Mice develop normally without tenascin

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Tenascin, an extracellular matrix protein, is expressed in an unusually restricted pattern during embryogenesis and has been implicated in a variety of morphogenetic phenomena. To directly assess the function of tenascin in vivo, we generated mutant mice in which the tenascin gene was nully disrupted by replacing it with the *lacZ* gene. In mutant mice, *lacZ* was expressed in place of tenascin, and no tenascin product was detected. Homozygous mutant mice were, however, obtained in accordance with Mendelian laws, and both females and males produced offspring normally. No anatomical or histological abnormalities were detected in any tissues, and no major changes were observed in distribution of fibronectin, laminin, collagen, and proteoglycan. The existence of these mutant mice, lacking tenascin yet phenotypically normal, casts doubt on the theory that tenascin plays and essential role in normal development.

[*Key Words*: Tenascin/cytotactin; extracellular matrix; homologous recombination; gene targeting; LacZ expression]

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Tenascin (TN) (Chiquet-Ehrismann et al. 1986) is a large extracellular matrix (ECM) glycoprotein that shares several structural and biological features with other ECM molecules such as fibronectin (FN) and laminin (LN). The macromolecule consists of 180- and 230-kD subunits, and it appears as a hexabrachion with six arms extending from a central core united by disulfide linkages at the amino termini (Vaughan et al. 1987). TN binds to FN, chondroitin sulfate proteoglycan (Hoffman et al. 1988), and a variety of cells including fibroblasts and neurons; the binding sites have been mapped in the distal portion of the arms (Friedlander et al. 1988; Spring et al. 1989). TN subunits have a multidomain: heptad repeats in the amino termini, epidermal growth factor (EGF)-like repeats, FN type III (FN3)-like repeats, and fibrinogen homologous sequences in the carboxyl termini (Gulcher et al. 1989; Jones et al. 1989; Spring et al. 1989; Saga et al. 1991; Siri et al. 1991; Weller et al. 1991). Although the polypeptide subunits that are expressed during development in different organs vary in molecular mass, all of these polypeptides appear to be the products of a single gene after alternative splicing in the FN3 repeat domain (Jones et al. 1989; Gulcher et al. 1991; Saga et al. 1991). TN is also known by the synonyms cytotactin (Grumet et al. 1985), hexabrachion (Erickson and Inglesias 1984), J1 glycoprotein (Kruse et al. 1985), myotendinous antigen (Chiquet and Fambrough 1984), and glioma mesenchymal ECM antigen (GMEM) (Bourdon et al. 1983).

Attention has been centered on the differential contributions to morphogenesis of ECM proteins TN, FN, and LN, which are present in overlapping spatial distributions in vivo. For example, they are all located in neural crest pathways and in a variety of basement membranes (Halfter et al. 1989). In general, however, LN and FN are distributed more widely in the embryo, and TN has a restricted distribution that changes during development (Crossin et al. 1986; Saga et al. 1991). In contrast, in the central nervous system (CNS), TN is present at high levels in a wide distribution, whereas LN is found only at certain sites and, except for basement membranes, FN may not be present at all (Grumet et al. 1985; Liesi 1985; Halfter et al. 1989). On the basis of its unique expression pattern, inhibition studies with monoclonal antibodies, and in vitro experiments, TN is believed to have a prominent role in such developmental processes as embryonic pattern formation, cell adhesion and migration, epithelial shedding and renewal, epithelial–mesenchymal interface formation, organogenesis, and somatic growth regulation. Recent studies have emphasized the important role of TN during neuronal development (Faissner and Kruse 1990; Husmann et al. 1992; Mege et al. 1992). However, the results have been contradictory (Stern et al. 1989; Tan et al. 1991; Bartsch et al. 1992), and the functional role of TN in vivo still remains a matter of conjecture.

One of the most promising approaches to the identification of gene function involves analysis of carrying lesions in specific genes. In this study with homologous recombination, we produced mutant mice in which TN gene expression was completely disrupted. Unexpectedly, the homozygous null mutant mice were born live, and no defects were apparent.

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Results

TN targeting in mouse embryonic stem cells

The introduction of *lacZ* into specific gene loci has proved useful for analysis of interactions among genes that participate in developmental regulatory networks in *Drosophila* and *Caenorhabditis elegans*. By moving the *lacZ* gene, which replaced TN, into different mutant backgrounds, we were able to examine the effects of other genes, for example, a homeo box gene *Evx-1* (Jones et al. 1992), on the expression of TN during embryogenesis. Accordingly, this gene was replaced by the *lacZ* gene by use of homologous recombination in mouse embryonic stem (ES) cells. Figure 1A shows the vector for TN gene targeting. The *lacZ* gene was inserted just before the translational initiation codon in exon 2 of the TN gene, deleting about two-thirds of exon 2 as well as a part of intron 2, so that it would be expressed under the control of the regulatory unit of the TN gene. The following steps were also taken in constructing the vector. The 5-kb homologous region was disrupted with a nonhomologous *lacZ–neo* insert and was 0.8 kb in the 5′ end to allow the identification of homologous recombinants with the polymerase chain reaction (PCR) (Joyner et al. 1989). The *neo* (neomycin phosphotransferase) gene with the PGK gene promoter and polyadenylation signal (Boer et al. 1990) was placed in an orientation opposite that of *lacZ*; the mutation in the *neo* gene was corrected to the wild-type form (Yenofsky et al. 1990). At the 3′ terminus of the homologous region, the thymidine kinase (TK) gene of herpes simplex virus was inserted to negatively select random integrates (Mansour et al. 1988).

The targeting vector was linearized at a unique *Xho*I site, and it was tranfected to CCE (Kuehn et al. 1987) or TT2 ES cells, that we recently established. After G418 and 1-(2-deoxy-2-fluoro-β-D-arabino-furanosyl)-5-iodouracil (FIAU) (Borrelli et al. 1988) double selection, resistant colonies were screened in pools of 10–15 colonies. Clones of positive or quasipositive pools were then analyzed individually by PCR (Fig. 1B; Joyner et al. 1989; Yagi et al. 1990). The frequency of targeting was very low: only 2 (B21-6 and D21-2) of 2550 resistant colonies with CCE cells and 1 (TN-F18) of 872 resistant colonies with TT2 ES cells. No enrichment was apparent by FIAU selection. The homologous nature of the PCR-positive clones was confirmed by Southern blot hybridization analyses after digesting their DNA with *Sac*I using probe A, which was not included in the targeting vector, and probe B, which was included (Fig. 1C). The endogenous allele was expected to yield a 5.0-kb fragment, and the homologously recombined allele was expected to yield a 7.0-kb fragment. Clones D21-2 and TN-F18 produced DNA fragments appropriate for the normal allele and homologously recombined allele at an equimolar ratio, but clone D21-2 showed extra bands recognized by probe B, indicating the presence of additional nonhomologous integration. In B21-6 cells, the intensity of the homologous allele was much lower than the endogenous allele, suggesting that the homologous recombinant constituted a minor population of the cells.

B21-6, D21-2, and TN-F18 cells were tested for their germ-line differentiating potency by making chimeric mice. Sixty-four and 48 pups were generated with the B21-6 and D21-2 cells, respectively, after injecting them into C57BL/6 blastocysts (Schwartzberg et al. 1989); but no chimeras were obtained with a coat color dominance of >50% with ES-derived cells. Among 32 pups born with TN-F18 from TT2 ES cells (after injecting them into ICR mouse embryos of the eight-cell stage), however, 27 were male, and 23 were composed primarily of the ES-derived cells, as judged by coat color. Eighteen of the latter were chosen at random, and they were mated with BALB/c females. The genotype of coat color is agouti for TT2 derived from an embryo resulting from a cross between C57BL/6 and CBA mice, and albino for ICR and BALB/c mice. ES-derived offspring were expected to have agouti coat color and host embryo-derived offspring albino coat color. In total, 322 pups were obtained from these 18 males, all with agouti color derived from mutant ES cells. In these pups, a Mendelian inheritance frequency of ~50% for the targeted allele was confirmed by *lacZ* staining of the tail as well as by PCR and Southern blot hybridization analyses of tail DNA.

lacZ *expression precisely manifested TN expression*

Several studies (Crossin et al. 1986; Prieto et al. 1990) have shown TN expression in the pericardium in early embryo and successively in somite and cartilage. *lacZ* staining was performed on these embryos to confirm that β-galactosidase was expressed in place of TN from the mutated allele. As shown in Figure 2, the staining pattern was consistent with the in situ hybridization analyses. Detailed comparison of the expression pattern was made on the sections prepared from lung and cerebellum. In lung of postnatal day 2, TN mRNA was detected in relatively large cells located on the alveolar walls adjacent to type-I alveolar cells and at the apex of protrusion of the alveolar septa (Fig. 2; Tsukamoto et al. 1991). In embryonic day 18 cerebellum, TN mRNA was distributed in Bergmann glial cells in the developing Purkinje cell layer (Tsukamoto et al. 1991). *lacZ* expression appeared to be identical except that processes of the glial cells were stained more heavily. This may be the result of wide distribution of β-galactosidase protein in contrast with the restricted localization of mRNA in the cell body. Overall, the results were consistent with the expectation that the TN gene was replaced by *lacZ* and that *lacZ* was expressed under the control of TN regulatory elements.

TN-deficient mice are apparently normal

Heterozygous mice were normal by all criteria, and the females were mated with parental males to examine the role of the TN gene in embryogenesis under conditions of a homozygously and nully mutated background. Pregnant females were sacrificed at embryonic day 18, and 12 embryos, all of normal size and morphology, were tested for genotype by PCR with liver DNAs (Fig. 3). Five of the

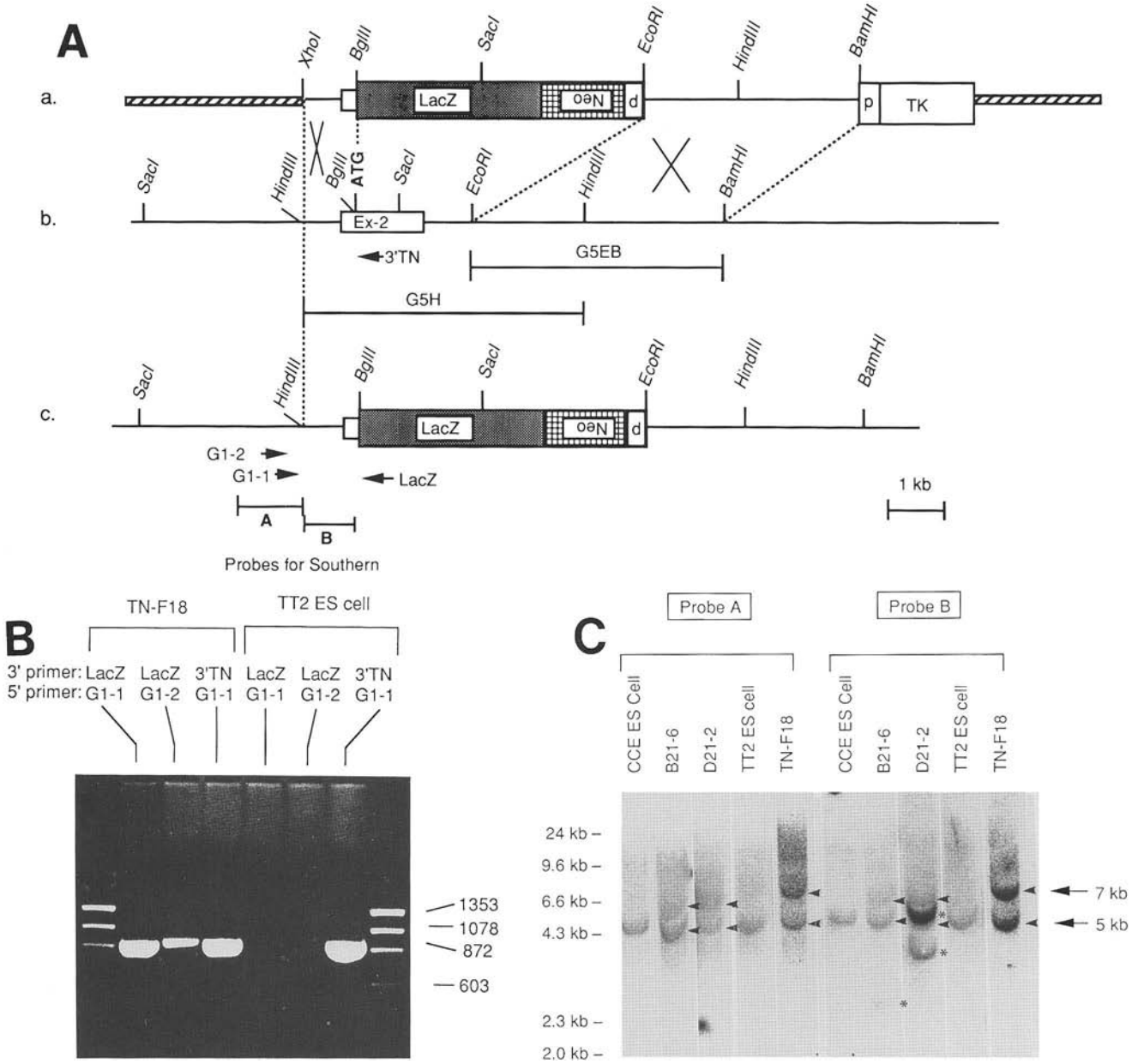

Figure 1. Strategy for mutagenesis of the TN gene with *lacZ* and its identification by PCR and Southern blot analyses. (*A*) Schematic representation of the structure of the targeting vector (*a*), endogenous allele (*b*), and homologously mutated allele (*c*). Primers used for the PCR analyses are indicated (arrows). Probes A and B for Southern blot analyses are indicated in *c*. Probe A was a 1.2-kb *Hin*cII–*Hin*dIII fragment, which was not included in the targeting vector and thus detected only endogenous and homologously recombined alleles. Probe B was a 0.8-kb *Hin*dIII–*Bgl*II fragment that was present in the targeting vector and thus detected nonhomologous integrates as well. Hatched boxes in *a* indicate sequences derived from the Bluescript II SK(+) vector. (*B*) An example of PCR amplifications in the homologous recombinant TN–F18. (*C*) Southern blot analysis of the parent CCE and TT2 cells and the three PCR-positive clones. The 5- and 7-kb bands represent endogenous and homologously recombined alleles, respectively, and are marked by arrowheads in each lane. Asterisks indicate bands probably due to nonhomologous integration.

12 embryos were homozygous, 5 were heterozygous, and 2 were wild genotypes, indicating that there was no death of homozygous embryos. At the same time, RNA was extracted from brains and analyzed for TN and *lacZ* expression by Northern blotting (Fig. 3). As expected, homozygous embryos did not express TN RNA but did express *lacZ*; heterozygous embryos expressed both, and wild-type mice expressed only TN. Another 103 offspring were examined at 3 weeks of age. The frequency of homozygous mutants was 31%, and all of these animals were apparently normal.

TN protein is known to be most abundant in thymus,

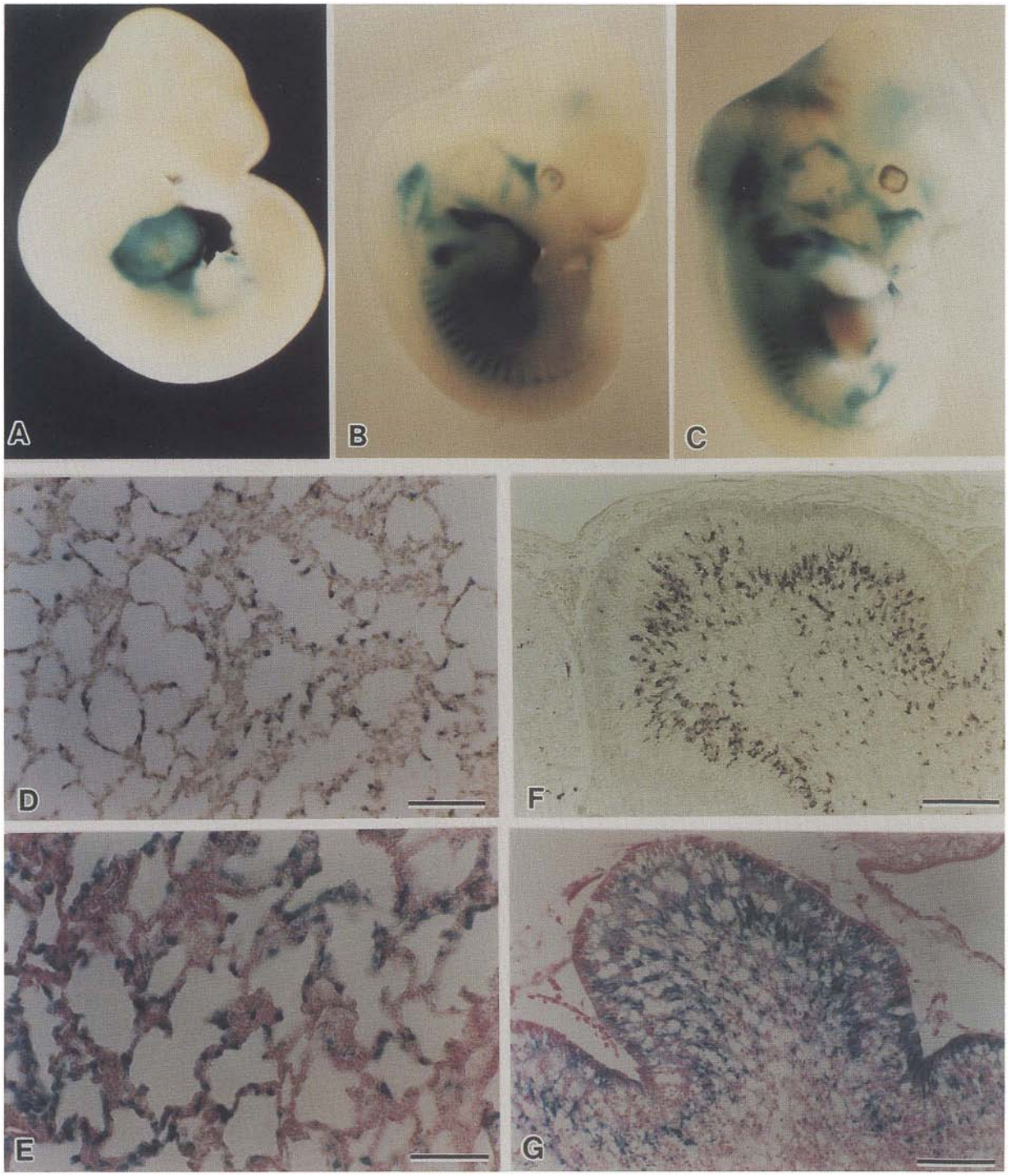

Figure 2. *lacZ* expression by histochemical staining and TN expression by RNA in situ hybridization in heterozygous embryos and mice. (*A–C*) Lateral views of whole-mount embryos at embryonic days 10–12; (*D,E*) sagittal sections of lung prepared on postnatal day 2; (*F,G*) cerebellum prepared on embryonic day 18; *A–C*, *E*, and *G* were stained by *lacZ* reaction; *D* and *F* were stained by in situ hybridization. Bars, 100 μm.

lung, and cerebellum (Saga et al. 1991); therefore these tissues were immunohistochemically examined for TN expression in homozygous mice. As shown in Figure 4, immunoreactive TN molecules observed in heterozygous mice were not present in homozygous mice. On the contrary, *lacZ* was expressed more heavily in the tissues of homozygous mice as expected. No difference in cell architecture was observed by hematoxylin–eosin stain-

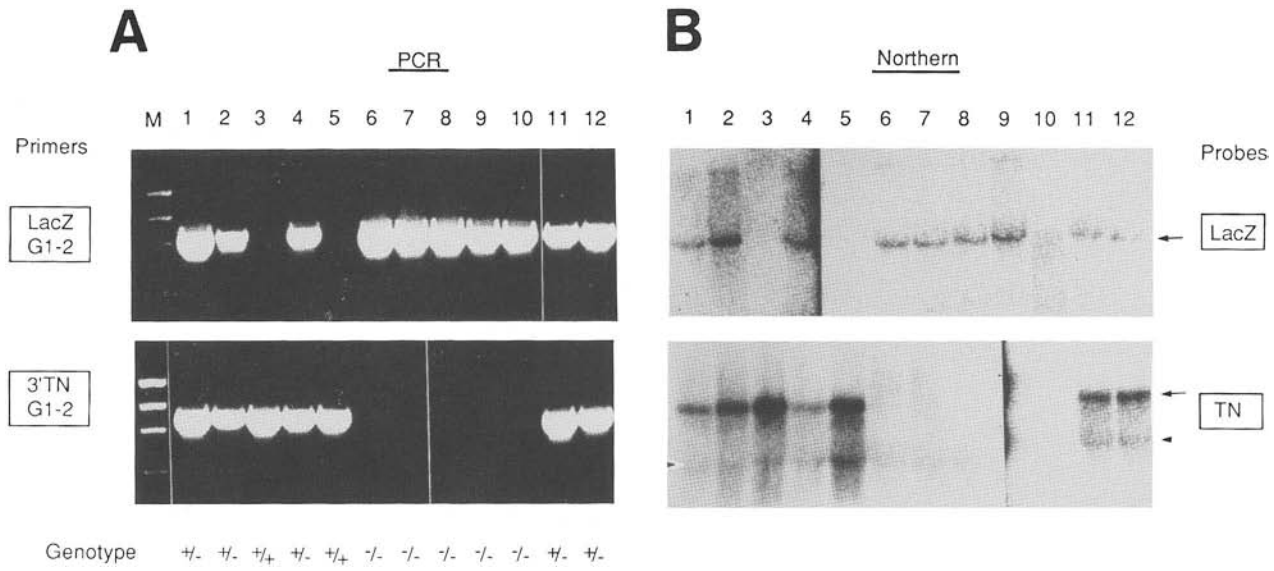

Figure 3. PCR analyses of genotypes and Northern blot analyses of TN transcript in embryos derived from crosses of heterozygous mice. (*A*) The presence or absence of the transgene (*top*) and the endogenous TN gene (*bottom*) by PCR analyses; (*B*) the presence of *lacZ* (*top*) and TN (*bottom*) transcripts in the corresponding individuals by Northern blot analysis. Livers and brains of embryos at 18 gestation days were used for preparing DNAs and RNAs, respectively. PCR was performed by use of either a TN primer or a *lacZ* primer as the 3′ primers in combination with 5′ TN primer G1-2. The endogenous TN gene encodes a 7-kb transcript (arrow) in brain (Saga et al. 1991); the *lacZ* transgene encodes a 4-kb transcript (arrow). Weak cross-hybridization of TN with 285 rRNA is marked by arrowheads.

ing in the tissues nor was any histological abnormality apparent in any other tissues of the body. The absence of TN protein was confirmed by Western blotting in the lung. A polyclonal antibody against human TN antibody recognized in 230- and 180-kD TN isoforms (Fig. 5) in wild-type and heterozygous mice. Neither a corresponding band nor a cross-reactive unique band, was detected, however, in tissues from homozygous mice. This seems to rule out the possible production of a truncated TN protein that might substitute for TN functionally and account for the normal development of the homozygous mutant mice.

Possible compensation by another matrix molecule

Apparently TN seems to be dispensable in the mouse. One possible explanation may be that other proteins take over the functions normally provided by TN. A TN-like molecule, TN–major histocompatibility complex (TN–MHC), has been reported recently in humans (Matsumoto et al. 1992); its gene is located in the MHC region and has the heptad, EGF repeats, FN3 repeats, and a fibrinogen domain, which are characteristic of the TN gene. The percent homology between the fibrinogen domains of human TN and TN–MHC is 51.4% at the amino acid level. We have identified a mouse homolog of the TN–MHC gene. The extent of its homology is 52.3% with mouse TN and 94.9% with human TN–MHC (K. Saga, unpubl.). The expression patterns overlapped in some tissues between TN and TN–MHC, but little expression of TN–MHC was observed in brain. Because no histological abnormality was observed in the brains of homozygous mutant mice, the possibility of induced expression of the TN–MHC molecule was examined. No such expression was detected on Northern blots (Fig. 5), nor was there any change in the TN–MHC expression in thymus, heart, or lung.

TN is known to interact with FN and a chondroitin sulfate proteoglycan (Hoffman et al. 1988). These components, including LN, collagen, and other proteoglycans, are thought to constitute a network of ECM components regulating cell adhesion, cell migration, and tissue pattern formation in a variety of ways. Therefore, we examined the distribution of these ECM components in the thymus and brain of TN-deficient mice (Fig. 5B). No major change in the tissue localization of LN, type-IV collagen, proteoglycan, or FN was visible by immunohistochemical staining; LN and type-IV collagen sometimes appeared to be arranged in smaller or less aggregated bundles in thymus of mutant mice. This observation is being investigated in more detail.

Discussion

TN has been identified recently in amphibian (Onda et al. 1991; Riou et al. 1991), as well as in leech (Masuda-Nakagawa et al. 1991). The sequence homology between chicken and newt TN is comparable to that between chicken and mouse or chicken and human. Sequence conservation and the unique expression in similar cell

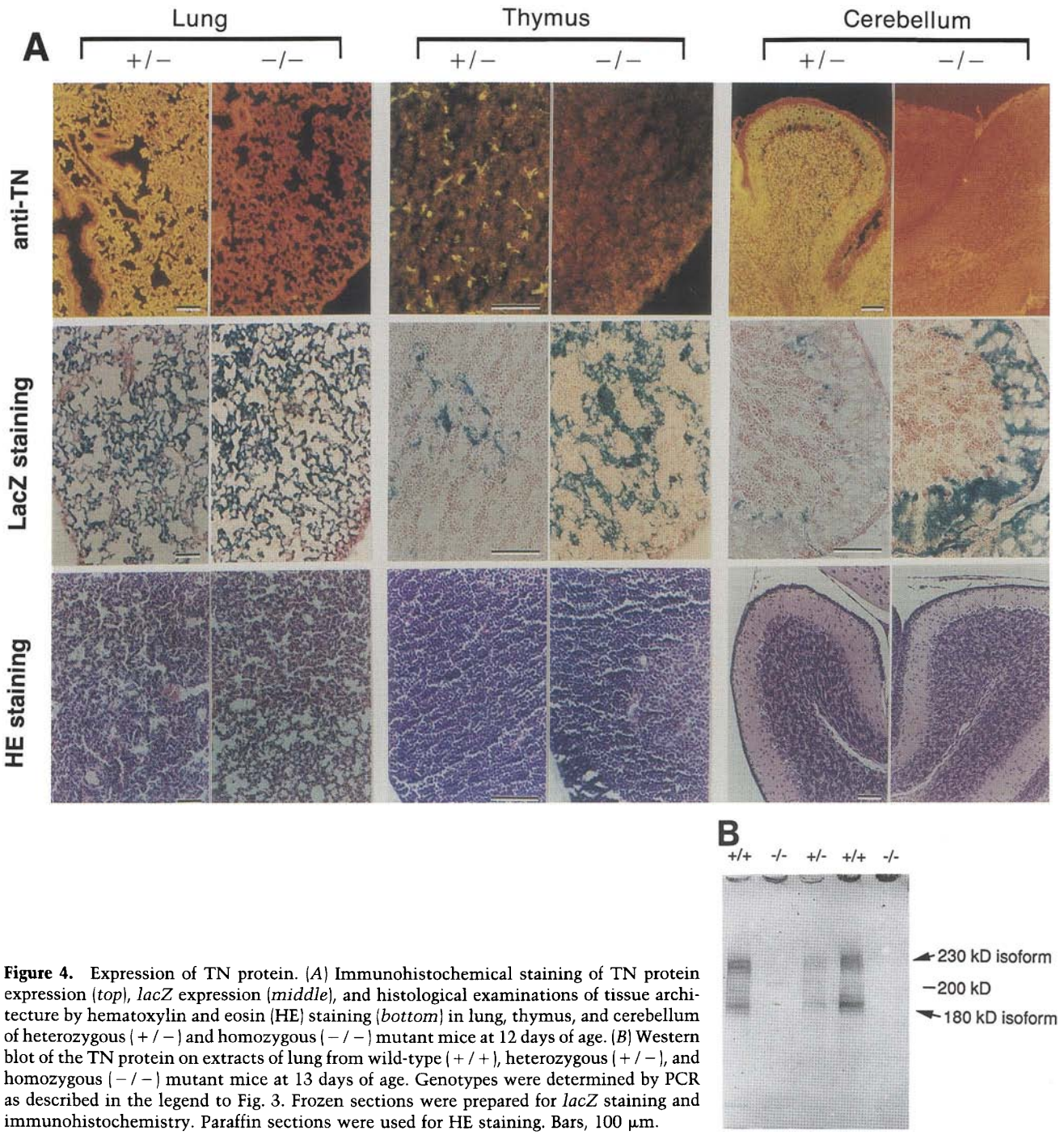

Figure 4. Expression of TN protein. (*A*) Immunohistochemical staining of TN protein expression (*top*), *lacZ* expression (*middle*), and histological examinations of tissue architecture by hematoxylin and eosin (HE) staining (*bottom*) in lung, thymus, and cerebellum of heterozygous (+/−) and homozygous (−/−) mutant mice at 12 days of age. (*B*) Western blot of the TN protein on extracts of lung from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mutant mice at 13 days of age. Genotypes were determined by PCR as described in the legend to Fig. 3. Frozen sections were prepared for *lacZ* staining and immunohistochemistry. Paraffin sections were used for HE staining. Bars, 100 μm.

types during the divergence of these species suggest a vital role for TN during development. A number of studies have emphasized the importance of TN in embryogenesis, especially morphogenetic events involving epithelial–mesenchymal interactions. TN is expressed only in the mesenchyme surrounding the epithelia undergoing differentiation. Examples are in mammary gland (Chiquet-Ehrismann et al. 1986) and tooth bud formations (Thesleff et al. 1987), early tubulogenesis in kidney (Aufderheide et al. 1987), and urogenital sinus at the sites of formation of prostatic buds and vagina (Takeda et al. 1988). These morphogenetic events, however, proceeded normally in the homozygous mutant mice described here. It has also been suggested that TN is a substrate required for migration of granule cells along the processes of Bergmann radial glia in the developing

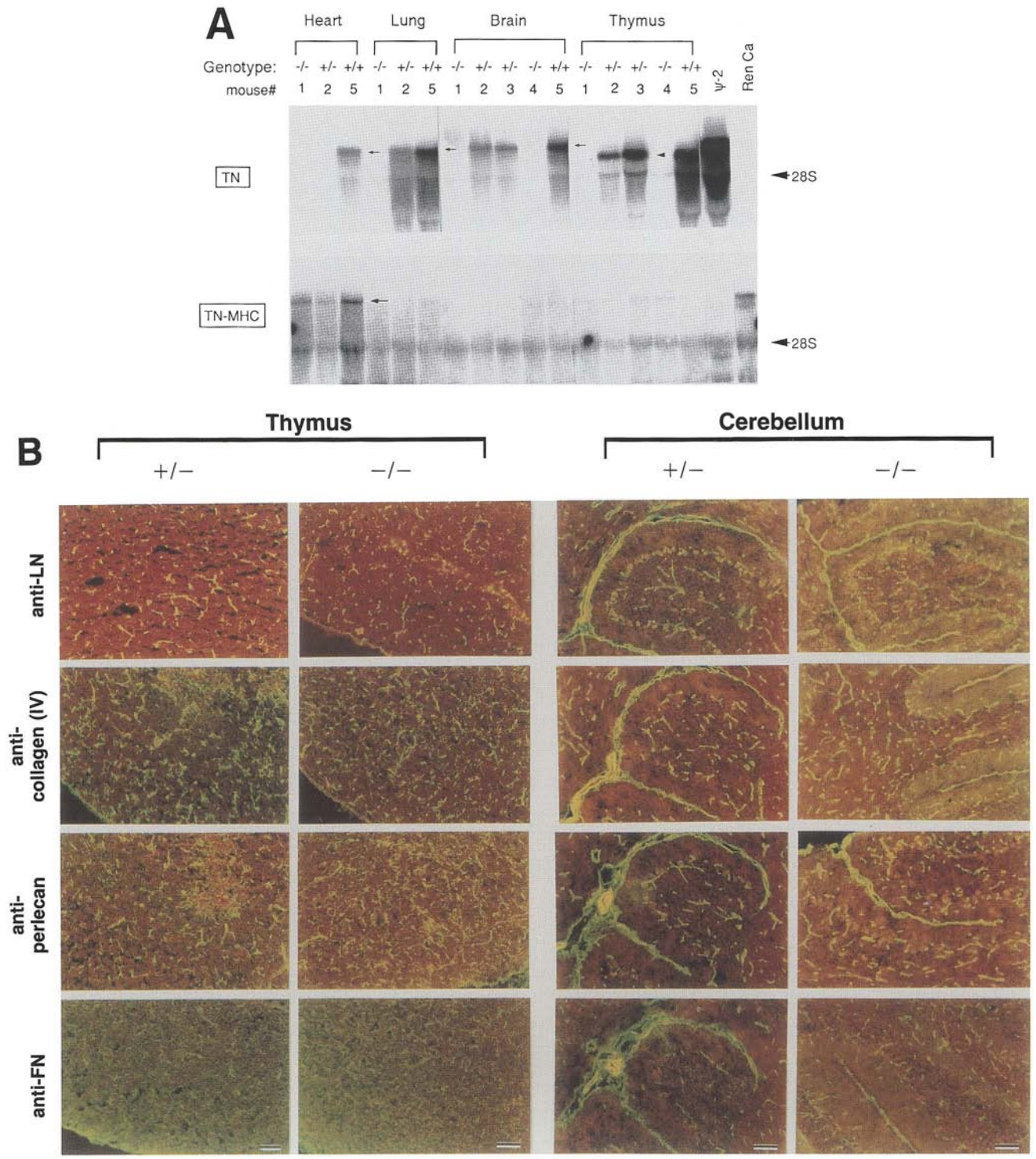

Figure 5. Expression of TN–MHC, FN, proteoglycan, LN, and type-IV collagen in TN-deficient mice. (*A*) Northern blot TN–MHC mRNA expression in heart, lung, brain, and thymus compared with TN expression. RNA blots of total RNAs from postnatal day 2 mice were hybridized with the TN probe. The same blots were rehybridized with a 550-bp DNA fragment of a fibrinogen homologous region in the TN–MHC gene. The large TN isoform (7 kb, arrows) was detected in heart, lung, and brain, and the small isoform (5.5 kb, arrowhead) was detected in the thymus of normal and heterozygous mice at 2 days of age. In the early postnatal period, the large isoform predominates in lung (Saga et al. 1991). The TN–MHC transcript (13 kb, arrow) was detected only in heart. RNAs from Ψ-2 cells (obtained from RIKEN Cell Bank, Tsukuba, Japan) and a renal carcinoma cell line (obtained from Dr. H. Yagita, Jyuntendou University, Tokyo) were used as positive controls for TN and TN–MHC, respectively. (*B*) The immunohistochemical distribution of LN, collagen, proteoglycan (perlecan), and FN in thymus and brain of heterozygous (+/−) and homozygous mutant (−/−) mice. The same specimens used for TN detection (Fig. 4) were tested. Bars, 100 μm.

cerebellum (Husmann et al. 1992). Histological analyses revealed that all of the major cell types were present in their normal locations in the mutant cerebellum. The thickness and density of both granular cell layer and the Purnkinje cell layer were also normal. Homozygous mutants showed no sign of cerebellar ataxia, nor did they exhibit any behavioral abnormalities. In addition, the migration of neural crest cells was apparently normal.

TN is normally present in small intestine (Aufderheide and Ekblom 1988), brain, and other neural derivatives (Crossin et al. 1989; Prieto et al. 1990; Bartsch et al. 1992), myotendinous junctions, smooth muscle (Grumet et al. 1985), condensing mesenchyme of cartilage and bone (Prieto et al. 1990), skin (Jiang and Chuong 1992), limb bud (Wehrle-Haller et al. 1991), and hair follicle (Chiquet-Ehrismann et al. 1986), but no abnormalities were found in any of these tissues. TN is expressed at high levels in the normal thymus as shown here, yet there were no irregularities in T-cell populations: CD4-, CD8-, CD3-, T-cell receptor αβ-, T-cell receptor γδ-, Thy-1- and IL2 receptor-positive or negative cells (data not shown). The uterus expresses TN during the estrous cycle (Hanazono et al., pers. comm.) and pregnancy (Castellucci et al. 1991), but offspring were normally obtained from homozygous mutant females as well as males. TN is prominent in healing wounds (Mackie et al. 1988). Nevertheless, wounds healed normally in amputated tails and injuries following fighting. TN expression is also known to be associated with tumorigenesis (Chiquet-Ehrismann et al. 1986; Mackie et al. 1987), peripheral nerve regeneration (Daniloff et al. 1989), and a whisker-related pattern formation in barrel-field cortex (Crossin et al. 1989); these processes are now being examined.

TN–MHC is not likely to compensate for TN, at least in the brain. Production of mutant mice lacking the TN–MHC gene is in progress. These mice should clarify whether TN–MHC compensates for TN. It is also possible that the multifunctional domains of the TN molecule comprising 14.5 EGF repeats, 13 FN3 domains, and a fibrinogen-like domain (Saga et al. 1991) were compensated by separate molecules with functions complementary to each domain.

During the preparation of this manuscript, the existence of another TN-like molecule was reported in chicken (Norenberg et al. 1992). The molecule, called restrictin, also shares the typical structural feature with TN and the expression is restricted in brain. The possibility that this molecule might compensate for TN function in the CNS is now under investigation.

Finally, the human TN gene is localized on chromosome 9 in band q32–q34 (Rocchi et al. 1991), and it has been suggested as a candidate for a tuberous sclerosis (TSC) gene (Fryer et al. 1987; Gulcher and Stefansson 1991). Major organs affected in TSC include the skin, CNS, kidneys, and heart. However, no lesions were observed in these tissues from TN-deficient mice. In sum, the results presented here strongly refute the vital role of TN. Nonetheless, these mutant mice may provide a valuable resource for further experiments to clarify the dynamic network of ECM that function during development.

Materials and methods

Construction of the targeting vector

A genomic DNA clone containing exon 2 of mouse TN was isolated from a genomic library generated from B10 mouse DNA partially digested with *Sau*3A in λJ1 vector (Loenen and Brammar 1980) by screening with a mouse 5′ cDNA probe. Targeting was designed to replace the *Bgl*II–*Eco*RI region of the TN gene with *lacZ* and *neo* genes (Fig. 1A). For this, 5.0-kb *Hin*dIII (G5H) and 4.5-kb *Eco*RI–*Bam*HI (G5EB) fragments of the TN gene were individually subcloned in a Bluescript II SK(+) vector. The *Nco*I–*Bam*HI *lacZ* fragment from pactβgal (Maekawa et al. 1991) was end filled and converted to the *Bgl*II site by ligating the *Bgl*II linker (New England Biolabs, cat. no 1036); the *Bam*HI site remained intact. The fragment was inserted into the *Bgl*II site of G5H yielding G5H-lacZ. The *Xho*I–*Eco*RI *neo* gene fragment was obtained from pKJ2 (Boer et al. 1990); the *Xho*I site was converted to *Eco*RI site by endfilling and ligating *Eco*RI linker (New England Biolabs, cat. no. 1020), and the fragment was inserted into *Eco*RI site of G5EB yielding Neo-G5EB. The *Bam*HI fragment of G5H-lacZ was inserted in the *Eco*RV site of Neo-G5EB ligated with a *Eco*RV–*Bam*HI adaptor (New England Biolabs, cat. nos. 1106 and 1140), yielding G5–LacZ–Neo. Finally, the *Bam*HI TK gene fragment from pPTK (kindly provided by Dr. N. Nakatsuji, National Inst. of Genetics, Mishima, Japan) was inserted in the *Not*I site of G5–LacZ–Neo by use of the *Not*I–*Bam*HI adaptor (New England Biolabs, cat. nos. 1160 and 1157), thus yielding the targeting vector G5–LacZ–Neo-TK.

Cell cultures and transfections

The ES cells were cultured on mitomycin C-treated feeder cells in Dulbecco's modified Eagle medium (high glucose) supplemented with 20% fetal bovine serum, 0.1 mM 2-mercaptoethanol, nonessential amino acids (Flow Laboratories), 1 mM sodium pyruvate, and leukemia inhibitory factor [LIF (1000 U/ml)]. The CCE cells were kindly provided by Dr. Elizabeth Robertson (Columbia University, New York). TT2 cells were established from an F_1 embryo of C57BL/6 and CBA mice (T. Yagi, pers. comm.). The targeting vector was linearized at a unique *Xho*I site and transfected into either CCE or TT2 ES cells by electroporation with a Bio-Rad Gene Pulser (25 μF, 1.35 kV). Cells were selected against 150 μg/ml of G418 and 0.4 μM FIAU (kindly provided by Bristol Myers) for 9 days, and resistant colonies were propagated and analyzed as as described previously (Yagi et al. 1990).

DNA analysis

PCR was performed for 35 cycles using *Taq*I DNA polymerase; each cycle consisted of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and polymerization at 72°C for 2 min. The sequence of the primer 3′TN was 5′-AAGATGCCTGGCAGTAGCCAGGTCAC-3′; *lacZ*, 5′-CTCCATGCTTGGAACAACGAGCGCAGC-3′; G1-1, 5′-GGTACCTGATTCCGAAGTGCATTGTCACGT-3′; G1-2, 5′-GAAGTCACTAGAAACTAGTGGACAACTC-3′. For Southern blot analysis, 10 μg of DNA was digested with *Sac*I, electrophoresed on a 0.7% agarose gel, blotted onto nitrocellulose filters, and hybridized with ^{32}P-labeled probe A or B.

Generation of chimeras

Chimeras were generated as described previously except that TT2 cells were injected into eight-cell-stage ICR embryos (Yagi et al. 1990).

Northern blotting

Total RNAs were electrophoresed on a formaldehyde gel, blotted on a nitrocellulose filter, and hybridized with a TN cDNA probe (Saga et al. 1991), a *lacZ* probe (the 3-kb DNA fragment covering the entire coding region), or a TN–MHC 550-bp PCR fragment.

Histological analysis

For LacZ staining of whole embryo, samples were fixed in a solution containing 2% paraformaldehyde–0.2% glutaraldehyde–0.02% NP-40 in 1× PBS for 60 min at room temperature and stained at 37°C for 1–3 hr in a solution containing 1 mg/ml of X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 2 mM $MgCl_2$ in 1× PBS. For cryostat sections, samples were incubated in 30% sucrose for 6 hr after fixation and embedded in OCT compound. The 10-μm frozen sections were used for *lacZ* staining and immunohistochemical detection. For immunofluorescent staining, the sections were incubated sequentially with primary antibodies (1 : 100–1 : 200 dilution of serum in PBS) and fluorescein-conjugated goat anti-rabbit IgG (1 : 100 dilution), counterstained with Evans blue, and mounted in 90% glycerol/PBS. Anti-TN polyclonal antiserum was prepared by injection of purified human TN into rabbit. The specificity of this anti-serum was confirmed by comparison with the staining patterns of a monoclonal antibody, MTn-12, kindly provided by Dr. P. Ekblom (Aufderheide and Ekblom 1988). Anti-mouse LN polyclonal antibody, anti-mouse type-IV collagen polyclonal antibody, and anti-mouse perlecan antiserum were provided by Dr. K. Kimata (Kimata et al. 1985), and anti-mouse FN polyclonal antibody was from Dr. M. Obara (RIKEN). In situ hybridization was performed as described (Tsukamoto et al. 1991).

Western blotting

One gram of each sample was mechanically homogenized in 1 ml of extraction buffer [50 mM Tris-HCl (pH 7.6)/1 M NaCl/1 M urea] and centrifuged. Supernatants were electrophoresed on 4–15% polyacrylamide gradient gels containing 0.1% SDS and blotted on an Immobilon-P membrane (Millipore, Bedford, MA). The blot was immersed sequentially in anti-human TN membrane (1 : 200), goat anti-rabbit IgG conjugated with alkaline phosphatase (1 : 200), and developed in chromogen solution [337.5 μg/ml of nitroblue tetrazolium salt, 175 μg/ml of 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in 100 mM Tris-Cl, 100 mM NaCl, 50 mM $MgCl_2$ (pH 9.5)].

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