Two Silencers Regulate the Tissue-specific Expression of the Collagen II Gene*

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Collagen II, the major component of cartilage, is synthesized primarily by chondrocytes and by certain cells in the eye. Previously, we have studied the regulatory regions of the collagen II gene by DNA transfection assays (Horton, W., Miyashita, T., Kohno, K., and Yamada, Y. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 8864-8868). These studies show that both the promoter and an enhancer sequence in the first intron are required for high transcriptional activity in chondrocytes. These elements do not show significant activity in cells which do not synthesize collagen II, such as in muscle cells and fibroblasts. In this report, we have constructed plasmids containing various deletions of the promoter of the collagen II gene, fused to a reporter gene for chloramphenicol acetyltransferase (CAT) and transfected them into both chick embryonic fibroblasts and HeLa cells. We have found that silencer elements in the collagen II promoter region reduce CAT activity 11-fold in fibroblasts, while not affecting the enhancer-mediated transcription in chondrocytes. Deletions in the promoter showed that most of the silencing activity was localized in two sites, between -360 and -460 base pairs and between -520 and -700 base pairs. Furthermore, a fragment containing these two sequences in a thymidine kinase promoter CAT construct reduced the activity of the promoter in an orientation independent fashion. Sequence analysis revealed that the two silencer regions are homologous and contain consensus motifs for silencer elements found in other genes. Gel retardation experiments showed that nuclear factors from HeLa cells bind specifically to a DNA fragment containing the silencer, whereas chondrocyte nuclear extracts did not show any activity. Thus, our study indicates that the expression of the collagen II gene is controlled by both negative and positive elements to ensure that the gene is only expressed in suitable cells.

Gene transcription is regulated by multiple cis-acting elements, such as promoters, enhancers, and silencers. Promoter elements are needed in all cases for transcription initiation. The transcription level is also regulated by both enhancer and silencers, located either close to the promoter or at distant sites, such as in introns. Enhancers are required in most cases to obtain significant transcription activity, whereas silencers turn off or reduce transcription activity. These DNA sequences are recognized by trans-acting factors, which bind to specific sites with variable affinity. This DNA-binding complex results in the enhancement, or the inhibition of the transcriptional complex initiating in the promoter region (1-3).

Cartilage is composed of specific components including collagen II, cartilage proteoglycan, and link protein (4-6). In cartilage, these components appear to be synthesized in a coordinate fashion. The coordinate expression of these elements could be due to some common regulatory elements shared by their genes. We have used the a1(II) collagen gene as a model for the study of gene regulation, because its expression is restricted mainly to chondrocytes in cartilage, although it is also present in some other tissues, including the vitreous and nucleus pulposus (7).

We have earlier identified and characterized regulatory regions of the rat collagen II gene, which include a promoter and an enhancer (8, 9). These studies show that a promoter-enhancer construct is needed in order to have a high level of transcription in chondrocytes (9). This enhancer, located in the first intron, is active only in chondrocytes. Thereafter, we have examined if the promoter could also control the tissue specificity of collagen II gene expression. In this report, we have identified silencing elements in the 5′-region which suppress activity of the collagen II promoter in various cell types, but not in chondrocytes.

MATERIALS AND METHODS

Plasmids Constructs—Collagen II promoter-CAT constructs were derived from pCII 1 (9) which contains 5′-sequence of the collagen II gene (-1780 to +110) fused to the CAT gene. To obtain deletions of the promoter, a HindIII fragment (-1780 to +110) excised from pCII 1 was subcloned in pUC 19. After linearization by Ava I, this plasmid was digested for various times with Bal 31 exonuclease. Following digestion, NdeI linkers were added and were digested with NdeI and HindIII. Fragments were fractionated by electrophoresis on a agarose gel. After elution from the gel, the fragments were cloned into the NdeI-HindIII site of pSVOCAT resulting in pCII-52 (-52 to +110), pCII-560 (-560 to +110), pCII-560 (-560 to +110), pCII-880 (-580 to +110), pCII-620 (-620 to +110), pCII-700 (-700 to +110), pCII-510 (-510 to +110), pCII-600 (-600 to +110), pCII-950 (-950 to +110), pCII-977 (-977 to +110) (Fig. 1).

To construct internal deletions, pCII-977 was linearized by HindIII digestion, and treated with exonuclease Bal 31. NdeI linkers were added and digested by Nde I. Fragments were cloned into pCII-52, or pCII-312, at Nde I site. pCII ID1, pCII ID2 and pCII ID3 contain deletions of -156 to -92 bp, -400 to -312 bp, and -400 to -52 bp respectively.

A heterologous promoter, from the thymidine kinase gene, was also fused with the 5′-sequences from the collagen II gene in order to study whether the silencers lost promoter specificity. To obtain 3′CII/pTK and 5′(inv)CII/pTK constructs, the 5′-sequence of the collagen

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† The abbreviations used are: CAT, chloramphenicol acetyltransferase; bp, base pairs; CEY, chick embryonic fibroblasts.
II gene was excised from pCI-2 by HpaII digestion and cloned in both orientations in the Cdv site of pTK.

The plasmids containing collagen II enhancer pCII-312E and pCII-977E were constructed by cloning the 1.2-kilobase pair BamHI fragment of pCII-4-T into the BamHI site of pCI-312 and pCII-2 (9), downstream of the CAT gene.

Cell Culture—Chondrocytes were isolated from sterna of 15-day-old chicken embryos as described (9) and grown in Ham’s F12 medium containing 10% fetal calf serum. Chondrocytes were transplanted on the same day as they were prepared. Chick embryonic fibroblasts (CEF) were derived from subcutaneous tissue of 10-day-old chicken embryos by using trypsin-EDTA treatment. CEF were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and used at passage 5. HeLa cells were obtained from the American Type Culture Collection and grown in Eagle’s minimum essential medium with nonessential amino acids and Earle’s salts and containing 10% fetal calf serum.

Transfection and CAT Assay—Collagen II-CAT constructs were transfected into fibroblasts, chondrocytes or HeLa cells by the calcium-phosphate method (10) using 10 μg of DNA for each sample. Precipitates were removed after 3 h (chondrocytes and fibroblasts) or after 15 h (HeLa cells). The cells were harvested 2 days after transfection, and CAT activity was assayed using the phase extraction method (11). Briefly, after scraping in phosphate-buffered saline, cells were pelleted and then lysed by sonication in 0.25 ml 0.1 M Tris-hydroxymethylamine (pH 7.5), 50 μl of 0.1% SDS, 5 μl of 1M acetyl-CoA (0.42 mM, 0.1 μCi, Du Pont-New England Nuclear), and 5 μl acetyl-CoA (4.4 mM, Pharmacia LKB Biotechnology Inc.). The mixture was overlayed with 5 μl of water insoluble scintillation counting solution (Bicinchoninic acid-2, Du Pont). Vials were incubated at 37°C and were counted at different times until 24 h. The final ratios used data obtained during the linear expansion of counting. These ratios were typically reached after 3-h incubation. Each construct was tested in duplicate, in at least three independent assays.

β-Galactosidase Assay—Construct pCH110 includes the β-galactosidase, under the control of the SV40 early promoter (Ref. 12, Pharmacia LKB Biotechnology Inc.). Plasmid (5 μg) was precipitated with calcium phosphate as described (10) and cotransfected with various CAT constructs. After 48 h, cells were incubated with the substrate p-nitrophenyl-β-D-galactopyranoside (3.2 mM) for different times. Supernatants absorbance was read after 4-h incubation on a spectrophotometer at 405 nm to estimate β-galactosidase activity (13), when the cells were scraped for CAT assays.

Gel Retardation—To prepare a 100-bp fragment containing CII51 (−700 to −601), pCII-700 was linearized with Ndel, treated with bacterial alkaline phosphatase, and was radiolabeled with [γ-32P]ATP and T4 kinase. A 100 bp fragment one end-labeled at the Ndel site was prepared by FokI digestion, followed by purification on an acrylamide gel. A 170-bp competition fragment from the collagene II enhancer was obtained by BamHI/XhoI digestion from pCII-C2, an enhancer-containing construct described previously (9). Nuclear extracts were prepared by the procedure described by Dignam et al. (14), from HeLa cells, chondrocytes, or fibroblasts cultures or rat swimm chondrosarcoma (9) tumor which was dissociated previously by 0.2% collagenase treatment (Type II, Worthington) for 3 h at 37°C. They were incubated with the radiolabeled DNA fragment, with or without competitor DNA, in a 40-μl reaction containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 5% glycerol, and 4 μg of poly[dI-dC]. After 30-min incubation at room temperature, reactions were analyzed on a nondenaturing 5% acrylamide gel containing 89 mM Tris-HCl (pH 8), 89 mM boric acid, and 2 mM EDTA. The gel was electrophoresed for 2 h at 8 V/cm and then dried and autoradiographed.

RESULTS

Deletions in the Collagen II Promoter Region Identify Two Negative Regulatory Regions—In order to determine important regulatory elements in the collagen II promoter, various 5’-upstream sequences from the collagen II gene were transplanted into CEF and HeLa cells, using the calcium phosphate method. CAT activity of the construct (pCII-977) containing the longest 5’-upstream region (−977 to +110 bp) was increased by 85% compared to the activity of the construct (pCII-52) containing the shortest promoter region (−52 to +110 bp) (Fig. 1). Successive constructs elongating from −52 to 5’-direction showed decreased activity. Two main regions were involved in this decrease, which each inhibited the CAT activity by 20%. The first region was located between −700 and −620 bp (CII51) and the second between −460 and −360 bp (CIIS2). Similar results were obtained when these constructs were transfected into HeLa cells (Fig. 1).

Plasmids including internal deletions of the promoter region were also prepared, and their activity was examined in CEF and HeLa cells (Fig. 1). Deletion from −110 to −52 (pCII ID1) did not modify significantly the silencer activity, compared with the control construct pCII-977. However, deletion from −400 to −312 (pCII ID2) increased more than 4-fold in CEF the CAT activity, compared with pCII-977. This increase was even higher when deleting the −400 to −52-bp region (pCII ID3), both in CEF and in HeLa cells. The results suggested that these deletions were removing a silencer element.

To confirm that the silencer activity detected in these experiments was not due to different transfection efficiency, plasmid pCH110 containing lacZ gene for β-galactosidase, under the control of the SV40 early promoter was cotransfected with collagen II/CAT constructs in CEF (Fig. 2). The three CAT constructs, pCII-312, pCII-880, and pCII-912E, which include the collagen II enhancer, gave similar β-galactosidase activity, whereas CAT activity of pCII-880, but not pCII-312, was strongly decreased as shown in Fig. 1. The inclusion of the enhancer to pCII-312 did not change CAT activity in CEF. These results indicate that transfection efficiency was similar for these constructs. The activity of the constructs was also checked by ribonuclease protection experiments. RNAs from transfected cells were hybridized with a RNA probe encoding the collagen II 5’untranslated region region, and CAT activity was assayed after 48-h incubation. Three more experiments. Standard error was below 8% in all cases. nd denotes not determined.

FIG. 1. Promoter activity of 5’-flanking region of the collagen II gene in CAT plasmids in CEF and HeLa cells. Various fragments from the 5’-flanking region of the collagen II gene were cloned in the pSVOCAT vector. Resulting constructs pCII-52 (−52 to +110), pCII-95 (−95 to +110), pCII-312 (−312 to +110), pCII-360 (−360 to +110), pCII-460 (−460 to +110), pCII-510 (−510 to +110), pCII-560 (−560 to +110), pCII-580 (−580 to +110), pCII-620 (−620 to +110), pCII-700 (−700 to +110), pCII-880 (−880 to +110) and pCII-977 (−977 to +110) were transfected into CEF and HeLa cells and a CAT assay was performed after 48-h incubation. Three more constructs including internal deletions comprising the secon region between −110 to −52 bp (pCII ID1), −400 to −312 bp (pCII ID2), and −400 to −52 bp (pCII ID3) were transfected. Activity was expressed in all cases as a percentage of the activity obtained with pCII-52. Transfections were performed in duplicate in three separate experiments. Standard error was below 8% in all cases. nd denotes not determined.
negative regulatory regions (respectively, pCII-977E and pCII-312E) were transfected into chick chondrocytes and more than 13 times higher than pCII-312 or pCII-977, the silenced constructs, was decreased by about 30% in fibroblasts. The transcription level of pCII-977, which includes the collagen II enhancer either with or without the silencer elements, was weakly functional in chondrocytes, where the enhancer appears to be essential for the transcription.

Results indicate that the negative regulatory elements are functionally active in chondrocytes compared to pCII-312E, which did not include them (Fig. 3). However, the two constructs gave an activity that was 1.47-fold higher than pCII-312E in CEF, and the activity was normalized to pCII-312E. Burs indicate ± S.D. from four cotransfections.

Transfections were performed in duplicate in at least three separate experiments. The relative activity of pCII-312E in CEF was 1.47-fold higher than pCII-312, which did not contain the collagen II enhancer. Activity was expressed as a percentage of the activity obtained with pCII-977E.

The relative activity of pCII-312E in CEF was 1.47-fold higher than in chick embryonic chondrocytes (CEC), for the same protein amount. Transfections were performed in duplicate in at least three separate experiments.

Collagen II Negative Regulatory Elements Inhibit Expression of a Heterologous Promoter—To study the promoter specificity of these negative regulatory elements, a 482-bp sequence (–783 to –301 bp) including the two silencing regions was coupled to the thymidine kinase promoter, in both orientations. When these constructs were transfected into CEF, CAT activity was reduced to a tenth of the expression observed with the thymidine kinase promoter alone (Fig. 4). However, all of them had similar activity in chick embryonic chondrocytes (data not shown). These results show that the collagen II negative regulatory elements also function with a heterologous promoter and are independent of the orientation. They also confirm the silencers are inactive in chick embryonic chondrocytes.

Gel Retardation Analysis—A 32P-labeled 100-bp fragment containing the silencer CII1S1 was incubated with nuclear extracts from different sources (Fig. 5). The DNA fragment was shifted with HEK cells nuclear extracts indicating the formation of complexes. The retarded band was competed by the unlabeled 100-bp fragment, but not by a 176-bp fragment from the first intron, containing the collagen II enhancer, indicating specific DNA-protein interactions (Fig. 5, lanes 2–4). Chick chondrocytes or rat chondrosarcoma nuclear extracts did not show any detectable activity (Fig. 5, lanes 5 and 6).

Sequence Comparisons—The two negative regulatory elements were analysed for their homology with previously described regulatory elements (Fig. 6). CII1S1 displays a 7-bp fragment CACCTCC found in CII1S1. CII1S2 also contained two regions of inverted homology. Consensus sequence is found in CII1S1, ACCCTCTCT, which occurs in a long interspaced rat repetitive element (16), as well as in another interspaced repetitive region, called the CR1 element, in the avian genome (17, 18). Its negative regulatory function has been shown in the chick lysozyme gene (19), rat insulin gene (16), mouse IgH gene (20), human β-interferon gene (21), human α-globin gene (22), and in the rat growth hormone gene (23). Homologous motifs are also found in the human c-myc promoter region (24, 25), close to a region found to have an inhibitory activity on transcription (26, 27). Interestingly, another motif, TACTCACAGG, localized in c-myc gene in the same negative regulatory element (26, 27), shows a homology of 80% with a motif in the opposite orientation included in CII1S1. Finally, a different motif, CCCCCATCC, found in the CR1 element (18), was present in CII1S2, but not in CII1S1.

Collagen II gene

5' CIIS1

5' CIIS2

TK PROMOTER

CAT

CAT

Relative CAT activity

1

1

10

10

0

5'CIIS1/pTK

5'(inv)CIIS1/pTK

5'CIIS2/pTK

Fig. 4. Inhibitory activity of the collagen II negative regulatory elements on thymidine kinase promoter CAT construct in CEF. A 5'-flanking region fragment (–783 to –301) was cloned into both orientations in a thymidine kinase promoter/CAT construct pTK. Resulting constructs 5'CIIS1/pTK and 5'(inv)CIIS1/pTK were transfected into CEF. CAT activity is expressed as a percentage of the activity of pTK, transfected in CEF. Transfections were performed in duplicate in three separate experiments.
Silencer Elements in Collagen II Gene

Fig. 5. Gel retardation analysis of CIIS1. Radiolabeled fragment (2 ng) including CIIS1 was analyzed on a 5% acrylamide gel, either alone (lane 1) or after incubation with 10 µg of nuclear extracts from HeLa cells (lanes 2-4), from chondrocytes (lane 5), or from chondrosarcoma (lane 6). Some experiments were performed in presence of 10 ng of competitor cold DNA, using either a fragment from collagen II enhancer (lane 3) or the fragment including CIIS1 (lane 4). All experiments were performed in presence of 4 µg of poly(dI-dC).

DISCUSSION

We describe here two negative regulatory elements in the 5' upstream region of the collagen II gene which reduced the transcriptional activity of the collagen II promoter when transfected in either fibroblasts or HeLa cells. These sequences were also active in either orientation on a thymidine kinase promoter, and at least one of them binds to nuclear factors in HeLa cells.

A variety of silencers have been characterized (16, 19-23, 26-40). Some appear to be promoter-specific, like the 5'-upstream element regulating the gamma crystallin gene (30). This specific activity for the homologous promoter may be due to the silencer's flanking regions. However, the collagen II silencers do not appear to be promoter-specific, since the thymidine kinase promoter was also inhibited by a fragment containing both CIIS1 and CIIS2. Several other reports have described consensus negative regulatory sequences which were active on heterologous promoters including a sequence found in the genes for lysozyme (19), insulin (16), and interferon (21), and growth hormone (20). This sequence, ACCCTCTCT, is also included in a long interspaced rat repetitive element (16) and growth hormone (23). This sequence, ACCCTCTCT, is also included in a long interspaced rat repetitive element (16) and in the repetitive CR1 element in the avian genome [17]. Interestingly, more than 50,000 copies of this repetitive element are found in the rat genome. Although their physiological function is still unclear, these repetitive elements are likely to be involved in gene regulation, possibly by preventing the transcription of different portions of the genome. We also found homologous motifs repeated in both orientation in the c-myc gene promoter region, close to a distinct sequence described as a silencer (26, 27). Moreover, the sequence TACTCACAGG, included in the c-myc silencer, appears highly homologous to an inverted motif found in CIIS1. Since these two consensus sequences are located close one to each other in CIIS1, they could interact by participating in the formation of a silencing complex with nuclear factors. The formation of this complex could also involve the two inverted repeats found in CIIS1, which concern both of the silencer consensus sequences. A distinct sequence, CACCTCC, is shared by CIIS1 and CIIS2 and is located one helix turn from the consensus sequence ACCCTCTCT in CIIS1. As this region does not share homology with any described consensus sequences, it could have a cartilage-specific activity, although this has not been shown directly.

Silencers are likely to be binding sites for nuclear factors. Our data suggest such soluble binding factors are present in nuclei from nonproducing HeLa cells, but are missing in nuclei from chondrocytes or chondrosarcoma cells that synthesize collagen II. Such factors could be specific for silencers or be similar to factors binding to activation sites like enhancers. In yeast, a transcriptional factor RAP 1 has been shown to bind to the upstream activation sites of different sets of genes, as well as to a silencer (31). In these cases, silencers could be considered as nonoperative enhancers which mimic the first steps of enhancer-mediated transcription and then block it at a further stage. A similar situation has been described for the c-myc gene. The c-fos protein has been found to be part of a complex binding to a c-myc negative regulatory element which includes partially the region showing homology with CIIS1 (27). This complex also involves the transcriptional factor AP1, encoded by c-Jun (41, 42). However, its DNA-binding site appears to include also a sequence recognized by the well described octamer-binding protein involved in the transcriptional control of immunoglobulin genes (26). These different factors could mediate activation or repression of the gene by modifying their relative binding affinities. Such a "push and pull" mechanism has also been proposed for the regulation of low density lipoprotein receptor gene, where the sterol-dependent binding of a protein to a regulatory sequence could inhibit the activity of an adjacent Sp1 binding site (32). This is more likely to occur when negative regulatory regions are located close to enhancer sites. Such a localization has been reported in several other cases including the murine IgH (20), the T cell receptor α/β locus (43), and the mouse α1(I) collagen gene. The α1(I) and α2(I) collagen gene shows some homologies with the α1(II) collagen gene. The 5'-upstream region and the first intron include negative regulatory elements (33, 34), as well as enhancer elements (33, 44, 45). Furthermore, we also found a negative regulatory region, located upstream from the 800-bp enhancer complex in the first intron, which represses the activity of the collagen II promoter, in a position-dependent manner.

The function of silencers in vivo is still unclear. They could be responsible at least partially for tissue-specific protein expression. Collagen II is expressed mainly by chondrocytes. Our data suggest that collagen II silencers express their activity in non-chondrocytic cells where the collagen II promoter would be active otherwise, as these cells do not require the collagen II enhancer to start transcription from the promoter. Therefore, silencers are not functional in chondrocytes, likely because these cells do not provide functional nuclear factors binding to the silencer, as suggested by our gel retardation experiments. Conversely, chondrocytes and other cells producing collagen II could be the only cells that provide nuclear factors able to bind specifically to the collagen II enhancer and initiate a transcription complex as suggested by gel retardation experiments.

2 P. Savagner, T. Miyashita, and Y. Yamada, unpublished data.
FIG. 6. Consensus sequences in the collagen II silencers. The sequence of the two collagen II silencers is shown, with an identical 7-bp motif CACCTCC (rounded rectangle). The silencer consensus sequence ACCCTCTCTT found in CR1 element, c-myc gene, and in several genes is in a rectangle (plain line with arrow). A CIIS1 sequence is homologous to c-myc gene when inverted (thick arrow). A homologous sequence CCCCATCC between CIIS2 and the CR1 element is surrounded in a dotted rectangle. An inverted repeat is found in CIIS1 sequence (dashed arrow). The partial sequences of CR1 (nucleotides 95-195 in the consensus element, Ref. 15) and c-myc (-350 to -194 bp from start site, Refs. 21 and 22) display the regions of homology.

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