Identification of a Minimum Enhancer Sequence for the Type II Collagen Gene Reveals Several Core Sequence Motifs in Common with the Link Protein Gene*

(Received for publication, July 18, 1995, and in revised form, November 2, 1995)

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The type II collagen gene (Col2a1) is expressed primarily in chondrocytes. Transcription of Col2a1 is mediated by cell-specific regulatory elements located within the promoter and first intron. Here, we map a minimal enhancer and identify elements that determine cartilage-specific Col2a1 expression by analyzing the activity of a series of chimeric genes consisting of rat Col2a1 first intron deletion mutants ligated to the chloramphenicol acetyltransferase reporter gene. We show that a 100-base pair (bp) segment within the first intron is the minimum size necessary for high level, cell type-specific expression of Col2a1. Sequence analysis of this 100-bp Col2a1 enhancer revealed several sequence motifs similar to motifs present within the regulatory region of the link protein gene, another cartilage gene. These motifs include an AT-rich element, a C1 motif and a C3 motif. Deletion of any of these elements reduced Col2a1 enhancer activity in chick embryo chondrocytes. We also tested enhancer-mediated activity in CCF2 cells which differentiate to a chondrogenic phenotype and begin to express type II collagen mRNA after extended culture. In stably transfected CCF2 cells, constructs containing the 100-bp enhancer were activated during the transition from prechondrogenic to chondrogenic cell populations and deletions within the enhancer strongly down-regulated activity. Chondrocyte-specific DNA-protein complexes were identified using nuclear extracts prepared from chick embryo chondrocytes and 32P-labeled oligonucleotides from these regions of the first intron. These results suggest that interaction of chondrocyte specific nuclear factors with multiple core elements from a small region within the first intron are important for cell-type specific Col2a1 enhancer activity.

Type II collagen is the primary collagen in cartilage. Although type II collagen is present in the notochord, nucleus pulposa of intervertebral disks and vitreous of the eye, type II collagen is most abundant in hyaline cartilage (1–4). In hyaline cartilage, type II collagen, aggrecan, and link protein form a collagen structure that functions to absorb shock and to resist compression and shearing. Disruption of collagen expression in cartilage due to inflammatory or genetic influences may lead to degenerative joint diseases and a variety of chondrodysplasias (5, 6). For example, defects in the type II collagen gene are associated with spondyloepiphyseal dysplasia, achondrogenesis type II, and the Stickler syndrome (7–10). Defects in type II collagen are also associated with Kniest dysplasia (11). Together, these observations suggest that the fidelity of type II collagen expression is essential for maintaining the normal structure and function of cartilage.

The type II collagen gene (Col2a1) encodes an α1(II) polypeptide chain that is assembled into a homotrimeric molecule. Regulation of Col2a1 is complex and involves both transcriptional and post-transcriptional mechanisms (12–17). Transcriptional regulation of Col2a1 is dependent on the binding of several nuclear factors to both the 5′-flanking region and an enhancer located within the first intron. Transcription of Col2a1 requires the presence of a minimal promoter that includes at least one functional Sp1 binding site (17). Previously, the majority of enhancer activity was localized to a 1.5-kb region in the central region of the first intron (15). In addition, the binding of chondrocyte nuclear factor(s) to a helix-loop-helix consensus site has been demonstrated in this region (18).

In this study, we used two different but complementary transfection strategies to further delineate the Col2a1 enhancer and determined the minimal sequence necessary for full enhancer activity. In addition, we show that chondrocyte-specific nuclear factors bind to these functionally important regions. Several sequence motifs including an AT-rich element were identified in the enhancer. Deletion of these motifs reduced enhancer activity in both transient and stably transfected chondrocytes. Interestingly, three of these motifs are also present in the regulatory region of the cartilage link protein gene, suggesting that cartilage genes may share common elements for transcriptional regulation.

MATERIALS AND METHODS

Plasmid Constructs—A schematic representation of the Col2a1-CAT constructs is shown in Fig. 1. pDAS1BB5 was prepared by cloning a 1.5-kb enhancer-containing intron fragment from the rat Col2a1 gene into the BamHI site of pCI12 (15) harboring a promoter segment from –977 to +110 in the pSVOCAT vector (19). All other plasmids were derived from pCI14 containing a promoter sequence –312 to +110 from the rat Col2a1 gene in pSVOCAT (15). pDAS1BE4 (pCI14-B) was prepared by cloning a 700-bp BamHI-EcoRI fragment from the 1.5-kb BamHI fragment into the BamHI site of pCI14. The EcoRI end was filled in with the Klenow fragment polymerase and ligated to a BamHI linker. Deletions of other intron sequences were created by either recombination.

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1 The abbreviations used are: Col2a1, gene for the type II collagen α1 chain; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; CEC, chick embryo chondrocyte; FCS, fetal calf serum; bp, base pair(s); kb, kilobase pair(s).
striction enzyme digestion or Bal-31 digestion and were cloned into the BamHI site or the Apal-BamHI sites of pCI4-B. Exact point
ofsternasof15-day-oldchickenembryosasdescribedpreviously(15).

Briefly, sternas were rinsed twice in Hank’s balanced salt solution and were incubated for 2.5 h in 0.4% collagenase B (Boehringer Mannheim) as described previously (15).

Oligonucleotide probe A (Col2a1 enhancer 311–340 bp), 5'-AAGCCCTGCGCTGTTTAAACCTGGGCTGC-3'; probe B (Col2a1 enhancer 375–410 bp); 5'-CTCGAGAAGGCCCCATCTGAGGAGCGCCATGCC-3'; mutant probe A (mA), 5'-AACCCCTGCGCCCATGCAGC AAAGCCGTC-3'. The nucleotide base substitutions for the Col2a1 oligonucleotide probe mA are underlined.

For electrophoretic mobility shift assays, 3 μg of nuclear extract was incubated for 30 min at room temperature in mobility shift buffer (12 mM Hepes (pH 7.9), 50 mM KCl, 4 mM MgCl₂, 1 mM EDTA, 0.1 mM ZnSO₄, 1 mM diithiothreitol, 5% glycerol and 2 μg poly(dI-dC)poly(dI-dC) with 30,000 cpm of 3²P end-labeled double-stranded DNA probe in a 30-μl volume. DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel containing 4.5 mM Tris-HCl (pH 7.5), 4.5 mM boric acid, and 1 mM EDTA. The gels were dried and exposed to x-ray film (Amersham Corp.).

RESULTS

Expression of Col2a1-CAT Constructs in CEC—Previous work has shown that the Col2a1 enhancer resides in a 1.5-kb BamHI fragment located within the first intron of the Col2a1 gene (15). The studies reported here were designed to identify the minimal enhancer elements required for chondrocyte-specific expression of Col2a1. The DNA constructs generated to examine enhancer activity were prepared by cloning various 3' and 5' deletions of the 1.5-kb BamHI enhancer fragment down-stream of the collagen II promoter in Col2a1-CAT constructs. The Col2a1-CAT constructs were transiently transfected into CEC and COS cells and CAT activity was determined (Fig. 1).

The construct pE55D, a 5' DNA deletion to 205 (the EcoRI site in the BamHI fragment was arbitrarily designated +1) retained almost full enhancer activity while deletion of sequences to 361 (pE80D) decreased CAT activity to 12% of the full-length construct. A deletion of the 3' portion of the BamHI fragment (pDAS10BE4) reduced CAT activity to 8% of full-length activity. Further deletion constructs, pE6S, pE5I, and OS-30 showed that a common minimum enhancer fragment of 126 bp could confer full enhancer activity while deletions within this region to generate pE6D8X reduced activity to 2% (Fig. 1). A deletion mutation to 311 (OS-55) maintained full enhancer activity, whereas deletions to 317 (OS-60) and to 331 (OS-35) decreased activity to 68 and 10%, respectively. Together, activity from these 5' and 3' deletion mutants suggest that the enhancer activity resides within the 205- to 331-base region (Fig. 1). A deletion mutation to 311 (OS-55) maintained full enhancer activity, whereas deletions to 317 (OS-60) and to 331 (OS-35) decreased activity to 68 and 10%, respectively. Together, activity from these 5' and 3' deletion mutants suggest that the enhancer activity resides within the 205 to 331-base region (Fig. 1).

Nuclear Extract Sequences of the Enhancer Containing Segment—The nucleotide sequence of the 822-bp EcoRI-BamHI fragment was sequenced by a combination of automated and manual methods (Fig. 2). There are several interesting motifs within the 100 bp (311–410) enhancer region. An AT-rich element (TATTTATTTTA) within this functionally important region of the Col2a1 gene is located between 322 and 331. A sequence similar to the C1 motif (AAACTGGTTC, 332–340) is found 60 bp upstream of the AT-rich element in the link protein gene (986 to –976) (25). In addition, a sequence similar to an ATTCTAGG sequence (C3 motif, 390 to 398) is present in the promoter of the link protein gene (–985 to –889) (25). There are also two almost perfect inverted repeat sequences (C2 motif) located in the minimal enhancer region of Col2a1;
Fig. 1. Map of the Col2a1-CAT plasmid constructs and CAT activity in transfected CEC. A, schematic representation of the rat Col2a1 first intron. The EcoRI site within the BamHI fragment is arbitrarily designated +1. B, several 5′ and 3′ first intron deletion mutants were transfected into CEC. CAT activity was analyzed 48 h after transfection. CAT activity was expressed as a percentage of the activity obtained with pDAS1BB5. Transfections were performed in duplicate in at least three independent experiments. *Values are expressed relative to the level of CAT activity of pDAS1BB5 in CEC. #Values are expressed relative to the level of CAT activity of pcDNA3/CAT in COS-7 cells.

TGAATCGGGCT (355–365) and AGCCCCATTCA (384–394).

To evaluate the significance of these sequence motifs, internal deletions and base substitution mutations within the 100 bp enhancer were created and examined for their enhancer activity (Fig. 3). Deletions of the AT-rich element (OS-3) and C1 motif (OS-12) reduced CAT activity to 64 and 53% of the 100-bp enhancer, respectively. OS-9, a deletion of 10 bp downstream of the C1 motif, did not significantly alter CAT activity. Elimination of one C2 motif (OS-83) decreased CAT activity to 10%, while a deletion of C3 (OS-80) decreased CAT activity to 8%. A 2-bp substitution (OS-20, TT to GG) in the overlapping region of the C2 and C3 motifs reduced CAT activity to only 85%. These results suggest that all the identified sequence motifs are critical for full enhancer activity.

Expression of Col2a1-CAT Constructs and Type II Collagen mRNA in Differentiating CFK2 Cells—The role of distinct regions of the Col2a1 enhancer in mediating the expression of the CAT reporter gene during chondrocyte differentiation was examined in a differentiating cell culture system. CFK2 cells are an established cell line derived from fetal rat calvariae (20). During the course of extended culture, CFK2 cells undergo a progressive developmental maturation to a chondrocytic phenotype (24). Therefore, using differentiating CFK2 cells as a model, distinct regions of the Col2a1 enhancer could be evaluated during the transition from a prechondrogenic (CFK2) to a chondrogenic (dCFK2) cell phenotype. Four independent cell lines were generated by stably transfecting Col2a1-CAT constructs pIE55D, OS-30, OS-35, and OS-80 into CFK2 cells.

CAT activity expressed by pIE55D and OS-30 transfected cells increased 6-fold during the course of differentiation of CFK2 to dCFK2 cells (Fig. 4). The increase in CAT activity from
The C3 motif of the duration of the culture period. When the AT-rich element or the was expressed and expression continued to increase for the mean 6 express detectable cell culture (Fig. 5). After 9 days in culture, CFK2 cellsdid not increase in endogenous either pIE55D or OS-30 transfected CFK2 cells paralleled the transfected cells was less than 20 counts/min/h /

Figure 6 shows ethidium bromide staining of 28S and 18S ribosomal RNAs. cells which express type II collagen (CEC) and those which do not. Binding activity to double-stranded oligonucleotide probes A, corresponding to the AT-rich element and the C1 motif (311–340 bp), and B, corresponding to the downstream motifs C2 and C3 (375–410 bp), was analyzed by EMSA. EMSA revealed a specific set of protein-DNA complexes when nuclear extracts prepared from CEC were incubated with 32P-labeled oligonucleotide probes A and B (Fig. 6, A–C). Two intense, and a third slower migrating complex were bound to oligonucleotide probe A (311–340 bp). The specificity of these protein-DNA interactions was shown by complete inhibition of binding in the presence of a 100-fold molar excess of unlabeled oligonucleotide A and minimal inhibition in the presence of excess unlabeled oligonucleotide B. These complexes likely represent cell type-specific interactions since no complexes were formed with nuclear extracts from human tongue fibroblasts, HeLa cells or the osteoblastic cell line MC3T3-E1 (Fig. 6A). Both the Col2a1 gene and the link protein gene share similar AT-rich elements in their regulatory regions, and specific mutations in the link protein AT-rich promoter abrogate its function. Thus, we created a mutant analogous to the link protein promoter to further explore the relationship between functional activity and specificity of nuclear factor binding to the AT-rich element in the Col2a1 gene (Fig. 6B). The mutant probe A (mA) was generated by base substitutions in the AT-rich element (see "Materials and Methods"). Mutation of the AT-rich element abrogated the specific binding of CEC nuclear factors. Taken together, the enhancer activity residing in the AT-rich element, as shown in both transient and stable transfections, and the specificity of nuclear factor binding provide compelling evidence for the presence of positive regulatory activity in the AT-rich element of the Col2a1 gene. We next studied the downstream enhancer region (379–410 bp) of Col2a1 which was also required for enhancer activity. Incubation of 32P-labeled oligonucleotide B with nuclear extracts from CEC resulted in the formation of three protein-DNA complexes (Fig. 6C). The formation of these complexes was inhibited by the inclusion of a 100-fold excess of unlabeled oligonucleotide B. Nuclear extracts from HeLa cells formed a high molecular weight complex with the B probe, however, the faster migrating complexes observed with CEC nuclear extracts were not observed (Fig. 6C). Together, these results suggest that CEC express nuclear binding factors that specifically interact with functionally important regions of the Col2a1 enhancer, whereas no binding activity was observed with the nonchondrocytic cells that we tested.
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Collagen II Gene Enhancer

\[ \text{Proteins} \rightarrow \text{Nucleic Acids} \]

**A**

| Nuclear Extract | Competitor |
|-----------------|------------|
| CEC            | A, B       |
| CEC            | A, B       |
| CEC            | A, B       |
| HTF            | A, B       |
| OB             | A, B       |
| HeLa           | A, B       |

**B**

| Nuclear Extract | Competitor |
|-----------------|------------|
| CEC            | A, mA, CEC |
| CEC            | A, mA, CEC |

**C**

| Nuclear Extract | Competitor |
|-----------------|------------|
| CEC            | A, B, HeLa |
| CEC            | A, B, HeLa |

**Free Probe**

1 2 3 4 5 6 7

**Fig. 6.** EMSA shows cell type-specific complex formation between the Col2a1 enhancer fragments and nuclear factors. A, the \(^{32}\)P-labeled probe A (311–340) was incubated with nuclear extracts (3 μg) obtained from chick embryo chondrocytes (CEC, lanes 2, 3, and 4), human tongue fibroblasts (HTF, lane 5), osteoblastic MC3T3-E1 cells (OB, lane 6), and human epithelial tumor cells (HeLa, lane 7). Competition studies were performed with 100-fold molar excess of either the A (lane 3) or B probe (lane 4). The filled arrowheads mark the cell type-specific DNA-protein complexes whose formation can be abrogated by the addition of unlabeled probe A, but are unaffected by nonspecific competition with the B probe. No specific DNA-protein complexes are formed with nuclear extracts from nonchondrogenic cells. Open arrowheads represent DNA-protein complexes that are not cell type-specific. B, EMSA of a \(^{32}\)P-labeled probe A and nuclear extracts from CEC. Lane 1, free probe; lane 2, probe A; lane 3, mutant probe A containing nucleotide substitutions within the AT-rich element. C, DNA-protein complex formation and competition with B fragment (375–410 bp) and nuclear extracts from CEC (lanes 1, 2, and 3) and HeLa cells (lanes 4, 5, and 6).

**DISCUSSION**

In the present study, we have defined a 100-bp segment within the first intron of the Col2a1 gene as the minimum size for cell type-specific enhancer activity. Further, several core enhancer elements were identified within this 100-bp segment. We have used two different, but complementary, transfection strategies to map the minimal chondrocyte-specific enhancer of the Col2a1 gene. Transient transfection of CEC with constructs containing various deletion mutants of the enhancer and 310 bp of the Col2a1 promoter revealed that first intron sequences from 311 to 410 were required for enhancer activity. To confirm the deletion mutation analysis in transiently transfected CEC, we tested a limited number of Col2a1-CAT constructs in stably transfected CFK2 cells. Extended culture of CFK2 cells results in an increase in phenotypic markers of a differentiated chondrocyte, including the appearance of glycosaminoglycans in discrete nodules and increases in mRNA levels for type II collagen, link protein, and aggrecan (24). Therefore, CFK2 cells provide a differentiating cell culture model in which Col2a1-CAT expression can be studied. The pattern of CAT expression in stably transfected CFK2 cells complements the results in transient transfection experiments in CEC and confirms the requirement for two regions within the first intron for cell differentiation-dependent expression of Col2a1. Moreover, these studies also displayed the differential use of Col2a1 enhancer elements during the course of development to a chondrocytic phenotype. During the initial cell culture period (7 days), all four Col2a1-CAT constructs expressed low CAT activity in CFK2 cells. The low level of CAT activity after 7 days in culture was consistent with the low to undetectable levels of endogenous Col2a1 mRNA expressed after a similar time in culture. However, as CFK2 cells differentiated into dCFK2 cells, primary enhancer activity was observed in the pL55D and OS-30 constructs. CAT activity also increased in the OS-35 and OS-80 transfected cells during extended cell culture, but the activity from these deletion mutants was low and remained between 4- and 6-fold less than that expressed by OS-30-transfected cells. The observation that CAT activity from the OS-30 construct was similar to the activity expressed from the much larger pL55D construct, supports the notion that the DNA elements within the 100-bp enhancer contain all of the elements essential for enhancer mediated-expression of Col2a1.

Sequence analysis revealed that the enhancer segment contains several motifs that are similar to sequences also found in the regulatory region of the promoter for the link protein gene. These include an AT-rich element, and C1 and C3 motifs. An identical 9-bp AT-rich element is present at −906 to −914 in the minus strand of the link protein gene (25). A comparable AT-rich element is also found in the promoter of the growth hormone (26) and the muscle creatine kinase genes (27) (Fig. 7). Deletion of the AT-rich element from the Col2a1 enhancer...
significantly reduced enhancer activity. Furthermore, mobility shift assays suggest that the Col2a1 AT-rich element binds nuclear factors present in CEC. A similar reduction in the promoter activity of the link protein gene was found when the AT-rich element was deleted or mutated by base substitutions (25). Thus, the AT-rich element is likely to be functionally important for transcriptional regulation of both the Col2a1 and link protein genes. It has been shown that Mhox, a mesodermally restricted homeodomain protein, and myocyte enhancer factor-2, binds to an AT-rich element in the muscle creatine kinase enhancer. Binding of myocyte enhancer factor-2 to the site in the muscle creatine kinase gene is required for enhancer activity in muscle cells (27). Equivalent factors, specific for chondrocytes, may bind to the AT-rich element of the Col2a1 and link protein genes. It is interesting to note that the C1 and C3 motifs are also present in the regulatory region of the link protein gene. These elements appear to be important for transcription of the link protein gene since deletions of these elements reduce promoter activity in the link protein gene (25).

The two C2 motifs that are located in an inverted orientation in the Col2a1 enhancer are not found in the link protein promoter. The rat sequence of this 100-bp enhancer region is highly conserved with both mouse and human (28, 29). The rat sequence shared a 98 and 92% identity with the mouse and human sequences, respectively. All motifs identified in this study are perfectly conserved, suggesting that these motifs may play significant roles in the type II collagen and link protein genes.

Initiation of transcription involves complex interactions between nuclear proteins and cis-acting elements located in the promoter and enhancer regions (30). It is becoming increasingly appreciated that these protein-DNA interactions occur at multiple sites in a given gene and may involve the interaction of accessory factors that may not directly bind DNA (31–33). Several published studies show that the Col2a1 gene shares this complex transcriptional regulation. Transcriptional activity has been identified in sequences within the 5’-flanking sequences and the first intron of the Col2a1 gene (15, 17, 18). Together, our findings, as well as previously published data, suggest that multiple functional elements are found within a relatively narrow region of the first intron. The function of discrete elements may depend on culture conditions or the assay system used. Alternatively, multiple DNA-binding proteins may be functioning through these elements. Our demonstration of enhancer activity in both transient and stable transfections strengthens the notion that the minimum 100-bp enhancer is functional. Since there are limitations of the interpretations of the results obtained in vitro, an in vivo assay using a transgenic mouse model will be help clarify this complex transcriptional regulation.

The use of multiple regulatory sites in transcriptional regulation is recognized for many genes. Because our current knowledge of possible cooperative or synergistic interactions in this region of the enhancer remains incomplete, the control of Col2a1 transcription in chondrocytes will likely involve other regulatory elements in addition to the regions described here. However, since we define an enhancer region that is operational in both transiently transfected chondrocytes and stably transfected CFK2 cells in which the transgenes have been incorporated into chromatin, we believe that this provides evidence for a minimal enhancer sequence required for cell type-specific expression of Col2a1. Because transfection experiments do not always define which regulatory elements will be functional in vivo (34, 35), the regulatory sites identified in this study will be used to generate transgenic mice in order to study regulation of Col2a1 in the context of a developing animal.

Acknowledgments—We are grateful to Drs. Suzanne Bernier and Robert Laffrenchie for critical reading of the manuscript. We thank Dr. John Harrison, University of Connecticut Health Center, for providing the nuclear extracts from MC-3T3E1 cells.

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