Analysis of the collagen $\alpha 1(I)$ promoter

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ABSTRACT

The collagen $\alpha 1(I)$ gene is regulated at a developmental and tissue specific level. We have previously demonstrated that only 220bp of the promoter region of the collagen $\alpha 1(I)$ gene are required for efficient expression in NIH 3T3 cells. DNase I protection assays demonstrated 4 footprinted segments in the promoter region. Deletional analysis revealed that the 3 most proximal footprints were required for maximal expression. The most proximal footprint contains a CCAAT sequence and a 12bp segment that forms a direct repeat with the preceding footprint. Ligation of the proximal footprint sequence to a heterologous promoter enhanced transcription of the reporter gene. These studies, therefore, identify and characterize elements in the promoter region of the collagen $\alpha 1(I)$ gene that interact with DNA binding proteins and are required for efficient expression.

INTRODUCTION

Type I collagen, the most abundant protein in the body, is the product of two independent genes, collagen $\alpha 1(I)$ and $\alpha 2(I)$. Two chains of collagen $\alpha 1(I)$ polypeptide and one chain of collagen $\alpha 2(I)$ polypeptide form a heterotriple helix (1). The expression of the type I collagen genes is controlled at both a developmental and a tissue-specific level. The over-expression of type I collagen genes is clinically significant in fibrogenic diseases such as hepatic cirrhosis (2,3). Understanding the regulation of type I collagen expression would provide insight into both normal development and pathological states.

Cis-acting regulatory elements are required for proper expression of eukaryotic genes, as demonstrated by *in vitro* mutagenesis and DNA-mediated gene transfer experiments. These DNA segments are located either within the promoter region close to the site of initiation of transcription, or at more distant sites, as with enhancer elements. The *cis*-acting elements interact with DNA binding proteins, as demonstrated by gel retardation and footprinting assays. This interaction regulates the induction, tissue-specificity and developmental expression of a gene (recently reviewed in 4 and 5).

The expression of the murine collagen $\alpha 2(I)$ gene is regulated through multiple DNA binding sites. Positive *cis*-acting elements have been demonstrated in the promoter regions including binding sites for CAAT binding proteins (6,7,32,34) and the inducible *trans*-acting factor NF-1 (8). In addition to the *cis*-acting elements in the promoter region, the first intron of the collagen $\alpha 2(I)$ gene also contains a cell-specific transcriptional enhancer (9).

We have previously analyzed a large segment of the murine collagen $\alpha 1(I)$ gene by gene transfer experiments (from -3700 to +1400 bp) (10). These experiments revealed that the promoter region (220 bp upstream from start of transcription) was sufficient for efficient

expression of a reporter gene in NIH 3T3 murine fibroblasts, cells which produce an abundance of type I collagen. Further experiments failed to reveal a significant enhancing activity within the first intron (10). Independent studies of transcriptional regulation of the human collagen $\alpha 1(I)$ gene produced conflicting results as to the enhancing activity of the first intron (11–14).

The goal of the present study was to identify the interactions between DNA binding proteins and specific segments of the collagen $\alpha 1(I)$ promoter region. This analysis has identified sites of the minimal DNA segment required for efficient transcription and four sites of protein DNA interaction.

METHODS

Plasmid Constructions

The plasmid pColCAT 2 containing the collagen $\alpha 1(I)$ gene segment –2500 to +115 and the plasmid pColCAT 3 containing the segment –220 to +115, were constructed as previously described (10) from the murine collagen $\alpha 1(I)$ genomic clone (15). The plasmid pColCAT 2 was digested at the unique Bgl II site at –220 in the collagen gene and subjected to Bal 31 digestion. The resulting deleted DNAs were digested with the restriction endonuclease XbaI and recloned into a promoterless plasmid containing the chloramphenicol acetyl transferase (CAT) gene and SV40 polyadenylation signal (pUCCAT). The vector DNA sequences were identical in all the constructed plasmids. The extent of the Bal 31 deletions were determined by dideoxy sequencing.

The double-strand oligonucleotide Col-1 was synthesized to contain the collagen $\alpha 1(I)$ nucleotides –103 to –82, corresponding to the proximal DNase I protected segment (footprint 1):

CTAGCTGATTGGCTGGGGGCCGGGCT
GACTAACCGACCCCCGCCCCGACTAG

Col-1 was cloned into the Bam HI and Xba I restriction endonuclease sites of the plasmid pBLCAT2 (33) which contains a TK promoter ligated to a CAT reporter gene, generating p(–103/–82)ColTKCAT.

Two plasmids were constructed in order to generate end-labelled probes for mobility shift and DNase I protection assays. Plasmid p(–103/–82)Col was constructed by ligating the Col-1 double-strand oligonucleotide into a Bam HI and Hind III restriction endonuclease digested pGem-blue 3 plasmid (Promega Corporation). Plasmid p(–220/+23)Col was constructed by digesting pColCAT 2 (10) with Bgl II and Ava I, incubating the fragment with Klenow to form blunt ends, and ligating this fragment into the SmaI site of pGem-blue 3. Radiolabelled probes were produced by digesting the respective plasmids with a restriction endonuclease to linearize the plasmids, filling in with reverse transcriptase in the presence of [32 P] α -dCTP and [32 P] α -dATP, digesting with a second restriction endonuclease to separate the inserts, and purification of the radiolabelled probes by gel electrophoresis. The end-labelled probes had specific activities of $\sim 10,000$ cpm/ng DNA.

Reporter gene assays

NIH 3T3 mouse fibroblast cells were plated at 5×10^5 cells per 100mm dish in DMEM containing 10% fetal calf serum in 90% air/ 10% CO₂ at 37°C. Hep G2 human hepatocarcinoma cells were plated at 1×10^6 cells per 100mm dish in RPMI 1640 containing 10% fetal calf serum in 95% air/5% CO₂ at 37°C. The next day, 8 μ g of a pColCAT plasmid and 2 μ g of the pSV2 β -gal plasmid pCH110 (16) were cotransfected into the cultured cells by the calcium phosphate precipitation method followed by a glycerol shock (17). After 24 hours the cells were harvested and CAT activity and β -galactosidase

activity were assayed using 50–100 μ g of protein extract per assay. CAT activity was measured by a modification (10) of the method of Gorman et al (17). β -galactosidase activity was measured using 2-nitrophenyl- β -D-galactopyranosid (ONPG) as a substrate (16) in order to correct for differences in transfection.

DNase I protection assays

Whole cell extracts were prepared from confluent NIH 3T3 cells grown in 15 850cm² roller bottles (18). The harvested washed cells had a packed cell volume (PCV) of 5ml. The cells were resuspended in 4 PCVs of solution 1 (10mM Tris pH 7.9, 1mM EDTA, 5mM DTT, and 2mM MgCl₂). The cells were allowed to swell for 20 minutes on ice and were homogenized with 10 strokes of a Dounce homogenizer. The suspension was gently stirred on an ice-water bath in 4 PCVs of solution 2 (50mM Tris pH 7.9, 10mM MgCl₂, 2mM DTT, 25% sucrose, and 50% glycerol). This solution was incubated with 1 PCV of saturated ammonium sulfate for 20 minutes, centrifuged at 240,000 g for 80 min., and the resulting supernatant collected. While the solution was stirring on ice, 0.33g of pulverized ammonium sulfate per ml of supernatant was added. Then 33 μ l of 1M NaOH per ml of supernatant plus 1 μ l of DTT per ml of supernatant were added and incubation on ice for 30 minutes. The resulting precipitant was collected by centrifugation at 58,000 g for 10 min and the pellet was resuspended in 1/10 of the original supernatant volume of buffer 3 (20mM Hepes pH 7.9, 100mM KCl, 12.5mM MgCl₂, 0.1mM EDTA, 1mM DTT, and 20% glycerol). The solution was dialyzed for 8 hours against buffer 3. The dialysate was clarified by centrifugation at 48,000 g for 10 min and then flash-frozen in liquid nitrogen. This whole cell extract was fractionated by applying 30mg of protein extract to a 2ml heparin agarose column. The column was washed with 2 column volumes of 0.1M KCl in TM (50mM Tris pH 7.9, 1mM EDTA, 1mM DTT, 12.5mM MgCl₂, and 20% glycerol), then eluted with 2 column volumes of 0.4M KCl in TM and 2 column volumes of 1M KCl in TM.

Each DNase I footprinting reaction (19) contained 1 μ g poly d(I-C), 2% polyvinyl alcohol, and 20,000cpms of radiolabelled probe DNA (2ng) in a 25 μ l final volume. Protein extract in 25 μ l of 0.1M KCl TM buffer was added, and the reaction was incubated for 15 minutes on ice and then for 2 minutes at room temperature. After 50 μ l of 5mM CaCl₂, 1.5mM EDTA was added, the DNA was digested with DNase I for 1.5 minutes at room temperature. The reaction was stopped with 100 μ l of 200mM NaCl, 20mM EDTA, 1% SDS, 50 μ g/ml tRNA, followed by extraction with phenol/chloroform/isoamylalcohol (25:25:1). The digested DNA was precipitated in ethanol, washed in 70% ethanol, and resuspended in formamide buffer, and electrophoresed in an 8% acrylamide, 7M urea gel at 1700 volts. The gel was dried and exposed to X-ray film at –70°C overnight.

Mobility shift assays

Radiolabelled probes were synthesized by kinasing the Col-1 oligonucleotide with [³²P]-ATP. Each 20 μ l reaction (20) contained 8 μ l of 2.5 $\times$ reaction buffer (25mM Hepes, pH 7.5, 10% glycerol, 150mM KCl, 1mM EDTA, 1mM DTT), 20,000cpm of DNA probe, 4 μ g of poly d(I-C), and 20 μ g of whole cell extract fraction. The reaction mixture was incubated 30 minutes on ice, and then electrophoresed in a 4% nondenaturing acrylamide gel in 0.25 $\times$ TBE at 10 volts/cm at 4°C. The gel was dried and exposed to X-ray film at –70°C overnight.

Primer extension assays

NIH 3T3 were transfected with pColCAT plasmids as described above. Total RNA was prepared and used as a template for the extension of a radiolabelled CAT oligonucleotide primer, according to the method of Mudryj and de Crombrughe (26).

RESULTS

Previous experiments using DNA mediated gene transfer and deletional analysis revealed that the plasmid p(-220/+115)ColCAT contained sufficient genetic information for efficient expression in NIH 3T3 cells (10). Therefore, DNase I protection experiments were performed using radiolabelled collagen $\alpha 1(I)$ DNA with NIH 3T3 extracts in order to identify the specific sites of interaction with DNA binding proteins. The extracts were partially purified with ammonium sulfate precipitations and heparin agarose chromatography. The 0.4M KCl elution fraction from the heparin agarose column contained DNA binding proteins which protected four distinct segments of a p(-220/+23)Col end-labelled probe (Fig 1A and 1B).

The DNA sequence of the protected segments were determined by simultaneously running Maxam and Gilbert sequencing markers. The two most proximal footprints (footprints 1 and 2) protected identical 12 base pair direct repeats; TGGGGGCCGGGC. Footprints 1 and 2 also contain an inverted CCAAT sequence. The double-strand oligonucleotide Col-1, which consists of the sequence of footprint 1 (-103 to -82) including the 12 bp direct repeat, competed with footprints 1 and 2 for DNA binding proteins (Fig 1A). This implies that the two most proximal footprints probably interact with the same DNA binding protein(s). Although to a lesser extent, the Col-1 oligonucleotide also competed with footprints 3 and 4 (Fig 1A), which might reflect related proteins binding to all four GC-rich footprints.

In order to independently assess protein binding to the (-103/-82)Col-1 oligonucleotide, mobility shift assays were performed. Incubating the radiolabelled Col-1 probe with NIH 3T3 whole cell protein fractions revealed that most of the DNA binding activity eluted in the 0.4 M KCl fraction (Fig 2). Excess cold Col-1 oligonucleotide completely eliminated gel retardation of the probe, demonstrating the specificity of the protein DNA binding. Since collagen $\alpha 1(I)$ gene expression in src-transformed NIH 3T3 cells is only 10% of that in NIH 3T3 cells (10), the DNA binding activity of src-NIH 3T3 cellular extracts to the Col-1 oligonucleotide was assessed. The 0.4M KCl fraction of the whole cell extraction from src-NIH 3T3 cells had markedly decreased binding activity compared to that of the identically prepared NIH 3T3 extracts. Therefore, the DNA-protein interactions of the collagen $\alpha 1(I)$ promoter as demonstrated by DNase I protection assays were confirmed and extended by these mobility shift assays.

The role of the four footprinted segments in the transcription of the collagen $\alpha 1(I)$ gene was determined by deletional analysis and DNA mediated gene transfer. A series of plasmids containing 5' deletions of this DNA segment were constructed by Bal 31 digestion. The resulting pColCAT plasmids were transfected into NIH 3T3 cells and the transfected cells

Fig 1. A. DNase I protection assay of the murine collagen $\alpha 1(I)$ promoter region. A DNA fragment containing the murine collagen $\alpha 1(I)$ promoter region (-220 to +23 bp) was labelled at the -220 end. Twenty thousand cpm of the probe was incubated with 25 μ g BSA ('0') (lanes 8 and 9), or 36 μ g of partially purified NIH 3T3 whole cell extract ('0.4'M KCl fraction) (lanes 1-7 and 10-13) in the absence of (lanes 7 and 10) or in the presence of competing cold DNAs. The competing DNAs were 10 or 50ng of the SV40 early region enhancer region (lanes 1-2), 1, 5, 10 or 50ng of the Col-1 oligonucleotide (-102 to -83 bp) (lanes 3-6), and 5, 10, or 50ng of an AP-2 binding site oligonucleotide (lanes 11-13). DNase I footprinting reactions were performed as described in Experimental Procedures and analyzed on an 8% acrylamide 7M urea gel. The regions of protection from DNase I digestion (footprints) are designated by the numbered boxes. B. Nucleic acid sequence of collagen $\alpha 1(I)$ promoter region. The lines over segments are DNase I protected footprints.

Fig 2. Mobility shift assay of the -103 to -82 bp segment of the collagen $\alpha 1(I)$ promoter region. A double strand oligonucleotide containing a section of the collagen $\alpha 1(I)$ promoter (-103 to -82 bp) (Col-I) was labelled with polynucleotide kinase. Twenty thousand cpm of the probe was incubated with no additions (lane 1), 12 μ g of the NIH 3T3 whole cell extract heparin-agarose flow through (lane 2), 12 μ g of the NIH 3T3 whole cell extract heparin-agarose wash (lane 3), 12 μ g of the NIH 3T3 whole cell extract heparin-agarose 1M KCl elution (lane 4), 12 μ g of the NIH 3T3 whole cell extraction heparin-agarose 0.4 M KCl elution (lanes 5 and 7) and 12 μ g of the src-NIH 3T3 whole cell extraction heparin-agarose 0.4 M KCl elution (lane 6). Competing cold Col-1 oligonucleotide was added to lane 7. Mobility shift assays were performed as described in Experimental Procedures and analyzed on a 4% nondenaturing acrylamide gel. The unbound probe and gel retarded bound probe were designated by arrows.

Fig 3. Collagen $\alpha 1(I)$ promoter deletion mapping. A series of overlapping fragments of the 5' flanking region of the collagen $\alpha 1(I)$ gene were assayed based on their capacity to promote CAT expression in NIH 3T3 cells. CAT activity for each plasmid is normalized to the β -galactosidase activity of the cotransfected plasmid pCH110 and expressed as a percentage of the activity of the plasmid p(-220/+155)ColCAT. The footprint map derived from DNase I protection assays is displayed above for orientation.

Fig 4. Transcriptional activity of collagen $\alpha 1(I)$ gene sequences -103 to -82 ligated to a heterologous promoter. Plasmids pBLCAT2, containing the thymidine kinase gene promoter (lanes 2 and 4), and p($-103/-82$)ColTKCAT, containing the collagen segment -103 to -82 ligated 5' of the thymidine kinase promoter, (lanes 3 and 5) were transfected into HepG2 cells (lanes 1–3) or NIH 3T3 cells (lanes 4–6). As controls, cells were mock transfected (lanes 1 and 4) and chloramphenicol was acetylated using an enzyme standard (lane 7). C designates unacetylated chloramphenicol and A designates acetylated forms of chloramphenicol.

assayed for CAT activity (Fig 3). The most distal footprint 4 was not required for efficient expression. Deletion of footprint 3 resulted in a 50% decrease in CAT activity, and deletion of the most proximal segment footprint 1 resulted in a 75% decrease from maximal CAT activity. Primer extension assays demonstrated that the ColCAT plasmids had the same transcriptional start site as the endogenous collagen $\alpha 1(I)$ gene (data not shown). These results demonstrate that the three most proximal footprinted segments contain *cis*-acting elements which are required for efficient expression.

In order to examine the effect on transcription of the proximal footprinted segment in more detail, the Col-1 oligonucleotide was inserted 5' of the herpes simplex virus thymidine kinase promoter creating the plasmid p($-103/-82$)ColTKCAT. In both NIH 3T3 cells and HepG2 cells the addition of the Col-1 oligonucleotide enhanced CAT activity five- to six-fold (Fig 4). Therefore, even in the context of a heterologous promoter, this DNA segment contains a *cis*-acting element that enhances transcription in cultured cells.

A literature search revealed that the collagen $\alpha 1(I)$ 12 bp direct repeat superficially resembles the DNA binding sites of two other *trans*-acting proteins, SP1 (21,22) and AP2 (23,24). However, a double-stranded oligonucleotide containing the AP2 binding site failed to compete for DNA binding with the intact collagen $\alpha 1(I)$ promoter, ($-220/+23$)Col (Fig 1), or the proximal footprinted region, ($-103/-82$)Col (Fig 5). The SV40 early promoter and enhancer region, which contains six SP1 binding sites and two binding sites for the CCAAT/core enhancer binding protein (C/EBP) (35,36), failed to compete with the NIH 3T3 cell extracts for binding to the intact collagen $\alpha 1(I)$ promoter (Fig 1) or proximal footprinted region (Fig 5). Finally, purified SP1 protein, which protected an extended footprint in the SV40 early promoter region, failed to protect the Col-1 oligonucleotide from DNase I digestion (Fig 5). Therefore, the DNA binding protein in the NIH 3T3 cell extract that interacts with the 12 bp direct repeats in footprint 1 and 2, does not appear to be a previously identified DNA binding protein.

Fig 5. DNase I protection assay of the -103 to -82 bp segment of the collagen $\alpha 1(I)$ promoter. A DNA fragment containing a murine collagen $\alpha 1(I)$ promoter (-103 to -82 bp) was labelled at the -103 bp end. The probe (20,000 cpm) was incubated with $25\mu\text{g}$ of BSA (lanes 1 and 5), 10ng of Sp1 (lanes 2 and 6), $14\mu\text{g}$ of partially purified HeLa cell extract (lane 3), and $24\mu\text{g}$ of NIH 3T3 whole cell extract heparin-agarose 0.4 M KCl elution (lane 4). Nonspecific cold competing d(I.C) DNA was present (lanes 1–4) or absent (lanes 5 and 6) in the reaction.

DISCUSSION

Deletional analysis of the $5'$ region of the collagen $\alpha 1(I)$ gene (from -3700 to $+1400$) has demonstrated that only 181 bp of the promoter is required for maximal expression of a reporter gene transfected into NIH 3T3 fibroblasts. This 181 bp promoter segment contains an intact TATA box, which is required for the proper initiation transcription, as well as additional upstream promoter elements. Further $5'$ deletions of the promoter resulted in decreased expression of the reporter gene. There are substantial differences in the organization of the promoter region in the murine fibrillar collagen genes. Collagen $\alpha 2(I)$ contains more distal positive *cis*-acting elements (25,32), while collagen $\alpha 1(III)$ contains only negative regulatory elements $5'$ to -80 bp (26). These studies do not eliminate the possibility of additional positive regulatory elements, such as far upstream of the start of transcription, within introns other than the first intron, or downstream to the termination of transcription.

DNase I protection assays revealed two footprinted segments in the collagen $\alpha 1(I)$ 120 bp promoter (footprints 1 and 2), which contain identical 12 bp direct repeats, TGGGGGCCGGGC. When these segments were deleted from the collagen $\alpha 1(I)$ promoter,

the expression of the reporter gene decreased in NIH 3T3 cells. When the footprint 1 segment was placed 5' to the thymidine kinase promoter, the expression of the reporter gene increased in NIH 3T3 and Hep G2 cells. Several experimental approaches demonstrated that these footprinted segments are not the DNA binding sites for SP1 (21,22) or AP2 (23,24), to which they bear a resemblance. Genuine DNA binding sites for SP1 and AP2 do not compete for binding activity with the collagen $\alpha 1(I)$ sites. Additionally, purified SP1 does not bind to the collagen $\alpha 1(I)$ footprint 1, and the (-103/-82)Col segment enhances transcription in Hep G2 cells, which lack the AP2 protein (23).

The collagen $\alpha 1(I)$ promoter contains a CCAAT motif in reverse orientation from positions -96 to -100. This sequence overlaps with the 5' end of DNase I footprint 1. The well characterized protein C/EBP binds to some CCAAT sites or to core enhancer elements (35,36). C/EBP is not the protein protecting collagen $\alpha 1(I)$ footprint I in that the footprint is not diminished by excess SV40 enhancer sequences, which contain 2 high affinity C/EBP binding sites. A recent study has provided evidence that a second CCAAT binding factor (CBF) interacts with this collagen $\alpha 1(I)$ reverse CCAAT sequence (34). Additional mutagenesis and transcriptional studies are required to define the relationship between CCAAT binding proteins and the 12 bp direct repeat binding protein.

If these 12 bp GC rich direct repeats are important in collagen $\alpha 1(I)$ gene regulation, we would expect conservation of this sequence through evolution. The chicken collagen $\alpha 1(I)$ gene (27) maintains 9 out of 12 bp in the distal element and 8 out of 12 bp in the proximal element; the rat collagen $\alpha 1(I)$ gene (31) perfectly conserves both elements, and the human collagen $\alpha 1(I)$ gene (11,12) conserves 12 out of 12 in the distal element and 10 out of 12 in the proximal element. Additionally, human HeLa cells synthesize a protein that binds to the proximal footprint 1 sequence.

Three DNase I hypersensitive sites have been demonstrated in the collagen $\alpha 1(I)$ gene in the chromatin of cells which transcribe this gene, designated as HS1 (within the body of the gene), HS2 (in the first intron), and HS3 (100 to 200 bp 5' of promoter) (28). Cells not transcribing collagen exhibit only HS1 and HS2 (28). The function of hypersensitivity sites is unknown, but hypersensitive chromatin sites are frequently present in cells that are actively transcribing a given gene and absent in cells that are not transcribing the gene (such as the collagen $\alpha 1(I)$ HS3 site). It has been postulated that hypersensitivity sites provide entry for polymerases or other *trans*-acting factors required for transcription (28,29). Since the 12 bp direct repeats are located at a transcriptional specific hypersensitivity site (HS3), this region might be critical in collagen $\alpha 1(I)$ gene expression.

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