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New Polymorphisms at the DXS98 Locus and Confirmation of Its Location Proximal to FRAXA by In Situ Hybridization

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Summary

The locus DXS98, detected with the 1.5-kb anonymous probe p4D-8, was recently shown to be closely linked and proximal to the locus for the fragile X syndrome, with θ = .05 at lod = 3.406, by utilizing a limited number of meioses informative for a two-allele *Msp*I RFLP. Because DXS98 may be the closest available marker to the fragile X locus (FRAXA), we sought to increase its utility for linkage studies by extending its PIC and confirming its localization to Xq27, proximal to FRAXA. We have isolated 15 kb of genomic DNA (λ4D8-3) from the DXS98 locus by using p4D-8 to screen a genomic phage library containing partial *Sau*3A-digested human DNA. Three additional RFLPs for the enzymes *Bgl*II and *Xmn*I were found by using the entire λ4D8-3 as probe. Combined heterozygosity for the four RFLPs in 25 unrelated females was 48%, as compared with only 28% when the *Msp*I RFLP alone was used. In situ hybridization of unique sequences from λ4D8-3 was performed on metaphase chromosomes of lymphocytes and lymphoblasts from patients with the fragile X syndrome. Grains on the X chromosome were significantly clustered at band Xq27. Following fragile site induction, all nine grains in the q27-28 region were proximal to the fragile site. Confirmation of the location of DXS98 proximal to FRAXA and the new RFLPs at this locus make DXS98 more useful for linkage analysis and physical mapping in the region of the fragile X mutation.

Introduction

Detection of the fragile X mutation in individuals known through pedigree analysis to be at risk is made difficult by a number of genetic and technical factors. The inadequate sensitivity of cytogenetic analysis for the presence of the fragile site at Xq27 is well documented (for summary, see Nussbaum and Ledbetter 1986). As an adjunct diagnostic tool, linkage analysis of flanking RFLPs has been useful in many families. Previous studies, most recently summarized in Human Gene Mapping 9 (1987) and by Opitz et al. (1988), have demonstrated measurable linkage of the fragile X locus (FRAXA) with DXS52 (St14), DXS15 (DXS13), and F8, which form a tightly linked cluster 10–15 cM distal to FRAXA, and with the proximal markers DXS51 (52A), F9, and the more recently described DXS105 (cpX55.7) (Hofker et al. 1987; Veenema et al. 1987; Arveiler et al. 1988).

The first report on the linkage of DXS98 with FRAXA was by Brown et al. (1987*b*). The probe 4D-8, isolated in our laboratory and detecting a two-allele *Msp*I RFLP (Boggs and Nussbaum 1984), was shown to have tight linkage to FRAXA, with a peak lod score of 3.406 at θ = .05 (95% confidence interval <14.5%). Because of the low heterozygosity (27% of females in their sample) at this locus, there were only 23 informative meioses among 33 families tested with the single *Msp*I RFLP. These data, if substantiated, would make DXS98 the closest available marker to FRAXA.

To expand the utility of the DXS98 locus for linkage analysis and physical mapping of the region surrounding FRAXA, we sought to increase the PIC of the locus

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and to confirm its localization to Xq27, proximal to the fragile site, by in situ hybridization.

Methods

Isolation of λ4D8-3 and λ4D8-15

DNA from GM1416, a 48,XXXX lymphoblastoid line (Institute for Medical Research, Camden, NJ) was subjected to partial *Sau*3A digestion. Fragments in the size range 15–23 kb were isolated and ligated to *Bam*HI-digested DNA from the substitution vector EMBL-3 after treatment with calf intestinal phosphatase. Recombinants were isolated by Spi selection on the P2 lysogen host NM539. The library contained 2×10^6 independent plaques and was subsequently amplified. The 1.5-kb *Hin*dIII insert of p4D-8 was isolated in low-melt agarose, radioactively labeled by the random priming technique (Feinberg and Vogelstein 1983, 1984), and used as a probe to screen nitrocellulose filters made from the plated genomic library grown in LE392. The filters were prewashed in 1M NaCl, 50 mM Tris pH 8.0, 1 mM EDTA, and 0.1% SDS at 42°C for 1 h, prehybridized in 50% formamide, 5 × SSPE, 10 × Denhardt's solution, 1% SDS, and 100 mg herring sperm DNA/ml overnight at 42°C, and then hybridized to the probe for another 18–24 h in the same buffer. Excess probe was rinsed in 2 × SSC/0.1% SDS for 40 min at room temperature and then for an additional 40 min at 65°C before autoradiography.

Of six positive clones obtained, two, λ4D8-3 and λ4D8-15, were plaque purified (Benton and Davis 1977). DNA was prepared from miniprep phage lysates via standard polyethylene glycol procedure. Before further study, λ4D8-3 and λ4D8-15 were both mapped to distal Xq by hybridization to a Southern blot panel containing *Taq*I-digested DNA samples from the following: (1) GM4025, a fragile X male (Institute for Medical Research, Camden, NJ), (2) 4.12, a somatic cell hybrid containing the entire X chromosome from GM4025 as the only human chromosome in a Chinese hamster (RJK88) background (Nussbaum et al. 1983), (3) X3000-11, a clone derived from 4.12 which contains the region Xq24-qter as the only human DNA (Nussbaum et al. 1986), (4) RJK88, the Chinese hamster V79 parent of 4.12 and X3000-11, (5) a normal 46,XY male, and (6) a normal 46,XX female. Both probes hybridized to all DNA samples except the hamster parent RJK88 and produced identical fragment sizes (data not shown).

Screening for New Polymorphisms

Southern blot panels of human genomic DNA containing 14 or 17 unrelated X chromosomes were screened for polymorphisms by using the entire λ4D8-3 as probe. Subsequent to RFLP detection, another 22 or 24 unrelated X chromosomes were studied to estimate population frequencies. Approximately 5 μg of DNA from each subject was digested with the following 14 enzymes: *Taq*I, *Msp*I, *Bgl*II, *Rsa*I, *Hin*dIII, *Pst*I, *Bcl*I, *Pvu*II, *Xmn*I, *Sac*I, *Bam*HI, *Eco*RI, *Hin*cII, and *Xba*I. Fragments were resolved by electrophoresis in 1% agarose gels at 2 V/cm for 16–20 h and transferred in 10 × SSC to Zetabind filters. Prehybridizations were carried out overnight in buffer containing 0.5% nonfat dry milk, 50% formamide, 4 × SSPE, 1% SDS, and 100 mg herring sperm DNA/ml at 42°C. Hybridizations to oligo-labeled DNA from λ4D8-3 (containing a 15-kb *Sal*I/*Bam*HI insert) were performed in the same buffer with 10% dextran sulfate for 24–36 h after preannealing of the probe to an excess of human placental DNA (Litt and White 1985). Oligo-labeled λ4D8-15 was also hybridized to *Taq*I, *Msp*I, *Bgl*II, *Rsa*I, *Hin*dIII, and *Pst*I digests in the same manner.

Unbound probe was rinsed in 2 × SSC/0.1% SDS at room temperature for 40 min, in 2 × SSC/0.1% SDS at 65°C for 15 min, and then in 0.1 × SSC/0.1% SDS at 65°C for an additional 30 min. Filters were air-dried and autoradiographed for 3–7 days at −80°C using an intensifying screen.

In Situ Hybridization of p4D8-IV

A peripheral blood sample and a lymphoblast cell line from two unrelated fragile X–positive males were used. Fragile X expression was induced by conditions of thymidylate stress as described elsewhere (Ledbetter et al. 1986).

The probe was labeled by random oligonucleotide priming with all four tritiated nucleotides to a specific activity of 10^8 cpm/μg. Hybridization to metaphase chromosomes was carried out by the method of Harper and Saunders (1981). Slides were dipped in Kodak NTB2 emulsion and exposed at 4°C for 4–5 wk. After standard development, slides were banded by the sodium borate method of Cannizzaro and Emanuel (1984).

Results

New Polymorphisms and Restriction Mapping of the DXS98 Locus

λ4D8-3 detected three new polymorphisms (table 1)

Table 1

Polymorphisms and Allele Frequencies at DXS98

Enzyme (No. of X Chromosomes) and Allele	Size (kb)	Frequency
*Msp*I (55):		
A1	18	.84
A2	10 + 8	.16
*Bgl*II (53):		
B1	4.4	.81
B2	3.8	.19
*Xmn*Ia (55):		
C1	8.5	.96
C2	5.6	.04
*Xmn*Ib (53):		
D1	1.75	.96
D2	1.8	.04

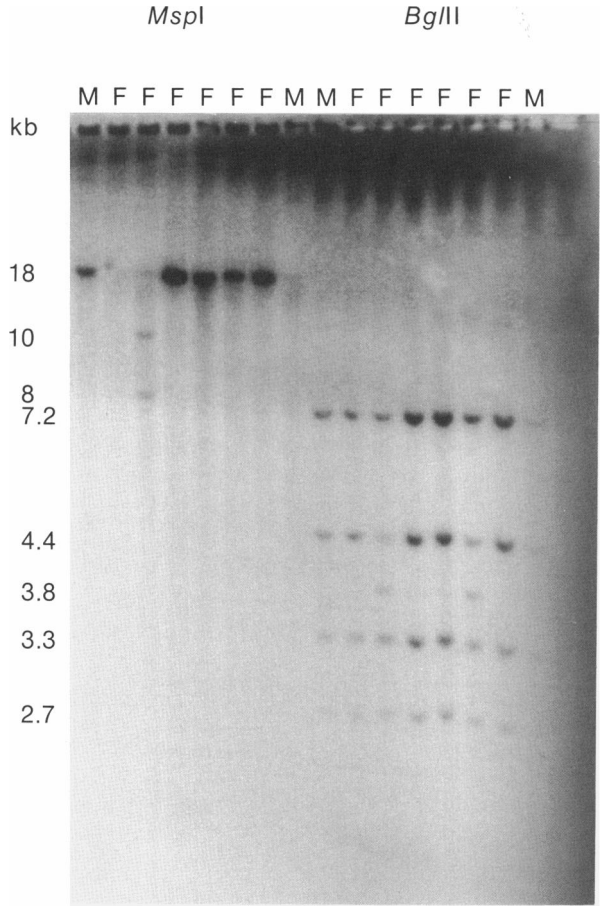

Figure 1 Autoradiograph of *Msp*I and *Bgl*II polymorphisms detected on a Southern blot screening panel, using the 15-kb λ4D8-3 preannealed to an excess of human placental DNA as the hybridization probe.

in the panels of 14 or 17 unrelated X chromosomes in addition to the original *Msp*I polymorphism which was detectable as either an 18-kb allele (A1, previously reported as 25 kb) or as an allele consisting of two component fragments (A2 = 10 kb + 8 kb) (fig. 1). New polymorphisms were all confirmed to be segregating in an X-linked Mendelian pattern. *Bgl*II produced alleles of 4.4 kb (B1) or 3.8 kb (B2). Two different RFLPs were found with *Xmn*I. One of these (fig. 2) had alleles of either 8.5 kb (C1) or 5.6 kb (C2). This RFLP was also detectable by the original p4D8 probe. The other *Xmn*I polymorphism (fig. 3) was defined by either a 1.75-kb fragment (D1) or a 1.8-kb fragment (D2).

Population frequencies for the B1,B2 and C1,C2 polymorphisms (table 1) were estimated by screening totals of 53–55 unrelated X chromosomes, including those of 25 unrelated females. Frequencies were as follows: B1:B2 = .81:.19 and C1:C2 = .96:.04. The two alleles of the D1,D2 RFLP also had frequencies of .96 and .04, respectively.

Combined heterozygosity for all four RFLPs in the 25 unrelated females was 48%, a substantial increase over the 28% heterozygosity found when *Msp*I alone was used.

Although the sample size is too small for formal statistical evaluation, it appears that the *Bgl*II and *Msp*I RFLPs are in partial linkage disequilibrium, with 7/25 females heterozygous for both rare alleles and 1/25 homozygous for both rare alleles (A2,A2/B2,B2). There was, however, one woman heterozygous for only the *Bgl*II polymorphism (A1,A1/B1,B2).

Two women were heterozygous only for C1,C2, and another two were heterozygous only for D1,D2. Thus, these two *Xmn*I allelic systems were primarily responsible for the overall increase in heterozygosity at the DXS98 locus and are seemingly in complete linkage equilibrium both with each other and with the *Msp*I and *Bgl*II alleles, despite the low frequencies of their rarer alleles.

λ4D8-3 was subcloned into pUC19 as an 8-kb *Sal*I/*Bam*HI fragment, p4D8-B, and a unique-sequence 7-kb *Bam*HI/*Bam*HI fragment, p4D8-IV, which contains only part of the original 1.5-kb *Hin*dIII p4D8 fragment at one end (fig. 4). p4D8-B still required preannealing for use as a probe for Southern blot hybridizations. One *Bam*HI/*Xba*I subfragment of p4D8-IV (p4D8-IVe) was isolated from low-melt agarose gels to more clearly detect the D1,D2 polymorphism. This polymorphism was best seen when electrophoresis was carried out in 1.5% agarose gels in which the 1.35-kb *Hae*III fragment of PhiX174 DNA had migrated at least 16 cm.

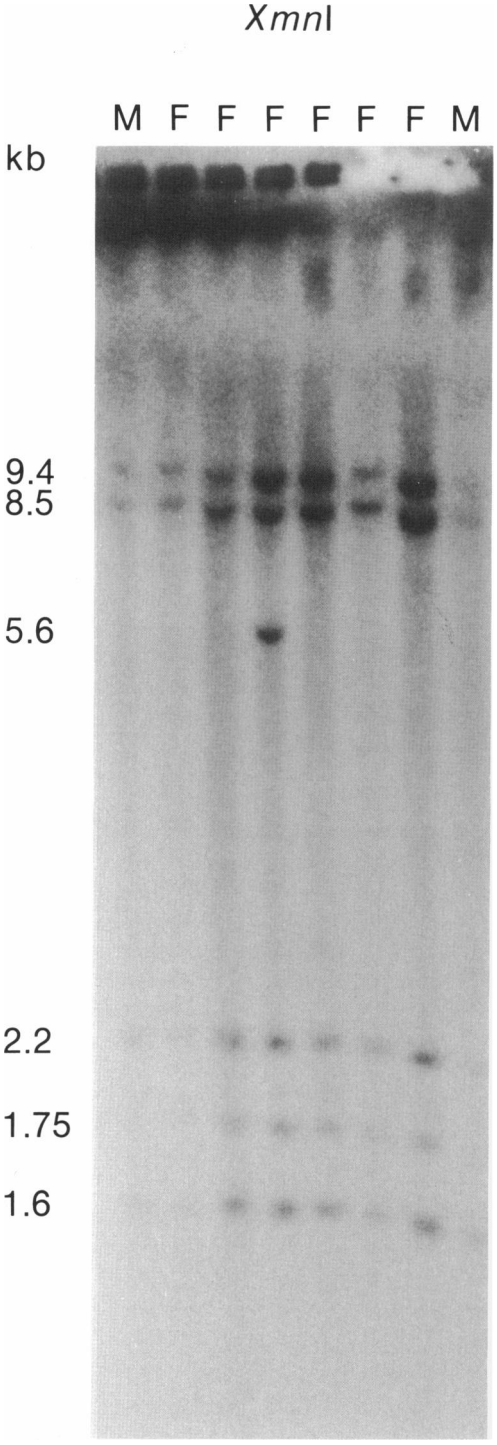

Figure 2 Autoradiograph of the C1,C2 *Xmn*I polymorphism, using preannealed λ4D8-3 as hybridization probe.

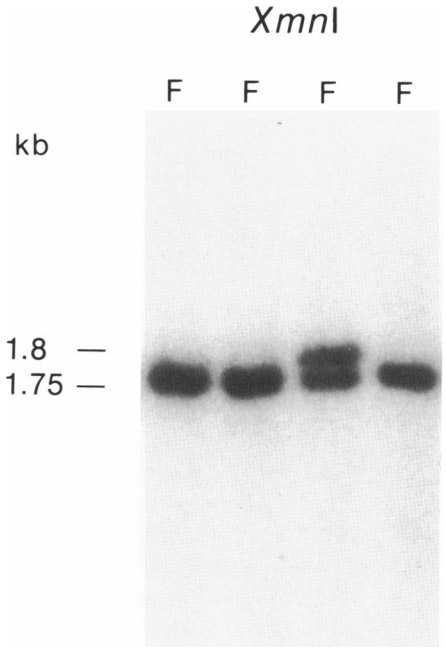

Figure 3 Autoradiograph of the D1,D2 *Xmn*I polymorphism. Here, the *Bam*HI/*Xba*I fragment p4D8-IVe was used as the probe on a blot made after a long run on a 1.5% gel.

With the enzymes tested, λ4D8-15 did not detect any polymorphisms that were not detected by λ4D8-3, and the probe appeared to contain a larger amount of repetitive sequence.

In Situ Hybridization of p4D8-IV

Only grains on the X chromosome were scored as to band localization. Of 24 grains identified on the X

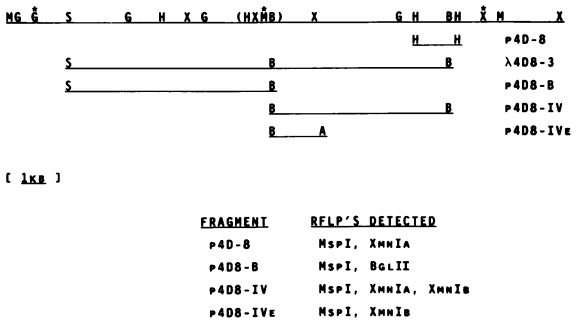

Figure 4 Partial restriction map of the DXS98 locus and polymorphisms detected by the subclones. * = Polymorphic site; M = *Msp*I; X = *Xmn*I; B = *Bam*HI, G = *Bgl*II; A = *Xba*I; H = *Hin*dIII; S = *Sal*I. The D1,D2 polymorphism is located at one of the two *Xmn*I sites surrounding the *Bam*HI site used to subclone the phage. Note that only part of the original p4D-8 probe is present at one end of the phage clone.

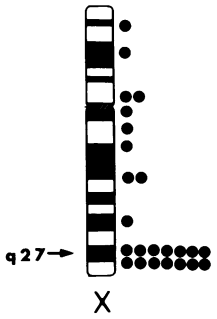

Figure 5 In situ hybridization of p4D8-1V. A total of 24 grains are plotted on an ideogram of the X chromosome at approximately the 400-band stage of resolution (Harnden and Klinger 1985).

chromosome, 14 (58%) were at band Xq27 (fig. 5). A total of nine cells that were both fragile X positive and had a grain in the region of Xq27 were observed. In all nine cells, the grain was proximal to the fragile site.

Discussion

Expansion of the DXS98 locus through the technique of chromosome walking described herein has resulted in the detection of three new allelic systems in addition to the original *Msp*I polymorphism. These new polymorphisms will make linkage analysis applicable to a significantly greater number of families than was previously possible. The issue of genetic heterogeneity of the fragile X syndrome (Brown et al. 1985, 1986, 1987*a*) will also be more easily addressed. Our verification by in situ hybridization of the location of DXS98 proximal to the fragile site at Xq27 allows for its consideration as a close flanking marker to FRAXA when more distal loci such as DXS52, DXS15, and F8 are also evaluated. If subsequent linkage analysis confirms the 5-cM recombination distance between DXS98 and FRAXA as initially determined by Brown et al. (1987*b*), DXS98 will be the closest available probe to the fragile X locus. The locus will also be more applicable to linkage analysis of other diseases in this region of the X chromosome.

The basis of the linkage disequilibrium that we observed between the *Bgl*II and *Msp*I RFLPs is not clear. These two polymorphic sites are separated by about 9 kb (fig. 4) while the two *Xmn*I RFLPs (one located quite near to the *Msp*I site and the other separated from it by about 7 kb) are seemingly in equilibrium in the small sample studied.

More recent linkage studies of the DXS98-FRAXA interval, as well as physical relationships in this region, were summarized by Davies et al. (1988) and Opitz et al. (1988). Linkage analysis has been limited by the previously low heterozygosity of the probe.

One large study of over 300 individuals in four reference pedigrees by using eight polymorphic markers from distal Xq including 4D-8 (Murphy et al. 1987) was unable to localize DXS98 in the region around FRAXA. However, other studies seem to confirm the initial data of Brown et al., with an average estimated recombination distance of 5 cM (Davies et al. 1988). N. J. Carpenter (personal communication) reported one individual that was recombinant between DSX105 and DXS98, but not between DXS98 and FRAXA. In addition, in her multipoint analysis, the order F9-DXS105-DXS98 had a relative odds ratio of 229 compared with the order F9-DXS98-DXS105. In a newer study, multipoint analysis in a study of 40 fragile X families (Brown et al. 1988) localized DXS98 between F9 and FRAXA, but only with a relative odds ratio of 17 compared with the order DXS98-F9-FRAXA or of 7.7 compared with the order F9-FRAXA-DXS98. In their two-point analysis, the same group calculated a θ of .06 at a maximum lod score of 3.12 for DXS98 with FRAXA. Mulley et al. (1988) estimate a θ of .01 at a maximum lod score of 4.79 for DXS98 and FRAXA in eight informative kindreds. Thus, DXS98 appears to be the closest locus to FRAXA, as established by linkage analysis, despite the limitations imposed by its low heterozygosity.

Physical mapping of p4D-8 to Xq26-qter was initially achieved using a somatic cell hybrid panel (Boggs and Nussbaum 1984). However, the precise physical location of DXS98 with respect to the fragile site at Xq27.3 had not been confirmed prior to the present study, although there is other suggestive evidence concerning its physical location. Schwartz et al. (1987) reported an individual with karyotype 46,X,inv dup(X) (pter→q27::q27→q21). Dosage analysis on Southern blots of DNA from this individual suggested that DXS51 and F9 were present in three copies, DXS105 in two copies, and DXS98 and DXS52 in only one copy each. Patterson et al. (1987, 1988*b*) recently reported two individuals with terminal Xq deletions in the region Xq26-q27 who retained DXS105 and DXS98 while losing more distal markers including DXS52, thus confirming the more proximal location of these loci. Another terminal-deletion patient retained F9 but lost DXS105 and DXS98.

As recombination across FRAXA may be disproportionate to physical distance (Hartley et al. 1984), an observed recombination distance of 5 cM between DXS98 and FRAXA may represent a physical distance

of substantially less than 5,000 kb. This genetic distance may be amenable to further study by techniques such as chromosome jumping (Poustka and Lehrach 1986) and additional analysis by pulsed-field gradient gel electrophoresis (Anand 1986) in the hope of isolating sequences even closer to, or responsible for, the fragile X syndrome.

Note added in proof.—Since submission of this article, Patterson et al. (1988*a*) reported a large fragile X kindred in which θ for the DXS98-FRAXA interval was .03 at a maximum lod score of 4.95 (95% confidence interval .00–.16), thus providing further support for close linkage between DXS98 and the fragile X syndrome.

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