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Familial Skewed X Inactivation: A Molecular Trait Associated with High Spontaneous-Abortion Rate Maps to Xq28

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Summary

We report a family ascertained for molecular diagnosis of muscular dystrophy in a young girl, in which preferential activation (⩾95% of cells) of the paternal X chromosome was seen in both the proband and her mother. To determine the molecular basis for skewed X inactivation, we studied X-inactivation patterns in peripheral blood and/or oral mucosal cells from 50 members of this family and from a cohort of normal females. We found excellent concordance between X-inactivation patterns in blood and oral mucosal cell nuclei in all females. Of the 50 female pedigree members studied, 16 showed preferential use (⩾95% cells) of the paternal X chromosome; none of 62 randomly selected females showed similarly skewed X inactivation ($P < .0003$). The trait for skewed X inactivation was maternally inherited in this family. A linkage study using the molecular trait of skewed X inactivation as the scored phenotype localized this trait to Xq28 (DXS1108; maximum LOD score [Z_{max}] = 4.34, recombination fraction [θ] = 0). Both genotyping of additional markers and FISH of a YAC probe in Xq28 showed a deletion spanning from intron 22 of the factor VIII gene to DXS115-3. This deletion completely cosegregated with the trait ($Z_{max} = 6.92$, $\theta = 0$). Comparison of clinical findings between affected and unaffected females in the 50-member pedigree showed a statistically significant increase in spontaneous-abortion rate in the females carrying the trait ($P < .02$). To our knowledge, this is the first gene-mapping study of abnormalities of X-inactivation patterns and is the first association of a specific locus for recurrent spontaneous abortion in a cytogenetically normal family. The involvement of this locus in cell lethality, cell-growth disadvantage, developmental abnormalities, or the X-inactivation process is discussed.

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Introduction

In normal XX female mammals, one of the two X chromosomes in each cell is inactivated at an early stage in development (Lyon 1961), to ensure dosage equivalence with males (Ohno 1967; Gartler and Riggs 1983; Lyon 1989). The X-inactivation process is subject to numerous influences both during normal development and in response to cytogenetic or single-gene pathologies. It is well documented that cytogenetically detectable deletions and duplications result in the preferential inactivation of the *abnormal* X chromosome in clinically unaffected females, presumably because of multigenic cell lethality (Thode et al. 1988; Wells et al. 1991). On the other hand, balanced translocations or insertions result in the preferential inactivation of the *normal* X chromosome, presumably because of lethal dosage abnormalities in autosome fragments (Mattei et al. 1982).

Nonrandom patterns of X inactivation may also result from single-gene mutations where there is a strong selection for the normal X chromosome, often in specific cell lineages requiring the protein function of the mutant gene. Inherited immune deficiencies are an excellent example of this kind of mechanism. Female carriers of Bruton X-linked agammaglobulinemia (XLA) show use of the normal X chromosome in all peripheral B cells, whereas T cells show random X inactivation (Alterman et al. 1993; Allen et al. 1994). In X-linked severe combined immunodeficiency (XSCID), skewed patterns of X inactivation are present in both T cells and B cells but not in granulocytes (Goodship et al. 1988; Conley et al. 1990; Puck et al. 1992). In X-linked thrombocytopenia, lymphocytes show a skewed pattern of X inactivation whereas polymorphonuclear lymphocytes do not (De Saint-Basile et al. 1991). Skewed X inactivation has also been seen in female carriers of certain X-linked recessive disorders (Orstavik et al. 1996).

Some X-linked disorders show inheritance that is consistent with a male-lethal trait. Carrier females in these families exhibit some symptoms, and most have the normal X chromosome active in nearly all cells (Migeon et al. 1989; Harris et al. 1992; Lindsay et al. 1994). Examples of these disorders are incontinentia pigmenti and 12 other X-linked male-lethal disorders, although no

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genes have been identified to date. Since "affected males" are generally not seen in these disorders, it remains possible that these do not reflect maternal inheritance of X-linked male-lethal traits but, instead, a paternally inherited new mutation from the father's germ line (Thomas 1996).

In many X-linked recessive disorders, rare female carriers can be found who show clinical symptoms of disease. Such "manifesting carriers" are perhaps best studied in Duchenne muscular dystrophy (DMD), for which we and others have shown that the clinical manifestation of muscular dystrophy in these females is due to "skewed" X inactivation (preferential inactivation of the normal X chromosome) (Pegoraro et al. 1994; Azofeifa et al. 1995). It has long been assumed that manifesting carriers of DMD and other X-linked disorders result from random statistical variations of X-inactivation patterns, with the incidence of skewed X inactivation in random carriers reflecting the incidence of skewed X inactivation in the random population. However, in a recent study of a cohort of 13 manifesting carriers of DMD, we showed that 90% of these girls and women received the dystrophin-gene mutation as a new paternally derived mutation: this observed inheritance pattern was at 27-fold odds with expectations based on Bayesian probabilities (Pegoraro et al. 1994). The variance between our observed inheritance patterns and theoretical predictions implied that there was a nonrandom occurrence of skewed X inactivation, as well as a possible relationship between skewed X inactivation and paternal inheritance of dystrophin-gene mutations. We hypothesized that skewed X inactivation in females with isolated dystrophinopathy could be due to inheritance of a distinct Mendelian trait resulting in skewed X inactivation, and, consistent with this hypothesis, most families of manifesting carriers tested to date show a strong clustering of skewed X inactivation in females (Azofeifa et al. 1995; Hoffman et al. 1996).

A single family has been reported with familial skewed X inactivation with preferential use of the paternal X chromosome (Naumova et al. 1996). This family was of insufficient size to permit genetic mapping of the trait or to establish inheritance patterns. Here we report a large pedigree showing familial skewed X inactivation. The family reported here, including 50 female members in four generations, was studied for X-inactivation patterns in both blood and oral mucosal cell DNA; results were compared with those in randomly selected females; and the genetic locus was characterized.

Subjects and Methods

Index Family

DNA was collected from 50 female family members in four generations. Quantitative X-inactivation analyses were done by use of DNA extracted from blood of 29 female relatives and from oral mucosal cell DNA of 48 female relatives. Twenty-seven female family members provided both oral mucosal cell DNA and blood DNA. Telephone interviews were conducted to assess past medical and family history for each family member.

Controls

DNA from 64 unrelated females was studied. Peripheral blood was collected and DNA was isolated from 44 unrelated females. Fourteen of these feamles were from myotonic dystrophy pedigrees, 5 were from a rippling-muscle-disease pedigree, 5 had Alzheimer dementia, 2 were minimally affected by an idiopathic myopathy, and 18 were normal. Oral cell DNA of the same 18 normal women was studied. Muscle DNA from 20 unrelated females referred to this laboratory for neuromuscular-disease studies was studied. All of these patients had minimally abnormal muscle histopathology and normal immunostaining for dystrophin.

DNA Extraction

Peripheral blood DNA.—Blood was collected in EDTA tubes, and DNA was isolated from peripheral blood, as described elsewhere (Pegoraro et al. 1994).

Muscle DNA.—DNA was isolated from cryosections of muscle biospy, as described elsewhere (Pegoraro et al. 1995).

Oral mucosal cell DNA.—Mouthwashing for 30 s with a 3% sucrose solution (∼10 ml) was done, and the solutions were collected in tubes containing 10 mM EDTA as a preservative (Lawton et al. 1992). Mouthwashes were pelleted at 14,000 *g* for 20 min, and the cells were solubilized in lysis buffer (Vogelstein et al. 1987). Proteinase K digestion in PCR buffer (Miller et al. 1988) was done at 60°C for 2 h. Samples were extracted with phenol-chloroform and chloroform, and the DNA was concentrated by use of Amicon Microcon microconcentrators.

X-Inactivation Study

X-inactivation patterns in peripheral blood DNA, muscle DNA, and oral mucosal cell DNA were quantitated by use of flurorescent PCR as described elsewhere (Allen et al. 1992; Pegoraro et al. 1994). In brief, the methylation status of the androgen-receptor promoter adjacent to a highly polymorphic CAG repeat in the 5′ end of the coding region of the androgen-receptor gene was determined by use of methylation-sensitive restriction enzymes *Hpa*II/*Cfo*I. PCR products, both before and after digestion, were electrophoresed on an ABI 373A automated sequencer, and peak heights were analyzed by use of GeneScan software (Applied Biosystems). Corrections for preferential PCR of alleles, as well as quantitation of X inactivation, were done as described

elsewhere (Pegoraro et al. 1994). Ratios of 95%:5% were used as a cutoff point for classification of the patient as showing skewed patterns of X inactivation. Twenty-two of 50 females were studied by two or more separate assays, and the values were averaged. Standard errors were generally 5%.

Statistical Analyses

Correlation of X-inactivation results in oral cell DNA versus blood DNA was done by use of the Pearson correlation coefficient and the Spearman correlation coefficient. Normality of the distributions of X-inactivation values was determined by S-W statistics. Data files were generated by use of the X-chromosome value $\geq 50\%$ as the measurement for each individual; however, this created an artifact in which normality of distribution was 50%–100%, with 50% being the mean. Thus, we duplicated the data, using the formula ($100\% - n\%$), so that the data distributions were 0%–100%, with 50% remaining as the mean. Coefficient of variance was also used. A nonparametric Mann-Whitney test was used to compare the difference between the values of X inactivation in the family versus those in control females. χ^2 Analysis was used to compare the sex ratio and the spontaneous-abortion rate in the affected and unaffected females. All statistical analyses were done by use of the SAS (Statistical Analysis System) software package.

Hypervariable-Repeat Analysis

Nineteen highly informative $(CA)_n$ loci were selected from the Weissenbach set, as described elsewhere (Kobayashi et al. 1995). Additional CA repeats were tested in Xq13 and Xq28, to provide denser allele data in the Xic region and telomeric regions of the X chromosome (DXS1124, DXS441, DXS1209, and DXS1222 in Xq13 and DXS1177, G6PD, factor VIII gene [F8C], and DXS1108 in Xq28) (Freije and Schlessinger 1992; Fain et al. 1995). Genotypes for $(CA)_n$ loci were determined by use of PCR methods as described elsewhere (Stephan et al. 1994; Kobayashi et al. 1995).

Linkage Analyses

For phenotype determination, blood DNA was used when available. If blood DNA was not available, then values of X inactivation in oral cell DNA were used.

Both the relative order of microsatellite markers along the X chromosome and their genetic distances (in cM) were derived from published maps (Nelson et al. 1995). The LINKAGE package (Lathrop et al. 1984) was used for two-point and multipoint linkage analyses.

The "disease" model that we used assumed that abnormal X-inactivation patterns were inherited as a Mendelian trait segregating in an X-linked dominant fashion, with complete penetrance. We also tested a model with reduced penetrance. We defined the trait of extremely skewed X-inactivation values ($\geq 95\%$:5%) as an affection-status locus. We also tested different affection-status criteria, and we also modified the disease frequency and the phenocopy rate in concordance with the specific affection-status criteria.

Cytogenetic Analysis

Cytogenetic studies were performed on cultured peripheral blood lymphocytes from an affected female (IV-18; fig. 1), by use of standard methods (Seabright 1971; Gosden et al. 1992). FISH analysis was performed by use of a YAC probe that maps to Xq28 (gift of D. Schlessinger, Washington University, St. Louis). The probe was labeled with biotin by use of a nick-translation kit (Gibco BRL). The labeled YAC probe was mixed with an excess unlabeled Cot1 DNA to suppress nonspecific binding of the probe to repetitive sequences and was denaturated at 80°C for 15 min. Hybridization between the denatured target DNA (slide) and the probe was performed at 37°C for 24 h. Posthybridization washes were done at 43°C, and FITC-labeled avidin was used for detection, with a DAPI counterstain to aid in identification of the chromosomes. Slides were viewed under a Zeiss Axiophot microscope equipped with an HBO (Osram) 100/2W mercury lamp. The images were acquired and analyzed by use of the Probe Vision FISH Imaging System (Applied Imaging).

Physical Mapping

Four polymorphisms within F8C were used: (1) a CA repeat in intron 22, (2) a CA repeat in intron 13, (3) a *Hin*dIII site in intron 19, and (4) a *Taq*I site in the 5′ flanking region (Antonarakis et al. 1995). Anonymous probe 767 for DXS115 (gift of E. Bakker, Leiden University) was also used; this recognizes three sites within Xq28 (Freije and Schlessinger 1992). PCR amplification, restriction-endonuclease digestion, and Southern analysis were done according to standard methods.

Results

Familial Skewed X-Inactivation Family

The proband (V-15; fig. 1) was a 2-year-old female who presented with proximal muscle weakness and calf hypertrophy. Serum creatine kinase measurements were markedly elevated (21,000 U/liter; normal <200 U/liter). The patient's biopsy was referred for dystrophin analysis, to rule out a primary dystrophinopathy. Dystrophin immunofluorescence showed a variable and faint reaction pattern in all muscle fibers, without definite dystrophin-negative fibers. Dystrophin immunoblotting showed dystrophin of normal size and amount, although the smaller (~400 kD) isoform of dystrophin was missing. A Becker carrier status could not be ruled out, and the patient underwent X-inactiva-

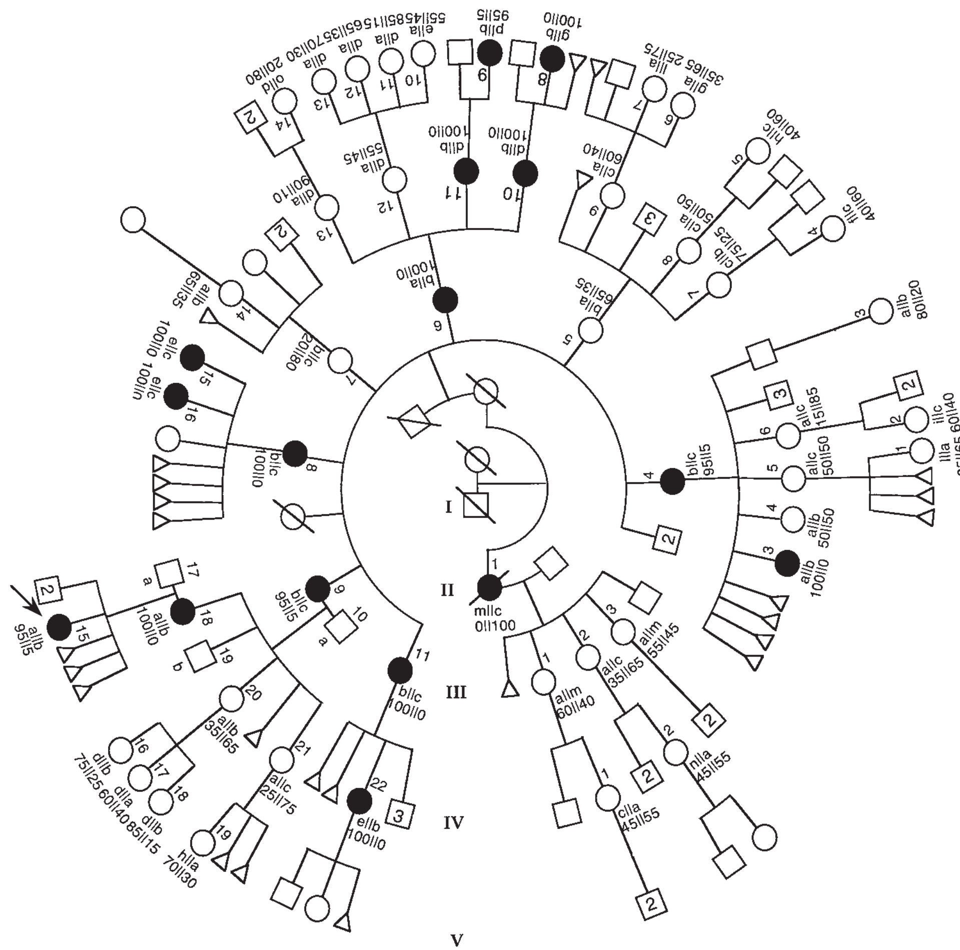

Figure 1 Pedigree of the family segregating familial skewed X inactivation. Unblackened symbols denote family members with random patterns (values <95%:5%) of X inactivation; and blackened symbols denote female members with skewed patterns of X inactivation (values ⩾95%:5%). Under each symbol, androgen-receptor alleles are shown. Also provided are the percentage of cells showing each X chromosome active. For X-inactivation assays, peripheral blood DNA was used when available; otherwise, results from oral mucosal DNA are shown.

tion studies to gather further evidence of an X-linked myopathy. X-inactivation analysis showed a completely skewed pattern, with one of the proband's X chromosomes active in >95% of her peripheral blood cells (fig. 1). To rule out cytogenetic abnormalities as the cause of skewed X inactivation in the proband, high-resolution chromosome banding was done; results were normal. We and others have previously observed familial clustering of skewed X inactivation, suggesting that it may be inherited as a Mendelian trait. To test whether the proband's family showed patterns of X inactivation differing from those found in the normal population, 50 females in four generations were studied.

DNA samples were collected from 50 female family members (oral mucosal cell DNA from 48; both peripheral blood DNA and oral mucosal DNA from 27). Between blood DNA and oral mucosal DNA, there was highly statistically significant correlation of X-inactivation results (Pearson correlation coefficient $R = .8818$, $P = .0000$; Spearman correlation coefficent $R = .8759$, $P = .0000$). Ratios between the active and inactive X chromosome were calculated as follows: 12 (41%) of 29 samples of peripheral blood DNA and 21 (44%) of 48 samples of oral mucosal cell DNA had 50%–70% of one X active; 4 (14%) of 29 blood DNA samples and 8 (17%) of 48 oral cell DNA samples had 70%–85% of one X active; and 10 (34.5%) of 29 blood DNA samples and 16 (33.5%) of 48 oral cell DNA samples had 95%–100% of one X active (figs. 1–3).

The set of family members in our index family for whom both blood DNA samples and oral cell DNA samples were available showed similar patterns of X inactivation in the two tissues (6.7% ± 3.3%), but a 0%–20.7% range of variability was observed (fig. 3*B*). However, when only the subset of females with values of X inactivation >95%:5% was considered, the difference between the patterns of X inactivation in the two tissues was extremely low (1.78% ± 2.11%) (fig. 3).

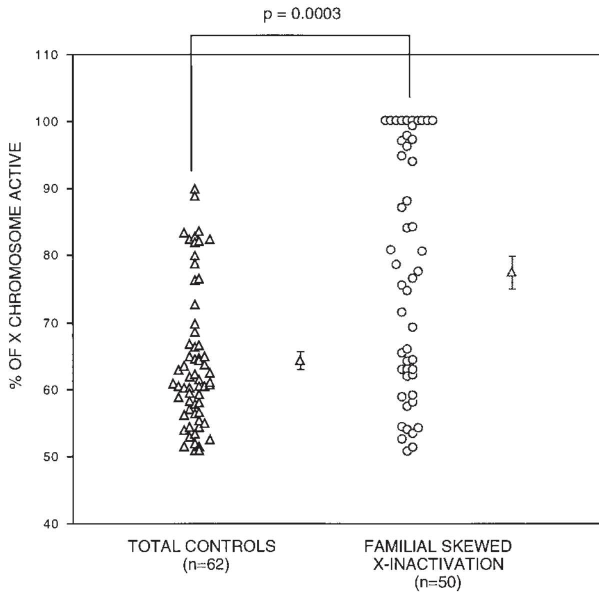

Figure 2 X-inactivation patterns showing statistically significant differences between the familial skewed X-inactivation group and the control group.

A statistical test for the normality of the distribution of the values of X inactivation in the female family members was done, and the distribution was found to deviate from a normal distribution ($P = .0001$). Analysis of kurtosis also showed a statistically significant long-tailedness of the distribution (kurtosis = 2.05). The coefficient of variance was 23.5%.

Female Control Population

Of the 64 female controls, 62 were informative at the androgen-receptor locus and were tested for X-inactivation patterns. Peripheral blood DNA from 18 normal females and from 24 female patients with various non–X-linked disorders was studied. Muscle DNA from 20 female patients showing minimally abnormal muscle histopathology with no evidence of dystrophin abnormalities was studied. Distributions of the X-inactivation patterns are shown in figure 2. Forty-eight (77.5%) of 62 DNA samples had 50%–70% of one X active; 10 (16%) of 62 DNA samples had 70%–85% of one X active; and 4 (6.5%) of 62 DNA samples had >85% of one X active. No female in the control groups had >95% of cells with one X active (fig. 2). Similar distributions were observed when control-group subsets (nor-

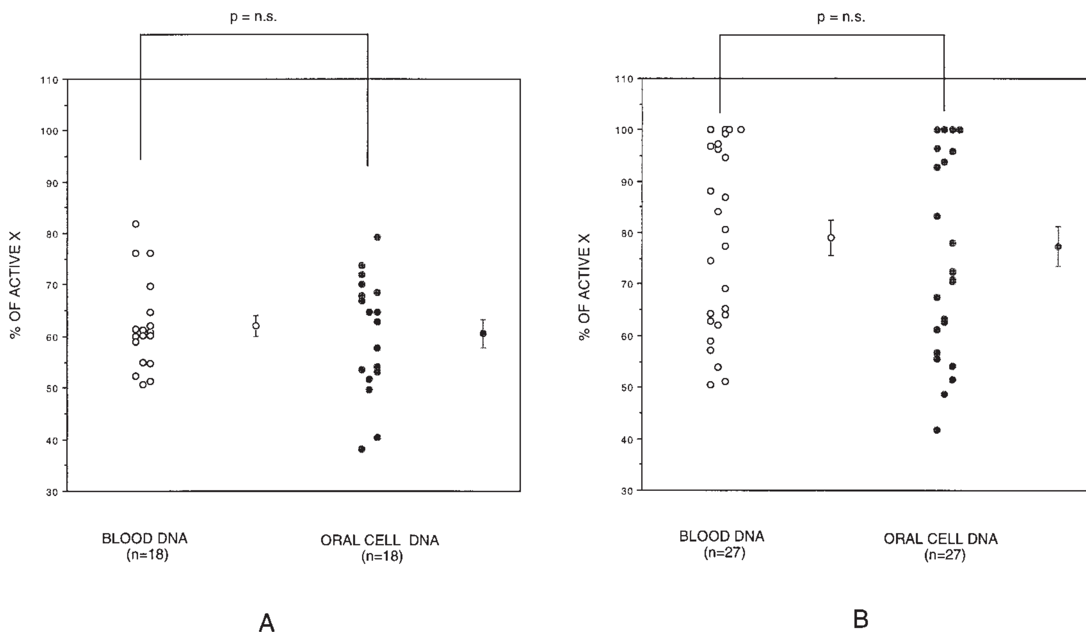

Figure 3 Peripheral blood DNA and oral cell DNA showing similar patterns of X inactivation in the familial skewed X-inactivation group and the control group. Shown is the comparison of X-inactivation patterns in peripheral blood DNA and oral mucosal cells DNA, in 18 normal females (*A*) and 27 female members of the index family (*B*). A concordant pattern of X inactivation, between blood DNA and oral cell DNA, is shown in the two groups. The average difference between the more active X chromosome in blood DNA and the more active X chromosome in oral cells DNA was 6% in the control group and 6.5% in the index family, with a variability range of 0%–20.7%. In the subset of extremely skewed (⩾95%:5%) females, the variability was reduced to 1.78%. There was excellent concordance between oral cell DNA and blood DNA from the same patient.

mal, myopathic, and other non–X-linked disorders) were compared (data not shown). The distribution of the values of X inactivation were not statistically different from an expected normal distribution ($P = .0626$). Kurtosis was also not statistically different from a normal distribution (kurtosis = .5). The coefficient of variance was 16.5%.

Eighteen normal female controls were studied for both blood DNA and oral mucosal cell DNA. Correlation of blood X-inactivation values and oral cell X-inactivation values was highly statistically significant (Pearson correlation coefficient $R = .7484$, $P = .0003$; Spearman correlation coefficient $R = .7589$, $P = .0003$) (fig. 3*A*). The difference between the percentage of cells having a given X chromosome active in peripheral blood DNA versus that in oral mucosal DNA was, on average, 6% (5.77% ± 1.9%), range 0.46%–14.14%.

Genetic-Linkage Analyses

Phenotype assignment.—We defined the "phenotype" as skewed X inactivation, in which individuals with values of X inactivation >95%:5% were scored as "affected" (fig. 1). Although it is well documented that females in the normal population can occasionally show skewed X inactivation, none of our female controls had patterns of X inactivation with <5% of cells with one X active (<95%:5%). Thus, the phenocopy rate could be set at 0. On the basis of this criterion, 16 of 50 females in the pedigree were scored as affected. Given the statistical concordance between X-inactivation values of blood DNA and those of oral cell DNA, either measurement was used for phenotype assignment; in no case did "affection status" change between blood and oral cell results.

Inheritance pattern.—Pedigree analysis showed that all affected females were born to affected mothers; there was absence of any male-to-female transmission, and there were no "isolated" cases (i.e., affected female but nonaffected mother). Given this inheritance pattern, we decided to test an inheritance model of a fully penetrant monogenic X-linked dominant trait causing inactivation of the X chromosome harboring the trait.

To test this model, we conducted an X-chromosome linkage search using 27 hypervariable repeats spaced an average of 10 cM apart on the X chromosome. Markers were typed on an ABI automated sequencer, by use of fluorescent multiplex analyses. Two-point linkage analysis was performed, and LOD scores were computed. A statistically significant LOD score (>2 for X-linked disorders) was obtained for marker DXS1108 (Maximum LOD score [Z_{max}] = 4.34 at recombination fraction [θ] = 0) in Xq28. Further testing of markers in Xq28 showed that an intronic CA repeat in intron 13 of F8C showed a lack of inheritance of alleles between all informative affected female family members (figs. 4 and 5), suggestive of a deletion. The highest two-point maximum LOD score (Z_{max}) was observed for marker F8C between the trait and this putative deletion (Z_{max} = 6.92 at θ = 0). All females who carried the deletion (either by noninheritance of F8C, or linkage to DXS1108, or both) showed highly skewed X inactivation (⩾95%:5%). In addition, all the females scored as "unaffected" did *not* inherit the deleted X chromosome.

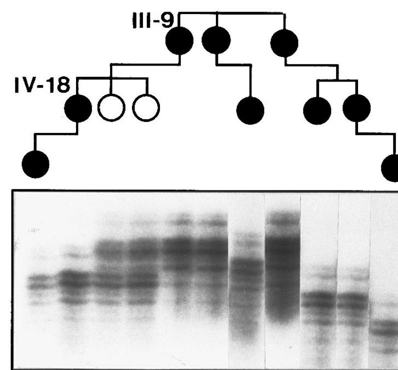

Figure 4 Inheritance of a putative deletion mutation in F8C in the affected females in the familial skewed X-inactivation pedigree. Shown is a subset of the family's pedigree, showing both lack of inheritance at F8C in the affected members and proper inheritance patterns in the unaffected members; for example, III-9 (lane 5) is homozygous for the larger F8C allele, the two unaffected daughters IV-20 and IV-21 (lane 3 and 4) are heterozygous, but IV-18 (lane 2) is homozygous, having only the paternal allele. IV-18 inherited a deleted allele, in the F8C region, from her mother.

No other marker outside the Xq28 region showed a positive LOD score. Specifically, markers in Xq13, where XIC maps, showed consistently negative LOD scores at all computed distances.

We tested the effect that alteration of the stringency of "affection status" had on the LOD scores. Affection status was changed from 95%:5%, to 90%:10%, 85%:15%, and 80%:20% (table 1). The penetrance in heterozygous affecteds was decreased slightly, from 1.0 to .999, although changing of the penetrance had little effect on LOD scores. In the pedigree, 90%:10% resulted in one additional affected family member, 85%:15% in four additional affected family members, and 80%:20% in seven additional affected family members (see X-inactivation values in fig. 1). The phenocopy rate was changed for each affection-status criterion, on the basis of the frequency of observation of that X-inactivation pattern in our control population (.00 for 95%:10%, .03 for 90%:10%, .06 for 85%:15%, and .12 80%:20%) (table 1). Reducing the stringency of affection status resulted in a lowering of Z_{max}'s; however, F8C showed LOD scores >2.0 for all models tested.

Analysis of the Pedigree

Sex ratios and spontaneous-abortion frequencies were determined through telephone interviews with all family

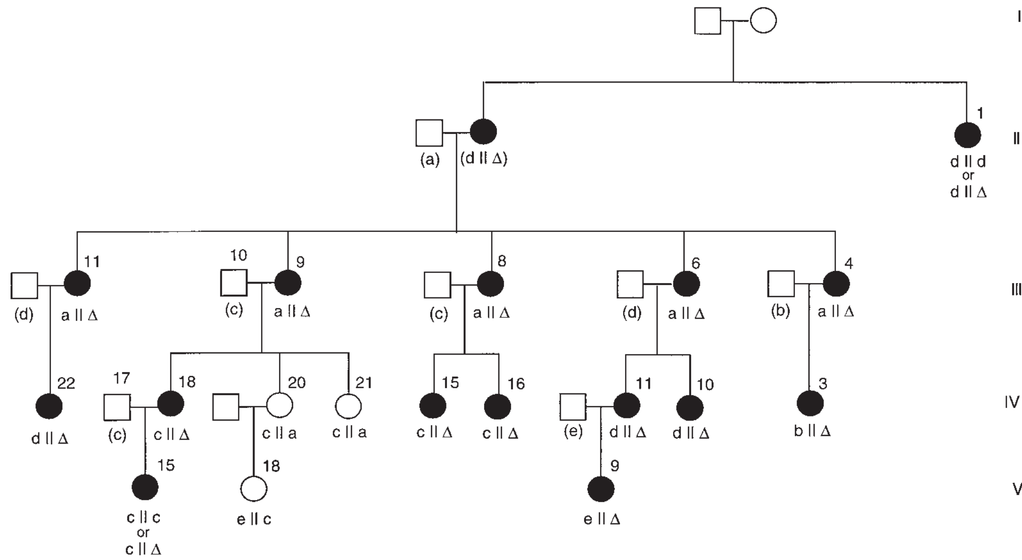

Figure 5 Deletion mutation at F8C, matching with phenotype assignment of the skewed X-inactivation trait. Shown are haplotype data for the F8C locus, for a subset of the pedigree. The affected females in this pedigree inherited a deletion mutation, at F8C, from their mother. In V-15 the marker was uninformative, but inheritance of the deletion could be inferred on the basis of DXS1108 genotyping.

members. Recorded were the number of female and male offspring from each union, the number of known spontaneous abortions, and any pertinent disease history. Other than in the index case, no inherited neuromuscular disorders were reported in this family.

The 10 affected females in the pedigree generated 22 females and 14 males, with a female:male ratio of 1:.64. Offspring of the 18 unaffected females were 24 females and 20 males, with a female:male ratio of 1:.83. Although there was a lower frequency of male offspring from affected females, this difference did not reach statistical significance ($\chi^2 = 0.349$ with 1 df, P not significant).

In the affected females there were 17 miscarriages, for a spontaneous-abortion rate of 32%. Gestational age of the miscarriages was 2 mo, in those that were known (2/2). In the 18 unaffected females there were 8 spontaneous miscarriages of unknown sex, for a spontaneous-abortion rate of 15%. The difference, in the spontaneous-abortion rate, between the two groups was significant ($P < .02$, $\chi^2 = 5.50$ with 1 df).

Table 1

Effect of Varying Threshold of Affection Status for X Inactivation, on Linkage Analyses

Affection Status/ Phenocopy Rate and Marker	LOD Score at θ =						
	0	.01	.05	.10	.20	.30	.40
⩾95%:5%/.00							
DXS1177	−∞	2.6	3.0	3.0	2.5	1.8	1
F8C	6.9	6.8	6.3	5.8	4.6	3.2	1.6
DXS1108	4.3	4.2	4.0	3.6	2.8	1.9	.8
⩾90%:10%/.03							
DXS1177	.3	1.4	2.1	2.3	2.1	1.6	.9
F8C	5.6	5.6	5.4	5.1	4.1	2.9	1.5
DXS1108	3.0	3.0	3.0	2.9	2.4	1.6	.7
⩾85%:15%/.06							
DXS1177	1.2	1.6	2.1	2.2	1.9	1.5	.8
F8C	4.8	4.8	4.8	4.6	3.8	2.7	1.4
DXS1108	2.2	2.3	2.4	2.4	2.0	1.4	.6
⩾80%:20%/.12							
DXS1177	.4	.6	1.0	1.2	1.2	.9	.5
F8C	3.4	3.4	3.2	3.0	2.5	1.8	.9
FXS1108	.8	.9	1.0	1.2	1.1	.8	.2

Identification of the Boundaries of a Deletion Responsible for Fetal Wastage and Skewed X Inactivation

In order to define the bounderies of the deletion in this family, three polymorphisms within F8C were typed in the proband, her parents, and a subset of affected females. A *Taq*I polymorphism in the 5′ flanking region was uninformative, and a *Hin*dIII polymorphism in intron 19 showed lack of inheritance between the affected family members, whereas the CA repeat inside intron 22 showed the expected diparental inheritance (data not shown). These data place the proximal boundaries of the deletion mutation between introns 19 and 22 of F8C (fig. 6).

To define the distal boundaries of this mutation, Southern analysis was done with probe 767 for the DXS115 locus. This probe has been shown to detect three homologous copies in Xq28: one in intron 22 of

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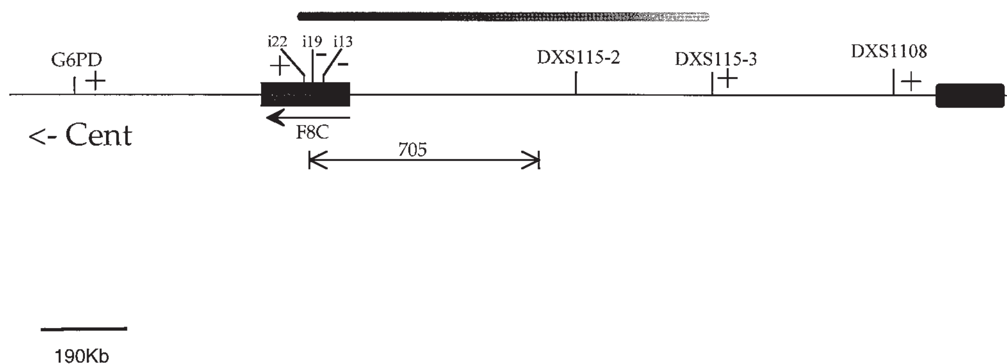

Figure 6 Diagram of the Xq28 region containing the locus for skewed X inactivation and recurrent pregnancy loss in the familial skewed X-inactivation pedigree. The deletion segregating in this family is shown, above the schematic, as a solid line defining an 800-kb interval. The proximal deletion breakpoint is between introns 22 and 19 of F8C, whereas the distal breakpoint is bordered by DXS115-3. The YAC clone 705 position used for FISH is shown (Palmieri et al. 1994).

F8C and two more that are upstream of the 5′ end of the gene and that are detected by *Pst*I and *Bst*XI (Kenwrick et al. 1992). Southern analysis showed the *Bst*XI to be heterozygous in affected females, whereas *Pst*I digestion was uniformative (data not shown). These data place the distal boundaries of the deletion in a 800-kb interval upstream of the 5′ end region of F8C (fig. 6).

Further confirmation of this deletion was attained by FISH studies using Xq28 YAC probe 705. Only one signal was detected with YAC probe 705 in metaphase spreads from the "affected" female IV-18, confirming a deletion mutation in Xq28 (fig. 7).

Discussion

Here we report what is, to our knowledge, the first successful genetic-linkage study of abnormal patterns of X inactivation. Most X-linked recessive disorders can show "manifesting carriers," in whom the mutant X chromosome is preferentially active. These patients have previously been thought to be the consequence of random statistical variations in the X-inactivation process. To the contrary, we and others have recently shown that skewed X inactivation may be an inherited molecular trait in some families (Azofiefa et al. 1995; Hoffman et al. 1995, 1996; Naumova et al. 1996). Indeed, our recent observation that ∼90% of isolated manifesting carriers of DMD showed paternal inheritance of a new dystrophin-gene mutation (at 27-fold variance with Bayesian predictions) has suggested an underlying genetic mechanism for skewed X inactivation (Pegoraro et al. 1994). Here we have described a 53-member pedigree that segregates preferential use (⩾95% of cells) of the paternal X chromosome inherited as a maternally transmitted X-linked dominant trait.

To show this, we first assigned a molecular phenotype to each female, on the basis of quantitative analysis of X-inactivation patterns, classifying the phenotype as "affected" if X-chromosome inactivation ratios were ⩾95%:5%. We found none of 62 normal females and 16 of 50 females in the pedigree to be "affected" on the basis of this criterion ($P < .0003$ by Mann-Whitney). We found positive evidence of linkage in Xq28, with marker DXS1108 ($Z_{max} = 4.34$, $\theta = 0$). Further genotyping of markers in Xq28 showed, between affected mothers and affected daughters, multiple cases of noninheritance of alleles of F8C, indicating a deletion mutation

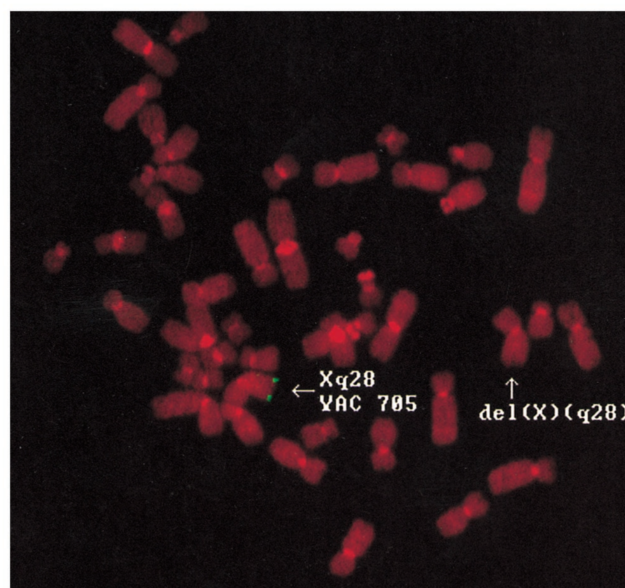

Figure 7 FISH results confirming deletion of a region of Xq28. Shown is a metaphase spread from IV-18, hybridized with a biotin-labeled YAC probe that localizes to Xq28. The image demonstrates a deletion at Xq28 on one of the X chromosomes. YAC probe 705 was detected on the nondeleted chromosome by use of FITC-labeled avidin and was counterstained with DAPI. The image was printed with use of an "apparent" red counterstain, to enhance the contrast.

covering at least part of F8C in Xq28. The deletion showed complete segregation with the skewed X-inactivation phenotype ($Z_{max} = 6.92$, $\theta = 0$). The boundaries of the deletion were defined as <800 kb, on the basis of the correlation between genetic and physical map data for Xq28.

Previous reports have addressed the incidence of skewed X inactivation in normal females; however, the results have varied, depending on (*a*) the methods used to quantitate X inactivation and (*b*) the definition of "skewed" X inactivation. The number of females showing skewed values of X inactivation in previous reports (3.7%–9.5% [Gartler and Riggs 1983; Harris et al. 1992; Puck et al. 1992; Alterman et al. 1993; Naumova et al. 1996]) is generally higher than we have found in the present study; however, many of these studies used Southern-blotting techniques that did not allow precise quantitation of the X-inactivation patterns. Moreover, the value of 20%:80% was the most often used cutoff between "random" and "skewed" patterns of X inactivation. In our study, we used quantitation on automatic sequencers, and, for classification as "abnormal," we used the more-restrictive criterion of ⩾95% of cells having one X active. Both altering the stringency of affection status in our pedigree and concomitantly increasing phenocopy rate lowered LOD scores; however, linkage to Xq28 remained statistically significant.

With this criterion, the number of skewed females in our family was significantly greater than that in the normal population: 32%, compared with 0% in our control group. To rule out tissue-specific abnormalities of X inactivation, X-inactivation patterns were tested in cells representative of both mesoderm (peripheral blood cells) and endoderm (oral mucosal cells), and patterns were found to be similar for any individual, whether in the index family or in control group of normal females. Thus, the trait causing skewed X inactivation in this family is unlikely to be due to cell- or tissue-specific factors.

The skewed X inactivation in the pedigree could be explained by a model in which the mutation is lethal to all cells (cell-lethal trait) or results in a growth disadvantage; both would be expected to cause a male-lethal phenotype, with skewed X inactivation in female carriers. Consistent with these mechanisms, we found no males with factor VIII deficiency, and the male:female ratio of offspring was lower in the affected females (1:.64) than in the unaffected females (1:.83). In these models, heterozygous female zygotes would show loss of cells with the abnormal X active, with either subsequent "catch-up growth" of the remaining cells (cell lethal), as has been hypothesized for MZ twinning (Nance 1990), or overgrowth of the normal cells (growth disadvantage). These females would be expected to show all cells with the paternal X active, as we found in our family. If the Xq28 locus were in fact a cell lethal, then the affected male zygote would be expected to die soon after fertilization, and the pregnancy would go undetected; this is *not* consistent with our data, in which there was a statistically significant increase in spontaneous-abortion rates in carrier females. Also more consistent with a growth disadvantage, rather than with a cell-lethal situation, is our observation that a small minority of cells in affected females do in fact show the abnormal X active. In the family that we studied, 6 of the 16 females with skewed X inactivation showed a residual activity, of 0.6%–5%, of the preferentially inactive X chromosome; a cell-autonomous-lethal mutation should invariably show 0% cells with the abnormal X active. It is possible that these low levels could be due to experimental error of the X-inactivation assay; however, our assay shows very high reproducibility (Pegoraro et al. 1994). Alternatively, this may reflect somatic recombination between the androgen-receptor locus, assayed for X-inactivation patterns, and the Xq28 locus; however, such somatic recombination is thought to be exceedingly rare.

An alternative novel mechanism that may explain our findings is a mutation that alters the probability that the chromosome harboring that allele will be inactivated (i.e., the mutated gene is directly involved in the X-inactivation process). Such mutations have been identified in the mouse (i.e., in X-chromosome controlling elements [Xce]) (Cattanach et al. 1969, 1970, 1991; Johnston and Cattanach 1981; Rastan 1982; Simmler et al. 1993; Courtier et al. 1995). Indeed, the region of the human X which is syntenic to the murine Xce locus, is Xq13, which also harbors XIST. Arguing against the hypothesis that the Xq28 locus is directly involved in X inactivation is the lack of mouse data suggesting an X-inactivation locus at the syntenic region of Xq28.

An alternative hypothesis is that multiple genes are located in a <800-kb interval deleted in this family, including genes causing male fetal loss and skewed X inactivation in females. This region is unusual in having a particularly low GC content (De Sario et al. 1996) and is thought to be very gene poor. No disease loci previously have been mapped within this small area. Two genes 70 kb telomeric to F8C have been partially characterized; however, patients having a deletion of F8C and these genes show a simple factor VIII–deficiency phenotype (Kenwrick et al. 1992). Thus, the genes responsible for the distinct male and female phenotypes are located in a <700-kb region, and this region is currently under study to identify novel transcript units.

Recurrent spontaneous pregnancy loss is an important women's-health issue, with 15%–20% being the frequency of clinically recognized spontaneous abortion in the general population (Roth 1963; Warburton and Fra-

ser 1964). There are many known cytogenetic, anatomical, endocrine, infectious, or immunological abnormalities associated with spontaneous abortion; however, the responsible abnormality can be identified in only approximately half of pregnancy losses (Stirrat 1990). Inherited genetic defects are assumed to cause much of the recurrent spontaneous pregnancy loss, although no genes or genetic loci have previously been identified. Identification of the specific responsible gene(s) in the deletion segregating in the family studied here will provide insights into the genetics of pregnancy loss.

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