Separable *cis*-regulatory Elements that Contribute to Tissue- and Site-specific α2(XI) Collagen Gene Expression in the Embryonic Mouse Cartilage

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Abstract. Type XI collagen is a structural component of the cartilage extracellular matrix and plays an important role in skeletal morphogenesis. As a step toward defining the molecular mechanisms responsible for the regulation of type XI collagen expression, we characterized the promoter region of the mouse α2(XI) collagen gene (*Col11a2*). We also generated transgenic mice harboring various fragments of the promoter and the first intron of *Col11a2* linked to the *Escherichia coli* β-galactosidase gene to identify the *cis*-acting elements responsible for tissue- and site-specific expression during development. Cloning and sequence analysis of the 5′ flanking region of *Col11a2* showed that the putative 3′ end of the retinoid X receptor β gene was located 742 bp upstream of the *Col11a2* start site. This suggested that the promoter region of *Col11a2* was localized within this 742-bp sequence, which contained multiple consensus regulatory elements. Examination of the transgenic mice revealed that the longest DNA construct (containing the entire promoter and first intron sequences) directed *lacZ* expression in the notochord as well as in the primordial cartilage throughout the body, with the pattern of expression mimicking that of endogenous *Col11a2* transcripts. On the other hand, deletion of the upstream ~290 bp resulted in the elimination of *lacZ* expression in the primordial cartilage of the carpals, tarsals, and vertebral bodies, whereas *lacZ* expression in the notochord and in the other primordial cartilage elsewhere was not affected. Deletion of the first intron sequence also resulted in the loss of *lacZ* expression in the primordial cartilage of the carpals, tarsals, and vertebral bodies, as well as in the notochord. These results demonstrate that the upstream 742-bp and first intron segments of the mouse *Col11a2* gene contain the necessary information to confer high level tissue-specific expression in mouse embryos. In addition, our observations suggest the presence of site-specific *cis*-acting elements that control *Coll11a2* gene expression in different cartilaginous components of the skeleton.

THE collagen superfamily consists of various collagens, which play an important role as structural components in the connective tissue. Among them, types II, IX, and XI collagens are predominantly found in hyaline cartilage, where they form heterotypic fibrils that confer tensile strength and provide a scaffolding for matrix proteoglycans (Kielty et al., 1993). The type XI collagen molecule is thought to coassemble with type II collagen molecules to form fibrils, whereas type IX collagen is associated with the surface of the fibrils (Mendler et al., 1989; Vaughan et al., 1988). The type XI collagen molecule is composed of three distinct subunits: α1(XI), α2(XI), and α3(XI) (Morris and Bächinger, 1987). The α3(XI) chain is believed to be a posttranslational variation product of the α1(II) collagen gene (Furuto and Miller, 1983), whereas the α1(XI) and α2(XI) chains are distinct gene products (Bernard et al., 1988; Kimura et al., 1989).

The function of type XI collagen is still obscure. However, emerging evidence suggests that it may influence the collagen fibrillar network in cartilaginous tissue by regulating the lateral growth of the fibrils (Mendler et al., 1989; Petit et al., 1993). This putative function is supported by the recent identification of an α1(XI) null mutation in *cho/cho* (chondrodysplasia) mice, where the cartilage lacking α1(XI) collagen chains showed abnormally thick collagen fibrils (Li et al., 1995). Furthermore, the identification of α2(XI) collagen mutation as a cause of certain forms of human osteochondrodysplasia, Stickler syndrome, and otospondylo-megaepiphyseal dysplasia, indicates that type XI collagen is intimately involved in skeletal morphogenesis (Vikkula et al., 1995).

Expression of the three subunits of the type XI collagen molecule follows a distinct tissue-specific pattern (Lui et al., 1995). In addition to its presence in cartilage, the mRNA

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encoding the α1(XI) collagen chain is found in a variety of non-cartilaginous tissues such as the bone, vitreous, heart, and cerebral neuro-epithelium (Yoshioka et al., 1995*a*). In some of these tissues, α1(XI) collagen has been suggested to form hybrid trimers with chains of type V collagen, indicating that type V and XI collagens may not necessarily be separate collagen entities (Mayne et al., 1993). The mRNA of the type II collagen gene, which encodes the α3(XI) chain as well as α1(II) chain, is also detected in various tissues in addition to cartilage (Cheah et al., 1991). On the other hand, expression of the α2(XI) collagen gene seems to be more restricted. Although low levels of alternatively spliced variants of α2(XI) transcripts are detected in various tissues (Tsumaki and Kimura, 1995), the major transcript is predominantly found in cartilage. However, the sequences responsible for this tissue-specific expression of the *Col11a2* gene have not been identified.

In the present study, we attempted to define the *cis*-regulatory regions which control α2(XI) collagen gene expression during development. For this purpose, we first characterized the 5′ portion of the mouse α2(XI) gene (*Col11a2*)[1]. Then, to investigate the ability of the *Col11a2* promoter to direct tissue-specific expression, we generated transgenic mice that harbored various combinations of *cis*-elements linked to the *Escherichia coli* β-galactosidase gene (*lacZ*). Our results indicated that a sequence from −742 to +380 combined with the first intron of *Col11a2* were sufficient to direct tissue-specific expression that was similar to that of the endogenous α2(XI) transcript. Interestingly, we also found that distinct sets of *cis*-elements directed *lacZ* expression in the different components of the cartilaginous skeleton.

Materials and Methods

Characterization of the Genomic Clone

Fragments from the λNT8 mouse genomic clone (Tsumaki and Kimura, 1995), which contained the 5′-flanking region of *Col11a2*, were subcloned into pBluescript II SK$^+$ (Stratagene Inc., La Jolla, CA) for nucleotide sequence analysis. Sequence analysis of double-stranded DNA was performed using *Taq* Dye Primer and Dye Deoxy Terminator cycle sequencing kits (Applied Biosystems Inc., Foster City, CA) together with an Applied Biosystems 373A DNA Sequencer.

Determination of the Start Site

To determine the sequence of the 5′ portion of α2(XI) collagen mRNA, rapid amplification of cDNA ends (RACE) was done as described previously (Tsumaki and Kimura, 1995). Briefly, Poly(A)$^+$ RNA was extracted from the thorax of 1-d-old mice and was reverse-transcribed using a *Col11a2*-specific primer (5′ CGTTGTGGGGGCACTCTTCT 3′, which was complementary to nt +373–+392) (see Fig. 1 for the location of the primers). After poly(dC) tailing, the cDNA was used as a PCR template by employing an adapter primer and another *Col11a2*-specific primer (5′ TCGGTGGCCGCTACTGTGT 3′, complementary to nt +335–+353) located just upstream of the reverse transcriptase primer. Then aliquots of the RACE products were purified by agarose gel electrophoresis, cloned into the plasmid vector, and sequenced as described above.

S1 nuclease protection was done by the method described for double-stranded DNA probes (Sambrook et al., 1989). Using the genomic fragment from λNT8 as a template, a ^{32}P-labeled DNA probe (397 bp in size) was generated by PCR with a 5′-end-^{32}P-labeled oligonucleotide (5′ ATGGTACACCCCTACTCCTG 3′, complementary to nt +147–+166) and an un-labeled oligonucleotide (5′ CAATTAGCACGTCACCCAT 3′, nt −231 to −213) as the primers. The end-labeled probe was purified using SupRec (Takara Inc., Shiga, Japan), and was hybridized for 16 h at 57°C with either 25 μg of total RNA from the rib cartilage of 4-wk-old mice or yeast tRNA. Then digestion was done with different concentrations of S1 nuclease (Takara Inc.). The reaction products were fractionated on denaturing 6% polyacrylamide gel for sizing relative to a sequence reacted with the same end-labeled primer used to obtain the double-stranded probe, after which bands on the dried gels were detected by autoradiography.

For the primer extension experiments, an end-labeled oligonucleotide primer (5′ ATCTTCTTCAGCCCCTTCAC 3′, complementary to nt +72–+91) was synthesized by attaching a fluorescent dye, 6-FAM amidite (Applied Biosystems Inc., Foster City, CA), to the 5′ end. One picomole of the primer was combined with 7 μg of poly(A)$^+$ RNA from the thorax of 3-d-old mice, and extended with reverse transcriptase according to the previously described method (Sambrook et al., 1989). The extension products were separated on denaturing 6% polyacrylamide gel for sizing relative to a sequence reacted with the same fluorescent dye-labeled primer. Then signals from the bands were recorded and analyzed using Applied Biosystems 373A and GENESCAN software (Applied Biosystems Inc.).

Construction of Col11a2-lacZ Reporter Genes

The expression vector used was pNASSβ (Clontech Inc., Palo Alto, CA), which contains a polylinker site, an SV40 RNA splice site, the *lacZ* reporter gene, and a SV40 polyadenylation signal. As described in Results, the putative 3′ end of the retinoid X receptor β gene (*Rxrb*) was located 742-bp upstream of the *Col11a2* transcription start site, suggesting that the promoter region of *Col11a2* was localized within this 742-bp sequence.

Two DNA segments of the *Col11a2* promoter were used: an 1,122-bp fragment between −742 and +380 was generated by PCR with the genomic subclone from λNT8 as the template using primers P-1 and Ex-1, while a 833-bp fragment between −453 and +380 was generated using the primers P-2 and Ex-1 (see Fig. 3 for the positions of the primers). To facilitate insertion of these DNA fragments into the polylinker site of pNASSβ, an 11-bp sequence containing the EcoRI site was added to the 5′ end of primers P-1 and P-2. Similarly, a sequence containing the XhoI site was added to the 5′ end of primer Ex-1. After digestion with EcoRI and XhoI, the 1,122-bp and 833-bp DNA fragments were cloned into the EcoRI/XhoI polylinker region located upstream of the SV-40 splice sites of pNASSβ to create two constructs (742*lacZ* and 453*lacZ*, respectively).

A 2.3-kb DNA fragment covering most of the first intron of *Col11a2* was also generated by PCR with the genomic subclone from λNT8 as the template using primers In-1 and In-2. These primers were tagged with 11-bp sequences containing the SalI and PstI sites, respectively. After digestion with SalI and PstI, the fragment was cloned into the SalI/PstI polylinker region located downstream of the SV-40 polyadenylation signal of 742*lacZ* and 453*lacZ* to create two more constructs (742*lacZ*Int and 453lacZInt, respectively). The PCR primers used were as follows: P-1, GTCGCGAATTCAATAGCCATTTTCTGGACTGAGA; P-2, TTCCCGAATTCTCTTGGTCACTTCAGCTTTCACC; Ex-1, TACGTCTCGAGACTCTTCTTGTCAGTGCTGCTTG; In-1, TGGTAGTCGACTGTCAGTCAGTTGAAGGTTGTAGCATC; and In-2, CAGCTCTGCAGCCAGATCTCAGTCTCCCTGACATTCTC.

Generation of Transgenic Mice

The plasmids 742*lacZ*, 453*lacZ*, 742*lacZ*Int, and 453*lacZ*Int were digested with EcoRI and PstI to release the inserts from their vector sequences. Transgenic mice were produced by microinjecting each of the inserts into the pronuclei of fertilized eggs from F1 hybrid mice (C57BL/6 × C3H) as described previously (Hogan et al., 1994). Founder mice were identified by PCR assays of genomic DNA extracted from the placenta or tail. The DNA was subjected to *lacZ*-specific PCR with a set of primers (sense, GCATCGAGCTGGGTAATAAGCGTTGGCAAT; antisense, GACACCAGACCAACTGGTAATGGTAGCGAC) to amplify an 822-bp product (Hanley and Merlie, 1991). Positive founder mice were mated with wild-type mice to generate hemizygous embryos, and positive embryos were identified by PCR using placental DNA.

1. *Abbreviations used in this paper*: *Col11a2*, the gene encoding murine α2(XI) collagen; nt, nucleotide(s); *lacZ*, the *Escherichia coli* β-galactosidase gene; p.c., postconception; RACE, rapid amplification of cDNA ends; *Rxrb*, the murine retinoid X receptor β gene; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Staining for β-Galactosidase Activity

β-galactosidase activity was detected in transgenic founder embryos or embryos obtained from transgenic mouse lines by staining with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Sigma Chem. Co., St. Louis, MO) according to the previously described technique (Bonnerot and Nicolas, 1993). Briefly, the embryos were fixed in 4% paraformaldehyde for 15–40 min according to their size, and were stained with X-gal for 2–7 h at 30°C. The reaction was stopped by washing in phosphate-buffered saline and postfixing in 4% paraformaldehyde. In some embryos more than 13.5 d postconception (p.c.), the spinal columns and limbs were dissected and processed in the same manner. All solutions used were buffered at pH 7.4 to eliminate the endogenous β-galactosidase activity. For histological examination, stained embryos and dissected tissues were embedded in Tissue-Tek® O.C.T. compound (Miles Inc., Elkhart, IN) for cryostat sectioning, or were dehydrated, embedded in paraffin, and sectioned on a microtome. Then the sections were counterstained with eosin.

Results

Structural Analysis of the 5′-Portion of Col11a2

Previously, several cDNA clones of mouse α2(XI) collagen have been isolated (Tsumaki and Kimura, 1995). In the present study, we used the RACE method to obtain a cDNA clone which further extended 507 bp upstream of the ATG codon (Fig. 1). Comparison of the sequences of the cDNA clones with those of genomic subclones from λNT8 suggested that the untranslated sequence resided in a single exon. The 5′ end of this exon was mapped by S1 nuclease protection using an end-labeled 397-bp DNA fragment and 166-170 nt protected fragments were found (Fig. 2), indicating that the end of the exon was located 516–520 bp upstream of the translational start codon. To confirm the 5′ boundary of exon 1, a primer extension experiment was performed using a primer located 426–445 bp upstream of the translational start codon. The primer yielded a single major extension product of 91 bp (Fig. 2). From these results, we determined that the G residue located 516 bp upstream of the translational start codon was the major transcriptional start site.

Sequence analysis of the 5′ flanking region of *Col11a2* showed that the putative 3′-end of the retinoid X receptor β cDNA sequence (Hamada et al., 1989) was located 742 bp upstream of the transcriptional start site (Fig. 1). Therefore, we speculated that the promoter region of *Col11a2* was localized within this 742-bp stretch, which was identical to the recently reported mouse *Col11a2* sequence with the exception of 2 bp and showed marked homology to that of the human gene (Vuoristo et al., 1995). Comparison of the sequence of the *Col11a2* promoter with consensus regulatory elements showed that there was no apparent TATA box or CCAAT element. However, there

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-780  CCATCTTTCA CTTGCACATA AAGTTGCTTT CCAGTTAAAA TAGCCATTTT CTGGACTGAG
-720  ATAACTATAT GGGCGGTGGG CACCAGGGCA TCTACCTGTG GGGATATCCG GTGAGAAACC
-660  CTGACATGTT CCCTGGATGC TGCCACGGCC TGAGGTTCCC TGGGCCTACA GAGCCGGTGC
-600  AGGGTGGGGG ACACAAGGCG TGCTTTGTCG GGAGGGGGAG AGGGTGTGCC AGGTTGGGGG
-540  CGGGGCTGCC CAGGGAGGAG GGAGAGCGGC TGCTTTTCAA AGGCGCCTTG TTTGCCGGTG
-480  CCCTTCCAGC GCCAGCGGGC GGCGGCGTCT TGGTCACTTC AGCTTTCACC CTGGCTTCGC
-420  CACAGGCTCC TGGCAGACCC GGCACTGTGC CCACGCTTAG GGGTGCGCTG CTCTCAGCGA
-360  TACCCTCCTA GAACGGCCAC GACCCTGAGT GACCCTCTTT TGCCAGACCT TTCCATCAGC
-300  ATCGCCCCCC CCCACCCCGT ACCCCTTTTC ATTCTGCTTC ACCTAGTCCA GTATTTTTAT
-240  CTTTGGGGGC AATTAGCACG TCACCCATCT CAGCTTTCTC CGTTCCGCAG GGCGACCCTA
-180  CGCCCCCCAC CTTTGGGTCG CTATCTATAG CTGGGTCTAT AAACACCCCG CCCAGGACTG
-120  CTCTGTAATT ACATGTGGCA GGACGAGGGA GGTAATTATG GAGAGCTGAT TACGAGTGGG
 -60  CGTGGAACGG TCGCCCGTAG GCCCGCCCCT TCTGTCCCAC TGTGCCCTGA GTCCCTCCCC
  +1  GGACTCTTCA TAGGGGCTCT GGGCCCCCAA GGGCAGCTGC CGTGGTGTGA AAGGACTATA
 +61  GGATCATAAA AGTGAAGGGG CTGAAGAAGA TATATTCTGT TCCCACAAAT GCCATCTGAG
+121  GGGGCACGCG AGCTACCTAA TCCCTCCAGG AGTAGGGGTG TACCATTCCA GGGCTAGCGA
+181  GTGAGGAAGG TTTCCAACCC TCACACTCTC CTGCCCAGGT GGAGATCGGG GTGGGGTTTC
+241  CCCTTGGTGG CTGTGGGCGG GCGTCAGGAG GCGGGAGCTG GACCTAGGGA CGGGGGCGAG
+301  CTGGGGTCTC TGAGCTGCCA GGGTGGCCAG GGACACACAG TAGCGGCCAC CGAAGAGCAA
+361  GCAGCACTGA CAAGAAGAGT GCCCCCACAA CGGCACCTTG CTGCATGGAG CTGGGCCACT
+421  GAAAGCTGCT GACATCCCGG GGTCCTGGTT CCGAGGCTCA GCTGGCTTCT GTCTGCTGTC
+481  AGCACCAGGA CTGCCTGGGA TCTGGCATTC TGAGCCATGG AGCGGTGCAG CCGCTGCCAC
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Figure 1. Nucleotide sequences of the mouse α2(XI) collagen promoter and 5′-untranslated region. The transcription start site is shown as +1 and the 5′ end of the primer extension product is indicated by the asterisk. The 5′ ends of the S1 nuclease-protected bands are indicated by black dots. Note that there is neither a proper TATA box nor a CCAAT box within the first 100-bp sequence. The putative SP1 recognition sequences are marked with boxes. The potential Pax-1 recognition sites are underlined. The closed triangle denotes the putative 3′-end of the retinoid X receptor β gene sequence. The putative translation start codon is underscored by bold line. The horizontal arrows show the positions of the nested primers used for RACE. The vertical arrow indicates 5′ end of the RACE product. The horizontal half-arrow marks the location of the oligonucleotide primer used for primer extension analysis. These sequence data are available from GenBank/EMBL/DDBJ under accession number D84066.

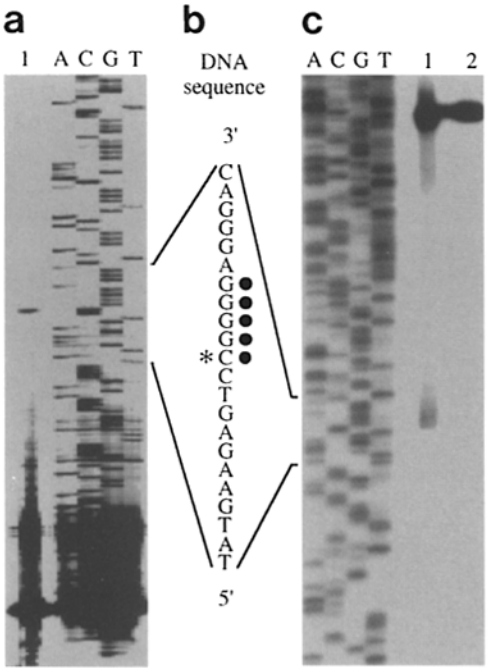


Figure 2. Determination of the transcription start site of *Col11a2*. (*a*) Primer extension mapping using mRNA from mouse thorax. The position of fluorescent-labeled primer used for the experiment is shown in Fig. 1 and begins at +91. After separation on denaturing polyacrylamide gel, signals were recorded and analyzed using Applied Biosysems 373A and GENESCAN software. The extension products are shown in *lane 1*, while the adjacent lanes show a sequence ladder of the corresponding genomic sequence processed using the same primer. (*b*) The results of primer extension and S1 nuclease protection studies. Along the DNA sequence of the antisense strand, the 5′ end of the extension product is indicated by an asterisk and the S1 protected bands by black dots. (*c*) S1 nuclease mapping using a 397-bp end-labeled DNA probe (nt −231 to +166 in Fig. 1). The probe was annealed with RNA from mouse costal cartilage (lane *1*) and with yeast tRNA (lane *2*). Sequencing reactions using the primer beginning at +166 are shown in the adjacent lanes.

were several potential SP1 binding sites (Fig. 1) typical of such TATA-less class promoters (Kageyama et al., 1989).

Production of Transgenic Mice Bearing Col11a2-lacZ Constructs

To identify the *cis*-regions responsible for the tissue-specific expression of *Col11a2*, we prepared DNA constructs containing various *Col11a2* fragments linked to the *lacZ* reporter gene. In addition to the promoter sequence, the first intron sequence of *Col11a2* was used to generate transgene constructs (Fig. 3), since previous studies have shown that other fibrillar collagen genes, including the α1(I) gene (Hartung et al., 1986), α2(I) gene (Rossi and de Crombrugghe, 1987), and α1(II) gene (Horton et al., 1987) possess elements with enhancer activity in the first intron. The constructs 742*lacZ* and 453*lacZ* contained the 742 and 453 bp promoter sequences, respectively, while 742*lacZ*Int and 453*lacZ*Int also harbored the first intron sequence in addition to the respective promoters.

By microinjecting these DNA constructs into fertilized

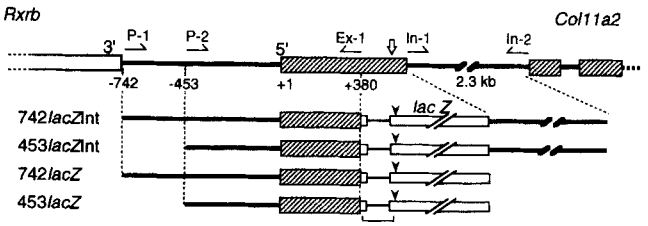


Figure 3. Diagrams of the DNA constructs used to generate *Col11a2-lacZ* transgenic mice. The gene structure around the mouse *Rxrb/Col11a2* junction is shown at the top. Boxes indicate the coding regions and solid lines denote non-coding sequences. Horizontal arrows indicate the positions of the primers used for construction. The open arrow marks the location of the translation initiation site of *Col11a2*. The various transgenes are shown below the genomic map. A bracket represents the SV-40 intron cassette, and arrowheads indicate ATG codon for *lacZ*.

eggs, independent transgenic mouse lines were generated (Table I). Five of the six transgenic lines bearing 742*lacZ*Int exhibited detectable β-galactosidase activity on X-gal staining. All four transgenic lines bearing 453*lacZ*Int also expressed detectable β-galactosidase activity. Although there were variations in the level of transgene expression between these lines, the overall pattern of β-galactosidase expression was similar. For 742*lacZ* and 453*lacZ*, one line each was found to express β-galactosidase activity. Therefore, additional X-gal staining analysis was performed in founder embryos. For 742*lacZ*, three transgenic founder embryos showed β-galactosidase activity and all three had a pattern of expression that was similar to each other and to that of the established transgenic line. In the case of 453*lacZ*, however, four out of seven transgenic founder embryos showed β-galactosidase activity, and the pattern of expression was variable.

α2(XI) Collagen-lacZ Transgene Expression during Murine Development

To assess the pattern of expression of the transgenes, we stained transgenic embryos with X-gal. The different *lacZ* expression patterns directed by DNA constructs containing various *cis*-regions are shown in Fig. 4. On 11.5 d p.c., 742*lacZ*Int, containing the entire promoter and first intron sequences, directed *lacZ* expression exclusively in the notochord (Fig. 4 *a*). Deletion of the 289-bp upstream sequence of the promoter region (453*lacZ*Int) did not affect the pattern of *lacZ* expression (Fig. 4 *b*). On the other hand, deletion of the first intron sequence (742*lacZ*) led to

Table I. Production Frequency of Transgenic Mice

Transgene construct	Number of embryos obtained	Number of transgenic mice	Number of transgenic lines expressing β-galactosidase*	Number of directly analyzed transgenic founder embryos‡
742*lacZ*Int	35	9	5/6	ND
453*lacZ*Int	31	7	4/4	ND
742*lacZ*	23	5	1/2	3/3
453*lacZ*	43	8	1/1	4/7

* *lacZ*-expressing lines/transgenic lines established.
‡ *lacZ*-expressing embryos/founder embryos stained with X-gal.
ND, not done.

the elimination of *lacZ* expression (Fig. 4 *c*). With 453*lacZ*, there was also no *lacZ* expression in the notochord (not shown).

By 13.5 d p.c., chondrogenesis has commenced in the limbs, axial skeleton, and thorax. The scapula, humerus, ulna, and radius of the forelimb are recognizable at this stage, and the primordial cartilage of the hindlimbs is also present. Three *Col11a2-lacZ* constructs (742*lacZ*Int, 453*lacZ*Int, and 742*lacZ*) directed similar overall *lacZ* expression pattern in these cartilaginous components of the skeleton (Figs. 4, *d–f*) despite some variations in the level of transgene expression between lines. In mice bearing 742*lacZ*, however, staining of the limb and rib cartilage tended to be weaker, and the future petrous temporal bone was more marked than in the mice bearing other constructs (Fig. 4 *f*).

Although 742*lacZ*Int, 453*lacZ*Int, and 742*lacZ* initially mediated similar β-galactosidase expression pattern in limb cartilage, marked differences were noted by 15.5 d p.c., which is when the distal components of the limbs, such as the carpals, metacarpals, and phalanges, undergo chondrification. As shown in Fig. 4 *g*, the construct 742*lacZ*Int containing the entire promoter and first intron sequences directed *lacZ* expression throughout the entire primordial cartilage of the forelimbs. On the other hand, deletion of the 289-bp upstream sequence of the promoter region (453*lacZ*Int) resulted in specific loss of expression in the primordial cartilage of the carpals (Fig. 4 *h*). The expression in the primordial cartilage of the metacarpals and phalanges remained unaffected, and the mineralized diaphyseal regions seemed to show more β-galactosidase activity. Similarly, deletion of the first intron sequence (742*lacZ*) from 742*lacZ*Int resulted in loss of expression in the carpals (Fig. 4 *i*) In the hindlimbs, each transgene directed similar, but slightly less distinct, patterns of *lacZ* expression to those seen in the forelimbs (Figs. 4, *j–l*).

Transgenic embryos bearing 453*lacZ* did not show *lacZ* expression in cartilage. In these embryos, the staining intensity was weak and variable among four founder mice and mice from one established lines (not shown). This result was probably influenced by the site of transgene integration (Allen et al., 1988), and suggested that the 453-bp promoter in the absence of the first intron lacked tissue-specific activity.

Anatomical Analysis of Transgene Expression during Spinal Column Development

To investigate transgene expression in the axial skeleton during embryonic development, we dissected the spinal columns out of transgenic mice at various stages of gestation, stained them with X-gal, and viewed the specimens from the ventral side. As already demonstrated in Figs. 4, *a–c*, 742*lacZ*Int and 453*lacZ*Int directed *lacZ* expression mainly in the notochord on 12.5 d p.c. (Figs. 5, *a* and *b*). On the other hand, deletion of the intron sequence (742*lacZ*) eliminated *lacZ* expression in the notochord (Fig. 5 *c*).

On 13.5 d p.c., the notochord began to show segmental degeneration where sclerotome cells formed the cartilaginous precursors of the vertebral bodies. Cartilaginous elements also appeared in the precursors of the ribs and the neural arches extending posteriorly along the lateral surface of the spinal cord. The longest construct (742*lacZ*Int) directed *lacZ* expression in the notochord remnant and in the primordial cartilage of the vertebral bodies, neural arches, and ribs (Fig. 5 *d*). On the other hand, deletion of the 289-bp upstream sequence of the promoter region (453*lacZ*Int) resulted in remarkable loss of *lacZ* expression in the cartilage of the vertebral bodies, whereas expression in the notochord remnant and cartilage of the neural arches and ribs remained unaffected (Fig. 5 *e*). Similarly, lack of the intron sequence (742*lacZ*) resulted in almost complete loss of *lacZ* expression in the cartilage of the vertebral bodies, as well as in the notochord remnant (Fig. 5 *f*).

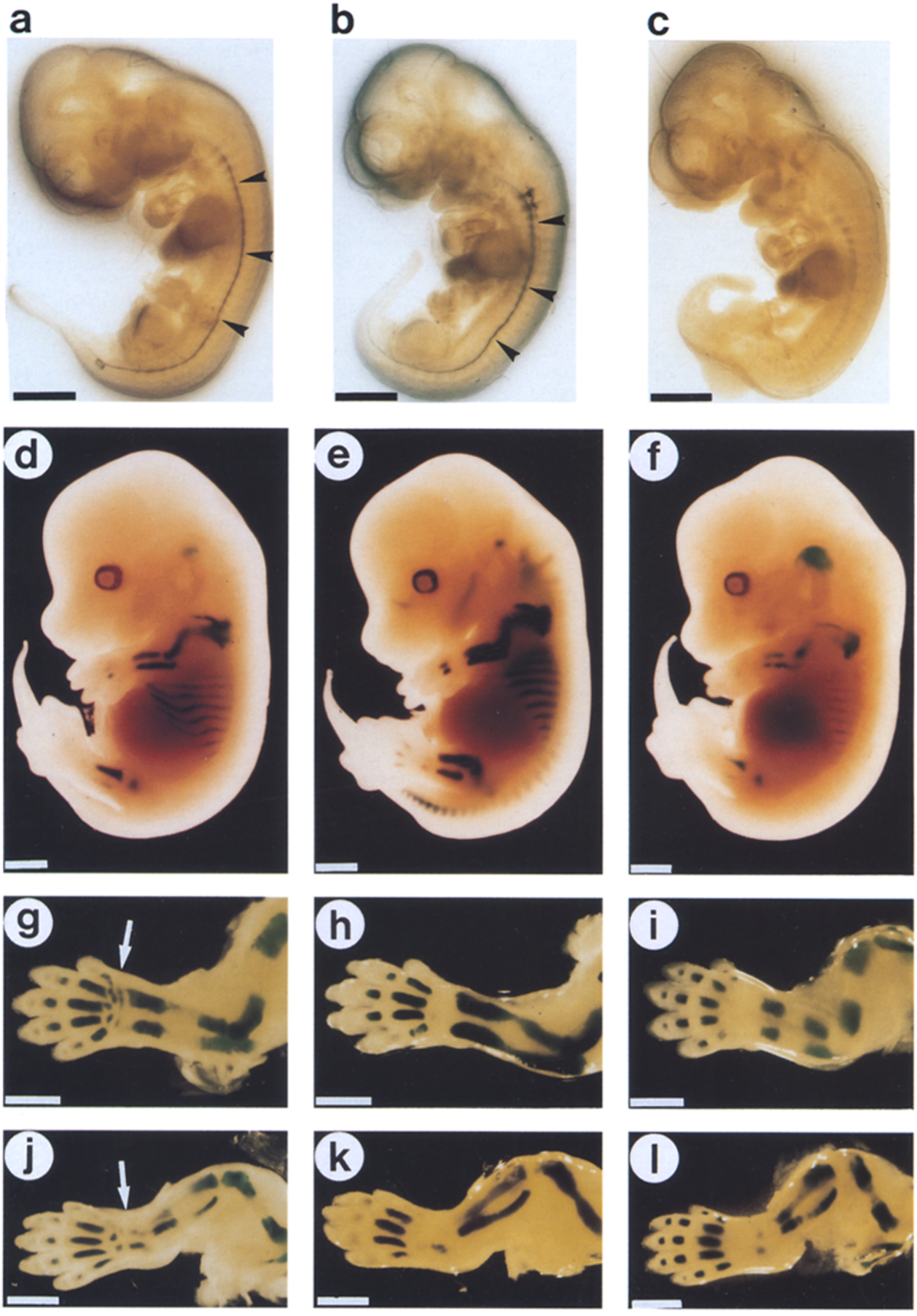
On 15.5 d p.c., both 742*lacZ*Int and 453*lacZ*Int maintained the expression of *lacZ* in the nucleus pulposus, which developed from the notochord remnant (Figs. 5, *g* and *h*). However, 742*lacZ* did not direct *lacZ* expression in the nucleus pulposus (Fig. 5 *i*).

Histological Analysis of Transgene Expression

Macroscopic observation revealed that the entire promoter and the first intron sequences were sufficient to confer *lacZ* expression in the primordial cartilage of the skeleton and in the notochord/nucleus pulposus. To further determine the nature of the cells which showed β-galactosidase activity in the primordial cartilage, we performed histological examination of transgenic embryos bearing 742*lacZ*Int. By 12.5 d p.c., 742*lacZ*Int started to direct β-galactosidase expression in distinct portions of the limb buds in addition to the notochord (Fig. 6 *a*). Examination of transverse sections indicated that the condensing cells showed X-gal staining (Figs. 6, *b* and *c*), suggesting that 742*lacZ*Int was activated in mesenchymal cells after they aggregated into condensations.

As the condensations differentiated into primordial cartilage, various types of chondrocytes became recognizable. Histological examination of the forelimb on 15.5 d p.c. showed that 742*lacZ*Int directed *lacZ* expression in cells ranging from resting to hypertrophic chondrocytes with the most intense staining being noted in the proliferating chondrocytes (Fig. 6 *d*). The pattern of expression directed by 742*lacZ*Int was similar to that of endogenous α2(XI) collagen as determined using in situ hybridization (Sugimoto, M., T. Kimura, N. Tsumaki, Y. Matsui, K. Nakata, Y. Kitamura, S. Nomura, T. Ochi, manuscript in preparation), except that expression of the transgene was slightly stronger in the hypertrophic zone.

The differences in the patterns of *lacZ* expression directed by each of transgene construct were confirmed by histological examination of the spinal columns. Parasagittal sections of the spines from 14.5 d p.c. embryos showed that 742*lacZ*Int directed *lacZ* expression in primordial cartilage of the vertebral bodies as well as in the nucleus pulposus and the diminishing notochord (Fig. 7 *a*). On the other hand, deletion of the 289-bp upstream sequence of the promoter region (453*lacZ*Int) resulted in the loss of detectable expression in the primordial cartilage of vertebral bodies, whereas expression in the nucleus pulposus and diminishing notochord remained unaffected (Fig. 7 *b*). Deletion of the intron sequence (742*lacZ*) similarly elimi-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nated expression in the primordial cartilage of the vertebral bodies, and also led to the loss of expression in the nucleus pulposus and diminishing notochord (Fig. 7 *c*).

Discussion

In this study, we defined the ability of the *Col11a2* gene promoter to direct tissue-specific expression in transgenic mice. Firstly, we cloned and sequenced the 5′ flanking region of *Col11a2* and determined the transcription start site. Our findings, as well as those recently reported by others (Vuoristo et al., 1995), indicated that *Col11a2* was located close to the *Rxrb* gene in a head-to-tail arrangement. The distance between the putative 3′ end of *Rxrb* and the transcription start site of *Col11a2* was ~742 bp, suggesting that the *Col11a2* promoter was located within this segment.

The transgenic mouse experiment indicated that the −742-bp promoter (742*lacZ*) contained information which conferred strong tissue specificity of expression. This segment was active in most cartilaginous tissues during embryonic development, while the shorter segment, a −453-bp promoter (453*lacZ*), was much less active in these tissues. In addition, the shorter promoter by itself showed variability among founder embryos in the pattern of expression, probably reflecting the influence of the site of integration (Allen et al., 1988). It is therefore likely that part of the putative cartilage-specific element is located between −742 and −453. Although the −742-bp promoter seemed to confer a high level expression on the reporter *lacZ*, the spatiotemporal profile of expression was not identical to that of endogenous α2(XI) collagen. Specifically, transgenic mice bearing the −742-bp promoter did not express the transgene in the notochord and the transgene was also distinctively inactive in the primordial cartilage of the vertebral bodies and of small bones including the carpals and tarsals.

The first intron of several collagen genes seems to contain elements that are required to obtain a high level of expression and/or tissue-specific expression. For example, the first intron of the α1(II) collagen gene has been shown to contain an enhancer element which is important for strong tissue-specific expression in chondrocytes (Zhou et al., 1995; Krebsbach et al., 1996). Our present data also support this concept. The transgene bearing the −742 promoter and first intron sequences (742*lacZ*Int) initially directed β-galactosidase expression in the notochord on 11.5 d p.c. and then in all of the cartilaginous tissues (including the carpals, tarsals, and vertebral bodies) after chondrogenic differentiation occurred in the embryos. The spatiotemporal pattern of expression driven by 742*lacZ*Int largely mimicked that of the endogenous gene during embryonic development. Thus, the first intron seemed to contain elements that are necessary for mediating the expression of this gene in the notochord and certain cartilaginous tissues.

The first intron sequence was also capable of mediating a high level expression together with the −453 promoter, although the latter had only a low level of activity by itself. In the case of 453*lacZ*Int, there was initial expression in the notochord and then in the notochord remnants/nucleus pulposus and most of the cartilaginous skeleton, except for the carpals, tarsals, and vertebral bodies. Taken together, our findings suggest a modular arrangement of the *cis*-acting elements in the *Col11a2* promoter and first intron, which control the expression of this gene in different parts of skeleton during embryonic development. There appeared to be several different cell-specific elements. First, an element that promoted a high level gene expression in most chondrocytes was located between −742 and −453 bp. Second, an element necessary for a high level of expression in the notochord and nucleus pulposus was located within the 2.3-kb first intron. Third, there was an element in the first intron that directed a high level of expression in most chondrocytes in cooperation with the −453-bp promoter. Fourth, an element of the first intron, in cooperation with an element between −742 and −453 bp, specifically directed a high level of expression of in the primordial cartilage of the carpals, tarsals, and vertebral bodies.

The distinctive absence of *lacZ* expression in the carpals of the transgenic mice bearing 742*lacZ* or 453*lacZ*Int suggests that a close regulatory relationship may exist between the *Col11a2* and *Hox* genes. Hox genes encode transcription factors that specify regional information along the embryonic axes (Krumlauf, 1994). During limb development, these genes are activated sequentially to create a transcriptional cascade from the *Hox-9* to *Hox-13* paralogues in the limbs. Davis et al. have proposed that the order of activation and interaction of these paralogues specifies the pattern of prechondrogenic condensation along the proximodistal axis (Davis et al., 1995); *Hox* group 9 genes may specify the scapula, *Hox-10* genes specify the humerus, *Hox-11* genes specify the radius and ulna, *Hox-12* genes specify the carpals, and *Hox-13* genes specify the metacarpals and phalanges. Considering the spe-

Figure 4. Transgenic embryos and limbs at various stages of development illustrating expression of the 742*lacZ*Int, 453*lacZ*Int, and 742*lacZ* transgenes. (*a–c*) Whole-mount embryos (11.5 d p.c.) stained with X-gal. (*a*) A 742*lacZ*Int transgenic embryo with transgene expression in the notochord (*arrowheads*). (*b*) A 453*lacZ*Int transgenic embryo also expressing *lacZ* exclusively in the notochord (*arrowheads*). (*c*) A 742*lacZ* transgenic embryo showing no expression throughout the body. (*d–f*) Whole-mount embryos (13.5 d p.c.) stained with X-gal. An identical pattern of *lacZ* expression is seen in the primordial cartilage of the limb bones and ribs in each transgenic embryo harboring 742*lacZ*Int (*d*), 453*lacZ*Int (*e*), and 742*lacZ* (*f*). (*g–i*) Forelimbs of 15.5 d p.c. transgenic embryos. (*g*) The 742*lacZ*Int transgene directed *lacZ* expression in the primordial cartilage of the carpal bones (*arrow*) as well as in the cartilage of the humerus, ulna, radius, metacarpals, and phalanges. On the other hand, 453*lacZ*Int (*h*) and 742*lacZ* (*i*) failed to direct β-galactosidase expression in the primordial cartilage of the carpal bones, although cartilage of the other limb bones showed X-gal staining. (*j–l*) Hindlimbs of 15.5 days p.c. transgenic embryos. (*j*) The 742*lacZ*Int transgene directed *lacZ* expression in the primordial cartilage of the tarsals (*arrow*) as well as in the cartilage of the femur, tibia, fibula, metatarsals, and phalanges. On the other hand, 453*lacZ*Int (*k*) and 742*lacZ* (*l*) caused minimal β-galactosidase expression in the cartilage of the tarsal bones, while that of other limb bones showed strong X-gal staining. Bars, 1 mm.

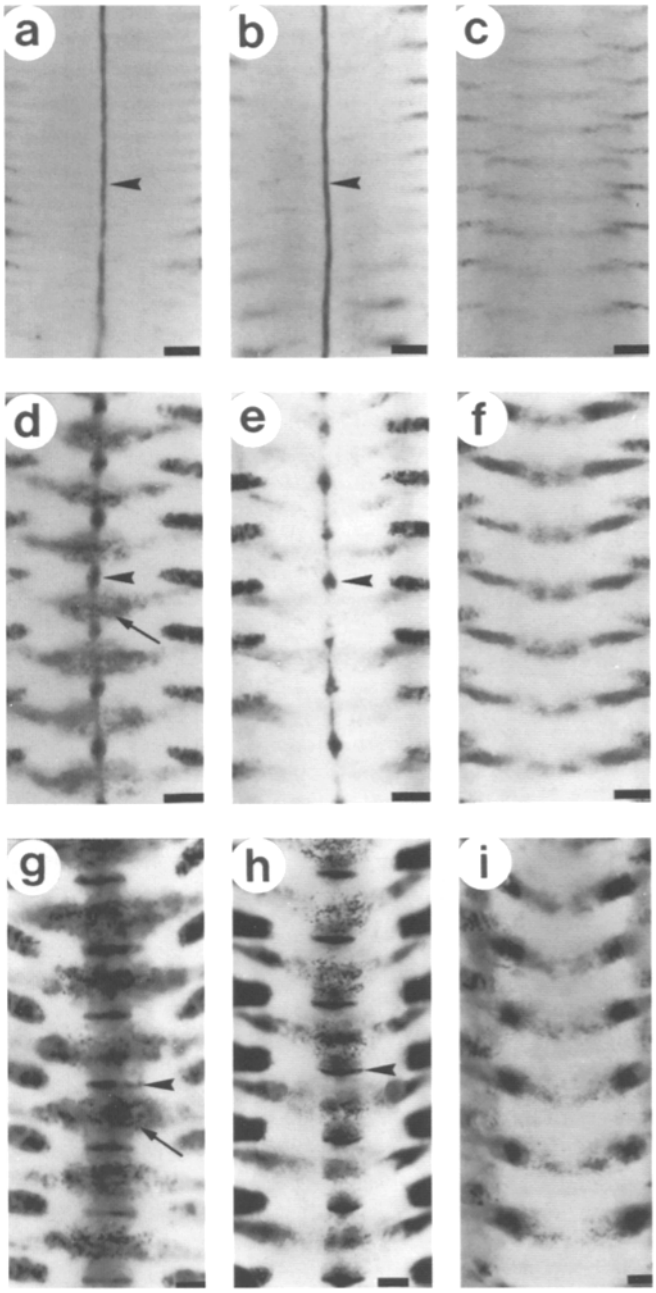

Figure 5. β-galactosidase expression directed by the 742*lacZ*Int, 453*lacZ*Int, and 742*lacZ* transgenes in the spine at different stages of development. The spinal columns with ribs were dissected out, stained with X-gal, and viewed from the ventral side. (*a–c*) Spinal columns of 12.5 d p.c. transgenic embryos. *lacZ* expression is mainly directed in the notochord (*arrowhead*) by 742*lacZ*Int (*a*) and 453*lacZ*Int (*b*), but little X-gal staining is visible in the 742*lacZ* transgenic mouse (*c*). (*d–f*) β-galactosidase expression in the spinal columns of 13.5 d p.c. transgenic embryos. (*d*) X-gal staining is visible in the notochord remnant (*arrowhead*) and primordial cartilage of the vertebral bodies (*arrow*), neural arches, and ribs in transgenic mice bearing 742*lacZ*Int. (*e*) On the other hand, 453*lacZ*Int did not direct β-galactosidase activity in the cartilage of the vertebral bodies, although the notochord remnants (*arrowhead*) and cartilage of the neural arches and ribs showed X-gal staining. (*f*) Furthermore, 742*lacZ* conferred no expression on the notochord remnants and weak expression on the vertebral body precursors, whereas the primordial cartilage of the neural arches and ribs showed β-galactosidase expression. (*g–i*) Spinal columns of 15.5 d p.c. transgenic embryos. (*g*) There is obvious X-gal staining in the primordial cartilage of vertebral bodies (*arrow*) and in the nucleus pulposus (*arrowhead*) of the 742*lacZ*Int transgenic embryo. (*h*) Isolated expression of *lacZ* is directed in the nucleus pulposus (*arrowhead*) by 453*lacZ*Int. (*i*) The spinal column of a 742*lacZ* transgenic mouse showing no β-galactosidase expression in the primordial cartilage of the vertebral bodies and in the nucleus pulposus. Bars, 200 μm.

cific loss of *lacZ* expression in the carpals of our transgenic mice bearing 742*lacZ* or 453*lacZ*Int, it is tempting to assume that certain element(s) may reside between −742 and −453 bp and within the first intron of *Col11a2* which directly or indirectly interact with Hox proteins. Activation of *Hox* genes such as *Hox-12* in the limb buds may cause cells to differentiate and to express genes encoding cartilage matrix molecules, including α2(XI) collagen, thus forming the primordial cartilage of the carpals.

The distinctive absence of *lacZ* expression in the vertebral bodies of transgenic mice bearing 742*lacZ* and 453*lacZ*Int, on the other hand, reminds us of the Pax-1 mouse mutants, *Undulated short-tail*, in which whole *Pax-1* locus is deleted. Vertebral bodies are virtually absent in these Pax-1-negative mice, while the neural arches are fairly normal, suggesting that Pax-1 is essential for ventral sclerotome differentiation into primordial cartilage of vertebral bodies (Wallin et al., 1994). Since *Pax-1* encodes a transcriptional factor, it is possible that certain elements may exist in the upstream sequence of promoter region and within the first intron of *Col11a2* which directly or indirectly interact with *Pax-1* gene products. Indeed, we found two sites of GTTCC core motifs recognized by Pax-1 (Chalepakis et al., 1991) within the 289 bp upstream sequence of the promoter (−653 to −649 and −626 to −622) and seven sites within the first intron (data not shown) of *Col11a2*.

Gross examination of the promoter sequence of the *Col11a2* showed only low overall homology with other genes which are predominantly expressed in cartilage including the mouse α1(II) collagen gene (Metsäranta et al., 1991), the human α1(XI) collagen gene (Yoshioka et al., 1995*b*), the rat aggrecan gene (Doege et al., 1994), and the rat link protein gene (Rhodes et al., 1991). However, close examination searching for elements with a length of several nucleotides revealed that the sequence at −142 to −135 of *Col11a2* was quite similar to the complementary sequence of the AT-rich element in the first intron of the type II collagen gene. This element is essential for cartilage-specific expression of the gene, and is a nuclear-factor binding site (Krebsbach et al., 1996). In addition, the DNA element located at −74 to −68 of *Col11a2* resembles the nuclear protein-bound sequence (−387 to −381) in the promoter of the α1(XI) collagen gene (Yoshioka et al., 1995*b*). However, the dissimilarity of other regions may account for the distinctive pattern of α2(XI) chain expression among the subunits of the type XI collagen molecule and other matrix proteins specifically expressed in cartilage.

This study provides a starting point for understanding the mechanism by which expression of the α2(XI) collagen gene is regulated. Experiments are in progress to elucidate further details of arrangement of the *cis*-elements of the

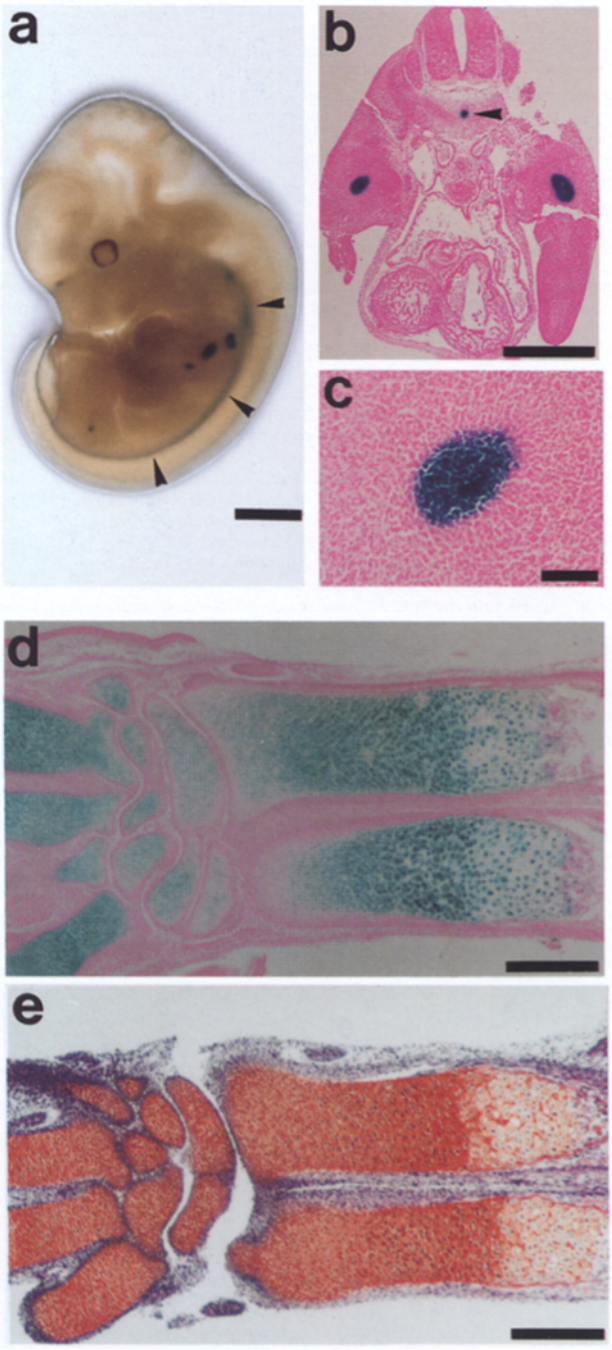

Figure 6. Transgenic mice bearing 742*lacZ*Int. (*a*) The whole-mount embryo shows β-galactosidase expression in distinct portions of the limb buds in addition to the notochord (*arrowheads*). (*b*) A transverse section shows that 742*lacZ*Int directed β-galactosidase expression in the notochord (*arrowhead*) and in the cluster of cells in the center of the forelimbs. The section was stained with X-gal and counterstained with eosin. (*c*) A higher power view reveals that the X-gal-stained cells in the limbs are mesenchymal cells aggregating into condensations. (*d*) Section of a forelimb from a transgenic mouse bearing the 742*lacZ*Int construct. (*e*) Section of a forelimb from a control mouse stained with safranin-O. The primordial cartilage of the radius, ulna, and carpals shows red staining. (*a–c*), 12.5 d p.c. (*d*, and *e*), 15.5 d p.c. Bars: (*a*) 1 mm; (*b*) 500 μm; (*c*) 50 μm; (*d* and *e*) 200 μm.

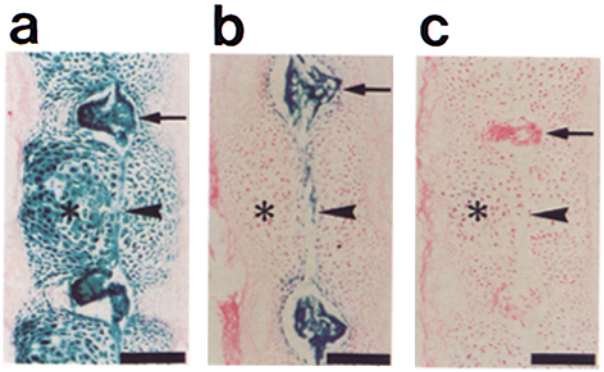

Figure 7. Sagittal sections through the spinal columns of 14.5 d p.c. transgenic mice bearing various *Col11a2-lacZ* constructs. (*a*) The transgenic mice bearing 742*lacZ*Int expressed *lacZ* in the primordial cartilage of the vertebral bodies (*asterisk*) as well as in the diminishing notochord (*arrowhead*) and the nucleus pulposus (*arrow*). (*b*) On the other hand, mice bearing 453*lacZ*Int only showed X-gal staining in the diminishing notochord (*arrowhead*) and nucleus pulposus (*arrow*), but not in the cartilage of the vertebral bodies (*asterisk*). (*c*) Mice bearing 742*lacZ* expressed the reporter gene in neither the cartilage of the vertebral bodies (*asterisk*) nor the diminishing notochord (*arrowhead*) and nucleus pulposus (*arrow*). Bars, 100 μm.

Col11a2 gene using transgenic mice bearing various DNA constructs. The information may provide additional insights into the mechanisms controlling the morphogenesis of various skeletal components.

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