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RESEARCH ARTICLE

Isolation of the Chicken *Lmbr1* Coding Sequence and Characterization of Its Role During Chick Limb Development

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In the developing amniote limb, anteroposterior (A/P) patterning is controlled through secretion of the Sonic Hedgehog (SHH) protein by cells in the zone of polarizing activity (ZPA) located in the posterior mesoderm. In the chicken mutant *oligozeugodactyly* (*ozd*), *Shh* is expressed normally in the entire embryo with the exception that it is undetectable in the developing limbs; this results in the loss of specific bones in wings and legs. The *ozd* phenotype is similar to that of humans affected with acheiropodia (ACHR), and the ACHR mutation has been mapped to a deletion of exon 4 and portions of introns 3 and 4 in the *LMBR1* gene. We have cloned the chick ortholog of *LMBR1*, *Lmbr1*, and report that, in chick, *Lmbr1* is expressed within the ZPA. Although the *ozd* phenotype is similar to ACHR, the open reading frame of *Lmbr1* is normal in *ozd*. Sequence analysis of *Lmbr1* intron 3 demonstrated that this particular genomic region segregates with the *ozd* phenotype. In addition, overexpression of *Lmbr1* throughout the developing limb mesoderm resulted in morphologically normal limbs. Collectively, these data suggest that the *Lmbr1* coding sequence is not required for normal chick limb development. We propose that the *ozd* mutation is linked to the genomic region containing *Shh* and *Lmbr1*. *Developmental Dynamics 229:520–528, 2004.*

Key words: limb development; Sonic hedgehog; *Lmbr1*, *oligozeugodactyly* (*ozd*); zone of polarizing activity (ZPA); acheiropodia

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INTRODUCTION

Development of the vertebrate limb is a complex and dynamic process that has been studied extensively. Classic experiments, such as surgical manipulations, combined with more recent genetic approaches have established that proper outgrowth and patterning of the developing limb are dependent upon the actions of three distinct signaling centers (Mariani and Martin, 2003; Niswander, 2003; Tickle, 2003). Many growth factors and signaling molecules are known to affect patterning and growth of the developing limb by acting through one or more of these signaling centers, and interactions among the different signaling centers are important for proper limb development. Patterning along the anteroposterior (A/P, thumb to little finger) axis in the limb is controlled by the activity of the Sonic Hedgehog (SHH) protein, secreted by a small group of cells in the posterior mesoderm called the zone of polarizing activity (ZPA; Riddle et al., 1993; Lopez-Martinez et al., 1995; Marti et al., 1995; McMahon et al., 2003).

Analysis of mouse embryos lacking SHH protein has provided insight into the importance of *Shh* function during limb development. Normal limbs consist of a stylopod (humerus/femur), zeugopod (radius and ulna/tibia and fibula), and autopod (wrist and hand/ankle and foot). The limbs

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of the *Shh*$^{-/-}$ mutant mice, however, present severe defects in both the forelimb and hindlimb (Chiang et al., 2001; Kraus et al., 2001). *Shh*$^{-/-}$ limbs consist of a normal stylopod, a reduced zeugopod, and the complete absence of the autopod (in the forelimb) or the absence of the autopod except for an identifiable first digit (in the hindlimb) (Chiang et al., 2001; Lewis et al., 2001).

Humans affected with acheiropodia (ACHR) also exhibit severe limb-specific defects, including posterior deletions and distal truncations of the limbs, and loss of all but one digit, with no other apparent defects. This phenotype is analogous to the *Shh*$^{-/-}$ limb phenotype, suggesting that ACHR may be caused by a limb-specific loss of *Shh*. Recently, people affected with this disorder were reported to have a deletion of exon 4 and several kilobases of flanking intronic sequence in the *LMBR1* gene (for Limb Region 1; Clark et al., 2000); in affected individuals, the *LMBR1* transcript is smaller (Ianakiev et al., 2001). It is unclear whether the ACHR phenotype is caused by a disruption of LMBR1 protein function, a loss of regulatory elements within intronic regions of the deleted sequence, or some other undetermined cause. These observations have attracted attention to the *LMBR1* locus and its possible role in limb development.

The mouse *Lmbr1* gene has been mapped to chromosome 5 in a region 800 kilobases upstream of *Shh* (Clark et al., 2000), and this chromosomal arrangement and spacing is conserved in humans. The *Lmbr1* transcript is predicted to encode a putative multi-pass transmembrane protein with little homology to other known proteins and no known function. *Lmbr1*$^{-/-}$ mice have been generated, and the defects consisted of mild posterior deletions in the limbs (Clark et al., 2001). However, less than 1% of mice homozygous for this mutation exhibited limb phenotypes, and it is unclear whether these mice truly lack *Lmbr1* function.

Several other mouse mutants with limb defects have provided further insight into a possible role for the *Lmbr1* gene during limb development. The polydactylous mouse mutant *Sasquatch* (*Ssq*) contains a reporter gene insertion within intron 5 of the *Lmbr1* gene (Lettice et al., 2002). Through an elegant *cis/trans* genetic experiment where *Ssq* and *Shh*$^{-/-}$ mice were crossed, the *Ssq* mutation was found to disrupt *Shh* expression in the limb only when located in *cis* with a functional *Shh* allele. These data were interpreted to mean that the *Ssq* mutation interferes directly with a long range *cis*-regulatory element for *Shh*, suggesting that genomic regions of *Lmbr1* provide long distance regulation of *Shh* specifically in the limb (Lettice et al., 2002). Two other mouse mutations have also been proposed to be within the *Lmbr1* region. Like *Ssq*, the mouse mutant *Hemimelic extra-toes* (*Hx*) exhibits dominant preaxial polydactyly. A single base pair change within intron 5 of the *Lmbr1* gene has been isolated recently in *Hx* mutant mice. It is proposed that this base pair change causes the *Hx* phenotype, although there is no direct evidence to support this hypothesis (Lettice et al., 2003). In addition, the *Hammertoe* (*Hm*) mouse mutant exhibits syndactyly in the limbs, and this mutation has been mapped to a candidate region containing the *Lmbr1* gene (Clark et al., 2000; Lettice et al., 2003). Together, these studies suggest that the *Lmbr1* gene may be important for regulating multiple aspects of limb development.

Of interest, the phenotype of a novel recessive chicken mutant, *oligozeugodactyly* (*ozd*), resembles the phenotype of individuals affected with ACHR and the limbs of *Shh*$^{-/-}$ mice (Smyth et al., 2000). *Shh* is expressed in its normal domains throughout developing *ozd* embryos except in the developing limbs, where *Shh* expression is undetectable (Ros et al., 2003). The *ozd* mutants develop normally except for displaying a limb phenotype similar to but less severe than *Shh*$^{-/-}$ mice, including loss of posterior distal skeletal elements in the wing and leg. When SHH is exogenously delivered to the *ozd* limb bud, downstream mediators of *Shh* signaling are expressed normally and the limb skeleton is rescued, consistent with the view that the phenotype of these mutants is caused by a loss of *Shh* function in the developing limb (Ros et al., 2003). Because we were struck by the similarity of the *ozd* phenotype to ACHR, we have investigated the role of *Lmbr1* during limb development in the *ozd* mutant as well as in wild-type chickens.

Previous studies have not thoroughly characterized the role of *Lmbr1* during vertebrate limb development. Here, we analyzed and explored the function of *Lmbr1* throughout limb development in the chick embryo. We isolated the chicken *Lmbr1* ortholog and found that chick *Lmbr1* is expressed within the ZPA in a dynamic pattern consistent with a potential role in limb development. However, when *Lmbr1* was overexpressed in the developing chick limb using a retroviral misexpression system, no changes in skeletal patterning were observed. Lastly, although the *Lmbr1* coding region is not mutated in *ozd*, sequence analysis and comparison of *Lmbr1* intron 3 demonstrated that the *ozd* mutation is likely within the *Lmbr1-Shh* genomic region. Taken together, these results suggest that, although overexpression of *Lmbr1* transcripts does not affect normal chick limb development, noncoding regions of *Lmbr1* may be important for regulation of *Shh* expression in the developing limb.

RESULTS

Chick *Lmbr1* Is Highly Homologous to Mouse *Lmbr1* and Human *LMBR1*

An expressed sequence tag (EST) from the Delaware chicken EST database containing a fragment of a chicken gene highly homologous to portions of the mouse *Lmbr1* gene was used to screen a stage18–24 limb bud cDNA library to obtain the full-length chicken *Lmbr1* transcript. The chicken *Lmbr1* transcript contains an open reading frame (ORF) of 1467 nucleotides and is predicted to encode a protein of 488 amino acids (GenBank accession no. AY316689). Sequence comparison of full-length chick *Lmbr1* with mouse *Lmbr1* and human *LMBR1* reveals that the amino acid sequence

A

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cLmbr1          MEA-DEVSIREQNFHSQVREYTICFLLFAVLYIVSYFIITRYKRKADEQEDEDAIVNRIS 59
hLMBR1          MEGQDEVSAREQHFHSQVRESTICFLLFAILYVVSYFIITGYKRKSDEQEDEDAIVNRIS 60
mLmbr1          MEGQDEVSAREQHFHSQVRESTICFLLFAILYIVSYFIIIRYKRKSDEQEDEDAVVNRIS 60
                **. **** ***:******* ********:**:******  ****:********:*****

cLmbr1          LFLSTFTLAVSAGAVLLLPFSIISNEILLSFPQNYYIQWLNGSLIHGLWNLASLFSNLCL 119
hLMBR1          LFLSTFTLAVSAGAVSLLPFSIISNEILLSFPQNYYIQWLNGSLIHGLWNLASLFSNLCL 120
mLmbr1          LFLSTFTLAVSAGAVLLLPFSIISNEILLAFPHNYYIQWLNGSLIHGLWNLASLFSNLCL 120
                *************** *************:**:**************************

cLmbr1          FVLMPFAFFFLESEGFAGLKKGIRARILETLVMLILLALLILGIVWVASALIDNDAASME 179
hLMBR1          FVLMPFAFFFLESEGFAGLKKGIRARILETLVMLLLLALLILGIVWVASALIDNDAASME 180
mLmbr1          FVLMPFAFFFLESEGFAGLKKGIRARILETLVMLLLLALLILGMVWVASALIDSDAASME 180
                *********************************:********:*********.******

cLmbr1          SLYDLWEFYLPYLYSCISLMGCLLLLLCTPVGLSRMFTVMGQLLVKPTILEDLDEQMYII 239
hLMBR1          SLYDLWEFYLPYLYSCISLMGCLLLLLCTPVGLSRMFTVMGQLLVKPTILEDLDEQIYII 240
mLmbr1          SLYDLWEFYLPYLYSCISLMGCLLLLLCTPVGLSRMFTVMGQLLVKPAILEDLDEQIYMI 240
                *************************************************:********:*:*

cLmbr1          TLEEEAIQRKLNGISSTLENQTVELERELEKVKCKKTNLERRKKASAWERNLVYPAVMIL 299
hLMBR1          TLEEEALQRRLNGLSSSVEYNIMELEQELENVKTLKTKLERRKKASAWERNLVYPAVMVL 300
mLmbr1          TLEEEALQRRLHGLSSSVEYNVMELEQELENVKILKTKLERRKKASAWERNLVYPAVMVL 300
                ******:**:*:*:**::* : :***:***:**  **:**********************:*

cLmbr1          LLIETSISVLLVAFNILYLLVDETAMPKGSGGPGIGNASLSTFGFVGAALEIILIFYLMV 359
hLMBR1          LLIETSISVLLVACNILCLLVDETAMPKGTRGPGIGNASLSTFGFVGAVLEIILIFYLMV 360
mLmbr1          LLIETSISVLLVACNILCLLVDETAMPKGTRGPGIGSASLSTFGFVGAALEIILIFYLMV 360
                ************* *** ***********: *****.***********.***********

cLmbr1          SSVVGFYSLRFFENFIPRKDDTTMTKIIGNCVSILVLSSALPVMSRTLGITRFDLLGDFG 419
hLMBR1          SSVVGFYSLRFFGNFTPKKDDTTMTKIIGNCVSILVLSSALPVMSRTLGITRFDLLGDFG 420
mLmbr1          SSVVGFYSLRFFGNFTPKKDDTTMTKIIGNCVSILVLSSALPVMSRTLGITRFDLLGDFG 420
                ************ ** *:*******************************************

cLmbr1          RFNWLGNFYIVLSYNLLFAIMTTLCLVRKFTSAVREELLKALGLDKLHLSNNPRDSE-TK 478
hLMBR1          RFNWLGNFYIVLSYNLLFAIVTTLCLVRKFTSAVREELFKALGLHKLHLPNTSRDSETAK 480
mLmbr1          RFNWLGNFYIVLSYNLLFAIMTTLCLIRKFTSAVREELFKALGLHKLHLSDTSRDSETTK 480
                ********************:*****:***********:*****.****.:..**** :*

cLmbr1          PSANGHQKTL 488
hLMBR1          PSVNGHQKAL 490
mLmbr1          PSANGHQKAL 490
                **.*****:*
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B

% Iden / % Hom	cLmbr1	hLMBR1
mLmbr1	90/94	95/97
hLMBR1	90/94	

Fig. 1. Lmbr1 is highly conserved among chick, human, and mouse. **A:** Amino acid alignment of chicken (c) Lmbr1 with mouse (m) Lmbr1 and human (h) LMBR1. Asterisks represent identities; colons and dots represent high and low degrees of homology, respectively. **B:** Table showing percentage identity and percentage homology between Lmbr1 proteins in different species.

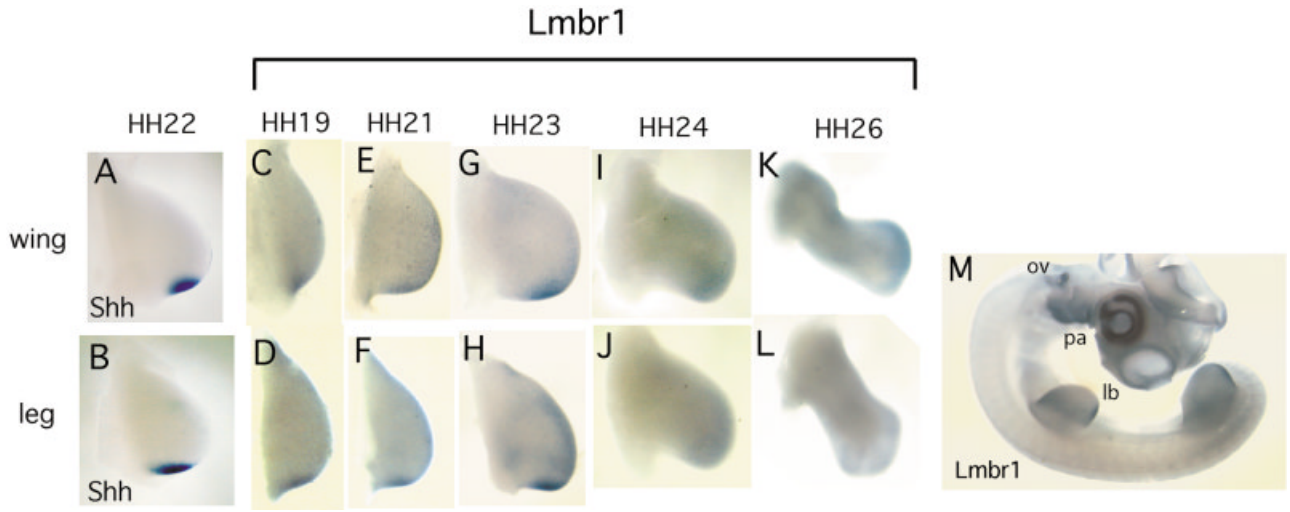


Fig. 2. Expression of *Lmbr1* in the developing chick limb by whole-mount in situ analysis. Localization of transcripts in the wing (A,C,E,G,I,K) and leg (B,D,F,H,J,L). **A–L:** Expression of *Shh* at stage 22 (A,B) and *Lmbr1* at stages 19 (C,D); 21 (E,F); 23 (G,H); 24 (I,J); and 26 (K,L). **M:** Regions of high *Lmbr1* expression in the developing embryo include the otic vesicles (ov), pharyngeal arches (pa), and limb buds (lb).

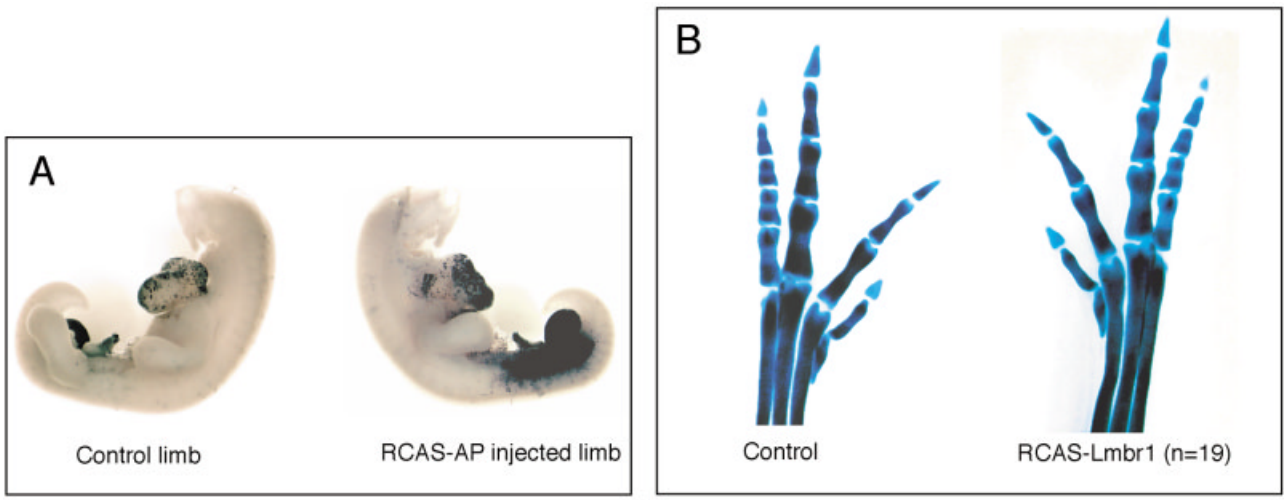


Fig. 3. Overexpression of *Lmbr1* in the developing chick limb using replication-competent avian sarcoma virus (RCAS) system. **A:** Embryos electroporated with an RCAS-alkaline phosphatase (RCAS-AP) DNA construct were harvested 48 hr after electroporation and AP activity was analyzed. A high percentage of experimental embryos (10 of 12) showed AP activity throughout the entire limb, suggesting that viral misexpression was efficient. **B:** Embryos electroporated with an RCAS-*Lmbr1* DNA construct at stages 12–14 were harvested 7 days after electroporation and stained for skeletal analysis. No differences were detected between experimental limbs (right) and contralateral control limbs (left, n = 19).

is greater than 90% identical among all three orthologs (Fig. 1A,B).

Lmbr1 Expression in the Developing Chick Limb

We began our analysis of *Lmbr1* function in chick limb development by characterizing its expression pattern by using whole-mount in situ hybridization. Although *Lmbr1* appeared to be expressed ubiquitously at low levels throughout the developing embryo, higher levels of *Lmbr1* transcript were apparent in specific regions in the embryo. In the developing limb, *Lmbr1* expression was first detected throughout the limb mesoderm as the limb bud emerged from the body wall (stages 17–18, data not shown). As limb outgrowth proceeded, the *Lmbr1* transcript became progressively up-regulated along the posterior margin of the developing limb bud (stages 19–23; Fig. 2C–H). Cells with this higher level of *Lmbr1* expression were located within the posterior portion of the *Shh* expression domain that marks the ZPA (compare Figs. 2A,B with Figs. 2G,H). Although *Shh* was first detected along the posterior of the developing limb at stage 17 (data

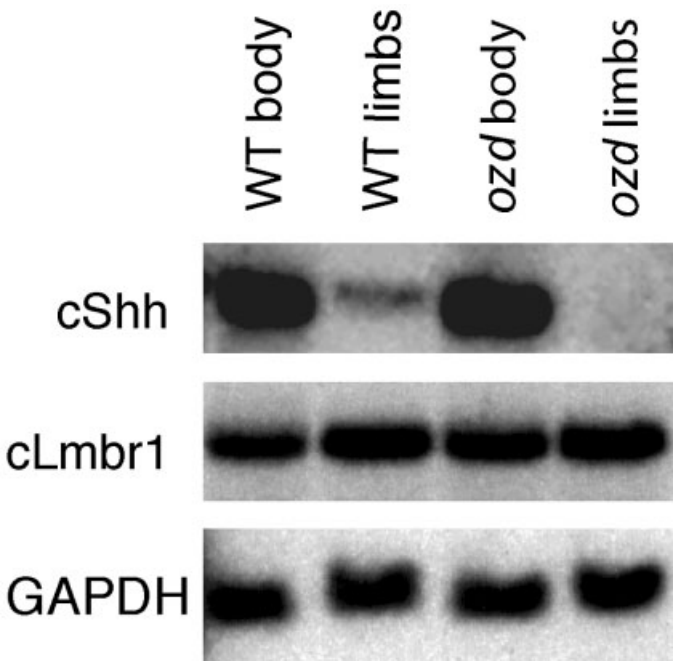


Fig. 4. Detection of *Lmbr1* transcripts in wild-type (WT) and *ozd* samples by Northern blot analysis. Expression of *Shh* (top panel), *Lmbr1* (middle panel), and *GAPDH* (bottom panel) in RNA isolated from WT bodies (lacking head and limbs), WT limbs, *ozd* bodies, and *ozd* limbs harvested at stages 26–27. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was analyzed as a loading control.

not shown) and low levels of *Lmbr1* expression were detectable throughout the limb bud at this stage (data not shown), enhanced *Lmbr1* expression at the posterior of the limb bud was not detected until stage 19 (Fig. 2C,D). This finding makes it unlikely that *Shh* is dependent upon *Lmbr1* expression for its normal ZPA expression in the limb. At later stages of limb development (stages 24–26, Fig. 2I–L), ZPA-localized *Lmbr1* expression was down-regulated and eventually became undetectable. This down-regulation occurs before the loss of *Shh* expression in the posterior limb. High levels of *Lmbr1* transcripts were also detectable in the otic vesicles and pharyngeal arches (see Fig. 2M).

Overexpression of *Lmbr1* in the Developing Limb

The dynamic expression pattern of *Lmbr1* in the ZPA suggested that *Lmbr1* might play a role in patterning the developing limb. To investigate this possibility, we designed a replication-competent avian sarcoma virus (RCAS) overexpression construct that contained the complete coding sequence of *Lmbr1*. Either the RCAS-*Lmbr1* DNA construct or a control expression construct containing alkaline phosphatase (RCAS-AP) was electroporated into the right prospective leg region of stage 12–14 chicken embryos.

We harvested several embryos 48 hr after electroporation to determine the extent of RCAS viral spread. We found that viral transcripts were present at high levels throughout the limb in RCAS-*Lmbr1* electroporated embryos by whole-mount in situ analysis (data not shown). Likewise, control embryos electroporated with RCAS-AP also showed a high level of reporter gene activity throughout the experimental limbs (n = 12, Fig. 3A), further indicating that the RCAS infection was efficient. We then harvested embryos electroporated with RCAS-*Lmbr1* seven days after electroporation to analyze the effects of *Lmbr1* overexpression on skeletogenesis. Overexpression of *Lmbr1* had no detectable effect on limb skeletal patterning (n = 19, Fig. 3B). In addition, there were no gross morphologic defects apparent in muscle or tendon development, but these structures were not examined in detail. Although we found that *Lmbr1* is expressed in a spatial and temporal manner consistent with an involvement in limb development, overexpression of *Lmbr1* in the developing chick limb has no detectable effect on skeletogenesis and does not interfere with normal chick limb development.

Analysis of *Lmbr1* Transcript in *ozd*

Because *ozd* is phenotypically similar to ACHR and ACHR is known to be a deletion within the human *LMBR1* gene, we examined the *Lmbr1* transcript in *ozd* to determine whether *Lmbr1* was similarly affected in this mutant. By using whole-mount in situ hybridization, we found that the wild-type ZPA-associated expression of *Lmbr1* was absent in *ozd* limbs at all stages analyzed (stages 21–25, data not shown). This correlates with the absence of *Shh* expression in the limb buds of this mutant (Ros et al., 2003).

We hypothesized that the *Lmbr1* transcript in *ozd* mutant embryos may be absent or truncated, because the *Lmbr1* transcript is smaller in ACHR patients and we were unable to detect localized *Lmbr1* expression in the posterior limb of *ozd* mutant embryos. We performed Northern blot analysis to further characterize the relative abundance and size of *Lmbr1* transcripts in *ozd* as compared with wild-type siblings. Total RNA was prepared from wild-type and *ozd* limbs and bodies at equivalent stages of development, and expression of *Shh*, *Lmbr1*, and *GAPDH* transcripts was analyzed in these samples. As expected, *Shh* expression cannot be detected in *ozd* limbs but is expressed in wild-type limbs and in both wild-type and *ozd* bodies (Fig. 4, top panel). It is notable that the *Lmbr1* transcript appeared to be expressed at relatively similar levels in all wild-type and *ozd* samples analyzed (Fig. 4, middle panel), which is consistent with the diffuse and ubiquitous low expression of *Lmbr1* detected throughout the embryo by whole-mount in situ analysis. Furthermore, the size of the

Lmbr1 transcript was comparable in wild-type and *ozd*, suggesting that the *Lmbr1* transcript does not contain a large deletion in *ozd*. To test the possibility that the *ozd* defect is due to either a small deletion or point mutation within the *Lmbr1* ORF, we cloned and sequenced the ORF of the *ozd Lmbr1* gene by reverse transcriptase-polymerase chain reaction (RT-PCR) and found no mutations in the coding region. With this information, it is apparent that neither the *Lmbr1* ORF (this report) nor the *Shh* ORF (Ros et al., 2003) is defective in the *ozd* mutant.

Genetic Analysis of *ozd* Mutation Shows Linkage to *Lmbr1* Genomic Region

The ACHR mutation is caused by a deletion of exon 4 and several kilobases of sequence from introns 3 and 4 of the *Lmbr1* gene (Ianakiev et al., 2001). Since we have shown that the *Lmbr1* coding sequence does not contain any mutations in *ozd*, we have begun a systematic sequence analysis of the corresponding intronic regions in chicken, and here we report our initial findings.

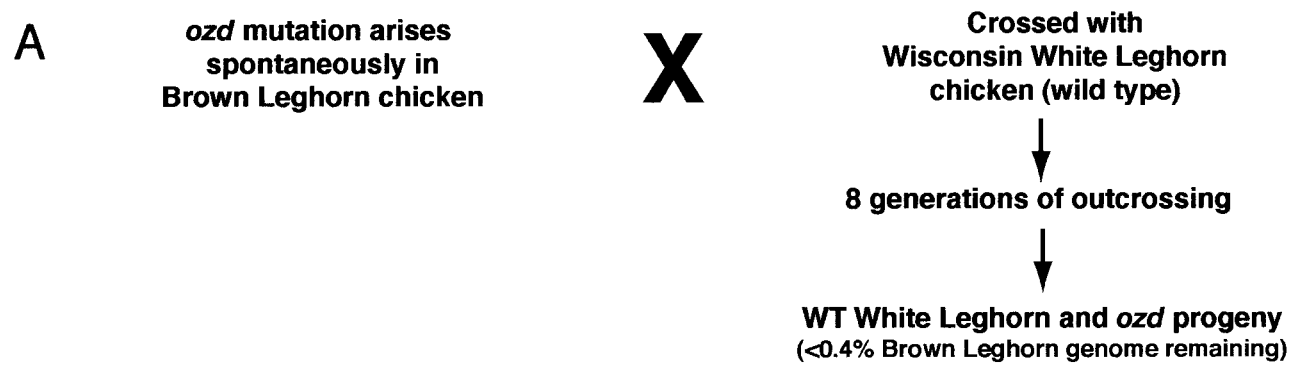
We cloned and analyzed *Lmbr1* intron 3 from the following relevant samples: *ozd* (n = 14), wild-type White Leghorn siblings of *ozd* (n = 16), wild-type Brown Leghorn chickens (n = 10), and known *ozd* carriers (n = 22). We chose to analyze these samples because the *ozd* mutation originated in the Brown Leghorn strain of chicken but has been outcrossed to the University of Wisconsin White Leghorn strain for eight generations (Fig. 5A). After eight generations, it is expected that less than 0.4% of the original Brown Leghorn genome would be present in the carrier flock, distributed randomly in the *ozd* mutant. Of interest, we found identical polymorphisms in *ozd* and wild-type Brown Leghorn samples but not in wild-type White Leghorn samples (Fig. 5B shows a portion of *Lmbr1* intron 3 that contains nearly all of the polymorphisms, where differences are underlined), suggesting that this chromosomal region from Brown Leghorn is retained in the White Leghorn *ozd* carrier flock. In addition, all *ozd* carriers were heterozygous for the intron 3 polymorphisms, containing one copy of each intron 3 variant (Brown Leghorn/*ozd* and wild-type White Leghorn). These polymorphisms do not cause the *ozd* phenotype, because the wild-type Brown Leghorns with the same intron 3 polymorphisms are phenotypically normal. However, these data are informative because they indicate that this Brown Leghorn genomic region segregates with the *ozd* phenotype and suggest that the *ozd* mutation may lie within the *Shh-Lmbr1* region.

DISCUSSION

In this report, we explored the role of the *Lmbr1* transcript during chick limb development. We show that the chicken ortholog of Lmbr1 is greater than 90% identical to human LMBR1 and mouse Lmbr1 at the amino acid level. In addition, we provide evidence that *Lmbr1* is expressed at the posterior margin of the developing chick limb throughout stages of limb development. This expression pattern suggests a possible role for *Lmbr1* in limb development. However, we demonstrate that overexpression of *Lmbr1* in the developing chick limb does not interfere with normal skeletal development. We also show that, although the chicken mutant *ozd* is phenotypically similar to patients affected with the human disorder ACHR, the *Lmbr1* transcript, while disrupted in ACHR, is not mutated in *ozd*. Lastly, we provide evidence through sequence comparison of intron 3 in *ozd* and other wild-type chickens that the *ozd* mutation may lie within the *Shh-Lmbr1* genomic region.

Although *Lmbr1* is expressed in a pattern that is spatially and temporally consistent with a potential role in limb development in the chicken, several lines of evidence suggest that the *Lmbr1* transcript is not important during limb development. Localized expression of *Lmbr1* cannot be detected in developing mouse limbs as it is in the chicken, even though, similar to the chick, *Lmbr1* is expressed ubiquitously at low levels throughout the mouse embryo (Hill et al., 2003). It is notable that, in mice homozygous for a *Lmbr1* loss-of-function allele, *Lmbr1* transcripts were detected by Northern blot, although they were not the size of *Lmbr1* transcripts in wild-type mice (Clark et al., 2001). It is possible that these smaller transcripts encoded truncated or nonfunctional proteins, because the gene targeting strategy only eliminated exon one. One possibility is that these shortened Lmbr1 proteins might interfere with normal *Lmbr1* function, acting as dominant negative proteins. Another possibility is that the shortened transcripts might provide partial function, producing a hypomorphic effect. Although less than 1% of *Lmbr1*$^{-/-}$ mice exhibited limb defects (Clark et al., 2001), provocatively, the limb defects present consisted of posterior digit losses, a milder version of the ACHR and *ozd* phenotypes. This observation leaves open the formal possibility that Lmbr1 protein does function in limb development. However, our data suggest that overexpression of *Lmbr1* in the developing chick limb has no effect on limb development. These data provide evidence that the *Lmbr1* coding sequence is not necessary for proper patterning of the vertebrate limb, as shown in both mouse and chicken.

In addition, we found that, although enhanced *Lmbr1* expression was detected at the posterior of the developing chick limb within the *Shh* expression domain in wild-type chick embryos, this localized posterior expression was lost in *ozd* limbs. Although it is possible that changes in the *Lmbr1* expression pattern contribute directly to the *ozd* phenotype, several more likely explanations exist. One possibility is that cells that normally express *Shh* and *Lmbr1* are not present in the *ozd* mutant limb, because Gli3R, which is normally excluded from cells in the ZPA, is expressed across the entire *ozd* limb (Ros et al., 2003). Another more enticing possibility is that a common regulatory element normally controls posterior limb expression of both *Shh* and *Lmbr1* and is disrupted in *ozd*. Our observation that the *Lmbr1* ORF is intact in *ozd* rules out a mutated Lmbr1 protein as the cause of the *ozd* phenotype.



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WL      AGAGGTGAGTGGGGAGAAAACAGTCAAACTGCTGTAGCATCTTAGAAGACAGTGTGTTAA 1380
ozd     AGAGGTGAGTGGGGAGAAAACAGTCAAACTGCTGTAGCATCTTAGAAGACAGTGTGTTAA 1379
BL      AGAGGTGAGTGGGGAGAAAACAGTCAAACTGCTGTAGCATCTTAGAAGACAGTGTGTTAA 1379

WL      GTTTTGCAGTTCTGGCTTAGAGTAATTCAGTATCCTAAATGAAAAGAAAAAAAACACCAA 1440
ozd     GTTTTGCAGTTCTGGCTTAGAGTAATTCAGTATCCTAAACAAAAAGAAAAAAC-CACCAA 1438
BL      GTTTTGCAGTTCTGGCTTAGAGTAATTCAGTATCCTAAACAAAAAGAAAAAAC-CACCAA 1438

WL      CCAAAGCATAC-AGTGGTACCTTC-TTCTGAAATGAAATTAGCCACACTGACTTTCTTGT 1498
ozd     CAAAAGCATACCACTGGTACAGTGGTTCTGAAATGAAATTAGCCACACTAACTTTCTTGT 1498
BL      CAAAAGCATACCACTGGTACAGTGGTTCTGAAATGAAATTAGCCACACTAACTTTCTTGT 1498

WL      GTAAATTAAGTGCTGTGGAATGAATAATAGATTTGCAGAGACTATTATGGAAAAGTAAGA 1558
ozd     GTAAATTAAGTGCTGTGGAATGAATAATAGATTTGCAGAGACTATTATGGAAAAGTAAGA 1558
BL      GTAAATTAAGTGCTGTGGAATGAATAATAGATTTGCAGAGACTATTATGGAAAAGTAAGA 1558

WL      CTTCTGTAAGTAGCTTATTGACTTTCCTGTTACAAACTCAGATAATTCCCAATTTCTTTG 1618
ozd     CTTCTGTAAGTAGCTTATTGACTTTCCTGTTACAAACTCAGATAATTCCCAATTTCTTTG 1618
BL      CTTCTGTAAGTAGCTTATTGACTTTCCTGTTACAAACTCAGATAATTCCCAATTTCTTTG 1618

WL      TTATGCAGCTTTGTTCTGTCA--GGATTTTGTTCTGTAGGAATGTTTTTGCCTTTTTTTC 1676
ozd     TTAGGCAGCTTTGTTCTGTCAGTGGATTTTGTTCTGTAGGAATGTTTTTGCCTTTTTTTC 1678
BL      TTAGGCAGCTTTGTTCTGTCAGTGGATTTTGTTCTGTAGGAATGTTTTTGCCTTTTTTTC 1678
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Fig. 5. Comparison of *Lmbr1* intron 3 in wild-type (WT) chickens and *ozd* mutants. **A:** Diagram depicting genetic background of *ozd*. **B:** A portion of *Lmbr1* intron 3 from wild-type White Leghorn (top), *ozd* (middle), and wild-type Brown Leghorn (bottom) chickens was compared. Identical polymorphisms were found in *ozd* and wild-type Brown Leghorn but not in wild-type White Leghorn chickens (differences are underlined). Known *ozd* carriers were found to contain both the Brown Leghorn/*ozd* and White Leghorn intron 3 polymorphisms.

In addition, we found that there is conservation of the original Brown Leghorn intron 3 in *ozd*. These data suggest that the *ozd* mutation lies within the *Shh-Lmbr1* chromosomal region and further support the hypothesis that the *ozd* mutation, and possibly ACHR, may disrupt a region necessary for *Shh* expression in the limb. It is relevant to this line of reasoning that discrete *cis*-regulatory elements necessary for *Shh* expression in specific regions of the developing central nervous system have been identified within the *Shh* promoter region through in vivo reporter assay studies (Epstein et al., 1999, 2000). Yet it is remarkable that no limb-specific regulator of *Shh* expression was found in these studies. However, analysis of the polydactylous mouse mutant *Ssq*, which contains a transgene insertion within *Lmbr1* intron 5, has led to the hypothesis that this mutation disrupts a long range *cis*-acting element regulating *Shh* function in the limb (Lettice et al., 2002). Recent analysis of *Lmbr1* intron 5 has led to the discovery of a roughly 800 base pair region within this intron that is highly conserved in many different species. Lettice et al. (2003) demonstrated that when this conserved region was tested in an in vivo reporter assay in mouse, it directed reporter gene expression to the correct posterior location of endogenous *Shh* in the ZPA. This result suggested that this region is a posi-

tive regulator for limb-specific *Shh* expression and led to the hypothesis that this highly conserved region contains a long-distance limb-specific regulator of *Shh* (Lettice et al., 2003). Therefore, based on the human ACHR mutation and our data, both *Lmbr1* intron 4 and intron 5 are possible candidate regions for the *ozd* mutation, and experiments are currently in progress to test this hypothesis. This strategy leads to the exciting possibility that *ozd* could be used as an important and unique tool to further explore and more precisely define the *cis*-regulatory regions necessary for *Shh* expression in the developing limb.

EXPERIMENTAL PROCEDURES

Cloning of Chick *Lmbr1* and Sequence Comparison

A chick EST containing sequence highly homologous to a portion of mouse *Lmbr1* was obtained from the Delaware chicken EST database. This fragment was used to screen a stage 18–24 chick limb bud cDNA library (gift from C. Tabin). Several positive clones were obtained that contained portions of chick *Lmbr1*, but none contained the most 5′ sequence. The remainder of the coding sequence of *Lmbr1* was obtained by performing primary and nested 5′-rapid amplification of cDNA ends (RACE) reactions using the FirstChoice RLM-RACE kit (Ambion), according to the manufacturer's instructions. Protein sequences for chick, human, and mouse Lmbr1 were compiled using ClustalW alignment program.

Embryos

Wild-type and *oligozeugodactyly* (*ozd*) embryos were obtained from mating flocks maintained at the University of Wisconsin Poultry Science Department (Madison, WI). For RCAS viral misexpression experiments, pathogen-free eggs were obtained from SPAFAS (North Franklin, CT). Eggs were incubated, opened, and staged as described (Hamburger and Hamilton, 1951; Ros et al., 2000).

Whole-Mount In Situ Hybridization

A digoxigenin-labeled antisense riboprobe specific for *Lmbr1* was generated by cloning a 1.1-kb fragment of *Lmbr1* containing the most 3′ 381 bp of the coding region and ~700 bp of 3′UTR and transcribing. Whole-mount in situ hybridization analysis was performed according to standard procedures (Nieto et al., 1996).

Construction of RCAS-*Lmbr1* Overexpression Construct

Full-length *Lmbr1* was generated by PCR using oligonucleotides containing restriction sites to allow in frame cloning into the *Nco*I site of the pSLAX13 vector. Positive clones were screened and sequenced, and a resulting error-free construct was obtained. This construct was then subcloned into RCAS-BP(A), a replication-competent retroviral vector, at the *Cla*I site. Orientation was checked by restriction analysis and the resulting construct was used in subsequent misexpression experiments (Logan and Tabin, 1998).

Electroporation Experimental Conditions

For electroporation experiments, eggs were opened after 2 days of incubation, corresponding to HH stage12–14. DNA was resuspended at a concentration of approximately 1 μg/μl in 10 mM Tris-HCl, pH 8.0. A small amount of Fast Green dye was added to visualize the solution while injecting. DNA solution was injected into the presumptive right hindlimb region of embryos, and several drops of chick Ringer's solution were dropped onto the embryo after DNA injection. Electrodes were placed above (cathode) and below (anode) the hind limb region containing injected DNA, and 1 round of electroporation was performed per limb to incorporate DNA throughout the entire limb. Conditions used for electroporation were three pulses of 15 V, 70 msec pulse length. Experimental embryos were harvested 48 hr after electroporation (for whole-mount in situ analysis or reporter gene activity assays) or 7 days after electroporation (for staining of skeletal elements). Visualization of skeletal elements was achieved through routine staining with Victoria Blue and tissue clearing with methyl salicylate (Ros et al., 2000).

Northern Blot Analysis

Limb buds or bodies (lacking limbs and heads) collected from either wild-type or *ozd* mutant embryos at HH 26–27 were harvested into TRI-Reagent (Sigma), and total RNA was isolated according to manufacturer's instructions. Twenty μg of total RNA for each sample was analyzed. Northern blot analysis was performed as previously described (Mitchell and Sheets, 2001). Radiolabeled double-stranded DNA probes were synthesized with random hexamers for *Shh*, *Lmbr1*, and a 450-bp fragment of *GAPDH* and used for hybridization.

Intron 3 Analysis

The entire *Lmbr1* intron 3 was cloned from *ozd*, wild-type White Leghorn, and wild-type Brown Leghorn chickens. Once it was determined that the Brown Leghorn strain-specific polymorphisms segregated with the *ozd* mutation, PCR primers were designed flanking a region of intron 3 containing nearly all of these polymorphisms. This region was amplified by using High Fidelity Polymerase (Roche) from wild-type Brown Leghorn (n = 10), wild-type White Leghorn (n = 16), *ozd* (n = 14), and known *ozd* carrier (n = 22) samples. Genomic DNA was isolated from blood or tissue by using GenomicPrep kit (Amersham) or by standard methods. The resulting PCR products were gel purified and sequenced, and comparisons were made by using ClustalW.

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