Use of Restriction Fragment Length Polymorphism Analysis for Detecting Carriers of “Fragile X” Syndrome

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Recombinant DNA technology promises to play an increasingly important role in the future of medicine. Application of this technology to the study of human disease will help us to define and clarify the molecular pathology of many clinical disorders, provide new diagnostic tools and approaches, and, finally, will provide new therapeutic agents including gene-replacement therapy. We have begun to exploit these powerful new techniques to aid in the laboratory diagnosis of several genetic disorders for which reliable assays are currently not available, such as “Fragile X” syndrome.

Additional Keyphrases: DNA · heritable disorders · mental retardation · screening

Mental retardation continues to be one of the most common lifelong handicaps in our society. Considering the complexity of the problem, it is not surprising that many different genetic and (or) environmental conditions are known to produce or to be associated with mental retardation (1). The prevalence of mental retardation in the general population is approximately 3%. Most of these individuals (90%) are mildly retarded, with the remainder being more severely affected. Moreover, mental retardation is unequally distributed between men and women (2). It is estimated that there are 25%–30% more affected males than females, the majority of which are thought to have disorders linked to the X chromosome. Thus, nearly 17 out of every 1000 males will be affected with some form of mental retardation, and two to three of these will have an X-linked disorder.

In 1943, Martin and Bell (3) documented the first kindred with an X-linked form of mental retardation (the mentally retarded individuals in this family were later shown to exhibit fragile sites on the X chromosome plus the associated features of macro-orchidism and the characteristic facial features). The investigation of X-linked mental retardation (XLMR) increased dramatically in the following 20 years as the importance of these diseases was being recognized and, in 1969, several authors reported on an apparent association of macro-orchidism with XLMR (4, 5). There was an important advance in the study of XLMR in this same year, when Lubs (6) described a fragile site on the long arm of the X chromosome and its apparent association with XLMR. Ten years later, with the advent of more reliable cytogenetic procedures for the expression of fragility, the marker X chromosome and large testes were observed to occur together. Thus, by the late 1970s it was becoming apparent that one particular syndrome, now commonly referred to as “Fragile X” syndrome, could be identified by both its distinct clinical features and by the presence of the marker X chromosome (for general reviews, see refs. 7, 8).

Although a host of conditions give rise to XLMR, the most common cause appears to be Fragile X syndrome. Data from several studies (9, 10) suggest that this syndrome accounts for nearly half of all XLMR, and while the prevalence of this syndrome has been difficult to estimate, current calculations from recent studies (6) indicate that about one in 1100 males and one in 700 females will carry the gene for Fragile X syndrome. These are incredible statistics and, although further studies are required to confirm these values, it is becoming increasingly evident that Fragile X syndrome is one of the leading chromosomal aberrations leading to mental retardation.

Clinical Features

Males with Fragile X syndrome exhibit a wide range of intellectual function. The degree of cognitive defects ranges from severe learning disabilities to profound retardation, with most patients showing moderate to severe retardation. Individuals with gentle, sweet natures are described in the literature, but violent behavior can also occur. A characteristic facial appearance includes a high and prominent forehead, prognathism, and protruding ears. Macro-orchidism, the hallmark of the Fragile X syndrome, is detected in approximately 80% of the patients and is most consistently found during and after puberty. Other common features of this syndrome include hyperactivity, hand biting, autism, hyperextensible joints, and mitral valve prolapse (7).

The female carriers of Fragile X syndrome have not been studied as extensively as a group as have the affected males. In general, these individuals range from normal to mildly retarded and usually lack the physical features that characterize the affected male. There are isolated reports, however, of affected females with long or prominent ears, broad foreheads, and prominent jaws. Although most of the females carrying the gene for Fragile X syndrome are thought to be normal, many may have learning disabilities that, until recently, have not been appreciated (7, 11, 12). The degree to which a female is affected clinically has also been shown to be somewhat related to the percentage of lymphocytes that express the fragile site on the X chromosome: the greater the percentage, the more severe the clinical involvement (13).

Cytogenetics

As noted above, the identifying marker on the X chromosome was first described by Lubs in 1969. This marker, a fragile site on the long arm of the X chromosome (q27.3), is seen as a nonstaining gap or break in the chromatin structure. As first shown by Sutherland (14), critical control of cell-culture conditions is essential to maximize the expression of the fragile site. Sutherland’s initial experiments showed that fragility was dependent on the concentration of folate acid in the culture media, low concentrations being correlated with increased expression of fragility. Currently, special media and culture conditions—including extended culture times, high pH, low folate acid, and the incorporation of fluorodeoxyuridine—are utilized to maximize the expression of the Fragile X marker (7).
Even under optimal conditions, fragility is not expressed in all cells derived from affected individuals. In fact, the frequency of expression can often be very low. In males with Fragile X syndrome, approximately 100 mitoses of cultured lymphocytes are usually examined, and fragility typically occurs in 10% to 40% of the cells. Furthermore, in approximately 5% of affected individuals, only 1%-4% of the cultured cells may express fragility. In addition to the low expression of the Fragile X marker in some of the affected males, current evidence suggests that nonpenetrance (absence of clinical features) of the disease (including fragility) may be as high as 20% (15).

Examination of the heterozygote female (obligate carrier) has revealed at least two groups. The first includes those individuals who are affected clinically who have a high percentage of their cultured cells expressing the Fragile X site, whereas the second are relatively unaffected, with few or no cells expressing fragility. According to a study by Sherman et al. (16), approximately 50% of obligate carriers with a normal IQ will express fragility in <4% of their cells. Unfortunately, these and other studies have shown that nearly half of the obligate carriers cannot be identified on the basis of either chromosomal abnormalities or mental status.

In light of the high prevalence of Fragile X syndrome, the accurate detection of female carriers and nonpenetrant males becomes extremely important. Until recently, clinical presentation, family history, and chromosomal analysis have been the only means of confirming the diagnosis of Fragile X syndrome. However, chromosomal analysis does not reliably identify unaffected males or female carriers: about 20% of the males and 50% of the females who carry the gene for this syndrome are cytogenetically negative for the Fragile X marker. Thus, a screening method with increased sensitivity is required for proper genetic counseling.

Recombinant DNA Technology

Recombinant DNA techniques, all resulting from the development of screening methods with increased sensitivity and specificity, are now becoming available for the diagnosis of a wide variety of genetic diseases. These powerful and exciting new tools are being used to define clinically useful assays for a number of diseases that are currently difficult to diagnose, in particular Fragile X syndrome.

Two major approaches are utilized for detecting a mutant gene at the DNA level. Direct screens can be used when the genetic defect for a particular disease is well characterized, such as sickle cell anemia (point mutation) or a-thalassemia (deletion). An unambiguous diagnosis of the disease can usually be made by directly examining the DNA from the affected individual for the presence or absence of the particular mutation. Often, however, a disease process is not the result of a single, easily detected mutation. Rather, many diseases are the result of various mutations, each one being different but at the same time giving rise to a similar if not identical clinical condition. One of the best-characterized examples of this phenomenon is β-thalassemia (17). β-Thalassemia comprises a group of β-globin gene disorders, involving well over 25 distinct mutations in and around the β-globin gene. Given the number of mutations involved, the diagnosis of β-thalassemia would be very difficult to make if each mutation known to cause this particular disease had to be systematically assessed. The problem is further complicated by the fact that the presence of other, as yet unknown mutations cannot be ruled out at the time of the analysis. Finally, for many diseases, such as Fragile X syndrome, the gene(s) responsible for the disease has not yet been described.

When simple, direct probing techniques are insufficient—as is the case with most genetic defects—a more global approach to the problem is necessary. The approach most widely utilized is a general linkage analysis, with recombinant DNA technology again providing new and exciting tools. In this approach, a mutant gene is detected indirectly by establishing a linkage relationship between a well-defined DNA marker and the gene of interest, even if that gene is not well characterized. The markers used for this purpose are naturally occurring variations in the nucleotide sequence of DNA (DNA polymorphisms). These polymorphic sites occur randomly throughout the human genome (estimates range from one to 10 sites per 1000 base pairs), have no apparent phenotypic effect, and are inherited in mendelian fashion. We can detect these polymorphic variants in DNA sequences and then use them as genetic markers. Most variants observed, because of their ease of detection, are polymorphisms that create or destroy a recognition site for a restriction enzyme. Restriction endonucleases are enzymes that recognize specific DNA sequences and cut the DNA only at those specific sites. The creation or destruction of a restriction site by a single nucleotide change (polymorphic variant) can then be identified by monitoring the resulting restriction fragments for alterations in their size. Currently, these sequence variants are detected by the method of Southern (18), where a recombinant DNA probe defines the region of interest and the restriction enzyme defines the observed sequence variant (Figure 1). An analysis of the DNA described in Figure 1, therefore, would show this particular individual to be heterozygous for the polymorphic marker (i.e., the recognition site for the enzyme is present on one of the X chromosomes and absent on the other), with the fragments detected by the probe being assigned to two alleles, A and a.

The laboratory approach to detecting these polymorphic sites is well established and straightforward (Figure 2). Briefly, human DNA is isolated from peripheral blood and then digested with a restriction endonuclease. The resulting DNA fragments are separated by electrophoresis, transferred to a solid support (such as nitrocellulose paper), and then challenged with a radiolabeled DNA probe derived from the particular chromosomal region that carries the polymorphic marker. The labeled DNA probe, which will hybridize only to complementary sequences, can then be viewed on x-ray film after autoradiography. Using these techniques, one can directly determine the inheritance of

![Fig. 1. Recognition sites for a restriction enzyme on a pair of homologous X chromosomes](image)
each variant and therefore track the inheritance of a specific region of the chromosome surrounding that polymorphism.

To be useful, however, a polymorphic marker must be located close to the defective gene, so that the two loci will co-segregate in a mendelian fashion. When these two loci are sufficiently close together on a chromosome such that the segregation of their alleles in family members is not randomized by genetic recombination, then these loci are said to be linked. Theoretically, any genetic disease (regardless of whether the gene or genetic defect has been characterized) can be diagnosed by establishing a useful linkage relationship between a polymorphic marker and a defective gene. Several hundred genetic markers distributed throughout the human genome have now been characterized (19,20) and are now available for these types of studies. Many of these were found during the characterization of specific cloned genes, but most have been derived from a systematic search of arbitrary cloned DNA fragments not associated with any particular genetic locus.

Figure 3 illustrates a simplified linkage study of an X-linked disorder such as Fragile X syndrome with use of a marker having alleles A and a. The following components are necessary for a successful linkage analysis: (a) there must be an affected member of the family (in this case a son) with an unambiguous phenotype; (b) the female obligate carrier must be a heterozygote for the sequence variant (i.e., A/a) so that the two chromosomes can be distinguished from each other; and (c) the recombination frequency between the marker and the disease locus must be very low (ideally being zero). An examination of the DNA from the affected male in this case reveals that allele A is associated with the chromosome carrying the defective locus. Thus, any offspring of the obligate carrier (mother) who inherits allele A will also inherit the mutant gene. The female sibling in this family would then be identified as a carrier for the disease in question, even in the absence of any clinical evidence for the disease, because she has inherited allele A from her mother. Remember that the marker and the disease locus will co-segregate only in the absence of any recombinational events between them.

Current Investigations

As indicated earlier, we are currently investigating the utility of the approach for the carrier detection of Fragile X syndrome. Some of the markers that have been localized to the distal portion of the X chromosome (Figure 4) and that would thus be potential markers for this syndrome include the genes for glucose-6-phosphate dehydrogenase (EC 1.1.1.49), for hypoxanthine phosphoribosyltransferase (EC 2.4.2.8), and for coagulation Factors IX and VIII (F9, F8), and the arbitrary DNA fragments 52A, DX13, St14, 4D-8, and cx55.7 (20). This list is far from complete (new markers are emerging at an incredible rate!); it is meant only to illustrate the power of this technique.

Initially, the most promising marker appeared to be a Taq I polymorphism identified within the gene for F9 (Factor IX). Data from Camerino et al. (21) suggested that the recombination frequency between Factor IX and the Fragile X locus P(x) was very likely to be <10%. Several studies (22-28), however, have shown a greater number of recombinants, indicating that the two loci are farther apart than was first thought, and more recently, significant genetic heterogeneity has been demonstrated on the centromeric side of the Fragile X locus (28). To date, the markers that appear to map closest to the Fragile X locus include F9, cx55.7, 4D-8, and St14 (26, 29). Despite tremendous efforts to identify closer markers, nearly all of the polymorphisms examined so far have been mapped as being very close to either the F9 locus or to the St14 locus.

We have selected a number of families with a history of Fragile X syndrome to study the linkage relationship of five polymorphic markers (52A, F9, cx55.7, 4D-8, and St14) to the Fragile X locus (ms. submitted for publication). Our primary objectives for this study have been to define more clearly (by adding to the current data base) the linkage map for these loci and to examine in more detail the question of genetic heterogeneity. The recombination frequencies for these various markers must be defined with a high level of confidence if we are to use them as diagnostic markers of inheritance.

Figure 5 shows the results of a representative Southern analysis for the polymorphic markers 52A, F9, and St14. A total of 17 pedigrees (nearly 300 specimens) have now been examined for all five of these markers (ms. submitted); an example of one of the pedigrees is shown in Figure 6. Our data are consistent with the observations of other investiga-
Although linkage was demonstrated by the molecular techniques described earlier, our goal was to continue to explore new markers, closer to the Fragile X locus, with the hope that a routine assay capable of identifying all carriers of this syndrome will soon be available. Unquestionably the use of recombinant DNA technology is revolutionizing the study of human disease.

Fig. 5. Southern blot analysis of DNA from three individuals as performed with the DNA probes 52A, F9, and St14. DNA was isolated, digested with Taq I, and subjected to Southern blot analysis. The numbers to the left of each panel represent the fragment size for the various restriction fragment length polymorphism alleles. The 52A probe detects a 1.3 and (or) a 0.7 + 0.6 Kb fragment (B and b, respectively), F9 detects a 1.8 and (or) a 1.3 Kb fragment (A and a, respectively), and St14 defines a highly polymorphic locus with approximately 10 separate alleles (between 3 and 8 Kb long).

Fig. 6. A Fragile X pedigree, showing segregation of three DNA markers (52A, B and b; F9, A and a; and St14, 4 and 8) to the Fragile X locus. □, ○ normal male and female, respectively; □, ○ individuals that demonstrate the Fragile X in cultured lymphocytes, the number of positive cells being shown below each individual (e.g., 29/100 = 29 cells demonstrated fragility out of 100 cells examined); ◯, ◯ mentally retarded; and ○ learning disabilities.

tors, and suggest that the linkage between F9 or St14 and the Fragile X locus is not as tight as one would like to see, both recombining with the Fragile X locus with a frequency of 15%–20%. Moreover, the linkage between F9 and F(x) shows substantial genetic heterogeneity, with some families exhibiting very tight linkage while others show very loose linkage. The average is close to the recombination fraction of 0.15 reported earlier. Figure 7 schematically represents this phenomenon, first described by Brown et al. (26).

Fig. 7. Genetic map of several DNA markers flanking the fragile X locus [FRA(X)]. "Tight" and "loose" pedigrees show tight or loose linkage between the F9 and Fragile X locus. All of the recombinant fractions shown were derived from two-point linkage analyses. The numbers below the lines refer to the recombination fraction for each of the linkage groups shown. [Reproduced by permission of W. T. Brown (26)].

Clearly one must be very careful in interpreting linkage data for clinical purposes, because the true recombination frequencies between these markers and the Fragile X locus are still not adequately described, and the rate of double recombinants (obtained by using both flanking markers) may be much higher than anticipated.

Although these markers may not be close enough to the Fragile X locus to provide the best possible information for genetic counseling, these findings do contribute new and useful information concerning the molecular basis of this disease. Our long-term goal is to continue to explore new markers, closer to the Fragile X locus, with the hope that a routine assay capable of identifying all carriers of this syndrome will soon be available. Unquestionably the use of recombinant DNA technology is revolutionizing the study of human disease.

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References