The Role of a Single Formin Isoform in the Limb and Renal Phenotypes of *Limb Deformity*

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ABSTRACT

Background: Mutations of the murine *limb deformity* (*ld*) locus are responsible for a pleiotropic phenotype of completely penetrant limb malformations and incompletely penetrant renal agenesis and/or dysgenesis. The *ld* locus encodes a complex family of mRNA and protein isoforms.

Materials and Methods: To examine the role of one of the more prominent of these isoforms, isoform IV, we specifically eliminated it by gene targeting.

Results: Unlike other mutant *ld* mice, homozygous mice bearing this isoform IV disruption display incompletely penetrant renal agenesis, but have perfectly normal limbs. Whole mount in situ hybridization demonstrated that this targeted disruption was specific for isoform IV and did not interfere with the expression of other *ld* isoforms. The isoform IV–disrupted allele of *ld* does not complement the renal agenesis phenotype of other *ld* alleles, in a manner consistent with its penetrance, and like the isoform IV–deficient mice, these compound heterozygotes have normal limbs. Sequence analysis of formin isoform IV in other *ld* mutant alleles did not detect any amino acid changes relative to the strain of origin of the mutant allele.

Conclusions: Thus, the disruption of isoform IV is sufficient for the renal agenesis phenotype, but not the limb phenotype of *ld* mutant mice. Structural mutations in this isoform are only one of several genetic mechanisms leading to the renal phenotype, since amino acid changes in this isoform were not detected. These results demonstrate that this gene is *limb deformity*, and that variable isoform expression may play a role in generating the pleiotropic *ld* phenotype.

INTRODUCTION

A number of murine and human complex dysmorphic syndromes are inherited in a Mendelian fashion (1,2), suggesting the action of a single gene. While the pleiotropic nature of such single-gene disorders may result from multiple factors, tissue-specific control of gene expression is one likely mechanism for pleiotropy. For example, the activity of a single-gene product can be regulated in a tissue-specific manner at the level of transcription or translation. In addition, multiple gene products can be variably produced in different tissues by alternative promoter utilization and/or differential splicing, resulting in the localized expression of distinct proteins.

The mouse *limb deformity* locus (*ld*) provides a good model for the study of factors involved in the generation of complex dysmorphic syndromes from single genes. Five recessive mutants (ld^{J}, ld^{OR}, ld^{TgHd}, ld^{TgBri}, and ld^{In2}) of this locus have previously been described (3–9). Homozy-

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Molecular Medicine, Volume 3, Number 6, June 1997 372-384

gous mutant mice have oligosyndactyly with fusion of the long bones of the limbs, as well as renal agenesis, hypoplasia, and dysplasia. The limb phenotype is completely penetrant in homozygotes, but the renal agenesis phenotype shows incomplete penetrance and variable expressivity. Each of the five known alleles have differences in penetrance of the renal phenotype, which vary from 98% in ld^J/ld^J mice (10) to 21% in ld^{ln2}/ld^{ln2} mice (9). These differences may be a consequence of the strength of each allele and/or strain background differences (10).

The gene for the *ld* locus was cloned via a transgene insertion in the ld^{TgHd} allele (7). The gene is composed of at least 24 exons spanning over 400 kb (11a), and has at least four different mRNA isoforms that are differentially expressed during development (see Fig. 1A) (11). Isoforms I–III contain a common 5′ exon that codes for a basic protein domain, while isoform IV contains a unique 5′ terminal exon encoding an acidic domain. Isoforms I–III are coordinately expressed in the developing kidney and central and peripheral nervous systems, but they are expressed only at very low levels in the limb buds. Isoform IV is expressed at high levels in the limb buds, particularly the apical ectodermal ridge (AER), developing kidneys, notochord, branchial arches, somites, and tail buds, as determined by RNase protection (11) and in situ hybridization analysis (12). In the chicken, a similar pattern of expression was seen using antibodies to the isoform IV product (13). In addition, there are at least two distinct promoters in the *ld* locus: one at the 5′ end of isoforms I–III and an internal promoter for isoform IV. The existence of the internal promoter was demonstrated using a *lacZ* reporter assay in transgenic mice (12). These observations suggest distinct roles for isoforms I–III and IV in the *ld* phenotype.

Given that *ld* is a complex locus giving rise to multiple protein isoforms, we were interested in determining whether components of the pleiotropic *ld* phenotype might be the consequence of a mutation affecting one of these isoforms. To test this, we have investigated the role of isoform IV in the *ld* phenotype by isoform-specific gene targeting, and by sequence analysis of this isoform in spontaneously occurring *ld* mutants. Our results indicate that the disruption of isoform IV results in the occurrence of one of the mutant *ld* phenotypes, renal agenesis/dysgenesis phenotype, but not the limb phenotype that accompanies other mutations at the *ld* locus.

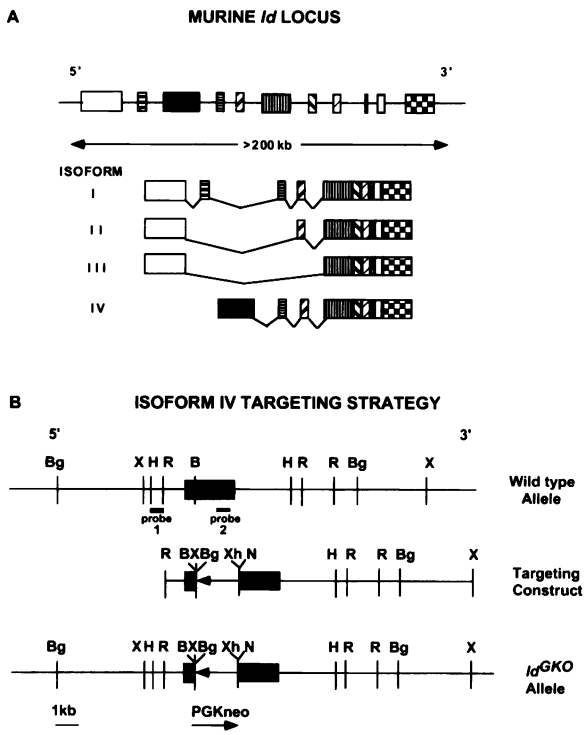

FIG. 1. Disruption of isoform IV of the *ld* gene

(A) Murine *ld* locus. The alternative splicing of isoforms I–IV are shown (after Jackson-Grusby et al. 1992). Not all 24 exons are shown, only those important for distinguishing the four isoforms. (B) Targeting strategy. Top: genomic organization of the wild-type *ld* gene (129 strain) around the 5′-terminal exon (black box) of isoform IV. Middle: replacement-type targeting construct for isoform IV. A PGK-*neo* gene (arrow, indicating direction of transcription) was inserted into this exon at a unique *Bam*HI site of isoform IV, in the opposite orientation relative to transcription, and introduced stop codons in all three reading frames of isoform IV. Bottom: structure of the mutant locus (ld^{GKO} allele) after homologous recombination with the targeting construct. The location of the flanking (probe 1) and internal (probe 2) probes used for Southern analysis are indicated by bars under the wild type allele. Restriction enzyme sites: B, *Bam*HI; Bg, *Bg*III; H, *Hin*dIII; N, *Not*I; R, *Eco*RI; X, *Xba*I; Xh, *Xho*I.

MATERIALS AND METHODS

Gene Targeting

The 129Sv genomic clone for the 5′-terminal exon of isoform IV was isolated from a 129Sv female liver genomic library (Stratagene). The targeting construct was made in the vector pPNT (14), and J1 ES cells (15) were used for transfection. The growth, transfection, and isolation of potential targeted clones were carried out as pre-

viously described (16). One hundred ninety-six clones were analyzed for homologous recombination by nested polymerase chain reaction (PCR) (data not shown), and by Southern analysis using a flanking primer and a *neo* primer. Eight PCR positive clones were expanded and correct targeting in six clones was confirmed by Southern blot analysis with five different restriction enzymes, using either a flanking probe (Fig. 1B, probe 1) or an internal probe (Fig. 1B, probe 2) from the exon. After blastocyst injection, one targeted line gave germ-line transmission. The production of chimeric animals and mating of chimeric males with NIH Black Swiss (Taconic) females to test for germ-line chimerism has been described elsewhere (16). F1 heterozygotes were intercrossed to generate all genotypes, and the line was maintained in this mixed background.

Skeletal Stains

Skeletal stains were done as described previously (17). The axial skeletons of ld^{GKO}/ld^{GKO} animals were normal (data not shown), as previously reported for all other *ld* alleles (3–9,18,19).

Whole Mount in Situ Hybridization Analysis

Experiments were performed as described previously (20), with the exception that embryos were permeablized with detergents (21), as opposed to proteinase K treatment when examining AER staining patterns (12). Probes for isoforms I–III and isoform IV were carried out as described elsewhere (12).

Immunoblot Analysis

For immunoblot analysis, protein samples were separated on 6% acrylamide Laemmli gels, transferred to Immobilon-P (Millipore), and hybridized with the appropriate antiserum. Adult animals were genotyped by Southern analysis of tail DNA, while embryos were the result of homozygous matings, and therefore of uniform genotype. An entire litter (8–9 embryos) was dissected for limb bud samples, while only two heads or two bodies (devoid of head and limb buds) were used for those samples. After dissection, the embryo samples were quickly frozen at −80°C. Samples were disrupted by Dounce homogenization in PBS with protease inhibitors. One-half of each sample was loaded in each well. Each genotype from each embryonic fraction was repeated at least twice, with similar results (data not shown). Polyacrylamide gel electrophoresis, immunoblotting, and stripping of filters were performed as described elsewhere (22).

Sequence Analysis

cDNA representing the entirety of the coding sequence of isoform IV were synthesized from homozygous kidney samples from FVB, ld^{J} and ld^{OR} mice, using SuperScript (GIBCO/BRL). Five primer pairs were used to amplify overlapping 800- to 1,000-bp fragments from isoform IV (sequences and PCR conditions available upon request). PCR products were cloned by the T-vector kit (InVitrogen), and two clones of each fragment were sequenced on one strand each to give complete coverage for isoform IV. Automated sequencing was performed on an Applied Biosystems, Inc., machine, using conditions recommended by the manufacturer. Sequence polymorphisms were confirmed by repeat sequencing of both strands of the same fragment. If confirmed, the relevant region of isoform IV was directly amplified from genomic DNA. The PCR fragments were directly sequenced, or the presence of polymorphic restriction enzyme sites from the polymorphic nucleotides were determined.

RESULTS

Targeted Disruption of Isoform IV

Unique 5′ exons are used for isoforms I–III and isoform IV (Fig. 1A). A replacement-type targeting construct was designed to disrupt isoform IV (Fig. 1B), using a 129-Sv genomic clone of the *ld* locus surrounding the 5′-terminal exon of isoform IV for the targeting construct. This exon is adjacent to the isoform IV promoter and is not utilized in isoforms I–III (Fig. 1A). A PGK-*neo* cassette was inserted 195 bp 3′ of the start codon (65 amino acids into the open reading frame) of this exon, in the opposite orientation relative to transcription. Stop codons were introduced in all three reading frames. This insertion into the 5′-terminal acidic exon of isoform IV is predicted to result in either a severely truncated, nonfunctional protein from the isoform IV transcript, or no protein product, and should have no effect on the expression of isoforms I–III.

This construct was introduced into J1 em-

bryonic stem cells (15), and nine clones containing the disrupted allele (ld^{GKO}) were identified using a nested PCR strategy, with *neo* primers and primers from the flanking region 5′ of the targeting construct (data not shown). These nine positive clones were analyzed by Southern blotting, using a flanking probe (probe 1, Fig. 1B) or an internal probe (probe 2, Fig. 1B). After *Xbal* digestion, the predicted polymorphic band of 2 kb and the wild-type 12-kb band were observed in eight clones using probe 1 (Fig. 2A), demonstrating appropriate targeting. Using probe 2 after *Hin*dIII digestion, correct targeting was confirmed by the presence of a polymorphic 8-kb band in seven clones, in addition to the wild-type 6-kb band (Fig. 2B). Using several different enzyme digestions and these probes, five of the clones had completely correct targeting (data not shown). However, the other PCR-positive clones had bands of inappropriate sizes (see Fig. 2A, lane 2 and Fig. 2B, lanes 2 and 5), suggesting that targeting was aberrant in these clones. One of three correctly targeted clones resulted in germline transmission after injection into blastocysts, establishing the ld^{GKO} isoform IV mutation in mice.

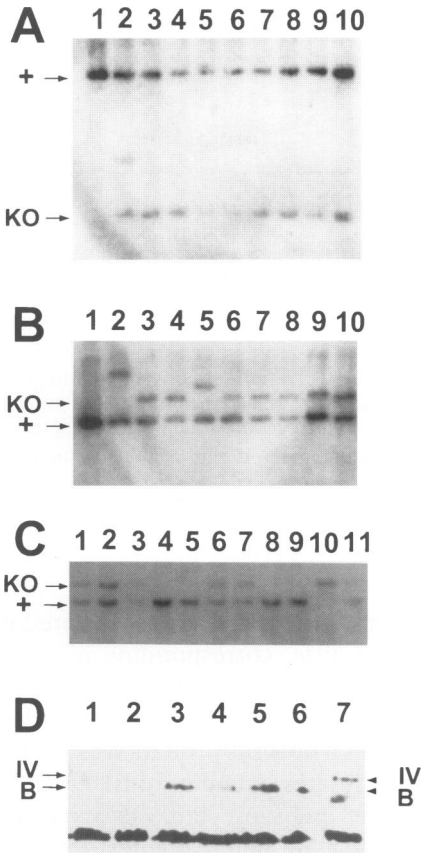

FIG. 2. Southern and immunoblot blot analysis of ES clones and animals

(A) Southern analysis of DNA from targeted ES clones, after *Xba*I digestion, and using a flanking probe (probe 1 from Fig. 1B). Lane 1, control J1 ES cell DNA; lanes 2–10, PCR-positive clones; +, wild-type allele (12 kb); KO, ld^{GKO} allele (2 kb). (B) Southern analysis of the same ES clones, after *Hin*dIII digestion, using an internal probe (probe 2 from Fig. 1B). +, wild-type allele (6 kb); KO, ld^{GKO} allele (8 kb). (C) Southern analysis of mice containing the ld^{GKO} allele. Tail DNA samples from the F2 offspring of a heterozygous cross (lanes 1–11) were analyzed by Southern blot analysis after *Eco*RI digestion, using the internal probe 2. +, wild-type allele (6 kb); KO, ld^{GKO} allele (8 kb). Pups 4, 5, 8, 9, and 11 were +/+, pups 1–3, 6, and 7 were ld^{GKO}/+, and pup 10 was ld^{GKO}/ld^{GKO}. (D) Immunoblot analysis of lysates from bodies, dissected away from head and limb buds, of E10.5 embryos of different genotypes. The genotypes analyzed were: ld^{GKO}/ld^{GKO} homozygotes (lanes 1,2); ld^{GKO}/ld^{TgBri} compound heterozygotes (lanes 3–6); and ld^{GKO}/+ heterozygote (lane 7). The relevant protein bands are indicated by arrows: IV refers to the 165-kDa isoform IV protein; B refers to the 155-kDa ld^{TgBri} truncated protein. A background band of about 120 kDa was shown to demonstrate relative protein loading in each lane.

Immunoblot Analysis of Isoform IV in Mutant Animals

F1 ld^{GKO}/+ heterozygotes were crossed, and all possible *ld* genotypes were observed in the offspring (Fig. 2C). Over 100 offspring resulting from heterozygous crosses were genotyped, and all genotypes were present in the expected 1:2:1 (+/+:ld^{GKO}/+:ld^{GKO}/ld^{GKO}) ratio. To determine if the disruption resulted in total loss of isoform IV protein, we performed Western blot analysis of embryos that resulted from heterozygous crosses. The different isoforms of the *ld* locus potentially encode a family of proteins, termed the formins (23). In vitro translation studies demonstrated that isoforms I, II, and IV encode proteins of 180, 160, and 165 kDa, respectively, and isoform III encodes a protein of 78 kDa (24). To examine the expression of formin isoform IV, we used a rabbit antiserum that was made to the 5′ end of the proline-rich exon of the mouse protein (α-proline antiserum). This exon is common to all four isoforms; however, this antiserum only detects a 165-kDa protein corresponding to isoform IV in embryonic samples (see below).

Immunoblots of kidney lysates from wild-type animals, using the α-proline antiserum,

TABLE 1. Limb and Kidney Phenotypes of F1 Compound Heterozygotes from Homozygous Crosses

Genotype	Normal limbs	Normal kidneys			Abnormal (%)	Kidney abnormality
		0	1	2		
$ld^{GKO}/+$	92/92	0	0	92	0	None
ld^{GKO}/ld^{GKO}	105/105	1	5	99	6%*	Agenesis
ld^{GKO}/ld^{TgBri}	62/62	0	1	61	2%	Agenesis
ld^{GKO}/ld^{ln2}	43/43	1	0	42	2%	Multicystic dysplastic

Matings were between homozygous parents to give uniform genotypes in each litter. Pregnant females were checked twice daily for litters to minimize the chance of missing pups that died at birth. All pups were scored for limb phenotype and for the number of kidneys by inspection under a dissecting microscope.

*$p < 0.05$, when compared with $ld^{GKO}/+$ neonates by Fisher's exact test.

demonstrated the presence of a predominant band of 165 kDa, corresponding in size to isoform IV, which was undetectable in lysates of ld^{GKO}/ld^{GKO} homozygote kidneys (data not shown). This 165-kDa protein was also missing from crude lysates of bodies of E10.5 ld^{GKO}/ld^{GKO} homozygote embryos, but, as expected, was present in $ld^{GKO}/+$ heterozygotes (Fig. 2D). Similar results were obtained using an antiserum raised against the 5'-terminal exon of isoform IV (data not shown), demonstrating that this protein represents the translation product of isoform IV and that it is absent in the null embryos. The ld^{TgBri} allele, resulting from a transgene insertion (8,19), codes for a truncated (155 kDa) protein of isoform IV (D.C. Chan, unpublished observations). Body lysates from ld^{GKO}/ld^{TgBri} compound heterozygotes contained only this truncated protein, and did not contain detectable levels of the 165-kDa protein (Fig. 2D, lanes 3–6), providing additional evidence that the isoform IV disrupted allele does not make any of the normal isoform IV 165-kDa protein. Identical results were obtained from immunoblot analysis of dissected limb buds and head samples of E10.5 embryos (data not shown). These results demonstrate that no isoform IV protein was produced from the ld^{GKO} allele and confirm that we have eliminated this isoform in these mice.

Limb and Kidney Phenotypes of Mutant Mice

All ld^{GKO}/ld^{GKO} animals appeared grossly normal by inspection and were healthy and fertile. To assess the phenotype of ld^{GKO}/ld^{GKO} homozygotes and compound heterozygotes formed between ld^{GKO} and other *ld* mutant alleles, ld^{GKO}/ld^{GKO} homozygous males were mated to +/+, ld^{GKO}/ld^{GKO}, ld^{TgBri}/ld^{TgBri}, and ld^{ln2}/ld^{ln2} homozygous females, and neonates were scored for limb and kidney abnormalities. All heterozygous $ld^{GKO}/+$ and homozygous ld^{GKO}/ld^{GKO} neonates had normal limbs by inspection (Table 1) and skeletal staining (Fig. 3A,B). In particular, all animals had normal numbers of digits, absence of syndactyly, and absence of radioulnar and tibiofibular synostosis, which are the hallmarks of the *ld* limb phenotype. ld^{GKO}/ld^{TgBri} and ld^{GKO}/ld^{ln2} compound heterozygotes also had entirely normal limbs (Table 1). These findings demonstrate that the disruption of isoform IV is not sufficient for the limb phenotype of the *ld* locus and that the ld^{GKO} mutant allele complements the limb phenotype of other *ld* alleles.

In contrast to the absent limb phenotype, 6% (6/105) of ld^{GKO}/ld^{GKO} homozygous mutants had unilateral or bilateral renal agenesis (Table 1, Fig. 3C,D). The adrenal glands were always present bilaterally. In some cases, blind-ended ureters originating from the bladder were present, while in others, the ureters were absent on the ipsilateral side (Fig. 3C,D). Of 62 ld^{GKO}/ld^{TgBri} compound heterozygote neonates, one animal (2%) had unilateral agenesis (Table 1). Also, one ld^{GKO}/ld^{ln2} compound heterozygote neonate had bilateral multicystic dysplastic kidneys (Table 1, Fig. 3E–G), a phenotype within the spectrum of renal agenesis and seen previously in ld^{ln2}/ld^{ln2} mutants (9,10). The tubules of such kidneys are characterized by developmental malformations, resulting in huge urine-filled cysts

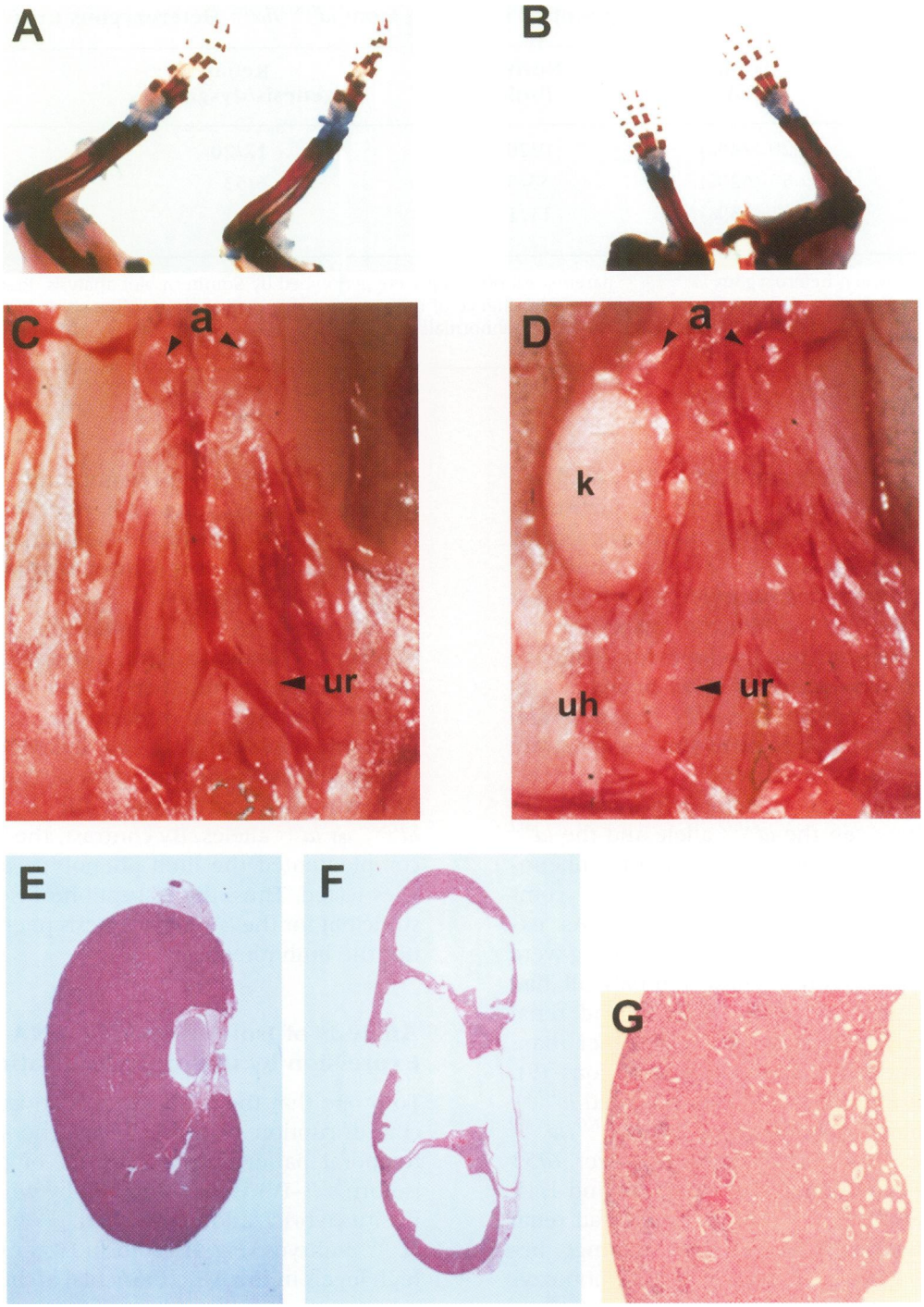

FIG. 3. Analysis of mutant mice

(A) and (B) Forelimbs after skeletal staining. Note that both limbs have the normal number of digits, and both have paired long bones (radius and ulna). Left, $ld^{GKO}/+$ limb; right, ld^{GKO}/ld^{GKO} limb. (C) ld^{GKO}/ld^{GKO} animal with bilateral renal agenesis. The adrenal glands (a) were present bilaterally, but the ureter (ur) was present only on the left side. (D) ld^{GKO}/ld^{GKO} animal with unilateral (left) renal agenesis. The adrenal glands (a) were present bilaterally, but the ureter (ur) was present only on the side with the kidney (k) and distinguished from the uterine horn (uh) on the same side. (E) Kidney from a 2-week-old +/+ mouse, sectioned and stained with hematoxylin and eosin. (F) Kidney from a ld^{GKO}/ld^{ln2} 2-week-old littermate with bilateral multicystic dysplastic kidneys, sectioned and stained with hematoxylin and eosin. Note the massive cysts, due to maldevelopment of the excretory system. (G) Part of kidney section in F (higher magnification). The small cysts on the right of the figure reflect the maldevelopment associated with the multicystic dysplastic kidney phenotype.

TABLE 2. Limb and Kidney Phenotypes of F2 Offspring from ld^{GKO}/ld^{ln2} Heterozygous Crosses

Genotype	Total (%)	Normal limbs	Renal agenesis/dysgenesis	Abnormal (%)
ld^{ln2}/ld^{ln2}	20 (24%)	0/20	12/20	60%
ld^{GKO}/ld^{ln2}	53 (62%)	53/53	6/53	11%
ld^{GKO}/ld^{GKO}	11 (13%)	11/11	1/11	9%

Matings were between heterozygous ld^{GKO}/ld^{ln2} parents. All offspring were genotyped by Southern blot analysis. Pregnant females were checked twice daily for litters to minimize the chance of missing pups that died at birth. All pups were scored under a dissecting microscope for limb phenotype and for kidney abnormalities.

from defective tubulogenesis (Fig. 3F, compared with a normal kidney in Fig. 3E). At high magnification (Fig. 3G), even the normal-appearing parenchyma at the margin of this multicystic dysplastic kidney showed evidence of smaller cysts, reflecting the hindrance of urine flow resulting from tubular maldevelopment. We have not observed renal agenesis in 92 neonatal heterozygous $ld^{GKO}/+$ mice (Table 1), nor in several hundred wild-type animals in this mixed strain background (data not shown).

To examine in more detail the noncomplementation between the ld^{GKO} allele and the ld^{ln2} allele, we analyzed the limb and kidney phenotypes of F2 offspring of an F1 ld^{GKO}/ld^{ln2} compound heterozygous cross (Table 2). As expected, approximately 25% of the offspring were ld^{ln2}/ld^{ln2} homozygotes. These animals all had abnormal limbs, and 60% of them had renal agenesis or dysgenesis, which was higher than, but consistent with, previous observations (9). The remaining neonates, which included ld^{GKO}/ld^{ln2} compound heterozygotes and ld^{GKO}/ld^{GKO} homozygotes, had normal limbs. However, 6/53 (11%) of the compound heterozygotes and 1/11 (9%) of the ld^{GKO}/ld^{GKO} homozygotes had renal agenesis/dysgenesis. In separate matings between wild-type mice and ld^{ln2}/ld^{ln2} homozygotes, out of 42 offspring, none had renal agenesis/dysgenesis, demonstrating that the renal phenotype observed in these mice was caused only by the *ld* alleles. There was a relatively lower number of ld^{GKO}/ld^{GKO} homozygotes (13%). We have not yet determined whether this occurred by chance or was due to aneuploidy from segregation of the abnormal chromosomes in the ld^{ln2} mice (9). We have not observed any other evidence that the isoform IV disruption had an effect on embryonic viability. In support of this interpretation, the litter sizes of matings between mice that were homozygous for the isoform IV disruption averaged between 9 and 10 mice, which is similar to wild-type litter sizes (data not shown).

These results indicate that the disruption of isoform IV results in renal agenesis/dysgenesis, with penetrance somewhat lower than that observed with other *ld* mutant alleles. Consistent with this observation, a low incidence of noncomplementarity of the renal agenesis phenotype was observed between the ld^{GKO} and the ld^{TgBri} or ld^{ln2} alleles. By contrast, the ld^{GKO} allele complemented the limb phenotype of all *ld* alleles tested. Thus, the disruption of isoform IV is sufficient for the *ld* renal agenesis phenotype, but not the limb phenotype.

Analysis of Isoform-specific RNA Expression by in Situ Hybridization

To prove that the ld^{GKO} allele is an isoform-specific disruption of the *ld* locus, the spatial and temporal patterns of expression of the formin isoforms (I–IV) were examined by whole-mount in situ hybridization analysis in +/+ and ld^{GKO}/ld^{GKO} embryos (Fig 4). Isoform IV is expressed at high levels in the AER, branchial arches, and in a punctate pattern in the somites of E9.5 and E10.5 embryos, but not in their dorsal root ganglia (DRG) or trigeminal ganglia (TG). In contrast, isoforms I–III are expressed in the DRG and TG of E10.5 embryos, but not the AER or somites (12). Therefore, the AER, somites, DRG and TG represent areas of unique expression of *ld* isoforms. In addition, isoforms I–III and IV are coordinately expressed in the mesonephros, the ureteric bud, and the branching derivatives of the bud (12).

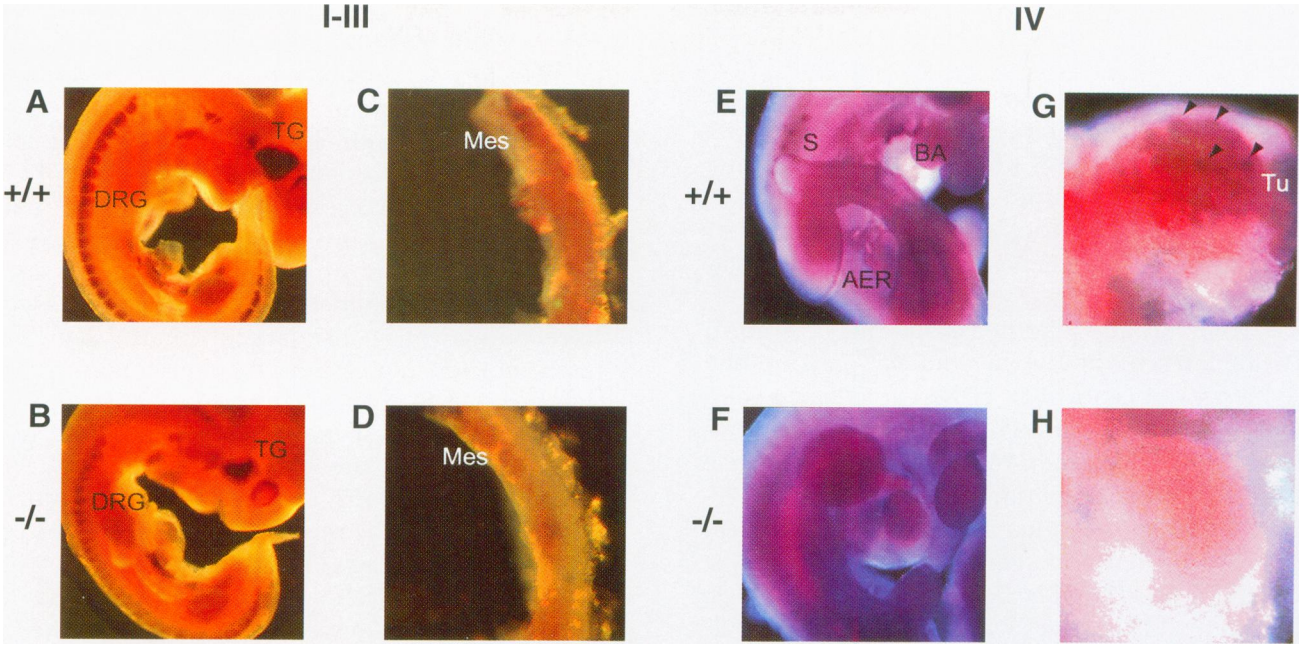

FIG. 4. Whole-mount in situ analysis of isoforms I–III and IV in wild-type and mutant mice
+/+ (A) and ld^{GKO}/ld^{GKO} (−/−) (B) E11.5 embryos, and dissected mesonephroi from +/+ (C) and ld^{GKO}/ld^{GKO} (−/−) (D) E10.5 embryos were hybridized with an isoform I–III RNA antisense probe. +/+ (E) and ld^{GKO}/ld^{GKO} (F) E10.5 embryos, and dissected kidneys from +/+ (G) and ld^{GKO}/ld^{GKO} (H) E13.5 embryos were hybridized with an isoform IV RNA antisense probe. AER, apical ectodermal ridge; S, punctate staining in the somites; DRG, dorsal root ganglia; TG, trigeminal ganglia; Mes, mesonephric tubules; Tu, metanephric tubules (arrowheads); BA, branchial arches.

Using an isoform I–III–specific antisense RNA probe, the staining patterns of the DRG and TG were identical between E11.5 +/+ (Fig. 4A) and ld^{GKO}/ld^{GKO} embryos (Fig. 4B), as well as in E9.5 and E10.5 embryos (data not shown). In contrast, using an isoform IV–specific antisense RNA probe, there was intense staining of the AER, somites, and branchial arches of +/+ E9.5 (data not shown) and E10.5 embryos (Fig. 4E). However, this staining pattern was absent in ld^{GKO}/ld^{GKO} E9.5 (data not shown) or E10.5 embryos (Fig. 4F).

The pattern of expression of isoforms I–III and IV was examined in the developing kidneys. The expression of isoform I–III in the mesonephric ducts and tubules was identical between +/+ (Fig. 4C) and ld^{GKO}/ld^{GKO} (Fig. 4D) embryos. In addition, the ureteric bud and branching derivatives were stained with a probe to isoforms I–III in embryos of both genotypes (data not shown). However, isoform IV was expressed in the developing kidneys only in +/+ embryos. Isoform IV was detectable in the mesonephric ducts and tubules, ureteric bud (data not shown), and in a punctate pattern from branching of the ureteric bud in older (E13.5) kidneys (Fig. 4G). In ld^{GKO}/ld^{GKO} embryos, no expression of isoform IV was detectable in mesonephroi, ureteric buds (data not shown), or E13.5 kidneys (Fig. 4H). Thus, the spatial and temporal expression of isoform IV RNA is specifically disrupted in the ld^{GKO} allele, whereas the expression of RNA from isoforms I–III is unaffected.

No Isoform IV Mutations in the ld^{J} or ld^{OR} Alleles

The ld^{TgBri} and ld^{In2} alleles are the result of a transgene insertion and a chromosomal translocation, respectively, at the 3′-end of the *formin* gene (9,19), which disrupt all known isoforms (I–IV) of the *ld* locus. As expected, these disruptions result in the production of slightly truncated isoform IV proteins as assessed by immunoblot analysis (D. C. Chan and P. Leder, unpublished observations, and results presented above). In contrast, the molecular basis of the mutations responsible for the ld^{J} and ld^{OR} alleles

have not been described. These spontaneously occurring alleles are likely to contain point mutations or small deletions or insertions, since no genomic rearrangements have been detected by Southern blot analysis (data not shown) and the proteins produced from these alleles are unaltered in size or quantity by immunoblot analysis (D. C. Chan and P. Leder, unpublished observations).

To determine whether mutations in isoform IV are present in the ld^J and ld^{OR} alleles, the sequences of isoform IV cDNAs for these alleles were determined and compared with the published sequence from FVB mice (11). Reverse transcription polymerase chain reaction (RT-PCR) products encompassing the ORF of isoform IV were cloned and sequenced from wild-type (FVB), ld^{OR}/ld^{OR} and ld^J/ld^J kidney RNA samples. No nucleotide changes were detected in the coding sequence of isoform IV in the wild-type allele of FVB mice. No mutations or polymorphisms were found in the ld^{OR} allele, either. However, we found four differences resulting in amino acid changes in isoform IV of the ld^J allele: (*1*) a 3-bp deletion from 2304-2306, resulting in the deletion of a proline residue (ΔP694); (*2*) an A → G transition at position 1372 (A1372G), which results in an arg → gly amino acid substitution at amino acid 383 (R383G); (*3*) a G → A transition at position 1322 (G1322A), which results in a gly → asp amino acid substitution at amino acid 366 (G366N); and (*4*) an A → G transition at position 3450 (A3450G), which results in a cys → trp amino acid substitution at amino acid 1076 (C1077W). To determine if these changes occur in the strain of origin of the ld^J allele, the ΔP694 region and the region surrounding amino acid 1077 were sequenced from CBA/Ca mice, which is the strain in which the ld^J allele arose. The G1322A and A1372G mutations introduced novel restriction enzyme recognition sites. PCR fragments surrounding these sites were amplified and digested with the appropriate enzymes from ld^J/ld^J and CBA/Ca DNA. All four mutations were present in CBA/Ca, indicating that they are strain polymorphisms. Since CBA/Ca mice have normal limbs and kidneys, these polymorphisms cannot be responsible for the abnormal function of the ld^J allele. Therefore, there are no pathological isoform IV mutations in the ld^J or ld^{OR} alleles, demonstrating that isoform IV mutations are not necessary for the limb and kidney phenotypes of these spontaneous mutants of the *ld* locus.

DISCUSSION

In an effort to dissect the molecular basis for individual components of the complex *ld* phenotype, we have examined the role of an individual isoform of the *ld* locus in the *limb deformity* phenotype. Isoform IV is expressed from an internal promoter (12) and is an independent transcriptional unit, distinct from isoforms I–III. By disrupting the 5′-terminal exon of isoform IV, we have created mice that lacked this isoform but retain normal expression of isoforms I–III. Mice that are selectively and completely deficient for isoform IV manifest incompletely penetrant renal agenesis, yet they have normal limbs. In addition, the isoform IV–disrupted allele does not complement the renal agenesis phenotype of two *ld* mutant alleles. Thus, loss of function of isoform IV is sufficient for the low incidence of the renal agenesis/dysgenesis phenotype of the *ld* locus, but not the limb phenotype. However, whereas elimination of isoform IV is sufficient to produce the renal phenotype, mutations in this isoform are not necessary for either the limb or kidney phenotype of *ld*, since no isoform IV mutations were found in either the ld^J or ld^{OR} alleles, which produce both manifestations of the *ld* phenotype. Our results uncover an important role for an individual isoform of the *ld* locus in the generation of the complex *limb deformity* phenotype and suggest that variable isoform expression may play a role in this pleiotropic phenotype. In addition, we have created a new *ld* mutant that can be used to study factors involved in the penetrance of the renal agenesis phenotype.

Renal Agenesis, but no Limb Phenotype, in Isoform IV–Deficient Mice

We have demonstrated that the disruption of a specific isoform of the *ld* locus can isolate the limb and kidney phenotypes of a complex dysmorphic syndrome in mice. The loss of function of isoform IV is sufficient for the renal agenesis phenotype of the *ld* locus, albeit at a low penetrance, providing compelling evidence for a role for this isoform and the 165-kDa protein that it encodes in kidney development. We have also definitively shown that this gene is involved in the *ld* phenotype, since the targeted disruption of isoform IV did not complement the renal agenesis phenotype of two other *ld* alleles. The noncomplementation of the kidney phenotype between ld^{GKO} and ld^{In2} is particularly compelling in this respect. However, isoform IV–deficient mice

have normal limbs, demonstrating that the loss of this isoform is not sufficient for the *ld* limb phenotype. One potential explanation for the segregation of the limb and kidney phenotypes is that the ld^{GKO} allele is weaker than the other known *ld* alleles; those mutant alleles in which the mutations have been characterized have disruptions of all of the known isoforms, whereas the isoform IV targeted allele specifically disrupts only this isoform, leaving intact the expression of isoforms I–III. This explanation is supported by the low penetrance of the renal phenotype in the isoform IV deficient mice. However, this cannot be a complete explanation. Although we do not know the molecular lesions of the ld^{J} or ld^{OR} alleles, the ld^{J} and ld^{OR} alleles have no mutations in isoform IV and hence do not disrupt all *ld* isoforms, either. It is also possible that the molecular actions of the *ld* gene products are distinct in the developing limb and kidney and that the pleiotropic phenotype is the result of variable isoform expression. This is supported by the different spatial and temporal patterns of expression of the RNA and protein isoforms of *ld*.

The developing AER is defective in *ld* mutants with limb abnormalities (25), which suggests that the origin of the limb defects in *ld* mutants resides in the AER. Consequently, the expression pattern of *ld* isoforms was examined, with the goal of identifying those isoforms which, by virtue of their expression in the AER, might contribute to the *ld* limb phenotype. Isoform IV is expressed at high levels in the AER of developing limb buds (11,12), yet no limb phenotype was observed in the isoform IV–deficient mice, suggesting that additional isoforms (and the proteins they encode) must complement a deficiency of isoform IV in the limb. Axial patterning in the developing limb bud is a result of reciprocal inductive interactions between the mesenchyme and ectoderm (for a recent review, see ref. 26). Molecularly, these interactions appear to result from a feedback loop in the mesenchyme and ectoderm, consisting of *Shh*, *fgf-4*, and *Wnt-7a* (27–34). This feedback loop is disrupted in *ld* mutants (12). Thus, it is possible that defects in the AER of *ld* mutants could result from primary abnormalities in the AER or mesenchyme. In support of the importance of the mesenchyme in the *ld* phenotype, wild-type mesenchyme can rescue the AER defect of *ld* ectoderm in chimeric mouse/avian limbs (35). Consequently, isoforms that are expressed in the mesenchyme may play an important role in the *ld* phenotype. Taken together, these experiments suggest that an *ld* isoform that is expressed anywhere in the developing limb could potentially be involved in the mutant limb phenotype. For example, low levels of RNA of isoforms I and II are present in the limb bud (10) and contain the COOH-terminal region of the *ld* gene disrupted in the ld^{TgHd}, ld^{TgBri}, or ld^{In2} alleles. These isoforms are unaltered in the isoform IV–deficient mice. Other candidate isoforms have been identified in embryonic limb bud ectoderm (11).

It is also clear that missense mutations of isoform IV are not necessary for either the limb or renal phenotypes of *ld*, since sequence analysis of two alleles (ld^{J} and ld^{OR}) failed to detect physiologically significant alterations in isoform IV protein structure. Both of these alleles have fully penetrant limb defects and highly penetrant renal agenesis. For example, the ld^{J} allele has 98% penetrance, while the ld^{OR} allele has 70% penetrance of renal agenesis (10). All of these results are best explained by a model in which isoforms other than isoform IV are necessary and sufficient for the limb phenotype of *ld*, whereas mutations in isoform IV are sufficient, but not necessary, for the renal agenesis phenotype.

The formation of the kidneys is a result of a reciprocal inductive interaction between the ureteric bud and metanephric blastema. At about E11 in the mouse, the ureteric bud grows out from the mesonephric duct into the metanephric blastema, resulting in the induction of the blastema to form kidney parenchyma. The differentiating blastema mesenchyme in turn signals the ureteric bud to continue to grow and branch. Renal agenesis can result from defects in either the ureteric bud or metanephric blastema. Delays in ureteric bud outgrowth appear to be the primary defect in *ld* mutant homozygotes, leading to renal agenesis (10). We have previously shown that isoforms I–III and isoform IV are expressed in the pronephros, mesonephric tubules and ducts, the ureteric bud of the developing metanephros, and ureteric bud derivatives of the developing kidney by whole-mount in situ hybridization analysis (12). Thus, the *ld* isoforms, including isoform IV, are expressed in the developing kidney in a pattern consistent with an important role in ureteric bud outgrowth.

The phenotype of the isoform IV–deficient mice demonstrates that isoform IV is necessary for normal renal development and implies that this isoform is involved in ureteric bud outgrowth. In situ hybridization analysis of the *ld* isoforms showed that isoform IV was not expressed in the ureteric bud of isoform IV–defi-

cient mice, but isoform I–III expression was expressed normally. Although the presence of all other *ld* isoforms is not sufficient for completely normal renal development, we have not ruled out roles for other *ld* isoforms in renal development. Isoforms I–III may also be important for the normal development of the kidneys, a hypothesis that is supported by their pattern of expression in the embryo. It is possible that the other isoforms compensate somewhat, but not completely, for the loss of isoform IV in the ld^{GKO}/ld^{GKO} mice.

Penetrance and Renal Agenesis

The renal agenesis phenotype of all *ld* mutations displays incomplete penetrance and variable expressivity (10). In the isoform IV–deficient mice, this phenotype may have reduced penetrance relative to other *ld* alleles because the ld^{GKO} allele is a weaker allele, as discussed above, or because of strain differences in penetrance. This low penetrance is nevertheless consistent with the renal agenesis phenotype of other *ld* mutants, where the penetrance ranges from 21% to 96% (10). The difference in penetrance among other *ld* alleles may be due to differences in the rate of ureteric bud outgrowth, or to the sensitivity of particular strains to variations in the rate of outgrowth. For example, the ld^{J} allele, with the highest penetrance, may result in a uniform delay in the rate of ureteric bud outgrowth such that nearly every animal has renal defects, while the ld^{GKO} allele, with relatively low penetrance, may have a less severe effect on the rate of ureteric bud outgrowth. It has not been possible to test directly whether ureteric bud outgrowth is delayed in the isoform IV–deficient mice because of the low penetrance of renal agenesis. Complicating this analysis is the fact that all alleles of *ld* arise and are maintained in different strain backgrounds. We are using these differences in penetrance to determine the role of individual alleles and strain-specific modifying alleles in determining the penetrance of renal agenesis in *ld* mutants.

Disruptions of other genes in mice have resulted in completely penetrant, recessive renal agenesis/dysgenesis: the Wilms tumor–associated transcription factor *WT-1* (36); the c-*ret* receptor tyrosine kinase (37); the c-*ret* ligand GDNF (38–40); the secreted signaling molecule *Wnt-4* (41), and the transcription factor *Pax-2* (42). *WT-1* (43,44) and *Wnt-4* (41) are expressed in the mesenchymal condensations surrounding the growing ureteric bud and are likely involved in receiving the inductive signal in the metanephric blastema from the ureteric bud. Interference with mesenchymal induction in these mutants would be expected to result in completely penetrant renal agenesis. c-*ret* is expressed in the ureteric bud (45), and the elimination of this gene or its ligand GDNF must result in a more severe disruption of ureteric bud function than the described mutations of *ld*, since they result in 100% penetrant phenotypes. *Pax-2* is expressed in both the epithelial and mesenchymal compartments of the developing kidney, and its loss also results in the loss of genital tracts, suggesting that *Pax-2* is central to development of mesonephric as well as metanephric derivatives. *ld* mutants have defects in ureteric bud outgrowth only, but not in the later development of the kidney, since careful examination of the normal-appearing kidneys in *ld* mutant animals have revealed no abnormalities (G. Ryan and A. Wynshaw-Boris, unpublished observations). Thus, the defects in renal development in *ld* mutants most likely precede ureteric bud outgrowth and probably occur in the mesonephric ducts. We are currently examining the expression patterns of markers for mesonephric ducts and ureteric buds in ld^{J}/ld^{J} mutants to address these issues.

Renal agenesis is also incompletely penetrant and variably expressed in humans, although it is unclear whether it is inherited in an autosomal-dominant and/or autosomal-recessive manner (46–48). By isolating the renal phenotype of *ld* from the limb abnormalities, the isoform IV–deficient mouse provides a model for human renal agenesis and suggests a candidate gene for mutation examination.

ACKNOWLEDGMENTS

We thank En Li and Rudolph Jaenisch for providing the J1 ES cells, Cathie Daugherty and Anne Harrington for excellent technical assistance, and Lee Niswander for sharing unpublished data. We are grateful to Les Biesecker, Bill Pavan, and members of the Leder and Wynshaw-Boris labs for useful discussions and comments on the manuscript. A. W.-B. was a Howard Hughes Medical Institute Physician Research Fellow.

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Communicated by P. Leder. Accepted April 3, 1997.