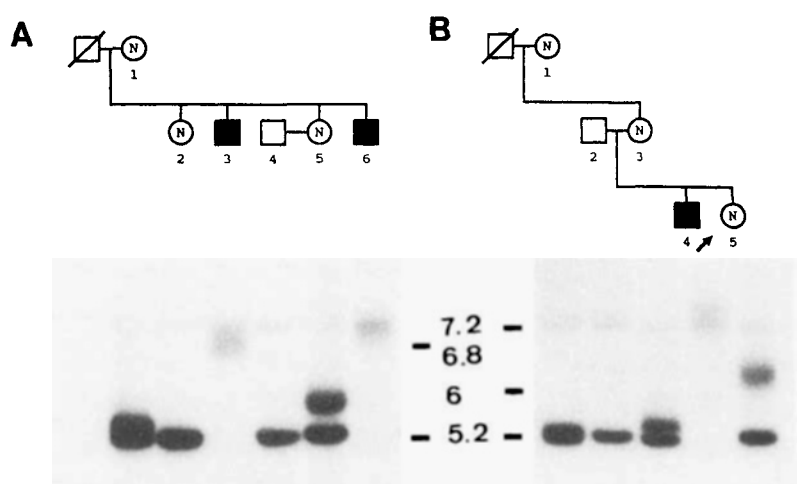

Fig. 1 Pedigrees of fra(X) families. The following symbols are used: Unaffected ○ female and □ male; Normal Ⓝ female and [N] male, without cytogenetic expression of the fragile X site; ■ Mentally retarded fragile X male, with cytogenetic expression of the fragile X site; ◑ Female with cytogenetic expression of the fragile X site.

In this family it is clear that the fragment increases in size from the mother A1 to her children A5,3,6. Daughter A5 inherits the fra(X) allele from her mother and is therefore a carrier of the syndrome. Daughter A2 has inherited the normal allele and thus is not a carrier.

This phenomenon of a fragment increasing in size is often observed in fra(X) families. It is not clear how and when this takes place. The most likely explanation would be that the initial increase in size is produced during oogenesis. The germ cell of the mother carries one abnormal fragment, but to account for the mosaicism in the patients it has to be assumed that during embryogenesis the CGG repeat "grows" in length. Clearly there is a great variability in the size in the CGG repeat that is passed on from the mother to her different children.

Fig. 1B shows an example of a family where the mutation changes in size through 3 generations. None of the 3 women in this pedigree showed any cytogenetic expression of the fragile site. Marker analysis had not been conclusive to establish the carrier status of B5. However, a clearcut result was obtained by Southern blot analysis using probe pE5.1. The grandmother B1 shows a fragment with a very small increase in the CGG repeat that barely resolves from the normal allele on the Southern blot. By PCR analysis using primers flanking the CGG repeat [Fu et al., 1991] it was confirmed that B1 had an increase in size of 108 bp [Nelson, unpublished results]. In her daughter B3 the fragment became larger, continuing its growth in the granddaughter B5 and her patient brother B4, who had 20% of cytogenetic fra(X) expression.

In the pedigree in Fig. 2A the individual A3 is a fra(X) patient. He has low (4 %) cytogenetic fra(X) expression and is mentally retarded. On a Southern blot analysis he shows a band of near normal size and in addition a smear with a population of larger fragments. This type of mosaicism, first observed by Oberlé et al. [1991], is different from the one observed in the patients A3 and A6 in family 1A. Other members in this pedigree were tested. The woman A6 shows a normal allele as well as one that is increased in size. This latter allele is passed on to her son A7. This fragment which is characterized by a small increase in size, is associated with lack of clinical and cytogenetic expression, which marks A7 as a normal transmitting male. A7 is related to A3 through their grandfathers, which strongly suggests that they too were normal transmitting males. This was supported by pE5.1 probing of DNA from their spouses, who were shown to have normal 5.2 kb alleles (A5, data not shown).

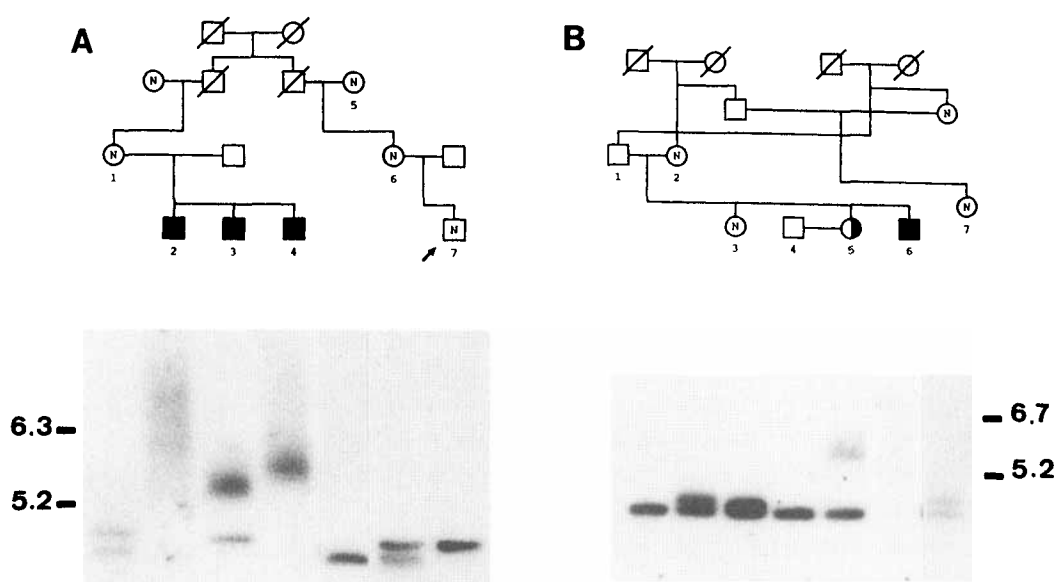

Fig. 2. Pedigrees of Fragile X families. For explanation of the symbols, see legend Figure 1.

Transmitting males, who have no cytogenetic or clinical expression can be detected by using probe pE5.1. They show fragments slightly increased in size. When this fragment is passed on to their daughters, often a change is seen in the size, although it stays in the premutation range, with fragment sizes increased roughly between 150 and 300 bp [Oberlé et al., 1991; Fu et al., 1991]. Patients in the next generation show much larger fragment lengths. Apparently, the increase in size in the CGG repeat has to pass a certain critical size to result in the fra(X) phenotype. When this limit is reached is not clear yet, but it is thought to be around a size of 500 bp [Oberlé et al., 1991].

In Fig. 2B, a pedigree is shown which illustrates that passage of the fra(X) allele from one generation to another is not always accompanied by an increase of the fragment length. In the woman B2, besides a normal allele, an allele with an increased size is observed. Two of her children, B4 and B5 have cytogenetic fra(X) expression and both show smears composed of different enlarged fragments. In the daughter B3 a clearly visible increased fragment is seen that is, however, smaller than in her mother. In this case we find a reduction in the fragment size. In B6 also an allele of increased size is found indicating her as a carrier.

Our analysis shows that the detection of abnormal fragments using pE5.1 is a powerful tool in fra(X) diagnosis. Abnormal patterns can be seen in normal transmitting males, female carriers and patients.

In addition, if there is doubt after Southern analysis about the presence of a fragment with a very small increase in size, amplification by PCR of the CGG repeat should give a clearcut answer [Fu et al., 1991]. It should be pointed out, that no amplification by PCR is found if the increase in the CGG repeat is too large. Using both techniques reliable genotyping is possible resulting in very much improved genetic counseling in families affected by fra(X) syndrome.

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