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Molecular Analysis and Test of Linkage between the FMR-1 Gene and Infantile Autism in Multiplex Families

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Summary

Approximately 2%–5% of autistic children show cytogenetic evidence of the fragile X syndrome. This report tests whether infantile autism in multiplex autism families arises from an unusual manifestion of the fragile X syndrome. This could arise either by expansion of the (CGG)n trinucleotide repeat in FMR-1 or from a mutation elsewhere in the gene. We studied 35 families that met stringent criteria for multiplex autism. Amplification of the trinucleotide repeat and analysis of methylation status were performed in 79 autistic children and in 31 of their unaffected siblings, by Southern blot analysis. No examples of amplified repeats were seen in the autistic or control children or in their parents or grandparents. We next examined the hypothesis that there was a mutation elsewhere in the FMR-1 gene, by linkage analysis in 32 of these families. We tested four different dominant models and a recessive model. Linkage to FMR-1 could be excluded (lod score between −24 and −62) in all models by using probes DXS548, FRAXAC1, and FRAXAC2 and the CGG repeat itself. Tests for heterogeneity in this sample were negative, and the occurrence of positive lod scores in this data set could be attributed to chance. Analysis of the data by the affected-sib method also did not show evidence for linkage of any marker to autism. These results enable us to reject the hypothesis that multiplex autism arises from expansion of the (CGG)n trinucleotide repeat in FMR-1. Further, because the overall lod scores for all probes in all models tested were highly negative, linkage to FMR-1 can also be ruled out in multiplex autistic families.

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Introduction

Autism is a severe developmental disorder that usually occurs within the first 3 years of life. It is characterized by marked social deficits, delay in language development, and a restricted range of stereotyped repetitive behaviors. The prevalence of autism is ~1/2,000 births, and the ratio of affected boys to affected girls is ~3:1 (Smalley et al. 1988). Although the cause of autism is unknown, family and twin studies strongly support a genetic etiology in a subset of cases, particularly in families in whom multiple cases occur (for reviews, see Folstein and Piven 1991; Smalley 1991). The recurrence rate among siblings of autistic individuals is ~3% and is 50–100 times higher than the risk in the general population (Smalley et al. 1988), but in families with multiple autistic members the recurrence risk for subsequent siblings is 8.6% and the relative risk may be >200 times higher (Ritvo et al. 1989). The concordance rate in twins, pooled across several studies, is 64% in MZ twins and 9% in DZ twins (Smalley et al. 1988; Steffenburg et al. 1989). The mode of transmission of autism is unknown, but it does not follow classical Mendelian inheritance. The inability to establish a diagnosis of autism in adults makes classical segregation analyses extremely difficult (for review, see the work of Lotspeich and Ciaranello [1993]; also, in various reports, autosomal dominant, autosomal recessive, and multifactorial models have all been implicated [Smalley et al. 1988; Jorde et al. 1991]).

Several disorders of known genetic etiology have been reported to be associated with autism. Among these, fragile X-linked mental retardation is the most common. The proportion of fragile X-positive cases among autistic individuals varies widely from study to study. Rates as high as 50% and as low as 0% have been reported (for reviews, see Payton et al. 1989; Bolton and Rutter 1990). These large discrepancies appear to be due to differences in ascertainment strategy, diagnostic criteria for autism, varying thresholds for the cytogenetic diagnosis of fragile X (Piven et al. 1991), and possibly variability in the diagnosis of autism among centers. To date, only one study has used a standardized instrument for diagnosing autism (Piven et al. 1991). In that study, a fragile X prevalence of 2.7% was

reported in a sample of 75 autistic individuals, compared with a 0.1% prevalence in the general population (Webb et al. 1986).

The molecular biology of the fragile X site has now been well established, and a candidate gene, FMR-1, has been isolated (Oberlé et al. 1991; Verkerk et al. 1991). The vast majority of fragile X subjects have an amplification of a (CGG)n trinucleotide repeat that occurs in the 5′-UTR of the FMR-1 transcript, as detected by either Southern blotting or PCR analysis (Rousseau et al. 1992). Males in whom the (CGG)n repeat is >600 bp almost invariably show clinical and cytogenetic expression of the disease.

The fragile X syndrome has presented a confusing and anomalous cytogenetic and clinical picture, in which extreme variability has been the rule. Analysis of the molecular defect has clarified many matters, but much remains to be explained, and the picture remains highly variable. Males have been found who both have an expanded CGG repeat and show the fragile X phenotype clinically yet are cytogenetically normal (Tarleton et al. 1992). Amplification of the CGG repeat has been detected in individuals who did not show any signs of the fragile X phenotype, either cytogenetically or clinically (Macpherson et al. 1992). Finally, subjects have been described who had neither an amplification of the CGG repeat nor a fragile site by cytogenetic methods but who had clinical manifestation of the fragile X phenotype. In these individuals molecular analysis of the FMR-1 gene showed a deletion within the FMR-1 gene in one case (Gedeon et al. 1992) and a point mutation in another (De Boule et al. 1993).

Since nothing is known about genetic defects in autism, it is important to explore its association with known genetic disorders, for clues that might lead to understanding the biology of autism. Fragile X syndrome is particularly useful in this regard, because of both the preponderance of affected males and the occurrence of mental retardation in both disorders. The fragile X–positive/autistic subjects described so far have all been identified cytogenetically. However, as described above, the fragile X syndrome exhibits great diversity in its phenotypic, cytogenetic, and molecular expression. Thus, a tenable hypothesis to emerge from all these observations is that autism is a variant of the fragile X syndrome, a variant in which the usual phenotypic and cytogenetic manifestations may be absent. This could arise in one of the following ways: (*a*) from an increase in the CGG repeat in the FMR-1 gene, which for unknown reasons is not expressed in the usual cytogenetic or phenotypic fashion (Macpherson et al. 1992) but which instead exhibits the clinical features of autism; (*b*) from a mutation elsewhere in the fragile X site, a mutation that does not involve the CGG repeat; or (*c*) a deletion of segments of both the FMR-1 gene and an unknown neighboring gene that is involved in autism. A deletion spanning both genes would lead to both autism and the fragile X syndrome. The null hypothesis is that autism and fragile X are unrelated—and that their co-occurrence is simply coincidental and may be related to the mental retardation that both syndromes share (Fisch 1992).

These hypotheses are amenable to testing with existing methodologies, and we have examined two of them in this report. To do so, we studied 35 families with two or more autistic children each; we selected multiplex families because they are more likely to have a genetic form of autism than are families in which a single case occurs. To test the hypothesis that a mutation in the CGG region in the FMR-1 gene is involved in the etiology of familial autism, we examined the size of the CGG repeat in 79 autistic children from these families. To examine the possibility of a mutation elsewhere in the FMR-1 gene, we carried out a linkage analysis for autism, using microsatellite markers that are tightly linked to the FMR-1 gene.

Subjects and Methods

Multiplex Families

Since most autistic children do not show phenotypic or cytogenetic evidence of fragile X, testing the hypotheses described above required exclusion of families that had preexisting evidence of fragile X. Families were referred to the study when there was a presumption of a minimum of two clinically identified autistic children. Before enrolling a family in the study, we carried out a telephone interview with one or both parents, to determine (*a*) that the presumably autistic children had been evaluated by a trained physician or other clinician experienced in autism, (*b*) that medical records existed on each child and that they were available for our review, and (*c*) that fragile X syndrome had been ruled out either by cytogenetic testing or by lack of phenotypic presentation on pediatric examination.

Once these eligibility criteria were met, we performed independent diagnostic assessments on each child, as described by Spiker et al. (1994). Two standardized instruments were chosen to establish a diagnosis of autism: the Autism Diagnostic Interview (ADI; Le Couteur et al. 1989) and the Autism Diagnostic Observation Schedule (ADOS; Lord et al. 1989). For a positive diagnosis of autism, a child must have scores above the prespecified cutoff points in the four areas of the ADI that correspond to the ICD 10 system (i.e., social impairment, unusual interests and routines, language impairments, age at onset). Children were considered unaffected if their scores were below all cutoff points in all four areas. They were classified as being of uncertain status if their scores were above the cutoff point in one, two, or three areas. Because of the uncertainty in diagnosing mild forms of autism, and because of the possibility that individuals with mild forms of autism might still marry and reproduce, the phenotype of all parents was considered to be unknown. We recruited and evaluated 140 children from 42 presumed multiplex families. All evaluations were videotaped, so the diagnosis can be independently verified by a second diagnostician who is blind to any information about the child being evaluated. In-

terrater reliability was routinely checked; kappa coefficients were >.90 for the comparison of autistic versus non-autistic (i.e., affected vs. unaffected).

Of the 42 families volunteering for the study, 35 proved to have at least two autistic children by ADI/ADOS assessment and were included in the study. The other seven families were excluded because only one of their children fulfilled the criteria for autism as defined in ICD 10; usually his or her presumably autistic sibling met most but not all ADI cutoff points for autism and may have been given a clinical diagnosis of pervasive developmental disorder not otherwise specified (PDD-NOS). Three of the families consisted of cousin pairs in which father-to-son transmission was evident on inspection of the pedigrees. Accordingly, these families were not considered in the linkage analysis.

The pedigrees for the remaining 32 families are shown in figure 1. One family had five affected children, 3 families had four affected children, and 28 families had two affected children. Altogether, 73 children fulfilled the criteria for autism, 21 were not autistic (unaffected), and 5 were classified as uncertain. Another three children were below the age of 3 years and will be evaluated when they are older. One child was unavailable for evaluation when the family was visited. The mean ± SD age of the autistic subjects was 14.5 ± 9.6 years; that of the unaffected subjects was 18.3 ± 11.0 years; and that of the subjects classified as uncertain was 14.0 ± 8.6 years. The ratio of males to females was 2.04 for the autistic subjects, 1.62 for the unaffected subjects, and 0.67 for the subjects classified as uncertain.

Southern-Blot Typing

Blood was drawn from 208 children (79 of whom were autistic), their parents, and, if available, their grandparents. Lymphoblastoid cell lines were established, as described by Anderson and Gusella (1984), with minor modifications. DNA was extracted according to the protocol of Steffen and Weinberg (1978). Aliquots containing 5 μg of genomic DNA were digested overnight with the restriction enzymes *Eag*I and *Eco*RI, as described by Rousseau et al. (1992). Restriction fragments were separated by electrophoresis on 1% agarose gels for 16 h at 1–1.5 V/cm. Lambda phage–derived size markers were included in one lane, permitting sizing of the fragments. After denaturation with sodium hydroxide and neutralization for 1 h, the DNA was transferred to nylon filters by the method of Southern (1975). The probe StB12.3 was radiolabeled with [^{32}P]adenosine triphosphate (specific activity 3,000 Ci/mM; Amersham) by the oligolabeling method (Feinberg and Vogelstein 1983) for 5 h. The filters were prehybridized in 50% formamide, 5 × SSPE, and 1 × Denhardt's solution overnight at 42°C and hybridized for 24 h with 0.6–2.0 × 10^7 cpm of labeled probe. After being washed, filters were exposed to film (Kodak XAR-5) with intensifying screens (DuPont Lightning Plus) at −70°C for 3 and 14 d.

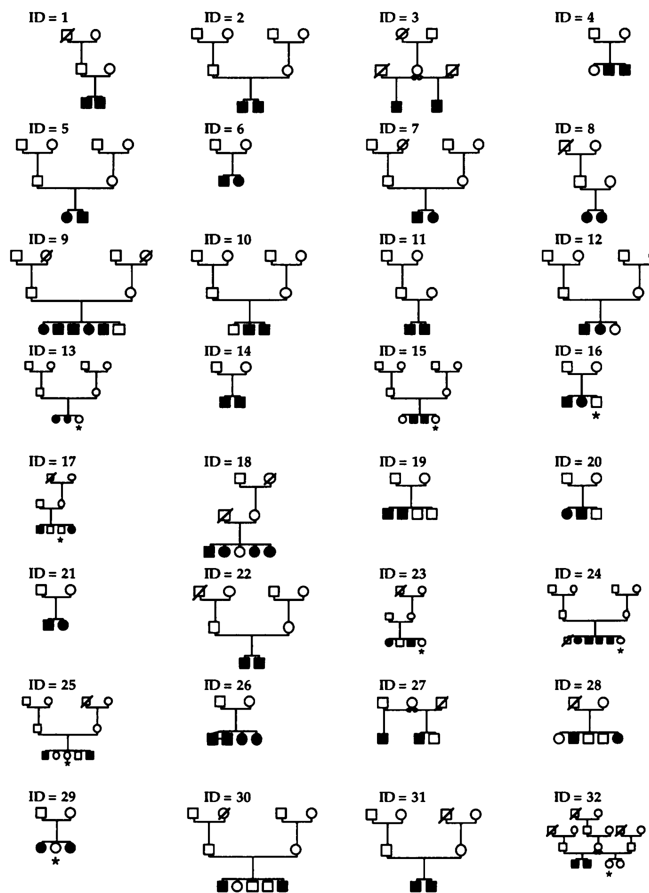

Figure 1 Family structure for 32 multiplex families. These were included in the linkage analysis. For a positive diagnosis of autism (blackened circles and squares), a child must have scores above the prespecified cutoff points in the four areas of the ADI that correspond to the ICD 10 system. Children were considered unaffected (unblackened circles and squares) if their scores were below all cutoff points in all four areas. Nine children were classed as unknown (indicated by an asterisk [*] under the symbol): five had uncertain diagnoses because of scores above the cutoff point in one, two, or three areas; three were below the age of 3 years; and one was unavailable for evaluation when the family was visited. The phenotype of all parents and grandparents was considered to be unknown in all calculations.

PCR Amplification of the CGG Repeat

For amplification of the CGG repeat, the primer sequences were FXCGGP1 5′-GAC GGA GGC GCC GCT GCC AGG-3′ and FXCGGP2 5′-GTG GGC TGC GGG CGC TCG AGG-3′ (Snow et al. 1993). PCR was performed as described below, with the following modifications: 75% of the GTP was substituted by 7-deaza-2′-deoxyguanosine5′-triphosphate, and 10% dimethyl sulfoxide (DMSO) was added. The PCR was started after 5 min preincubation of the DNA at 95°C (hot start), and amplification was accomplished by 30 cycles consisting of 94°C and 74°C for 90 s each.

Dinucleotide Polymorphism

The primer sequences for the loci amplified were the following: DXS548, RS46-CA1 5′-AGA GCT TCA CTA

Table I

Polymorphisms Typed

Locus	No. of Alleles	Heterozygosity
DXS548	8	.65
FRAXAC1	5	.43
FRAXAC2	9	.71
CGG	33	.82
DXS292	6	.58
DXS998	4	.47
DXS1126	8	.68
DXS15	8	.83

TGC AAT GGA ATC-3′, RS46-CA2 5′-GTA CAT TAG AGT CAC CTG TGG TGC-3′; FRAXAC1 (Richards et al. 1991), FRAXA.PCR1.1 5′-GAT CTA ATC AAC ATC TAT AGA CTT TAT T-3′, FRAXA.PCR1.2 5′-AGA TTG CCC ACT GCA CTC CAA GCC-3′, FRAXAC2 (Richards et al. 1991), FRAXA.PCR2.1 5′-GAC TGC TCC GGA AGT TGA ATC CTC A-3′, FRAXA.PCR2.2 5′-CTA GGT GAC AGA GTG AGA TCC TGT C-3′, DXS292 (Richards et al. 1991) VK14F 5′-TCA TAC CAT ACT GTA TGA TGA TT-3′, VK14R 5′-GAA CTA GCT CCT GCAT AGC ACT GG-3′, DXS998 (Weissenbach et al. 1992) AFM224-zg11a 5′-CAG CAA TTT TTC AAA GGC-3′, AFM224zg11m 5′-AGA TCA TTC ATA TAA CCT CAA AAG A-3′, DXS15 (Wehnert et al. 1993), 9120 5′-AGC ACA TGG TAT AAT GAA CCT CCA CG-3′, and 9121 5′-CAG TGT GAG TAG CAT GCT AGC ATT TG-3′. The number of alleles and the heterozygosity of the typed markers are given in table 1.

PCR (Saiki et al. 1986) was performed in a 25-μl volume containing 50–100 ng of human genomic DNA as template; 200 μM each dNTP, except that 90% of the dATP or the dCTP was replaced by 1 μCi α[P^{32}]-dCTP (NEN) or α[^{35}S]-dATP (Amersham); 12.5 pmol of each primer; 50 mM KCl; 10 mM Tris; 1.25 mM $MgCl_2$; and 1 U of *Taq* polymerase (Boehringer Mannheim). For specific amplification of the FRAXAC1 CA repeat, a higher $MgCl_2$ concentration, 5.5 mM, was required. For FRAXAC2, 0.01% gelatin and 10% DMSO were added, and the primers used were 62.5 pmol each. All reactions were overlain with mineral oil. Cycling conditions were as follows: 30 cycles of 94°C for 1 min, 60°C for 1 min (62°C for FRAXAC1), and 72°C for 1 min, for FRAXAC1, DXS1126, DXS15 and DXS548; 25 cycles of 95°C for 1 min, 55°C for 2 min, and 72°C for 1 min, for DXS998; and 4 min at 94°C, after which the *Taq* polymerase was added ("hot start"); then 30 cycles of 1 min at 94°C and 1 min at 55°C, for DXS292; and the "hot start" was followed by 30 cycles of 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min, for FRAXAC2. Four microliters of the amplified product was mixed with formamide sample buffer and was analyzed on a 6% denaturing polyacrylamide sequencing gel. Samples were electrophoresed at 55 W for 2–4 h, were dried without fixation, and were exposed to XAR film (Kodak) overnight. Genotype assignments were done by investigators who were blinded to the affection status of the subjects.

Linkage Analysis

For linkage analysis, genotypes were available from 177 individuals, including 73 autistic children and 21 unaffected children. For the statistical analysis, autism was treated either as an X-linked dominant trait (models 1–4) or as an X-linked recessive trait (model 5). The different genetic models are shown in table 2. Since the penetrance of the disease gene in autism is unknown, calculations were performed with five different penetrance values (table 2). For the dominant models, penetrance was fixed in males, and gene frequencies were calculated on the basis of a 4/10,000 incidence of autism. Female penetrance was calculated assuming a sex ratio of 3:1 males:females. For the recessive model, penetrance was fixed at 50% for the homozygote and the male hemizygote. The gene frequency was assumed to be .02. These numbers were arbitrarily chosen. We also tested several other combinations of penetrance and gene frequency; the effect of varying these was to increase or decrease the negativity of the lod scores, but in no case were the conclusions changed. The affection status of the parents was considered unknown in all models. Individuals with the diagnosis "uncertain" were omitted from the calculations. Linkage analysis was performed using the software package LINKAGE (version 5.03; Lathrop and Lalouel 1984). Two-point analysis was performed using the program MLINK; multipoint analysis was performed using the program LINKMAP.

Results

Southern-Blot Analysis of the CGG Repeat

Using the *Eco*RI/*Eag*I double digest allows us to analyze both amplification of the CGG repeat and methylation of DNA (Rousseau et al. 1992). When DNA from unaffected individuals is digested with *Eco*RI/*Eag*I and probed with StB12.3, one or two bands are typically seen. Females show characteristic 5.2- and 2.8-kb bands, with the 5.2-band arising from the failure of *Eag*I to cut DNA from the methylated (inactive) X chromosome and with the 2.8-kb band arising from the nonmethylated (active) X. Males show a single 2.8-kb band. The analysis of methylation is useful for distinguishing between large premutations without clinical expression and small full mutations. Premutations in males are detected by an increase in size of the unmethylated fragment, by 70–∼500 bp. In females, premutations give a very distinctive four-band pattern that is easily detected. Full mutations are generally in the 1–3-kb range and are rarely overlooked.

In none of the subjects examined did we detect any increase in the size of the 2.8- or 5.2-kb fragment. All females showed the expected 5.2/2.8-kb band pattern, whereas males showed only the 2.8-kb band. Fragile X–positive

Table 2

Genetic Models for Which Lod Scores Were Calculated

	Model 1	Model 2	Model 3	Model 4	Model 5
Male penetrance ...	.80	.50	.3	.10	.50
Female penetrance:					
Heterozygote	.13	.083	.05	.016	.0
Homozygote	.90	.90	.90	.90	.50
Gene frequency	.000375	.0006	.001	.003	.02

For the dominant models (models 1–4), the male penetrance values were fixed and the female penetrances were calculated on the basis of a male:female autism ratio of 3:1. Gene frequencies were calculated assuming a 4/10,000 prevalence of autism. For the recessive model (model 5), penetrance was fixed at 50% for the homozygote and the male hemizygote. The gene frequency was assumed to be .02.

controls (received from the Cytogenetics Laboratory at Stanford University Medical Center) could easily be detected by an increase in the size of the fragment. Thus we found no molecular evidence for the occurrence of fragile X syndrome in this sample of autistic children.

PCR Analysis of the CGG Repeat

The Southern-blot method used above may not detect small mutations or premutations. During the course of this work, we evaluated an autistic child with the phenotypic features of fragile X. He, his carrier mother, and his infant sister all had CGG repeats of 65, as measured by PCR analysis; this repeat would not have been detected by Southern analysis. Accordingly, to insure that we were not overlooking small repeat expansions, we directly measured repeat length by PCR analysis in all the subjects who were part of the linkage study. The PCR results confirmed those obtained by Southern-blot analysis, without exception. The range of repeat length in the unaffected group was 17–46 repeats, and that in the autistic group was 20–43 repeats. No difference between autistic and nonautistic individuals was observed in the average repeat length. None of the subjects had a repeat length in the premutation size range.

Linkage Analysis

No recombination was observed between the markers FRAXAC1, FRAXAC2, and DXS548 and the CGG repeat. Therefore, haplotypes were used for calculations of lod scores. Two-point lod scores between haplotypes and autism are shown in table 3. In all four dominant models, linkage between the fragile X locus and autism could be excluded with high confidence in 21 families (table 4). In these families, a maximum negative lod score was observed at 0% recombination. Results for the remaining 11 families were inconclusive; 3 of these families were uninformative, 5 showed presumptive evidence of linkage (lod score 1.15), and 3 showed slightly negative results. Similar results were obtained for the recessive model, with overall lod scores being less negative (table 4).

To examine this further, we typed additional markers on the families that were inconclusive or positive (table 5). DXS998 and DXS1126 showed no recombination to the previous markers typed. Inclusion of the typing in the haplotype analysis did not change the lod scores. Two-point lod scores for markers located 5 cM (centromeric DXS292) and 12 cM (telomeric DXS15) away from the FMR-1 gene resulted in negative lod scores. Multipoint analysis between the markers resulted in negative log-likelihood differences across the region of the FMR-1 gene in the models specified. However, they did not reach the level of statistical significance, and no additional conclusions could be drawn.

Discussion

The occurrence of the fragile X syndrome has been described in a small number of cases of autism; although the

Table 3

Two-Point Lod Scores between Autism and Haplotypes of DXS548, FRAXAC1, FRAXAC2, and the CGG Repeat

	Lod Score at Recombination Fraction of					
Model	.000	.050	.100	.200	.300	.400
1	−62.12	−14.39	−8.36	−3.21	−1.17	−.26
2	−55.06	−13.39	−7.66	−2.86	−1.04	−.20
3	−50.01	−13.13	−7.56	−2.84	−1.06	.23
4	−40.86	−12.93	−7.56	−2.84	−.98	−.22
5	−27.26	−12.48	−7.60	−3.07	−1.01	−.26

Table 4

Lod Scores for Linkage Analysis between Haplotypes of FRAXAC1, FRAXAC2, DXS548, and CGG Repeat Length and Autism in 32 Multiplex Families

	Lod Score						
	Model 1, at Recombination Fraction of			Model 5, at Recombination Fraction of			
Family	.0	.01	.05	.0	.01	.05	Affected Siblings Share Allele of Mother
1	−3.13	−1.39	−.72	−1.27	−1.04	−.63	No
2	−3.13	−1.39	−.72	−1.27	−1.04	−.63	No
3	−3.13	−1.39	−.72	−1.27	−1.04	−.63	No
4	−3.25	−1.40	−.72	−1.28	−1.04	−.63	No
5	−2.82	−1.39	−.72	−1.27	−1.04	−.63	No
6	.00	.00	.00	.00	.00	.00	Noninformative
7	−2.82	−1.39	−.72	−1.27	−1.04	−.63	No
8	−.18	−.17	−.14	−1.27	−1.04	−.63	No
9	−2.22	−1.23	−.58	−.89	−.75	−.45	No
10	.52	.51	.46	.52	.51	.46	Yes
11	−3.13	−1.39	−.72	−1.27	−1.04	−.63	No
12	−3.13	−1.39	−.72	−1.44	−1.13	−.66	No
13	.00	.00	.00	.00	.00	.00	Noninformative
14	−3.13	−1.39	−.72	−1.27	−1.04	−.63	No
15	−3.25	−1.4	−.72	−1.28	−1.04	−.63	No
16	−2.82	−1.39	−.72	−1.27	−1.04	−.63	No
17	−3.30	−1.40	−.72	−1.74	−1.24	−.69	No
18	.78	.76	.70	.68	.67	.61	Yes
19	−.65	−.63	−.52	−.64	−.62	−.51	Yes
20	.52	.51	.46	.52	.51	.46	Yes
21	−2.82	−1.39	−.72	−1.27	−1.04	−.63	No
22	.30	.29	.26	.29	.28	.25	Yes
23	−2.82	−1.39	−.72	−1.74	−1.24	−.69	No
24	−2.22	−1.08	−.46	−.74	−.61	−.33	No
25	−3.43	−1.40	−.72	−1.44	−1.13	−.66	No
26	−2.22	−2.12	−1.38	−.74	−.73	−.67	No
27	−.18	−.17	−.14	−.17	−.16	−.13	Yes
28	.00	.00	.00	.00	.00	.00	Noninformative
29	.12	.12	.10	.29	.28	.25	Yes
30	−4.20	−1.64	−.91	−2.21	−1.54	−.90	No
31	−3.13	−1.39	−.72	−1.27	−1.04	−.63	No
32	−3.25	−1.40	−.72	−1.28	−1.04	−.63	No

Note.—Calculations were performed for model 1 (see table 2) and for model 5, at the recombination fractions given.

Table 5

Log-Likelihood Differences between Autism (Model 1) and a Fixed Map of DXS292-DXS15 in the Region of FMR-1, for 11 Families That Were Inconclusive in the Two-Point Analysis

Family	Log-Likelihood Difference
1	.00
2	−.14
3	.50
4	−.08
5	−1.50
6	−.12
7	.00
8	.18
9	−.14
10	−.27
11	−.14

occurrence varies across studies, the best estimates indicate that 2%–5% of autistic children exhibit cytogenetic evidence of the fragile X syndrome. There is abundant evidence that some proportion of cases of autism are genetic, but the fraction of these is probably small, compared with the fraction of nongenetic cases. Thus, in theory, fragile X syndrome could account for a substantial portion of the genetic cases of autism. Since the FMR-1 gene involved in fragile X syndrome has been cloned and characterized, it is important to establish whether the relation between autism and fragile X syndrome is biologically meaningful. If it is, then knowledge about the pathobiology of fragile X syndrome might contribute important insights to our understanding of genetic forms of autism.

This study tested two hypotheses concerning autism and the fragile X syndrome; the underlying premise in both is that genetic cases of autism represent an "occult" form of the fragile X syndrome, in which the typical cytogenetic

and phenotypic characteristics of fragile X are not always expressed. The first hypothesis is that autism is an uncommon variant of fragile X, in which only amplification of the CGG repeat is present; the phenotypic and cytogenetic manifestations of the disorder are absent. The second hypothesis is fundamentally the same but places the putative mutation elsewhere in the FMR-1 gene. To test the first hypothesis, we examined the FMR-1 gene in multiplex autistic families. We found no evidence for an expanded trinucleotide repeat in the 79 autistic individuals in these families. Thus there is no evidence to support the hypothesis of a (CGG)n amplification in autism.

We also did not find evidence for premutations in the FMR-1 gene. The result of our genotyping analysis is in agreement with cytogenetic studies in autistic children from simplex families (for review, see Einfeld and Hall 1992).

We also examined the hypothesis that a mutation lies elsewhere within the FMR-1 region. To test this, we first performed a linkage analysis using microsatellite markers DXS548, FRAXAC1, and FRAXAC2. All of these markers are located <150 kb from the FMR-1 gene. Additionally, we typed the CGG repeat itself, which is highly polymorphic. No recombination between the markers was observed. Linkage analysis was performed using haplotypes (tables 3 and 4) for five different modes of transmission with varying degrees of penetrance. The overall observed heterozygosity was 90.6%. Assuming a dominant model, we could unequivocally rule out linkage in 21 families; 3 families were uninformative; and 5 families showed slightly positive lod scores. We would expect that some families would show positive lod scores by chance; the number of positive families would be dependent on the degree of heterogeneity. To examine this, we calculated the expected lod scores for varying degrees of heterogeneity. The expected cumulative probabilities are shown in figure 2. These results predict, under the null hypothesis, that no family is linked to FMR-1 and that ≤ 15 families could show lod scores ≥ 0, while we observed 8 such families. It would thus appear that, in these families, positive lod scores occur with no greater frequency than would be predicted by chance, and there is therefore no basis for invoking heterogeneity as an explanation of our results. Varying the penetrance estimates did not change these conclusions, for any model.

We obtained similar results in testing a recessive model. Six of the families had slightly positive lod scores. As in the dominant model, the number of these families could be accounted for by chance. Tests for heterogeneity by using the program HOMOG (Ott 1991) were negative for all dominant and recessive models. Additional typing of flanking markers in the same subset of families that gave inconclusive results in the two-point analysis did not show evidence for linkage. In multipoint analysis, log likelihood differences were negative in the region of the FMR-1 gene.

Since the results of linkage analysis are dependent on the correct specification of the mode of transmission, we also performed a sib-pair analysis using affected siblings only. No significant sharing of alleles was detected. Genotyping data were available for siblings as well as for at least the mother in all pedigrees. The number of alleles sharing identity by descent was less than expected by chance, whether only male pairs were analyzed or whether females were also included in the analysis.

Although our data indicated that heterogeneity was absent in this sample and that linkage to FMR-1 could be excluded for the sample taken as a whole, we attempted to determine the maximum proportion of families that could theoretically be linked. A total of 22 families displayed discordance of maternal alleles (table 4). Using the maximum likelihood method, we estimated α, the proportion of linked families (see Appendix). The maximized α value is 0, indicating both the absence of heterogeneity and that no families are linked. The α value that gives a likelihood 100 times lower than 0 ($\log L = -2$) is .27. However, for several different models and ways of analyzing the empirical data, our results consistently demonstrate that linkage to FMR-1 can be ruled out in multiplex autistic families.

The initial observation of a high comorbidity of autism and fragile X was greeted with interest because research in the etiology of autism had turned up so few conclusive causes, and a genetic cause seemed highly plausible. However, as work in this field has unfolded, it now appears that only a small percentage ($\sim$3%) of autistic individuals exhibit the fragile X phenotype. Our data indicate that fragile X is almost certainly not the cause of autism in families likely to have a genetic form of this disorder. This would indicate that a search for other genes causing autism will be necessary.

Our data do not, however, address whether, when autism and fragile X co-occur, autism arises from a different mutation, either in FMR-1 or in the genes linked to the FRAXA and FRAXE markers, both of which have been recently implicated in related forms of the fragile X syndrome (Knight et al. 1993). To examine this hypothesis, we have recently begun collecting families in which two or more members are fragile X and autistic. Genotyping studies on these families are underway.

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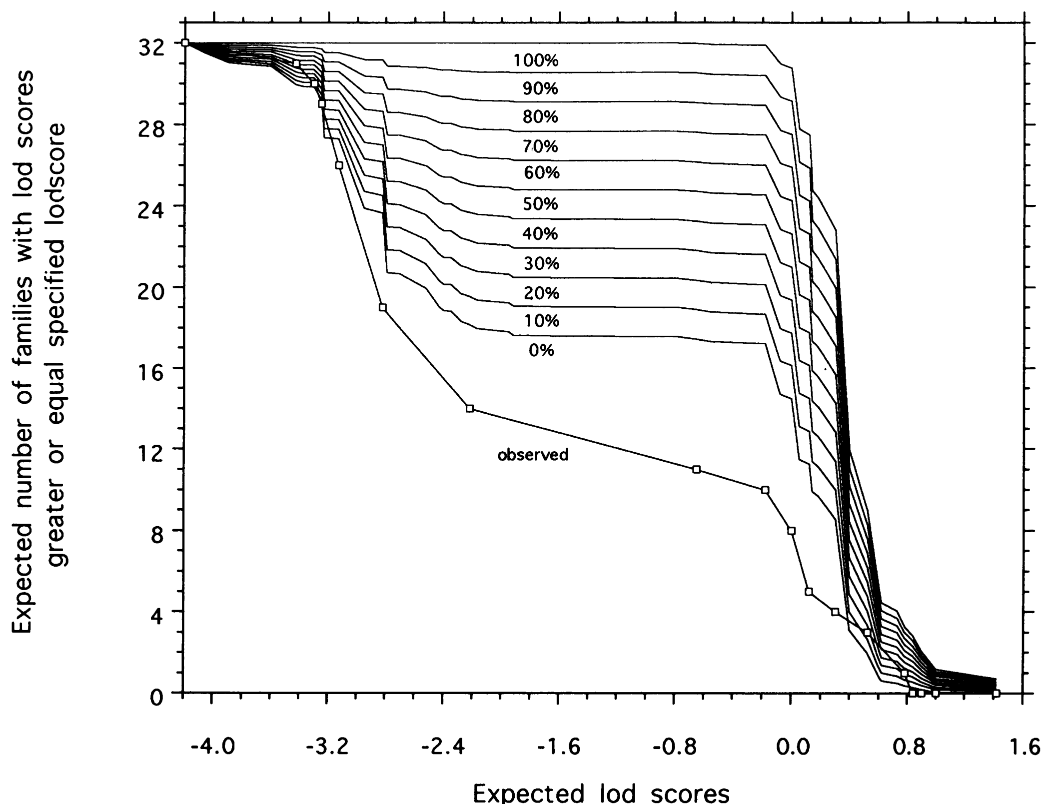

Figure 2 Number of families whose lod scores were expected to be greater than or equal to specified lod score. Expected lod scores (model 1) for the families tested in the linkage analysis at 0% recombination fraction were calculated for varying degrees of interfamilial heterogeneity (0%, 10%, and 20%) and were compared with the observed number of families (unblackened squares). Calculations are based on a marker heterozygosity of 90.6%.

The authors wish to express their gratitude to Dr. Neil Risch for performing the calculation described in the Appendix.

Appendix

A total of 22 families display discordance of maternal alleles. In 19 of these families, the prior probability of sharing is $^1/_2$; in 1 family it is $^1/_4$; in 1 family it is $^1/_8$; and 1 family it is $^1/_{16}$.

There are seven families where the mother is heterozygous but the sibs are concordant. For six of these the prior probability of concordance is $^1/_2$; in the seventh it is $^1/_4$.

In any family with discordance the hypothesis of linkage is excluded; in the families with concordance it is not excluded. If we assume that a priori a proportion, α, of families are linked to FMR-1, the likelihood of the above results is

$$L = [(1-\alpha)^1\!/\!_2]^{19}[(1-\alpha)^1\!/\!_4][(1-\alpha)^1\!/\!_8][(1-\alpha)^1\!/\!_{16}] \times [\alpha + (1-\alpha)^1\!/\!_2]^6[\alpha + (1-\alpha)^1\!/\!_8]\,, \quad \text{(A1)}$$

if we ignore the $^1/_2$ it follows that

$$L = (1-\alpha)^{22}(1+\alpha)^6(1+7\alpha)\,;$$

$$\log L = 22\log(1-\alpha) + \log(1+\alpha) + \log(1+7\alpha)\,.$$

log L is maximized at $\alpha = 0$ (log L = 0).

The value of α that gives a likelihood 100 times lower than the value corresponding to $\alpha = 0$ is the solution of the equation

$$22\log(1-\alpha) + 6\log(1+\alpha) + (1+7\alpha) = -2\,. \quad \text{(A2)}$$

This solution is $\alpha = .27$.

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