The murine limb deformity (ld) locus encodes a set of proteins, termed formins, that are required for embryonic limb and kidney development. Previous studies had indicated that these proteins are located in the nucleus and cytoplasm and have biochemical properties consistent with an action within the nucleus. To test the notion that nuclear localization is crucial for formin function, we carried out molecular and biochemical studies on one ld allele. We show that two transgene-induced alleles, ldTgBri and ldTgHd, generate similar COOH-truncated formins that lack the terminal 110 amino acids, while a third allele, ldIn2, generates a less extensively truncated formin that lacks the terminal 42 amino acids. Using subcellular fractionation analysis, we find that wild-type formin is detected in both nuclear and cytosolic fractions; in contrast, the truncated formins encoded by ldTgBri and ldTgHd are strictly cytosolic. The less extensively truncated ldIn2 formin shows a similar, but less complete, localization defect. Consistent with this weaker cellular phenotype, hind limbs from ldIn2 mice have milder skeletal defects than those of ldTgBri mice. These observations define a small region in the carboxyl terminus that is required for nuclear localization and suggest that nuclear localization plays a role in formin action.

The murine limb deformity (ld) gene is required for normal limb and kidney development (1, 2). Mice homozygous for ld mutations exhibit fusion of the long bones of the limbs and fusion and reduction of the metacarpals/metatarsals and phalanges. This limb defect is first manifested in the embryonic limb bud as a failure of proper apical ectodermal ridge formation and a reduction in the width of the anteroposterior limb axis (3). These two defects are likely related to the loss of fgf-4 expression in the apical ectodermal ridge and the reduction of shh and HoxD expression in limb bud mesoderm (4, 5). In addition, mutant mice can suffer unilateral or bilateral renal agenesis or hypoplasia, although this phenotype is of variable penetrance and expressivity. All five alleles of ld (ldTgHd, ldBri, ldTgBri, ldIn2) display similar limb defects (1, 6–8), while the kidney defects differ in penetrance and severity among the alleles (9).

The recovery of a transgene insertion allele, ldTgHd, allowed the cloning of the ld locus by transgene tagging (1, 2). The ld locus produces a complex set of transcripts encoding novel proteins termed formins (2). Three ld alleles, ldTgHd, ldTgBri, and ldIn2, contain structural alterations in the 3’ end of formin transcripts (7, 10). The molecular bases of two other ld alleles, ldJ and ldOR, are unknown.

At least four major transcripts arise from the ld locus (Fig. 1A), and their embryonic expression patterns are consistent with the ld mutant phenotype. Isoforms I, II, and III share a common amino-terminal exon and are expressed coordinately (11). These three isoforms are expressed in the pronephros, mesonephros, and metanephros of midgestation mouse embryos but are absent from the embryonic limb buds (4). Like isoforms I, II, and III, isoform IV is present throughout renal development; in addition, it is expressed in early limb buds, with highest levels in the apical ectodermal ridge (4, 11). These differential expression patterns suggest that all four isoforms may play important roles during renal development, while isoform IV may play a role in the ld limb defects. RNase protection assays indicate that other, less well characterized ld transcripts are also present in the ectoderm of limb buds (11).

The biochemical function of formins is unknown, although recent studies have provided a number of clues. First, a central portion of the proteins encoded by isoforms I, II, and IV contains a large number of proline residues, and some sequences in this region match consensus sequences forSrc homology 3 ligands. Indeed, fusion proteins containing this region of formin have been demonstrated to bind in vitro to Src homology 3 domains (12, 13). This proline-rich region also binds in vitro to a class of novel proteins containing WW domains (12). These findings suggest that this proline-rich region probably mediates physical interactions of formin with associated proteins. Second, formins share some sequence similarity with the Drosophila genes diaphanos (14) and cappuccino (15), which are required for cytokinesis and egg polarity, respectively. Third, immunohistochemical studies indicate that chicken formin is localized in a punctate nuclear pattern (16). Finally, formins harvested from transfected COS cells are phosphorylated mostly on serine and bind to DNA-cellulose columns (17), suggesting that formins may interact directly or indirectly with nucleic acids.

To learn more about the function of formins, we have compared the structural and biochemical properties of wild-type formin to mutant formins encoded by several ld alleles. We find that three ld alleles encode COOH-truncated but stable proteins. Formins from ldTgHd and ldTgBri cells have identical truncations and are strictly cytosolic, whereas a large proportion of formin from wild-type cells is stably associated with nuclei. Formin from ldIn2 cells shows a less severe localization defect. In addition, morphological comparisons show that ldIn2 mice have a less severe limb phenotype than ldTgBri mice.

**EXPERIMENTAL PROCEDURES**

3’ Rapid Amplification of cDNA Ends—3’ Rapid amplification of cDNA ends was performed as described previously (18) to isolate mutant cDNAs. One μg of polyadenylated RNA (Pharmacia QuickPrep),
isoforms I and IV were generated with rabbit reticulocyte lysate (Pro trollous gift from Lewis Chodosh. Purified by affinity chromatography as described (19). Protein preparations were further purified by preparative SDS-polyacrylamide gel electrophoresis, and gel slices were injected into rabbits (Pocono Rabbit Farms). Rabbit bleeds were initially screened by an enzyme-linked immunosorbent assay, and bleeds with high titers were selected for further characterization. Antiserum A was purified on an affinity column (20) containing maltose-binding protein (New England BioLabs) fused to amino acids 519–641 of isoform IV. Affinity-purified antibody A was used at a 1:1000 dilution for Western Blots. Antiserum B was used as a crude serum at a 1:2000 dilution. Control Western blots on subcellular fractions were done using a 1:1 dilution of hybridoma supernatant containing anti-c-myc monoclonal antibody 3C7 (21), a generous gift from Lewis Chodosh.

**Immunoprecipitations—**2S-labeled in vitro translation products for isoforms I and IV were generated with rabbit reticulocyte lysate (Promega) as described (17). Ten μg of the lysate were incubated with 400 μg of radiolabeled precipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0), and 2 μg of either preimmune serum, affinity-purified antibody A, or antiserum B. After 1 h at 4°C, 20 μl of either preimmune serum, affinity-purified antibody A, or antiserum B was used as a crude serum at a 1:2000 dilution. Control Western Blots on subcellular fractions were done using a 1:1 dilution of hybridoma supernatant containing anti-c-myc monoclonal antibody 3C7 (21), a generous gift from Lewis Chodosh.

**Subcellular Fractionation—**For each fractionation experiment, five nearly confluent 15-cm plates of fibroblast monolayers (17) were washed twice with phosphate-buffered saline. Subsequently, all steps were performed on ice or at 4°C. Cells were scraped off with a rubber scraper and spun down at 1500 × g for 5 min. The cell pellet was resuspended in the hypotonic buffer (20 mM Hepes pH 7.4, 150 mM KCl, 1.5 mM MgCl2, 1 μg/ml aprotonin, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin) and allowed to swell on ice for 10 min. Cells were then lysed by vigorous Dounce homogenization (100 strokes) using a tight-fitting Dounce homogenizer. At this point and throughout the procedure, cell lysis and recovery of nuclei were monitored by phase microscopy. The homogenate was spun at 1500 × g for 5 min. The mixture was then spun at 15000 × g for 5 min, and both the supernatant containing anti-c-myc monoclonal antibody 3C7 and the pellet were analyzed by Western Blotting. Western Blots were performed using standard procedures (20) and detected using enhanced chemiluminescence (Amersham).

**Skeletal Stains—**Skeletal analysis was done on embryonic and adult limbs. To obtain embryos for staining, females were examined daily for vaginal plugs. Noon of the day of the vaginal plug was designated as embryonic day 0.5. Day 15.5 embryos were dissected and fixed overnight in 4% paraformaldehyde at 4°C. After a washing with phosphate-buffered saline (twice, 30 min each), embryos were treated with 5% trichloroacetic acid for 3 h. They were then washed with phosphate-buffered saline (twice, 5 min each), followed by 70% ethanol (5 min). Embryos were then stained overnight in 0.1% Alcian green in 70% ethanol. After washing (5 parts 70% ethanol: 1 part 5% trichloroacetic acid) for several hours and dehydrating in ethanol (2 times, 30 min), samples were cleared in methylsalicylate. Adult limbs were stained with Alcian blue and Alizarin Red S as described previously (22). After clearing, limbs were dissected to facilitate photography.

**RESULTS**

**Three Limb Deformity Alleles Encode COOH-truncated Formins—**Previous studies have shown that three ld alleles, ldTgBri, ldTgHd, and ldIn2, are associated with structural alterations within the formin gene locus (6, 7, 10). The sites of these alterations within formin genomic DNA are schematically shown in Fig. 1A. In the two transgene-induced alleles, ldTgHd and ldTgBri, the disruptions occur in the intron sequences 5’ of exon A, located close to the 3’ end of the formin gene (2, 7). In the ldIn2 allele, caused by a chromosomal inversion event, the rearrangement breakpoint occurs in intronic sequences 3’ of...
These epitopes are common to isoforms I, II, and IV. Isoform III does not encode these epitopes due to an alternative splice which results in early termination. A second polyclonal serum (B) was raised against amino acids 1–457 of formin isoform IV. This second antiserum recognizes epitopes unique to isoform IV and was used in this study to confirm results obtained using antiserum A. To test the isoform specificity of our antisera, we used these antisera to immunoprecipitate in vitro translated isoforms I and IV. As expected, antiserum A precipitated both isoforms I and IV, whereas antiserum B precipitated only isoform IV (Fig. 2B). No immunoreactivity was obtained from preimmune serum.

We used affinity-purified antibody A to probe Western blots containing total cell lysates from wild-type and ld fibroblasts (Fig. 2C). Consistent with previous immunoprecipitation experiments using anti-peptide antibodies (17), wild-type fibroblasts contained a predominant immunoreactive band migrating at ~165 kDa. This band has the same molecular weight as in vitro translated isoform IV, which is the major isoform in fibroblasts (17). This 165-kDa band is also detected using antiserum B (Fig. 3B), further supporting its identification as the formin gene product. Most definitively, mutant mice containing a targeted disruption of formin isoform IV lack this 165-kDa protein. In Western blots probed with antibody A, there is also a prominent band at ~110 kDa; at present we do not know the identity of this polypeptide. This 110-kDa band is not recognized with antiserum B and is not altered in the ld mutants, suggesting that it is likely to be a nonspecific band. In the experiment shown in Fig. 2C, the 110-kDa band is apparently decreased in the ld sample, but this result is not seen in other experiments and is unlikely to be significant. In both ld and ld fibroblasts, the 165-kDa band is absent; instead there is a smaller band migrating at ~152 kDa (Fig. 2C). In ld lysates, the 165-kDa band is replaced by one at ~160 kDa. Mutant tissue lysates from brain, kidney, and muscle also showed truncated bands when compared with wild-type tissues (data not shown). Taken together, these results identify formin isoform IV as a 165-kDa protein and demonstrate that the alleles ld, ld, and ld produce stable, truncated formins consistent with their mutant cDNAs.

A Large Portion of Wild-type Foramin Is Stably Associated with Nuclei in Fibroblasts—Since the truncated ld formins are stable and show normal tissue distribution, we sought to uncover biochemical differences between wild-type and mutant formins. We used biochemical subcellular fractionation techniques (see “Materials and Methods”) to localize wild-type formin in primary (Fig. 3) and established cell lines (data not shown). Briefly, fibroblast monolayers were swelled in a hypotonic solution and lysed by Dounce homogenization. Nuclei and unlysed cells were pelleted by a 1000 × g spin. Purified nuclei were obtained from this crude nuclear pellet by two washings with a Nonidet P-40 containing buffer. The original supernatant (nonnuclear fraction) was spun at 100,000 × g to obtain the soluble 100 fraction (cytosol) and the pellet P100 fraction (large complexes and cellular membranes). When Western blots containing these fractions were probed with antisera A or B (Fig. 3, A and B), we found that a substantial portion of the total formin protein is retained in the nuclear fraction under these separation techniques. At 100 mM NaCl most of the nuclear formin remains stably associated with nuclei, but at 250 mM NaCl the majority is eluted from the nuclei (Fig. 3C).

Our cell fractionation experiments also show that much of the nonnuclear formin sediments into the P100 fraction. This

A. Wynshaw-Boris, D. Chan, and P. Leder, manuscript in preparation.

D. Chan and P. Leder, unpublished results.
result raises the possibility that the nonnuclear formin may be associated with a large macromolecular complex. We find that the amount of formin present in the S100 fraction can vary substantially in different experiments, suggesting that it is sensitive to experimental conditions.

IdTgHd and ldTgBri Formins Are Cytosolic—We performed subcellular fractionation experiments on primary fibroblasts cultured from homozygous mutant animals. With both ldTgBri and ldTgHd fibroblasts, we obtained a fractionation profile strikingly distinct from that of wild-type fibroblasts (Fig. 4). There are two major differences in the localization of ldTgBri and ldTgHd formins compared to wild-type formins (Fig. 4, A–C). First, the mutant formins are absent from the nuclear fraction and instead are found almost entirely in the S100 fraction. Second, very little of the formin in the nonnuclear fraction sediments during the 100,000 × g centrifugation (P100), suggesting that these truncated formins are not associated with a large macromolecular complex. These differences were reproducible on multiple experiments. ldBri cells showed a similar but less dramatic localization defect. ldIn2 formin is found predominantly in the S100 and P100 fractions, but a smaller portion is present in the nuclear fraction (Fig. 4D).

We checked our fractionation protocol by light microscopy at various stages of the procedure and found clean separations in each of the cell lines (data not shown). Furthermore, control experiments using the nuclear marker c-Myc showed that the nuclear localization defect that we observed in mutant cells is specific for formin. C-Myc is detected only in the nuclear fraction of wild-type and mutant cells (Fig. 4E).

To rule out the possibility that these strikingly different fractionation profiles were due to stochastic differences among primary cell lines or to experimental variability, primary cell lines from heterozygous ldTgBri/+ and ldIn2/+ mice were derived (we were unable to perform similar experiments with ldTgHd mice because this mutant is no longer available). Immunoblot analysis demonstrates that the heterozygous ldTgBri/+ cell line contains both the wild-type 165-kDa formin and the mutant 152-kDa formin (Fig. 5A). Thus, both the wild-type and mutant isoforms can be analyzed within the same cell line and within a single fractionation experiment. With the ldTgBri/+ cell line, a substantial portion of the wild-type formin is present in the nuclear fraction, while the truncated, mutant formin is found solely in the S100 fraction (Fig. 5A). Similarly, the truncated ldIn2 formin in the ldIn2/+ cell line is found in proportionately larger quantities in the S100 fraction than wild-type formin, although the difference is not as great as with ldTgBri formin (Fig. 5B). These results agree well with the results obtained from homozygous cells.

ldIn2 Mice Have Less Severe Hind Limb Defects Than ldTgBri Mice—As described above, ldIn2 formin contains a less severe COOH truncation than ldTgBri and ldTgHd formins. In addition,
ldIn2 formin has a less dramatic localization defect than ldTgBri and ldTgHd formins. This correlation between degree of truncation and severity of localization defect raises the possibility that ldIn2 may be a weaker allele than both ldTgBri and ldTgHd. Consistent with this view, we noticed that ldIn2 mice could usually be distinguished from ldTgBri mice by inspection of their hind limbs. The distal portion of ldIn2 hind limbs is typically thicker than that of ldTgBri hind limbs, which appear to contain fewer digits. To define this observation further, we performed skeletal stains on adult and embryonic limbs from these two mutants (Table I; Fig. 6). ldTgBri hind limbs typically (95%) contained only a single digit, resulting in a distal hind limb that is very narrow (Fig. 6C). In contrast, only 16% of ldIn2 hind limbs displayed such a severe defect, with most hind limbs (84%) showing two or more distinct digits (Fig. 6B). No significant differences were observed in the forelimbs.

DISCUSSION

We have shown that three ld alleles result in COOH-truncated formins. Two such alleles, ldTgHd and ldTgBri, encode similarly truncated formins that show abnormal subcellular localization. Whereas a substantial proportion of wild-type formin is stably associated with nuclei, both these mutant formins are localized almost exclusively to the cytosol. Furthermore, the mutant formins are not sedimented by 100,000 × g centrifugation, suggesting that they are not part of a large macromolecular complex. We conclude that the COOH residues deleted in ldTgHd and ldTgBri are essential for nuclear localization and possibly for interactions with other molecules. A third allele, ldIn2, leads to a smaller truncation, and the corresponding formin shows a less complete localization defect.

At present the precise role of the highly basic COOH region (pI = 9.6 for the COOH-terminal 110 residues) in nuclear localization is unclear. Inspection of this sequence reveals a deleted in centromere (Drosophila) homology to formins, and potentially for interaction with other molecules. A third possibility is that the COOH-terminal residues are essential for nuclear localization, but rather that other critical portions of the molecule suffer conformational changes due to the truncations.

Work on cappuccino, a Drosophila gene with sequence homology to formins, also suggests an important role for the carboxy region of formin. Cappuccino belongs to a family of related genes containing two regions of homology (termed FH1 and FH2) to formins (15). One of these homology regions, FH2, is located near the COOH terminus of formin and ends slightly amino terminal to the breakpoint junction found in ldTgHd and ldTgBri. The homology between cappuccino and formins, however, is particularly high and extends beyond FH2 and into the extreme COOH terminus. Significantly, two cappuccino mutants, capBr/ and capHd, contain mutations in the carboxy region (15), close to the corresponding region on formin where the breakpoint junctions of ldTgHd, ldTgBri, and ldIn2 reside. These results reinforce our general conclusion that the COOH region of formin plays a critical function, and in future work it will be interesting to determine whether defective subcellular localization occurs in these two cappuccino mutants.

The observation that ldIn2 shows both a less severe truncation and a less complete localization defect when compared with either ldTgHd or ldTgBri raises the issue of allelic strength. Since the site of formin action is thought to reside in the nucleus (16, 17), our results suggest that ldTgHd and ldTgBri are severe loss-of-function alleles, possibly null alleles, and that ldIn2 is a weaker allele. Consistent with this view, the hind limbs of ldIn2 mice show less severe digit reductions than those of ldTgHd and ldTgBri mice. Since these ld alleles are on mixed genetic backgrounds, it remains possible that these phenotypic differences reflect strain differences. Similar comparisons, unfortunately, could not be performed on ldTgHd hind limbs, since this line is no longer available.

The renal phenotype of ldIn2 mice also appears to be signifi-
significantly less severe than that of \(ldTgHd\) or \(ldTgBri\) mice. First, the incidence of renal aplasia ranges from 57% with \(ldTgHd\) and 33% with \(ldTgBri\) to 2.5% with \(ldIn2\) mice (6, 7, 9, 24). Second, unlike the other \(ld\) alleles, in which renal aplasia is the predominant phenotype, the most common renal phenotypes in \(ldIn2\) mutant mice are hydronephrosis and hydroureter. Renal agenesis in \(ldIn2\) mice is much rarer (6). The renal aplasia in \(ld\) animals is due to failure of proper ureteric bud outgrowth into the metanephric cap (9). Hydronephrosis and hydroureter, seen in \(ldIn2\) mice, may be less severe manifestations of the same underlying developmental defect. These observations are consistent with the model that \(ldTgBri\) and \(ldTgHd\) are more severe loss-of-function alleles than \(ldIn2\). As with the discrepancies in limb phenotype, it is possible that these differences in penetrance and expressivity reflect the influence of modifying loci as well as the intrinsic strengths of the alleles. Mating experiments, however, have suggested that the differences in the renal phenotype are more likely a result of intrinsic allelic strength (9).

In summary, our biochemical analyses of wild-type and mutant formins define a region required for the nuclear localization of formins and emphasize the nucleus as the site of action of formins. Our results provide genetic evidence that formins function within the nucleus. Consistent with these results, previous studies showed that chicken formin is present in a punctate nuclear pattern (16) and that murine formins are nuclear phosphoproteins that bind to DNA-cellulose (17). While the precise biochemical function of formins remains to be determined, the recent identification of two classes of proteins that potentially interact with formins should provide new avenues toward discovering their function within the nucleus (12).

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