Transcriptional Regulation of the Human $\alpha 2(I)$ Collagen Gene

COMBINED UPSTREAM STIMULATORY AND INHIBITORY CIS-ACTING ELEMENTS

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This study identifies three regions of the human $\alpha 2(I)$ collagen promoter involved in the binding of nuclear factors. These regions include sequences from −173 to −155 (footprint I), −133 to −119 (footprint II), and −101 to −72 (footprint III). A novel positive cis-element containing a TCTCC motif was identified within footprint II. In addition, we demonstrated that a pyrimidine-rich region within footprint I is a binding site for a transcriptional repressor, and a CCAAT motif within footprint III is a binding site for a transcriptional activator. Comparative functional analysis of the cis-acting elements within the proximal 350 base pairs of this promoter, including previously characterized Sp1 binding sites at −300, indicates that constitutive activity of this promoter is regulated equivalently by the three positive cis-acting elements at −300, −125, and −80. Mutations in the repressor site at −160 increase constitutive activity by 4-6-fold. However, simultaneous mutations of the repressor site and the cis-regulatory element at either the −300 or −125 sites result in no increase in constitutive transcription activity suggesting interaction between the activators and repressor elements. In contrast, simultaneous mutation of the CCAAT motif and the repressor site results in about a 4-fold increase, suggesting that activation via the CCAAT motif may be independent of this repressor.

Collagen type I, the most abundant mammalian collagen, consists of two $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain, which are coordinately expressed (1, 2). The expression of type I collagen is strictly regulated during development and is tissue-specific (2). Excessive deposition of type I collagen is characteristic of many fibrotic disorders (3) and most likely results from transcriptional activation of collagen genes in response to cytokines and other factors present in the prefibrotic/inflammatory lesions.

Previous studies have characterized several responsive elements and cognate transcription factors involved in the regulation of collagen type I genes in murine fibroblasts (1). The most extensively studied transcription factor CBF is a heterotrimer consisting of subunits denoted A, B, and C, all of which are necessary for DNA binding (4, 5). CBF is a transcriptional activator of mouse $\alpha 1(I)$ and $\alpha 2(I)$ collagen genes (4, 5), where it binds to CCAAT motifs located between −80 and −84 in the $\alpha 2(I)$ promoter and −96 and −100 in the $\alpha 1(I)$ promoter (6, 7). Another transcriptional activator of the murine $\alpha 2(I)$ collagen promoter is a member of the CTF/NF1 family, which binds between −315 and −295 in the $\alpha 2(I)$ promoter (6) and mediates TGF-$\beta$ stimulation of this promoter (8). A third site in the mouse $\alpha 2(I)$ collagen promoter at −250 also contributes to basal promoter activity, but a cognate transcription factor has not yet been characterized (6). Both $\alpha 1(I)$ and $\alpha 2(I)$ collagen promoters in the mouse are negatively regulated by a transcriptional repressor termed IF1 that binds to two adjacent sites between −90 and −170 in the $\alpha 1(I)$ promoter and between −165 and −155 in the $\alpha 2(I)$ promoter (9, 10). In addition, a novel factor of unknown function has been shown to interact with the region between −419 and −399 in the mouse $\alpha 2(I)$ promoter (11).

Initial studies of the human $\alpha 2(I)$ promoter have indicated that the −376 to −108-bp promoter segment is sufficient to direct a high level of transcription in human fibroblasts (12). However, transcriptional regulation of this promoter in human fibroblasts differs in some respects from transcriptional regulation of the murine $\alpha 2(I)$ collagen promoter. For example, it has been demonstrated that CTF/NF1 does not bind to the human $\alpha 2(I)$ collagen promoter (13). TGF-$\beta$ stimulation of human $\alpha 2(I)$ collagen promoter is mediated by a multiprotein complex that interacts with two distinct promoter segments (−330 to −286 and −271 to −255) termed TbRe (13). One of the proteins in this complex has been identified as Sp1 (13). Interestingly, TbRe also mediates inhibitory effects of tumor necrosis factor-α on transcriptional regulation of the human $\alpha 2(I)$ collagen promoter (14). In addition, Sp1 mediates basal activity of the human $\alpha 2(I)$ collagen promoter by binding to three responsive elements located between bp −303 and −271 (15). Two recent studies have also suggested the involvement of Sp1 family members in mediating TGF-$\beta$ effects in the mouse and human $\alpha 2(I)$ collagen promoters (16, 17). The human $\alpha 2(I)$ collagen promoter region downstream from the Sp1 and TbRe binding sites has not been characterized. However, previous studies have indicated that a human $\alpha 2(I)$ collagen promoter segment with a deleted or mutated Sp1-responsive element still maintained low levels of promoter activity suggesting the presence of additional regulatory sites in the downstream promoter region (15). Moreover, several response elements were

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1 The abbreviations used are: TGF-$\beta$, transforming growth factor-$\beta$; bp, base pair(s); DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; DMS, dimethyl sulfate; LMPCR, ligation-mediated polymerase chain reaction; TbRe, TGF-$\beta$ response element.
Functional Analysis of Human \( \alpha 2(1) \) Collagen Promoter

We have previously analyzed the \(-353\) to \(-234\) region of the human \( \alpha 2(1) \) collagen promoter and have demonstrated the presence of three binding sites for a transcription factor related to Sp1 in this promoter region (15). To characterize DNA-protein interactions in the promoter region downstream from the Sp1 binding sites, we performed a DNase I protection assay using a promoter fragment from bp \(-235\) to \(-205\) and the nuclear extract from human fibroblasts. Three protected DNA segments were observed, which included two strongly protected regions located between bp \(-173\) and \(-155\) (footprint I), bp \(-133\) and \(-119\) (footprint II), and a broad weakly protected region between bp \(-101\) and \(-72\) (footprint III) (Fig. 1).

In Vivo Footprinting—Human fibroblasts were grown to confluence in DMEM, \(10\%\) FCS. The medium was then replenished with DMEM, \(10\%\) FCS containing \(0.1\%\) dimethyl sulfoxide (DMS) and incubated for 2 min. Cells were rinsed once with phosphate-buffered saline at \(37^\circ\) C, followed by three washes with phosphate-buffered saline for \(30\) s each with gentle shaking at \(37^\circ\) C. Cells were lysed on the plates using \(1.5\) ml \(50\) mM Tris-HCl, \(10\) mM EDTA, \(0.4\) mg/ml proteinase K, and a broad weakly protected region between bp \(-101\) and \(-72\) (footprint III) (Fig. 1).

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Functional Analysis of Human α2(I) Collagen Promoter

![Diagram of DNase I footprinting analysis of the α2(I) collagen promoter](Image)

**Fig. 1.** DNase I footprinting analysis of the α2(I) collagen promoter. A 5'-end-labeled fragment encompassing the promoter region between −235 and −34 was used as a probe in the binding reactions containing nuclear extract (120 μg/lane) from human foreskin fibroblasts. GA reaction of the coding strand is shown; −, DNA without nuclear extract; +, DNA with nuclear extract. Protected regions are shown diagrammatically, with the limits of each footprint indicated.

![Graph of functional analysis](Image)

**Fig. 2.** DMS in vivo footprint of the −234 and −34 α2(I) collagen promoter region. The in vivo footprint patterns of the noncoding strand (A) and coding strand (B) are shown. Lane 1, naked DNA from in vitro DMS-treated samples; lane 2, DNA from in vivo DMS-treated human fibroblasts. The location corresponding to the sequence ladder (not shown) with respect to the transcription start site (23) is indicated at the left side of each panel. Promoter segments indicating DNA-protein interaction are shown diagrammatically at the right side of each panel.

![Characterization of the CCAAT motif by DNA mobility shift assay](Image)

**Fig. 3.** Characterization of the CCAAT motif by DNA mobility shift assay. Nuclear extract (5 μg/lane) prepared from human fibroblasts was incubated with a 5'-end-labeled α2(I) collagen fragment from bp −234 to −34 (202-mer) (lanes 1–5) or with a mutated 202-mer, containing a single substitution mutation in the CCAAT motif (lane 6). Lane 1, no nuclear extract; lane 2, no competitor; lane 3, with addition of 200-fold molar excess of cold 202-mer; lane 4, with addition of wild type 21-mer (containing CCAAT motif; see text); lane 5, with addition of mutated 21-mer (see text). Specific protein-DNA complexes are indicated by arrows.

with DMS in vitro (lane 1) and chromosomal DNA treated with DMS in vivo (lane 2) on the mRNA coding strand in the promoter region between bp −179 and −148 and bp −133 and −119. It appears that in vitro footprints correlate with the footprints I and II obtained in the DNase I protection assay (Fig. 1). A second set of primers was used to visualize binding to the noncoding strand (Fig. 2B). Protection and hypersensitivity sites were observed between bp −81 and −74 that correlate with the footprint III in Fig. 1. Thus, the three footprints identified by DNase I protection assays colocalsed with in vivo footprints. The footprint III contains an inverted CCAAT motif between bp −84 and −80 that has been previously shown to bind the heterotrimeric transcription factor CBF in the mouse α2(I) collagen promoter (6). The sequences corresponding to footprints I and II have not been rigorously analyzed in either mouse or human α2(I) collagen promoters.

**The Function of the CCAAT Motif Is Conserved between the Mouse and the Human α2(I) Collagen Promoters**—To test binding to the CCAAT motif in the human α2(I) collagen promoter, we employed a mobility shift assay using a 202-bp DNA fragment (from −235 to −34) and human fibroblast nuclear extracts. Two strong protein-DNA complexes (denoted 2 and 3 in Fig. 3, lane 2)) and two relatively weak complexes (denoted 1 and 4, Fig. 3, lane 2) were observed. Formation of one of the complexes (denoted 3) was abolished by the addition of excess cold 21-mer containing the CCAAT motif (CAGCCCTCCCATTTGAGGAGG) (lane 4) but not by the 21-mer containing a single substitution mutation within the CCAAT motif (CAGCCCTCCCATTTTGGAGG) (lane 5). Such mutation has been previously shown to abolish binding of CBF to the mouse collagen promoter (6). Furthermore, the smallest complex did not form when a 202-mer containing the same substitution mutation in the CCAAT motif was used as a probe (lane 6). These data indicate that a factor from human fibroblasts, probably a human homolog of murine CBF, binds specifically to the CCAAT motif in the human α2(I) collagen promoter. Previous studies in the mouse α2(I) collagen promoter have demonstrated that the CCAAT motif at position −80 contributes significantly to the constitutive activity of this promoter (6). We therefore tested the effects of substitution mutation in the CCAAT motif (CCAAA) on the activity of the human promoter. The substitution mutation introduced into the −353 bp α2(I) collagen promoter fragment linked to the CAT reporter gene resulted in a 90% reduction of the basal promoter activity when compared with the wild type promoter (set arbitrarily at 100%) (Fig. 4). Thus, similar to the mouse promoter, the CCAAT motif in the human promoter is the binding site for an activator, most likely a human homolog of CBF.

**The TCCTCC Motif within Footprint II Mediates Binding of a Transcriptional Activator**—To characterize binding of the
nuclear factors to the promoter region corresponding to footprint II, we performed DNA mobility shift assay using the promoter fragment from −135 to −116 and nuclear extracts from foreskin fibroblasts. Three specific protein-DNA complexes were observed (Fig. 5B, lane 2). To further characterize DNA sequences involved in formation of these DNA-protein complexes, a series of oligonucleotides containing substitution mutations (see Fig. 5A) were used as competitors in the DNA mobility shift assay. M3 and M4 oligonucleotides, containing substitution mutations in the TCCTCC motif located in the middle of the protected region, failed to inhibit formation of the DNA-protein complexes, while formation of all three complexes was abolished by an excess of M1, M2, and M6 oligonucleotides and reduced by an excess of M3 oligonucleotide. These results indicate that the pyrimidine-rich sequence CCTTCCCCC within footprint I mediates binding of the nuclear proteins to this promoter region, and the flanking regions may be important for forming stable DNA-protein complexes.

To analyze the contribution of the TCCTCC motif to the basal collagen promoter activity, we introduced substitution mutations into TCCTCC motifs using a −353 promoter construct. TCCTCC motifs were mutated separately in the same fashion as oligonucleotides M3 and M4 used in the gel shift assay (Fig. 5). The promoter constructs carrying substitution mutations were analyzed by transient transfection assays (Fig. 4). Mutations in either TCCTCC motif, when compared with the wild type promoter, resulted in significant reduction of the basal promoter activity, although mutation of the proximal site had a reproducible stronger effect (10.9 ± 2.9%) than mutation of the distal site (17.8 ± 2.8%).

Transcriptional Repressor Binds to the Footprint I in the Human a2(I) Collagen Promoter—To test binding to the promoter region corresponding to footprint I, we performed a mobility shift assay using the promoter fragment from bp −176 to bp −153 and nuclear extract from newborn foreskin fibroblasts. Three specific protein-DNA complexes were observed (Fig. 6B, lane 2). To further characterize DNA sequences involved in formation of these DNA-protein complexes, a series of oligonucleotides containing substitution mutations (see Fig. 6A) were used as competitors in the DNA mobility shift assay. M4 and M5 oligonucleotides, containing substitution mutations in the “TCCCCC” motif located between −164 and −159 in the promoter region, failed to inhibit formation of the DNA-protein complexes, while formation of both complexes was abolished by an excess of M1, M2, and M6 oligonucleotides and reduced by an excess of M3 oligonucleotide. These results indicate that the pyrimidine-rich sequence CCTTCCCCC within footprint I mediates binding of the nuclear proteins to this promoter region.

To analyze the contribution of this pyrimidine-rich motif to the basal promoter activity, we introduced substitution mutations into the TCCCCC motif using a −353 promoter construct. Proximal and distal triplets were mutated separately in the same fashion as oligonucleotides M5 and M4 used in the gel shift assay (Fig. 6A). The promoter constructs carrying substitution mutations were analyzed together with the wild type promoter by transient transfection assays. Mutations in either triplet, when compared with the wild type promoter, resulted in a significant increase in the basal promoter activity (Fig. 4).

Functional Analysis of the Cis-regulatory Elements in the Human a2(I) Collagen Promoter—Previous analyses of the human a2(I) collagen promoter have demonstrated a strong positive cis-regulatory element located between −319 and −267. This element, composed of three GC-rich motifs, has been characterized as a binding site for transcription factor Sp1. Thus, three positive and one negative cis-acting regulatory elements are present within the 350-bp fragment of the human a2(I) promoter. The three positive cis-acting elements at −300, −125, and −80 appear to contribute equally to promoter activity, as substitution mutation in each of these elements decreased promoter activity about 10-fold (Fig. 4). To test the nature of the interactions between the Sp1 response element and the two proximal positive response elements at −125 and
Previous studies of the human \( \alpha_2(1) \) collagen promoter have demonstrated that the 350-bp upstream promoter segment directs high constitutive promoter activity (12, 15). The \(-303 \) to \(-255 \) region has been shown to contain the binding site for the transcription factor Sp1 and also the TGF-\( \beta \) response element (13, 15). In this study we have identified three additional regions of the human \( \alpha_2(1) \) collagen promoter that bind nuclear factors by using in vitro and in vivo footprinting assays (Figs. 1 and 2). These regions include sequences from \(-173 \) to \(-155 \) (footprint I), \(-133 \) to \(-119 \) (footprint II), and \(-101 \) to \(-72 \) (footprint III) (Fig. 8).

Footprint II contains a novel cis-regulatory element that is not present in the mouse \( \alpha_2(1) \) collagen promoter. Functional analysis of this response element indicates that the sequence TCTCTCC (between \(-128 \) and \(-123 \)) contributes significantly to the basal promoter activity (Fig. 4). The nature of the binding proteins is presently unknown. A similar responsive element has been found in the osteonectin promoter, and a cognate factor termed “GGA factor” has been demonstrated by UV cross-linking to be a single 40-kDa protein (20). Binding to the TCTCTCC motif in the human \( \alpha_2(1) \) collagen promoter appears to be more complex as indicated by the three DNA-protein complexes in the gel shift assay (Fig. 5). Purification of the transcription factors from human nuclear extracts that bind to

**DISCUSSION**

### Footprint II

Footprint II contains a novel cis-regulatory element that is not present in the mouse \( \alpha_2(1) \) collagen promoter. Functional analysis of this response element indicates that the sequence TCTCTCC (between \(-128 \) and \(-123 \)) contributes significantly to the basal promoter activity (Fig. 4). The nature of the binding proteins is presently unknown. A similar responsive element has been found in the osteonectin promoter, and a cognate factor termed “GGA factor” has been demonstrated by UV cross-linking to be a single 40-kDa protein (20). Binding to the TCTCTCC motif in the human \( \alpha_2(1) \) collagen promoter appears to be more complex as indicated by the three DNA-protein complexes in the gel shift assay (Fig. 5). Purification of the transcription factors from human nuclear extracts that bind to
Footprints are indicated by the pattern shown in the text. At −353 to +56, all three GC boxes were mutated as described previously (15). Mutation of the CCAAT motif was used to construct double mutants (see text). At −125, the M4 mutation was used to construct double mutants (see text). At −80, the CCAAT motif was mutated as described in the text. The CCAAT motif is critical for activation of the collagen type I gene in fibroblasts. For example, the CBF-like transcriptional activator, Sp1, and other activators of collagen transcription with the basal transcription machinery and with each other in the regulation of the human α2(1) collagen promoter.

FIG. 7. Activities of the α2(1) collagen promoter constructs carrying double substitution mutations. Substitution mutations were introduced into the plasmids containing the −353 to +56 α2(1) collagen promoter fragment cloned upstream from the chloramphenicol acetyltransferase reporter gene. At −300, all three GC boxes were mutated as described previously (15); at −160, the M5 mutation was used to construct double mutants (see text); at −125, the M4 mutation was used to construct double mutants (see text). At −80, the CCAAT motif was mutated as described in the text. These plasmids were used in transient transfection assays as described under “Materials and Methods.” The diagram on the left indicates the mutated responsive elements (black boxes). The bar graph on the right shows the promoter activity of each mutated construct relative to the wild type −353 promoter construct, which was arbitrarily set at 100. The means ± S.E. for separate experiments are shown at the right. The number of experiments used to calculate the mean is shown in parentheses. Asterisks indicate statistically significant results relative to the wild type promoter (p < 0.01, Mann-Whitney U test). × indicates statistically significant results relative to the single mutant in the CCAAT motif (p < 0.01).

FIG. 8. Summary of the protein/DNA interactions detected in the human α2(1) collagen promoter. Boundaries of the in vitro footprinting analysis are indicated by boxes. Asterisks indicate protected G residues based on in vivo footprinting analysis.

The TCCTCC motif will facilitate further analysis of this novel responsive element.

Footprint III contains an inverted CCAAT motif, which was previously shown to bind CBF, an activator of the murine α2(1) and α1(1) collagen promoters (6). In this study we have demonstrated specific binding to the CCAAT motif in the human α2(1) promoter (Fig. 3). Functional analysis of the CCAAT motif in human fibroblasts indicates that a transcriptional activator, possibly a homolog of CBF, binds to this sequence (Fig. 4). Analysis of footprint I reveals that a pyrimidine-rich motif located between −164 and −159 is a binding site for a transcriptional repressor (Figs. 4 and 6). The homologous regions in murine α1(1) and α2(1) collagen promoters also have been shown to bind a transcriptional repressor termed IF1 (10, 21). Thus, the CCAAT motif and the pyrimidine-rich element between −164 and −159 are functionally conserved between human and mouse promoters. On the other hand, the Sp1 binding sites at −300 (15) and the TCCTCC motif within footprint II appear to be specific for the human α2(1) collagen promoter. Interestingly, we have not observed any protection in either in vitro or in vivo footprinting analyses in the −250-bp region in the human α2(1) collagen promoter (data not shown). The corresponding region has been shown to mediate activation of the murine α2(1) collagen promoter (6) and could be the locus of additional differences between the regulatory mechanisms of the human and mouse collagen genes.

Functional analysis of the four response elements identified in the 350-bp region of the human α2(1) collagen promoter reveals a complex interaction between the positive and negative regulatory elements. Mutation in the Sp1 binding sites decreases promoter activity by a factor of 10 (15). Likewise, mutations in the CCAAT and TCCTCC motifs result in a similar reduction of promoter activity (Fig. 4), indicating that these three response elements contribute equivalently to the constitutive promoter activity. This is further demonstrated by double mutants in either Sp1 and CCAAT sites or Sp1 and TCCTCC sites, which reduce the promoter activity by an additional factor of 2 (Fig. 7), suggesting that proteins recognizing these sites have an additive effect in activating transcription. It is possible that combined actions of the three independent positive cis-regulatory elements are necessary to provide high levels of expression of the collagen type I gene in fibroblasts. Previous studies demonstrated that activation of transcription by Sp1 involves direct interaction with one of the subunits of the transcription factor TFIID, the 110-kDa polypeptide TAFII 110 (22). A similar interaction was recently reported for CBF (5). Future studies are needed to elucidate possible interactions of CBF, Sp1, and other activators of collagen transcription with the basal transcription machinery and with each other in the regulation of the human α2(1) collagen promoter.

Stimulation of transcription by CBF, Sp1, and other activators is counteracted by the action of the transcriptional repressor. Mutations in the TCCCCC motif located between −164 and −159 in the collagen promoter increase constitutive transcription levels by 5–6-fold. While the nature and mechanism of action of this repressor are presently unknown, our data suggest that it may work by interfering with the activity of the factors bound to the −300 and −125 sites. Interestingly, CBF seems to work independently of this repressor. These results indicate that under different physiological conditions, different combinations of available factors may dictate the expression levels of the α2(1) collagen gene. For example, the CBF-like factor may provide a pathway for bypassing the repressor activity or for finetuning the expression level.

In conclusion, this report presents a detailed analysis of the cis-regulatory elements of the human α2(1) collagen promoter. Characterization of the nuclear proteins interacting with these sequences is currently under way, but much work remains to be done to fully understand the basal and cytokine-stimulated transcriptional regulatory mechanism of the collagen type I gene in human fibroblasts. The in vivo footprinting assay pro-
vides an important tool to confirm *in vitro* observations in living cells.

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