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Evaluation of *Lama5* as a Candidate for the Mouse Ragged (*Ra*) Mutation

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The laminin α5 chain is a component of the basement membranes of many developing and adult tissues. The mouse laminin α5 chain gene (*Lama5*) has been mapped close to the locus of the semidominant ragged (*Ra*) mutation on distal chromosome 2. The cause of the *Ra* mutation, which is usually lethal in the homozygous state, has not been determined. We have investigated whether a defect in *Lama5* is responsible for the ragged mutation, using the Ra^J strain. No differences in the level of the laminin α5 chain transcript were found in placental RNA from homozygous Ra^J mutant embryos compared to normal littermates. Antiserum raised against a recombinant laminin α5 chain polypeptide stained the basement membranes of both normal and homozygous mutant embryos to a similar extent. More precise mapping of *Lama5* on an interspecific *Ra* backcross indicated that *Lama5* is proximal to the *Ra* locus. These results exclude *Lama5* as a candidate gene for the *Ra* mutation.

The laminins are basement proteins composed of three non-identical subunits, the α, β, and γ chains. Multiple isoforms of each chain exist in vertebrates (α1-α5; β1-β3; γ1-γ2), which can assemble to form at least 11 different laminin molecules that have distinct tissue distributions and diverse biological functions (1-3). The most recently identified laminin subunit, the α5 chain, is widely expressed in rodent and human tissues (3-7). Immunohistochemical studies in rodents have shown that the α5 chain is present in many developing and adult epithelial basement membranes, including those of the lung and kidney, in smooth muscle basement membranes, and in some endothelial basement membranes (3, 8, 9). Laminin α5 chain immunoreactivity was found in embryonic skeletal muscle basement membranes but in adult muscle was restricted to the neuromuscular junctions (8,10). A similar distribution pattern for the laminin α5 chain has been shown in human tissues using the 4C7 monoclonal antibody (11-15), previously thought to recognize the α1 chain but now known to react with the α5 chain (16, 17). Several biological activities have been attributed to laminin isoforms containing the α5 chain; laminin 11 ($\alpha5\beta2\gamma1$) inhibits the outgrowth of neurites from motor neurons (10) and the migration of Schwann cells (18), and laminin 10 ($\alpha5\beta1\gamma1$)/laminin 11 has been shown to promote cell attachment via the $\alpha3\beta1$ integrin (19).

Insight into the crucial functions performed by specific laminin chains has come from the identification of inherited disorders caused by mutations in the laminin α2, α3, β3, and γ2 chain genes and from the targeted disuption of the β2 chain in mice (reviewed in ref. 20). The human laminin α5 chain gene (LAMA5) on chromosome 20q13.2 has not yet been linked to any genetic disease (7), but we and others have mapped the mouse gene (*Lama5*) to distal chromosome 2 close to the locus of the semidominant Ragged (*Ra*) mutation (3, 7). Heterozygous *Ra* mice have thin, ragged fur due to the incomplete development of some hair follicle types, while homozygous *Ra* mice are nearly hairless (21). *RaRa* embryos and newborns are frequently non-viable and suffer from generalized edema (21, 22), suggesting that in the homozygous state the *Ra* mutation may lead to defects in organs such as the heart, lung, or kidney, in addition to the skin (22). The nature of the *Ra* mutation is not known, but due to the close linkage of *Lama5* and *Ra*, and the expression of the laminin α5 chain in tissues such as the skin, lung, and kidney, we have evaluated *Lama5* as a candidate for the *Ra* mutation.

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901  GTAAAGTCTCATGAGAGAACAACTCCCATGCTCCAACTA
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FIG. 1. Sequence of a segment of the mouse laminin α5 chain gene amplified from C57BL/6J mouse genomic DNA. The fragment was obtained by PCR using primers corresponding to nt 8839–8859 and 9452–9471 of the cDNA sequence (4). The genomic DNA has three small introns located after nt 8990, nt 9267, and nt 9398 of the cDNA sequence. Exon sequences are in upper case letters and intron sequences are in lower case letters. The sequences of the 178F (nt 1–21), 179R (nt 920–939), and 302R (nt 453–474) PCR primers are underlined. The sequence is available from the EMBL/GenBank database under the accession number AJ006993. Sequencing of the genomic DNA fragment amplified from *Mus spretus* DNA with the same primer pair revealed the following single base pair changes in the *M. spretus* allele: C at nt 193; A at nt 387; C at nt 416; T at nt 552; G at nt 570; and C at nt 894.

MATERIALS AND METHODS

Ragged mice. Breeding pairs of C3H/HeSnJ-*Ra*^{J} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The *Ra*^{J} mutation arose at The Jackson Laboratory, and the mice have a phenotype similar to that of the original *Ra* strain (Hope Sweet, The Jackson Laboratory, personal communication). From one breeding pair we obtained nine heterozygous *Ra*^{J} F1 sister and brother pairs. We were unable to obtain live homozygous *Ra*^{J} mice from the *Ra*^{J}/+ *Ra*^{J}/+ matings, and therefore sacrificed pregnant females to examine F2 embryos. A total of 73 late gestation embryos were examined. Several non-viable embryos were seen in each litter. Two embryos that were clearly edematous, and with macroscopic features typical of those described for homozygous *Ra* embryos (22), were used for RNA extraction and immunohistochemical analysis. Apparently normal littermates served as controls.

RNA extraction and Northern blotting. Total RNA was extracted from the placentae of normal and edematous mouse embryos using the TRIzol reagent (Gibco/BRL). Fifteen μg of total RNA per lane was denatured with formamide/formaldehyde and separated on a 1% agarose denaturing formaldehyde gel (23) and capillary-blotted onto Hybond N+ membranes (Amersham). The blots were hybridized to the 0.94 kb insert of p1121, which was obtained by PCR amplification of genomic DNA from C57BL/6J mice using primers derived from the mouse laminin α5 chain cDNA sequence (ref. 4; GenBank U37501): 178F (5′-GCGATCCAAGTGTTTCTATTG, nt 8839–8859) and 179R (5′-TAGTTGGAGCATGGGAGTTG, nt 9452–9471). The PCR product was subcloned into the pCR-Script vector (Stratagene) and sequenced as previously described (24). Labeling of the probe

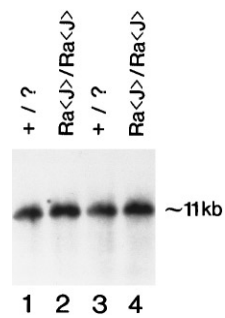

FIG. 2. Expression of laminin α5 chain mRNA in *Ra*^{J} embryos. Northern blot of total RNA (15 μg per lane) from the placentae of two apparently normal (+/?) embryos (lanes 1 and 3) and from two presumably *Ra*^{J} homozygous (Ra<J>/Ra<J>) embryos (lanes 2 and 4) hybridized to a mouse laminin α5 chain probe. Similar levels of the ≈11 kb laminin α5 chain transcript were found in both the normal and homozygous mice.

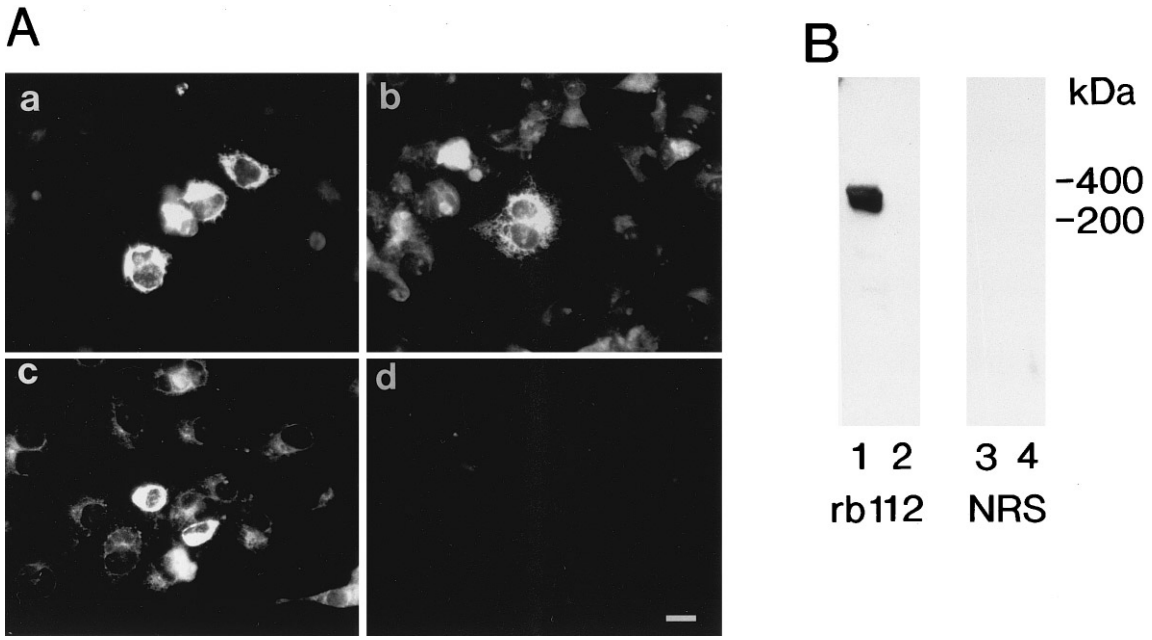

FIG. 3. Characterization of antisera to the laminin α5 chain. Recombinant laminin α5 polypeptides produced in *E. coli* were used as immunogens to raise polyclonal antisera to the mouse and human laminin α5 chains. Panel A shows that these antisera stain COS-7 cells transiently transfected with a human laminin α5 chain expression construct (p1150): (a) rb111 to human α5, (b) rb 112 to mouse α5, and (c) rb 114 to mouse α5 chain, but not cells transfected with the vector with no insert (d); scale bar is 12 μm. Panel B shows that these antisera react with a single protein band of M_r $\approx$350 kDa in Western blotting of clone A cell layer (lane 1), but apparently no α5-chain containing laminin is secreted into the medium (lane 2). In lanes 1 and 2 rb 112 antiserum and in lanes 3 and 4 normal rabbit serum has been used.

with [α-^{32}P]dCTP and hybridization and washing of the blots were performed using previously described methods (25).

Constructs for synthesis of mouse and human recombinant laminin α5 chain polypeptides. For production of a mouse laminin α5 chain recombinant polypeptide, a 0.53 kb cDNA fragment encoding 176 amino acids in the G3-G4 repeats of the C-terminal G domain of the mouse laminin α5 chain polypeptide (residues 3105–3280 of ref. 4) was obtained by RT-PCR. cDNA was synthesized using total RNA from newborn mouse lung as previously described (25) and then amplified with *Pfu* polymerase (Stratagene) and the primers 313F (5′-dGAGGATCCGTTTTATGAACCAAGAGGTG, nt 9311–9333 of the mouse cDNA sequence, plus a *Bam*HI linker) and 312R (5′-dAGAGGTGGAGCCTATTCCTGTGG, complimentary to nt 9819–9841). The PCR product was cut with *Bam*HI and subcloned into the *Bam*HI/*Sma*I site of pBluescript (Stratagene) and sequenced to confirm its identity. The insert was then excised as a *Bam*HI/*Pst*I fragment and cloned into the *Bam*HI/*Pst*I site of the 6′ His-tag bacterial expression vector pQE32 (Qiagen) to give p1141.

For production of a human laminin α5 chain recombinant polypeptide, a 0.60 kb *Bam*HI/*Pst*I fragment of the human laminin α5 chain cDNA clone p1047 (ref.7; nt 2081–2683 of GenBank Z95656) was subcloned into the *Bam*HI/*Pst*I site of pQE32, yielding p1122, which encodes 201 amino acids in the G4-G5 repeats of the G domain. The human and mouse laminin α5 chain expression plasmids were transformed into *E. coli* M15[REP] cells. Synthesis of the His-tagged recombinant laminin α5 polypeptides was induced with 1 mM IPTG, and the cell pellets were extracted with lysis buffer (6 M guanidine HCl/0.02 M Tris pH 7.9/0.5 M NaCl/5 mM β-mercaptoethanol). The His-tagged fusion proteins were bound to a TALON immobilized metal ion affinity resin (Clontech) in lysis buffer, washed with the same buffer, and eluted in lysis buffer containing 0.05 M Na_2EDTA.

For testing the specificity of the antisera, a human laminin α5

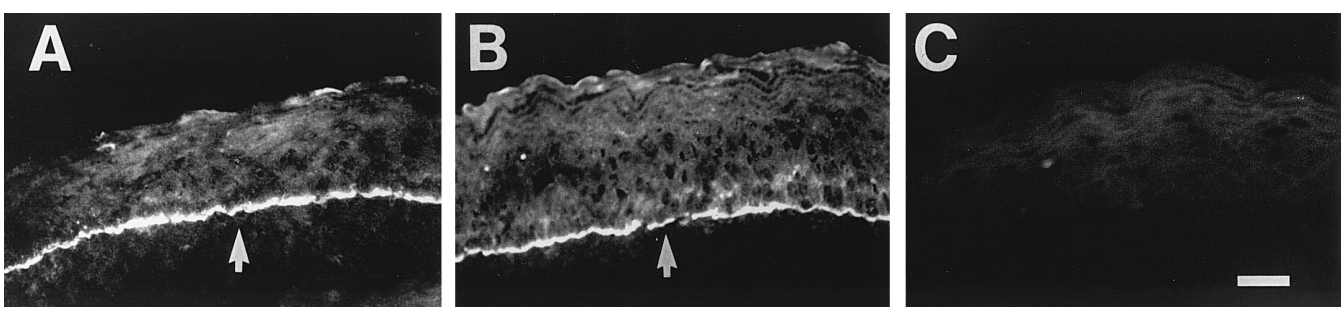

FIG. 4. Immunostaining of mouse embryo skin sections with antisera to the laminin α5 chain. The epidermal basement membranes (arrows) of a normal mouse embryo (+/?) (A) and of an Ra^J/Ra^J embryo (B) were stained with rb 114 (1:100). No staining was observed in a parallel section in which the antiserum was preabsorbed with the recombinant polypeptide used for immunization (C). Scale bar is 22 μm.

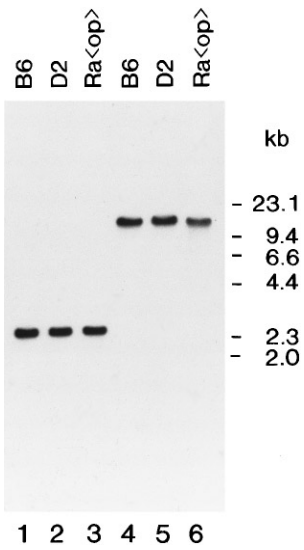

FIG. 5. Southern blot analysis of the laminin α5 chain gene in *Ra*^{op} mice. Southern blot of genomic DNA from C57BL/6J (B6), DBA2/J (D2) and B6D2-*Ra*^{op} (Ra<op>) mice digested with *Sst*I (lanes 1–3) and with *Eco*RI plus *Bam*HI (lanes 4–6) was hybridized to a laminin α5 chain probe. No differences were observed in the restriction fragment pattern of *Lama5* in the mutant strain and the two parental strains. The migration of the size markers (*Hin*dIII fragments of bacteriophage λ DNA) in kb is indicated.

chain eukaryotic expression vector encoding the entire 953-amino acid G domain was constructed from two overlapping α5 chain cDNA clones (7). The *Bgl*II site in the insert of p1140, located in the 3′ UTR of the α5 chain cDNA (7), was converted to a *Xho*I site, and the 2.4 kb *Sal*I/*Xho*I fragment (nt 573–2968 of GenBank Z95656) was subcloned into pBluescript. A 0.58 kb PCR product was amplified with *Pfu* polymerase using p1079 as a template and the primers 332F (5′-dCGAAGCTTCTCAGGGGTGCAGCTGC, nt 1–16 plus a *Hin*dIII linker) and 333R (5′-dCCGGTCGACTTGGAGCGG (complimentary to nt 564–581). The product was digested with *Hin*dIII and *Sal*I and ligated into the *Hin*dIII/*Sal*I site of the plasmid constructed in the previous step. The 3 kb insert was excised by digestion with *Hin*dIII and *Xho*I and subcloned into the *Hin*dIII/*Xho*I site of pSecTagA (In-Vitrogen) to give p1150, in which the α5 G domain is fused in-frame with the Igκ chain signal peptide and expressed under the control of the CMV promoter.

Production and characterization of polyclonal antibodies to recombinant human and mouse laminin α5 chain polypeptides. Rabbits were immunized with recombinant laminin α5 polypeptides derived from expression constructs p1141 (mouse) and p1122 (human) as previously described (24, 25). The sera from three rabbits were used in the present study, rb 112 and 114 to the mouse laminin α5 chain and rb 111 to the human laminin α5 chain. The antisera were characterized as described earlier (24, 25) using the following approaches: a) immunostaining of COS-7 cells transiently transfected with the laminin α5 G domain expression vector p1150 or with a vector control; b) Western blotting of authentic laminin produced by human clone A colon carcinoma cells in culture; and c) ELISA assays using as a substrate human placental laminin (Gibco-BRL 12163-010), which contains the α5 chain (19).

Laminin α5 immunohistochemistry on tissue sections. Cryostat sections were air-dried and fixed in pre-cooled acetone at 4°C for 15 min. Each of the respective antisera (diluted 1:100) were applied to the sections and incubated for 1 h at room temperature. Following a thorough rinse, the sections were incubated with fluorescein isothiocyanate-coupled secondary antibodies 1:50 (DAKO) for 30 min. Incubations with both primary and secondary antibodies were performed in 0.05 M Tris-HCl (pH 7.2) and rinses in 0.05 M Tris-HCl (pH 7.2) containing 0.15 M NaCl and 0.05 % Tween 20. On control sections, the specific antisera were omitted or replaced with preimmune rabbit antiserum. As a further control, the inhibitory effect of simultaneously incubating the antisera with the respective recombinant polypeptides (final concentration 20 μg/ml) was examined. The slides were mounted in buffered glycerol and examined using an Axiovert inverted microscope connected to a PentaMAX chilled charge-coupled device camera (Princeton Instruments, Inc., Trenton, NJ) and a Dell computer.

Southern blotting. DNA from the C57BL/6J, DBA/2J, and B6D2-*Ra*^{op} mouse strains was purchased from The Jackson Laboratory Mouse DNA Resource. DNA aliquots (3 μg per lane) were digested with *Bam*HI, *Eco*RI, and *Sst*I, separated on 1% agarose gels, and blotted onto Hybond N+ membranes. The blots were hybridized to the ^{32}P-labeled insert of p1121 as described above and also to a probe corresponding to nt 173–557 of the cDNA, generated by RT-PCR and subcloned into pCR-Script (p1232).

Mapping of Lama5 on the Ra backcross. *Lama5* had been mapped to distal mouse chromosome 2 by following the segregation of a *Stu*I polymorphism in mice from the Jackson Laboratory BSS backcross mapping panel (7). An attempt to map *Lama5* relative to *Ra* on the (*Ra*/+ ×′ *M. spretus*)F1′ C3H/HeH backcross described previously (26), which now has generated nearly 1000 progeny, revealed that the *Stu*I site was not informative in this cross. Consequently, genotyping of critical recombinants in the *Ra* backcross was performed by PCR amplification using the 178F primer described above and the 302R primer (5′-dCCTGGGTGCCACGAAAGCCAAA, complementary to nt 9214–9235), followed by sequencing of the amplification products to score for *M. spretus* -specific sequences at nt 193 and 416.

RESULTS AND DISCUSSION

The ragged mutation was first reported by Carter and Phillips (21) and described in more detail by Slee (22, 27, 28). A high proportion of homozygous *Ra* embryos die *in utero*, and newborn *Ra* homozygotes are often edematous and dyspneic, and most die before weaning (21, 22). About 21% of the post-12=4 day embryos from *Ra*/+ ×′ *Ra*/+ matings were found to have generalized edema and were presumed to be *Ra* homozygotes (22). Except for the skin and hair follicles, detailed histological studies of the tissues of the affected mice have not been performed (27, 28). As the physiological origin of the edema and the molecular nature of the *Ra* mutation have not been elucidated, we have asked whether the laminin α5 chain may be involved in the ragged phenotype.

In the present study, we examined the progeny of *Ra*^{J}/+ ×′ *Ra*^{J}/+ matings during late gestation. Two embryos with distinct high grade edema were assumed to be homozygotes. Several non-viable embryos were also observed; some of the latter showed mottled skin typical of homozygotes (22), but these were not studied further. The edema of the two presumptive *Ra*^{J} homozygotes appeared as a broad region of translucency beneath the epidermis and consisted of undifferentiated mesenchymal tissue, similar to that described for the original *Ra* strain (22). The two edematous embryos

and several normal littermates were used for analysis of the expression of the laminin α5 chain mRNA and protein.

To obtain a DNA probe for Northern and Southern blot analysis of the mouse laminin α5 chain mRNA and gene, we used PCR to amplify a segment of the genomic DNA that encodes nt 8839–9471 of the cDNA. The 939 bp PCR product contained three small introns that account for less than half of the genomic DNA sequence (Fig. 1). Total RNA from the placentae of normal and mutant embryos was fractionated by Northern blotting and hybridized to the mouse laminin α5 chain probe. Both the normal and mutant embryos contained high levels of the ≈11 kb laminin α5 chain mRNA (Fig. 2). This suggests that the expression of the laminin α5 chain transcript is not deficient in the homozygous Ra^J mutant embryos. The laminin α5 chain mRNA was also detected in several tissues from *Ra* mice, using RT-PCR and the mouse-specific primers described in ref. 7 (not shown).

To compare the distribution of the laminin α5 chain in both normal and Ra^J mice, we characterized antisera raised against a recombinant laminin α5 polypeptide produced in *E. coli*. The antisera stained COS-7 cells transfected with a human laminin α5 chain G domain expression vector but not cells transfected with a vector without insert (Fig. 3A). In ELISA assays, these antisera reacted with purified human laminin (not shown) and on Western blots reacted with an ≈350 kDa band in extracts of cultured clone A colon carcinoma cells, which contain high levels of α5 chain mRNA (7) (Fig. 3B). These laminin α5 antisera reacted with basement membranes of mouse embryos (E15) essentially as described (3, 8, 10), in that we observed a distinct immunostaining of the skin, skin appendages, and lung epithelial basement membranes, and heterogeneous staining of the developing skeletal muscles. The basement membranes of both normal and edematous Ra^J embryos were stained to a similar extent with antiserum against the laminin α5 chain, as typified by the strong staining of the skin basement membranes shown in Fig. 4. Little or no laminin α5 staining in normal adult mouse skin basement membrane was found using our antisera; this suggests that expression of the α5 chain is post-natally downregulated in the skin, as it is in the muscle basement membranes. The epidermis of the Ra^J homozygote shown in Fig. 4 is hyperplastic/acanthotic, which has also observed in the skin of *Ra* homozygotes (28). At other sites the epidermis appeared histologically normal. In the two late gestation edematous embryos no gross difference in hair follicle density was apparent (not shown), in accordance with previous reports (28).

There exists a third, more severe allele of ragged, the semidominant lethal ragged-opossum (Ra^{op}) mutation, which arose spontaneously in a C57BL/6J ×′ DBA/2J F1 offspring (29). The Ra^{op} heteozygotes have very sparse fur and low viability, and all homozygotes die *in utero* (29, 30).To test whether the more severe Ra^{op} strain may carry a null allele of *Lama5* associated with a large deletion or rearrangement of the gene, we used Southern blot hybridization to compare the restriction fragment pattern of *Lama*5 in genomic DNA from the Ra^{op} strain and the two parental strains. No differences were observed on blots of genomic DNA from the mutant and parental strains cut with *Sst*I or with *Bam*HI plus *Eco*RI and probed with a DNA fragment from the 3′ end of the gene (Fig. 5). Similar results were obtained when the blot was hybridized to a probe from the 5′ end of the gene (not shown).

Finally, we attempted to map the location of *Lama5* with respect to *Ra* more precisely using an interspecific *Ra* backcross (26). Since the restriction site polymorphism originally used to map *Lama5* to distal chromosome 2 was not informative in this cross, we employed sequencing of PCR products amplified from genomic DNA to type the progeny of the backcross and were thus unable to score all of the mice. Genotyping of eight critical recombinants in the backcross revealed two recombinants between *Lama*5 and *Ra*, indicating that *Lama5* and *Ra* do not co-segregate and that *Lama5* is proximal to *Ra* (data not shown).

The widespread distribution of the laminin α5 chain in the basement membranes of many tissues during mouse development implies that it plays an important role in organogenesis, and mutations in the gene would be anticipated to have deleterious consequences. *Lama5* appeared to be a promising candidate gene for the *Ra* mutation, as the low survival rates of *Ra* and Ra^J homozygotes and the embryonic lethality of Ra^{op} homozygotes indicate that the ragged locus encodes a gene essential for normal development. However, no deficiency of either the laminin α5 chain protein or the mRNA were found in Ra^J homozygotes, and no evidence for a deletion or rearrangement of *Lama5* in the more severe Ra^{op} mutant was obtained. These results do not rule out the possibility that the ragged mutation could be due to a point mutation or in-frame deletion in *Lama5* that impairs the function of the protein but does not alter its immunoreactivity or decrease the mRNA levels. However, more precise mapping of *Lama5* indicates that it is distinct from the *Ra* locus. The evidence presented here thus seems to rule out a mutation in the laminin α5 chain gene as the cause of the *Ra* phenotype. Further studies aimed at analyzing the function of the laminin α5 chain in various tissues during development *in vivo* may require the use of transgenic mice to introduce targeted mutations in the gene.

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