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ARTICLE

Stability and haplotype analysis of the FRAXE region

Anna Murray[1], Sarah Ennis[2], Sheila A Youings[1], Andrea J Sharrock[1], Catherine Lewis[1], Michelle C Pound[1], James N Macpherson[1], Nick R Dennis[3], Newton E Morton[2] and Patricia A Jacobs[1]

[1] *Wessex Regional Genetics Laboratory, Salisbury District Hospital, Salisbury;* [2] *Department of Human Genetics, Southampton University Hospital Trust;* [3] *Wessex Clinical Genetics Service, Princess Anne Hospital, Southampton, UK*

FRAXE full mutations are rare and appear to be associated with mild mental retardation. As part of a screening survey of boys with learning difficulties to determine the frequency of full and premutations, we have collected data on the frequency of instability at FRAXE for about 4000 transmissions and the haplotype for over 7000 chromosomes. The distribution of FRAXE repeats was similar to other English populations but differed from two North American Caucasian series. Observed instability at FRAXE was rare but increased with increasing repeat number, and there were no expansions into the full mutation range, except in pedigrees ascertained through a full mutation. Haplotype analysis suggested division into five groups with each group having a characteristic distribution of FRAXE repeats. Fourteen of the 15 full mutations occurred on a single haplotype and this haplotype also had a significant excess of intermediate-sized alleles, suggesting that full mutations originate from large normal alleles. However, a related haplotype also had a significant excess of intermediates but we observed no full mutations on this haplotype, suggesting either loss or gain of stability determinants on it. We suggest that whilst triplet repeat size is a significant predisposing factor for expansion at FRAXE other genetic determinants are also likely to be important. *European Journal of Human Genetics* (2000) **8**, 583–589.

Keywords: FRAXE; haplotype; stability; trinucleotide repeat

Introduction

The FRAXE fragile site was first identified in patients with folate sensitive sites at Xq27–28 but no evidence of CGG expansion at the FRAXA locus.[1,2] The molecular basis for the observation was an expanded GCC repeat approximately 600 kb distal to FRAXA.[3] The GCC repeat is at the 5' end of the *FMR2* gene and is polymorphic.[4] The normal size range is 5–30 repeats, while full mutations have over 200 repeats with concomitant methylation of the repeat and promoter region. In males the full mutation is associated with a variable degree of learning difficulty, whilst females appear unaffected. However, detailed phenotypic studies have been hampered by the scarcity of patients with the full mutation.[5–7] Premutations for FRAXE have over 60 repeats and are usually transmitted unstably, but are unmethylated. The boundary between the normal and premutation range is uncertain and therefore alleles between 31 and 60 repeats have been called intermediate.[8]

The processes that govern the stability of the FRAXE trinucleotide repeat are not understood. At FRAXA it is thought that the purity of the repeat has an important role in stability, such that AGG repeats interrupting the CGGs prevent slippage during replication and hence expansion.[9] The FRAXE repeat is not interrupted, suggesting there may be a simple relationship between size of the repeat and likelihood of expansion.[10] Haplotype studies have been extremely valuable in suggesting possible mechanisms of expansion for trinucleotide repeat diseases, in particular FRAXA. These studies suggest that flanking sequences may be important determinants for stability, and at FRAXA have led to the proposal of at least three independent mechanisms.[11] Similar studies at FRAXE have been limited, partly due to the paucity of suitable markers in the region. In an earlier publication we demonstrated allelic association between

Correspondence: Anna Murray, Wessex Regional Genetics Laboratory, Salisbury District Hospital, Salisbury, Wiltshire SP2 8BJ, UK.
Tel: +44 1722 425048; Fax: +44 1722 338095;
E-mail: a.murray@dial.pipex.com
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FRAXE and DXS1691, a dinucleotide repeat polymorphism within FMR2 but 5 kb distal to the repeat.[8] Expanded FRAXE alleles were associated almost exclusively with allele 38 (allele 3 in old nomenclature) at DXS1691. Limprasert *et al* also found association of FRAXE alleles larger than 21 repeats with allele 38 at DXS1691 (19 in their nomenclature), and with marker DXS8091 demonstrated differences in FRAXE repeat number on different haplotypes.[12] We have extended our analysis of this region by testing a second polymorphism, DXS6687, which lies less than 20 kb distal to the FRAXE repeat. The aim of this study was to correlate haplotype data with stability data from family studies to determine the evolutionary dynamics of the FRAXE repeat.

Materials and methods

The sample consists of 7012 chromosomes from Wessex and 13 FRAXE expansions from seven other laboratories in England, the Netherlands and America (see Acknowledgements). These chromosomes are all independent in the sense that identity by descent was excluded within families. The Wessex sample consisted of boys with learning disability from a fragile X screening survey, the nontransmitted chromosomes of their mothers, and chromosomes from pedigrees selected through FRAXA or FRAXE full, premutation, intermediate or minimal probands. Preliminary results on 1013 boys from the screening survey and 760 of their mothers have been reported previously.[8] FRAXE alleles are classified as minimal (M) if <11 repeats, common (C) if 11–30 repeats, intermediate (I) if 31–60 repeats, premutation (P) if 61–200 repeats and unmethylated, and full mutation (F) if >200 repeats and methylated. Details of the screening survey methodology can be found in Youings *et al*.[13]

DNA from individuals in the screening survey was from buccal smears, whilst all other DNA was from peripheral blood. All chromosomes were tested for FRAXE and DXS1691 (5 kb distal to FRAXE), and 2937 of the chromosomes were also tested for DXS6687 (20 kb distal to FRAXE). All genotyping was by fluorescent labelled PCR and analysed on an ABI 377 (PE/Applied Biosystems, Warrington, UK). FRAXE and DXS1691 PCR was performed as previously described[8] and DXS6687 was amplified separately using 0.6 μM of each primer, G8068 – 5' CTGAATGTACCACATTCAGGTTC 3' (FAM labelled) and G8238 – 5' GATCCAGGCAAAAGTCTCAGTG 3' (Sam Knight, personal communication, 1996), 2.4 μM $MgCl_2$, 0.2 mM dNTPs and 0.75 U of AmplitaqGold (Perkin Elmer, Warrington, UK) in a final volume of 10 μl, under the same PCR conditions as DXS1691. Allele nomenclature followed the proposal of Chiurazzi *et al*,[14] which uses the size in base pairs of the repeated sequence as the allele name.

Statistical analysis

Statistical analyses were performed using SAS (SAS Institute Inc, Cary, NC, USA). To examine the haplotype relations we first took all DXS1691-FRAXE haplotypes as a single sample. This was followed by analysis of DXS6687-FRAXE haplotypes and then the 3-locus haplotypes. We used linear regression for integers representing number of FRAXE repeats in the common range (11–30) and for minimal and expanded FRAXE alleles as attributes. Alleles were expressed as 0,1 variables. For a class with N_i observations we computed summary statistics on common FRAXE sizes: the mean $m_i = \sum_j X_{ij}/N_i$ and the variance $V = \Sigma (X_{ij} - m_i)^2/(N_i - 1)$, with degrees of freedom N_i and $N_i - 1$, respectively.

Results

Distribution of FRAXE

All of the 2785 untransmitted chromosomes from mothers of boys with learning difficulty were used to determine the population distribution of FRAXE alleles (Figure 1). The distribution has modes at 15 and 18 and a small mode at 23/24 repeats and is positively skewed. Fifteen alleles in the common size range were sequenced to confirm the repeat number, and all alleles analysed were composed of pure GCC without interruption. Sequencing of the –9 repeat allele revealed a deletion of 57 bp of sequence distal to a repeat tract of 16.[15] The deleted allele was associated with a FRAXA full mutation and segregated stably within the family. The region flanking the FRAXE triplet appears to be prone to deletion, as a number of cases have been reported.[16–19]

Stability of the FRAXE repeat

There were 4124 transmissions of the FRAXE repeat in our sample; 3824 female and 300 male. Transmissions were only counted when both the parent and offspring were tested for FRAXE. Transmissions to probands were included. There were 33 transmissions in which the repeat was unstable and the likelihood of a repeat being unstable increased with increasing repeat number (Table 1). The degree of instability in alleles with fewer than 60 repeats was restricted to changes of 1 or 2 repeats, with expansions of more than twice as common as contractions (Table 2). We were certain of phase from family data in all but two transmissions, for these two the parental allele nearest in size to the transmitted allele was taken. There were two families with relatively large alleles but no evidence of expansion to a full mutation: a male with 65 repeats transmitted a 60 repeat allele to his daughter, and she transmitted 58 repeats to one son and 65 repeats to a second son, and in the second family a 66 repeat allele increased by 21 repeats from mother to son.

Allelic association within the FRAXE region

DXS1691 and DXS6687 are 15 kb apart and, as expected, show significant allelic association. The full contingency table of 6 DXS1691 alleles by 14 DXS6687 alleles is sparse, and 71 per cent of the cells have expected counts less than 5 ($\chi^2_{65} = 1904$). Most of the significance is in the cells with large numbers of observations (Table 3, $\chi^2_{12} = 1837$). These DXS1691–DXS6687 haplotypes have different distributions

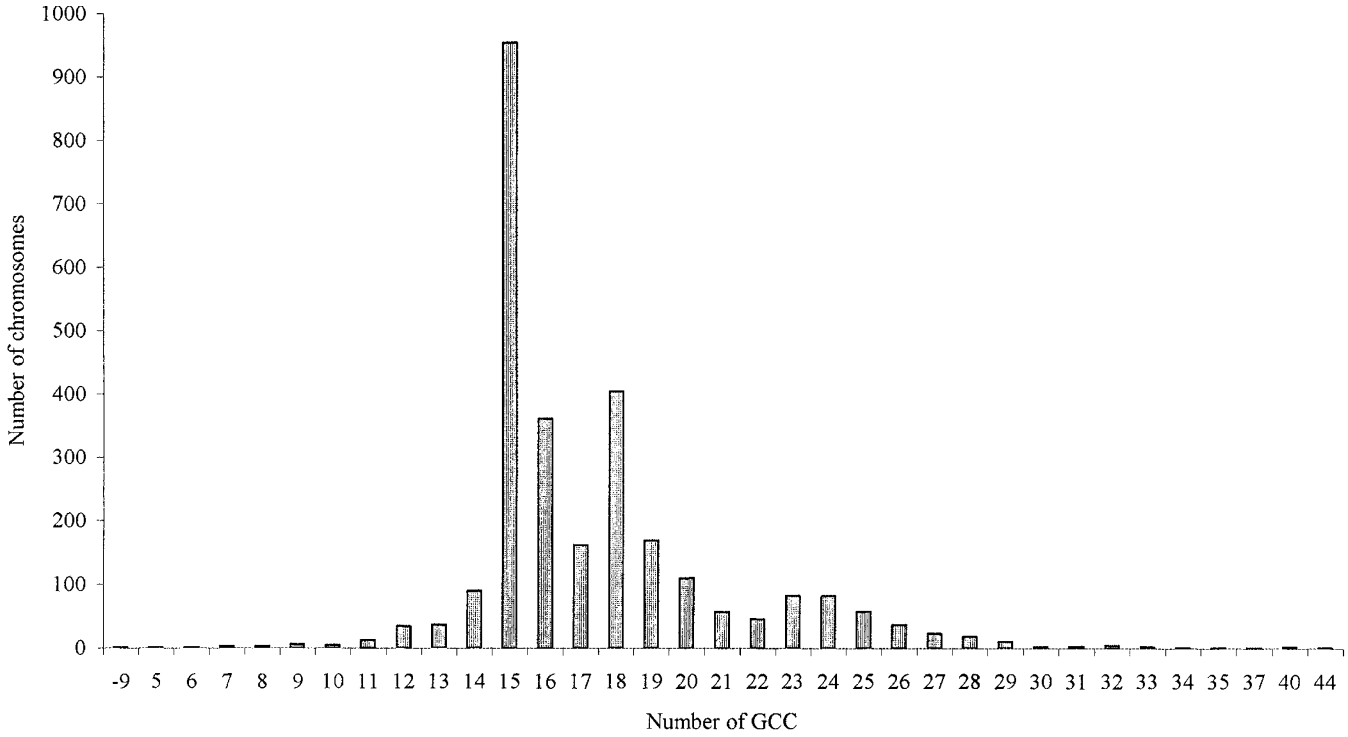

Figure 1 Distribution of FRAXE alleles (N = 2785).

of FRAXE repeats (Table 4). Similar or apparently related haplotypes were grouped and these five groups (Q–U) captured much of the variation in FRAXE, as follows.

Group Q Haplotye 40–24. The FRAXE distribution within the common range is compact and has a mode at 15 repeats, an elevated frequency of minimal alleles, and a low risk for FRAXE expansion.

Group R Haplotype 40-Y, where Y signifies any DXS6687 allele except 24. The FRAXE mode is greater than 15 and the distribution is compact, with a corresponding low frequency of minimal alleles and low risk for expansion.

Group S Haplotype 38–36. The FRAXE distribution within the common range is dispersed, with a mode at 15 and an excess of minimal and expanded alleles.

Group T Haplotype 38-Y, where Y signifies any DXS6687 allele except 36. The FRAXE distribution has a mode greater than 15 and an elevated risk of expansion.

Group U Haplotype X-Y, where X is any DXS1691 allele except 38 or 40 and Y is any DXS6687 allele. This small residual group has FRAXE modes at 15 for X-24 (suggesting origin from 40–24) and at 19 (suggesting mixed origin). The very small subgroup X-36, presumably derived from 38–36, has an excess of intermediate and large common alleles.

Table 1 Transmission of FRAXE alleles

GCC_n of transmitting parent	*Male to Female*		*Female to Male*		*Female to Female*	
	Stable	*Unstable*	*Stable*	*Unstable*	*Stable*	*Unstable*
<11	11	0	37	0	18	0
11–30	267	1	3158	1	539	1
31–40	15	4	31	2	12	0
41–60	0	0	2	5	2	1
>60	0	1	0	3	0	2
Full	0	1	0	6	0	5

The frequent haplotypes Q and S account for over 80% of minimal alleles (Table 4). Both have modes at 15 repeats, but the group Q distribution is much more compact. Haplotypes with modes at larger alleles have a low frequency of minimal alleles. Intermediate alleles usually have allele 38 at DXS1691, which has a distribution skewed toward expansion within the common class. Differences among haplotype groups are significant for minimal, common and expanded (intermediate, premutation and full mutation) FRAXE alleles.

DXS1691 alleles 36 and 38 are significantly associated with elevated numbers and variances of repeats in common FRAXE alleles (Table 5). Minimal FRAXE alleles are significantly associated with DXS1691 alleles 38 and 44 and expansions of FRAXE with DXS1691 alleles 36 and 38. The size of common FRAXE alleles has a significantly quadratic

Table 2 Unstable transmissions of FRAXE alleles

Family	Haplotype (FRAXE–DXS1691–DXS6687)	Allele changes	Transmission (F=Female, M=Male)
1	26–38–U	16 to 15 or 14	F→F
2	27–38–U	26 to 27	F→M
3	32–38–36	27 to 28	M→F
4	16–40–U	32 to 33	M→F
5	33–38–U	33 to 32	F→M
6	36–38–U	35 to 36	M→F
		35 to 36	M→F
7[8,29]	37–38–36	37 to 27/37	F→M
8[29]	40–38–36	40 to 41	M→F
9[29]	42–38–36	41 to 42	F→M
10	51–38–36	51 to 52	F→M
		51 to 53	F→F
		51 to 53	F→M
11	65–38–38	65 to 60	M→F
		60 to 65	F→M
		60 to 58	F→M
12[8,29]	66–38–42	66 to 87	F→M
13[29]	Full–38–36	Full to Pre	M→F
		Pre to Full	F→M
14[29]	Full–38–36	Full to Full	F→M
		Full to Full	F→F
		Full to Full	F→F
		Full to Full	F→M
		Full to Full	F→F
		Full to Full	F→F
		Full to Full	F→M
15	Full–38–36	Full to Full	F→M
16	Full–38–36	Full to Full	F→M
17	Pre–38–U	Pre to Pre	F→F
		Pre to P/F	F→F
		Pre to Full	F→M
18	Full–38–36	Full to Full	F→M
		Full to Pre	F→F

U=untested; references for previously published families are indicated in superscript.

regression on DXS6687 alleles, with a peak near the mode at allele 36, which has the highest frequency of minimal and expanded FRAXE alleles. Pairwise comparison of all three loci demonstrates that all correlations of allele size are highly significant, but DXS1691–DXS6687 and DXS1691–FRAXE are negative correlations (–0.161 and –0.232 respectively) and DXS6687–FRAXE is positive (0.338). In multiple regression the DXS6687 locus has a much greater association with common FRAXE size than DXS1691, although both are significant, $P < 0.001$ ($\chi^2 = 529.29$ and 30.42 respectively). On the contrary, minimal and expanded allele frequencies are associated more with DXS1691: when DXS1691 is conditional on DXS6687 the χ^2 for minimal alleles is 55.77 and for expanded alleles is 46.2, whilst when DXS6687 is conditional on DXS1691 the χ^2 are 10.57 and 19.32 respectively, which are not significant. This may be no more than a reflection of the proximity of DXS1691 to FRAXE, but then the contrary observation for common repeats is unexplained. Together with the positive correlation of FRAXE allele size with the number of repeats at DXS6687, these observations are consistent with a common cause of expansion, although an evolutionary accident cannot be excluded.

Discussion

FRAXE repeat distributions have been reported for several ethnic groups[8,10,20–22] and our data closely resemble other English distributions.[20,21] However, there are differences between populations, even among Caucasians. It is difficult to compare absolute repeat numbers between studies because of intra-laboratory variation, but the study by Ritchie *et al*,[20] in which allele sizing was presumably standardised for the five populations tested, clearly demonstrates the differences in modal repeat number: the predominant English mode being 15, whereas in the other four populations studied (African, Chinese, Greek and Indian) the mode is 16–18. Two studies of American Caucasians have similar FRAXE distributions but are markedly different from our data[10,22] and like us, Zhong *et al*[10] have sequenced a proportion of alleles thus allowing direct comparison. The mode is 16 in New York Caucasians compared with 15 in the present study. Our distribution appears almost trimodal with an antimode at 17 repeats, but this antimode is not seen in New York or Atlanta Caucasians. Although differences in repeat distribution between ethnically diverse populations are not unexpected, it is a surprising observation within similar ethnic groups, particularly when FRAXA repeat distributions and interspersion patterns among all Caucasians appear very similar.[8,21–28] This suggests that dynamic mutation at FRAXA and FRAXE is subject to independent evolutionary mechanisms and that FRAXE is perhaps more mutable than FRAXA.

However, we have studied over 4000 transmissions of the FRAXE repeat and changes in repeat number were remarkably uncommon. We found a bias towards expansion, but most changes, excluding full mutations, were of only one or two repeats. This mirrors our findings for FRAXA where changes of only a few repeats were most frequent.[29]

Table 3 DXS1691–DXS6687 haplotypes

DXS1691	DXS6687							Total
	24	34	36	38	40	42	Other	
38	20[a]	43[b]	669[b]	113[b]	34[a]	15[a]	7[a]	901
40	830[b]	3[a]	124[a]	89[a]	522[b]	256[b]	104[b]	1928
Other	33	0	12[a]	3	48[b]	9	3	108
Total	883	46	805	205	604	280	114	2937

[a]observed is less than expected, $\chi^2 > 10$; [b]observed is greater than expected, $\chi^2 > 10$; Other = 34, 36, 42,44 for DXS1691 and 20, 22, 26, 30, 32, 44, 46, 48 for DXS6687.

Table 4 Number of FRAXE repeats by DXS1691–DXS6687 haplotype

FRAXE Group	*DXS1691–DXS6687*	*M*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	*I*	*P*	*F*	*N*
Q	40–24	**14**	3	3	4	5	**624**	116	19	12	15	2	3	4	1	-	-	2	-	-	-	-	2	-	1	830
R	40–26	-	-	-	-	-	8	7	7	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	24
R	40–36	-	-	2	-	2	3	14	6	7	19	**52**	3	-	1	2	6	3	1	1	2	-	-	-	-	124
R	40–38	-	-	-	-	4	-	5	2	**53**	11	3	1	3	3	3	1	-	-	-	-	-	-	-	-	89
R	40–40	3	3	-	1	44	30	33	41	**196**	54	41	28	21	8	11	3	2	1	1	-	1	-	-	-	522
R	40–42	1	3	-	1	1	33	**143**	8	41	9	2	5	3	3	1	-	-	-	2	-	-	-	-	-	256
R	40–44	-	-	-	-	-	4	9	5	**16**	-	2	1	-	-	1	-	1	-	1	-	-	-	-	-	40
R	40–Y_1	-	-	-	1	-	15	**21**	2	-	-	-	1	-	-	1	1	-	-	1	-	-	-	-	-	43
S	38–36	**25**	4	21	21	19	**202**	12	46	46	26	5	5	4	37	**51**	41	20	19	15	6	2	**28**	-	**14**	669
T	38–24	-	-	1	-	-	**12**	2	1	1	1	-	-	-	-	-	-	-	-	-	1	1	-	-	-	20
T	38–34	-	1	-	1	-	4	-	**10**	-	-	-	1	9	3	3	2	5	1	-	-	-	3	-	-	43
T	38–38	2	-	-	-	-	3	4	**24**	2	9	-	-	4	**22**	8	8	5	4	2	1	-	**14**	1	-	113
T	38–40	-	-	-	-	-	2	-	1	**27**	1	1	2	-	-	-	-	-	-	-	-	-	-	-	-	34
T	38–42	-	-	-	-	1	4	5	-	-	-	-	-	-	1	2	-	-	-	-	-	-	1	1	-	15
T	38–Y_2	1	-	1	-	-	4	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7
U	42–40	-	-	-	-	2	-	-	-	11	**31**	2	1	-	1	-	-	-	-	-	-	-	-	-	-	48
U	X–36	-	-	-	-	1	1	-	1	-	1	-	-	-	-	-	-	1	-	2	2	2	1	-	-	12
U	X–24	2	-	-	-	1	**23**	4	1	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	33
U	Other	-	-	-	-	-	2	2	-	1	7	1	-	2	-	-	-	-	-	-	-	-	-	-	-	15

X = Any DXS1691 allele other than 38 and 40; Y_1 = Any DXS6687 allele other than 24, 26, 36, 38, 40, 42 and 44; Y_2 = Any DXS6687 allele other than 24, 34, 36, 38, 40 and 42; M = <11 repeats; I = 31–60 repeats; P = 61–200 repeats; F = Full mutation.

Table 5 FRAXE correlates of marker alleles

		FRAXE alleles				
DXS1691 allele	*Mean*	*Common Variance*	*Number*	*Minimal Number*	*Expanded Number*	*Total Number*
34	17.0	–	1	–	–	1
36	20.8[a]	35.5[a]	24	–	1	25
38	18.8[a]	21.7[a]	1884	39	76	1999
40	16.8	6.2	4698	28	8	4734
42	18.0	5.5	211	1	–	212
44	16.7	8.3	6	2	–	8
DXS6687 allele						
20	15.0	–	8	–	–	8
22	15.8	1.2	5	–	–	5
24	15.4	1.9	864	16	3	883
26	15.8	1.3	24	–	2	26
28	–	–	0	–	–	0
30	15.0	–	1	–	–	1
32	18.0	18.0	2	–	–	2
34	20.6[b]	17.9[b]	43	–	3	46
36	18.9[b]	21.2[b]	737	25	43	805
38	19.9[b]	11.1[b]	188	2	15	205
40	18.2[b]	5.7[b]	601	3	–	604
42	16.8[b]	4.6[b]	277	1	2	280
44	17.7[b]	7.4[b]	43	1	–	44
46	16.4	6.9[b]	24	–	–	24
48	15.8	0.3	4	–	–	4

[a]χ^2 >10 for comparison with DXS1691 allele 40; [b]χ^2 >10 for comparison with DXS6687 allele 24.

Changes of 10 repeats were also detected at FRAXA, albeit rarely, and there is one case of presumably mitotic contraction by 10 repeats in our series of FRAXE transmissions: a mother with 37 repeats had a son with 37 and 27 repeats (Klinefelter syndrome was excluded). As repeat number increased there was an increase in the frequency of instability, with an overall stability rate for alleles with less than 61 repeats of 0.36%. However, the rate of instability for alleles of 60 and under for male transmission was 1.68% compared with 0.26% for female transmission, and all of the male mutations were expansions, whilst only 60% of the female mutations were expansions. These data are not statistically significant but suggest that FRAXE expansions within the normal range may be more common in males, in contrast to full and premutations where female transmissions tend to be more unstable,[6] which may indicate that the mechanism of expansion for common/intermediate and premutation alleles may be different. All alleles with greater than 60 repeats were unstably transmitted, but we did not ascertain any full mutation carriers through a premutation proband, suggesting that progression to a full mutation is a gradual process even from a relatively unstable premutation sized allele. Premutation alleles previously described that have progressed to a full mutation have all been larger than 100 repeats[7,21,30] although in many families no premutations are seen and it has been suggested that there is a lower threshold for methylation than at FRAXA.[30]

FRAXE allelic associations with flanking markers have not been studied extensively. We have therefore correlated FRAXE repeat number with alleles at two microsatellite markers DXS1691 and DXS6687. These markers cover approximately 20 kb of genomic DNA and as expected we found significant linkage disequilibrium across the region. We classified the haplotypes into five groups, each group having a characteristic distribution of FRAXE alleles. Some haplotypes are associated with the high end normal/intermediate FRAXE alleles, for example those in groups S and T. Fourteen of the 15 FRAXE full mutations also had the haploptype 38–36 (group S). This suggests that most FRAXE expansions are derived from a pool of large normal alleles. This is analogous to one of the proposed mechanisms for

FRAXA expansion, where the 2-1-3 haplotype carries an increased risk for FRAXA expansion and is also significantly associated with intermediate alleles.[11] Intermediate and premutation FRAXE alleles are also found on rarer haplotypes in group T, which are presumably phylogenetically related to 38–36, differing only at the DXS6687 locus. However, we have no examples of group T haplotypes in full mutation carriers despite the number of intermediates/premutations being similar (17 in group T *vs* 28 in group S) and the proportion of the total for that group being greater (13% of group T are intermediate/premutation *vs* 4% of group S). A possible explanation for these data is that 38–38 haplotype diverged from 38–36 with concomitant acquisition of a stabilising element either within the triplet repeat or elsewhere. One candidate for such an element would be interruptions within the repeat, which at FRAXA are thought to confer stability.[9,31] Sequencing of FRAXE alleles has not detected any interruptions, however Zhong *et al*[10] sequenced only 21 alleles including one intermediate, and we have only sequenced 15 common alleles and no intermediates. It would therefore be interesting to sequence the intermediate alleles on the 38–38 haplotype and compare them with the 38–36 intermediates.

There are two ways in which allele association between disease susceptibility and a closely linked marker locus can arise: one or more marker alleles or other alleles with which they are in disequilibrium may cause instability, alternatively stochastic factors such as genetic drift accentuated by population bottlenecks may result in chance associations. Distinction between causation and historical accident can be extremely difficult, but it is worthwhile to make an attempt. Most of the variation within the common range at FRAXE is associated with alleles at DXS6687 but interestingly the distinction between minimal and expanded categories is best captured by DXS1691. This relatively low heterozygosity of DXS1691 may explain the association with expanded and minimal FRAXE alleles: if a single founder gave rise to the majority of expanded alleles and the mutation rate for DXS1691 was low, an association between the founder allele and expansions would be preserved. It is conceivable that minimal alleles are reciprocal products of an expansion event and therefore they too may be associated with this founder. The variation at DXS6687 is much greater and almost parallels that at FRAXE. Although a founder effect is still apparent in associations with expanded alleles, this has been diluted considerably by the more rapid co-evolution of this locus with FRAXE. The haplotype associations described, in the absence of stabilising factors, suggest the existence of external determinants of size variation in the FRAXE GCC repeat.

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