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A New Rett Syndrome Family Consistent with X-Linked Inheritance Expands the X Chromosome Exclusion Map

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Summary

Although familial recurrences of Rett syndrome (RTT) comprise only ~1% of the reported cases, it is these cases that hold the key for the understanding of the genetic basis of the disorder. Families in which RTT occurs in mother and daughter, aunt and niece, and half sisters are consistent with dominant inheritance and variable expressivity of the phenotype. Recurrence of RTT in sisters is likely due to germ-line mosaicism in one of the parents, rather than to recessive inheritance. The exclusive occurrence of classic RTT in females led to the hypothesis that it is X-linked and may be lethal in males. In an X-linked dominant disorder, unaffected obligate-carrier females would be expected to show nonrandom or skewed inactivation of the X chromosome bearing the mutant allele. We investigated the X chromosome inactivation (XCI) patterns in the female members of a newly identified family with recurrence of RTT in a maternal aunt and a niece. Skewing of XCI is present in the obligate carrier in this family, supporting the hypothesis that RTT is an X-linked disorder. However, evaluation of the XCI pattern in the mother of affected half sisters shows random XCI, suggesting germ-line mosaicism as the cause of repeated transmission in this family. To determine which regions of the X chromosome were inherited concordantly/discordantly by the probands, we genotyped the individuals in the aunt-niece family and two previously reported pairs of half sisters. These combined exclusion-mapping data allow us to exclude the RTT locus from the interval between DXS1053 in Xp22.2 and DXS1222 in Xq22.3. This represents an extension of the previous exclusion map.

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Introduction

Rett syndrome (RTT) is a neurodevelopmental disorder characterized by developmental regression with loss of speech and of hand use, gait abnormalities, and seizures (Rett 1966; Rett Syndrome Diagnostic Criteria Work Group 1988). The vast majority of cases are sporadic; however, twin data clearly support a genetic basis for the disorder (Zoghbi 1988). Because classic RTT has been described exclusively in females, it has been hypothesized that it is an X-linked condition with lethality or nonexpression in males and with variable expression in females, owing to X chromosome–inactivation (XCI) mosaicism (Zoghbi 1988). Dominant inheritance is supported by pedigrees with transmission from mother to daughter (Witt Engerstrom and Forslund 1992), with affected half sisters (Hagberg 1985; Zoghbi 1988), and with affected aunt-niece pairs (Hagberg 1985; Zoghbi 1988; Anvret et al. 1990; Curtis et al. 1993). The variability in expressivity that occurs even within monozygotic twin pairs (Coleman et al. 1987; Bruck et al. 1991) could reflect differences in inactivation of the X chromosome harboring the RTT mutation.

There are two alternative mechanisms that could explain the X-linked model of inheritance and the exclusive occurrence of the syndrome in females. The first is that RTT results from an abnormality in XCI (Riccardi 1986). However, studies of XCI patterns in sporadic cases have failed to show any consistent abnormalities (Zoghbi et al. 1990; Kormann-Bortolotto et al. 1992; Webb et al. 1993; Anvret and Wahlstrom 1994; Camus et al. 1994, 1996; Kormann-Bortolotto and Webb 1995). The alternative model is that RTT is an X-linked dominant disorder, the phenotypic expression of which is influenced by patterns of XCI. Skewing would lead to differences in phenotypic expression, hence providing an explanation for the milder so-called forme fruste phenotype observed in some patients. In RTT, the unaffected obligate-carrier female is likely to demonstrate nonrandom XCI (with the X chromosome carrying the *mutant* allele inactivated selectively), unlike in X-linked recessive disorders such as Duchenne muscular dystrophy, for which female heterozygotes show clinical signs because of nonrandom XCI (with the X chromosome

carrying the *normal* allele inactivated selectively), and random XCI is associated with unaffected status (Zoghbi et al. 1990).

The small number of familial recurrences of RTT has precluded standard linkage analysis as a method to define the chromosomal location of the disease gene. However, on the basis of the hypothesis that RTT is X linked, exclusion mapping of the X chromosome has been done by the genotyping of affected individuals from familial cases and by the determination of whether the probands are concordant or discordant at given loci (Archidiacono et al. 1991; Ellison et al. 1992; Curtis et al. 1993). The results of this analysis demonstrated that the RTT locus does not map between DXS704 in Xp21 and DXS456 in Xq22.

In this study, we have performed extensive exclusion-mapping analysis of a newly identified RTT family (RTT family 3), with an affected maternal aunt and niece and a mildly affected transmitting mother, and we have expanded the exclusion-mapping studies of the two previously reported families with affected maternal half sisters (RTT families 1 and 2). The results of the exclusion-mapping studies allow the expansion of the excluded region on the X chromosome, which now spans the interval between DXS1053 in Xp22.2 and DXS1222 in Xq22.3. In addition, we examined the XCI patterns in RTT family 3 and in the mother in RTT family 1. In RTT family 3, the transmitting mother is an obligate carrier and shows skewed inactivation of her maternally derived X chromosome. The grandmother, however, is likely to have germ-line mosaicism for the RTT mutation because she has random XCI in the somatic cells studied, although the shared regions of the X chromosomes of the two probands originated from her. In RTT family 1, the transmitting mother was found to have random XCI, suggesting that germ-line mosaicism is the most likely mechanism for repeated transmission of the RTT mutation to the daughters.

Materials and Methods

Sample Collection and DNA Extraction

RTT families 1 (Zoghbi 1988; Ruch et al. 1989) and 2 (Hagberg 1985), which consist of two pairs of affected half sisters who are related through their mothers, have been reported elsewhere. Lymphoblastoid cell lines (LCL) and/or peripheral blood leukocytes (PBL) were available from the female members of these families, and genomic DNA was extracted by use of standard techniques or by use of an automated DNA extractor (Applied Biosystems) (Ellison et al. 1992). For RTT family 3, informed consent was obtained from the subjects or from their guardian, by use of a protocol approved by the Institutional Review Board. Blood and/or skin samples were obtained and were used for DNA extraction, as well as to establish LCLs and fibroblast (FB) cell strains.

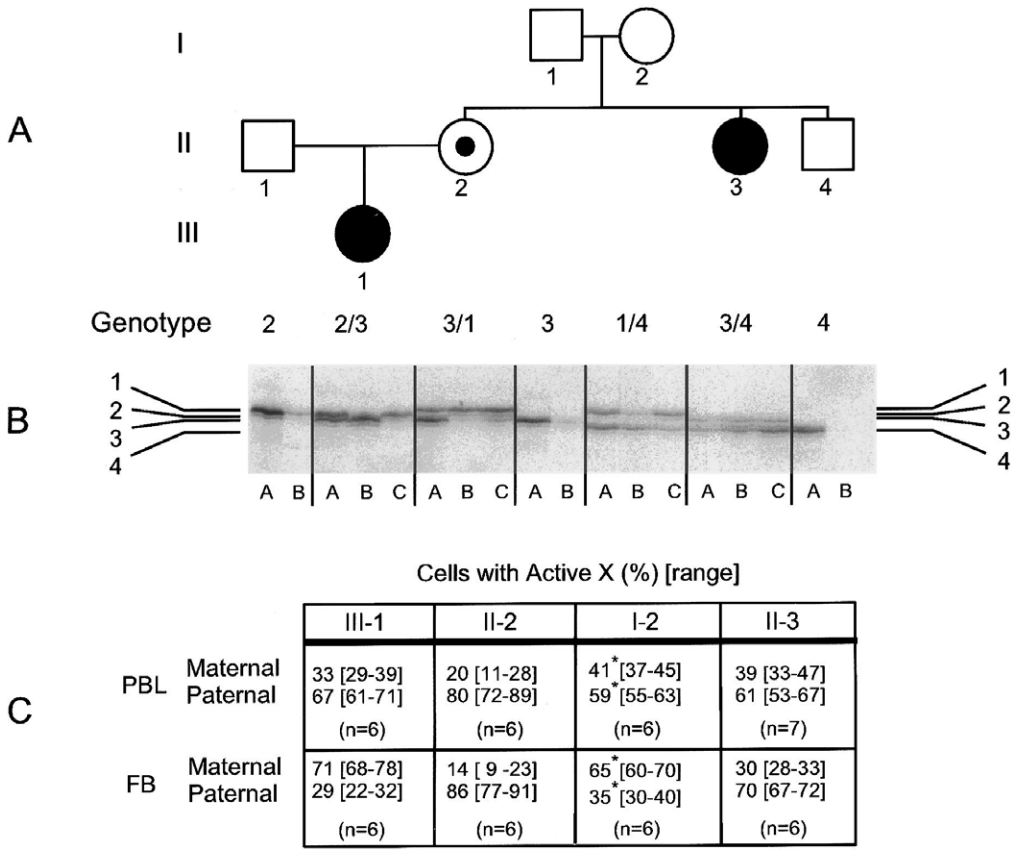
Microsatellite-Marker Typing

X-chromosomal microsatellite markers were tested by use of primers that are either commercially available (MapPairs; Research Genetics) or reported elsewhere (Simmler et al. 1987; Barker et al. 1989; Ellison et al. 1992; Weissenbach et al. 1992; Gyapay et al. 1994). PCR conditions for all markers used in RTT family 3 were a 20-μl volume in 1 × *Taq* Extender Buffer (Stratagene), 125 μM dNTP, 1 μM primer, 5 μCi ^{35}S-αdATP, 100 ng genomic DNA template, 1 unit Perkin Elmer *Taq* polymerase, and 1 unit *Taq* Extender. The initial denaturation cycle was at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 7 min. The products were separated on 5% Long Ranger sequencing gels (J. T. Baker) and then were autoradiographed overnight or for up to 18 d, by use of Hyperfilm (Amersham). Marker typing of RTT families 1 and 2 was performed as described elsewhere (Ellison et al. 1992).

Assay of Allele-Specific XCI

Genomic DNA (1 μg) from each individual in RTT family 3 and from the mother in RTT family 1 was digested overnight, with either *Hpa*II or *Msp*I (New England Biolabs), in a 50-μl volume, by use of the manufacturer's recommended buffer. PCR amplification using previously reported primers that amplify 280-bp products from the AR gene (Allen et al. 1992) was performed in the same conditions as described above, except that the cycle number was 28. The products were separated on a 5% Long Ranger sequencing gel, were dried, and were autoradiographed on Hyperfilm (Amersham). Because of problems with overlapping alleles and shadow bands, we also performed this assay using a ^{33}P end-labeled primer. Briefly, 1 μmol of the forward primer was kinased by use of γ-[^{33}P]-ATP and T4 polynucleotide kinase (Gibco) in a 50-μl volume, in the manufacturer's recommended buffer. Unincorporated nucleotides were removed with a Nuctrap column (Stratagene). The labeled primer was used at 0.1 μM in the final reaction volume including 0.9 μM unlabeled forward primer. The reactions were cycled and the products electrophoresed as described above. Alleles were quantitated by use of a model 300A Computing Densitometer with the ImageQuant software, version 3.15 (Molecular Dynamics), or by using a phosphorimager. Because the smaller alleles were amplified more efficiently in the undigested samples, a correction factor was generated from the undigested samples, to normalize them such that both alleles were represented equally. This factor then was applied to the digested samples to correct for differences in amplification that were based on the length of the amplimer (Naumova et al. 1996). The assay was performed three times for each sample.

Additionally, the PCR was performed on duplicate digested samples with a fluorescently labeled primer



		III-1	II-2	I-2	II-3
PBL	Maternal	33 [29-39]	20 [11-28]	41*[37-45]	39 [33-47]
	Paternal	67 [61-71]	80 [72-89]	59*[55-63]	61 [53-67]
		(n=6)	(n=6)	(n=6)	(n=7)
FB	Maternal	71 [68-78]	14 [9 -23]	65*[60-70]	30 [28-33]
	Paternal	29 [22-32]	86 [77-91]	35*[30-40]	70 [67-72]
		(n=6)	(n=6)	(n=6)	(n=6)

Figure 1 Quantitation of XCI patterns, by the androgen-receptor methylation assay. *A*, Pedigree of RTT family 3. *B*, Phosphorimage of ^{33}P end-labeled PCR products. PBL DNA from each individual was either used directly as a template (lane A) or digested with *Hpa*II prior to PCR amplification (lane B). For each female family member, *Hpa*II-digested genomic DNA from skin FBs also was used as a template (lane C). The undigested fragments remaining in the lanes of the *Hpa*II-digested samples represent the alleles present on inactive X chromosomes. *C*, Estimated proportion of cells bearing the same active X chromosome, on the basis of quantitation of the AR assay. The values represent the means of three independent experiments, with the ranges in parentheses. Asterisks (*) indicate that the parent of origin is not known; thus, the values indicate the proportion of cells with the upper versus lower set of allelic fragments from the active X chromosome.

kindly provided by Eric Hoffman at the University of Pittsburgh. PCR conditions were as described above, except that the primer concentration was dropped to 0.1 μM. Five microliters of the amplified products were dried and resuspended in 3 μl of loading buffer (50% formamide, 20 mg blue dextran/ml, 10 mM EDTA, and GeneScan size standard 350 ROX), then were separated on a 4% sequencing gel with an ABI 377 automated sequencer. Alleles were determined with the GeneScan analysis program (ABI). These assays were performed twice.

Results

Clinical Histories and Findings

RTT family 3 is of mixed European and Native American descent. There is no known consanguinity (fig. 1*A*). II-2 was born to a 23-year-old mother (I-2), following a term pregnancy, and weighed 7 lb 8 oz at birth. Spontaneous vaginal delivery was complicated by occiput posterior presentation. For II-2, the early developmental milestones were normal; however, when she began school, learning disabilities became apparent. She was noted to have poor coordination, particularly involving reciprocal movements. Until 6 years of age, she was unable to walk up stairs sequentially or to ride a tricycle. Extensive neuropsychiatric testing at 8 years of age was notable for mild hypotonia, abnormalities in postural integration, and an inability to cross the midline. She was noted to be apraxic in the Motor Accuracy and Graphesthesia testing. Neurologically, she displayed a fine tremor that worsened with stress or movement. Her performance on the WISC-R revealed a full-scale I.Q. of 71, with a performance I.Q. of 64 and a verbal I.Q. of 82. As an adult, she has specific difficulties with integration and problem solving. She is able to do only simple arithmetic. She has ongoing coordination problems, is unable to walk backwards, and still has difficulty performing activities that require her to cross the midline.

II-3 was born to I-2, following an uncomplicated pregnancy. Fetal movements were felt to be decreased, compared with previous pregnancies. II-3 was born by spontaneous vaginal delivery from an occiput posterior position, at 42 wk. Her weight (7 lb 2 oz), length (18 in), and head circumference (35.5 cm) were all normal. Her neonatal course was uneventful. Early developmental history was significant for smiling at 6 wk, finger feeding at 7 mo, rolling and combat crawling at 10 mo, and sitting unsupported at 1 year. She had at least four words at 1 year of age. Constipation began at 14 mo. At ~2 years of age, she developed seizures and, with the initiation of anticonvulsant therapy, had a significant regression of motor skills, losing her ability to sit unsupported as well as her pincer grasp. Her social activities changed, and she became less interested in her family and her toys. The rate of head growth slowed before 2 years of age, and her head circumference was below the 2d percentile by 8 years of age. She developed hand wringing, bruxism, and respiratory irregularities while awake, between 2 and 3 years of age. Scoliosis was present by 6 years of age. The diagnostic evaluation consisted of a metabolic screen and an ophthalmologic evaluation, which were normal. Her electroencephalogram showed seizure activity, and a noncontrasted computer-assisted tomography (CAT) scan of the head (done at 11 years of age) was notable for prominent ventricles but no other abnormality. Currently, at 27 years of age, she is nonambulatory and nonverbal, using eye pointing to communicate. She has nocturnal screaming and laughing episodes. Her feet are small and cold. She has moderate-severe scoliosis. She was diagnosed with RTT at 17 years of age, by V.A.H.

III-1 was the product of the first pregnancy of II-2. Decreased fetal movements were noted during the last 2 mo of the pregnancy. Birth occurred by spontaneous vaginal delivery at 40 wk and was complicated by a face presentation. She cried spontaneously and vigorously. Birth weight was 6 lb 9 oz, with a head circumference at the 15th percentile. Developmental abnormalities were first noted at 6–8 mo of age, when she could not sit without being placed in the position. Her acquisition of milestones was slow, but she continued to progress and was able to sit unsupported at 12–14 mo and to walk alone at 28 mo. At 34 mo, she lost her pincer grasp and the ability to pull to stand but was still able to stand when placed. She developed constipation at 14 mo of age. By 3 years of age, her head circumference was at the 2d percentile, and she developed seizures and hand stereotypies. Currently, at 4 years of age, she has three words, which she uses nonspecifically, respiratory irregularities with periodic apnea, and small cold feet. She walks with an awkward gait and has truncal ataxia. She has nocturnal screaming, laughing, and hand-wringing episodes. Diagnostic evaluations, including a urine metabolic screen, a noncontrasted head CAT scan, and a karyotype analysis, were normal. Her ophthalmologic examination was normal except for amblyopia. She has no neurocutaneous stigmata. She was diagnosed with RTT at the age of 3 years 2 mo, by V.A.H.

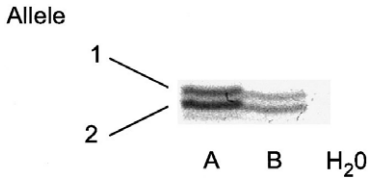


Figure 2 XCI pattern for the mother in RTT family 1, by the androgen-receptor methylation assay. Autoradiogram of the ^{33}P end-labeled products obtained by PCR amplification of the AR polymorphism, by use of either undigested DNA (lane A) or DNA digested with *Hpa*II (lane B) or by use of water as a template. Quantitation of the proportion of cells bearing the same active X chromosome was estimated by densitometric analysis of the autoradiogram and indicates a random pattern of XCI in PBL. The average ratio of inactivation of the X chromosome bearing allele 1 compared with the X chromosome bearing allele 2 is 46% (range 43%–50%; $n = 3$) to 54% (range 50%–57%; $n = 3$).

I-1, I-2, and II-4 are intellectually normal and have no neurologic abnormalities. II-1 has neurofibromatosis (type I) and has some cognitive impairments.

XCI Studies

The androgen-receptor assay for XCI was used to evaluate XCI patterns in the female members of RTT family 3 and the mother in RTT family 1 (Allen et al. 1992). This assay relies on the differential methylation of a cytosine residue just 5′ to the highly polymorphic trinucleotide repeat in the androgen-receptor gene on Xq11-q12. The site is consistently methylated on the inactive X chromosome and, therefore, is resistant to digestion by the methylation-sensitive restriction enzyme *Hpa*II. On the active X chromosome, the site is unmethylated and, thus, is susceptible to digestion. PCR amplification across the region, by use of *Hpa*II-digested genomic DNA as a template, allows determination of the relative ratio of methylation of each allele, corresponding to the ratio of inactivation. Because the methylation site is adjacent to the polymorphic triplet repeat, the alleles that amplify can be distinguished by their length. Digestion of the DNA, with *Msp*I, a methylation-resistant isoschizomer of *Hpa*II, serves as a control. The assay is considered semiquantitative (Allen et al. 1992). If the same X chromosome appears to be inactive in $\geqslant 80\%$ of cells, then the XCI pattern is considered skewed (Naumova et al. 1996).

This assay was performed on genomic DNA from PBL from each individual, as well as from cultured skin FBs from the four female family members in RTT family 3 (fig. 1*B* and *C*). Samples from the male family members amplified only if undigested by *Hpa*II, which is consistent with the presence of a single active AR allele. For I-2, results suggested random XCI in both tissues examined. In the

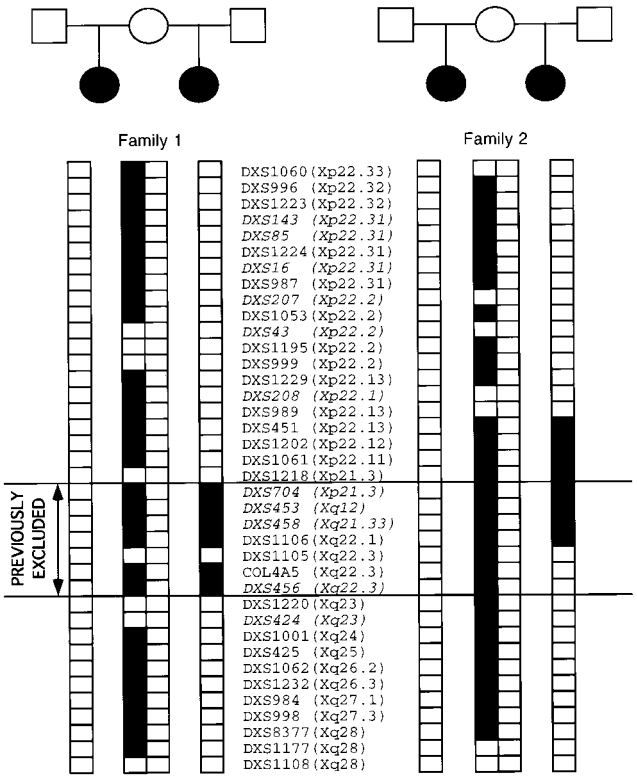


Figure 3 Exclusion-mapping data from RTT families 1 and 2. Data for loci in italics were reported elsewhere (Archidiacono et al. 1991; Ellison et al. 1992; Curtis et al. 1993). The maternal alleles are distinguished by black or white squares, with two white squares indicating homozygosity. Approximate cytogenetic band locations of the loci are from the report by Nelson et al. (1995). The new data for RTT family 2 extend the excluded region, on the short arm, through DXS451.

affected aunt (II-3) XCI was essentially random, although there was a slight preference toward inactivation of her maternal X chromosome, in both tissues studied. However, in the obligate carrier (II-2) preferential inactivation of her maternal X chromosome was observed in both tissues examined. In the obligate carrier's affected daughter (III-1), XCI was apparently random, with a slight tendency toward preferential inactivation of her maternal X chromosome in blood and of her paternal X chromosome in skin.

Examination of the XCI pattern in PBL from the mother in RTT family 1 previously had been unsuccessful when a *Bgl*I polymorphism in the PGK gene and a *Bam*HI polymorphism in the HPRT gene were used, because she was uninformative at these loci (Zoghbi et al. 1990). She was informative, however, for the AR polymorphism and demonstrated a random inactivation pattern in PBL (fig. 2). Thus, germ-line mosaicism is likely to be the mechanism by which she transmitted RTT to her two daughters.

Exclusion Mapping

We have performed extensive exclusion mapping of the X chromosome, for RTT families 1, 2, and 3. Data from RTT families 1 and 2 are the basis for the existing exclusion map and have been reported elsewhere (Archidiacono et al. 1991; Ellison et al. 1992; Curtis et al. 1993). To refine the boundaries of the excluded regions, we have typed 27 additional markers in RTT family 1 and 23 markers in RTT family 2. For RTT family 3, which had not been studied previously, a total of 67 microsatellite markers were typed for each individual.

In the previous studies of RTT families 1 and 2, the most distal discordant marker on Xp, for both families, was DXS704 in Xp21.3 (Ellison et al. 1992). Since the 6–10 markers telomeric to DXS704 were uninformative, the boundary of the discordant region was unclear. By typing additional markers on Xp, we have identified discordances for markers DXS1218 (Xp21.3), DXS1061 (Xp22.11), DXS1202 (Xp22.12), and DXS451 (Xp22.13), in RTT family 2 (fig. 3). RTT family 1 was either uninformative, or the probands were concordant for the maternal allele, at these loci. Additional markers also were typed on the long arm. DXS1106 was discordant in both RTT families, and COL4A5 was discordant in RTT family 1; however, no new discordances were identified distal to DXS456, in these two RTT families; thus, the excluded region on Xq was unchanged (fig. 3).

The genotypic analysis of RTT family 3 allowed further expansion of the exclusion map, on the basis of careful analysis of haplotypes and the parental origin of alleles. The results of the 49 markers that were informative are shown in figure 4. The density of the markers chosen was increased in the regions that had not been excluded previously in RTT families 1 and 2. For I-2, haplotypes were assigned with the assumption of the lowest number of recombination events in her children. Examination of the maternal haplotypes in II-2 and II-3, derived by subtraction of the identical paternal haplotypes, indicated a crossover between DXS207 and DXS1053, so that the sisters share the distal short-arm markers but are discordant for the centromeric markers. On the long arm, another recombination event occurred between DXS1222 and DXS1059, making the sisters discordant for the more proximal markers on Xq but concordant for a large portion of the long arm.

When the genotypes of the two affected individuals (II-3 and III-1) were compared, it was apparent that markers on the distal short arm are shared. Although the grandparental origin of the DXS1283E alleles was not clear, owing to the homozygosity of II-2, the alleles at DXS237 and DXS996 are of grandmaternal origin. Therefore, it is most likely that the entire DXS996–DXS207 region is inherited from her. The two probands are discordant for 12 loci on Xp proximal to and including DXS1053 on Xp22.2. Because II-2 is homozygous at several loci on the proximal short arm, the possibility of closely spaced double-recombination events cannot be excluded formally. Although it is possible that the probands share alleles of grandpaternal

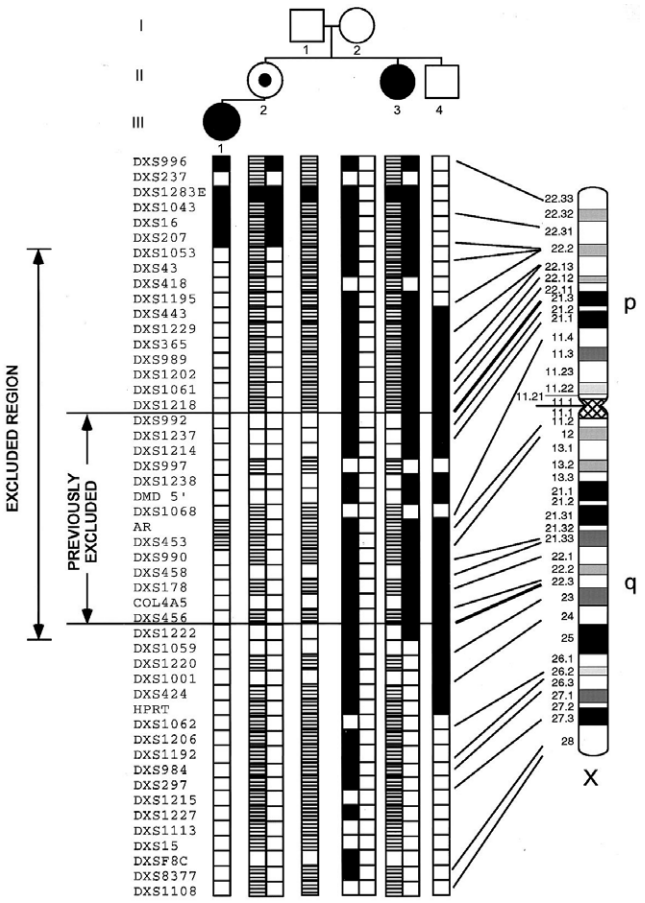


Figure 4 Exclusion mapping of X chromosome markers for RTT family 3. Data for 49 microsatellite markers are shown. The alleles of I-2 are indicated by either a white square or a black square. Markers for which she is homozygous are indicated by two white squares. The alleles of I-1 are indicated by a hatched square (▤), except when the allele is identical to an allele of I-2, which is indicated by either a black or a white square. For III-1, only the maternal haplotype, derived from studies of her father (data not shown), is shown. The approximate chromosomal band positions of some markers are indicated on the X chromosome ideogram.

origin in the region, the presence of interspersed alleles arising from I-2 makes it most likely that the entire region is of grandmaternal origin.

A recombination event that involves the proximal Xq causes the probands to be concordant for the I-1–derived alleles at the AR and DXS453 loci. Of note, this region as well as the uninformative region on proximal Xp have been excluded in previous studies (Archidiacono et al. 1991; Ellison et al. 1992; Curtis et al. 1993). The probands are discordant for DXS990, DXS178, and DXS456. II-2 is homozygous at DXS1222, but the flanking markers are derived from I-2; thus, it is most likely that they are discordant at this locus as well. They are concordant for the remainder of the long arm. With few exceptions, the regions that are inherited concordantly appear to originate from I-2. We also genotyped the unaffected male (II-4) and saw that he is discordant with one or both probands, for markers on Xp and proximal Xq. He does share the distal portion of Xq with his affected sister and niece.

Discussion

Since RTT was first described, there has been much debate about its pattern of inheritance and the possible chromosomal location of the causative gene. Diverse mechanisms of inheritance have been proposed, such as digenic inheritance (Buhler et al. 1990), aberrations in XCI (Riccardi 1986; Zoghbi et al. 1990; Webb et al. 1993; Camus et al. 1996), and uniparental disomy (Rivkin et al. 1992; Webb et al. 1993; Migeon et al. 1995), to account for the predominantly sporadic occurrence and sex-limited expression of RTT. The ascertainment of new familial cases is providing insight into the primary genetic basis of the disorder. The presence of the RTT phenotype in mother and daughter, in affected half sisters, and in affected aunt-niece pairs supports the existence of a single dominantly inherited disease gene, either X-linked or autosomal. The variability in age of onset and rate of progression and the exclusive occurrence in females is explained best by an X-linked mutation that may be lethal in males. The apparent lack of increased spontaneous miscarriages in families with RTT probands has been used to argue against male lethality. Paternally derived mutations, however, would lead to only affected females and would not be expected to give rise to lost male conceptions (Thomas 1996). Furthermore, if the majority of cases resulted from new mutations, no increased abortion rate would be expected (Comings 1986). Evidence of lost male conceptuses would only be expected in families with mothers who are either germ-line mosaics or RTT heterozygotes with favorably skewed XCI patterns. To date, few such families are known.

If RTT is an X-linked disorder, examination of XCI patterns in familial cases may give insight into the basis of phenotypic variability, particularly when unaffected transmitting females are examined. To gain useful information from these studies, it is necessary to examine families in which the parental origin of the RTT mutation is clear. Recurrence in sisters does not imply that the mother is an obligate carrier, as either maternal or paternal germ-line mosaicism of an X-linked mutation may result in affected daughters (Hunter 1992; Pegoraro et al. 1994; Thomas 1996). Therefore, examination of XCI patterns in the mothers of affected sister pairs may not provide conclusive data to refute or to support the localization of the RTT locus on the X chromosome (Webb et al. 1993; Kormann-Bortolotto and Webb 1995; Migeon et al. 1995; Camus et al. 1996). Furthermore, because the number of new mutations contributing to the population of RTT probands is likely to be high, one cannot conclude that the familial recurrences

in distantly related female individuals are the result of familial transmission of the same mutant or predisposing allele, rather than the chance occurrence of two new mutations within a kindred. Thus, only a few of the known familial cases would be suitable for the XCI and exclusion-mapping studies described here. These families include the affected half-sister pairs (which are related through maternal lineages), as well as the maternal aunt-niece pairs and, perhaps, the discordant monozygotic twins. Indeed, previous examination of the XCI patterns in blood from the normal mother of RTT family 2 disclosed extreme skewing, providing a mechanism for the reduced expressivity seen in that individual (Zoghbi et al. 1990). Nonrandom inactivation of the paternal X chromosome was identified in the unaffected twin in the only known pair of discordant monozygotic twins (Migeon et al. 1995). Although in this case the phenotypic discordance could have resulted from a postzygotic mutational event occurring in the affected twin, nonrandom inactivation of a paternally derived mutation also could be the basis for the normal phenotype in one twin.

We found that, in RTT family 3, the transmitting female (II-2) had preferential inactivation of her maternal X chromosome, the same chromosome from which concordant regions in the two affected probands are derived. These data and those reported by Zoghbi et al. (1990) demonstrate that transmitting females have preferential inactivation of one X chromosome. These findings are consistent with X-linked inheritance. Although patterns of XCI in neuronal tissue are most likely the major determinant of the phenotypic effects, a skewed pattern in peripheral tissue may suggest aberrant XCI in that individual. As demonstrated by the XCI patterns seen in blood versus skin FBs from III-1 in RTT family 3, there can be tissue-specific differences in XCI.

On the other hand, when the XCI pattern in the transmitting female in the aunt-niece pair reported by Anvret and Wahlstrom (1994) was examined by use of the M27ß probe in blood, XCI was reported to be random, although no quantification was performed. However, as noted in that report, the pattern in blood may not represent the pattern in the brain (Anvret and Wahlstrom 1994). In this family, it is unlikely that the transmitting female has germ-line mosaicism for the RTT mutation, because she is an obligate carrier. The finding of random XCI patterns in the mother in RTT family 1 and in I-2 in RTT family 3 does not exclude an X-linked locus but suggests that germ-line mosaicism or tissue-specific differences in XCI must account for some recurrent transmission of RTT.

Exclusion mapping can provide a useful approach to localization of a disease gene in collections of small families, for which standard linkage studies are unlikely to be fruitful. Exclusion mapping requires that appropriate families are utilized, to maximize the available information. For example, previous exclusion-mapping data derived from comparison of affected full sisters are inconclusive because sisters share the entire paternal X chromosome, and paternal origin of the mutation cannot be excluded (Curtis et al. 1993). It is best to confine conclusions about X chromosome exclusion mapping to families for which maternal inheritance can be demonstrated. The three families investigated in this study fulfill this criterion, and the data generated allowed the exclusion of a large portion of the X chromosome, between DXS1053 and DXS1222. Additionally, the combination of XCI data and exclusion-mapping data, for RTT family 3, suggests that the RTT mutation originated on the grandmaternal X chromosome, since the regions that are concordant in the probands are derived from I-2. Furthermore, the daughter who is an obligate carrier has preferential inactivation of her maternal X chromosome. At this time, although a large portion of the X chromosome is excluded, a sizable segment of Xq remains unexcluded. The fact that the normal male (II-4) in RTT family 3 shares a large portion of Xq with the two probands cannot be used to exclude the region, because his mother (I-2) is probably a germ-line mosaic for the RTT mutation, on the basis of her normal phenotype and random XCI. Thus, the mutation may not have been present on the X chromosome that was transmitted to II-4. Since the number of recombination events per meiosis is limited, more information can be obtained only from the study of additional families, rather than from additional examination of the same families, once the regions of the recombination events have been localized. Nevertheless, given the new exclusion-mapping data, the search for the RTT gene by use of a candidate-gene approach now can be limited to the newly defined unexcluded regions of the X chromosome.

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