Mutations in mouse Aristaless-like4 cause Strong’s luxoid polydactyly

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SUMMARY

Mutations that affect vertebrate limb development provide insight into pattern formation, evolutionary biology and human birth defects. Patterning of the limb axes depends on several interacting signaling centers; one of these, the zone of polarizing activity (ZPA), comprises a group of mesenchymal cells along the posterior aspect of the limb bud that express sonic hedgehog (Shh) and plays a key role in patterning the anterior-posterior (AP) axis. The mechanisms by which the ZPA and Shh expression are confined to the posterior aspect of the limb bud mesenchyme are not well understood. The polydactylous mouse mutant Strong’s luxoid (lst) exhibits an ectopic anterior ZPA and expression of Shh that results in the formation of extra anterior digits. Here we describe a new chlorambucil-induced deletion allele, lstAlb, that uncovers the lst locus. Integration of the lst genetic and physical maps suggested the mouse Aristaless-like4 (Alx4) gene, which encodes a paired-type homeodomain protein that plays a role in limb patterning, as a strong molecular candidate for the Strong’s luxoid gene. In genetic crosses, the three lst mutant alleles fail to complement an Alx4 gene-targeted allele. Molecular and biochemical characterization of the three lst alleles reveal mutations of the Alx4 gene that result in loss of function. Alx4 haploinsufficiency and the importance of strain-specific modifiers leading to polydactyly are indicative of a critical threshold requirement for Alx4 in a genetic program operating to restrict polarizing activity and Shh expression in the anterior mesenchyme of the limb bud, and suggest that mutations in Alx4 may also underlie human polydactyly.

Key words: Limb bud, Pattern formation, aristaless, Paired-type homeodomain, Zone of polarizing activity, sonic hedgehog, Evolution, Mouse

INTRODUCTION

The development of the vertebrate limb serves as an instructive paradigm for investigations of the genetic and cellular programs that specify the formation of patterned structures (Hinchliffe and Johnson, 1980). Classical embryological manipulations in amphibians and avian embryos have identified three morphogenetic signaling centers that act to coordinate the patterning and outgrowth of the limb along the dorsal-ventral (DV), proximal-distal (PD) and anterior-posterior (AP) axes: respectively, the dorsal ectoderm, the apical ectodermal ridge (AER), a group of columnar epithelial cells localized to the dorsal-ventral border of the limb bud distal tip, and the zone of polarizing activity (ZPA), a group of mesenchymal cells localized to the posterior margin of the limb bud mesoderm (Tickle and Eichele, 1994). The patterning functions of these signaling centers have been ascribed to their expression of specific signaling molecules. Specifically, Wnt-7a expressed in the dorsal ectoderm, fibroblast growth factors (FGFs) expressed in the AER and Shh expressed in the ZPA have been shown to be major determinants of the patterning functions of these signaling centers. In addition, recent molecular analysis has revealed the existence of positive feedback loops that serve to coordinate the growth and patterning of the limb bud in three dimensions over embryological time (Johnson and Tabin, 1997; Martin, 1998). The nature of the genetic hierarchy that acts to deploy and/or restrict these signaling centers within the limb bud remains an important area of inquiry.

Investigation of existing mutants identified by virtue of their phenotypes provides an opportunity to identify genes that direct limb patterning by combining experimental embryology with the power of molecular genetics and gene discovery (Niswander, 1997). There are a large number of spontaneous and induced mouse and chick mutants that exhibit as part of their phenotype a dysmorphic limb (Johnson, 1986). The study of these phenotypically identified mutants complements the approach of gene targeting in mice by presenting opportunities to both identify novel genes and to uncover allelic variation of proteins of a known class, thereby providing insight into structure-function relationships (Bedell et al., 1997; Eppig, 1997). The set of existing mutants that exhibit preaxial (anterior) polydactyly provide opportunities to gain insight into the genetic control...
of AP patterning and the restriction of polarizing activity and Shh expression.

A number of mouse mutants with preaxial polydactyly exhibit ectopic Shh expression in the anterior mesenchyme of the limb bud during development; these include Extra toes (Xt), Strong’s luxoid (lst), luicate, X-linked polydactyly, RIm4 and Hemimelic extra toes (Buscher and Ruther, 1998; Chan et al., 1995; Eppich, 1997; Masuya et al., 1995, 1997). Among these, only the Xt mutant, which is due to mutational inactivation of the GlI3 zinc-finger transcription factor gene, has been molecularly defined (Hui and Joyner, 1993; Schimmang et al., 1992; Vortkamp et al., 1992). Further evidence for a role for Gli3 in patterning the limb comes from the analysis of three human dysmorphic syndromes, Greig-cephalopolysyndactyly, Pallister-Hall and postaxial polydactyly type A, which define an allelic series of mutations in the Gli3 gene and which serve to illustrate phenotypic heterogeneity associated with the mutational spectra within a single gene (Biesecker, 1997; Vortkamp et al., 1991). The other phenotypic limb mutants define additional loci that may function together with, or independently of, Gli3 to restrict ZPA formation and Shh expression. We have focused on an identification of the lst gene to gain a better understanding of the molecular basis by which this locus acts to restrict the localization of the ZPA.

The Strong’s luxoid mutant, lst, was first noted in 1946 as a polydactylosus mouse (originally called the Springfieldville mouse) that arose in the course of 3-methylcholanthrene mutagenesis experiments performed by Leonel Strong in his investigations of chemical induction of mammary tumors (Strong, 1961; Strong and Hardy, 1956). A second allele, designated lst2, arose spontaneously at The Jackson Laboratory (P. W. Lane, personal communication). Both lst alleles exhibit semidominant inheritance, such that heterozygotes have preaxial polydactyly that is most often manifest as a broadened hallux or a single additional anterior triphalangeal digit on the hindlimb (Forsthoefel, 1962; P. W. Lane, personal communication). However, selection for modifiers has shown that the penetrance and expressivity of the lst limb phenotype is sensitive to genetic background (Forsthoefel, 1962, 1968; P. W. Lane, personal communication). In contrast, homozygous mutant mice have extensive preaxial polydactyly of all four limbs (up to nine digits), hemimelia (absence) of the tibia, craniofacial defects, dorsal alopecia, weakness of the ventral body wall and, in males, anomalies of the phallus and cryptorchidism (Forsthoefel, 1959, 1962, 1963). The lst limb phenotype is first morphologically evident at embryonic day 11 p.c. as an enlarged preaxial extension of the AER and overall broadened AP axis (Chan et al., 1995; Forsthoefel, 1963; Masuya et al., 1995). Heterozygous transplantation of lst anterior mesenchyme into the anterior aspect of recipient chick limb buds has demonstrated that the broadening across the AP axis is associated with the presence of an ectopic anterior ZPA (Chan et al., 1995). Consistent with this observation, molecular markers of anterior ZPA formation and downstream events, including ectopic Shh, ptc and 5’ Hex-d gene expression in the mesoderm, as well as a concomitant anterior extension of an Fgf4 expression domain in the AER, are detected in the limb buds of lst embryos (Chan et al., 1995; Goodrich et al., 1996; Masuya et al., 1997; Platt et al., 1997).

In addition to phenotypic analysis, genetic interactions of lst with two preaxial polydactyly mutants, Carter’s luxate (lx), Green’s luxoid (lu) and one oligosyndactyly mutant, formin limb deformity (Fmn1ld) have been investigated (Forsthoefel, 1959, 1962; Vogt and Leder, 1996). Analysis of mice segregating single, double or triple combinations of lst, lu and lx led to the interpretation that their interactions result in an additive effect on the phenotype and suggest that the lst, lu and lx genes may act in independent pathways (Forsthoefel, 1959, 1962). In outcrosses, Fmn1ld alleles were shown to suppress the lst polydactylyous phenotype (Vogt and Leder, 1996). Despite the detailed developmental and genetic analysis of lst mice, mechanistic interpretations of the defects in lst mutants and its genetic interactions have been limited by both the fact that the lst mutation is semidominant, making it difficult to discern whether it represents a gain-of-function or loss-of-function mutation, and by the lack of knowledge about the molecular identity of the affected gene (Chan et al., 1995; Vogt and Leder, 1996).

Here we describe the identification of a chlorambucil-induced deletion that uncovers a new lst allele, lstAlb. Genetic and physical mapping of the deleted interval identified Alx4 as a candidate gene for the lst mutation. Consistent with the possibility of alleleism between Alx4 and lst, mice homozygous for a targeted mutation of Alx4 (Alx4tm1qw) display preaxial polydactyly and ectopic Shh expression, as well as craniofacial and ventral body wall defects (Qu et al., 1997). In addition, we provide genetic and molecular evidence that demonstrate that Alx4 is the target of mutation in lst mice. Additional genetic evidence indicates that other genes interact with Alx4 to repress ZPA formation in the anterior limb bud mesenchyme in a way that strongly modifies the polydactyly observed in heterozygotes. Molecular and biochemical characterization provide structure-function information that demonstrates that the Strong’s luxoid phenotype is the result of loss-of-function mutations in Alx4.

MATERIALS AND METHODS

Mice

Procedures for chlorambucil (CHL) mutagenesis have been previously described (Flaherty et al., 1992). The mutation was observed in the progeny of a CHL-mutagenized C3H/HeJ (C3H) male, which was mated to a wild-type C57BL/6 (B6) female. The lstAlb mutation is, therefore, on a parental C3H chromosome. The stock has been maintained as a closed inbred colony. The lst mutant, which is due to mutational inactivation of Gli3, was first noted in 1946 as a polydactylosus mutant, lu, and one oligosyndactyly mutant, formin limb deformity (Fmn1ld) have been investigated (Forsthoefel, 1959, 1962; Vogt and Leder, 1996). Analysis of mice segregating single, double or triple combinations of lst, lu and lx led to the interpretation that their interactions result in an additive effect on the phenotype and suggest that the lst, lu and lx genes may act in independent pathways (Forsthoefel, 1959, 1962). In outcrosses, Fmn1ld alleles were shown to suppress the lst polydactylyous phenotype (Vogt and Leder, 1996). Despite the detailed developmental and genetic analysis of lst mice, mechanistic interpretations of the defects in lst mutants and its genetic interactions have been limited by both the fact that the lst mutation is semidominant, making it difficult to discern whether it represents a gain-of-function or loss-of-function mutation, and by the lack of knowledge about the molecular identity of the affected gene (Chan et al., 1995; Vogt and Leder, 1996).

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SSLP analysis

Genomic DNA from tail clippings was extracted using a standard
proteinase K/phenol-chloroform/EtOH precipitation procedure (Qu et al., 1997). These DNAs were used as PCR templates to amplify simple sequence length polymorphisms (SSLPs); primer pairs for specific MIT SSLP markers were obtained from Research Genetics (Huntsville, AL). PCR was carried out essentially as described (Dietrich et al., 1996).

DNA analysis
For the molecular characterization of the lst<sup>Alb</sup> allele, DNA was isolated from newborns from a Alx4<sup>tm1qw</sup>/+ × lst<sup>Alb</sup>/+ cross and Southern blotting was performed as described (Qu et al., 1997). For the molecular characterization of the lst and lst<sup>s</sup> alleles, the protein-coding region of Alx4 was examined by PCR amplification and sequencing of products from first-strand cDNA and genomic DNA templates. RT-PCR was performed on first-strand cDNA synthesized from total RNA isolated from phenotypically homozygous mutant E11.5 embryos derived from Alx4<sup>tm1qw</sup>/+ × lst<sup>s</sup>/+ crosses. The use of primers that flanked the neo insertion site in the Alx4<sup>tm1qw</sup> targeted allele restricted amplification to the lst<sup>s</sup> allele (Qu et al., 1997). To obtain additional Alx4 genomic sequence a 129 genomic library in BACs (Research Genetics) was screened with an Alx4 cDNA probe (Qu et al., 1997). Hybridization-positive BACs were either sequenced directly or following subcloning to obtain intron DNA sequence (Shizuya et al., 1992). Primers and details on Alx4-positive BACs are available upon request. PCR was performed on genomic DNA isolated from phenotypically homozygous lst/slst and lst<s>/lst<s> mutants. The following primer pairs were used:

coding region of exon 1:

forward primer: 5¢ CGTCGCCCCGCGACGGCCA 3¢ (exon 5¢ of AUG)

reverse primer: 5¢ GGTCACTGACGTGACTC 3¢ (first intron sequence)

exon 2:

forward primer: 5¢ GGTGAGGAGGGACAGAC 3¢ (first intron sequence)

reverse primer: 5¢ GGTACATTGAGTGTGCTTCC 3¢ (second intron sequence)

exon 3:

forward primer: 5¢ CCTGCATTGGGTCAGACCTGC 3¢ (second intron sequence)

reverse primer: 5¢ CTGCGGACAGTGAAGGGAGA 3¢ (third intron sequence)

exon 4:

forward primer: 5¢ TCTCCCTCTACTGCTTCCAG 3¢ (third intron sequence)

reverse primer: 5¢ GGGGTTGAGGGACAGAGA 3¢ (exon sequence 3¢ of stop codon)

RT-PCR:

forward primer: 5¢ CCAGAGGTGGCTCCTAGACT CTG 3¢ (exon 2 sequence 5¢ of paired-homeodomain)

reverse primer: 5¢ GGAGTTGAGGGAGGCTTC 3¢ (exon 4 sequence 5¢ of stop codon).

DNA-binding assays
For in vitro DNA-binding studies, peptides containing the Alx-4 homeodomain (residues 185-268) or the corresponding R206Q mutant were expressed with a (His)<sub>6</sub> tag in E. coli BL21 and recombinant fusion proteins were purified on Ni<sup>2+</sup>-NTA agarose. Gel shift reactions contained 75 mM NaCl, 15 mM Tris pH 7.5, 1.5 mM EDTA, 1.5 mM DTT, 7.5% glycerol, 0.3% NP-40, 4 mM spermine, 4 mM spermidine, 0.8 μg poly(dIdC), and the appropriate [³²P]-labelled probe. For gel shifts using the P3 probe, tenfold more protein was used for each condition. Reactions were incubated on ice without the probe for 5 minutes, the probe was then added and samples were incubated at room temperature for 20 minutes. Samples were then resolved on 5% polyacrylamide gels in 0.5X TBE at 150 volts for 90 minutes. For competition experiments, a 50-fold excess of the appropriate unlabelled competitor was added during the preincubation. To test for the ability of R206Q to inhibit the DNA-binding activity of the wild-type Alx4 homeodomain, increasing amounts of the R206Q homeodomain (up to 32 ng per reaction) were mixed with a fixed amount of wild-type homeodomain (3.2 ng) in gel shift reactions using the P3 probe. DNA-binding activity from proteins expressed in cell culture used 4 μg of nuclear extract from transiently transfected 293 cells (Andrews and Faller, 1991).

Reporter gene studies
For transient expression and reporter activation assays, cDNAs encoding either Alx4 wild-type or R206Q versions were introduced into the mammalian expression vector pCMX (Umesono et al., 1991). Reporter constructs contained three tandem repeats of either the P3, P3 mutant or P1/2 sequences upstream of the chloramphenicol acetyl transferase (CAT) gene driven by the basal promoter of the adenovirus E1b gene. Human 293 cells were transiently transfected using Lipofectamine (Gibco) with 100 ng of expression construct, 500 ng of the indicated reporter construct and 1.0 μg of a β-galactosidase expression plasmid (pCH110) that served as a control for transfection efficiency. 40 hours after transfection, cell lysates were prepared and CAT activity was determined. For dominant negative experiments, 100 ng of pCMX-Alx4 (wild type) was mixed with increasing amounts, up to 500 ng, of pCMX-Alx4 R206Q. Nuclear extracts prepared from cells transiently transfected with the appropriate Alx4 expression plasmid were analyzed for expression of Alx4 protein by western blotting with an Alx4-specific antibody (Qu et al., 1997); detection was by enhanced chemiluminescence.

RESULTS

A chlorambucil-induced deletion on chromosome 2 uncovers Strong's luxoid

As the chemical and spontaneous origins of the lst and lst<sup>s</sup> alleles, respectively, suggested they were likely to harbor subtle mutations, we sought to identify additional alleles associated with chromosomal alterations that would facilitate identification of the lst gene. The chemotherapeutic drug chlorambucil (CHL) has been found to induce deletions and complex rearrangements at a high frequency (7.5×10<sup>-4</sup> per locus per gamete) in male postmeiotic germ cells (Russel et al., 1989; Rinchik et al., 1990). CHL-induced deletions are useful because they often result in complete loss-of-function alleles and are effective genetic tools for mapping, complementation analysis and positional cloning (Rinchik et al., 1993). One of us (L. A. F.) has been conducting a CHL-based mutagenesis screen for developmental mutants (Flaherty et al., 1992).

Progeny of a CHL-mutagenized C3H male were recovered that exhibited preaxial polydactyly of the hindlimb. The mode of inheritance of the polydactyly was indicative of a dominant mutation with incomplete penetrance. As a starting point for tests of allelism, the CHL-induced polydactyly mutation was first examined for linkage to mouse chromosomes 2 and 13 (locations of the lst and Xt mutations, respectively) by following the segregation of polydactyly with the SSLP markers D2Mit4 and D13Mit57. These preliminary mapping results excluded linkage to chromosome 13 and were consistent with linkage to chromosome 2, suggesting possible allelism with lst (n=1/15 animals recombinant with D2Mit4, 7/15 recombinant with D13Mit57).
Fig. 1. lstAlb fails to complement lst. (A) 1-month-old lstAlb/lst mouse exhibiting dorsal alopecia and polydactyly. Arrowhead highlights the dorsal-ventral boundary of alopecia (Forsthoefel et al., 1966). (B) Skeleton of an lstAlb/lst neonate stained with alizarin red and alcian blue to visualize bone and cartilage, respectively. (C,D) Close-up of forelimb (C) and hindlimb (D) of an lstAlb/lst neonate shown in B. lst homozygotes and the lstAlb/lst transheterozygotes display preaxial polydactyly of all four limbs and hemimelia of the tibia. (E) Genetic mapping data from the 163-animal backcross segregating lst (Vogt and Leder, 1996). Numbers refer to MIT SSLP markers on chromosome 2 (D2Mit**+) (Dietrich et al., 1996). The lstAlb deletion mapping data (see F below) was integrated with this cross. Four of the ten deleted SSLPs (indicated in red) were informative in the lst backcross, and these markers appear in red on the lst genetic map. Of the four deleted markers, three failed to recombine with lst and the fourth, D2Mit161, mapped 0.6 cM distal to the mutation. (F) The minimal CHL-associated lstAlb deletion interval. SSLP markers are indicated beneath physical contigs identified on the MIT Mouse Physical Map (Release 15, October 1997; http://www-genome.wi.mit.edu/cgi-bin/mouse/index). Markers scored in the lst genetic map are in green (centromeric) or yellow (telomeric). Markers indicated in black print are not deleted in lstAlb animals. The SSLPs D2Mit98, D2Mit101, D2Mit102, D2Mit163, D2Mit207, D2Mit442, D2Mit438, D2Mit474 and D2Mit476 were also found to not be deleted in lstAlb, but have not yet been placed on the physical map. SSLP markers indicated in red were found to be deleted in lstAlb (D2Mit221, D2Mit129, D2Mit386, D2Mit351, D2Mit350, D2Mit41, D2Mit253, D2Mit439, D2Mit15 and D2Mit161). The deletion of these markers was confirmed by genotyping the mutant offspring of an lstAlb×AJJ cross (data not shown). Nine of these SSLPs fall on a doubly-linked YAC contig; one marker, D2Mit161, was deleted but has not yet been placed on the physical map. One marker, D2Mit130, was not informative in the C3H and B6 backgrounds and therefore could not be scored for deletion. (G) SSLPs from the lst cross were used to define a candidate interval on the Jackson Laboratory’s (B6×SPRET)×SPRET community mapping panel (Rowe et al., 1994). The SSLP D2Mit130 does not recombine with lst. Several potential lst candidates fall within the defined interval: Xrf79, D2Xrf79, EST with homology to yeast CBF5; Wsu101e, anonymous cDNA from ectoplacental cone-specific library; Alx4; Xrf428, D2Xrf428, EST with homology to yeast CDC19; Xrf174, D2Xrf174, EST with homology to yeast YAK1; Xrf212, D2Xrf212, EST with homology to yeast PDA2 (Eppig, 1997). The SSLP D2Mit15, deleted in lstAlb had previously been mapped in the BSS cross to the same location as D2Mit14 (Peichel et al., 1996).

To directly address allelism of the CHL-induced mutation with lst, we used the difference in the severity of the lst/+ and lst/lst phenotypes as an assay in complementation tests. The pleiotropic phenotype observed in lst homozygotes has not been observed in either lst/+ or CHL-induced heterozygotes. Mice carrying the CHL-induced mutation were mated to lst/+ heterozygotes (both parents displaying only hindlimb polydactyly). The CHL-induced Albany mutation failed to complement lst, as indicated by the presence of the severe lst/lst phenotype in ~25% of the offspring (Fig. 1A-D). Based on this failure to complement and additional data described below, we refer to the CHL-induced mutation as lstAlb.

Deletion analysis and integration of mapping data

Although the lst homozygous phenotype was observed in transheterozygous animals, we have not observed the pleiotropic phenotype of profound skeletal and ventral body wall defects and dorsal alopecia in progeny of lstAlb/+×lstAlb/+ intercrosses. Since CHL is known to produce deletions, this suggested that lstAlb might be encompassed by a deletion sizable enough to uncover multiple loci, at least one of which may be required for viability (Rinchik et al., 1993). To determine if lstAlb was, indeed, a deletion allele, we took advantage of the high density of SSLP markers in the lst region. We examined the mutant F1 progeny of a lstAlb×B6 cross for absence of the C3H allele of SSLPs in the lst region. Two F1 mutants from different lstAlb parents were genotyped at each of the 62 SSLPs informative between B6 and C3H in a 3.3 cM interval flanking lst on the MIT F2 Intercross Map (Dietrich et al., 1996). By this methodology, 10 SSLP markers were found to be deleted in lstAlb (Fig. 1F). Integration of the genetic data with the MIT Mouse Physical Map demonstrated that the deleted markers span a large doubly linked YAC contig (Fig. 1F).
To anchor the lst genetic maps, we examined the SSLP markers deleted in lst<sup>hbb</sup> in a backcross segregating the original lst allele (Vogt and Leder, 1996) and the Jackson Laboratory Community Interspecific Backcross panel (Rowe et al., 1994) (Fig. 1E-G). First, the cohort of deleted markers was tested for polymorphism in the lst cross. Three informative markers (D2Mit386, D2Mit439 and D2Mit253) did not recombine with lst, and a fourth, D2Mit161, was placed 0.6 cM distal to lst. (Fig. 1E). We next anchored the lst cross to The Jackson Laboratory’s (B6×SPRET)xSPRET (BSS) community backcross mapping panel (Rowe et al., 1994), by scoring the relevant BSS recombinants at the SSLPs D2Mit14 (proximal to lst), D2Mit130 (non-recombinant with lst) and D2Mit43 (distal to lst). This integrated map defines a minimal lst region and suggested Alx4 as a candidate gene (Fig. 1G).

**lst and Alx4 fail to complement**

While mapping of the lst mutation was in progress, functional characterization also suggested Alx4 as a candidate gene for the lst mutation. Specifically, animals homozygous for a targeted mutation in the Alx4 gene, on an inbred 129 background, have preaxial polydactyly and ventral body wall defects, reminiscent of the lst homozygous phenotype. However, in contrast to lst mutants, Alx<sup>tm1qw</sup> homozygotes have only a single extra preaxial digit, and heterozygotes are unaffected (Qu et al., 1997). To test the possibility of allelism between lst alleles and Alx4, outcrosses between heterozygotes were performed. In each case, non-complementation, scored as the presence of profound polydactyly on all four limbs (Fig. 2) and ventral body wall defects (data not shown), was observed in the progeny. These results also led us to reassess the Alx<sup>tm1qw</sup> allele, which unlike the three lst alleles, does not exhibit semidominant hindlimb polydactyly (Qu et al., 1997).

The Alx<sup>tm1qw</sup> allele has been maintained on an inbred 129 genetic background; however, one generation of outcrossing of Alx<sup>tm1qw</sup> heterozygotes to other genetic backgrounds (e.g. C57BL/6J) produced Alx<sup>tm1qw</sup> heterozygous progeny with hindlimb preaxial polydactyly similar to that previously observed for lst heterozygotes (Fig. 3, compare to Fig. 2). Thus, as previously documented for the lst alleles (Forsthoefel, 1968), the penetrance of the Alx<sup>tm1qw</sup> heterozygous hindlimb polydactyly phenotype is sensitive to

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**Fig. 2.** Genetic interaction between lst alleles and Alx<sup>tm1qw</sup>. Skeletal stains of forelimbs (fore) and hindlimbs (hind) of progeny are shown with the genotypes indicated to the left. The appearance of fore- and hindlimb preaxial polydactyly and variably expressive radial and/or tibial hemimelia is indicative of the phenotype seen in homozygous lst and Alx<sup>tm1qw</sup> mice (Chan et al., 1995; Forsthoefel, 1962, 1968; Qu et al., 1997).

**Fig. 3.** Genetic background modifies the Alx<sup>tm1qw</sup> heterozygous phenotype. (A,B) Skeletal stains of the polydactylous hindlimbs of a (C57BL6J +/+ × 129/SvEvTac Alx<sup>tm1qw</sup> /+) F<sub>1</sub> progeny displaying preaxial polydactyly.
strain-specific modifying loci. The linkage of *lst* and *Alx4* and their failure to complement provide strong genetic evidence that the *lst* alleles harbor loss-of-function mutations in *Alx4* and prompted molecular analysis of the *Alx4* gene in *lst* mice.

**Alx4 is mutated in all *lst* alleles**

We looked for molecular alterations of *Alx4* in the three *lst* alleles (Fig. 4). In the 3-methylcholanthrene-associated *lst* allele, the coding sequence of *Alx4* has a single base change in codon 206 (CGA>CAA, R206Q), resulting in the substitution of glutamine for arginine at the fifth residue of the homeodomain (Fig. 4B,C). Sequencing of the *Alx4*-coding region in the spontaneously arising *lst* allele revealed a 16 base pair deletion within the paired-type homeodomain. This deletion disrupts the DNA recognition helix and results in a frame-shift of the downstream protein-coding sequence (Fig. 4B). Genomic Southern analysis of the CHL-associated *lstAlb* allele revealed that the *Alx4* gene is deleted (Fig. 4D). Thus, each of the three independently generated *lst* alleles have mutations in *Alx4*. Hereafter, we refer to the *lst* alleles as *Alx4*<sup>lstJ</sup>, *Alx4*<sup>lstAlb</sup> and *Alx4*<sup>lstAlb</sup>.

**The *lst* alleles represent *Alx4* loss-of-function mutations**

Previous models to explain the abnormal limb patterning in *lst* mice could not distinguish whether the *lst* mutations represented gain- or loss-of-function alleles (Chan et al., 1995; Vogt and Leder, 1996). The *Alx4*<sup>lstAlb</sup> gene deletion indicates that a loss of *Alx4* function can underlie the *lst*-associated polydactylosis phenotype. Because of their large effects on protein structure, the *Alx4*<sup>lst</sup> and the *Alx4*<sup>tm1qw</sup> alleles are also likely to result in a loss of function (Qu et al., 1997). In contrast, the *Alx4*<sup>lstR206Q</sup> mutation could represent either a loss-of-function or a dominant negative allele, and its further characterization would provide additional insight into structure-function relationships of the paired-type homeodomain. RNA expression of *Alx4*<sup>lst</sup> in mouse embryos at day 11 of gestation is indistinguishable from wild type, suggesting that the effect of the *Alx4*<sup>lst</sup> mutation would be post-transcriptional (B. Prabhakaran and T. F. V., data not shown; Qu et al., 1997). Therefore, we examined the effect of the *R206Q* mutation on the biochemical function of *Alx4* in more detail.

Both biochemical and crystallographic analyses have shown that the paired-type homeodomain binds cooperatively as a homodimer to specific palindromic DNA sequences (Wilson et al., 1993, 1995). The crystal structure of the paired-type homeodomain dimer bound to DNA shows that arginine 5 of the homeodomain (the residue altered in the *R206Q* mutant) makes specific DNA contacts in the minor groove (Fig. 4C) (Wilson et al., 1995). Thus, the substitution of the neutral glutamine for arginine at the fifth residue of the codon 206 (CGA>CAA, R206Q), resulting in the substitution of glutamine for arginine at the fifth residue of the homeodomain (Fig. 4B,C). Sequencing of the *Alx4*-coding region in the spontaneously arising *lst* allele revealed a 16 base pair deletion within the paired-type homeodomain. This deletion disrupts the DNA recognition helix and results in a frame-shift of the downstream protein-coding sequence (Fig. 4B). Genomic Southern analysis of the CHL-associated *lstAlb* allele revealed that the *Alx4* gene is deleted (Fig. 4D). Thus, each of the three independently generated *lst* alleles have mutations in *Alx4*. Hereafter, we refer to the *lst* alleles as *Alx4*<sup>lstJ</sup>, *Alx4*<sup>lstAlb</sup> and *Alx4*<sup>lstAlb</sup>.

The *lst* alleles represent *Alx4* loss-of-function mutations

*Strong’s luxoid* mice exhibit preaxial polydactyly, which is preceded during development by the formation of an ectopic ZPA and expression of *Shh* in the anterior mesenchyme of the limb bud. Our genetic, molecular and functional analyses demonstrate that loss-of-function mutations in *Alx4* cause the phenotype of *Strong’s luxoid* polydactylosis mutants.

**DISCUSSION**

*Strong’s luxoid* mice exhibit preaxial polydactyly, which is preceded during development by the formation of an ectopic ZPA and expression of *Shh* in the anterior mesenchyme of the limb bud. Our genetic, molecular and functional analyses demonstrate that loss-of-function mutations in *Alx4* cause the phenotype of *Strong’s luxoid* polydactylosis mutants.

**Chlorambucil-induced chromosomal deletion uncovers *lst***

Comparative genetic mapping and the identification of a chromosomal deletion that uncovered *lst* provided a genetic and physical framework to assess candidate genes and served to focus attention on *Alx4*. The deletion uncovering *Alx4*<sup>lstAlb</sup> is certain to uncover additional loci, one or more of which is likely to account for our failure to observe viable progeny homozygous for the deletion. The deletion spans a genetic distance of 2-3 cM, removes 10 SSLPs and is spread across multiple YACs on the MIT Physical Map, indicating that its size may be measured in the hundreds of kilobases. At this level of inspection, the CHL-induced deletion appears to be an interstitial deletion and not a complex rearrangement. The size of the deletion falls within the range (0.5 cM-15 cM) previously indicated by analysis of CHL-induced mutations centered around the *albino* locus on mouse chromosome 7 and serves to highlight the utility of integrating CHL-mutagenesis with the increasingly dense mouse genetic and physical maps (Bedell et al., 1997; Dietrich et al., 1996; Rinchik et al., 1993).

**Polydactylosis results from *Alx4* loss of function**

Prior studies of *lst* have been unable to distinguish whether the mutations represented a loss or gain of function. In conjunction with the gene-targeted disruption of the *Alx4* homeodomain, the present identification of the *Alx4*<sup>lstAlb</sup> chromosomal deletion and the *Alx4*<sup>lstJ</sup> allele’s 16 bp deletion of the homeodomain demonstrate that the phenotypes associated with
Alx4 mutations causes lst polydactyly

lst can be caused by loss-of-function mutations. The Alx4<sup>lst</sup> single amino acid R206Q substitution is also consistent with a loss of function. A number of lines of evidence lead us to conclude the R206Q mutation results in loss of function. Arginine 5 of the amino terminal arm of the homeodomain is one of the most conserved residues in the homeodomain (98% of 346 homeodomains surveyed, an exception is the divergent PrH/Hex proteins which have a glutamine at amino terminal position 5 and an arginine at position 7) (Burglin, 1994). A high resolution crystallographic analysis of the paired-type homeodomain bound as a dimer to the palindromic P3 element shows that arginine 5 is insinuated into the minor groove of the DNA, where it physically interacts with both strands (Wilson et al., 1993, 1995). Consistent with the evolutionary invariance and the structural analysis, our biochemical evidence demonstrates that the R206Q mutant behaves as a functional null in terms of DNA binding and transcriptional regulation. Furthermore, our observation that the R206Q homeodomain does not interact with DNA as a monomer corroborates the importance of arginine 5 in DNA binding and predicts that mutation of arginine in this position would result in a loss of function in many different classes of homeodomain proteins.

Consistent with our assignment of R206Q as a loss-of-function mutation, we were unable to find any evidence that the R206Q protein could inhibit either the DNA-binding or transcriptional properties of the wild-type protein. Although all biochemical properties of Alx4 that are dependent on DNA binding are absent in the Alx4 R206Q mutant, it remains possible the R206Q protein may exhibit a non-DNA-binding-dependent activity in some contexts (Benassayag et al., 1997). The biochemical profile of Alx4<sup>lst</sup> as a loss-of-function mutation is consistent with our genetic evidence that the in vivo behavior of R206Q as assessed by phenotype is largely indistinguishable from the null alleles. This interpretation is consistent with prior genetic evidence from Drosophila that the interaction of the paired homeodomain with palindromic elements is critical for biological activity (Bertuccelli et al., 1996). In summary, both biochemical and genetic analysis of Alx4<sup>lst</sup> demonstrate a critical role for arginine 5 in homeodomain function.

**Developmental and evolutionary control of ZPA formation**

Proper functioning of the ZPA demands that it be precisely localized in time and space (Johnson and Tabin, 1997). The identification of loss-of-function mutations in Alx4 mice, together with the expression of Alx4 in the anterior limb bud, demonstrates that Alx4 function is not required for the formation of the normal posterior ZPA; in contrast, Alx4 is required to repress both ZPA formation and Shh expression in the anterior aspect of the limb bud. This requirement for Alx4 function is similar to the requirement for Gli3 gene activity to repress ectopic Shh expression and ZPA formation (Buscher et al., 1997; Buscher and Ruther, 1998; Hui and Joyner, 1993). Based on molecular marker and genetic analysis, Alx4 currently cannot be positioned in a genetic pathway restricting anterior Shh expression (Dunn et al., 1997; Qu et al., 1997). Three different lines of evidence provide insight into the refinement of ZPA activity and subsequently Shh expression and serve to provide a framework for viewing Alx4 function. First, signaling by retinoids is implicated, as the engraftment of beads soaked in RA antagonists to the posterior aspect of the limb bud results in decreased Shh expression, and engrainment of RA-soaked beads to the anterior limb bud results in an ectopic anterior domain of Shh expression in the limb bud and the formation of an ectopic anterior ZPA (Lu et al., 1997). Although the mechanism by which retinoids exert this effect is unclear, it may relate to the known ability of RA to regulate AP positional specification by controlling axial Hox gene expression. Hoxb-8 expression in chick limb buds is altered in response to RA bead grafts (Stratford et al., 1997), and misexpression of Hoxb-8 in the anterior mouse forelimb bud results in anterior Shh expression and polydactyly (Charité et al., 1994). Ectopic forelimb expression of Hoxb-8 is not detected in either Alx4<sup>lst</sup> or Alx4<sup>tm1qw</sup> limb buds, suggesting that Alx4 is not required for restriction of Hoxb-8 expression (Chan et al., 1995; Qu et al., 1997). Whether Alx4 is a target of patterning by retinoids or Hox genes remains to be determined.

Second, the continued expression of Shh in the posterior mesoderm is contingent on the expression of Wnt-7a in the dorsal ectoderm and FGFs in the AER; this regulation presumably serves to coordinate patterning along the three axes (Johnson and Tabin, 1997). Prior genetic studies of the interaction of lst with recessive formin<sup>limb</sup> deformity mutant alleles have indirectly suggested a dependence of the ectopic ZPA on a preaxial extension of the AER and its reciprocal signaling for Shh maintenance (Vogt and Leder, 1996). Formin mutations cause an inability to form or maintain a proper apical ectodermal ridge that subsequently leads to a failure to maintain Shh expression, resulting in oligosyndactyly (Zeller et al., 1989). When Alx4<sup>lst</sup> is placed in trans to mutant formin alleles, the semidominant polydactyly of Alx4<sup>lst</sup> heterozygotes is suppressed and a wild-type limb results (Vogt and Leder, 1996). The genetic evidence that the Alx4 alleles are likely to represent severe hypomorphic or null alleles suggests that lst and formin are operating on distinct developmental pathways. Presumably, Alx4 loss leads to ectopic Shh expression in the anterior limb bud mesenchyme that is dependent on formin function for the anterior expansion of the AER that may be required to support ectopic Shh expression.

Third, the large number of mouse and human polydactyly conditions suggests that the genetic mechanisms that restrict Shh from the anterior mesoderm of the limb bud are complex. Prior studies examining genetic interactions between lst and the polydactyly mutants luxate and luxoid led to a view that the phenotypic consequences were additive (Forsthoevel, 1962). With respect to Gli3<sup>III</sup> and Alx4, it is not clear whether they participate in hierarchical or parallel pathways. At the level of RNA in situ expression analysis, neither Alx4 nor Gli3 expression are significantly altered in the respective mutant backgrounds, suggesting they function in separate biochemical pathways to repress Shh expression in the anterior limb bud mesenchyme (Qu et al., 1997). Whether Alx4 expression is altered in the limb buds of other preaxial polydactyly mutants remains to be examined.

During vertebrate evolution the transition of fins into limbs has been proposed to involve a developmental elaboration of digits as novel distal autopod elements (Daeschler and Shubin, 1998; Sordino et al., 1995). The molecular changes underlying this evolutionary process are not clear, but may reflect distal cell growth in response to distal Hox gene expression (Sordino et al., 1995; Zakany et al., 1997). Regardless of mechanism,
the fossil record suggests that the ancestral Devonian tetrapods had limbs with up to eight digits (Coates, 1994; Coates and Clack, 1990). The digits of polydactylous limbs of *Acanthostega* and *Ichthyostega* appear at some level to resemble the mirror-image polydactyly seen in *Alx4* alleles. This resemblance suggests that during the fin-to-limb transition, a branch of ancestral tetrapod limbs were in fact polydactylous due to the presence of symmetric anterior and posterior ZPAs (Raff, 1996). Subsequently, during evolution of the pentadactyl limb, the presence of an anterior ZPA would have become actively repressed. Based on this notion, the *Alx4* mutant alleles would represent atavistic mutations, suggesting that during tetrapod evolution *Alx4* may have been recruited or co-opted from a role in axial patterning as part of a genetic program to repress anterior ZPA formation. Alternatively, as may be argued from the restriction of *Shh* to the proximal posterior region of the fin bud in the teleost zebrafish (Krauss et al., 1993), the polydactyly in the anterior limb bud may instead be ancestral. An ancestral role for *Alx4* is also suggested by the expression of *Drosophila* relatives of *Alx4* and Gli3 (aristaless and *ci*, respectively) in the anterior compartment of the wing imaginal disc (Campbell et al., 1993; Schneitz et al., 1993; Shubin et al., 1997). Whereas in AP patterning of the fly wing *ci* appears to act downstream of hedgehog (Domínguez et al., 1996), the function of *aristaless* and its relationship to hedgehog and AP patterning is less well defined (Campbell et al., 1993; Schneitz et al., 1993). Analysis of molecular markers such as *Shh*, *Gli3* and *Alx4* expression in zebrafish and in more primitive species such as lungfish and coelacanths should provide information by which to evaluate these speculations.

### Modifiers of *Alx4* polydactyly and implications for human polydactyly

Previous analysis of the *Alx4* targeted mutation, which was carried out in 129/Sv inbred mice, failed to reveal hindlimb polydactyly in heterozygous animals as had been observed in *Strong's luxoid* heterozygous animals (Qu et al., 1997). Several lines of genetic evidence suggest that the differences in heterozygous phenotype are the result of strain-specific genetic changes and are not the result of different *Alx4* alleles. First, it has previously been established that the *lst* allele is subject to strain-specific modifying loci (Forsthoefel, 1962, 1968). Second, the nature of the *Alx4* alleles is all indicative of protein nulls. Third, although the *Alx4* targeted allele does not generate a heterozygous phenotype on the 129/Sv background, outcrossing the mutation to C57BL/6J mice or to the *lst* DP background results in the appearance of heterozygous polydactyly in the first generation. Fourth, when the strain background is the same (F1 progeny of an *Alx4*·*)lst* and *Alx4*tm1qw genotypes, polydactyly is observed.

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**Fig. 4.** *lst* alleles have mutations in *Alx4*. (A) Schematic representation of the *Alx4* open reading frame with the paired homeodomain indicated as a blue box and a 22 amino acid region conserved amongst aristaless-related family members termed the paired-tail indicated as a yellow box (Mathers et al., 1997). (B) The nucleotide and amino sequence of the *Alx4* homeodomain, showing the location of the mutations present in the *lst* (R206Q) base transition and *lst* (16 base pair deletion) alleles outlined in red. The inverted arrowhead indicates the position of intron 2. (C) The crystal structure of a paired-type homeodomain (Wilson et al., 1995) showing the position of the homeodomain R5 residue that is altered in the *Alx4*R206Q protein. The DNA is in space-filling representation, the paired-type homeodomain monomer is represented as a blue ribbon trace of the polypeptide backbone and the side chain of Arg5 is indicated in red depicting its contact with the DNA backbone in the minor groove (Wilson et al., 1995). (D) Genomic Southern analysis of *Alx4* in *lst* alleles. Genomic DNA prepared from the parental strains, *lst*+/+ and *Alx4*tm1qw +/+, and F1 progeny was digested with Apal, transferred to a nylon membrane and hybridized with an *Alx4* genomic probe (Qu et al., 1997). Deletion of *Alx4* sequences is observed in F1 progeny no. 5 as an absence of the upper wild-type *Alx4* hybridizing band. In all cases the observed phenotype of the F1 progeny was consistent with their genotype at *Alx4*.
Fig. 5. Functional characterization of the Alx4<sup>1st</sup> R206Q mutant protein reveals defective DNA binding. (A) DNA binding by the Alx4 homeodomain. Peptides containing either the wild-type Alx4 homeodomain, with flanking sequences, or the corresponding R206Q mutant protein, were expressed in E. coli and purified to homogeneity. The purified proteins were used in gel shift assays with either the P3 palindromic probe, which Alx4 binds as a homodimer, or the corresponding half-site probe (P1/2), which Alx4 binds as a monomer. Lane 1, probe only; lanes 2-4, increasing amounts of Alx4 wild-type homeodomain; lane 5, 50-fold excess of unlabelled P3 DNA as competitor; lane 6, 50-fold excess of unlabelled P3 mutant DNA as competitor; lane 7, probe only; lanes 8-10, increasing amounts of Alx4 R206Q homeodomain. D designates the migration of dimeric Alx4 bound to probe; M designates the migration of Alx4 monomer bound to probe. Tenfold more protein was used to examine monomeric DNA binding to the P1/2 probe than to assay dimeric binding to the P3 palindromic probe (see Materials and Methods). (B) Transcriptional activation by Alx4. Plasmids directing the expression of either wild-type Alx4 or Alx4 R206Q were co-transfected into human 293 cells along with the indicated reporter construct. Lysates were prepared 40 hours later and analyzed for CAT activity. Schematic diagrams of the reporter constructs are shown. Activity of wild-type Alx4 on the P3 reporter was assigned as 100%; values represent the average of four independent experiments. (C) Expression of Alx4 proteins in transiently transfected cells. 40 hours after transfection of 293 cells with either wild-type or Alx4 R206Q expression constructs, nuclear extracts were prepared and analyzed by Western blotting with an Alx4-specific antibody. (D) Nuclear extracts prepared from human 293 cells transiently transfected with either wild-type Alx4 or Alx4 R206Q were assessed for binding to a P3 DNA probe by gel shift assay. Lane 1, probe alone; lane 2, vector transfected cells; lane 3, wild-type Alx4 extracts; lane 4, Alx4 R206Q extracts. (E) DNA binding by Alx4 is not inhibited by Alx4 R206Q. Recombinant Alx4 homeodomain (3.2 ng) was used in gel shift assays with the P3 probe as above; increasing concentrations of Alx4 R206Q homeodomain were added into the reaction. The amount of R206Q in highest concentration (32 ng) was tenfold more than wild-type Alx4 (3.2 ng). (F) Transcriptional activation by Alx4 is not inhibited by Alx4 R206Q. Expression plasmids directing the expression of wild-type Alx4 and increasing amounts of Alx4 R206Q were co-transfected into human 293 cells along with P3 reporter construct. Lysates were prepared 40 hours later and analyzed for CAT activity. Activity of wild-type Alx4 on the P3 reporter was assigned as 100%; values represent the average of four independent experiments.

cross), animals heterozygous for the Alx4<sup>1st</sup> and Alx4<sup>tm1qw</sup> alleles exhibit a similar degree of polydactyly. Taken together, these data indicate that strain-specific genetic changes strongly modify Alx4-dependent polydactyly in heterozygotes. By extension, it seems reasonable to infer that differences in the homozygous phenotypes, including the polydactyly, hemimelia, ventral body wall defects and alopecia are also related to strain-specific rather than allele-specific effects.

The strain-specific changes in phenotype make it possible to genetically define other genes that play a role in AP patterning in the limb bud by genetic mapping. Among the candidate modifiers are genes that encode proteins that are highly related to, and possibly functionally overlapping with, Alx4. These include other aristaless-related genes (Cart-1 and Alx3) and members of the paired-type homeodomain gene class. In this context, the Cart-1 gene functions in a partially redundant
manner to suppress ZPA formation in the anterior aspect of the limb (Zhao et al., 1996) (S. Q., R. W., and B. de Crombrugghe, unpublished data). In addition, the possible interaction of Alx4 with transcriptional co-activators may serve to modify functional activity and therefore patterning. The finding that mutations in the transcriptional coactivator CBP underlie the human Rabinstein-Tabyi syndrome and its associated preaxial limb phenotype of a broadened digit 1 (Petrij et al., 1995) are consistent with a model in which limb patterning is very sensitive to alterations in gene dosage or stoichiometric relationships of transcriptional regulators.

The demonstration that mouse Alx4 mutant alleles are haploinsufficient and dissociate polydactyly from the ventral body wall defects suggests that human dysmorphic syndromes that include polydactyly, with or without abdominal wall and craniofacial defects, could be due to mutations in human Alx4 (McKusick, 1997). The map position of Alx4 on mouse chromosome 2 is contained in a region of conserved synteny that predicts that human Alx4 and any associated syndromes would most likely map to chromosome 11p12-q12 (Eppig, 1997). A search of the OMIM database did not reveal any human syndromes mapping to this region that display the full range of lst phenotypes. However, one mapped disorder, Bardet-Biedl syndrome type I, localizes to human chromosome 11q13 and displays postaxial polydactyly (Bruford et al., 1997). This is in contrast to the preaxial polydactyly observed in lst; however, as noted above, the allelic series of mutations in human GLI3 can result in either preaxial or postaxial polydactyly (Biesecker, 1997). Because the vast majority of human polydactyly mutations have not yet been mapped, there exists a large set of candidates for identity with Alx4 or genes acting within an Alx4-dependent pathway.

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