Modular Arrangement of Cartilage- and Neural Tissue-specific cis-Elements in the Mouse α2(XI) Collagen Promoter*

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Type XI collagen, a heterotrimeric collagen, plays an important role in cartilage morphogenesis. We analyzed various α2(XI) collagen promoter-lacZ reporter gene constructs in transgenic mice to understand tissue-specific transcriptional regulation. The −530 promoter sequence was sufficient to direct reporter gene expression specifically to cartilage. Further deletion to −500 abolished reporter gene expression in cartilage but activated the expression specific to neural tissues such as brain and neural tube. An additional 47-base pair deletion resulted in random tissue expression patterns. A 24-base pair sequence from −530 to −507 of the α2(XI) promoter was able to switch the activity of the heterologous neurofilament light gene promoter from neural tissues to cartilage. These results suggest that the α2(XI) collagen gene is regulated by at least three modular elements: a basal promoter sequence distal to −453, a neural tissue-specific element (−454 to −500), and a cartilage-specific element (−501 to −530), which inhibits expression in neural tissues and induces expression in cartilage.

Cartilage is a highly specialized tissue that serves as the template for the development of skeleton and lines the joint surface. Cartilage consists of an abundant extracellular matrix maintained by chondrocytes. The collagen network provides a scaffolding for proteoglycans in the extracellular matrix and confers tensile strength important for resisting compression and shearing loads in cartilage. Cartilage collagen fibrils are heterotypic fibrils composed of types II, IX, and XI collagens.

The type XI collagen molecules co-assemble stoichiometrically with the major collagen, type II collagen, to form cartilage fibrils, whereas type IX collagen is associated with the surface of the fibrils (1, 2). The type XI collagen molecule is composed of three distinct subunits: α1(XI), α2(XI), and α3(XI) (3). The α3(XI) chain is a post-translational variant of the α1(II) chain (4), whereas the α1(XI) and α2(XI) chains are distinct gene products (5, 6).

Type XI collagen likely regulates the diameter of cartilage collagen fibrils (1, 7). This putative function is supported by a null mutation in the α1(XI) gene of cho/cho (chondrodysplasia) mice, in which the cartilage lacks the α1(XI) collagen chain and perhaps collagen XI trimer molecules resulting in abnormally thick collagen fibrils (8). Furthermore, association of α2(XI) gene mutations with certain forms of human chondrodysplasia, Stickler syndrome, and otospondylo-megaepiphyseal dysplasia indicates that type XI collagen is intimately involved in skeletal morphogenesis (9). These observations suggest that the fidelity of spatiotemporal expression of type XI collagen is crucial for the development and maintenance of the normal structure of cartilage.

The expression patterns of the three subunits of type XI collagen molecules differ from each other. In addition to its presence in cartilage, mRNA for the α1(XI) chain is also found in a variety of non-cartilaginous tissues, including the bone, vitreous, heart, and cerebral neuro-epithelium (10). Similarly, mRNA for the α3(XI) chain is detected in various tissues besides cartilage (11). On the other hand, expression of the α2(XI) gene appears to be more restricted. Although low levels of alternatively spliced variants of α2(XI) transcripts are detected in non-cartilaginous tissues, the major transcript is predominantly found in cartilage (12, 13). In addition, the level of α2(XI) mRNA is lower than that of α1(XI) mRNA and much less than that of the α1(II) collagen gene (14). Together, these results suggest that expression of the α2(XI) gene may be rate-limiting for the trimer formation of type XI collagen molecules that is critical for the subsequent lateral growth of cartilage collagen fibrils.

Recently, we cloned and sequenced the 5′-flanking region of the mouse α2(XI) collagen gene (Col11a2) and found that the retinoic acid receptor β gene (Rarb) is located 742 bp upstream of the transcriptional start site of the Col11a2 gene (15). We also showed that the −742-bp promoter sequence of the Col11a2 gene contains information for expression specific to the primordial cartilage of long bones and ribs (15). In the present study, we further delineated cis-regulatory elements of the Col11a2 promoter necessary for cartilage-specific expression using reporter gene constructs in transgenic mice. We found that the −530-bp sequence was sufficient for the cartilage-specific expression of the Col11a2 gene. Deletion of a sequence between −530 and −500 abolished reporter gene expression in cartilage. Interestingly, deletion of this sequence induced neural tissue-specific expression. We also showed that a 24-bp sequence in this region could activate reporter gene expression in cartilage when it was linked to the heterologous human neu-

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§ The abbreviations used are: Col11a2, the gene encoding murine α2(XI) collagen; d.p.c., days postconception; lacZ, the Escherichia coli β-galactosidase gene; NF-I, the human neurofilament light gene; Rarb, the murine retinoid X receptor β gene; X-gal, 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside.
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rofilament light gene (NF-L) promoter. Our data suggest a modular arrangement in which a basal promoter, a neural tissue-specific cis-element, and a cartilage-specific cis-element regulate transcription of tissue-specific expression of the Col11a2 gene.

EXPERIMENTAL PROCEDURES

Construction of Col11a2-lacZ Transgenes—The transgene constructs, 742lacZ and 453lacZ, which bear 742- and 453-bp Col11a2 promoter sequences, respectively, linked to the β-galactosidase reporter gene, were described previously (15). Other transgene constructs containing various lengths of the Col11a2 promoter region were prepared by cloning into the expression vector pNASSβ (CLONTECH), which contains a SV40 RNA splice site, the β-galactosidase gene, and a SV40 polyadenylation signal. All constructs were confirmed by DNA sequencing.

For construction of 680lacZ, 530lacZ, 518lacZ, 530lacZ, and 500lacZ, DNA fragments spanning −680 to +380, −530 to +380, −518 to +380, −530 to −517 and −500 to +380, and −500 to +380 of the Col11a2 promoter, respectively, were generated by PCR with a genomic subclone from AN8 (12) as a template using specific forward primers containing an EcoRI site and an Ex-1 reverse primer with a XhoI site as described previously (15). After digestion with EcoRI and XhoI, the PCR fragments were cloned into the EcoRI/XhoI sites upstream of the SV40 splice site of pNASSβ. The forward primers used for synthesis of each DNA construct were as follows: 680lacZ, GTCCGGAATTCGGATCATCGGTTGAGAACCCTGTA; 530lacZ, GTCCGGAATTCGGAGGAGGACGTTCCCTGCTC; 518lacZ, TTAATAGATCAGCCGCTCTCTTCAAAACGGGC; 530lacZ, TTAATAGATCAGCCGCTCTCTTCAAAACGGGC; 500lacZ, GTCCGGAATTCGGAGGAGGACGTTCCCTGCTC; 500lacZ, GTCCGGAATTCGGAGGAGGAGGACGTTCCCTGCTC.

For the reporter gene construct containing the promoter of the human neuronfilament light gene (16), a DNA segment between −292 and +15 of NF-L was amplified by PCR with human genomic DNA as a template using primers Ne-1 containing an EcoRI site and a XhoI site and primer Ne-2 containing the SV40 splice site of pNASSβ. The PCR fragment was cloned into the EcoRI/XhoI sites of pNASSβ to create DNA construct NF-lacZ. For generation of the fusion promoter construct, complementary oligonucleotides 24-1 and 24-2 containing three tandem copies of a sequence (−530 and −507) of Col11a2 were synthesized and annealed to generate a double-stranded DNA fragment. The annealed fragment was cloned into the EcoRI/XhoI sites upstream of the SV40 promoter to create the promoter sequence (−292 to +15) of the human neuronfilament light gene (NF-L). For construction of 24×3-NF-lacZ, three copies of 24-bp fragment from −530 to −507 were placed upstream of the NF-L promoter.

Expression of the lacZ Reporter Gene Driven by Col11a2 Promoter Constructs in Transgenic Mice—The 742lacZ construct containing the −742-bp promoter showed expression in the cartilage of transgenic mice, which is inconsistent with our previous finding (Fig. 2A) (15). Further 5’-deletions of the Col11a2 promoter revealed that the constructs, 680lacZ and 530lacZ, containing −680- and −530-bp promoter, respectively, were also capable of directing reporter gene expression specifically to the primordial cartilage in limbs and in ribs (Fig. 2, B and C). Histological analysis confirmed that X-gal staining of these mice was restricted to chondrocytes (Fig. 2D). All reporter gene expression patterns were similar expression specifically in cartilage (Table I).

A 5’-12-bp deletion of the −530-bp promoter (518lacZ) resulted in no reporter gene expression in cartilage, but expression was activated in neural tissues such as brain and neural tube (Fig. 2, E and F). Strong staining was observed in the roof of the neopallial cortex, in the primitive ectomeninx, and in a medial portion of the neural tube. An internal 16-bp deletion between −516 and −501 (530lacZ) in the 530lacZ construct also abolished expression in cartilage but induced neural tissue-specific expression (Fig. 2, G and H). The entire roof of the neopallial cortex, roof of the midbrain, and neural tube showed strong staining. A 5’-30-bp deletion from 530lacZ (500lacZ) also resulted in the expression of β-galactosidase in neural tissue, but not in cartilage (Fig. 2, I and J). Intense X-gal staining was detected in the roof of the neopallial cortex. The overall β-galactosidase expression patterns of transgenic embryos for these three constructs, 518lacZ, 530lacZ, and 500lacZ, were limited to neural tissues, although there were some variations in the relative staining intensity. Histological analysis confirmed specific expression of the reporter gene in neural tissue in transgenic mice bearing these constructs (Fig. 2K). The 453lacZ construct, which had an additional 57-bp deletion from 500lacZ at 5’-end, showed β-galactosidase expression in various tissues. No consistent tissue-specific reporter gene expression patterns
were found in the five transgenic mice with this construct, agreeing with previous reports (Fig. 2L; Ref. 15). These results suggest that the whole 30-bp sequence (−530 to −500) of the Col11a2 gene is required for cartilage-specific expression.

Expression of the lacZ Reporter Gene by the Fusion Promoter of the Col11a2 and NF-L Genes—Because a sequence between −530 and −500 of the Col11a2 promoter region appeared to be capable of converting reporter gene expression from neural tissue-specific to cartilage-specific expression, we investigated this activity with an heterologous promoter from the NF-L gene, which can direct expression in neural tissues. The 24×3-NF-lacZ construct was prepared by placing three copies of a 24-bp sequence spanning −530 to −507 of the NF-L promoter (−292 to +15). The NF-L promoter alone (NF-lacZ) specifically expressed β-galactosidase in the nervous system (Fig. 3, A and B), in agreement with a previous report (16). Inclusion of the 24-bp segment to the NF-L promoter (24×3-NF-lacZ) showed activation of the reporter gene in the primordial cartilage of ribs, scapula, and neural arch. Although reporter gene expression was observed in some other tissues such as the primordium of follicle of vibrissa at nose and some surface ectoderm in the limbs, the 24-bp sequence down-regulated neural tissue-specific expression directed by the NF-L promoter and activated the expression predominantly in cartilage.

DISCUSSION

In this study, we have defined the minimal sequences responsible for cartilage-specific expression of the Col11a2 gene using a series of deletions in transgenic mice. We found that the −530-bp promoter sequence was sufficient to direct reporter gene expression specifically to cartilage. Further deletions showed that the −500-bp promoter sequence exclusively directed expression to the neural tissue, whereas the −453-bp promoter sequence expressed the reporter gene in various tissues. These results suggest that the Col11a2 promoter consists of three modular elements: the basal promoter sequence located downstream from −453 conferring ability to activate transcription without any tissue specificity, the neural tissue-specific cis-element between −454 and −500, and the cartilage-specific cis-element between −501 and −530 of the a2(XI) promoter.

The neural tissue-specific expression patterns of transgenic mice bearing 518lacZ and 530lacZ suggest that the 30-bp sequence (−530 to −500) of the Col11a2 gene is necessary for cartilage-specific expression. A GGAGGAG sequence (−527 to −521) in this region is likely important, because a homologous sequence was found in the complementary sequence of a silencer of the α1(II) collagen gene (19). At the 3′-end of the 30-bp region of the a2(XI) promoter, there is a potential Sox9 binding motif for SRY and SOX proteins (TTCAAG, −505 to −499). Sox9 was shown to bind to the enhancer of the α1(II) collagen gene and to activate its expression (20, 21). Thus, this site may be important for activation of the a2(XI) gene.

It was surprising to find that a sequence within the a2(XI) promoter directed the expression of the reporter gene in neural tissues, because Col11a2 expression is restricted to cartilage and is hardly found in neural tissues (13). Inclusion of the

| Transgene construct | Number of transgenic embryos | Number of transgenic embryos expressing β-galactosidase | X-gal-stained tissue |
|---------------------|-------------------------------|-------------------------------------------------------|---------------------|
| 742lacZ             | 5                             | 4                                                     | Cartilage           |
| 680lacZ             | 3                             | 2                                                     | Cartilage           |
| 530lacZ             | 6                             | 3                                                     | Cartilage           |
| 518lacZ             | 3                             | 2                                                     | Neural tissue       |
| 5303lacZ            | 3                             | 2                                                     | Neural tissue       |
| 500lacZ             | 5                             | 4                                                     | Neural tissue       |
| 453lacZ             | 8                             | 5                                                     | Random              |
| NF-lacZ             | 6                             | 3                                                     | Neural tissue       |
| 24×3-NF-lacZ        | 2                             | 2                                                     | Cartilage, other tissues |
30-bp sequence to the −500-bp promoter changed the expression pattern from neural tissues to cartilage. These results may suggest that the 30-bp cartilage-specific cis-element has dual activities, one for inactivation of transcription in neural tissues and the other for activation of transcription in cartilage. These activities were partly supported by the finding that the reporter gene expression was active in cartilage but inactive in neural tissues in transgenic mice bearing the fusion promoter construct, 24×3-NF-lacZ, containing the 24-bp sequence and the NF-L promoter. Because both cartilage and neural tissues are highly differentiated tissues, it is possible that genes expressed in these tissues may share common regulatory motifs and binding factors, which are responsible for tissue-specific gene expression. Nuclear factors bound to these sites remain to be elucidated.

In addition to the 30-bp sequence in the promoter, the first intron sequence of the Col11a2 gene is also important for cartilage-specific expression. The first intron sequence induces cartilage-specific expression when combined with the −453-bp basal promoter of the gene. It also enhances the expression in cartilage when combined with the −742 promoter sequence (15). It is of interest to note that homologous sequence motifs to the 30-bp sequence are found within the first intron sequence. (15). It is of interest to note that homologous sequence motifs to the 30-bp sequence are found within the first intron sequence. This diversity of the regulatory mechanisms between the two cartilage genes may have been evolved because different levels of α2(XI) collagen may be necessary for optimal function in different cartilage tissues.

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