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X chromosome-linked Kallmann syndrome: Stop mutations validate the candidate gene

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ABSTRACT **Kallmann syndrome represents the association of hypogonadotropic hypogonadism with anosmia. This syndrome is from a defect in the embryonic migratory pathway of gonadotropin-releasing hormone synthesizing neurons and olfactory axons. A candidate gene for the X chromosome-linked form of the syndrome was recently isolated by using a positional cloning strategy based on deletion mapping in the Xp22.3 region. With the PCR, two exons of this candidate gene were amplified on the genomic DNAs from 18 unrelated patients affected with the X chromosome-linked Kallmann syndrome. Three different base transitions—all leading to a stop codon—and one single-base deletion responsible for a frameshift were identified. We thus conclude that the candidate gene is the actual *KAL* gene responsible for the X chromosome-linked Kallmann syndrome. Furthermore, unilateral renal aplasia in two unrelated patients carrying a stop mutation indicates that the *KAL* gene is itself responsible for this Kallmann syndrome-associated anomaly. The gene is, therefore, also involved in kidney organogenesis. Additional neurologic symptoms in Kallmann patients are also discussed.**

Kallmann syndrome, originally described in 1856 by Maestre de San Juan (1), is defined by the association of hypogonadotropic hypogonadism with anosmia (lack of smell). Hypogonadism is from insufficient release of gonadotropin-releasing hormone (GnRH) from the hypothalamus (2), whereas anosmia has been related to agenesis of the olfactory bulbs (3). The first familial cases were reported by Kallmann *et al.* in 1944 (4). Subsequently, segregation analysis revealed several transmission modes: X chromosome-linked, autosomal recessive, and autosomal dominant (5–8). The incidence of Kallmann syndrome has been estimated at 1 male out of 10,000 males (9). The 5- to 7-fold excess of affected males versus females (9) suggests that the X chromosome-linked form (catalog number 308700; ref. 10) is the most frequent form. Using a positional cloning strategy, we have recently isolated a candidate gene accounting for the X chromosome-linked form of the syndrome (11). The strategy was based on deletion-mapping that defined an interval for localization of the gene in the Xp22.3 region. This interval (KAL), 67 kilobases (kb) in length, was limited by the chromosomal breakpoints in two males, one being affected with the Kallmann syndrome, whereas the other was not. The gene has been called ADMLX for adhesion molecule-like from the X chromosome because of sequence homologies between the putative 680-amino acid encoded protein and adhesion molecules. The same candidate gene has been isolated by another group (12).

That the ADMLX gene was the actual *KAL* gene was supported by strong evidence. (*i*) No other gene was found in the KAL interval despite exhaustive exon screening. (*ii*) An additional deletion, involving only the 5′ part of the gene, was detected by Southern blot analysis in one patient affected with Kallmann syndrome (11). (*iii*) Sequence homologies of the putative ADMLX protein were consistent with a predicted role for the Kallmann gene in the embryonic migration pathway of GnRH-synthesizing neurons and olfactory axons (13–16). However, final validation of this candidate gene required the finding in Kallmann-affected patients of point mutations or small deletions in that gene.

We have, therefore, undertaken a search for such mutations in the coding exons and flanking splicing sites in 18 unrelated patients affected with familial X chromosome-linked Kallmann syndrome. We report here three different base transitions, all leading to a stop codon, and one single-base deletion responsible for a frameshift, in two contiguous exons of the candidate gene.

In addition to hypogonadism and anosmia, other variable abnormalities have been reported in Kallmann patients. These abnormalities include neurologic disorders, such as mirror movements, abnormal visual spatial attention, ocular motor abnormalities, sensory neural hearing loss, cerebellar dysfunction, and pes cavus deformity (17, 18), suggesting that the *KAL* gene(s) might also be involved in the embryonic pathway of other neurons. More enigmatic is the unilateral renal aplasia described in several X chromosome-linked Kallmann patients (19). This anomaly was recently seen in two unrelated patients with a deletion in the Xp22.3 region (J.-P.H., J.L., P.B., C.P., J. Young, M. Pholsena, G. Schaison, unpublished data). Whether this anomaly was from alteration of the *KAL* gene itself or from simultaneous deletion of a contiguous gene could only be solved by finding mutations within the *KAL* gene in Kallmann patients with unilateral renal aplasia. Neurologic and renal symptoms of the four patients carrying a mutation will be discussed in respect to these questions.

MATERIALS AND METHODS

Patients. X chromosome-linked transmission of Kallmann syndrome in the selected families was assumed from the presence of at least one affected male in the maternal family of the propositus, absence of affected females, and absence of consanguinity.

Exon Positions and Intron–Exon Boundaries. To establish intron–exon boundaries, genomic DNA of the corresponding regions was sequenced (I.d.C. and M.C.-S., unpublished data).

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Abbreviations: GnRH, gonadotropin-releasing hormone; ADMLX, adhesion molecule-like from the X chromosome.

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The precise limits of the two exons were deduced from the comparison between genomic and complementary DNA sequences and according to the AG/GT rule for splicing acceptor/donor sites (20). Exons A and B extend, respectively, from position 542 to 726 and from position 727 to 856 on the 2040-base-pair (bp) open reading frame of the ADMLX cDNA (11). Sequences of intron boundaries are shown in Fig. 1.

Genomic DNA Isolation. Epstein–Barr virus-transformed lymphoblastoid cells from patients were cultured in RPMI 1640 medium (Flow Laboratories)/10% fetal calf serum (Jacques Boy, Reims, France). Genomic DNA was prepared from lymphoblastoid cell lines.

Southern Blot Analysis. Restriction enzyme digests (*Eco*RI, *Hin*dIII, and *Pst* I) were carried out with 15–20 units of enzyme per μg of genomic DNA. Digestions of DNAs, electrophoresis, and membrane transfer (N+Hybond, Amersham) were done as described (22). Twelve probes covering the entire ADMLX cDNA were derived by using PCR amplification. Hybridizations were done at 65°C, as described by Church and Gilbert (23). Membranes were washed at 65°C in 2× standard saline/citrate (SSC)/0.1% SDS.

Genomic PCR. Two sets of specific primers, 21 nucleotides in length, were chosen in the intronic sequences flanking each of the two exons (Fig. 1). An *Eco*RI site was added to the 5′ end of each primer for cloning purpose of PCR products. The PCRs were done as follows: 94°C denaturation, 1 min; 55°C annealing, 1 min; 72°C extension, 1 min; 30 cycles.

DNA Cloning and Sequencing. The PCR-amplified fragments (266 bp and 217 bp in length) from genomic DNAs of patients were cloned in M13mp18 at the *Eco*RI site. Transformation was performed in *Escherichia coli* strain TG1, according to Hanahan (24). The M13 single-strand templates were sequenced according to Sanger *et al.* (25) with fluorescently tagged M13 primers with *Taq* DNA polymerase (NBL) on Applied Biosystems 370A DNA sequencers.

A single sequence from one or the other strand was considered sufficient to conclude normality when no mismatch was found compared with the corresponding cDNA sequence. Otherwise, the sequences from both strands were compared. Each putative mutation was confirmed, starting from a second independent PCR amplification.

RESULTS AND DISCUSSION

Xp22.3 Deletions in Isolated X Chromosome-Linked Kallmann Syndrome. During the cloning procedure of the ADMLX candidate gene, the genomic DNAs from 20 unrelated patients affected with only X chromosome-linked Kallmann syndrome had been systematically hybridized to ADMLX cDNA probes on Southern blots. We thereby identified two large deletions that both included the entire ADMLX gene (data not shown).

Large Xp22.3 deletions have been described for several years in patients affected with both X chromosome-linked ichthyosis and Kallmann syndrome (26). We recently reported a deletion that only included the 5′ end of the ADMLX gene in another patient (RM254) (11). Because we found only two deletions among the 20 families analyzed, such deletions appear uncommon in patients affected with the X chromosome-linked Kallmann syndrome alone.

Identification of Single-Base Mutations. Two ADMLX exons were amplified from genomic DNAs of the 18 unrelated X chromosome-linked Kallmann patients in whom no deletion of the ADMLX gene had been observed. Four patients carried different mutations in either exon A or exon B (Fig. 2): patients PAR366, LON36K4, and LON77A10 are carrying single-base transitions, replacing the normal corresponding codon (11) with a TGA stop codon; patient TOU351 has a single-base deletion, which causes a frameshift (11). Each mutation was confirmed in two independent PCR-cloning experiments and by sequencing both DNA strands. Sequencing of the corresponding exon from the obligate carrier mothers of the four patients revealed both normal and mutated alleles. No other mutation was found in these exons among the 14 remaining patients.

The finding in four unrelated Kallmann patients of three nonsense mutations and of one single-base deletion, located between codons 237 and 277 of the ADMLX open reading frame (680 codons), demonstrates that this candidate gene is, indeed, responsible for the X chromosome-linked Kallmann syndrome. Similar analysis of the other exons of the *KAL* gene should point out the mutations responsible for the Kallmann syndrome in the 14 remaining patients. A hot-spot for mutations cannot yet be excluded, especially in the 5′ end of the transcript, which is particularly rich in CpG dinucleotides (24 in the first 200 coding nucleotides). This cluster of CpG has been shown to be methylated (11, 27) and thus does not correspond to an *Hpa* II tiny fragments island. Because C → T transitions resulting from 5-methylcytosine deamination in CpG dimers are not efficiently corrected, these sites are potential hot-spots for mutation (28). The CGA → TGA mutation reported here in patient PAR366 is consistent with such a mechanism. It should be noted that, in the absence of a functional test for KAL protein, establishing that some rare missense mutation causes the disease might not be easy, unless the mutation occurs *de novo* in a Kallmann patient. Finally, direct analysis of the KAL transcript might be required to find some putative mutations, such as those creating additional splicing sites or mutations in the promoter region responsible for low level (or absence) of transcription of the *KAL* gene.

***KAL* Gene and Clinical Features.** The pedigrees of the four families in which mutations were found are shown in Fig. 3.

Patient PAR366 was examined at birth for micropenis and bilateral cryptorchidism. At 11 yr of age, he was anosmic, had

EXON A

5′ CAGATTGTTTTAATTGATACGGTCCTGTTTCAGGTGTCC...GCCCAGGTAAGAACCTCATATAGACTTTGATTTTCATCCTGGAGGTAGTGTCTGC 3′
542 726

EXON B

5′ AGTGACATGTTCCCTGTGCTCTGTGACATGTCCCCTCAGACCACA...CCAAAGGTCAGTCTTCCTTTTCTTCCCAGACACAGAATACTATCCTTGCTACCAG 3′
727 856

FIG. 1. Sequence of intron–exon boundaries for exons A and B. Boldface letters indicate exonic sequences. Arrowheads indicate the first and the last nucleotide of each exon; numbers refer to the position in the ADMLX open reading frame. Splicing junction sequences follow the 5′ and 3′ current consensus (21). The intronic primer sets used for PCR amplification of these exons are underlined, and their sequences are as follows: A1, 5′-CAGATTGTTTTAATTGATACG-3′; A2, 5′-GCAGACACTACCTCCAGGATG-3′; B1, 5′-AGTGACATGTTCCCTGTGCTC-3′; B2, 5′-CTGGTAGCAAGGATAGTATTC-3′.

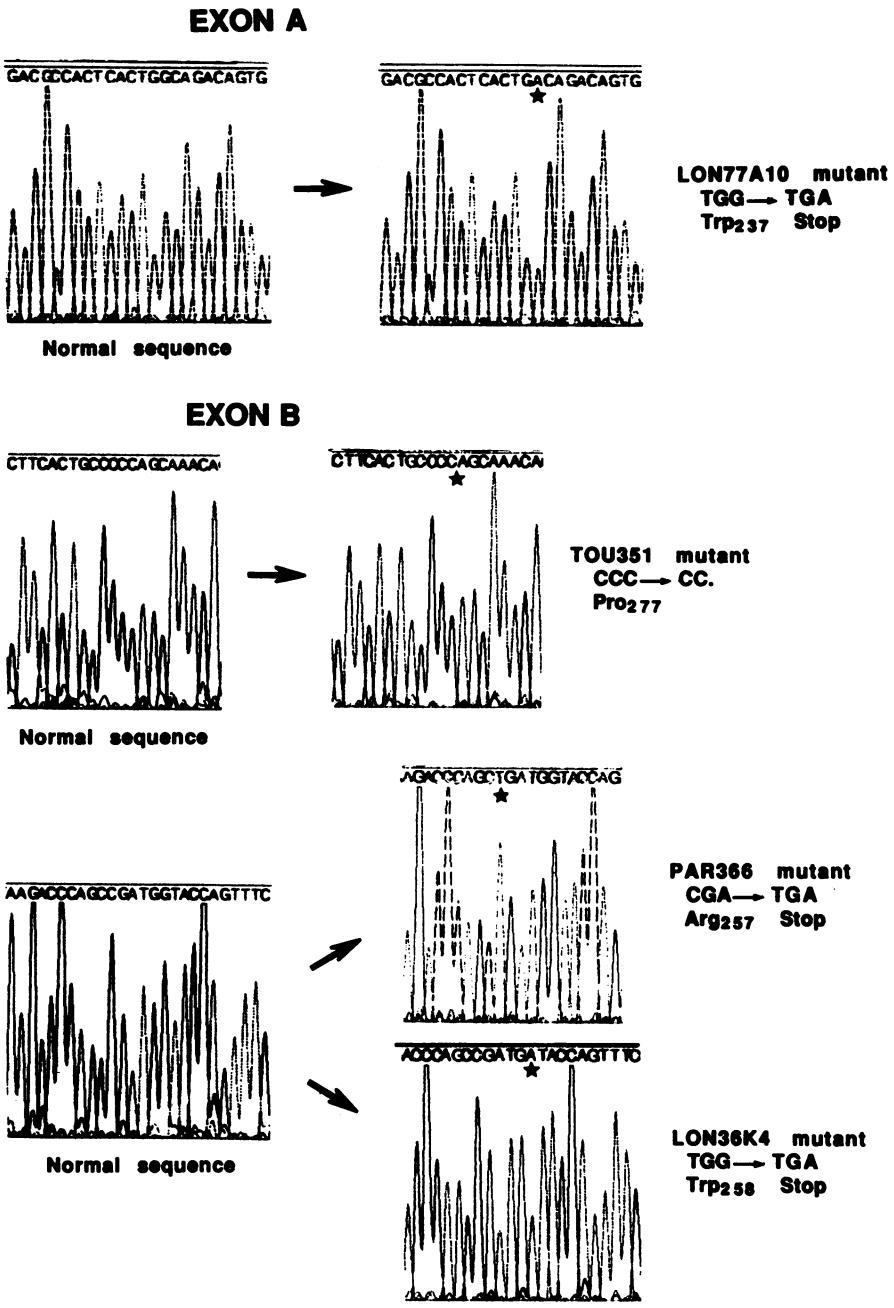

FIG. 2. Mutations detected in the four propositus individuals. For each mutation, part of the corresponding exon sequence is shown together with the normal sequence. Point mutations are indicated with stars. Changes in the corresponding codons and amino acids with their positions deduced from the ADMLX open reading frame are indicated at right.

typical mirror movements of hands (bimanual synkinesia), and mild bilateral pes cavus deformity (high-arched foot). Several pigmented naevi were noted on the skin. No abnormal ocular movements or cerebellar ataxia was found. An audiogram was normal. Echography showed the presence of two kidneys. A maternal great-uncle is also affected with Kallmann syndrome.

Patient LON36K4 presented at 11 yr of age with unilateral cryptorchidism and was found to be anosmic. He had, in addition, left ptosis (droopy eyelid), synkinesia, and unilateral renal aplasia. Two brothers and a maternal uncle are affected with Kallmann syndrome. One of these two brothers has, in addition, left congenital hemiparesis.

Patient LON77A10 was examined at 4 yr of age for bilateral cryptorchidism. He was anosmic, had synkinesia, and suffered from minor motor epilepsy. He had, in addition, unilateral renal aplasia. One cousin, three uncles, and two great-uncles are affected with Kallmann syndrome. A brother who died at 1 day of age had only one kidney.

Patient TOU351 was first examined at 8 yr of age for micropenis and bilateral cryptorchidism. He was anosmic and had bilateral pes cavus deformity. Neither mirror movements nor cerebellar ataxia were found. His brother is also affected with Kallmann syndrome and has marked pes cavus deformity. In addition, both have a high-arched palate. Both siblings have normal renal echography. One cousin, an uncle, a nephew, and two cousins of the mother are affected with Kallmann syndrome.

A number of additional neurologic symptoms have been described in Kallmann patients (17, 18). The present data establish that, at least, two symptoms, mirror movements and pes cavus deformity, can occur as a consequence of mutation in the *KAL* gene. In contrast to hypogonadism and anosmia, no embryological data are presently available to

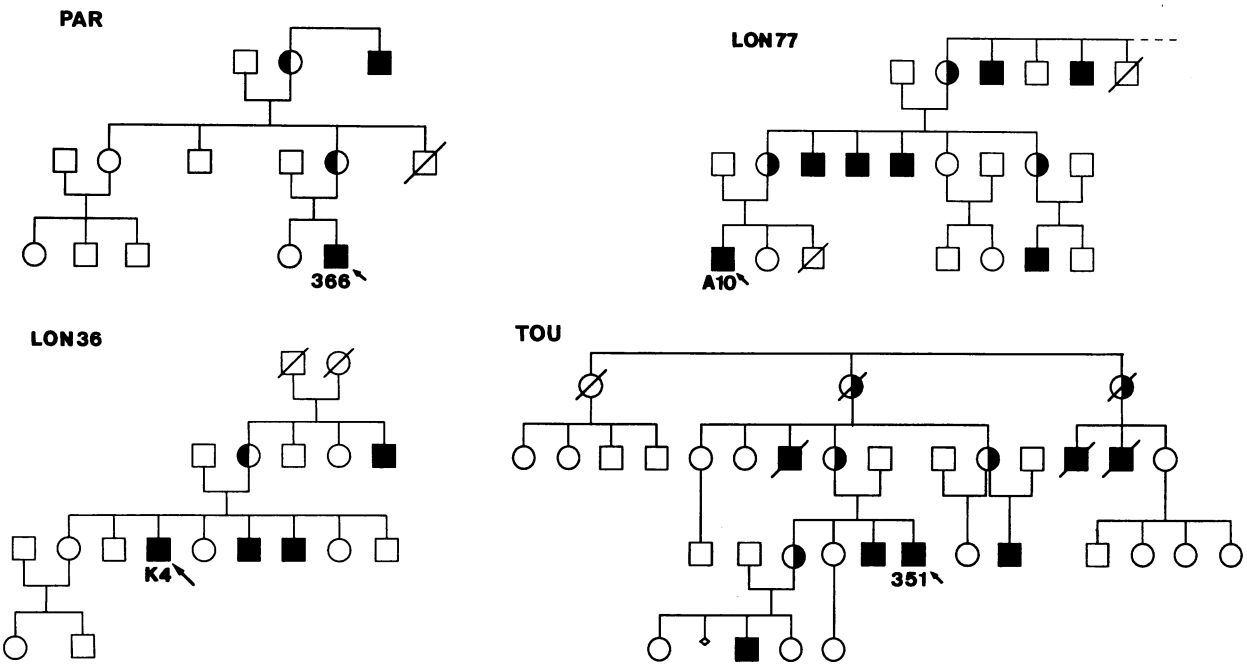

FIG. 3. Pedigrees of the four families carrying mutations. ■, Affected male; □, normal male; ◐, obligate carrier mother; ○, homozygous normal female or female with undetermined carrier status; ◪, ◪, and ⊘, deceased individual; ◇, stillborn, sex unknown. In each family, the propositus is indicated with an arrow.

explain pathogenesis of these symptoms. Although some, such as mirror movements and cerebellar ataxia have been ascribed to a central nervous system midline defect (17), it seems unlikely that they result from a defect in a common embryonic pathway because they are seldom associated in one patient. Synkinesia are the more frequent and have been attributed to a lack of inhibitory fibers connecting the two hemispheres through the corpus callosum (29). Other neurologic symptoms reported here, epilepsy and congenital hemiparesis, have not yet been described in Kallmann patients to our knowledge. Whether they might be related to Kallmann syndrome or not requires other clinical reports of such associations.

The finding in two unrelated patients of stop mutations associated with unilateral renal aplasia provides strong evidence that this symptom is related to the abnormal *KAL* gene itself and not to a putative contiguous gene. The role of the *KAL* gene, therefore, extends to development of nonneuronal tissues. However, the observation that not all Kallmann patients from one given family exhibit this additional abnormality indicates that some genetic and/or epigenetic factors are required in addition to the *KAL* gene alteration. Moreover, the presence of one normal kidney in affected patients emphasizes the role of local developmental factors in the achievement of renal aplasia. However, one would also expect bilateral renal aplasia to occur in these families, causing oligohydramnios and neonatal death.

Further associated genetic defects and clinical symptoms in patients affected with Kallmann syndrome could have important implications for investigating the physiological function(s) of the *KAL* gene.

Previous reports have established the common embryonic origin and migratory pathway for GnRH-synthesizing neurons and olfactory axons (13–15). The observation in a Kallmann fetus that both GnRH neurons and olfactory nerves were absent from the brain and had accumulated in the nasal area suggested that the *KAL* gene might be implicated in the guidance of GnRH neurons and olfactory axons (16). Sequence analysis of the KAL putative protein revealed a characteristic signal peptide but no evidence of either a transmembrane domain or a hydrophobic C terminus for phosphatidylinositol anchorage. Thus, the KAL protein is likely secreted as an extracellular-matrix molecule. Sequence homologies were found with both the whey acidic protein (WAP) motif (30, 31) shared by several serine protease inhibitors (32–35) and the fibronectin type III repeat (36) present in several other extracellular-matrix proteins, such as tenascin (37, 38), undulin (39), and collagens VI, XII, and XIV (40, 41). Some of the latter are involved in cell adhesion (42, 43). Both functions, antiprotease and adhesion, are consistent with a role of the *KAL* gene in neuronal guidance (44–46). Such a pattern of homologies also constitutes an original feature of the KAL putative protein, suggesting that it might interact with extracellular serine protease(s) and cell-surface receptor(s). Interestingly, the type VI collagen α_3 chain also contains both types of domains, one homologous to the fibronectin type III repeat and one homologous to serine protease inhibitors of the Kunitz type (40). Further experimental data are required to validate adhesion and antiprotease functions of the KAL protein.

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