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Report

Haploinsufficiency of *ALX4* as a Potential Cause of Parietal Foramina in the 11p11.2 Contiguous Gene–Deletion Syndrome

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Heterozygous mutations in *MSX2* are responsible for an autosomal dominant form of parietal foramina (PFM). PFM are oval defects of the parietal bones that are also a characteristic feature of a contiguous gene–deletion syndrome caused by a proximal deletion in the short arm of chromosome 11 (Potocki-Shaffer syndrome). We have identified a human bacterial artificial chromosome (BAC) clone mapping to chromosome 11, containing a region homologous to the human homeobox gene *MSX2*. Further sequence analysis demonstrated that the human orthologue (*ALX4*) of the mouse *Aristaless-like 4* gene (*Alx4*) is contained within this 11p clone. We used FISH to test for the presence—or for the heterozygous deletion—of this clone in two patients with the 11p11.2-deletion syndrome and showed that this clone is deleted in these patients. *ALX4* and *Alx4* were shown to be expressed in bone and to be absent from all other tissues tested. The involvement of *Alx4* in murine skull development, its bone-specific expression pattern, the fact that *Alx4* is a dosage-sensitive gene in mice, and the localization of a human genomic clone containing *ALX4* to 11p11.2, with hemizygosity in patients with deletion of 11p11.2 who have biparietal foramina, support the contention that *ALX4* is a candidate gene for the PFM in the 11p11.2-deletion syndrome.

Parietal foramina are the result of delayed or incomplete ossification of the parietal bones of the skull. Parietal foramina can occur as either an isolated autosomal dominant trait (PFM [MIM 168500]) or as part of a syndrome. One such syndrome is the contiguous gene–deletion syndrome (CGDS) caused by monosomy of 11p11.2 (Potocki-Shaffer syndrome) (Shaffer et al. 1993; Bartsch et al. 1996; Potocki and Shaffer 1996; Wuyts et al. 1999). The Potocki-Shaffer syndrome (PSS [MIM 601224]), includes biparietal foramina, multiple exostoses (MIM 133701), dysmorphic features, and mental retardation (Shaffer et al. 1993; Bartsch et al. 1996; Potocki and Shaffer 1996; Wuyts et al. 1999).

Recently, autosomal dominant, isolated parietal foramina were found to be the result of mutations in the homeobox gene *MSX2,* which maps to 5q34-q35 (Wilkie et al. 2000; Wuyts et al. 2000). Mutations resulting in PFM were either entire-gene deletions or loss-of-function mutations, which supports the hypothesis that haploinsufficiency of this protein results in PFM. This possibility is distinct from the previously described gain-of-function mutation in *MSX2,* which is associated with craniosynostosis (Jabs et al. 1993; Ma et al. 1996). Mice with loss-of-function homozygous mutations in *Msx2* display a number of anomalies, including defects in skull ossification (Satokata et al. 2000).

CGDS refers to conditions caused by haploinsufficiency of multiple, functionally unrelated yet physically contiguous loci (Schmickel 1986; Schinzel 1988). The challenge in studying any CGDS is in proving that haploinsufficiency of one particular gene within the deletion interval causes a specific clinical feature of the syndrome. Few examples exist whereby a gene has been shown, through identification of mutations in individuals or families showing the isolated clinical anomaly, to be the causative gene of a single feature (reviewed in Shaffer et al. 2001).

Given that haploinsufficiency of *MSX2* results in PFM, we hypothesized that an *MSX2* homologue could be responsible for the PFM in the 11p11.2-deletion syndrome. Our approach was to use the *MSX2* sequence

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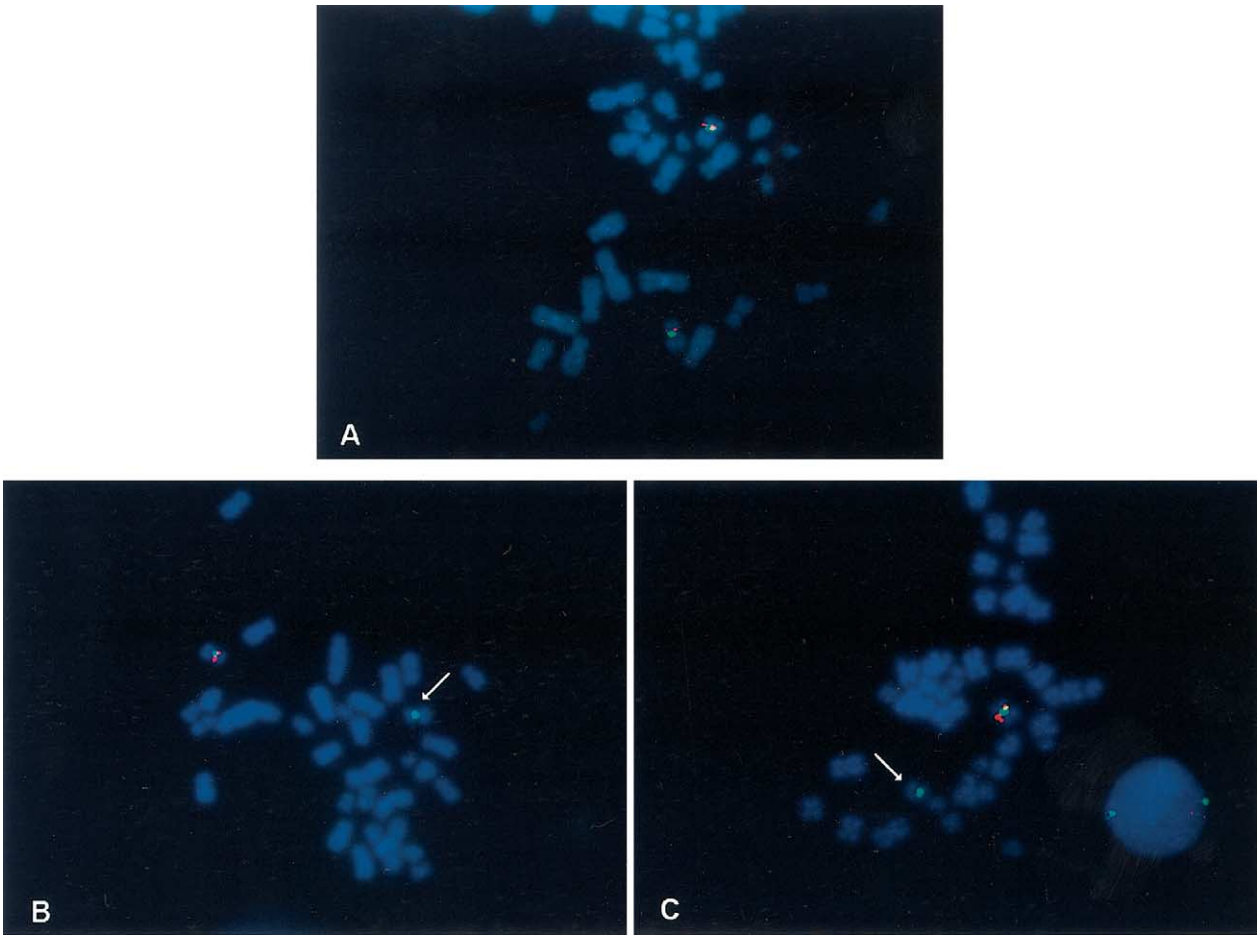

Figure 1 Representative FISH results. BAC 706A13 is labeled with digoxigenin and is detected with anti-digoxigenin conjugated to rhodamine (fluoresces red). The centromere of chromosome 11 is identified by an alpha-satellite probe labeled with biotin and is detected by avidin conjugated to fluorescein (fluoresces green). *A,* Control metaphase showing localization of clone 706A13 to the proximal short arm of chromosome 11. *B* and *C,* Cell lines derived from patients with 11p11.2 deletions and with PSS. *B,* Patient originally reported by Shaffer et al. (1993). *C,* Patient originally reported by Potocki and Shaffer (1996). Arrows indicate the deleted chromosome for each patient.

to identify the 11p-specific gene responsible for PFM in PSS.

The complete coding sequence of human *MSX2* was used to perform a BLASTN homology search against the high-throughput genome-sequence database (BLAST). The human clone RP11-706A13 (GenBank accession number AC019143), previously mapped to chromosome 11, was identified and obtained from the BAC-PAC Resources at Children's Hospital Oakland Research Institute (CHORI). The region of homology in clone 706A13 corresponds to 26 bp of 100% identity to *MSX2,* in only the homeodomain region (E value = 9×10^{-4}).

Two-colored FISH, using 706A13 labeled with digoxigenin 11-dUTP and a chromosome 11 centromeric probe (ONCOR) labeled with biotin 16-dUTP, was performed simultaneously on denatured metaphase spreads of chromosomally normal control individuals. The FISH analysis showed that clone 706A13 localized to the proximal short arm of chromosome 11 (fig. 1*A*). Two patients with dysmorphic features, biparietal foramina, multiple exostoses, and mental retardation were ascertained by clinical geneticists at Baylor College of Medicine, as has been reported elsewhere (Shaffer et al. 1993; Bartsch et al. 1996; Potocki and Shaffer 1996). FISH analysis of these two cell lines showed that 706A13 was deleted in both cases (fig. 1*B* and *C*).

A homology search using sequence flanking the homeobox domain on clone 706A13 was performed and resulted in the identification of significant homology to the mouse *Alx4* coding sequence. *Alx4* is a homeobox gene, which has been shown to be involved in skull and limb development in mice (Qu et al. 1997*b,* 1998). A BLASTN search using the full mouse *Alx4* cDNA identified four, unordered, nonoverlapping fragments con-

tained within BAC 706A13 (fig. 2). Sequence comparison of *Alx4* and these BAC fragments showed extensive and significant homology, with the exception of certain specific regions as shown in figure 2. This finding indicates that the human orthologue of *Alx4* (*ALX4*) is contained within clone 706A13.

ALX4 primers were designed from BAC 706A13 sequence between base pair 151246 and base pair 151803, with the PCR product corresponding to the coding region from amino acid 1 to amino acid 156, and from sequence between base pair 25355 to base pair 25582, corresponding to the coding region from amino acid 382 to the last codon. These regions represent sequence present in the BAC but not corresponding to the mouse cDNA (fig. 2). Nested reverse transcriptase–PCR (RT-PCR) (SUPERSCRIPT First-Strand Synthesis System; Gibco) was performed to obtain the sequence residing between these primer pairs. β-actin was used as a positive control. The PCR products were sequenced and confirmed that *ALX4* is contained within clone 706A13, which allowed for the full length of human *ALX4* cDNA to be assembled and compared with the *Alx4* cDNA (fig. 2). The full-length *ALX4* cDNA is 1,583 bp and codes for a 410-amino-acid protein (start codon at base pair 105 and stop codon at base pair 1337). The exon/intron boundaries of *ALX4* were obtained by sequence comparison between the cDNA and the genomic BAC clones (table 1). The gene contains at least four exons (fig. 2). The nucleotide-sequence comparison showed 88% identity between the orthologues. Predicted amino acid composition (BLASTX) showed 80% identity and 83% similarity between the two predicted proteins (fig. 3).

The tissue-specific expression patterns were investigated, for both *Alx4* and *ALX4*. For all experiments, β-actin was used as a positive control. On the basis of northern blot analysis (Clontech), we confirmed that *Alx4* is not expressed in mouse heart, brain, spleen, lung, liver, skeletal muscle, kidney, or testis (data not shown) (Qu et al. 1997*a*); however, *Alx4* expression was evident in cultured mouse osteoblasts as assayed by RT-PCR. This expression pattern is consistent with that reported for the whole mount in situ of mouse embryos, in which expression is seen in both the craniofacial region and the anterior aspect of the developing limb buds (Qu et al. 1997*b*). Expression studies of human *ALX4* demonstrated no detectable transcript on a northern blot assay of human heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, uterus, small intestine, colon, or peripheral blood leukocytes (Clontech) (data not shown). However, nested RT-PCR with primers to *ALX4* on parietal bone, obtained from a newborn who died of arrhythmia, demonstrated expression (fig. 4). Thus, *ALX4* expression is likely to be restricted to bone, and we have demonstrated such expression specifically in parietal bone, the region of defect in PFM.

Table 1

***ALX4* Splice Junctions and Surrounding Sequence**

Splice Acceptor	Flanking Exon Sequence (5′→3′)	Splice Donor
	ATG AAT GCT...CCT GCT ACG	gtgagtgcacg
tcctccctcag	CTA AAG AGA...CGC GTG CAG	gtcagtgaggg
gcgtcttgtag	GTC TGG TTC...TAC GCC CAG	gtaagtcccgc
tcctctcccag	ATT CAG AAC...GCC ACA TGA	

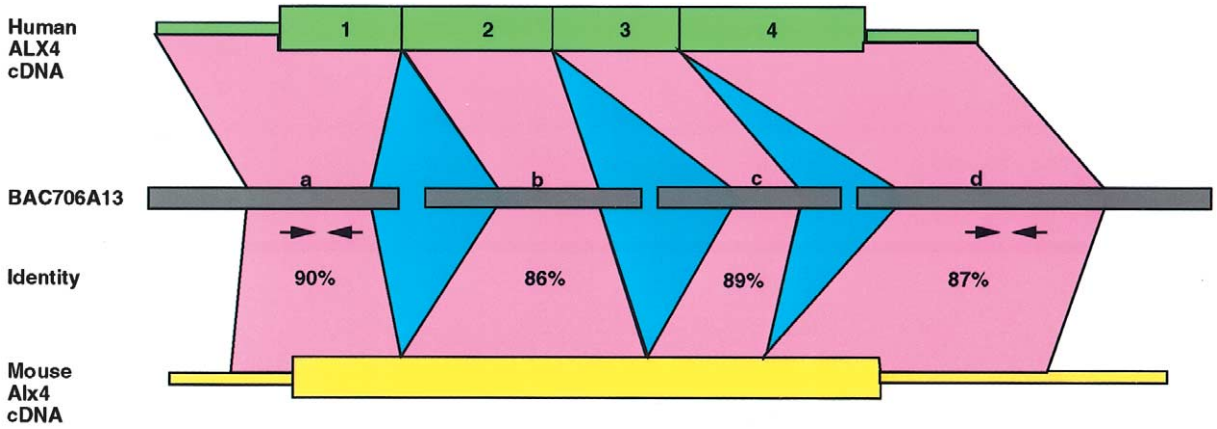

Figure 2 Diagrammatic representation of regions of homology between human *ALX4* sequence (*top*, *green*) and mouse *Alx4* (*bottom*, *yellow*). BAC 706A13 is indicated in gray (*middle*). Pink areas denote coding regions, and the percentages of identity between *ALX4* and *Alx4* are given in each area. Blue regions denote intronic sequence. Letters "a"–"d" indicate four different BAC 706A13 fragments used to assemble the human cDNA. Black arrows indicate position and direction of primers designed to obtain the human sequence, not present in the mouse cDNA (see text).

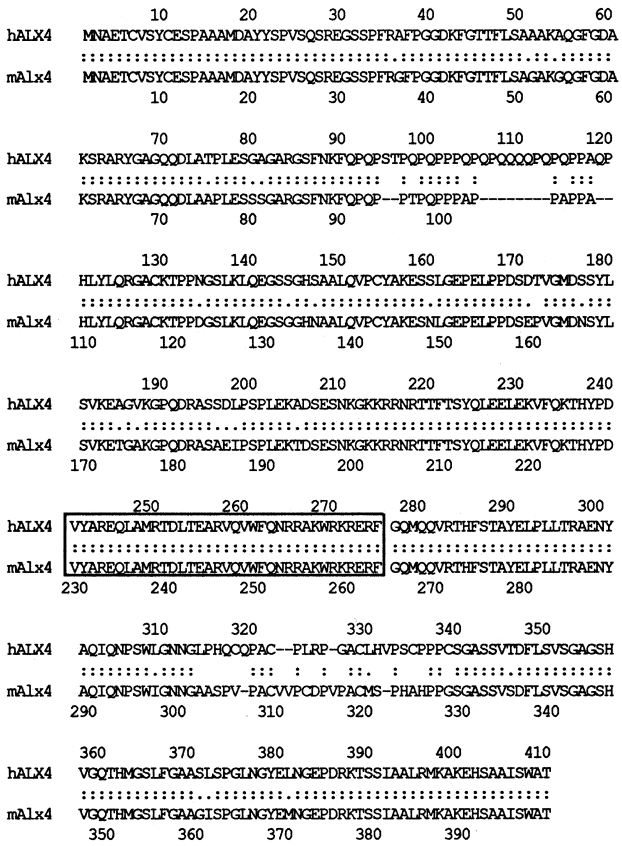

Figure 3 Comparison of predicted protein sequence of human ALX4 (hALX4) to mouse Alx4 (mAlx4). Identical amino acids are denoted by two vertical dots, similar amino acids are denoted by a single dot, and blank spaces denote nonidentity. Dashed lines denote regions of ALX4 not represented in Alx4. The boxed area indicates the location of the homeobox domain.

Herein, we report the identification of a human genomic clone containing a homeobox gene orthologous to mouse *Alx4*. This clone maps to proximal 11p (band p11.2), the region deleted in PSS. This clone was identified on the basis of a very short region of homology to *MSX2* and was shown to contain the sequence encoding the human *ALX4* gene. *ALX4* encodes a putative transcription factor that is among the paired-class homeoproteins (Qu et al. 1997*a*). This gene is similar to the *Drosophila* gene *aristaless,* which binds palindromic DNA sequences (5′-TAAT-3′) as either homodimers or as heterodimers with other family members (Tucker and Wisdom 1999). The murine orthologue, *Alx4,* is expressed at several sites during development, including the craniofacial and limb-bud mesenchyme (Qu et al. 1997*b*). Homozygous null mutants of *Alx4* show ventral body-wall defects, preaxial polydactyly, and decreased size of the parietal plate of the skull (Qu et al. 1997*b*). In these *Alx4*-mutant mice, ossification of the parietal bone did not extend over the superior aspect of the skull (Qu et al. 1997*b*). This is in contrast to what was seen in wild-type mice and may be due to a delay in bone development (Qu et al. 1997*b*). For the most part, the skeletal anomalies seen in the *Alx4*-mutant mice are abnormalities of either membranous ossification or endochondral ossification (Qu et al. 1997*b*). As is often seen when a correlation between a mouse mutation and the human condition is made, the analysis of *Alx4*-targeted mutations failed to reveal any phenotypic alteration in heterozygous mice. However, the penetrance of the polydactyly phenotype in heterozygotes appears to be dependent on the specific mouse strain (Qu et al. 1998). This same phenomenon may apply to the other aspects of the homozygous phenotype, including the craniofacial anomalies (Qu et al. 1998). The *Strong's luxoid* mutant, *1st,* recently has been shown to be the result of loss-of function mutations in *Alx4* (Qu et al. 1998). In addition to craniofacial defects, polydactyly, and other anomalies, the male mice have anomalies of the phallus (Forsthoefel 1963; Qu et al. 1998). This includes both reduction of the pubic bone and a shortened phallus that is not well separated from the scrotal swellings (Forsthoefel 1963). Forsthoefel (1963) speculated that this is the result of retarded growth of the underlying mesenchyme of the pubic bone and phallus. This might be the correlate of the micropenis seen in

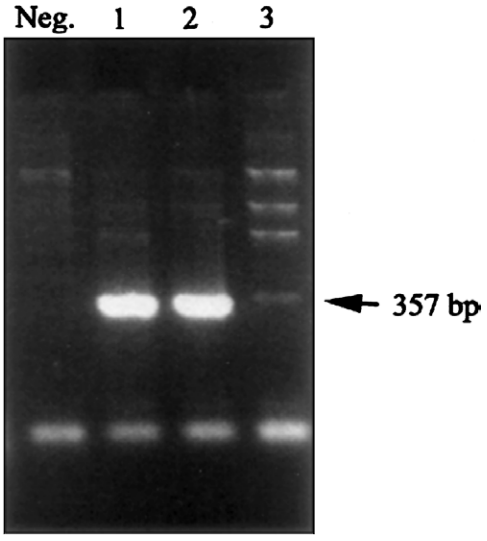

Figure 4 Results of nested RT-PCR with primers to *ALX4,* showing strong expression in parietal bone (*lanes 1 and 2*) and weak expression in a lymphoblast cell line of a normal individual (*lane 3*). Outside primers are as follows: ALX4OF, 5′-CTCCTCGCCTCCCCAAACTC-3′; and ALX4OR, 5′-CCTCCCTCCCAGCAGTCCAC-3′. Inside primers are as follows: ALX4IF, 5′-GGGGCCAGCAGCGTCACC-3′; and ALX4IR, 5′-CTTGGGGCGGCTGAAAGTG-3′.

males with PSS (Shaffer et al. 1993). Moreover, *Alx4* maps to mouse chromosome 2, a region of conserved synteny with human 11p12q12 (Mouse Genome Informatics). This predicts that human *ALX4*—and any syndromes that may result from mutation at this locus—would likely map to the pericentromeric region of chromosome 11 (Qu et al. 1998; also see Mouse Genome Informatics). Given that the mouse *Alx4* is involved in craniofacial development and that mice that are mutant for this gene show a decrease in the size of the parietal bone (Qu et al. 1997*b*), *ALX4* is a good candidate for PFM in cases of del(11)(p11.2p12).

Elsewhere, we have described a CGDS due to a microdeletion of the proximal short arm of chromosome 11 (Shaffer et al. 1993; Potocki and Shaffer 1996). Some of the features of this CGDS can be found as isolated Mendelian traits—specifically, the multiple exostoses that are caused by haploinsufficiency for the *EXT2* gene (Stickens et al. 1996; Wuyts et al. 1996; Ligon et al. 1998). PFM is another clinical finding of this CGDS. There are several lines of evidence indicating that human *ALX4* is responsible for the PFM seen in patients with the 11p11.2-deletion syndrome. First, the murine orthologue, *Alx4,* is expressed in craniofacial mesenchyme during embryogenesis and is involved in skull development. Second, mutations in this gene in mice cause a decrease in the size of the parietal bone, among other craniofacial and developmental features. Third, as demonstrated by the present report, a gene showing significant homology with mouse *Alx4* maps to proximal 11p and is deleted in two patients with craniofacial anomalies, including parietal foramina. Fourth, *ALX4* and *Alx4* appear to be expressed exclusively in bone, and *ALX4* has been demonstrated to be expressed in parietal bone. Finally, *ALX4* has been identified through homology searches using *MXS2,* a gene that encodes a homeobox protein and that, by mutation analysis, has been shown to cause parietal foramina in some families.

Further functional studies and identification of isolated mutations in patients with autosomal dominant forms of PFM will be required in order to allow characterization of the role of *ALX4* in the PFM defect seen in 11p-deletion patients. Patients with the PSS deletion show multiple exostoses caused by haploinsufficiency of *EXT2,* PFM, and mental retardation. It is likely that *ALX4* is indeed the cause of PFM in this syndrome. Complete characterization of the molecular basis of PSS awaits the identification of a mental retardation locus within 11p11.2.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

BLAST, http://www.ncbi.nlm.nih.gov/blast
GenBank, http://www.ncbi.nlm.nih.gov/Genbank (for BAC 706A13 [accession number AC019143] and human ALX4 cDNA sequence [accession number AF294629])
Mouse Genome Informatics, http://www.informatics.jax.org
Online Mendelian Inheritance in Man (OMIM), http://www3.ncbi.nlm.nih.gov/Omim (for multiple exostoses [MIM 133701], PFM [MIM 168500], and PSS [MIM 601224])

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