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Binding of Upstream Stimulatory Factor to an E-box in the 3′-Flanking Region Stimulates α1(I) Collagen Gene Transcription*

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Since several lines of evidence implicate the 3′-flanking region in regulating α1(I) collagen gene transcription, we analyzed 12.4-kilobase pairs of 3′-flanking sequence of the murine α1(I) collagen gene for transcriptional elements. A region of the 3′-flanking region stimulated expression of the heterologous β-globin gene promoter in an enhancer trap plasmid and of the α1(I) collagen gene promoter in a collagen-luciferase reporter gene construct when located 3′ to the luciferase reporter gene. DNase I footprinting analysis demonstrated the presence of three regions where DNA binding proteins specifically interact within this 3′-stimulatory region. Inspection of the DNA sequence revealed a consensus E-box, a binding site for basic helix-loop-helix proteins, in one of the protein binding sites. Mobility shift assays demonstrated that upstream stimulatory factors (USF) USF-1 and USF-2 bind to this E-box. Mutating the E-box in the context of the 3′-flanking region confirmed that it contributes to the enhancement of transcriptional activity of the α1(I) collagen gene promoter. Mutations in all three protein binding sites abolished transcriptional activation by the 3′-flanking region, suggesting a complex interaction among the *trans*-acting factors in enhancing transcriptional activity. Thus, a region of the 3′-flanking region of the α1(I) collagen gene stimulates transcription of the α1(I) collagen gene promoter, and USF-1 and USF-2 contribute to this transcriptional stimulation.

Type I collagen, the most abundant protein in vertebrates, has diverse biological functions. It promotes cell migration, differentiation, and tissue morphogenesis during development. Additionally, it provides tensile strength to connective tissues such as bone, tendons, and skin, and forms a supporting framework of connective tissues in all major internal organs and the vascular system. Type I collagen is also the major protein produced during repair of tissue injuries and wound healing. Excess deposition of type I collagen occurs in fibrogenic diseases, such as hepatic fibrosis (1), pulmonary fibrosis (2), primary systemic sclerosis (3), and eosinophilic myalgia syndrome (4). Type I collagen is the product of two genes, the α1(I) and the α2(I) collagen genes, whose products form a heterotrimeric protein composed of two α1(I) and one α2(I) polypeptide chains. Although located on different chromosomes, both genes are generally coordinately regulated in a developmental and tissue-specific manner. Expression of the type I collagen genes is active in many cell types and under various physiological conditions, and their regulation is accordingly complex (5–8). Type I collagen gene expression is also modulated by agents such as cytokines and chemical or viral transformation.

Transcriptional regulatory elements have been previously identified in the 5′-flanking region, the promoter region, and the first introns of both type I collagen genes in several species (9–19). While sequences in the minimal promoter, within 220 bp[1] upstream of the start site of transcription, appear to be sufficient for the basal activity and partial tissue specificity of the α1(I) collagen gene promoter in transient transfection assays, the precise function of distal 5′-flanking sequences and the first intron is less clear. Moreover, several lines of evidence suggest that, in addition to these regulatory elements, sequences in the 3′-flanking region may contribute to transcriptional regulation of the α1(I) collagen gene. First, the 5′-regulatory elements were not always sufficient for precisely regulated, tissue-specific, high level expression of the gene when tested in transient transfection experiments or in transgenic mice (10, 20–23). Second, in the transgenic HucII mouse strain, a single copy of the human α1(I) collagen gene, which included 1.6 kb of 5′-flanking region, the entire structural gene, and 20 kb of 3′-flanking sequence, was expressed as efficiently as the endogenous collagen gene in an appropriate tissue-specific manner (24) and was induced appropriately during hepatic fibrogenesis (7). Finally, the human α1(I) collagen gene contains several DNase I-hypersensitive sites located immediately 3′ of the structural gene (25), which are often indicative of regulatory elements.

Therefore, we initiated a systematic analysis for regulatory elements located within the 3′-flanking region of the murine α1(I) collagen gene. We located a segment of the 3′-flanking region which was found to enhance expression of the heterologous β-globin gene promoter as well as the endogenous α1(I) collagen gene minimal promoter in NIH 3T3 fibroblast cells. DNase I footprinting analysis demonstrated the location of three sites of DNA-protein interactions within this transcriptional stimulatory region. One of the binding sites contained a consensus E-box, to which both USF-1 and USF-2, two basic

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U50767.

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[1] The abbreviations used are: bp, base pair(s); USF, upstream stimulatory factor; kb, kilobase pair(s); PCR, polymerase chain reaction; bHLH, basic helix-loop-helix.

helix-loop-helix proteins (bHLH), were found to bind. When the E-box was mutated within the context of surrounding wild-type 3′-flanking sequence, reduced levels of reporter gene activity were observed. However, when all three *cis*-acting elements were mutated a complete loss of transcriptional stimulatory activity was obtained. These results demonstrate that transcription of the α1(I) collagen gene is stimulated by a region within the 3′-flanking region of the gene and that USF-1 and USF-2 participate in the stimulation of transcriptional activity of the α1(I) collagen gene promoter.

MATERIALS AND METHODS

Plasmid Construction and DNA Sequencing—Fragments of the genomic clones pCE4 and pCE5 (kindly provided by K. Harbers) which contain the 3′-flanking sequences of the murine α1(I) collagen gene (26) (Fig. 1) were cloned into the unique *Sph*I site of the enhancer trap plasmid pβe$^-$ (27). The entire α1(I) collagen sequence present in pCE5 was sequenced. This fragment contains the last 12 codons of the α1(I) collagen gene. As a reference, the first base following the translational stop codon is designated as +1. The complete genomic insert in pCE5 (4.6 kb) was cloned into pβe$^-$, creating plasmid pβCE5, and a series of deletions shown in Fig. 1*B* were created by digestion with *Bal*31 exonuclease utilizing unique *Pst*I and *Hin*dIII sites in the plasmid. The plasmid pβCOL-(+1899−4597) was created by digesting pβCE5 with *Pst*I, gel-purifying the large DNA fragment, and religating using T4 DNA ligase. A partial *Hinc*II digestion of the pCE4 genomic insert produced two fragments (5.4 and 2.4 kb) which were individually cloned into the *Sph*I site of pβe$^-$. The reporter gene pGLCOL3 was constructed by ligating the α1(I) collagen promoter (−220 to +116) into the *Bgl*II and *Hin*dIII sites of the luciferase reporter gene pGL2-Basic (Promega, Madison, WI). Reporter genes pCOL-(+1899−4597) and pCOL(+4597 to 1899) were constructed by ligating the 2698-bp 3′ α1(I) collagen fragment from plasmid pβCOL-(+1899−4597) (Fig. 1) into the blunted *Sal*I site of pGLCOL3, which is located at the 3′ end of the luciferase gene, in both orientations. Plasmid pCOL-(+1899−4597) contains the 3′-flanking region in the 5′ to 3′ orientation, with respect to the direction of transcription from the collagen promoter, while in plasmid pCOL-(+4597 to 1899) the 3′-flanking sequence is positioned 3′ to 5′, with respect to the direction of transcription from the collagen promoter. Plasmids pCOL-(+3590−4597) and pCOL-(+4597 to 3590) were created by digesting pβCOL-(+1899−4597) with *Hin*dIII and *Pst*I, blunting the ends of the isolated 1-kb fragment, and ligating the fragment into pGLCOL3 which was digested with *Bam*HI and the ends blunted. This places the α1(I) collagen 3′-flanking sequence 3′ with respect to the luciferase gene. Plasmids pCOL-(+4090−4597) and pCOL-(+4597 to 4090) were constructed by generating a PCR product using primers that hybridized to positions +4090 to +4113 (5′-ATC CGG ATC CGT AAC CTA AAG ATG GTG GGT TTT C-3′) and positions +4597 to +4577 (5′-ATC CGG ATC CGA ATT CCC ACT AGT GCG GGG G-3′) of the α1(I) collagen 3′-flanking sequence. The PCR reaction contained 10 mM Tris-HCl, pH 8.9, 50 mM KCl, 2.5 mM $MgCl_2$, 2 μM dNTPs, 1 μM each primer, 100 ng of pCE5 as the template, and 2.5 units *Taq* DNA polymerase (Boehringer Mannheim). The reaction was cycled as follows: 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min for 30 cycles, an extension incubation at 72 °C for 10 min, followed by incubation at 4 °C, using a GeneAmp PCR System 9600 (Perkin-Elmer). The PCR fragment was digested with *Bam*HI, using the *Bam*HI recognition site designed in the primers, and cloned into the *Bam*HI site of pGLCOL3 in both orientations. To construct plasmids containing the 3′-footprinted regions, oligonucleotides were synthesized and complementary strands annealed. The annealed oligonucleotides were ligated together and the ligation reaction subsequently electrophoresed in polyacrylamide gels. Bands representing three and five copies of the ligated oligonucleotides were excised and eluted from the gel. The oligonucleotides were then ligated into the *Bam*HI site of pGLCOL3. The oligonucleotides used for the three footprinted regions were as follows, 3′FP1 top strand: 5′-GAT CCG CGG CTG TCA CGT GGC ATG GGC TGG TAT GTG CTC TAA ATA-3′, bottom strand: 5′-GAT CTA TTT AGA GCA CAT ACC AGC CCA TGC CAC GTG ACA GCC GCG-3′; 3′FP2 top strand: 5′-GAT CCT CCT TTC CGC TGA CAT CAT TGC TGC CA-3′, bottom strand: 5′-GAT CTG GCA GCA ATG ATG TCA GCG GAA AGG AG-3′; and 3′FP3 top strand: 5′-GAT CCC TTT GGG GAG GGA CCT GGA GCA A-3′, bottom strand: 5′-GAT CTT GCT CCA GGT CCC TCC CCA AAG G-3′. In creating the construct p3′FP1M, where the E-box was mutated while in the context of surrounding wild-type 3′-flanking sequence in plasmid pCOL(+3590−4597), the overlap extension method described by Ho *et al.* (28) was used. Briefly, two oligonucleotides were synthesized which contained the 6-bp mutation (underlined) (primer 1: 5′-CAC CCC GCA GCG GCT GTT GTA CAG CAT GGG CTG GTA TGT GCT-3′; primer 2: 5′-AGC ACA TAC CAG CCC ATG CTG TAC AAC AGC CGC TGC GGG GTG-3′). Two additional primers were synthesized which flanked the 5′ end (primer 3: 5′-ATC CGG ATC CTG CGC CTG AAA ATC TAT ACA TAT AC-3′) and 3′ end (primer 4: 5′-ATC CGG ATC CGA ATT CCC ACT AGT GCG GGG G-3′) of the α1(I) collagen 3′-flanking sequence of the pCOL-(+3590−4597) insert. Two PCR reactions were performed using the conditions as described above, one using primers 2 with 3 and the second PCR reaction with primers 1 and 4. An aliquot of each PCR reaction was combined in a second round PCR reaction along with primers 3 and 4 using the same PCR conditions described above. The product of this second PCR reaction was then digested with *Bam*HI and subsequently cloned into the *Bam*HI site of pGLCOL3.

To mutate all three of the footprinted regions in the 3′-flanking region while in the context of plasmid pCOL-(+3590−4597), a similar mutagenesis approach, as described above, was utilized. The plasmid p3′FP1M was used as a template in PCR reactions using primers designed to mutate 3′FP2 (primer 1: 5′-TGA ACC CAA GCC CTC CTT TCT ATC AGT GCT GCC GCT GCC TTA AAT ACA GAT GCC-3′; primer 2: 5′-GGC ATC TGT ATT TAA GGC AGC GGC AGC ACT GAT AGA AAG GAG GGC TTG GGT TCA-3′; the mutated nucleotides, +3797–3809, are underlined) along with the 5′-flanking primer (primer 3 above) and 3′-flanking primer (primer 4 above). After the second PCR reaction the 3′FP1 and 3′FP2 mutated fragment was used as a template in PCR reactions to generate mutations in 3′FP3 as described above using 3′FP3 M primers (primer 1: 5′-TTG GAA TCC AAG TCC CTT TGG AAG AAA GTT CGG AGC ATG GTC ACT CCT GG-3′; primer 2: 5′-CCA GGA GTG ACC ATG CTC CGA ACT TTC TTC CAA AGG GAC TTG GAT TCC AA-3′; the mutated nucleotides, +4050−4059, are underlined) along with the 5′-flanking primer (primer 3 above) and 3′-flanking primer (primer 4 above). After the second PCR reaction the PCR product was digested with *Bam*HI, gel-purified, and cloned into pGLCOL3, digested with *Bam*HI, creating p3′FP1-2-3M.

DNA sequencing was performed by the dideoxy method using the Sequenase version 2.0 kit (Boehinger Mannheim) according to the manufacturer's recommended protocol to confirm the presence of the mutations in the respective footprinted regions.

Transfections and Reporter Gene Assays—NIH 3T3 fibroblast cells and HeLa cells were cultured in 150-mm plates with Dulbecco's minimum essential medium (Life Technologies, Inc.) supplemented with 10% calf serum and grown in a 5% CO_2, 95% air atmosphere at 37 °C. Transfections using the β-globin reporter gene were performed using the calcium phosphate precipitation method as described (13, 19). In addition to 20 μg of the pβe$^-$ based reporter gene constructs, each transfection contained 20 μg of the pα reporter gene (27) which served as an internal control for transfection efficiencies. Cells were treated with 75 μM chloroquine during the transfection, and after 4 h the transfection mixture was removed, and the cells were shocked for 1 min using 10% glycerol. The cells were harvested 36−48 h after transfection, and total RNA was prepared by the acid-phenol method (29). Radiolabeled antisense β-globin and α-globin RNA probes were generated by SP6 and T7 RNA polymerase, respectively. T7 transcription of pα creates a 244-nucleotide transcript of which 131 nucleotides are protected (30). SP6 transcription of pβe$^-$ creates a 500-nucleotide transcript of which 350 nucleotides are protected (27). Total RNA samples were analyzed by RNase protection assay as described (31), and α- and β-globin transcripts were quantitated by scanning with an image analyzer or direct counting of the bands. Transient transfections using the luciferase reporter gene plasmids were performed using LipofectAMINE (Life Technologies, Inc.). NIH 3T3 cells were seeded into 6-well dishes at a density of 9×10^4 cells per well. The day after seeding 0.5 μg of luciferase reporter plasmid, 0.5 μg of pRSV-βgal and 1.1 μg of carrier DNA, pUC19, was added to the cells using 11 μg of LipofectAMINE reagent per well following the recommended protocol of the manufacturer. Liposomes were incubated with the cells for 8 h. The RSV-βgal reporter gene plasmid was co-transfected to normalize for transfection efficiencies. Luciferase and β-galactosidase reporter gene assays were performed as described previously 36−48 h after transfection (13, 19).

DNase I Footprinting and Mobility Shift Assays—DNase I footprinting analysis and mobility shift assays were performed as described previously (13). Nuclear proteins were obtained using the method described by Schreiber *et al.* (32). Supershift assays were performed, as described previously (13), using USF-1 and USF-2 polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The oligonucleotides used in the mobility shift assays representing 3′FP1 were top strand:

5′-GAT CCG CTG TCA CGT GGC ATG GGC TGA-3′; bottom strand: 5′-GAT CTC AGC CCA TGC CAC GTG ACA GCG-3′. The oligonucleotides that contained a mutation within the consensus E-box, 3′FP1M were top strand: 5′-GAT CCG CTG TTG TAC AGC ATG GGC TGA-3′; bottom strand: 5′-GAT CTC AGC CCA TGC TGT ACA ACA GCG-3′ (the mutated nucleotides are underlined).

Oligonucleotides—Oligonucleotides were synthesized using a Cyclone Plus Oligonucleotide Synthesizer (Milligen, Novato, CA) and were gel-purified after electrophoresis in 10% polyacrylamide gels.

DNA Sequence—The DNA sequence described in this paper has been deposited into the GenBank data base.

RESULTS

The 3′-Flanking Region of the α1(I) Collagen Gene Contains a Region That Stimulates Transcription in an Enhancer Trap Plasmid—To investigate the presence of transcriptional regulatory elements located in the 3′-flanking region of the α1(I) collagen gene, we cloned several fragments representing 12.4 kb of this region into the enhancer trap plasmid, pβe$^-$ (27). This plasmid contains the human β-globin promoter and structural gene with a unique *Sph*I cloning site located 2.2 kb downstream (or 3.3 kb upstream) of the β-globin transcriptional start site. The recombinant plasmids were transiently transfected into NIH 3T3 cells along with a plasmid containing the human α-globin gene, to serve as an internal control for transfection efficiency. The relative levels of β- and α-globin mRNA were assessed by RNase protection assays using total RNA from transfected cells. Insertion of the entire 4.6 kb genomic fragment of pCE5 into the enhancer trap plasmid stimulated the expression of the β-globin gene 1.7-fold compared with expression of pβe$^-$ alone, whereas α1(I) collagen gene fragments derived from the pCE4 plasmid did not exhibit any stimulatory effects on β-globin expression (Fig. 1*A* and summarized in Fig. 1*B*). Therefore, we concentrated our efforts on α1(I) collagen 3′-flanking sequences in pCE5 to further localize the position of the enhancing activity using deletional analysis. Fig. 1*B* summarizes the results of two to six transfection experiments carried out with independently purified plasmid preparations. The smallest construct containing enhancing activity was pβCOL-(+2643–4597) which reproducibly stimulated β-globin expression 2.8-fold as compared to pβe$^-$. This level of stimulation by the α1(I) collagen 3′ fragment is comparable with the stimulation of pβe$^-$ by the strong SV40 early gene enhancer in NIH 3T3 cells (27). To eliminate the possibility that the expression of the α-globin gene interferes with or affects the β-globin expression, the β-globin constructs were also transfected alone in a separate experiment with identical results (data not shown).

To determine whether this enhancing activity is cell type-specific, transient transfections were performed using the same reporter gene constructs in HeLa cells, which express no or low levels of type I collagen (33). None of the α1(I) collagen 3′-flanking genomic sequences stimulated β-globin gene expression in HeLa cells (Fig. 1*C*). In fact, the α1(I) collagen derived fragments in plasmids pβCOL-(+1899–4597) and pβCOL-(+2643–4597) had a slight inhibitory effect on β-globin gene expression relative to the control pβe$^-$ plasmid in these cells.

The α1(I) Collagen Gene 3′-Flanking Region Stimulates Expression of the α1(I) Collagen Gene Promoter—Since a segment of the α1(I) collagen gene 3′-flanking region stimulated expression from the heterologous β-globin promoter, we wanted to determine if this region could also stimulate expression from the homologous α1(I) collagen gene promoter. Therefore, we cloned the α1(I) collagen genomic insert from the plasmid pβCOL-(+1899–4597) in both orientations, behind the luciferase reporter gene in pGLCOL3, in which the luciferase gene is driven by the α1(I) collagen minimal promoter (−220 to +116), creating the plasmids pCOL-(+1899–4597) and pCOL-(+4597 to 1899) (Fig. 2*A*). When these plasmids were transiently transfected into NIH 3T3 cells, and transfection efficiencies were normalized by co-transfection with pRSV-βgal, a significant increase in transcriptional activity of the α1(I) collagen promoter was observed when the 3′-flanking sequence was in the 5′ to 3′ orientation. However, when positioned in the opposite orientation (3′ to 5′) an inhibitory effect was observed (Fig. 2*B*) indicating the presence of an inhibitory element. To further localize the stimulatory element within the α1(I) collagen 3′-flanking region, the shorter collagen sequence in pβCOL-(+3590–4597) was inserted behind the luciferase reporter gene in the pGLCOL3 plasmid, in both orientations, creating the plasmids pCOL-(+3590–4597) and pCOL-(+4597 to 3590) (Fig. 2*A*). Transient transfections with these plasmid constructs into NIH 3T3 cells also demonstrated a stimulatory effect of this region on the α1(I) collagen gene promoter when

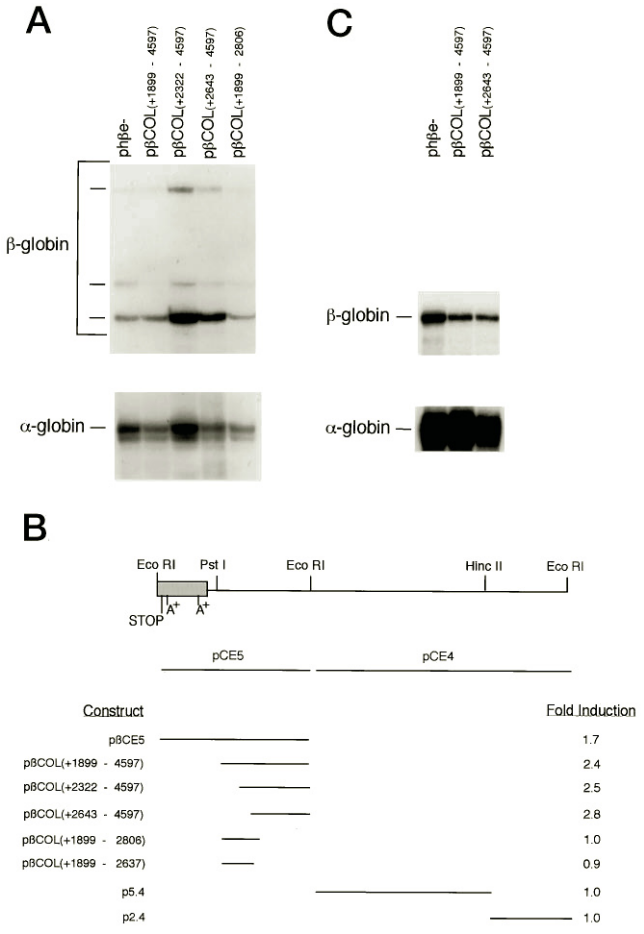


FIG. 1. **The 3′-flanking region of the α1(I) collagen gene contains a region that enhances transcription from a heterologous promoter.** *A,* NIH 3T3 cells were transiently transfected by the calcium-phosphate method and expression levels transfected the plasmids determined by RNase protection assays. The plasmid, pα, was used as an internal reference to normalize for transfection efficiency. Several independent plasmid preparations gave the same results of β-globin transcriptional activation. *B,* summary of expression of pβe$^-$-derived plasmids in NIH 3T3 cells. The *first line* shows the 3′ region of α1(I) collagen with stop codon, polyadenylation sites, the last exon (*stippled box*), and selected restriction sites. The 4.6- and 7.8-kb fragments contained in pCE5 and pCE4 plasmids, respectively, are shown in the *second line*. The fragments used in transfection assays and the levels of β-globin RNA expressed relative to the level produced by the enhancerless pβe$^-$ plasmid and corrected for transfection efficiency by normalizing for α-globin mRNA levels are shown. *C,* HeLa cells were transiently transfected by the calcium-phosphate method with the indicated plasmids, and RNase protection assays were performed as with NIH 3T3 cells.

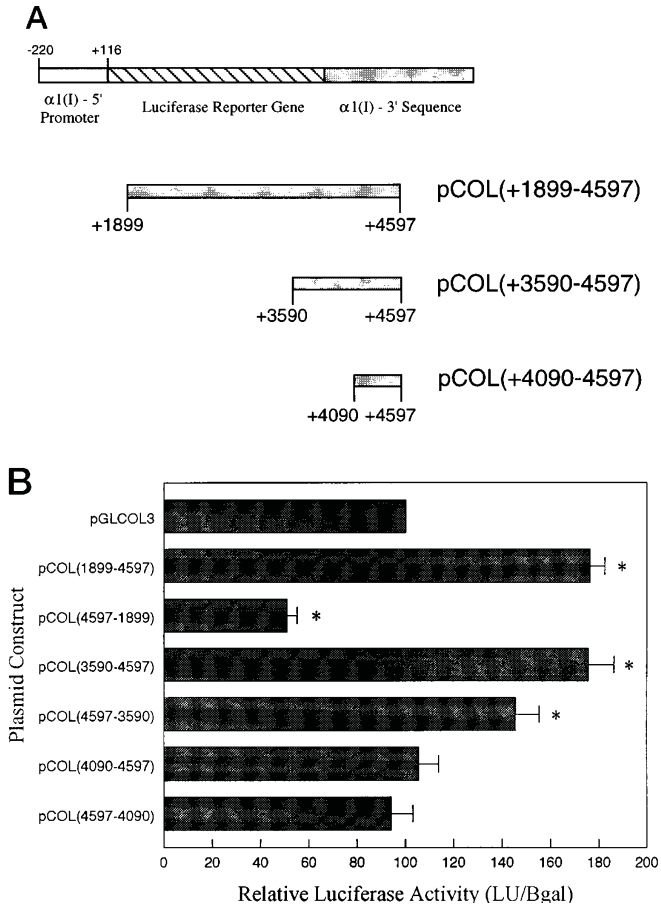


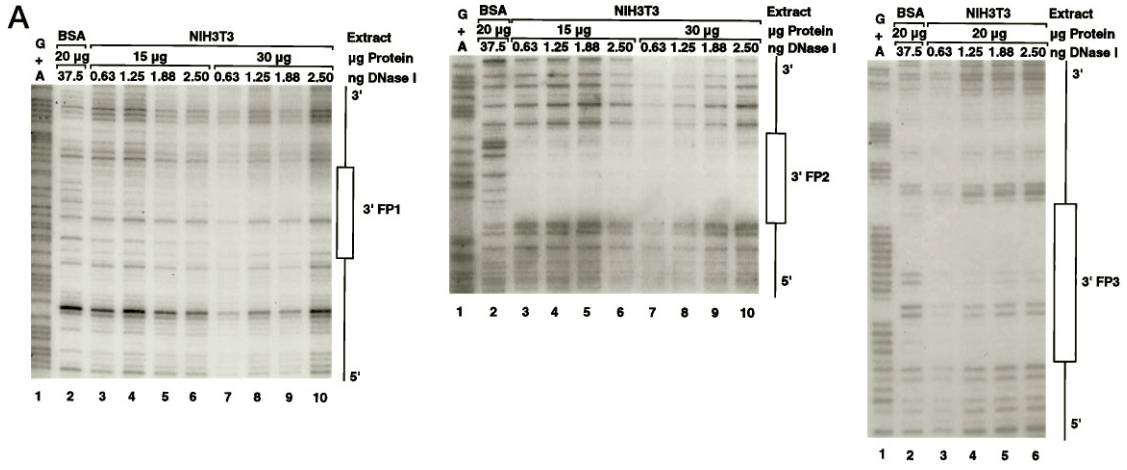
FIG. 2. **The 3′-flanking region of the α1(I) collagen gene stimulates transcription from the α1(I) collagen gene promoter.** *A,* general structure of reporter genes containing the α1(I) collagen promoter-luciferase reporter gene and α1(I) collagen 3′ sequence. The specific α1(I) collagen 3′ sequence used in each construct is designated below. *B,* plasmids were transfected into NIH 3T3 cells by the lipofection method and relative luciferase activity normalized to the activity of the co-transfected pRSV-βgal plasmid. Data represent the average of at least eight transfections. Those plasmids in which the activity was significantly different compared with pGLCOL3, using the Wilcoxan signed ranks statistical analysis, are designated by an *asterisk*; $p < 0.05$. *Error bars* represent S.E.

positioned in either orientation (Fig. 2*B*). However, further deleting 3′-flanking sequence to nucleotides +4090 to +4597 (plasmids pCOL-(+4090–4597) and pCOL-(+4597 to 4090)) eliminated the stimulatory effect. Together, these data indicate that a transcriptional stimulatory element located in the α1(I) collagen gene 3′-flanking region is positioned between nucleotides +3590 to +4089.

DNA Binding Proteins Specifically Interact at Three Locations within the α1(I) Collagen Gene 3′-Flanking Region—To determine the locations of DNA binding proteins in this stimulatory element in the 3′-flanking region of the α1(I) collagen gene, we performed DNase I footprinting analysis using NIH 3T3 nuclear extracts. Three sites of specific DNA-protein interactions were located and called 3′FP1, 3′FP2, and 3′FP3 (Fig. 3*A, left, middle,* and *right*, respectively; and summarized in Fig. 3*B*). To determine the effect that each of the footprinted regions exerts on expression of the α1(I) collagen promoter region, we cloned one copy, in both orientations, as well as three and five copies of the oligonucleotides representing each footprint into the *Bam*HI site of pGLCOL3. We investigated single and multiple copies of the binding sites in order to optimize their effects on transcriptional activation. The plasmid constructs were transiently transfected into NIH 3T3 cells and transfections normalized to the activity of the co-transfected pRSV-βgal plasmid. Although no stimulatory effect on transcriptional activity of the α1(I) collagen gene minimal promoter was observed when one copy of 3′FP1 was present in either orientation, the 3′FP1 sequence stimulated expression nearly 4-fold when three copies were present and approximately 6-fold when five copies were present (Fig. 4). On the other hand, 3′FP2 slightly stimulated expression of the α1(I) collagen gene promoter with only one copy present but not with five copies (Fig. 4). 3′FP3 slightly stimulated expression of the parental pGLCOL3 plasmid when either a single copy or with three copies of the footprinted regions were positioned 3′ of the luciferase reporter gene (Fig. 4).

USF-1 and USF-2 Interact at a Consensus E-box Binding Site Located in the α1(I) Collagen 3′-Flanking Region—Since 3′FP1 stimulated expression of the α1(I) collagen gene minimal promoter, we wished to identify the protein(s) that interacts with this *cis*-acting element. Mobility shift assays were performed with a radiolabeled 3′FP1 oligonucleotide using NIH 3T3 nuclear extracts. A DNA-protein complex was formed in the mobility shift assay (Fig. 5, *lane 2*) which was specifically competed with cold unlabeled 3′FP1 oligonucleotide (Fig. 5, *lane 3*). Examination of the nucleotide sequence of 3′FP1 demonstrated a consensus E-box (CANNTG), a known binding site for bHLH proteins (34), located at nucleotide +3695 to +3700 in the α1(I) collagen 3′-flanking sequence. To determine if the consensus E-box is involved in protein binding to 3′FP1, we used a mutated 3′FP1 oligonucleotide in which the six nucleotides representing the consensus E-box were mutated in the context of surrounding wild-type sequence. This mutated oligonucleotide failed to compete for protein binding (Fig. 5, *lane 4*) demonstrating that the E-box is required for protein binding to the 3′FP1 footprint sequence. The nucleotide sequence of this E-box matches the USF-1/USF-2 consensus binding site (CACGTG) (35). Therefore, we used antibodies directed against USF-1 or USF-2 in the mobility shift assay to test if these two bHLH proteins interact with 3′FP1. Addition of either the USF-1 or the USF-2 antibody in the binding reaction supershifted the DNA-protein complex (Fig. 6, *lanes 4* and *5*), with the USF-1 antibody supershifting the complex to a greater degree than the USF-2 antibody. When both antibodies were included in the binding reaction (Fig. 6, *lane 6*), the entire complex was eliminated indicating that both USF-1 and USF-2 interact with 3′FP1 as either homodimers or heterodimers. Additionally, binding to 3′FP1 requires Mg^{2+} for binding with maximal binding activity requiring at least 5 mM $MgCl_2$ (data not shown). This is in agreement with a previous report demonstrating that decreased Mg^{2+} concentration reduces binding affinity of USF-1 to DNA (36). Binding activity of USF-1/USF-2 to 3′FP1 was not salt-dependent as binding occurs over a wide range of salt concentrations (26 to 200 mM NaCl) in the binding reaction (data not shown).

USF Participates in the Activation of the α1(I) Collagen Gene Promoter—To determine the functional role of USF interaction with this 3′ E-box in the stimulatory effect of the 3′-flanking region on the α1(I) collagen gene promoter, we generated a mutated reporter gene construct in which only the CACGTG (nucleotides +3695 to +3700) E-box site was mutated in pCOL-(+3590–4597) (see Fig. 2*A*), creating p3′FP1M. When p3′FP1M was transiently transfected into NIH 3T3 cells and transfection efficiencies normalized to the co-transfected pRSV-βgal plasmid, a significant decrease in reporter gene activity was observed compared with the wild-type parental pCOL-(+3590–4597) plasmid (Fig. 7). This indicates that at least part of the stimulatory properties of this region on the α1(I) collagen gene promoter are due to USF-1/USF-2 binding



B

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+3590   TGCGCCTGAA AATCTATACA TATACATACA TGTACACACG TGGATATACA CAAGGGCATG
                                                          3'FP1
+3650   TGCACCTTGT GATAGGCCTT GTTTGCAGCA CCCCGCAGCG GCTGTCACGT GGCATGGGCT
+3710   GGTATGTGCT CTAAATGTGT GTATAGAACA GGGACGGAGA CAACAAAGAG ATCATTGTAT
                                  3'FP2
+3770   CACACTCTGA ACCCAAGCCC TCCTTTCCGC TGACATCATT GCTGCCTTAA ATACAGATGC
+3830   CAGGCCCTGT TTCCAAGACC CTCACTGTCC CCTGTGTGCT AACACAGCAC CTACTCCCAG
+3890   CTCTGCTCTA TGACAGTCAC CTTCGTTTTT TGTGGGGAAG AGTAGTCTCT TGGAGCTGAT
+3950   ACTTCAGTGA AATCCAAGTG GAAAATCTAC AGGGAAAGAA GACTTCATTT TGACTTTTTT
                                                  3'FP3
+4010   TCTCTCTCTT GCTGTCCTTT TGGAATCCAA GTCCCTTTGG GGAGGGACCT GGAGCATGGT
+4070   CATCTCCTGG ATGGATTTGC +4089
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FIG. 3. **The 3′-flanking region of the α1(I) collagen gene contains the binding sites for three DNA binding proteins.** *A,* DNase I footprinting assays were performed using nuclear extracts obtained from NIH 3T3 cells and incubated with radiolabeled probes from the 3′-flanking region of the α1(I) collagen 3′-flanking region. DNase I footprinting assays were performed as described under "Material and Methods." G + A reactions were performed to locate the specific regions where proteins bind. Bovine serum albumin (*BSA*), 20 μg, was used to compare the banding pattern with nonspecific proteins. The footprint reactions for 3′FP1, 3′FP2, and 3′FP3 are positioned *right, middle,* and *left*, respectively. *B,* the locations of each of the three footprinted regions are *underlined* in the nucleotide sequence of the α1(I) 3′-flanking region. 3′FP1 is between nucleotides +3690 and 3710; 3′FP2 is between nucleotides +3790 and 3815; 3′FP3 is between nucleotides +4045 and 4064.

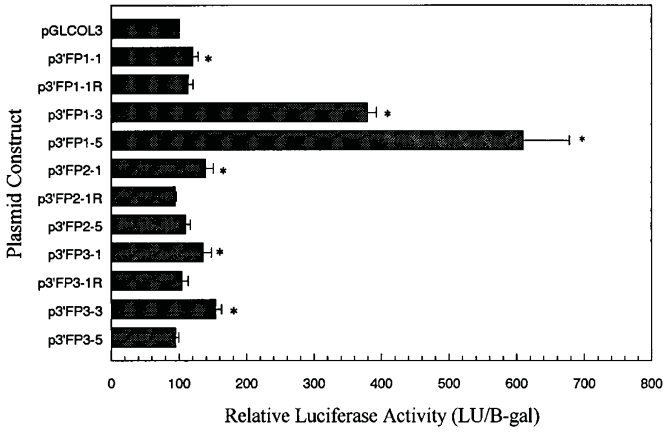

FIG. 4. **3′FP1 stimulates expression of the α1(I) collagen gene promoter in NIH 3T3 cells.** Each of the 3′-footprinted regions were cloned behind the luciferase gene in the pGLCOL3 plasmid with either one copy, in both orientations, three, or five copies. Each construct was transiently transfected into NIH 3T3 cells and luciferase activity measured and normalized to the activity of the co-transfected pRSV-βgal plasmid. Data represent the average of four transfections. Those plasmids in which the activity was significantly different compared to pGLCOL3, using the Wilcoxan signed ranks statistical analysis, are designated by an *asterisk*; $p < 0.05$. *Error bars* represent S.E.

to the E-box. To assess if the other footprinted regions, 3′FP2 and 3′FP3, participate in the stimulatory properties in the pCOL-(+3590–4597) plasmid, we created plasmid p3′FP1–2-3M which contained mutations in all three footprinted regions (nucleotides +3695–3700; nucleotides +3797–3809; nucleotides +4050–4059). When this plasmid was transiently transfected into NIH 3T3 cells, a complete inhibition of transcriptional activation was observed and, in fact, resulted in a reduction of expression compared with the parental pGLCOL3 plasmid (Fig. 7). This result supports data presented in Fig. 2*B* demonstrating an inhibitory element is present as shown in plasmid pCOL-(+4597 to 1899). Taken together, these data indicate that USF-1/USF-2 is involved in the stimulatory properties of the 3′-flanking region on the α1(I) collagen gene promoter; however, 3′FP2 and 3′FP3 also cooperate in stimulating transcription of the α1(I) collagen gene promoter.

DISCUSSION

The genes encoding the α1 and α2 polypeptide chains of type I collagen are regulated in a developmental, inducible, and tissue-specific manner. In previous studies of the human, murine, and rat α1(I) collagen genes using either transient transfections into cultured cells or transgenic animals, *cis*-regulatory elements have been identified in the 5′-flanking regions, the promoter region, and first introns of these genes (9–19). The precise functions of many of these *cis*-acting elements remain to be elucidated, and some conflicting results have been reported, perhaps reflecting the use of different reporter gene constructs and different cell types. Most studies support the notion that the 5′ region of the α1(I) collagen gene contains a strong proximal promoter which exhibits at least partial tissue specificity and which is modulated by additional regulatory elements.

Several lines of evidence suggested that the 3′-flanking region of the α1(I) collagen gene contains transcriptional regulatory elements. Therefore, we initiated a systematic analysis of the 3′-flanking region to locate important regulatory elements. Our analysis of the 3′-flanking region of the α1(I) collagen gene has located a fragment (between nucleotides +1899 to +4597

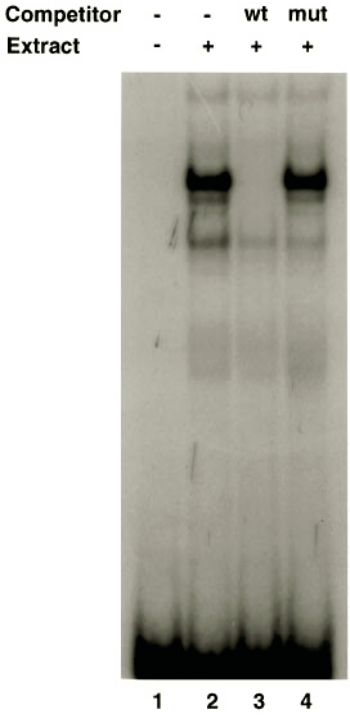

FIG. 5. **Protein binding to 3′FP1 requires an intact consensus E-box.** The mobility shift assay was performed using a radiolabeled probe representing 3′FP1 and 10 μg of NIH 3T3 nuclear extract. A specific DNA-protein complex is formed with the wild-type (*wt*) 3′FP1 oligonucleotide used in the mobility shift assay (*lane 2*), which is efficiently competed with wild-type unlabeled oligonucleotide (*lane 3*). A mutant (*mut*) 3′FP1 oligonucleotide, in which the consensus E-box is mutated, fails to compete for protein binding (*lane 4*). *Lane 1* is the probe incubated without nuclear extract. A 100-fold molar excess of unlabeled oligonucleotides was used in the competition reactions.

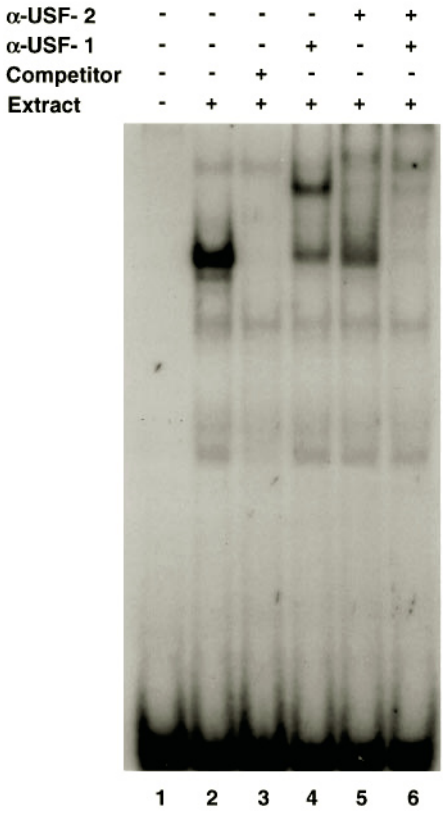

FIG. 6. **Both USF-1 and USF-2 bind to 3′FP1.** A mobility shift assay was performed using 3′FP1 as a radiolabeled probe and incubated with 10 μg of NIH 3T3 nuclear extract (*lanes 2–6*). *Lane 1* is the probe incubated without nuclear extract. A 100-fold molar excess of unlabeled 3′FP1 oligonucleotide was used as a competitor in the binding reaction (*lane 3*). Supershift studies were performed using either a polyclonal USF-1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in the binding reaction (*lanes 4* and *6*) or a polyclonal USF-2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in the binding reaction (*lanes 5* and *6*).

downstream of the translational termination site of the α1(I) collagen gene) that enhances transcription of the heterologous β-globin gene promoter in NIH 3T3 cells, a cell line that expresses moderate levels of type I collagen. Although the level of enhancement is not dramatic, it is comparable to the enhancement of the same reporter gene construct by the SV40 enhancer in the same cells (27). This fragment also stimulates transcription driven by the α1(I) collagen promoter in an orientation-dependent manner. Deletional analysis of the 3′-stimulatory region narrowed the enhancing region to within a 500-bp segment. DNase I footprinting assays demonstrated the presence of three regions that specifically interact with DNA binding proteins. Only one of the footprinted regions was capable of stimulating expression of the α1(I) collagen promoter when multiple copies were inserted 3′ of the luciferase reporter gene. Further analysis of this protein binding site demonstrated that both USF-1 and USF-2 interact with the nucleotide sequence of a consensus E-box, the binding site for basic helix-loop-helix (bHLH) proteins. A functional role of this site in stimulating expression of the α1(I) collagen gene promoter was assessed by mutating only the nucleotides comprising the consensus E-box while in the context of the wild-type pCOL-(+3590−4597) collagen containing sequence. The mutated collagen-luciferase reporter gene construct containing the mutated E-box had decreased transcriptional activity compared with the wild-type E-box. However, the transcriptional stimulatory properties of the 3′-flanking region was completely lost only when all three protein binding sites were mutated. This was surprising since 3′FP2 and 3′FP3 alone did not stimulate transcription (Fig. 4). These data suggest a complex interaction between the *trans*-acting factors interacting with the 3′-flanking region. In addition, when all three *cis*-acting elements were mutated, promoter activity was reduced compared with to the level of the α1(I) promoter-driven plasmid pGLCOL3. This indicates that a negative regulatory element is present between nucleotides +3590 and 4597. This finding is supported in the construct pCOL-(+4597 to 1899) where an inhibitory effect was observed on transcriptional activity (see Fig. 2).

The USF-1 binding site in the endogenous α1(I) collagen gene is located over 20 kb downstream from the promoter. Regulatory elements have also been demonstrated in the 3′-flanking region of several other genes, including the mouse cytosolic glutathione peroxidase gene (37), the human keratin 19 gene (38), the murine c-*fos* protooncogene (39), the human angiotensinogen gene (40), and the human tyrosine hydroxylase gene (41). One could speculate that looping of the chromatin would place the two regions in close enough proximity to one another for appropriate interactions to occur.

Both USF-1 and USF-2 are ubiquitously expressed, although their relative abundance varies in different cell types (42). In fact, USF was originally identified and characterized in HeLa cells (43–45). Thus, it was surprising that the 3′ region of the α1(I) collagen gene enhanced transcription in NIH 3T3 cells but not in HeLa cells. Perhaps differences in the USF proteins, transcriptional co-factors, or other DNA binding proteins allow for this difference in transcriptional activity between the two cell types. The expression of both USF-1 and USF-2 genes results in multiply spliced messages producing variations in the amino terminus of the protein (42). This, therefore, may give rise to proteins with different transcriptional stimulating

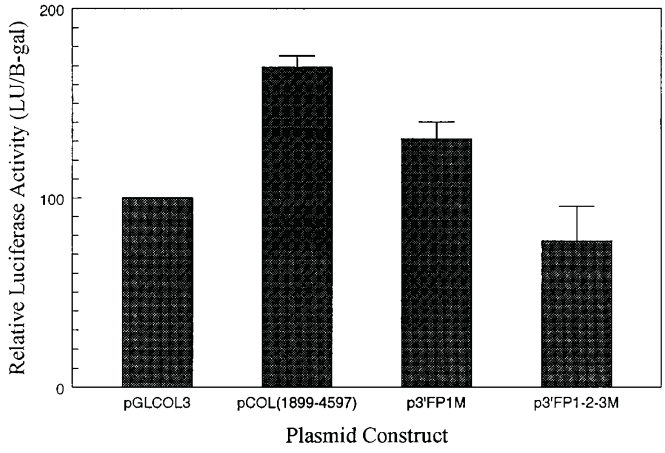

FIG. 7. **Mutating the consensus E-box in the context of surrounding 3′-flanking sequence reduces α1(I) collagen promoter activity.** Plasmids pGLCOL3, pCOL-(+3590−4597), p3′FP1M, and p3′FP1-2-3M were transiently transfected into NIH 3T3 cells. Luciferase activity was determined, and the data were normalized to the activity of the co-transfected pRSV-βgal plasmid. Data represent the average of four transfections. *Error bars* represent S.E. The activity of p3′FP1M was statistically lower than pCOL-(+3590−4597), $p = 0.027$, and the activity of p3′FP1-2-3M was statistically lower than pCOL-(+3590−4597), $p = 0.008$, as determined using the Student's paired *t* test.

activities since the amino-terminal portion of the proteins contain the *trans*-activating domain. Since the USF family members homodimerize and heterodimerize with each other, but do not heterodimerize with other bHLH proteins (46, 47), various ratios of multiple USF-1 and USF-2 proteins will generate complex sets of USF members with potentially different transcriptional activating capacities. Additionally, transcriptional co-factors may be absent in the HeLa cells that are necessary for USF to stimulate the α1(I) collagen gene promoter. USF does not appear to *trans*-activate effectively in highly purified *in vitro* systems (48) suggesting a role for additional interacting proteins. It is believed that USF-1 affects transcription by interacting with the multisubunit TFIID complex (43, 49) and specifically with the TFIID subunit $TAF_{II}55$ (50). Additional factor(s) also appear to be required for USF to stimulate transcription, one of which appears to be PC5, a novel co-factor which has been demonstrated to mediate transcriptional activation by USF-1 (51).

USF contributes to the regulation of multiple genes, some of which are expressed in a tissue-specific or inducible manner. The human β-globin locus control region (52), the murine type IIβ regulatory subunit gene of cyclic adenosine 3′,5′-monophosphate-dependent protein kinase gene (53), the murine p53 gene (54), the human cell cycle-dependent cyclin B1 gene (55), the chicken αA-crystallin gene (56), the rat cardiac ventricular myosin light chain 2 gene (57), and the rat preprotachykinin-A gene (58) are regulated in part by USF. USF has also been shown to be involved in the TGF-β1 responsive element in the human plasminogen activator inhibitor gene (59) and in the glucose responsive element in the pyruvate kinase gene (60). Our study demonstrates that the ubiquitous USFs contribute to the moderate high transcriptional rate of the α1(I) collagen gene in NIH 3T3 cells.

The present study, in conjunction with previous studies, demonstrates that the α1(I) collagen gene is regulated by a complex array of *cis*-acting elements in the 5′-flanking region, the promoter, the first intron, and 3′-flanking region. Like USF-1 and USF-2, which interact with the 3′-regulatory element identified in this study, most of the other factors involved in transcriptional regulation of the α1(I) collagen gene are ubiquitous factors. For example, the proximal promoter has been shown to interact with SP1, NF-I, CBF, and cKrox (13, 61, 62), none of which is present exclusively in collagen-producing cells. Several elements, however, have been shown to contribute to cell-specific expression of the α1(I) collagen promoter. These include a TGF-β response element located approximately 1.6 kb upstream of the transcriptional start site (63), a sequence required for expression in bone (64) and elements that modulate expression in dermal fibroblasts, osteoblasts, and odontoblasts, and in tendon and fascia fibroblasts (65). However, even without these elements the α1(I) collagen promoter exhibits a remarkable degree of tissue specificity (10, 65, 66). Taken together, these observations suggest that the various regulatory elements located in the 5′-flanking region, the first intron, the 3′-flanking region, and possibly additional sites so far unidentified act in concert to provide appropriate tissue specificity and high levels of activity of the α1(I) collagen gene promoter and that the correct spatial arrangement of the various elements is required for appropriate activity.

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