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## Evidence Consistent with Human L1 Retrotransposition in Maternal Meiosis I

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We have used a unique polymorphic 3' transduction to show that a human L1, or LINE-1 (long interspersed nucleotide element-1), retrotransposition event most likely occurred in the maternal primary oocyte during meiosis I. We characterized a truncated L1 retrotransposon with a 3' transduction that was inserted, in a Dutch male patient, into the X-linked gene *CYBB*, thereby causing chronic granulomatous disease. We used the unique flanking sequence to localize the precursor L1 locus, *LRE3*, to chromosome 2q24.1. In a cell culture assay, the retrotransposition frequency of *LRE3* is greater than that for any other element that has been tested to date. The patient's mother had two *LRE3* alleles that differed slightly in the 3'-flanking genomic DNA. The patient had a single *LRE3* allele that was identical to one of the maternal alleles; however, the patient's insertion matched the maternal *LRE3* allele that he did not inherit. Other data indicate that there is only a small chance that the father (unavailable for analysis) carries the precursor *LRE3* allele. In addition, paternal origin of the insertion would have required that an *LRE3* mRNA transcribed before meiosis II be carried separately from its precursor *LRE3* allele in the fertilizing sperm. Since the mother carries a potential precursor allele and the insertion was on the patient's maternal X chromosome, it is highly likely that the insertion originated during maternal meiosis I.

### Introduction

Preliminary analysis of the human genome has shown that transposable elements compose  $\geq 45\%$  of its mass (Lander et al. 2001). L1, or LINE-1 (long interspersed nucleotide element-1), retrotransposons are one of the most successful transposable elements, comprising 17% of the genome (Lander et al. 2001). Most of the  $\sim 500,000$  L1 copies present in the human genome are truncated at their 5' end, rearranged by inversion, and/or mutated. Only a small percentage are full length (Lander et al. 2001), and only 40–80 potentially are retrotranspositionally active (Sassaman et al. 1997). In humans, the youngest and most active elements belong to the Ta-1d subset (Boissinot et al. 2000). The insertion reported here is the 14th-known recent L1 insertion in humans (Ostertag and Kazazian 2001). However,  $>70\%$  of these insertions were ascertained by the presence of X-linked disease in male patients (Ostertag and Kazazian 2001). Estimates of the actual

frequency of L1 insertions range from 1 in 10 to 1 in 120 individuals (Kazazian 1999; Li et al. 2001).

An intact, full-length human L1 element is  $\sim 6.1$  kb and contains (1) a 5' UTR that is followed by two non-overlapping ORFs, (2) a 3' UTR with a polyadenylation signal, and (3) a polyA tail (Scott et al. 1987). The element encodes endonuclease and reverse-transcriptase activities in ORF2 (Mathias et al. 1991; Feng et al. 1996) and duplicates by the following mechanism (the details of which are largely unknown): First, the original retrotransposon is maintained, in situ, where it is transcribed (Whitcomb and Hughes 1992; Luan et al. 1993). Then, the transcript is reverse transcribed and integrated into a new genomic location by a process termed "target-primed reverse transcription" (Luan et al. 1993). Finally, on integration, the L1 element typically is flanked by target-site duplications (TSDs) of 6–20 bp; the integration site has a weak consensus sequence (5'-Py-Py/AAAA-3') (Cost and Boeke 1998), and L1 elements appear to be inserted at random locations in the genome (Ovchinnikov et al. 2001).

There are exceptions to the canonical retrotransposition event. For example, L1 elements often transduce DNA flanking their 3' ends. In  $\sim 20\%$  of cases, the L1 polyA signal is skipped in favor of a downstream polyA signal (Holmes et al. 1994; Goodier et al. 2000; Pickeral et al. 2000). In one previous instance, unique transduced sequence was used to find and characterize the precursor

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to a human L1 insertion (Holmes et al. 1994). Also, L1 elements can be inserted into the genome without a TSD (Narita et al. 1993; Jensen et al. 1994).

Little is known about either the timing of human retrotransposition events or the cell types in which such events occur. From an evolutionary standpoint, retrotransposition in a germline or a germline precursor is necessary. In tissue culture, it has been shown that retrotransposition from an episome is possible in several transformed cell lines—including feline G355.5 cells (Esnault et al. 2000); human HeLa (Moran et al. 1996), 143B TK– (thymidine kinase negative; American Type Culture Collection number CRL-8303), and H460 cells (E.O., unpublished data); and mouse L<sup>TK–</sup> cells (Moran et al. 1996; Naas et al. 1998). Our laboratory has failed to demonstrate retrotransposition in primary cell lines, including mouse embryonic fibroblasts and human fibroblasts (authors' unpublished data). In a transgenic mouse model, we have, by reverse-transcriptase PCR, found L1 mRNA in spermatocytes, and we have shown retrotransposition in male germ cells (Ostertag et al. 2000). It has also been shown that ORF1 is expressed in mouse testis (spermatocytes, myoid cells, and Leydig cells) and ovaries, as well as in steroidogenic cells (Branciforte and Martin 1994; Trelogan and Martin 1995). Finally, in one instance, an L1 insertion into the *APC* gene caused colon cancer in a human (Miki et al. 1992). However, to date, it has not been possible to define the timing and cell type of a heritable human retrotransposition event. Here, we present data that strongly suggest that an L1 insertion occurred during maternal meiosis I.

## Subject and Methods

### Patient History

The patient was born in 1980 and presented, in the 1st year of life, with multiple infectious complications—including recurrent pneumonia—as well as abscesses of the perianal region, liver, and lymph nodes. After diagnosis of chronic granulomatous disease (CGD [MIM 306400]) at 15 mo of age, prophylactic treatment with cotrimoxazole was initiated. Pneumonia caused by *Aspergillus pneumoniae* at the age of 22 mo led to treatment by the addition of itraconazole to the prophylactic protocol. Under this regimen, the patient remained infection free until 1999, when he presented again with pneumonia, leading to his hospitalization, that was caused by *A. pneumoniae*. Ensuing complications included several episodes of bacterial sepsis; fungal lesions in lungs, liver, and spleen; osteomyelitis; and proctitis. Currently, he is infection free and is followed as an outpatient.

### Diagnosis of CGD

Diagnosis of CGD was achieved by analysis of gran-

ulocyte function, nitroblue tetrazolium (NBT) test, determination of cytochrome *b*<sub>558</sub> content in whole and Triton X100-extracted cells, and monocyte hybridization, which were performed as described elsewhere (Weening et al. 1985).

### PCR

**Amplification of CYBB.**—The 13 exons of *CYBB*, including their adjacent intronic sequences (namely, exon 1 and promoter sequences), were amplified as described elsewhere (Meischl et al. 2000). In brief, genomic DNA (50–500 ng) was amplified by use of the Rapid Cyclor (Idaho Technology), with 50 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 15 s and slope S9. The reaction volume of 15  $\mu$ l contained 2 U of *Taq* DNA polymerase (Promega), 2 U of *Taq*Start antibody (Clontech Laboratories), 50 ng of each primer, 200  $\mu$ M of each of the deoxyribonucleoside triphosphates (Promega), and reaction buffer (50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1% Triton X100, 10 mM Tris, and 8% dimethyl sulfoxide, with pH 9.0 at 25°C) in 10- $\mu$ l glass capillaries (Idaho Technology).

**Amplification of insertion-containing exon 4 of CYBB.**—Products of the insertion-containing exon 4 from the patient were obtained, with primers 5'-GTTAACAATTACTATTCCATTCTTTCCCCC-3' and 5'-CTATGATAGAGGGAAGTCCCTGGTTCCAAG-3', by use of the Expand Long Template PCR System (Roche), according to the manufacturer's recommendations. In brief, the region of interest was amplified by Expand Long Template PCR on a PTC-0220 Peltier Thermocycler (MJ Research), with 50–200 ng of genomic DNA as a template, in a total reaction volume of 20  $\mu$ l. The PCR was run with the following settings: denaturing at 94°C for 30 s, annealing at 56°C for 40 s, and 30 cycles of extension at 68°C for 2 min.

**Amplification of LRE3 from both the mother and the patient.**—The unique primers LRE3F (5'-ATTTGGCGCCGCTACCCCCTACTGTTGTGCCTTG-3') and LRE3R (5'-CGTCTACCGAAAGTCCATACTACCC-3') were designed using the chromosome 2 sequence from AC067958 (GenBank). The first primer has a *Not*I site at the 5' end for cloning. Three additional unique pairs were also designed to verify that *LRE3* was present in the sequence on AC067958 (GenBank). The ~7,750-bp product was amplified by use of the Expand Long Template PCR System (Roche), according to the manufacturer's recommendations. In brief, the 7.7-kb region was amplified on a PTC-0220 Peltier Thermocycler (MJ Research), with 50–200 ng of genomic DNA as a template, in a total reaction volume of 20  $\mu$ l. The PCR was run with the following settings: denaturing at 94°C for 30 s, annealing at 67°C for 40 s, and 30 cycles of extension at 68°C for 6 min.

### Polymorphism Studies

The presence or absence of *LRE3* in 23 individuals from five different ethnic groups was determined using a two-reaction, three-primer technique (Boissinot et al. 2000). The Expand Long Template PCRs were performed in a PTC-0220 Peltier Thermocycler (MJ Research), with 50–200 ng of genomic DNA as a template in a total reaction volume of 20  $\mu$ l. The PCR was run with the following settings: denaturing at 94°C for 30 s, annealing at 64.8°C for 40 s, and 30 cycles of extension at 68°C for 2 min. In brief, in the first reaction, two unique flanking primers, A (5'-TTCCACCTCTGTGGTATCTATGGTC-3') and C (5'-CATGACAATGGTCTGGGCAGTG-3'), were used to yield a ~1,350-bp product, in the absence of the element; in the second reaction, the unique A primer and a reverse primer B in the 5' section of the L1 element (5'-TGTGGGATATAGTCTCGTGGTGC-3') were used to yield a ~1,330-bp product, in the presence of the element.

### Southern Blotting

Southern blotting, with 8  $\mu$ g of genomic DNA, was performed as described elsewhere (Sambrook et al. 1989).

### Cloning of *LRE3*

The single band obtained with primers *LRE3F* and *LRE3R* was cut from a 0.7% agarose gel and was purified using a Gene Clean Spin kit (BIO 101). The band was digested with *NotI* (in *LRE3F*) and *BstZ171* (in the 3' end of the L1 element). The full-length *LRE3* fragment, as a *NotI/BstZ171* fragment, was swapped into pL1<sub>RP</sub>-EGFP (with *BstZ171*) (full-length L1<sub>RP</sub> element tagged with the EGFP (enhanced green fluorescent protein) retrotransposition cassette; the pL1<sub>RP</sub>-EGFP construct was the same as that described by Ostertag et al. [2000] except that it was modified to contain an intact *BstZ171* site), to produce pLRE3-EGFP. This clone was then tested for retrotransposition activity in a cell culture assay.

### Sequencing and Sequence Analysis

Clones and purified PCR products were sequenced using L1-specific or 3' transduction-specific primers. Each sequencing reaction contained ~50 ng of PCR product or ~500 ng of plasmid DNA and ~3.2 pmol of oligonucleotide primer. All sequencing was performed on ABI 3100 automated sequencers.

### Retrotransposition Assay

Constructs pL1<sub>RP</sub>-EGFP, pL1<sub>RP</sub>(JM111)-EGFP (Ostertag et al. 2000), and pLRE3-EGFP were assayed for retrotransposition activity in human 143B TK- osteosarcoma cells. Cell culture, transfection of cells, antibi-

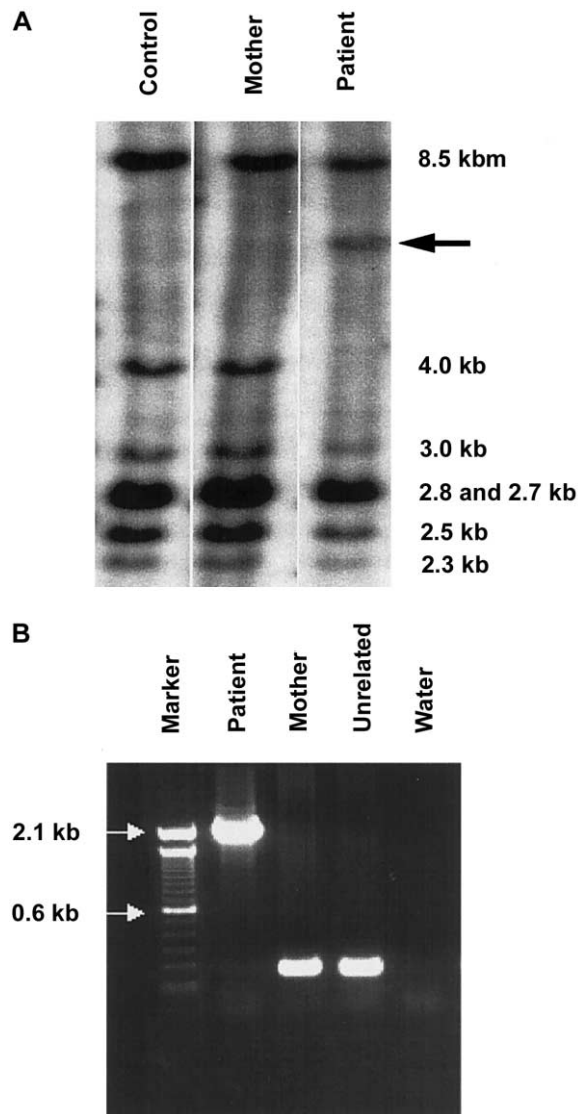
otic selection, and flow cytometry were performed as described elsewhere (Ostertag et al. 2000).

### Results

CGD is a disorder that is caused by a deficiency in the enzyme NADPH oxidase, which creates the reactive oxygen species that are used by phagocytes to kill bacteria and fungi. CGD is characterized by repeated infections. Age at onset and phenotype are quite variable. NADPH oxidase is composed of subunits that are encoded by several different genes. Defects in the X-linked gene *CYBB*, which encodes the gp91phox subunit of cytochrome *b*<sub>558</sub>, are the most common cause, but other autosomal forms exist (Roos et al. 1996). Analysis of the patient's granulocyte function revealed a normal chemotactic response but a reduced intracellular killing of *Staphylococcus aureus* (in the patient's granulocytes, 57% of the bacteria were killed after 30 min, whereas, in granulocytes from a control individual, 95% were killed after 30 min) and a greatly reduced oxygen consumption after addition of serum-treated zymosan (in the patient, 0.6 nmol/10<sup>6</sup> cells; in the control individual, 7.2 nmol/10<sup>6</sup> cells). A negative NBT test and the absence of cytochrome *b*<sub>558</sub> in both whole cells and Triton X100-extracted cells confirmed the diagnosis of CGD. Failure to complement the patient's oxidase system in monocytes with that of a known patient with X-linked CGD indicated the presence of a defect in the X-linked gene *CYBB*.

A Southern blot of *EcoRI*-digested genomic DNA probed with *CYBB* coding sequence (fig. 1A) revealed an increase of ~2 kb in the fragment that comprises exon 4 of *CYBB*. The aberrant band was not seen in either the patient's mother or an unrelated control individual (fig. 1A); the patient's father was unavailable for analysis.

To confirm the results of the Southern blot, we amplified exon 4 in the patient, his mother, and an unrelated control individual (fig. 1B). The patient lacked a normal exon 4 product but had an exon 4 that contained an extra 2,105 bp, which indicates that the patient had one X chromosome and that it contained an abnormal exon 4 of *CYBB*. Sequencing of the abnormal PCR product revealed an insertion, comprising the final 1,722–1,724 bp of an L1 element, followed by a 280-bp 3' transduction. When compared with L1<sub>RP</sub>, the most active element yet tested in the cell culture assay, the L1 fragment was found to contain a single-nucleotide change in the 3' UTR. The 3' transduction was composed of a 21-bp polyA tail followed by 259 bp with no homology to L1 or *CYBB* sequences. This sequence was followed by a polyA tail of 101 bp that led directly to the remainder of exon 4 of *CYBB*. Since the 5' two bases of the insertion were both A, it was impossible to de-



**Figure 1** Insertion analyses. *A*, *CYBB*-specific Southern blot with *EcoRI*-digested DNA—from an unrelated control individual, the patient's mother, and the patient—demonstrating the shift, in the patient's sample, of the exon 4-containing fragment. The sizes of the wild-type bands are given; the arrow ( $\leftarrow$ ) indicates the aberrant band. The probe was constructed with coding cDNA of *CYBB* as template. *B*, Expand Long Template PCR of exon 4, in the patient, the patient's mother, and an unrelated control. The arrows ( $\rightarrow$ ) indicate markers in the ladder.

termine whether they constitute a 2-bp TSD within a 2,103-bp insertion or whether they are part of a 2,105-bp insertion without a TSD (fig. 2). The 5' portion of the transduced sequence contained an imperfect 29-repeat GAAA section. However, the final 153 bp contained unique sequence. A BLAST search of the human genome, for the entire 153-bp unique sequence, produced one exact match, AC067958 (GenBank), on chromosome 2q24.1.

The AC067958 sequence (GenBank) contained a slightly different imperfect GAAA repeat section, upstream from the unique sequence, but no L1 sequence. Because active L1 elements are often polymorphic, we assumed that the precursor (i.e., LRE3) was absent from the Human Genome Project sequence. PCR using four unique primer pairs showed that, in this region, the patient's mother was homozygous for a ~6.1-kb LRE3 allele, whereas the patient was heterozygous (fig. 3). These results were confirmed using a third primer internal to the L1 element (data not shown).

On direct sequencing of the patient's LRE3 PCR product, the final 1,720 bp of L1 sequence were an exact match of the insertion on the X chromosome (fig. 2). However, the imperfect GAAA repeat section 3' from the patient's LRE3 allele had 28 repeats, whereas his insertion had 29 repeats. We hypothesized that the mother had a 29-repeat LRE3 allele that was the actual precursor to the insertion and a 28-repeat allele that was inherited by the patient. After cloning the maternal LRE3 PCR product, we sequenced 12 clones and found that seven contained (GAAA)<sub>3</sub>TAAATAAAAAGAAAA(GAAA)<sub>8</sub>-GAA(GAAA)<sub>11</sub>GAGAAAAGCAAAA (29 repeats) and that five contained (GAAA)<sub>3</sub>TAAATAAAAAGAAAA(GAAA)<sub>8</sub>GAA(GAAA)<sub>10</sub>GAGAAAAGCAAAA (28 repeats). The sequence of the maternal 29-repeat LRE3 allele perfectly matched all 2,105 bp of the patient's insertion.

A small possibility exists that a 29-repeat LRE3 transcript from the father was carried in the sperm and that the precursor to the insertion is paternal in origin. Since the father was unavailable for analysis, we observed the prevalence of the 29-repeat LRE3 allele in the white population, the most prevalent ethnic group in the Netherlands. Of 50 white haploid genomes, 9 contained an LRE3 element. We used PCR to obtain the 3' flank. On sequencing, we discovered that all nine LRE3 elements were followed by different repeat sections and that only one of the nine matched the patient's 29-repeat insertion. Thus, the allele frequency of the 29-repeat LRE3 allele in whites is ~1 in 50 haploid genomes, or ~2%. Since the patient is heterozygous for LRE3, his father has at least one chromosome 2 without an LRE3 allele. Therefore, if the father is white, then he has an ~2% chance of having a 29-repeat LRE3 allele.

LRE3 is located on chromosome 2q24.1. Because of the polymorphic, repeating nature of this location, it is impossible to determine unambiguously the "empty site," the exact point of insertion, or the length of the TSD. If we assume that an endonuclease-mediated insertion occurred, then the most likely point of insertion is 5' from the (GAAA)<sub>n</sub> repeat at 5'-TGTC/AAAAA-3'. This point of insertion follows the consensus 5'-Py-Py/AAAAA-3' sequence of the L1-endonuclease site (Cost and Boeke 1998). The TSD would then be 28 bp—that is,



**Figure 2** Insertion sequence analyses. *A*, Schematic representation of the *CYBB* insertion. PT = important sequence of a PCR product from the patient’s exon 4. *B*, *CYBB*-insertion sequence alignments. *CYBB* = GenBank sequence at the point of insertion, into exon 4, of *CYBB*; PT = sequence of a PCR product from the patient’s *CYBB* exon 4 (which includes the disease-causing insertion); *LRE3* = sequence of a PCR product from the patient’s chromosome 2q24.1; *L1<sub>RP</sub>* = GenBank sequence of the most active element known (the nucleotide number of the adjacent base is given in parentheses). A possible 2-bp TSD in the disease-causing insertion is underlined; differences between sequences are shaded; leader dots indicate sequence that is consistent with the *L1<sub>RP</sub>* sequence.

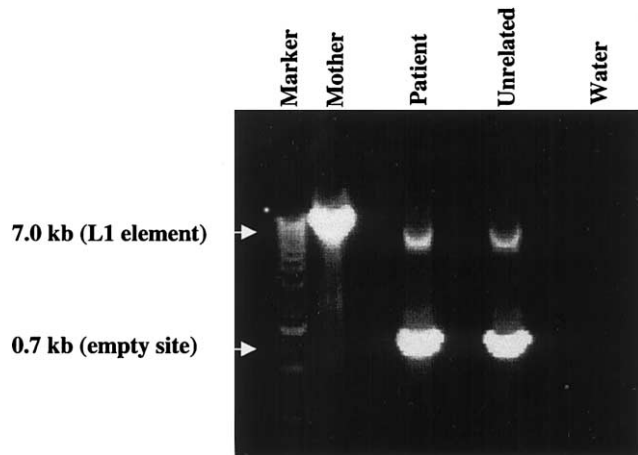
5'-AAAAAAAAAAAAAAAAAGAAAGAAAGAAA(G/T)AA-3', with one G/T base change near the 3' end (fig. 4).

*LRE3* has two ORFs, is a member of the Ta-1d subset of human retrotransposons, and is 99.9% identical to *L1<sub>RP</sub>*. Sequence comparison of *LRE3* with *L1<sub>RP</sub>* showed the following six differences: two in the 5' UTR; one in ORF1; one in the inter-ORF region; one, leading to an amino acid substitution, in ORF2; and one in the 3' UTR (fig. 4). The mother’s *LRE3* alleles are identical in sequence except for the 3' flank.

Since *LRE3* is 99.9% identical to *L1<sub>RP</sub>*, we predicted that the two elements would have similar retrotransposition activity in the cell culture assay. To test this prediction, we cloned both the mother’s and the patient’s PCR products directly into the pCEP4 expression vector, which contained an antisense EGFP sequence that was disrupted by an intron in the same orientation as the

*L1* element. On transfection into 143B TK- osteosarcoma cells, the EGFP retrotransposition marker is expressed only when the *L1* is transcribed, the intron is spliced out, and the *EGFP* gene is reverse transcribed and integrated into the genome (Ostertag et al. 2000). When tested, under the direction of their endogenous *L1* promoters, both the mother’s and the patient’s *LRE3* alleles had retrotransposition activities ~50% greater than that of *L1<sub>RP</sub>* (fig. 5). *LRE3* is therefore the most active human *L1* element yet characterized in a cell culture assay.

We assessed the frequency of *LRE3* in the general population, using DNA from 5 Indo-Pakistanis, 9 sub-Saharan Africans, 5 Pacific Islanders, 4 Chinese, and 25 white individuals. One or two individuals of all ethnic groups except the sub-Saharan Africans, in whom *LRE3* was not found, were heterozygous for *LRE3*. In sum,



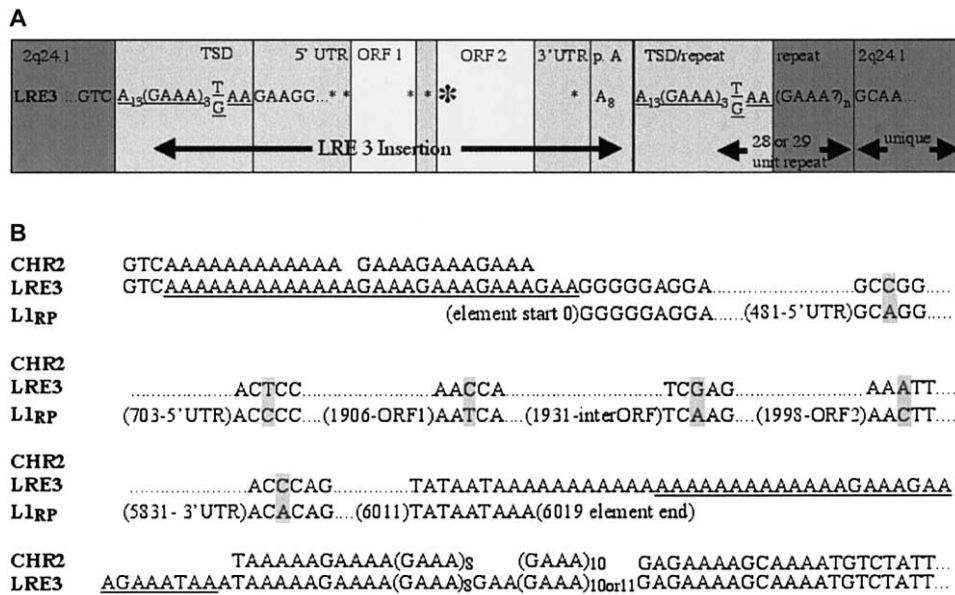
**Figure 3** Expand Long Template PCR with flanking primers, demonstrating the presence or absence of *LRE3* in the patient's mother, in the patient, and in an unrelated heterozygote. All bands were sequenced, to confirm findings.

*LRE3* was present in 16 of the 96 genomes, giving an allele frequency of 0.17 and further confirming the recent evolutionary nature of the Ta-1d subset (Boissinot et al. 2000) (fig. 6).

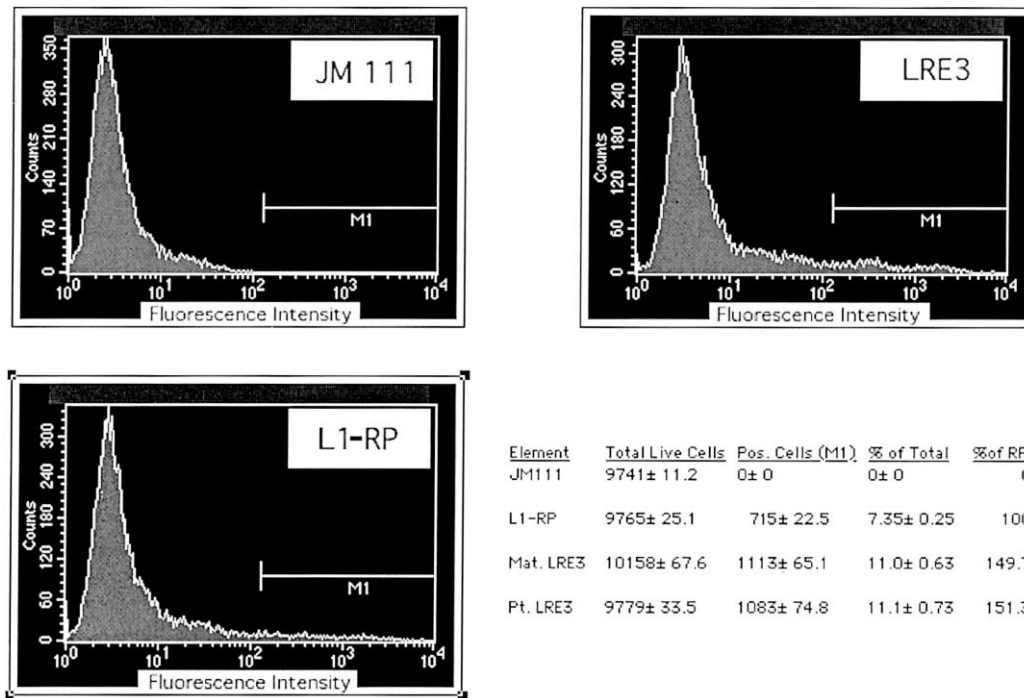
**Discussion**

We used a unique polymorphic 3' transduction to derive evidence that *LRE3* retrotransposed, into *CYBB*, in the primary oocyte during maternal meiosis I. The patient's 29-repeat *CYBB* insertion does not match his 28-repeat *LRE3* allele but does match the mother's 29-repeat *LRE3* allele. Since the patient's mother does not appear to have the *CYBB* insertion, the simplest explanation is that the 29-repeat *LRE3* allele in the mother was transcribed and retrotransposed into an X chromosome before the conclusion of maternal meiosis I. On ovulation, the separation of homologous chromosomes occurred, and both the 28-repeat *LRE3* allele and the X chromosome with the 29-repeat insertion became part of the secondary oocyte, whereas the actual precursor 29-repeat *LRE3* allele and the X chromosome without the insertion were lost in the extruded polar body. At fertilization, the sperm carried a Y chromosome and a copy, lacking *LRE3*, of chromosome 2. The fertilized egg produced a male offspring with a single 28-repeat *LRE3* allele on chromosome 2 and a 29-repeat insertion on the X chromosome.

Retrotransposition during maternal meiosis I provides the simplest explanation for our data. Since ma-



**Figure 4** *LRE3* sequence analyses. *A*, Schematic representation of the *LRE3* insertion into chromosome 2q24.1. *LRE3* = important sequence from *LRE3*. Potential TSDs are underlined; asterisks (\*) are placed at locations where differences exist between *LRE3* and *L1<sub>RP</sub>*. The larger asterisk indicates an amino acid change. *B*, *LRE3* sequence alignments. CHR2 = GenBank sequence's "empty site" at the *LRE3* locus; *LRE3* = sequence of a PCR product from both the patient's chromosome 2q24.1 and the patient's mother's chromosome 2q24.1; *L1<sub>RP</sub>* = GenBank sequence of the most active element known (the nucleotide number of the adjacent base is given in parentheses). A possible 28-bp TSD of *LRE3* is underlined; nucleotide differences are shaded; leader dots indicate sequence that is consistent with the *L1<sub>RP</sub>* sequence.



**Figure 5** Example of flow-cytometry results. A live/dead gate was set by use of forward and sideward scatter of cells, and ~10,000 live cells were assayed for fluorescence. Cells within the M1 marker were counted as positive. The M1 marker was zeroed using a single JM111 clone (L1<sub>RP</sub>, with two disabling ORF1 point mutations) and a single L1<sub>RP</sub> clone (*not shown*) that lacked the 3,832-bp *Afl*III fragment. Both negative controls were assayed in triplicate; one L1<sub>RP</sub> clone was assayed in triplicate, as a positive control; two *LRE3* clones, one derived from maternal genomic DNA and another derived from patient genomic DNA, were assayed in triplicate. Numbers give the average number, over three assays, of positive cells for each clone ± 1 SD.

ternal meiosis I occurs in every ovulating female and lasts from birth to ovulation, a rare retrotransposition event could take place at any time during this period. However, other scenarios that require the occurrence of an additional rare event are formally possible:

1. If the mother were mosaic, then the actual insertion into the X chromosome could have taken place as early as maternal embryogenesis. However, by PCR, we did not find any evidence for maternal mosaicism of lymphocyte DNA. In addition, at this time, there is no evidence that retrotransposition occurs during embryogenesis.

2. The patient's insertion could have come from his own precursor; instability during retrotransposition or replication at the polymorphic repeat locus could have resulted in an altered *LRE3* allele that matched a maternal sequence. However, any instability would have to be very precise, since one specific maternal allele would have been changed to match the other. The wide variety of repeat formats found in the nine individuals tested suggests that this is unlikely. Also, present knowledge indicates that the process of retrotransposition appears to be accurate. Two precursors of human L1 insertions have been exact matches to their respective insertions

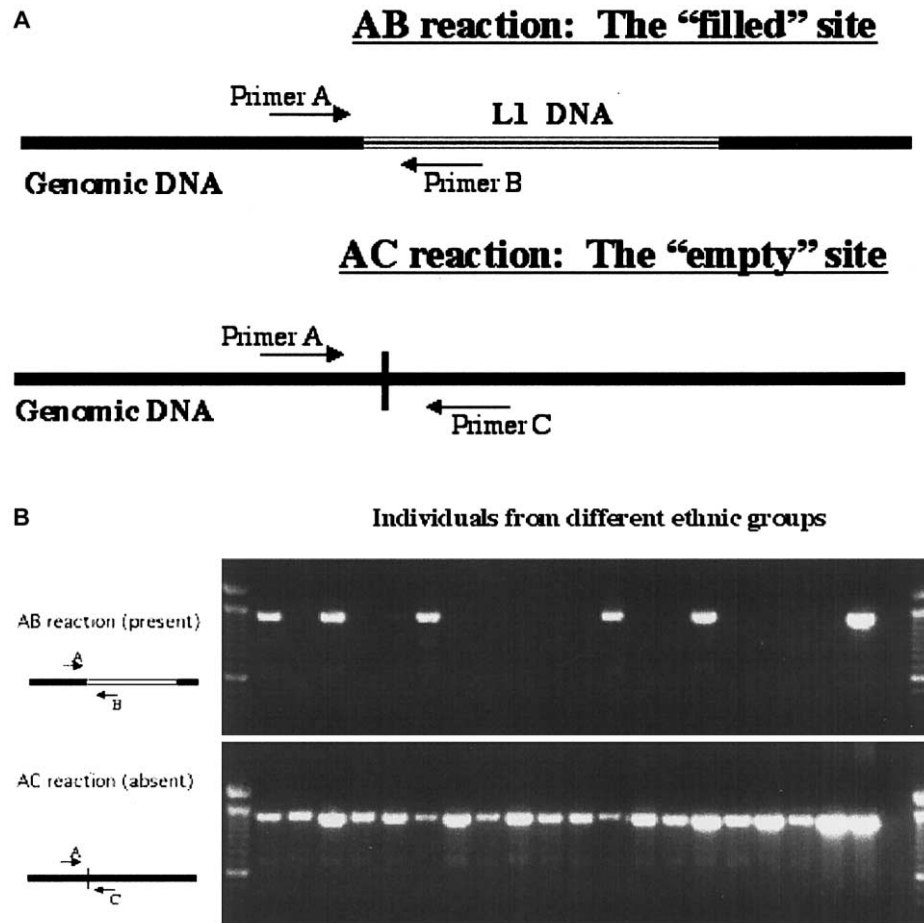
over a total of ~6 kb (Dombroski et al. 1991; Holmes et al. 1994).

3. The father had a small chance of carrying a 29-repeat *LRE3* allele and could have passed a lingering 29-repeat *LRE3* message, in his sperm, that retrotransposed into the maternal X chromosome. This scenario would require the confluence of three rare events: (a) he would need to have the correct *LRE3* message; (b) he would need to pass a stable full-length L1 mRNA, in his sperm, separate from its precursor chromosome; and (c) that message would need to be reverse transcribed and inserted into the mother's X chromosome postfertilization.

Because of the relative rareness of these alternative scenarios, a retrotransposition event during maternal meiosis I is the most straightforward explanation for our data.

From an evolutionary perspective, only retrotransposition in germ cells or germ cell precursors can be passed on to progeny; somatic retrotransposition, on the other hand, is an evolutionary dead end. The new insertion cannot be passed on, and, if the insertion harms the host, then the precursor to the insertion will not be passed on. Although ORF1 expression has been de-





**Figure 6** Assay and gel analyses. *A*, Schematic representation of the polymorphism assay that was used to determine the absence or presence of an element. *B*, Sample gel, showing results from 20 individuals of diverse ethnic backgrounds.

tected in somatic cells in mouse testis (Branciforte and Martin 1994), the only known example of somatic retrotransposition is an L1 insertion, into an *APC* gene, resulting in colon cancer (Miki et al. 1992). Oncogenesis, however, is a multistep process, and the colon cell may have been partially transformed at the time of retrotransposition. Although there is no in vitro evidence of retrotransposition in primary cell lines, retrotransposition is readily demonstrated in cultured transformed cell lines (Moran et al. 1996).

*LRE3*, the precursor to the insertion, is the most active human L1 element yet tested in our cell culture assay. When active L1 elements are used to create an amino acid consensus sequence for ORF1 and ORF2 (data not shown), the proteins encoded by *LRE3* ORF1 and ORF2 are exact matches. Therefore, *LRE3* is the first element tested that has a perfect amino acid consensus through both ORF1 and ORF2. *LRE3* and *L1<sub>RP</sub>* differ by one silent change in ORF1, four changes in noncoding regions, and one T→N amino acid change at position 5

of ORF2; however, *LRE3* is 50% more active. Further analysis may provide insight into domains that are important in the mechanism of retrotransposition.

The insertion, into exon 4, of the patient's *CYBB* gene appears to lack TSDs that have commonly been found in retrotransposition events. TSDs result when the L1 endonuclease cuts the two DNA strands at the insertion site in a staggered manner. At least two explanations for the apparent lack of TSDs are possible: First, L1-endonuclease-mediated insertion could produce very short TSDs of two bases (AA) or even no TSDs (Jensen et al. 1994); insertion, in this case, would have occurred at 5'-TC<sup>^</sup>AA-3', a potential L1-endonuclease site (fig. 2) (Cost and Boeke 1998). Second, it has been proposed, as an explanation for the rare cases of TSD-less L1 insertions, that L1 fragments play a role in the repair of preexisting double-strand breaks (Mager et al. 1985; Van de Water et al. 1998; Kondo-Iida et al. 1999; Browning et al. 2001). Either hypothesis could explain the findings in the patient.

*LRE3* is the second precursor L1 element that has been characterized using flanking sequence transposed in a de novo insertion (Holmes et al. 1994). Indeed, *LRE3* is only the third active precursor L1 locus that has been characterized overall (hence, its designation as “L1 retrotransposable element-3”). With the inclusion of the full-length elements that were isolated from genome analysis and that have retrotransposition activity in the cell culture assay (Moran et al. 1996; Ostertag et al. 2000), there are now 10 active human L1 elements known (Sassaman et al. 1997; Schwahn et al. 1998; Kimberland et al. 1999).

Overall, 211 *CYBB* mutations are listed in the Human Gene Mutation Database. One deletion was a non-homologous recombination that involves an L1 element that lies 5 kb upstream from *CYBB* (Kumatori et al. 1998). In addition, there has been a previous L1 insertion into an intron of *CYBB* (Meischl et al. 2000). More than half of known recent human L1 insertions have occurred in only three genes: *CYBB*, *factor VIII*, and *dystrophin* (Ostertag and Kazazian 2001). It would be interesting to determine whether these three genes are L1 hotspots or whether this seeming cluster of L1 activity is the result of an ascertainment bias.

The polymorphic 3' transduction of this insertion has allowed us to predict, with great confidence, both the timing and the parental origin of an in vivo retrotransposition event. *LRE3*, presently the most active human L1 element in cell culture, is also the first element with an active amino acid consensus sequence for both ORFs. It will be interesting to see whether departures from consensus or changes in noncoding nucleotides will further increase the retrotransposition activity of L1 elements. As the most active L1 element currently known, *LRE3* is now the best candidate for mutagenesis systems and other molecular biology applications.

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## Electronic-Database Information

The accession number and URLs for data presented herein are as follows:

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for *Homo sapiens* BAC clone RP11-81F8 [accession number AC067958])  
Human Gene Mutation Database, The, <http://archive.uwcm.ac.uk/uwcm/mg/search/120513.html> (for *CYBB* mutations)

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for CGD [MIM 306400])

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