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Four Chromosomal Breakpoints and Four New Probes Mark Out a 10-cM Region Encompassing the Fragile-X Locus (FRAXA)

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Summary

We report the validation and use of a cell hybrid panel which allowed us a rapid physical localization of new DNA probes in the vicinity of the fragile-X locus (FRAXA). Seven regions are defined by this panel, two of which lie between DXS369 and DXS296, until now the closest genetic markers that flank FRAXA. Of those two interesting regions, one is just distal to DXS369 and defined by probe 2-71 (DXS476), which is not polymorphic. The next one contains probes St677 (DXS463) and 2-34 (DXS477), which are within 130 kb and both detect *TaqI* RFLPs. The combined informativeness of these two probes is 30%. We cloned from an irradiation-reduced hybrid line another new polymorphic probe, Do33 (DXS465; 42% heterozygosity). This probe maps to the DXS296 region, proximal to a chromosomal breakpoint that corresponds to the Hunter syndrome locus (IDS). The physical order is thus Cen–DXS369–DXS476–(DXS463,DXS477)–(DXS296,DXS465)–IDS–DXS304–tel. We performed a linkage analysis for five of these markers in both the Centre d'Etude du Polymorphisme Humain families and in a large set of fragile-X families. This establishes that DXS296 is distal to FRAXA. The relative position of DXS463 and DXS477 with respect to FRAXA remains uncertain, but our results place them genetically halfway between DXS369 and DXS304. Thus the DXS463–DXS477 cluster defines presently either the closest proximal or the closest distal polymorphic marker with respect to FRAXA. The three new polymorphic probes described here have a combined heterozygosity of 60% and represent a major improvement for genetic analysis of fragile-X families, in particular for diagnostic applications.

Introduction

The cloning of sequences associated with the fragile-X mental retardation syndrome is an important issue, because of both the high frequency of the disease and the many questions raised by its peculiar inheritance (Nussbaum and Ledbetter 1986; Opitz et al. 1990). Very close probes are needed as starting points for chromosome-walking or -jumping strategies. The nearest proximal probe described up to now, RN1 (DXS369; Oostra et al. 1990), maps at about 5 cM from the disease locus, while the closest distal probe appears to be VK21 (DXS296; Suthers et al. 1989). The genetic distance between DXS296 and the fragile-X syndrome locus (FRAXA) is not precisely known, because of the relatively low heterozygosity of the probes, and its genetic location distal to FRAXA had yet to be definitively ascertained. We have undertaken to isolate new probes in this region to allow for a better physical analysis and to provide new polymorphic probes for diagnostic purposes. We report the validation of a hybrid cell panel which enables a rapid localization of probes in the DXS369–DXS296 interval. We have identified four new probes in this interval, of which three are polymorphic. By analysis of a large set of normal (Centre d'Etude du Polymorphisme Humain) and fragile-X families we have generated a multipoint genetic map that includes five markers within a 10-cM region around FRAXA.

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Material and Methods

DNA Probes

Probe St677 (DXS463) is a 3.5-kb *Eco*RI insert cloned into the *Eco*RI site of pBR329. The probe is slightly repetitive, hence it is preferable to use a 1.1-kb *Eco*RI-*Bst*XI subfragment. Probes 2-34 (DXS477) and 2-71 (DXS476) are *Eag*I-*Eco*RI fragments cloned in Bluescript (Maestrini et al., in press). Since they are highly repetitive, we isolated subfragments which can be hybridized without competition with an excess of human DNA. The 0.8-kb *Ava*II subfragment of 2-34 and the 1.6-kb *Acc*I subfragment of 2-71 give clear patterns. Probe Do33 (DXS465) was obtained by Alu-PCR amplification (Nelson et al. 1989; also see Results). After amplification the primers were eliminated with Centricon 100 (Amicon®), and the amplified DNA was digested by *Eag*I and cloned in the pBluescript KS+ vector. The repetitive sequences present in the 3.6-kb Do33 insert can be eliminated by *Eag*I-*Bst*NI double digestion, which yields a 2.1-kb fragment, smaller than the 2.3 vector fragment. RN1 (DXS369) was obtained from Dr. B. Oostra (Oostra et al. 1990). The polymorphic probes used for DXS304 and DXS296 have been described elsewhere (Suthers et al. 1989; Rousseau et al. 1990).

Linkage Analysis

Southern blot analysis was performed as described elsewhere (Rousseau et al. 1990), with blotting of digested DNA on diazobenzyloxymethyl paper or Hybond N+™ nylon membranes (Amersham) and hybridization at 42°C in 50% formamide, followed by two washing steps in 0.5 × SSC, 0.1% SDS at 65°C. Linkage analysis was performed with the LINKAGE package v.4.8 (Lathrop et al. 1985). Linkage parameters were those of Vincent et al. (1989). In some possible carrier females (with normal IQ) a very low frequency (1%–2%) of X chromosomes were scored as having the fragile-X marker, a frequency which is only slightly above background levels. We have performed alternate calculations which assume either that these carry the mutation or that their affection status at FRAXA is unknown.

Results

Probe Isolation

Three different cloning strategies were used to generate probes in the region of interest. A systematic search for probes around the FRAXA region was conducted by subcloning inserts from a phage library enriched in X chromosome sequences (ATCC catalog no. 57750) and by physical mapping using a panel of cell lines (see below). Probe St677 is one of 25 tested that map in Xq27-28. Probes 2-34 and 2-71 are part of a set of probes highly enriched in CpG islands and cloned from the somatic hybrid line X3000.1, which contains the Xq24-qter region as sole human contribution (Maestrini et al., submitted). To generate probes more specifically from the FRAXA region, we analyzed a series of 224 hybrid cell lines with 24 probes from the Xq26-q28 region. They were generated by X-irradiation and fusion (Benham et al. 1989; Cox et al. 1989) starting from the somatic hybrid line CL2D that contains a single X chromosome on a hamster background (P. Goodfellow and I. Oberlé, unpublished data). One of these cell lines containing flanking markers of FRAXA was used for DNA amplification between adjacent Alu sequences. As oligonucleotide primers, we used the TC65 sequence (Nelson et al. 1989) and another one derived from it as homologous to the Alu J subclass of sequences (Jurka and Smith 1988). Probe Do33 was cloned this way.

Physical Mapping

We have characterized over the past years an extensive panel of 22 cell lines with chromosomal breakpoints in Xq26-q28, by hybridization to about 40 DNA probes from this region. This allowed us to select three cell lines that are most appropriate for mapping probes in the FRAXA region. The lymphoblastoid cell line LL556 is derived from a male patient with hemophilia B and mental retardation whose X chromosome is deleted for a Xq26.2-q27.2 region, with one breakpoint proximal to the coagulation factor IX gene and the other distal to probe RN1 (DXS369) (Oberlé et al., in press). The somatic hybrid line PeCH N contains a translocation chromosome (Xpter-q27::21q11-qter) (Couturier et al. 1979) with a breakpoint between DXS369 and DXS296 (probe VK21; Suthers et al. 1989). In most experiments the PeCH A hybrid clone which contains the complementary translocation chromosome (21pter-q11::Xq27-qter) was also used for confirmation. The L10B somatic hybrid clone contains the translocated chromosome Xpter-q27.3::Yq11-qter as the sole human contribution, with a breakpoint between DXS98 and DXS369 (Oberlé et al., in press). More recently we used the hybrid line CY34A derived from a female patient with Hunter syndrome (Suthers et al. 1989) and containing the Xq25-q28 region proximal to the break-

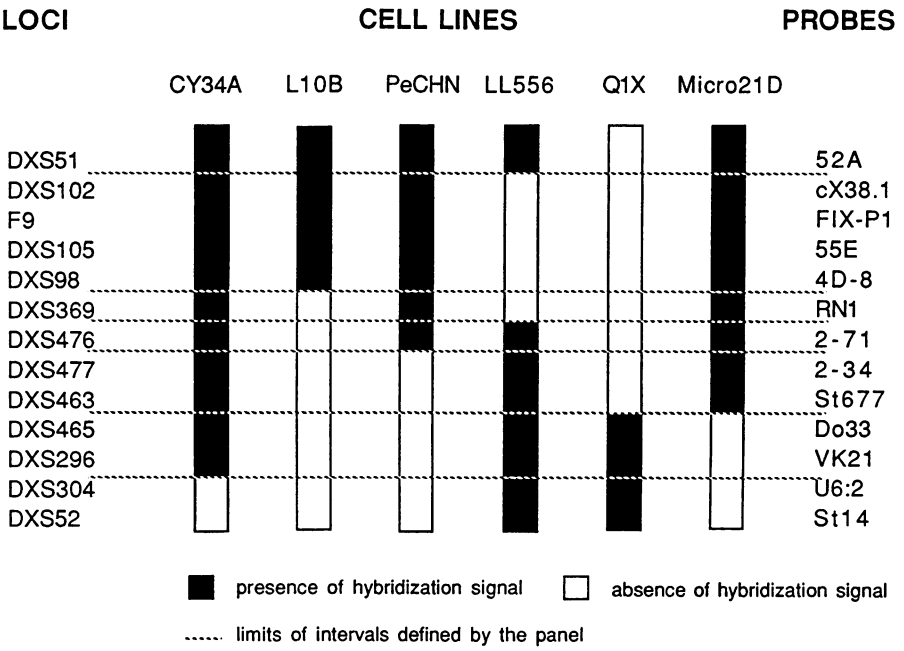

Figure 1 Schematic representation of hybridization data for cell lines panel

point and to the DXS304 locus (probe U6:2). We added to this panel two hybrid cell lines, with breakpoints at or very near the fragile site translocated to a rodent chromosome, generated by breaking the X chromosome from a fragile-X affected male (Warren et al. 1990). Clone Micro21D contains Xpter-q27.3, whereas clone Q1X has only retained Xq27.3-qter.

Using this smaller panel and our set of probes, we identified seven regions and placed four new probes between the FRAXA flanking markers DXS369 and DXS304 (fig. 1). The first region, defined by probe 52A (DXS51), is proximal to the deletion in LL556. A region including DXS102, F9, DXS105, and DXS98 is limited by the proximal breakpoint of LL556 and that of L10B. DXS369 is located between the breakpoints of L10B and LL556. Probe 2-71 (DXS476) was the first probe that allowed us to distinguish between the distal breakpoint of LL556 and the breakpoint in the PeCH lines. A similar result has been very recently obtained using a different probe (Suthers et al. 1990). We have mapped two new probes, St677 (DXS463) and 2-34 (DXS477), in the interval between the PeCH and Q1X or Micro21D breakpoints. No probe was found to distinguish the Q1X and Micro21D breakpoints. The two probes VK21 (DXS296) and Do33 (DXS465) are distal to these latter breakpoints but proximal to the CY34A–Hunter breakpoint. Probe U6:2 (DXS304) and all the more distal Xq28 loci define the seventh region. This panel has thus allowed us to place four probes in the interval between DXS369 (which is about 5 cM proximal to FRAXA) and DXS304 (which is about 5 cM distal) (Dahl et al. 1989; Vincent et al. 1989; Oostra et al. 1990; Oberlé et al., in press).

Probes St677, 2-34, and Do33 Detect RFLPs

Two RFLPs have already been defined at DXS296; they appear in very strong linkage disequilibrium, and the heterozygosity at this locus is 31% (Suthers et al. 1989; and our unpublished results). We have looked systematically for RFLPs for probes St677, 2-34, Do33, and 2-71 by using the rapid strategy described elsewhere (Rousseau et al. 1990). We found *TaqI* RFLPs detected by St677 and 2-34 and a *BglI* RFLP with Do33 (table 1). The RFLPs detected by St677, 2-34, Do33, and VK21A are in complete equilibrium (table 2).

Furthermore, the *TaqI* RFLPs detected by St677 and 2-34 can be assayed in a single hybridization and haplotyped, since we have recently shown that they are physically located within 130 kb (A.V., unpublished results); this simplifies their use in diagnostic or linkage analysis (fig. 2). Their combined heterozygosity reaches 30%. On the other hand, allele 1 detected by VK21A has a size very similar to that of allele 1 of St677, a fact which precludes their use in the same hybridization. The *BglI* RFLP detected by probe Do33 has 42% heterozygosity (table 1).

Table 1

RFLPs Detected by Three New Probes in the FRAXA Region

Probe (locus), RFLP	Allele Size (kb)	Observed Frequency	No. of Chromosomes Tested	Heterozygosity[a]
St677 (DXS463), *Taq*I:				
1	10.0	.91	134	.16
2	5.1	.09		
2-34 (DXS477), *Taq*I:				
1	2.8	.08	104	.15
2	2.0	.92		
Do33 (DXS465), *Bgl*I:				
1	23	.30	82	.42
2	16	.70		

[a] Calculated from allele frequency.

Genetic Mapping of Probes St677, 2-34, and VK21

We have tested the new *Taq*I RFLPs in the families from the Centre d'Étude du Polymorphisme Humain and in a series of fragile-X families. All these families have been previously typed by us using many polymorphic probes in the Xq26-q28 region, including all RFLPs at each of the DXS369 (Oostra et al. 1989; Oberlé et al., in press) and DXS304 (Dahl et al. 1989; Rousseau et al. 1990) loci that flank FRAXA. In all linkage calculations, we haplotyped the data obtained using St677 and 2-34. From now on we will refer to this cluster as DXS463/477.

No recombination was found (table 3) between DXS296 and DXS463/477 (lod score 5.95 [lod score − 1 confidence interval .00–.09]). Of the fragile-X families informative for St677 or 2-34, three showed evidence or high probability of recombination, and a maximum lod score of 7.32 was obtained at a recombination fraction of .038. However, this may be an overestimate due to the difficulty in genotyping at FRAXA. Two recombination events are dependent on the assumption that two women of normal intelligence with either 1% (1/100 mitoses) or 2% (4/200) of fragile sites are carriers, and they were coded as such in this analysis (mode A). A third possible recombination occurred in a young girl in whom no fragile sites were detected but who carries the disease-linked allele. When an alternate calculation (mode B) was performed by coding females with 1% or 2% fragile sites as unknown for affection status, maximum lod score at a recombination fraction of .00 was then obtained between DXS463/477 and FRAXA, indicating no evidence for obligate recombination. With respect to the initial characterization of DXS296, table 3 adds significant data concerning its genetic mapping in the FRAXA region. DXS296 showed evidence of recombination with FRAXA. In this case, for three probable recombination events we faced similar genotyping problems in women. Nevertheless we observed one obligate recombination in an affected boy (the family was not informative for DXS463/477). Depending on the coding of females with very low levels of marker chromosome, maximum lod score for DXS296–FRAXA was 10.42 at a recombination fraction of .057 (mode A) or 12.47 at .020 (mode B). DXS463/477 appears closer to the proximal locus DXS369 than to DXS296. The reverse is observed with the distal marker DXS304, where DXS463/477 is farther than DXS296. These data parallel the physical evidence that DXS463/477 lies proximal to DXS296, but their location with respect to FRAXA remains to be established. A very significant lod score was associated with the genetic distance between the flanking markers DXS369 and DXS304 (lod score 22.3 at a recombination fraction of .09; lod score − 1 confidence interval .049–.147). With good confidence, it can be said that,

Table 2

Pairwise Tests for Linkage Disequilibrium between New Markers in the Vicinity of FRAXA

	χ^2 (P),n[a]		
	DXS465	DXS296	DXS477
DXS463	.04 (.83), 63	.00 (.98), 90	.00 (.99), 95
DXS477	.20 (.65), 64	.40 (.52), 80	
DXS296	.02 (.88), 77		

[a] χ^2 estimated with Yates's continuity correction; $P > .05$ means no significant linkage disequilibrium; n = number of independent chromosomes tested.

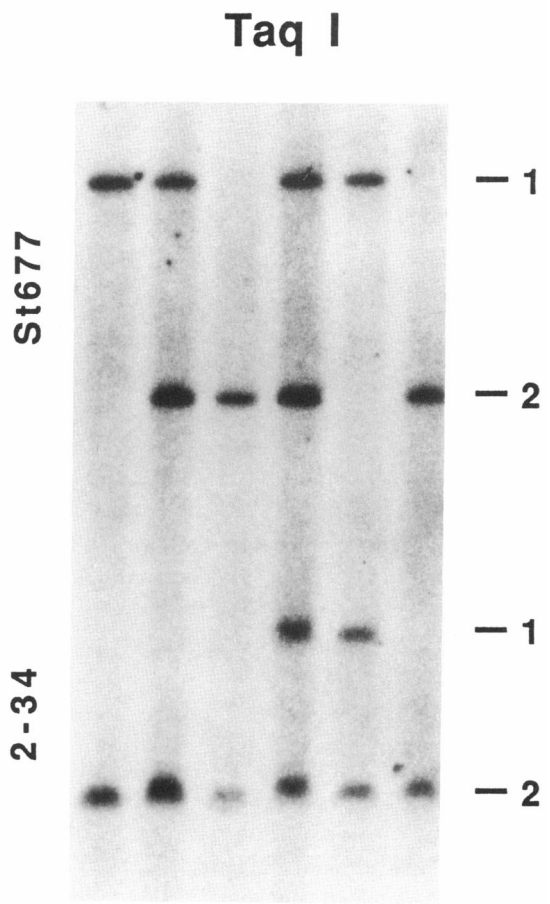

Figure 2 *Taq*I blot hybridized simultaneously to St677 and 2-34. Allele numbers are on the right side of the picture (see table 1 for allele sizes).

genetically, FRAXA lies about halfway between these two markers.

Even with lod scores greater than 10, lod score − 1 confidence intervals overlap and cannot be used to establish order. Thus, using the ILINK program of the LINKAGE package, we performed multipoint analysis to test the genetic order of FRAXA, DXS296, and DXS463/477 with respect to the other previously mapped loci (Rousseau et al. 1990; Oberlé et al., in press) (table 4). The odds in favor of a distal localization of DXS463/477 and DXS296 with respect to FRAXA were at least 57:1 and around 5,000,000:1 respectively, under the coding mode A. However, under mode B, while the distal localization of DXS296 was still favored by 1,200:1, the relative position of DXS463/477 and FRAXA could not be determined. To see whether the multipoint map distances between markers were sensitive to the problem of genotyping at FRAXA, we used the ILINK program to yield several genetic maps of the vicinity around FRAXA, including or not including FRAXA (fig. 3). Very similar map distances between flanking markers were obtained in all cases, which is a good indication of the robustness of our results.

Discussion

The cell hybrid panel we characterized has enabled us to rapidly map physically the new probes that either increase the informativeness of the closest distal region or identify new intervals between DXS369 and DXS304. Probe 2-71 lies in the most proximal of these intervals, but, unfortunately, up to now it has not been polymorphic.

Of the six cell lines described here, two have been specifically generated in conditions favoring the break at or near the Xq27.3 fragile site (Warren et al. 1990). Our data give a much more precise characterization of these breakpoints (fig. 1), which were previously shown to lie between DXS98 and DXS52, i.e., a region of about 30 cM. If no rearrangement has occurred in these lines, our results indicate that DXS463/477 should lie proximal to DXS296. The testing of the other cell lines generated in the experiment of Warren et al. (1990) will tell whether a majority of the breakpoints behave similarly with respect to the new probes described here.

The DXS296 locus was previously proposed to map distal to the fragile site by in situ hybridization. However, half of the grains were found hybridizing either proximally or over the chromosome gap (Suthers et al. 1989). In the absence of other data confirming the distal localization of DXS296 with respect to FRAXA, we undertook genetic analyses with this marker and with RFLPs detected by probes St677 and 2-34, to confirm the order given by physical mapping and to position FRAXA on the map of the region.

Both DXS296 and DXS463/477 showed evidence of recombination with FRAXA, although some of these events are dependent on the assumption that women with 1% or 2% fragile sites are true carriers. This may lead to an overestimation of the genetic distances. This uncertainty is a general problem in fragile-X linkage studies, since, because of the incomplete penetrance in both males and females, recombination can only be proven in affected males, in women with levels of fragile sites well above background, or in obligate carriers. For the loci DXS296, DXS304 and DXS369, our data on linkage with FRAXA can be combined with the first published reports (table 3). This leads to a doubling

Table 3

Two-Point Lod Scores in the FRAXA Region

Pairwise Loci	Mode A[a]								Mode B[b]	
	Maximum Recombination Fraction	Lod Score at Maximum Recombination Fraction	Lod Score at Recombination Fraction of						Maximum Recombination Fraction	Lod Score at Maximum Recombination Fraction
			.00	.01	.05	.10	.15	.20		
DXS369–FRAXA	.057	14.82	4.67	12.46	14.80	14.32	13.01	11.33	.060	14.25
FRAXA[c]	~.06	~30	. . .	26.34	30.09	28.95	26.30	22.88		
DXS463/477	.026	8.66	−∞	8.51	8.53	7.95	7.19	6.34		
DXS296	.107	4.40	−∞	1.79	3.98	4.40	4.28	3.91		
DXS304	.090	22.27	−∞	14.10	21.35	22.23	21.19	19.24		
DXS52	.166	18.99	−∞	−13.08	10.04	6.96	18.89	18.64		
FRAXA–DXS463/477	.038	7.32	5.78	7.05	7.30	6.94	6.36	5.65	.000	10.36
DXS296	.057	10.42	5.51	9.19	10.41	10.12	9.33	8.27	.020	12.47
DXS296[d]	.049	15.23	10.87	14.40	15.18	14.26	12.80	11.10		
DXS304	.067	14.46	6.01	11.79	14.34	14.17	13.04	11.44	.070	13.78
DXS304[e]	~.05	~20	11.87	–	19.61	18.82	. . .	14.82		
DXS52	.115	31.86	−13.43	13.93	28.21	31.73	31.25	28.85	.102	33.13
DXS463/477–DXS296	.000	5.95	5.95	5.85	5.44	4.91	4.36	3.78		
DXS304	.035	11.73	−∞	11.25	11.67	10.99	10.00	8.83		
DXS52	.178	9.02	−∞	−7.97	3.92	7.67	8.89	8.95		
DXS296–DXS304	.000	5.59	5.59	5.49	5.10	4.56	3.99	3.37		
DXS52	.115	9.43	−∞	2.63	8.21	9.39	9.25	8.52		

[a] Women with 1%–2% marker X chromosomes were coded as affected.

[b] These women were coded as affection status unknown.

[c] Lod-score curves obtained by pooling our data with those of Oostra et al. (1990).

[d] Lod-score curves obtained by pooling our data with those of Suthers et al. (1989).

[e] Lod-score curves obtained by pooling our data with those of Dahl et al. (1989).

Table 4

Order of DXS296 and DXS463/477 in the FRAXA Region

Tested Order of Loci	Mode A[a]		Mode B[b]	
	− 2ln Likelihood	Relative Likelihood	− 2ln Likelihood	Relative Likelihood
DXS98–DXS369–FRAXA–DXS304–DXS296–DXS52	7,266	5,020,000	7,243	1,036
DXS98–DXS369–FRAXA–DXS296–DXS304–DXS52	7,266	4,990,000	7,243	1,228
DXS98–DXS369–DXS296–FRAXA–DXS304–DXS52	7,297	1	7,257	1
DXS369–FRAXA–DXS463/477–DXS296–DXS304–DXS52	7,345	179	7,323	1.1
DXS369–FRAXA–DXS296–DXS463/477–DXS304–DXS52	7,347	57	7,323	1.0
DXS369–DXS463/477–FRAXA–DXS296–DXS304–DXS52	7,355	1	7,321	2.8

[a] Women with 1%–2% marker X chromosomes were coded as affected.
[b] These women were coded as affection status unknown.

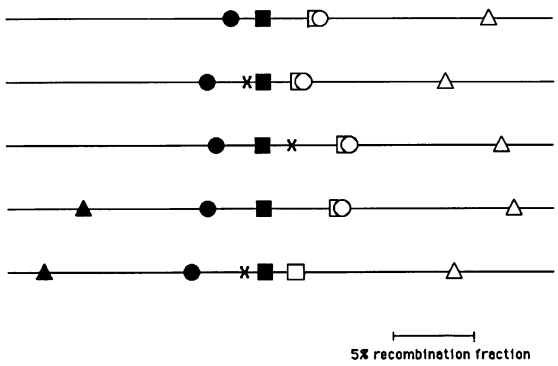

Figure 3 Genetic map of FRAXA region, based on multipoint analysis. Multipoint analyses were performed using the ILINK program under mode A. They included 5 or 6 of the following markers: DXS98 (▲), DXS369 (●), FRAXA (X), DXS463/477 (■), DXS296 (□), DXS304 (○), and DXS52 (△).

or tripling of lod scores—up to values of 15–30—and thus to a better estimate of recombination fractions. The genetic distance between the flanking markers had not been previously determined. This estimate is not subjected to the same uncertainty as for linkage data involving FRAXA. We provide very significant data (lod score 22.27 at a recombination fraction of .09) for the DXS369-DXS304 interval.

The large lod scores obtained in several two-point linkage analyses and the large number of multiply informative families enable reliable multipoint analysis (table 4). In one affected male a recombination observed between DXS296 and FRAXA allows us to place DXS296 distal to FRAXA. This establishes independently the location of DXS296 with respect to the disease. The order of DXS463/477 and DXS296 could not be determined genetically, since they do not recombine. When the physical mapping data are taken into account (DXS463/477 proximal to DXS296), the order FRAXA–DXS463/477–DXS296 is favored, under one mode of calculation but not under the other, over the order DXS463/477–FRAXA–DXS296. Given the fragile-X typing problems in the possible recombinant families, we feel that the exact location of DXS463/477 is still pending on recombinants found in unambiguously affected people. In any case, the DXS463/477 marker is at present the polymorphic one closest to FRAXA, either on the proximal or on the distal side. Our multipoint analysis yields also a better estimation of genetic map distances constrained by all the relationships between the tested markers, especially since some intervals are supported by very high lod scores (19 for DXS369–DXS52, 32 for FRAXA–DXS52, and

22 for DXS369–DXS304; table 3 and fig. 3). In particular, the distance between DXS369 and DXS304 appears well established at about 8–10 cM, with FRAXA and DXS463/477 lying genetically about halfway of these markers.

The new probes we have described significantly improve the genetic analysis of fragile-X families, which is important for diagnostic purposes and for the fine mapping of the disease locus. The *Taq*I RFLPs detected by probes St677 and 2-34 are especially convenient, since they can be haplotyped (combined heterozygosity 30%) and assayed, in a single hybridization step, to blots that can also be used with probes RN1 (Oberlé et al., in press), VK21A (Suthers et al. 1989), and U6:2 (Dahl et al. 1989). Probe Do33 (DXS465) enhances, from 30% to 60%, the heterozygosity of the DXS296 region and should facilitate the analysis of fragile-X as well as Hunter families. In preliminary studies, we did not find recombination between DXS296 or DXS463/477 and DXS465 in six informative Centre d'Etude du Polymorphisme Humain families (unpublished results).

It will be most interesting to compare the genetic mapping of probes in the FRAXA area with fluorescent in situ hybridization images obtained on induced fragile-X sites, as well as with the location of the breakpoints in the other hybrids lines generated, as Micro21D and Q1X, by breakage at or near the fragile site (Warren et al. 1990). A distal location of DXS463/477 would contrast with its location proximal to the breakpoints in Micro21D and Q1X. It is possible that the chromatin decondensation detected cytogenetically extends preferentially or variably in some direction from the mutated sequences, which would result either in constant or variable location of the nearest probes in the decondensated chromatin. The 2-71, Do33, and St677/2-34 markers introduce new landmarks in the FRAXA region which are good candidates for physical linking of the known FRAXA flanking loci or for detecting, by pulsed-field gel electrophoresis, potential genomic rearrangements in affected fragile-X patients. In this respect the probes 2-34 and 2-71 are especially interesting, since they are next to *Eag*I and *Not*I sites, respectively (Maestrini et al., in press).

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