Chondrodysplasia in Transgenic Mice Harboring a 15-Amino Acid Deletion in the Triple Helical Domain of Proα1(II) Collagen Chain

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Abstract. We have generated transgenic mice by microinjection of a 39-kb mouse proα1(II) collagen gene construct containing a deletion of exon 7 and intron 7. This mutation was expected to disturb the assembly and processing of the homotrimeric type II collagen molecule in cartilage. Expression of transgene mRNA at levels equivalent or higher than the endogenous mRNA in the offspring of two founder animals resulted in a severe chondrodysplastic phenotype with short limbs, hypoplastic thorax, abnormal craniofacial development, and other skeletal deformities. The affected pups died at birth due to respiratory distress. Light microscopy of epiphyseal growth plates of transgenic pups demonstrated a marked reduction in cartilaginous extracellular matrix and disruption of the normal organization of the growth plate. The zone of proliferating chondrocytes was greatly reduced whereas the zone of hypertrophic chondrocytes was markedly increased extending deep into the diaphysis suggestive of a defect in endochondral ossification. Electron microscopic examination revealed chondrocytes with extended RER, a very severe reduction in the amount of cartilage collagen fibrils, and abnormalities in their structure. We postulate that the deletion in the α1(II) collagen acts as a dominant negative mutation disrupting the assembly and secretion of type II collagen molecules. The consequences of the mutation include interference with normal endochondral ossification. These mice constitute a valuable model to study the mechanisms underlying human chondrodysplasias and normal bone formation.

TYPE II collagen, a homotrimer of α1(II) chains, is the predominant protein in cartilage matrix. The functional form of type II collagen in most hyaline cartilages is a thin fibril (10–50 nm in diameter) in which individual molecules associate laterally and head-to-tail with each other and with type XI and type IX collagen molecules (Mayne and von der Mark, 1983; Mendler et al., 1989; Hunziker and Herrmann, 1990). The fibrils confer tensile strength and provide a scaffolding network for proteoglycans. Like for other fibrillar collagens the important structural characteristics of type II collagen include the presence of glycine residues as every third amino acid throughout the triple helical domain and conservation of the length of the triple helical domain (Vuorio and de Crombrugghe, 1990; Metsäranta et al., 1991). These features are critical for the formation of the triple helix, its stability, and collagen fibril assembly. Any change in these properties is likely to disturb either the formation of the triple helix or fibrillogenesis, as appears to be the case with mutations in the genes coding for type I collagen, which cause various forms of osteogenesis imperfecta and Ehlers Danlos syndrome (Byers, 1989; Prockop et al., 1990). Given the many lateral associations between the polypeptide chains in the procollagen molecules and between collagen molecules in the fibrils, mutations in the triple helical domain of type II collagen are expected to be dominant.

In an adult organism type II collagen is mainly found in the hyaline cartilage of articular surfaces and the nucleus pulposus of the intervertebral discs. Earlier in life the growth of long bones, which largely determines skeletal size, occurs at growth plates by production of cartilage matrix by chondrocytes (Stockwell, 1979; Sandberg and Vuorio, 1987). In addition to these structural roles cartilage matrix also functions during embryonic development as a model for bone formation through endochondral ossification. During development transient type II collagen expression also occurs at several epitheliomesenchymal interfaces of the craniofacial system as well as in the tail tendon, heart, epidermis, and brain (Thorogood et al., 1986; Cheah et al., 1991; Wood et al., 1991). The significance of this transient synthesis is not yet understood.

The phenotypes of diseases in which linkage to the type II collagen gene has been identified are clearly related to either disturbed long bone growth or degeneration of articular

surfaces, i.e., osteoarthrosis (Francomano et al., 1987; Palotie et al., 1989; Knowlton et al., 1990). Genetic defects in type II collagen have been identified at the nucleotide level in some chondrodysplasias representing variable symptomatologies from lethal type II (Langer-Saldino) achondrogenesis (Vissing et al., 1989), to less severe syndromes such as hypochondrogenesis, spondyloepiphyseal dysplasia (SED), congenita (Lee et al., 1989; Tiller et al., 1990), and Stickler syndrome (Ahmad et al., 1991), and in osteoarthrosis (Ala-Kokko et al., 1990). The diversity of these phenotypic abnormalities clearly demonstrates that mutations in type II collagen can have very different consequences. In humans the functional domains of type II collagen can only be defined by characterizing rare and often sporadic mutations. Therefore transgenic mice are well suited to study the functional anatomy of the molecule in a more systematic way. Chondrocyte proliferation and production of extracellular matrix play central roles at growth plates during long bone growth, but the contribution of individual components to the process is poorly understood. The introduction of mutated type II collagen genes into the mouse germ line can generate abnormal phenotypes caused by loss of function of normal collagen, and provide a way to understand consecutive alterations in other matrix components. This was recently shown for a point mutation which changed the glycine residue at position 85 of the triple helical domain of type II collagen to a cysteine residue and caused a chondrodysplastic phenotype (Garofalo et al., 1991).

In this study we have generated transgenic mice strains by microinjection of a mouse type II collagen gene carrying a 150-bp deletion including the 45-bp exon 7. Expression of the transgene results in a very severe depletion of collagen fibrils in cartilages which presumably causes the pronounced short limbed dwarfism and other skeletal abnormalities, many of which resemble those seen in human chondrodysplasias. The histological and ultrastructural characteristics of these mice are distinctly different from those seen in mice harboring the glycine-85 substitution mutation.

Materials and Methods

Gene Constructions

The entire mouse proα1(II) collagen gene was sequenced before mutagenesis (Metsäranta et al., 1991). The single ClaI site within the gene and the ClaI site in cosmid pWE15 close to the cloning site made it possible to construct cosmid clones p8045 and p8023 containing the 5′ and 3′ portions of the gene with 3 and 7 kb of flanking sequences, respectively (Fig. 1). The mutation was introduced into cosmid clone p8045 by replacing the wild-type PstI-EcoRI fragment with the corresponding mutated fragment (Fig. 1, *A–C*). The 800-bp PstI-EcoRI fragment was first removed from a 2.2-kb wild-type KpnI-EcoRI genomic fragment (Fig. 1 *A*). The clone was digested with ApaI and the 150-bp ApaI fragment removed (Fig. 1 *B*). Generation of this deletion was based on the presence of two ApaI sites, one at the beginning of exon 7 and one at the beginning of exon 8, both in the same reading frame (gGGCCC, Metsäranta et al., 1991). This deletion also removed an NcoI site in exon 7. The resulting size difference and the NcoI polymorphism provided simple methods for detection of the transgene over the background of the endogenous type II collagen genes (Fig. 2). The shortened (650 bp) PstI-EcoRI fragment was recloned into the 2.2-kb KpnI-EcoRI fragment (Fig. 1 *C*) followed by insertion of a reconstituted 1.9-kb XhoI-XhoI fragment into the 13-kb NotI-ClaI subclone p8045 (Fig. 1 *D*). The mutation and the orientation of the fragment were confirmed by DNA sequencing using specific oligonucleotide primers. Reconstruction of the gene was completed by addition of the 26-kb ClaI-ClaI fragment of p8023 containing the 3′ half of the gene (Fig. 1 *E*). The orientation of the insert was verified by restriction mapping. The insert containing the reconstituted gene, 3 kb of promoter sequences and 7 kb of 3′ flanking sequences (Fig. 1 *F*) was liberated from the cosmid vector with NotI digestion, purified, and used for microinjection.

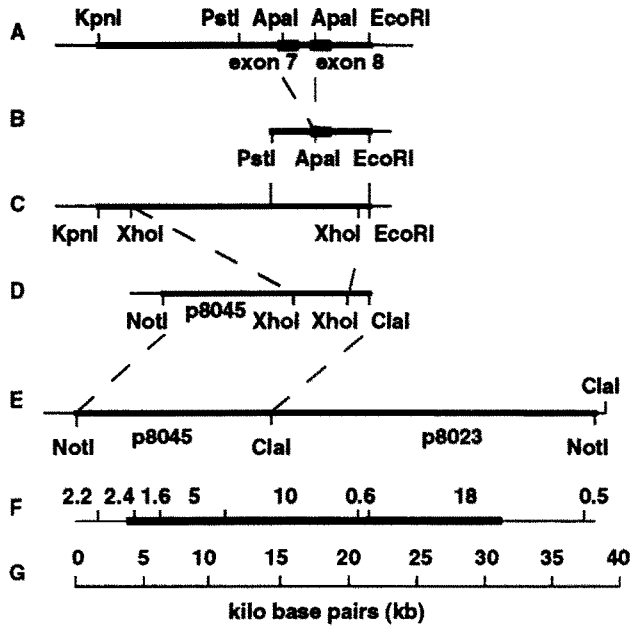

Figure 1. Schematic representation of the construction of the mutated transgene. The deletion of exon 7 was generated by removal of a 150-bp ApaI fragment containing exon 7 and intron 7 (*A* and *B*). The resulting shortened PstI-EcoRI fragment was subcloned back into the wild-type clone (2.2-kb KpnI-EcoRI fragment) followed by subsequent cloning of a 1.9-kb XhoI-XhoI fragment into p8045 containing the 5′ half of the mouse type II collagen gene (*C* and *D*). Reconstruction of the gene was completed by addition of the 26-kb ClaI-ClaI fragment p8023 containing the 3′ half of the gene (*E*). EcoRI restriction map of the reconstructed gene (*F* and *G*) shows the structural portion of the proα1(II) collagen gene as the solid rectangle. The insert containing the reconstituted gene was liberated from the cosmid vector with NotI digestion.

Generation and Identification of Transgenic Mice

Mouse oocytes were isolated from B6D2F1 females mated with B6D2F1 males as described (Hogan et al., 1986). DNA was microinjected into pronuclei of one-cell embryos and the surviving embryos were implanted into CD1 pseudopregnant foster mothers. The transgenic founders were initially identified by polymerase chain reaction (PCR)[1] analysis of genomic tail DNA using primers A4 in exon 6 and A5 in exon 8 which flank the deletion mutation (Fig. 2; Garofalo et al., 1991). The amplification conditions for PCR were 94°C for 1 min, 57°C for 2 min, and 72°C for 3 min for 30 cycles. The number of integrated transgene copies was determined by Southern analysis using the 800-bp PstI-EcoRI genomic fragment (Fig. 1 *A*) as probe. For Southern analyses of the EcoRI digested DNAs the entire type II collagen cosmid clone p804 was used as probe. Because of the presence of repetitive sequences within the gene mouse genomic DNA was added to the hybridization solution (Sambrook et al., 1989) to a final concentration of 100 μg/ml.

Preparation and Analysis of RNA

Total RNA was isolated from several mouse tissues by homogenization in 4 M guanidine isothiocyanate followed by sedimentation through 5.7 M cesium cloride (Chirgwin et al., 1979). Determination of the ratio of transgene derived mRNA and wild-type mRNA for proα1(II) collagen was per-

1. *Abbreviation used in this paper*: PCR, polymerase chain reaction.

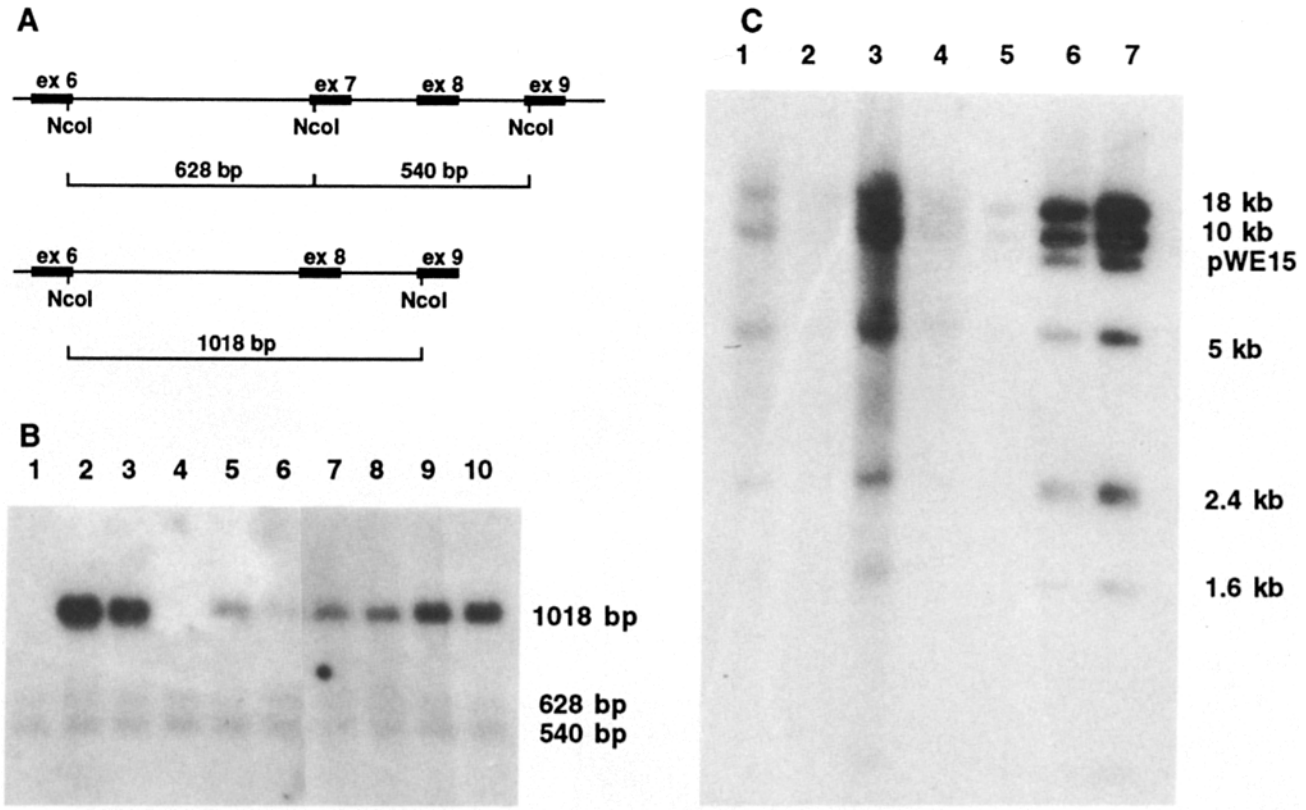

Figure 2. Identification of transgenic mice by Southern analysis. (*A*) Schematic representation of wild-type and mutated gene structures and NcoI sites near the mutation site. The presence of the transgene in the mouse genome results in a 1,018-bp NcoI fragment while the endogenous gene is represented by 628 and 540 bp fragments. (*B*) Detection of transgene by Southern analysis after NcoI digestion. Tail DNA samples (10 μg) were digested with NcoI, fractionated on a 1% agarose gel, and blotted onto a Hybond-N membrane. The blot was hybridized with a ^{32}P-labeled 800-bp PstI-EcoRI fragment (see Fig. 1 *C*) which spans from the middle of intron 6 to intron 8 (*A*). The samples in *B* are: (lane *1*) nontransgenic control; (lanes *2* and *3*) positive offspring of founder Del 3; (lane *4*) nontransgenic control; (lane *5*) founder Del3; (lane *6*) founder Del2; (lane *7*) founder Del1; (lane *8*) heterozygous offspring of Del1 founder; and (lanes *9* and *10*) homozygous offspring of Del1 founder. (*C*) Southern analysis of the entire type II collagen gene in the offspring of transgenic founder animals. Aliquots of transgene construct and tail DNA samples (10 μg) were digested with EcoRI, fractionated on a 0.7% agarose gel, and analyzed by Southern hybridization using the entire ^{32}P-labeled wild-type gene as probe. The samples are: founder Del1 (lane *1*); nontransgenic control (lane *2*); positive offspring of founder Del3 (lane *3*); founder Del2 (lane *4*); and aliquots of the transgene construct corresponding to 1, 10, and 20 copies per diploid genome (lanes *5–7*, respectively). The 2.2- and 0.5-kb terminal fragments of the construct fuse in a head-to-tail integration into a band migrating with the 2.4-kb fragment. In the wild-type gene the 5′ end migrates as a 4.7-kb EcoRI band with the 5-kb integral EcoRI fragment. *pWE15* marks the vector band which is only present in the copy number controls. The hybridization pattern confirms the presence of the entire transgene in the mouse lines with no indication of rearrangements.

formed using an RNase protection assay (Sambrook et al., 1989). A linearized cDNA clone covering sequences from exon 5B to exon 8 (Metsäranta et al., 1991) was used as a template for the synthesis of antisense RNA using T7 polymerase and ^{32}P-UTP. Full length transcripts were purified on a denaturing polyacrylamide gel. Solution hybridization was performed at 55°C using 2 × 10^5 cpm of robe and 10 μg of total RNA. After hybridization the samples were digested with RNase A and T1, and proteinase K, and precipitated for electrophoresis on denaturing 8% polyacrylamide gels. After fixation and drying the gels were exposed with x-ray films and quantified using a betascan counter.

Tissue Preparation, Histology, and Electron Microscopy

To obtain fresh tissue samples for light and electron microscopy embryos were removed by Cesarean section at 18.5–19.5 d. For visualization of the entire skeleton of transgenic mice and their nontransgenic littermates 19.5-d embryos were stained with alcian blue and alizarin red S (McLeod, 1980).

Routine histology was performed on tissues that were fixed in 4% buffered formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

The samples for EM were dissected immediately after the death of the mice. The forelimbs were immersed in 1.5% glutaraldehyde, 1.5% paraformaldehyde, and 0.5% ruthenium hexamine trichloride in 0.1 M cacodylate buffer, pH 7.3. During this initial stage of fixation the long bones were removed by dissection under a magnification of 6.4 and were then fixed for 3 h in a fresh aliquot of the primary fixative. Subsequently, after several brief rinses in cacodylate buffer, the long bones were postfixed in 1.0% osmium tetroxide in cacodylate buffer. The specimens were dehydrated, embedded in Spurr's resin, sectioned, and stained with 4% uranyl acetate and Reynold's lead citrate.

Results

Generation of Transgenic Mice

Three transgenic founder mice were generated by microinjection of the gene construct into fertilized oocytes. DNA was isolated from tails of putative founder animals for

screening with PCR and Southern hybridization. In PCR analysis two oligonucleotide primers (containing sequences in exons 6 and 8, respectively; Fig. 2 *A*) were used. The transgene fragment was easily detected because of its smaller size (750 vs 900 bp). The findings were confirmed by Southern blot hybridization which also allowed determination of the number of transgene copies integrated into the genome of founder animals. Digestion of genomic DNA with NcoI resulted in a diagnostic 1,018-bp transgene fragment and two endogenous 628 and 540 bp gene fragments in Southern hybridization (Fig. 2 *B*). To demonstrate the successful integration of the entire transgene into the genome of founder

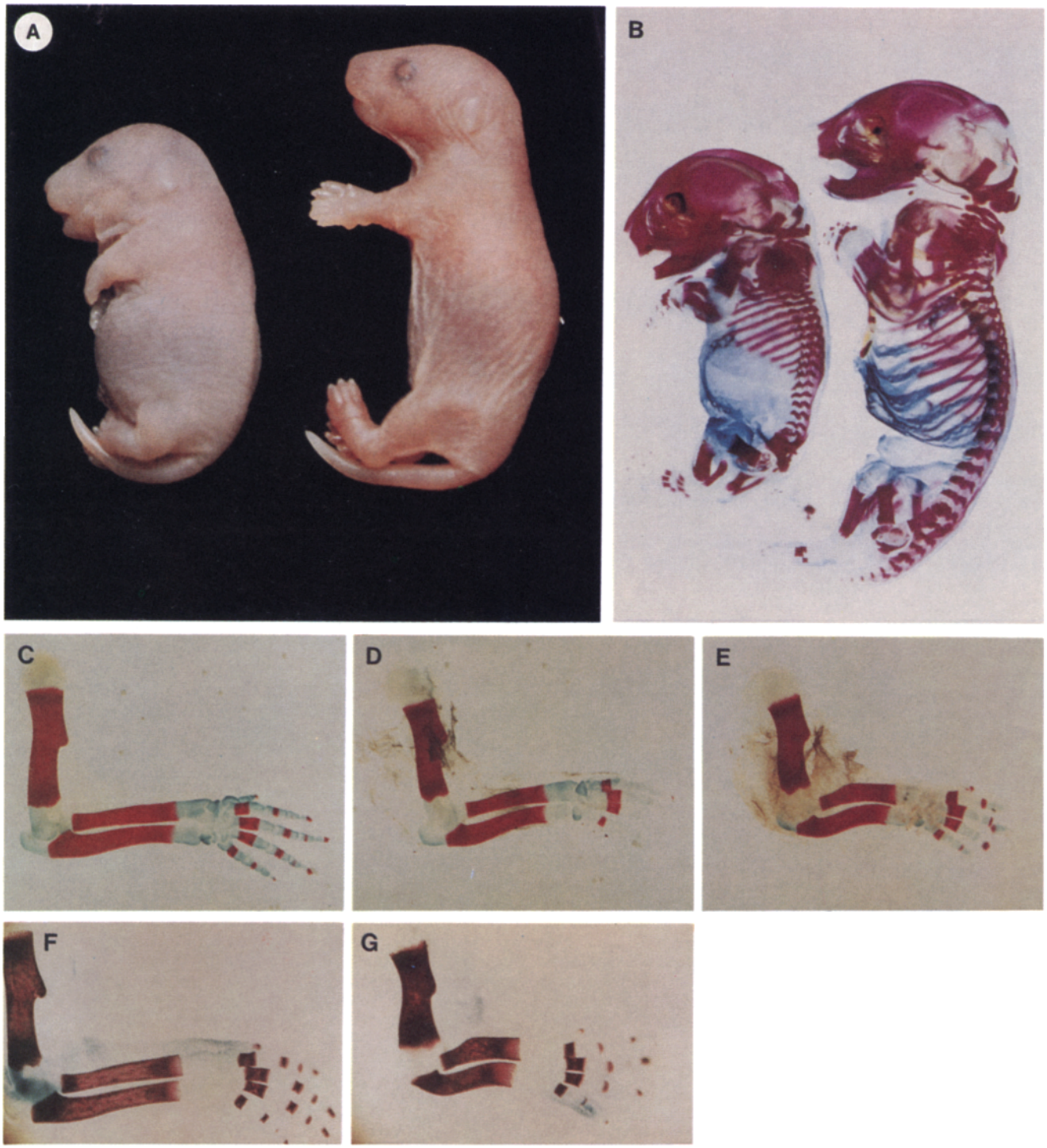

Figure 3. Chondrodysplastic phenotypes of transgenic mice. (*A*) A 19.5-d transgenic Del3 embryo (*left*) and its nontransgenic littermate (*right*). (*B*) Skeleton of 19.5-d Del3 embryo (*left*) and its nontransgenic littermate (*right*) stained with alcian blue and alizarin red S. Shortening of the long bones is evident in the forelimbs of 19.5-d heterozygous (*D*) and homozygous (*E*) Del1 embryos when compared with their nontransgenic littermate (*C*), and in the forelimb of a 19.5-d Del3 embryo (*G*) compared with its nontransgenic littermate (*F*).

animals Southern blot hybridizations of EcoRI digested genomic DNAs were performed. Using the entire mouse α1(II) collagen gene as probe no differences were observed in the genomic fragments when compared with the normal mouse α1(II) collagen gene or the cosmid clone, demonstrating that no gross rearrangement had occurred during the integration of the transgene in the mouse genome (Fig. 2 *C*). Three founder animals carrying the transgene were detected. In quantitative analysis of the Southern blots the female founder (Dell) was found to have approximately six copies of the transgene in its genome and the two male founders (Del2 and Del3) one and four copies, respectively (Fig. 2 *C*).

None of the founder animals exhibited any abnormal phenotype except for a smaller size of founder Dell. However, all transgenic offspring of founder Del3 had a severe form of short-limbed dwarfism (Fig. 3) and died shortly after birth due to respiratory distress. The animals attempted to breathe, but were unable to ventilate their lungs (Fig. 4 *D*). The animals became cyanotic and unresponsive to external stimuli and died shortly afterwards. In addition to short limbs these mice displayed a shortened axial skeleton, hypoplastic thorax, distended abdomen, short snout, protruding tongue, and cleft palate (Figs. 3 and 4). Southern analysis of the transgenic offspring of founder Del3 showed a higher copy number (15 copies) of the transgene than the founder (four copies; Fig. 2, *B* and *C*). These analyses proved that the Del3 founder was mosaic for the transgene, which probably explains its normal phenotype.

The Dell founder and its F1 offspring (six copies of the transgene) did not show any phenotypic abnormality other than a smaller size. Mating of positive F1 mice has generated mice homozygous for the transgene (12 copies) which all have a severe short-limbed dwarfism similar to that seen in Del3 offspring (Figs. 3 and 4). The offspring of Dell founder will subsequently be referred to as heterozygous and homozygous Dell pups, respectively. No abnormalities have been observed in the heterozygous and homozygous progeny of founder Del2; therefore, these have not yet been studied further.

Expression of the Mutant Transgene

To determine whether the transgene was specifically transcribed in the cartilage of the affected mice total RNA was isolated from limbs of normal and transgenic mice. Since the transgene is identical to the endogenous mouse gene except for the 150-bp deletion, the transgene derived mRNA had to be distinguished from the wild-type α1(II) collagen mRNA on the basis of its smaller size (45 nucleotides) using either RNAse protection experiments or amplification by PCR. Specific oligonucleotide primers bracketing the deletion

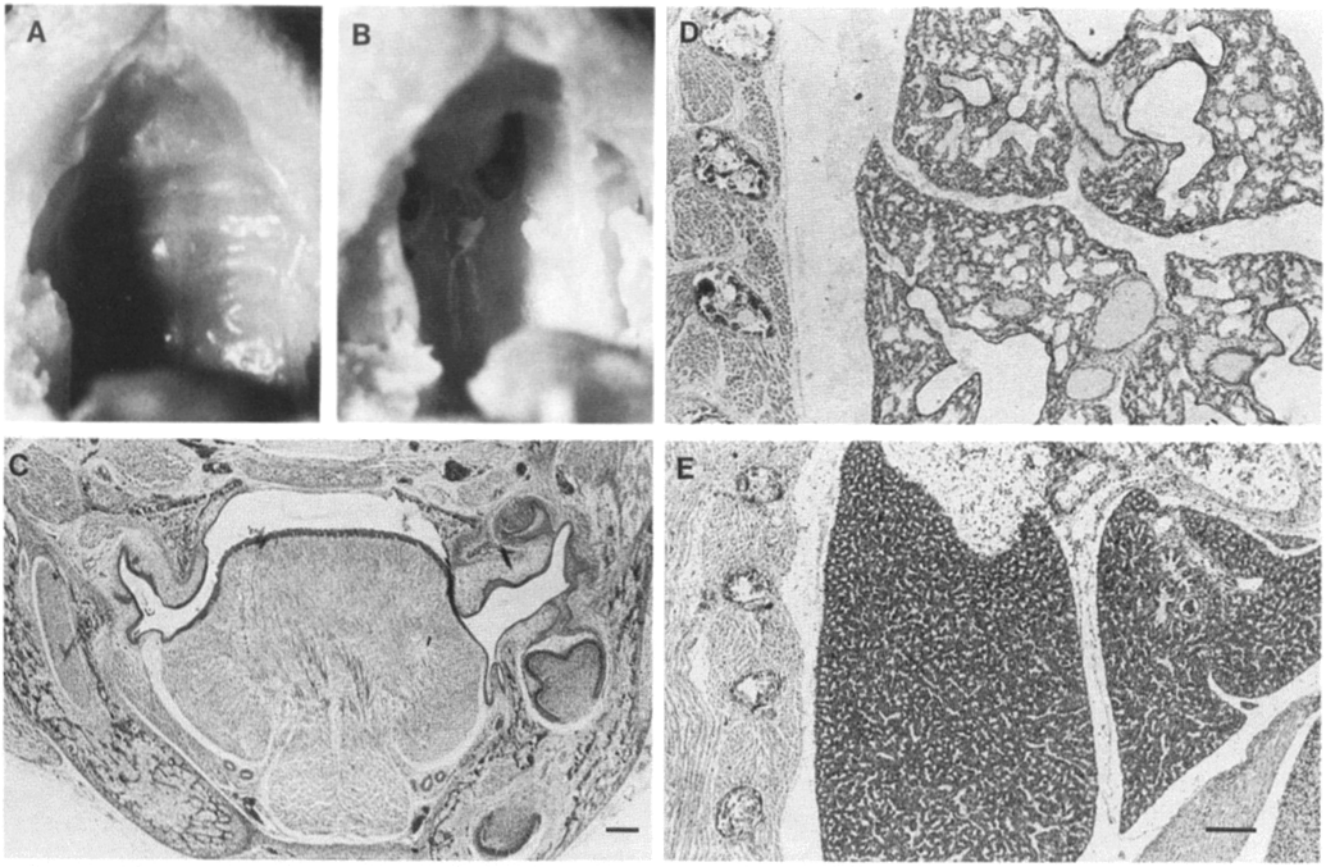

Figure 4. Additional phenotypic abnormalities in Del3 transgenic offspring. Cleft palate in a 19.5-d homozygous Dell embryo (*B*) and the normal palate of its nontransgenic littermate (*A*). A transverse section of the head of Del3 newborn mouse demonstrating the tongue obstructing the closure of palatal ridges (*C*). Histological section through the thorax reveals the inflated lung of a nontransgenic mouse (*D*), and the collapsed lung of a newborn Del3 transgenic mouse (*E*). Bars, 200 μm.

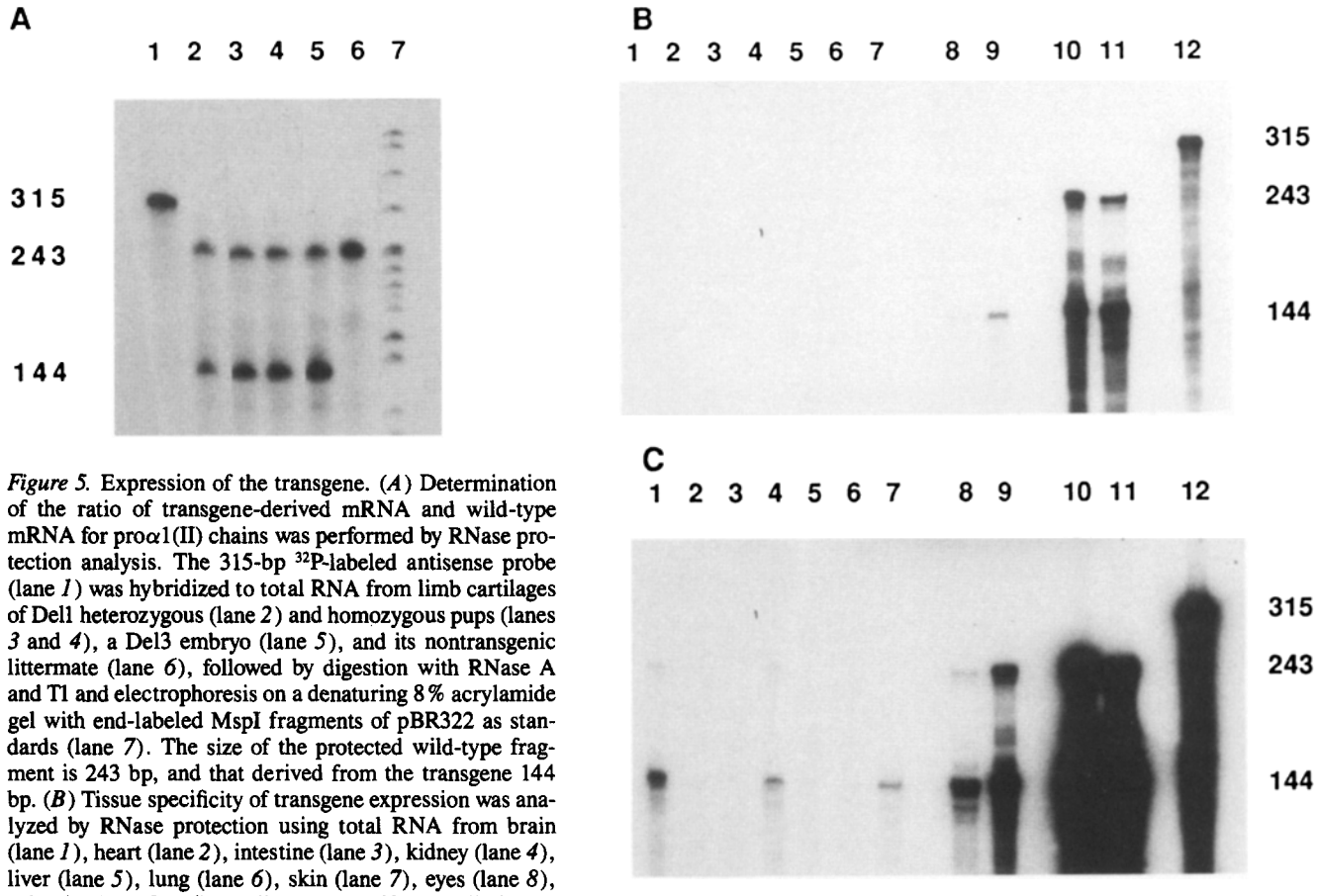

Figure 5. Expression of the transgene. (*A*) Determination of the ratio of transgene-derived mRNA and wild-type mRNA for proα1(II) chains was performed by RNase protection analysis. The 315-bp ^{32}P-labeled antisense probe (lane *1*) was hybridized to total RNA from limb cartilages of Del1 heterozygous (lane *2*) and homozygous pups (lanes *3* and *4*), a Del3 embryo (lane *5*), and its nontransgenic littermate (lane *6*), followed by digestion with RNase A and T1 and electrophoresis on a denaturing 8% acrylamide gel with end-labeled MspI fragments of pBR322 as standards (lane *7*). The size of the protected wild-type fragment is 243 bp, and that derived from the transgene 144 bp. (*B*) Tissue specificity of transgene expression was analyzed by RNase protection using total RNA from brain (lane *1*), heart (lane *2*), intestine (lane *3*), kidney (lane *4*), liver (lane *5*), lung (lane *6*), skin (lane *7*), eyes (lane *8*), calvaria (lane *9*), rib cartilages (lane *10*), and limb cartilages (lane *11*). The undigested probe was run in lane *12*. (*C*) Overexposure of the same filter to demonstrate low abundancy mRNAs.

were used in the PCR amplification of cDNA synthesized by reverse transcriptase for cartilage RNA of transgenic mice. These analyses suggested that expression of both the endogenous gene and the transgene occurred predominantly in cartilage and eye. However, low levels of both transcripts, probably related to illegitimate transcription (Chelly et al., 1989), were detected in all RNA samples studied (data not shown).

Quantitation of transgene expression was performed using an RNase protection assay. With an antisense RNA probe spanning the deletion the ratio of transgene-derived mRNA and endogenous proα1(II) collagen mRNA was determined in the same reaction (Fig. 5 *A*). The results indicate that in the severely affected Del3 pups the average ratio of transgene RNA to proα1(II) collagen mRNA was 1.9. The corresponding ratios for homozygous and heterozygous Del1 pups were 1.5 and 1.2, respectively.

Analysis of RNAs isolated from several other tissues of transgenic mice demonstrated the tissue specificity of transgene expression (Fig. 5, *B* and *C*). The highest levels of transgene- and endogenous gene-derived transcripts were seen in limb and rib cartilages, followed by calvaria and eye RNA. In skin the ratio of the transgene to endogenous gene expression was clearly increased over what was seen in cartilage. With this exception the construct used for microinjection appears to contain the elements necessary for tissue-specific expression.

Structure and Ultrastructure of the Cartilage

The most dramatic effect of the transgene in the affected offspring of Del1 and Del3 founders was the abnormal development of the bony and cartilaginous skeleton as demonstrated by staining of whole embryos with alcian blue and alizarin red S (Fig. 3 *B*). All skeletal structures were smaller in the transgenic offspring of the Del3 founder than in their nontransgenic littermates. This was particularly evident in the long bones of forelimbs and hindlimbs with an average reduction in the length/width ratio of 40% (Fig. 3, *F* and *G*). The homozygous offspring of founder Del1 demonstrated similar skeletal abnormalities in comparison with their nontransgenic littermates (25% average reduction). In the heterozygous Del1 offspring the corresponding length/width ratio was reduced by an average of 15% (Fig. 3, *C–E*). Since retarded growth of long bones was a distinct feature of the skeletal abnormalities we focused our attention on epiphyseal cartilages and growth plates. These tissues contain chondro-

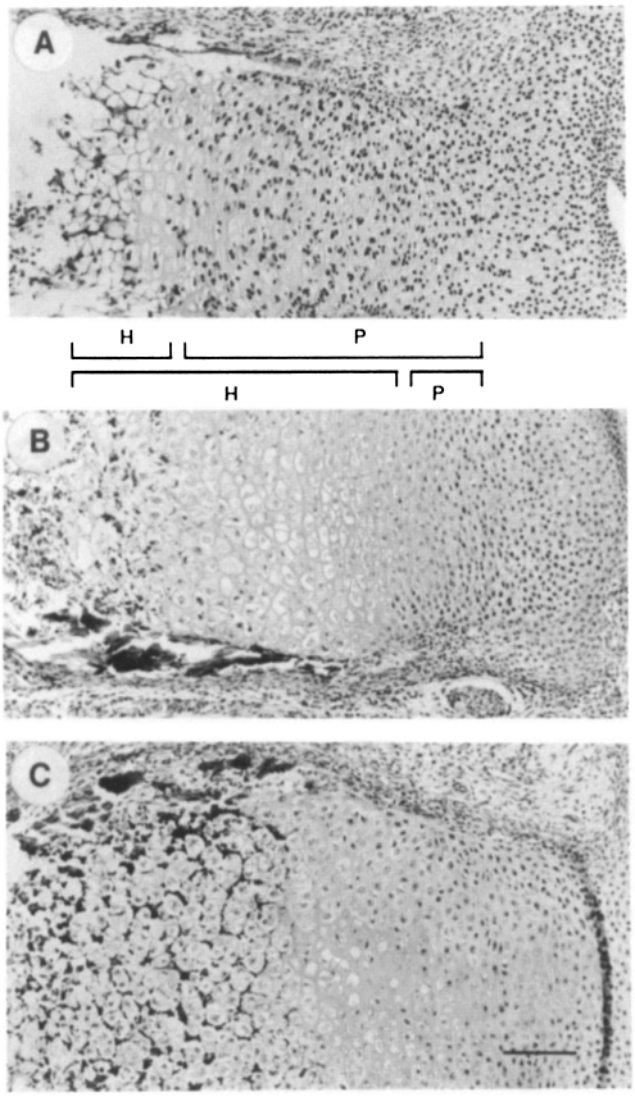

Figure 6. Light microscopy of a growth plate from a 19.5-d Del3 transgenic embryo (*B*) and its nontransgenic littermate (*A*). The forelimbs of the embryos were removed immediately after Cesarean section, fixed, and embedded. Thin sections were stained with hematoxylin and eosin. The figure shows representative views of distal ends of radial bones with the heights of the proliferative and hypertrophic zones indicated with *P* and *H*, respectively. Another view of a 19.5-d homozygous Dell transgenic growth cartilage (*C*) demonstrates an acellular area in the central part of the cartilage seen typically in several severe Del mutant cartilages. Bar, 100 μm.

cytes in various developmental stages with different proliferative and synthetic activities (Stockwell, 1979; Sandberg and Vuorio, 1987).

In light microscopy the most striking features in the epiphyseal and growth plate cartilages of Del3 offspring were the reduction in the cartilaginous extracellular matrix and disorganization of the characteristic architecture of the normal growth plate (Fig. 6). Compared with wild-type mice (Fig. 6 *A*) the Del3 and homozygous Dell offspring exhibited a marked reduction in the number of proliferating chondrocytes, an absence of the typical columnar arrangement, and a severalfold increase in the height of the zone of hypertrophic chondrocytes (Fig. 6, *B* and *C*). The sharp line of demarcation at the osteochondral junction was replaced by deep invaginations of hypertrophic chondrocytes into the diaphysis. In the transgenic mice the bone spicules were reduced in numbers suggesting impaired replacement of cartilage matrix by bone. On the contrary, bone formation along the periosteal collar of long bones was enhanced as evidenced by the thickened periosteal bone extending well beyond the osteochondral junctions. In Del3 and homozygous Dell cartilages chondrocyte hypertrophy was also observed in anomalous locations, e.g., central parts of the growth plate and epiphysis (Fig. 6 *C*). Strikingly, no thick bundles of collagen fibers, prominent in another transgenic mouse line with a Gly85→Cys point mutation in the same gene (Garofalo et al., 1991), were seen in the cartilaginous matrix of the Dell or Del3 offspring. Light microscopic changes in heterozygous Dell mice were much milder; the main difference seen was the increased number of hypertrophic and degenerating chondrocytes in growth cartilages and in anomalous locations.

Electron microscopic examination of the growth cartilages revealed a very severe reduction in the cartilage collagen fibrils in Del3 and homozygous Dell pups (Fig. 7). In addition the morphology of these fibrils was abnormal: instead of the thin straight fibrils the mutant cartilages contained disorganized fibrils which appeared curved or wavy (Fig. 7, *F* and *H*). Collagen fibrils in heterozygous Dell cartilages were only slightly reduced in amount; some of the fibrils had a wavy structure analogous to those seen in the more severe mutants (Fig. 7 *D*). In all transgenic cartilages the chondrocytes were found to contain prominent, dilated RER, suggestive of retention of newly synthesized molecules in the cells (Fig. 7, *C*, *E*, and *G*).

Discussion

The expression of an α1(II) collagen transgene with a deletion of exon 7 has dramatic effects on the development and growth of the skeleton. The mutation in the α1(II) collagen gene causes malfunction of the cartilage growth plates, which results in reduced size, retarded ossification, and disproportionate growth (Figs. 3 and 6). The malfunction of the growth plates is most likely caused by reduction in the amount of cartilage extracellular matrix, which is most likely itself a consequence of a very marked decrease in the collagen fibrils (Fig. 7). This reduction in the amount of cartilage fibrils, the final synthetic product of the type II collagen gene was clearly dependent on the level of transgene expression as determined by RNase protection experiments (Fig. 5). Indeed, the most severe phenotype was seen in Del3 offspring which has the highest expression level of the transgene, a somewhat milder symptomatology in homozygous Dell offspring with a somewhat lower level of transgene-derived mRNA, a much milder phenotype in heterozygous Dell offspring, and apparently normal phenotypes in homozygous and heterozygous Del2 offspring. The tissue specificity of transgene expression, as well as a correlation between gene copy number and the severity of the growth abnormalities, probably result from the use of the entire mouse gene with a substantial amount of flanking sequences for microinjections. Recent work on the rat type II collagen gene has suggested the presence of a tissue-specific enhancer

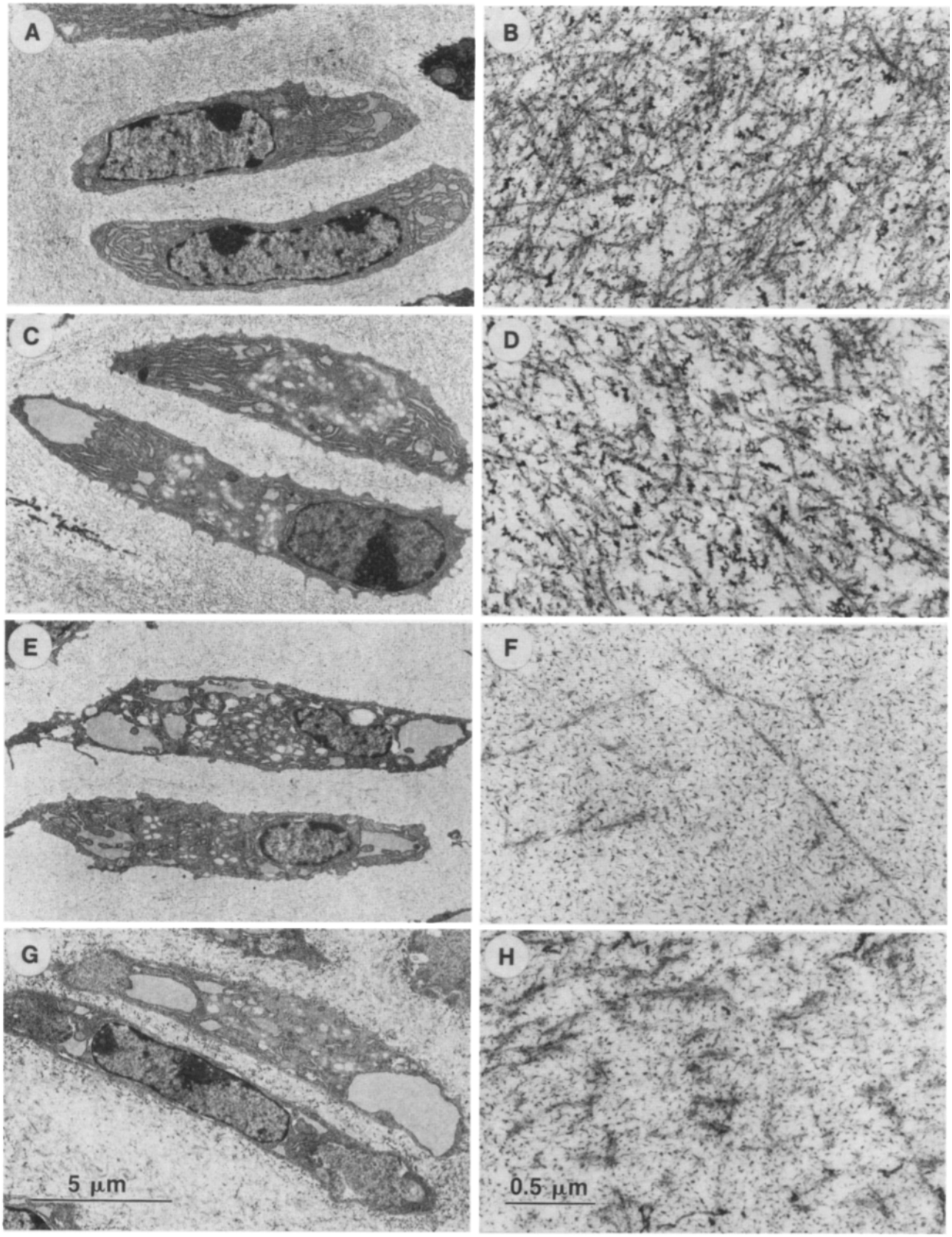

Figure 7. EM of growth cartilages of 19.5-d embryos. (*A*, *C*, *E*, and *G*) Chondrocytes in the proliferative zone of nontransgenic (*A*), Del1 heterozygous (*C*), and homozygous (*E*), and Del3 offspring (*G*) with the corresponding views of cartilage matrix in *B*, *D*, *F*, and *H*. The bars in panels G and H show the magnification for left and right columns, respectively. Bars: (*A*, *C*, *E*, and *G*) 5 μm; (*B*, *D*, *F*, and *H*) 0.5 μm.

in the first intron of the gene and two silencer elements in the promoter (Horton et al., 1987; Savagner et al., 1990). It is not known, however, whether the sequences in the first intron or other sequences in the gene are, in our experiments, responsible for the substantial level of tissue-specific expression of the transgene, which appears to be independent of the site of integration. Some regulatory elements are probably missing from our construct since >6–7 transgene copies are needed before a gross growth abnormality can be observed.

We believe that the reduction in the amount of extracellular matrix is a direct consequence of retarded secretion of type II collagen molecules containing shortened α chains. The stoichiometry of chain association in the homotrimeric type II collagen molecule makes it particularly vulnerable to such mutations. The presence of one shortened α chain probably reduces the stability of the triple helix and increases intracellular degradation of such mutant procollagen molecules. EM of chondrocytes in Del3 and homozygous Del1 cartilages demonstrated greatly extended RER, a finding that is also typical of analogous mutations in type I collagen. In this case the distension of RER correlates with retention of abnormal procollagen within the cell (Dombowski et al., 1989; Prockop et al., 1990). In the mild chondrodysplasia of heterozygous Del1 mice the extended RER was the most pronounced abnormality observed at electron microscopic level. Currently we cannot explain how these Del1 chondrocytes can sustain quasinormal growth while containing approximately equal amounts of endogenous and transgene mRNA since this stoichiometry suggests that considerable amounts of abnormal procollagen molecules are produced. The presence of abnormal curving or wavy fibrils seen in the cartilage matrix of the severely affected mice suggests a defect also in the extracellular assembly of type II collagen molecules into fibrils. This could be because of the presence of some heterotrimeric type II collagen molecules in these fibrils. Alternatively, homotrimeric molecules of shortened α1(II) chains may be secreted and incorporated into fibrils, their reduced length disrupting fibrillogenesis. Biochemical analyses on the composition of extracellular and intracellular type II collagen molecules are currently in progress to clarify this point.

The structure of cartilage collagen fibrils in the Del mutants is in striking contrast to the abnormalities seen in transgenic mice harboring three other mutations which affect either one of the two cross-linking lysine residues, one in the *N*-telopeptide and one in amino acid 87 of the triple helix, or the glycine-85 residue adjacent to lysine-87. These mutations result in the appearance of thick bundles of banded type II collagen fibrils throughout the cartilage matrices suggesting a major defect in the mechanism which controls the diameter of cartilage collagen fibrils (Garofalo et al., 1991; and unpublished observations). No such thick fibrils were observed in the Del mutant cartilages. Recently mice harboring a human type II collagen transgene with a large deletion of 12 triple helical exons were shown to have a short limbed dwarfism similar to the severe Del phenotype (Vandenberg et al., 1991). Histological analysis of these animals also revealed a marked reduction in cartilage collagen fibrils. No thick collagen fibrils typical for the Gly85 and cross-link mutants were observed.

The most interesting aspect of the expression of the mutant transgene is the marked disorganization of the normally well-defined columnar architecture of proliferating, hypertrophic, and degenerating chondrocytes in the growth cartilage (Sandberg and Vuorio, 1987; Hunziker and Herrmann, 1990). We propose that the shortening of the proliferative zone results from inadequate production of type II collagen, which prevents the clonally derived chondrocytes from separating from each other and forming typical columnar structures. This phenomenon probably explains the greatly reduced growth rate of long bones. A unique feature of the Del mutation in the growth plate is the severalfold increase in the size of the zone of hypertrophic chondrocytes extending deep into the diaphysis of long bones in contrast to the remarkably sharp line of demarcation between cartilage and invading bone in the normal growth plate. The histological picture suggests that replacement of cartilage matrix by bone is impaired in these mice. The severe reduction in collagen fibrils clearly causes an impairment of the growth plate architecture at several levels. The normal columnar organization of the proliferative chondrocytes, the size of the zone of hypertrophic chondrocytes, and the transition zone between hypertrophic zone and the osseous zone itself are all severely disrupted by the diminution of the number of collagen fibrils. Our results indicate that the structural integrity of these different parts of the growth plate are essential to assure normal skeletal growth. At least three extracellular matrix components have been proposed to play a role in mineralization and degeneration of hypertrophic cartilage. Type X collagen is a short-chain collagen with an extremely restricted expression pattern limited to hypertrophic chondrocytes (Schmid and Linsenmayer, 1987). In the growth plate, chondrocyte hypetrophy is also coincident with expression of mRNA for osteonectin (SPARC), a calcium binding protein (Metsäranta et al., 1989). The C-propeptide of type II collagen (chondrocalcin) is another component which has been suggested to be enriched in hypertrophic cartilage and to play a regulatory role (Poole et al., 1984). The production of the C-propeptide, which normally is cleaved extracellularly from secreted procollagen, must be severely affected in the mutant Del cartilage. Although we cannot at this time explain the mechanisms regulating the size of the hypertrophic zone, the abnormal growth plates in Del mice may be valuable for such investigations.

We believe that the phenotypic abnormalities in the Del3 and Del1 transgenic pups result from impaired cartilage production which results in retarded bone growth. The cleft palate is probably a secondary consequence of retarded mandibular growth which pushes the tongue upwards and prevents the normal closure of palatal ridges (Fig. 4, *A–C*) (Ferguson, 1988). The breathing difficulties probably result from obstructed air flow due to the marked weakening of cartilage structure. Many of the phenotypic abnormalities we have seen in the severe Del3 and Del1 transgenic mice resemble those seen in human chondrodysplasias. Indeed type II collagen mutations have recently been identified in some of these diseases with very different degrees of severity extending from perinatal lethal achondrogenesis type II to mild spondyloepiphyseal dysplasia with predisposition to osteoarthritis (Lee et al., 1989; Vissing et al., 1989; Tiller et al., 1990; Ala-Kokko et al., 1990; Ahmad et al., 1991). While most of the abnormalities in the Del mice (e.g., matrix deficiency, disorganization of the growth plate, short limbs, and other skeletal deformities) are typical for the spon-

dyloepiphyseal dysplasia (SED) group of chondrodysplasias, the persistence of hypertrophic chondrocytes is a characteristic finding of opsismodysplasia, a rare form of lethal chondrodysplasias (Maroteaux et al., 1984; Spranger and Maroteaux, 1990). In addition to delayed bone maturation the abnormalities in opsismodysplasia include thoracic and craniofacial abnormalities and muscular hypotonia. Several forms of the mouse chondrodystrophies also share features similar to those described for the Del mice (Johnson, 1986). Although the macroscopic phenotype in disproportionate micromelia and chondrodystrophy closely resembles that of severe Dell and Del3 mutants, the histologic findings are clearly distinct from ours. Further work is needed before the link between the human diseases, the mouse chondrodystrophies, and the transgenic mouse model can be established. However, our observations illustrate the usefulness of the transgenic mouse system to characterize both the functional topology of the type II collagen molecule and the dysfunction of growth cartilages in human chondrodysplasias.

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