Transcription Factors Nuclear Factor I and Sp1 Interact with the Murine Collagen $\alpha 1(I)$ Promoter

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The collagen $\alpha 1(I)$ promoter, which is efficiently transcribed in NIH 3T3 fibroblasts, contains four binding sites for *trans*-acting factors, as demonstrated by DNase I protection assays (D. A. Brenner, R. A. Rippe, and L. Veloz, *Nucleic Acids Res.* 17:6055-6064, 1989). This study characterizes the DNA-binding proteins that interact with the two proximal footprinted regions, both of which contain a reverse CCAAT box and a G+C-rich 12-bp direct repeat. Analysis by DNase I protection assays, mobility shift assays, competition with specific oligonucleotides, binding with recombinant proteins, and reactions with specific antisera showed that the transcriptional factors nuclear factor I (NF-I) and Sp1 bind to these two footprinted regions. Because of overlapping binding sites, NF-I binding and Sp1 binding appear to be mutually exclusive. Overexpression of NF-I in cotransfection experiments with the $\alpha 1(I)$ promoter in NIH 3T3 fibroblasts increased $\alpha 1(I)$ expression, while Sp1 overexpression reduced this effect, as well as basal promoter activity. The herpes simplex virus thymidine kinase promoter, which contains independent NF-I- and Sp1-binding sites, was stimulated by both factors. Therefore, expression of the collagen $\alpha 1(I)$ gene may depend on the relative activities of NF-I and Sp1.

Type I collagen, the most abundant protein in the human body, is the product of two genes, $\alpha 1(I)$ and $\alpha 2(I)$, located on different chromosomes. Two chains of $\alpha 1(I)$ and one chain of $\alpha 2(I)$ form a heterotrimeric helix molecule, and the two genes are generally coordinately regulated. Expression of the type I collagen genes is controlled at multiple levels, including developmental regulation (29, 43), tissue-specific expression (43, 47), and inducible expression by such agonists as transforming factor β (35) and the ethanol metabolite acetaldehyde (4). In addition, overexpression of type I collagen is clinically significant in fibrogenic diseases, such as hepatic cirrhosis (44), primary systemic sclerosis (28), pulmonary fibrosis (27), and the eosinophilic myalgia syndrome (46). Therefore, understanding the regulation of type I collagen expression would provide insight into normal development and pathological states.

Detailed studies of murine $\alpha 2(I)$ have identified two transcriptional factors which stimulate its expression (41). The CCAAT-binding factor (CBF) binds to the $\alpha 2(I)$ promoter to maintain high basal activity (25), and nuclear factor I (NF-I) stimulates $\alpha 2(I)$ transcription in response to transforming growth factor β (37). We (36) and others (24) have started to characterize the regulatory regions in the murine $\alpha 1(I)$ gene. The 220 bp upstream of the transcriptional start site in the $\alpha 1(I)$ promoter are sufficient for a high level of expression in collagen-producing NIH 3T3 cells and appropriate cell type-specific expression in transient transfection experiments (36). This segment contains four discrete regions protected from DNase I digestion by nuclear proteins extracted from NIH 3T3 cells (5, 24). The two more distal footprints interact with unidentified transcriptional inhibitors (24). Therefore, elements that interact with the two proximal footprints account for the high rate of transcriptional activity observed in the basal state. Each of the two proximal footprinted

regions contains a reverse CCAAT motif and a 12-bp G+C-rich direct repeat (see Fig. 1) (5).

The mechanisms by which the type I collagen genes are regulated are unknown. Since the murine $\alpha 1(I)$ gene promoter confers appropriate cell type-specific expression, this study examined the structural and functional roles of the DNA-protein interactions in the $\alpha 1(I)$ gene promoter. This analysis demonstrated that two transcriptional factors, NF-I and Sp1, bind to the two proximal footprinted regions and modulate $\alpha 1(I)$ gene expression.

MATERIALS AND METHODS

Construction of plasmids. Plasmid Col3luc was constructed by subcloning the *Bgl*II-*Xba*I (-220 to +116) fragment of the $\alpha 1(I)$ collagen gene into the *Bam*HI-*Xba*I sites of pBluescript SK⁺ (Stratagene, La Jolla, Calif.) and then cloning the *Hind*III-*Sst*I fragment into the identical restriction sites of the p19luc reporter plasmid (13, 45). Col2luc was constructed by cloning the *Hind*III-*Hind*III (-2500 to +116) fragment of pCol2- β Gal into the unique *Hind*III site of p19luc in the correct orientation. Col7luc was constructed by cloning the *Pst*I-*Xba*I (-1627 to +116) fragment of the $\alpha 1(I)$ gene into p19luc. The NF-I sense expression vector was prepared by first subcloning the NF-I cDNA *Eco*RI-*Xba*I fragment from pBSK⁺ into the same sites of pGem3Z (Promega, Madison, Wis.), then subcloning the *Eco*RI-*Hind*III fragment of the resulting plasmid into the same sites of pBluescript SK⁺, and finally cloning the *Hind*III-*Not*I NF-I cDNA fragment from pBluescript SK⁺ into identical sites in the Rous sarcoma virus *c-Jun* expression vector (8), replacing the *c-Jun* coding sequences.

Site-directed mutagenesis was performed by using the Amersham system version 2 (Amersham, Arlington Heights, Ill.). A *Pst*I-*Xba*I fragment (-1627 to +116) of the $\alpha 1(I)$ promoter was inserted into M13mp18 bacteriophage DNA for use as the template. Oligonucleotide primers containing

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FIG. 1. Schematic diagram of ColCAT7 reporter plasmids, showing the localization of the two proximal DNase I-protected sequences, FP1 and FP2. The reverse CCAAT motifs are bracketed, and the G+C-rich 12-bp direct repeats are underlined. Asterisks indicate the mutated sites. Each plasmid was cotransfected with pRSV- β Gal into NIH 3T3 fibroblasts. Chloramphenicol acetyltransferase activity was normalized for protein concentration and β -galactosidase activity and compared with the wild type (Wt) (=100%). Shown is the mean of three independent experiments, each performed in duplicate.

substitution mutations were synthesized. The resulting mutations in the $\alpha 1(I)$ promoter were subcloned into the ColCAT7 reporter gene, replacing the wild-type promoter (see Fig. 1). ColCAT7 contains -1627 to +116 bp of 5' regulatory sequences of the $\alpha 1(I)$ gene driving the chloramphenicol acetyltransferase gene (36). Mutations were confirmed by restriction mapping and DNA sequencing.

pSVSp1-F, which expresses functional Sp1, and pSVSp1-FX, which expresses nonfunctional frameshift mutant Sp1, were provided by J. Saffer (39). ptkluc, which contains the herpes simplex virus thymidine kinase (*tk*) promoter driving the luciferase reporter gene, was provided by G. Fey, and frameshifted, nonfunctional Rous sarcoma virus m-Jun was provided by M. Karin (8).

Cell transfection and reporter gene assays. NIH 3T3 fibroblasts were cultured in 35-mm-diameter plates with Dulbecco modified Eagle medium supplemented with 10% calf serum in a 10% CO₂-90% air atmosphere. Calcium phosphate-mediated gene transfer was performed by the method of Chen and Okayama (7). Cells were split to 30 to 40% confluency on the day before transfection. The cells were incubated with the DNA precipitate overnight at 3% CO₂ and washed with fresh 10% calf serum-Dulbecco modified Eagle medium on the next day. In dose-effect cotransfection experiments (see Table 2), NIH 3T3 cells were transfected with 1 μ g of the luciferase reporter gene plasmid (Col3luc or tkLuc), various amounts (0.01 to 1.0 μ g) of a sense or frameshifted expression vector, and 0.5 μ g of pRSV β -Gal. pUC18 was added to transfect a constant amount (2.5 μ g) of DNA per 35-mm plate. In the NF-I and Sp1 cotransfection experiments (see Table 3), 1.0 μ g of the sense NF-I expression vector (pRSV-NF-I) or the pRSV control vector (pRSV-mJun), 0.1 μ g of the sense Sp1 expression vector (pSVSp1-F) or the pSV control expression vector (pSVSp1-FX), 1.0 μ g of a luciferase reporter gene plasmid, and 0.5 μ g of pRSV β -Gal were transfected into NIH 3T3 cells. After overnight incubation in the calcium phosphate precipitant, fresh medium was added and the cells were harvested 24 h later.

Published procedures were used to measure firefly luciferase (13), chloramphenicol acetyltransferase (36), and

FIG. 2. Interaction of NIH 3T3 nuclear fibroblast extracts with the wild-type and mutant murine $\alpha 1(I)$ collagen promoters. The noncoding strand of the $\alpha 1(I)$ promoter region (-220 to +116) was labeled at the 5' end. A probe containing 15,000 cpm was incubated with 20 μ g of bovine serum albumin (BSA) (lanes 2, 7, 12, and 17) or 20 μ g of nuclear extracts (lanes 3 to 5, 8 to 10, 13 to 15, and 18 to 20). DNase I protection experiments were carried out as described in Materials and Methods. The protected regions are designated by the numbered boxes (FP1 to FP4). WT, wild type; 1m, 2Am, and 2Bm, mutant promoters indicated in Fig. 1. Maxam-and-Gilbert G+A sequencing reactions are shown in lanes 1, 6, 11, and 16.

β -galactosidase (17) activities. β -Galactosidase activity served as an internal standard to correct for differences in transfection efficiency. Luciferase activity is expressed as a ratio of the sense expression plasmid (pRSV-NF-I or pSVSp1-F) to the control expression plasmid (see Table 2). Luciferase activity for the reporter gene cotransfected with the control expression plasmids was defined as 1.00, and all other experiments were normalized with respect to this value (see Table 3). Results are expressed as means $\pm$ standard errors of the means ($n = 3$ to 8).

Preparation of nuclear extracts. Nuclear proteins from NIH 3T3 fibroblasts were extracted by the method of Shapiro et al. (42) or that of Dignam et al. (14), which produced

TABLE 1. Oligonucleotides used

Name	Sequence	Location and source	Reference
FP1	5'-CTAGCTGATTGGCTGGGGGCCGGGCT-3' 3'-GACTAACCGACCCCGGCCGACTAG-5'	-103 to -82, murine $\alpha 1(I)$ collagen gene	5
FP2	5'-AGCTTCCAAATTGGGGGCCGGGCCAG-3' 3'-AGGTTTAACCCCGGCCCGGTCTAG-5'	-131 to -110, murine $\alpha 1(I)$ collagen gene	5
NF-I	5'-GTTTTGGATTGAAGCCAATATGAG-3' 3'-AAAACCTAAGTTCGGTTATACTCG-5'	Adenovirus origin of replication	9
Sp1	5'-GATCGGGGCGGGGC-3' 3'-CCCCGCCCCGCTAG-5'	Simian virus 40 early promoter	15
CP-2	5'-AGCTGACCAGTTCAGCCACTCTTTAGTC-3' 3'-CTGGTCAAGGTCGGTGAGAAATCAGGATC-5'	-78 to -59, γ fibrinogen gene	34
MLTF	5'-AGCTGTAGGCCACGTGACCGGGT-3' 3'-CATCCGGTGCCTGGCCAGATC-5'	-66 to -48, adenovirus major late promoter	26
CBF	5'-GGGCCCCTAGCCCTAGCCCTCCCATTGGTGGAGACGTTTTGG-3' 3'-GGGGATCGGGATCGGGAGGGTAACCACCTCTGCAAAAACC-5'	-105 to -64, murine $\alpha 2(I)$ collagen gene	41
AP-1	5'-TAAAGCATGAGTCAGACACCTC-3' 3'-ATTTCGTACTCAGTCTGTGGAG-5'	-79 to -58, human collagenase gene	2

comparable extracts, as assessed by DNase I protection and gel retardation assays (data not shown).

Oligonucleotides. All oligonucleotides except Sp1 (a gift of J. Kadonaga) were synthesized with a Cyclone Plus oligonucleotide synthesizer (Milligen, Novato, Calif.) and purified by thin-layer chromatography. The DNA sequences of the oligonucleotides used in the experiments are listed in Table 1.

The Sp1 oligonucleotide was dimerized prior to labeling, and a concatemer was used in the competition experiments. The CP-1 fragment was derived from plasmid p108-MLP (9) by digestion with *Bam*HI-*Hind*III and isolation of the 74-bp fragment (containing -108 to -62 from the major late promoter cap site).

DNase I protection and gel retardation assays. DNase I protection assays were performed as described previously (5), with minor modifications. The noncoding strand of the murine $\alpha 1(I)$ collagen promoter (-220 to +116 bp) was radiolabeled at the 5' end by using a fill-in reaction of the overhanging end created by linearization with *Bgl*II. The labeled fragment was obtained by digestion with *Xba*I and purification in a 1% agarose gel. DNA-protein-binding reactions were performed on ice for 20 min. The reaction mixture contained 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.6), 10% glycerol, 50 mM KCl, 100 mM EDTA, 1 mM dithiothreitol, 2 μ g of poly(dI-dC), 5 mM MgCl₂, 2% polyvinyl alcohol, 20,000 cpm (approximately 1 ng of radiolabeled probe), and 20 μ g of nuclear protein (or an equal amount of bovine serum albumin). After incubation, an equal volume of 10 mM HEPES (pH 7.6) and 5 mM CaCl₂ was added, followed by addition of various amounts of DNase I. Digestion reactions were incubated at room temperature for 1.5 min and terminated by addition of an equal volume of stop mixture (200 mM NaCl, 20 mM EDTA, 1% sodium dodecyl sulfate, 50 μ g of yeast tRNA per ml). After phenol-chloroform-isoamyl alcohol (25:24:1) extraction and subsequent precipitation, the DNA was suspended in formamide dye and the fragments were separated in a standard 8% sequencing gel. Maxam-Gilbert G+A sequenc-

ing reactions (32) were electrophoresed as size markers. After electrophoresis, the gels were dried and autoradiographed at -70°C with an intensifying screen.

Binding reactions using purified recombinant Sp1 protein were modified by omitting poly(dI-dC), using a final MgCl₂ concentration of 1 mM, and reducing the DNase I concentration.

Gel retardation assays were performed as previously described (18). Supershift experiments were performed by incubating the nuclear extract with NF-I antiserum, Sp1 antiserum, or preimmune serum for 1 h at 22°C. After addition of the probe, the standard gel retardation protocol was used (18).

RESULTS

Functional analysis of the murine $\alpha 1(I)$ promoter. The two proximal DNase I footprints in the collagen $\alpha 1(I)$ promoter (FP1 and FP2) contain reverse CCAAT motifs and G+C-rich 12-bp direct repeats (Fig. 1A). To assess the functional significance of these elements, reporter genes driven by the wild-type or mutated $\alpha 1(I)$ promoter and 5'-flanking regions (-1627 to +54 bp) were transfected into NIH 3T3 cells. The mutant reporter gene plasmids contained 2- or 4-bp substitutions (Fig. 1). Chloramphenicol acetyltransferase activity was normalized with respect to the β -galactosidase activity of the cotransfected pRSV- β Gal plasmid. Mutations in the CCAAT motifs in both FP1 (FP1m) and FP2 (FP2Am) decreased expression about three- and twofold, respectively, compared with wild-type expression levels (Fig. 1).

DNase I protection assays of the mutant promoter fragments revealed alterations of the footprinting pattern by the 2-bp substitution in each CCAAT box (Fig. 2). Although the 4-bp substitution mutation in the 12-bp repeat of FP2 (FP2Bm) clearly abolished binding, no significant change of expression was observed in NIH 3T3 cells (Fig. 1).

Identification of nuclear proteins which interact with the two most proximal footprints of the $\alpha 1(I)$ promoter. To identify the nuclear proteins that interact with FP1 and FP2,

TABLE 2. Effects of NF-I and Sp1 overexpression on collagen $\alpha 1(I)$ and herpes simplex virus *tk* promoter activities in NIH 3T3 fibroblasts: response to increasing doses of NF-I or Sp1

Plasmid	Mean luciferase activity $\pm$ SEM with ^a :					
	pRSV-NF-I			pSVSp1-F		
	0.01 μ g	0.1 μ g	1.0 μ g	0.01 μ g	0.1 μ g	1.0 μ g
Col3luc	1.12 $\pm$ 0.04	2.63 $\pm$ 0.67	12.96 $\pm$ 3.78	0.96 $\pm$ 0.04	0.66 $\pm$ 0.08	0.73 $\pm$ 0.20
ptkluc	0.98 $\pm$ 0.05	1.93 $\pm$ 0.27	4.78 $\pm$ 1.87	3.03 $\pm$ 0.92	1.89 $\pm$ 0.39	1.82 $\pm$ 0.31

^a Results were calculated as described in Materials and Methods. Luciferase activity is expressed as the ratio of the cotransfected sense expression plasmid (pRSV-NF-I or pSVSp1-F) to the control expression plasmid.

gel retardation experiments were performed by using synthetic double-stranded oligonucleotides corresponding to FP1 and FP2 as radiolabeled probes and incubated with nuclear extracts from NIH 3T3 cells. Two major DNA-protein complexes, designated A (upper) and B (lower), were detected (Fig. 3A and B, lanes 2). A 200-fold molar excess of unlabeled FP1 oligonucleotide (Fig. 3A, lane 3, and B, lane 4) or FP2 oligonucleotide (Fig. 3A, lane 4 and B, lane 3) competed for both complexes, implying that similar factors bind to both oligonucleotides.

Since CCAAT-binding proteins have been classified by their binding affinities to different DNA-binding sites (9), synthetic oligonucleotides containing high-affinity CCAAT protein-binding sites were used to compete for the observed complexes. Sp1- and major late transcription factor (MLTF)-binding sites, which show some sequence homology to the G+C-rich 12-bp repeat, were also tested.

An oligonucleotide with the strong NF-I-binding site from the adenovirus origin of replication competed efficiently with the FP1 and FP2 probes for the B complex (Fig. 3A and B, lanes 5), while a concatemerized Sp1 oligonucleotide completely abolished the A complex (Fig. 3A and B, lanes 8). These results indicate that factors which also interact with NF-I- and Sp1-binding sites are involved in A and B complex formation, respectively, and that binding occurs for each factor independently. A 100-fold molar excess of the CBF oligonucleotide, which is involved in murine $\alpha 2(I)$ collagen gene regulation (25), weakly attenuated the A complex (Fig. 3A and B, lanes 7), perhaps because of a G-rich region 5' to the CBF-binding site. CP-2 (34)- and MLTF (26)-binding sites did not compete with the formation of complex A or B (Fig. 3A and B, lanes 6 and 9).

Since purified CBF was reported to bind to the first footprint of the murine $\alpha 1(I)$ collagen gene promoter (30), we

FIG. 3. Complexes formed between the FP1 [−103 to −82, $\alpha 1(I)$], FP2 [−131 to −110, $\alpha 1(I)$], or CBF [−105 to −64, $\alpha 2(I)$] probe and nuclear proteins extracted from NIH 3T3 fibroblasts. The gel retardation assays were performed as described in Materials and Methods. Panels A and B, FP1 and FP2 probes, respectively. Lanes: 1, probe alone; 2, 10 μ g of nuclear protein, which formed two specific complexes designated A and B; 3 and 4, competition with a 200-fold molar excess of unlabeled FP1 and FP2 oligonucleotides; 5 to 9, competition with a 100-fold molar excess of the NF-I, CBF, CP-2, Sp1, and MLTF cold oligonucleotides. Panel C, CBF probe with no extract (lane 1) or NIH 3T3 nuclear extract (lanes 2 to 11). Lanes: 4 and 5, competition with CP-1, a DNA fragment containing the sequence −108 to −62 from the adenovirus major late promoter, using a 100- and 500-fold molar excesses, respectively; 6 and 7, 8 and 9, and 10 and 11, competition with 100- and 500-fold molar excesses of the unlabeled CP-2, FP1, and FP2 oligonucleotides, respectively. comp, competitor.

FIG. 4. Mobility shift experiments with antisera to NF-I (α NF-I) and Sp1 (α Sp1). (A) Preincubation of NIH 3T3 nuclear extracts with α NF-I (lanes 2, 5, 8, and 11) or preimmune serum (lanes 1, 3, 4, 6, 7, 9, 10, and 12) was carried out as described in Materials and Methods. Gel-retarded complexes were formed with NF-I (lane 1), FP1 (lane 4), FP2 (lane 7), and AP-1 (lane 10) labeled probes, and their specificity was shown by competition with a 100-fold molar excess of the corresponding unlabeled oligonucleotides (lanes 3, 6, 9, and 12). (B) Preincubation of the nuclear extracts with preimmune serum (lanes 1, 3, 4, 6, 7, 9, 10, and 12) or α Sp1 (lanes 2, 5, 8, and 11) prior to a mobility shift assay. Lanes: 1, 4, 7, and 10, specific complexes formed between the nuclear extracts and dimerized Sp1, FP1, FP2, and AP-1 oligonucleotide probes, respectively; 3, 6, 9, and 12, competition with a 100-fold molar excess of the unlabeled concatemeric Sp1-binding site.

determined whether the NIH 3T3 nuclear extract contained CBF and estimated the affinity of this factor for the CCAAT-binding sites of the $\alpha 1(I)$ promoter. The CBF-binding site of the murine $\alpha 2(I)$ collagen promoter was used in these experiments; this was the same oligonucleotide used for affinity purification of this factor (31). A gel retardation experiment with the CBF oligonucleotide as a labeled probe demonstrated two complexes formed with nuclear extract from NIH 3T3 fibroblasts (Fig. 3C, lane 2). A 100-fold excess of the cold CBF-binding site competed for these complexes, indicating their specificity. Neither nucleotide FP1 nor FP2 competed for the complexes at a 100 or 500 molar excess (Fig. 3C, lanes 10 to 13). CP-1, a CCAAT-binding protein from the adenovirus major late promoter competed for CBF binding (Fig. 3C, lanes 6 and 7), as previously suggested (9, 31). An additional gel retardation experiment using a CP-1-binding site as a probe gave an identical result; only the CBF, not the FP1 or FP2, oligonucleotide was competitive (data not shown).

To investigate the identity of the NF-I- and Sp1-related factors further, mobility shift experiments using polyclonal antisera raised against recombinant human NF-I (α NF-I) or Sp1 (α Sp1) were performed. α NF-I recognized NF-I, as demonstrated by supershifting of the NF-I probe (Fig. 4A, lane 2). α Sp1 mainly interfered with Sp1 binding to the dimerized Sp1 consensus binding site, resulting in decreased complex formation, and supershifted some residual complex (Fig. 4B, lane 2). α Sp1 was previously demonstrated to inhibit formation of the Sp1 DNA complex (1). Using a monomer Sp1-binding site as a probe produced an identical

complex, but binding was less efficient than with the dimerized probe (data not shown).

Incubation of the nuclear extract with α NF-I prior to the standard gel retardation assay with an FP1 or FP2 probe resulted in decreased appearance of complex B and formation of a supershifted complex (Fig. 4A, lanes 5 and 8) compared with that observed with preimmune serum (Fig. 4A, lanes 4 and 7). An AP-1 probe did not show similar changes, demonstrating the specificity of the antiserum (Fig. 4A, lanes 10 to 12). Competition with the corresponding unlabeled oligonucleotides demonstrated the specificity of the complexes (Fig. 4A, lanes 3, 6, 9, and 12).

Preincubation with α Sp1 reduced A complex formation for both the FP1 and FP2 probes while not altering the B complex (Fig. 4B, lanes 5 and 8). Again, AP-1 probe reactions served as controls for the specificity of the antiserum (Fig. 4B, lanes 10 to 12). Competition with the cold concatemeric Sp1 oligonucleotide, as shown in lanes 3, 6, 9, and 12 (Fig. 4B), demonstrated the specificity of the Sp1 complexes formed. Therefore, NF-I and Sp1 interacted with both the FP1 and FP2 oligonucleotides, forming independent complexes in the gel retardation assays.

DNase I protection assays with competitor DNA. DNase I protection assays were performed to analyze the interactions between NF-I and Sp1 binding to the collagen $\alpha 1(I)$ promoter. Fig. 5A, lane 3, shows the footprinting pattern obtained by using NIH 3T3 extracts and 1 ng of the 5'-end-labeled $\alpha 1(I)$ promoter per reaction. Adding 5 (Fig. 5A, lane 4) or 50 (Fig. 5A, lane 5) ng of the unlabeled FP1 oligonucleotide to the binding reaction inhibited FP1 and FP2

FIG. 5. Analysis of NF-I and Sp1 interactions with the $\alpha 1(I)$ collagen promoter by DNase I protection. (A) The wild-type $\alpha 1(I)$ collagen promoter (–220 to –116) was end labeled on the 5'-noncoding strand and incubated with bovine serum albumin (BSA) (lane 2) or nuclear proteins from NIH 3T3 cells (lanes 3 to 15). Lane 3 contained a control reaction with no competitor (comp). A 1-ng sample of the labeled fragment (=20,000 cpm) competed with 5 or 50 ng, respectively, of the unlabeled oligonucleotides as indicated above the lanes. N+S, NF-I plus Sp1. The Maxam-and-Gilbert G+A sequencing reaction is shown in lane 1. The positions of the protected regions correspond to the numbered boxes. (B) DNase I protection of the same probe after incubation with increasing concentrations (0 to 2.5 ng) of purified human Sp1. The footprinting pattern obtained with NIH 3T3 nuclear proteins is shown by the boxes, and the positions of the 12-bp direct repeats (d.r.) are indicated.

protection. Reduction of the DNase I hypersensitivity site between FP1 and FP2 was also observed. When excess NF-I oligonucleotide was used as a competitor (Fig. 5A, lanes 7 and 9), the 5' part of FP1 progressively disappeared with increasing concentrations of the competitor, showing that NF-I accounts for part of the DNase I protection of FP1.

When the Sp1 oligonucleotide was used as a competitor (Fig. 5A, lanes 10 and 11), loss of FP2, as well as the hypersensitive sites bordering the 12-bp repeat of FP1, was observed. When both the NF-I and Sp1 oligonucleotides were used as competitors (Fig. 5A, lanes 12 and 13), the 3' end of FP1 was lost in addition to FP2, suggesting that Sp1 can bind to the proximal 12-bp repeat in the absence of NF-I binding. Combination of the NF-I and Sp1 oligonucleotides

had the same effect as using the FP1 or FP2 oligonucleotide as a competitor. The cold CBF oligonucleotide competed weakly for FP2, while neither FP1 nor the hypersensitive site between the two footprints was altered.

Further proof that Sp1 can bind to both G+C-rich 12-bp direct repeats was obtained by using purified recombinant Sp1. Since mobility shift assays detected maximal formation of complex A (Sp1) with low magnesium concentrations (data not shown), the DNase I protection experiments were performed under these optimized conditions. Purified Sp1 protected the entire FP2 and a small 3' part of FP1 encompassing the 12-bp direct repeat (Fig. 5B). Sp1 binding was also detected in G+C-rich footprints 3 and 4 but not in gel retardation assays using crude NIH 3T3 nuclear extracts (data not shown).

Effects of NF-I and Sp1 on $\alpha 1(I)$ expression. To study the regulation of $\alpha 1(I)$ expression by NF-I and Sp1, NF-I and Sp1 expression vectors were cotransfected with Col3luc, a plasmid containing 220 bp of the $\alpha 1(I)$ collagen promoter driving the firefly luciferase gene, into NIH 3T3 cells. The effect of increasing doses of each expression vector was compared with that of the same dose of similar expression vectors driving frameshifted nonfunctional cDNAs. For comparison, the NF-I and Sp1 expression vectors were cotransfected with a herpes simplex virus *tk* promoter luciferase gene plasmid. The *tk* promoter contains independent binding sites for NF-I and Sp1 (23). Overexpression of NF-I led to a dose-dependent increase of $\alpha 1(I)$ promoter activity, indicating the functional significance of the $\alpha 1(I)$ NF-I site for transactivation by NF-I. Overexpression of NF-I conferred a similar but weaker dose-dependent increase on *tk* promoter expression (Table 2).

Overexpression of Sp1 did not induce collagen $\alpha 1(I)$ promoter activity but instead showed decreased promoter activity compared with control transfections. In contrast, the *tk* promoter was inducible by overexpression of Sp1, indicating that endogenous Sp1 levels were not saturating for this promoter (Table 2). At increasing doses of the Sp1 expression vector, transactivation of *tk* decreased, which might result from squelching or nonspecific toxicity of the overexpressed Sp1 protein.

Coexpression of NF-I and Sp1 stimulated *tk* promoter activity in NIH 3T3 cells to a greater extent than expression of either transcriptional factor alone (Table 3). However, coexpression of NF-I and Sp1 actually inhibited the stimulatory effect of NF-I on $\alpha 1(I)$ promoter activity (Table 3). These results suggest that in unstimulated NIH 3T3 fibroblasts, NF-I is likely to be the critical transactivator of the $\alpha 1(I)$ promoter.

DISCUSSION

The murine collagen $\alpha 1(I)$ promoter contains two protein-binding sites (FP1 and FP2), each comprising a reverse CCAAT box and a G+C-rich 12-bp direct repeat. Expression of mutant promoter constructs in NIH 3T3 cells demonstrated the importance of the CCAAT boxes for high basal level promoter activity. Further analysis by gel retardation and DNase I protection assays identified NF-I and Sp1 as the proteins that interact with both FP1 and FP2. For both elements, Sp1 binding resulted in a higher-mobility shift complex and NF-I-binding resulted in multiple complexes with a lower-mobility shift. Within the context of the whole promoter, NF-I binds predominantly to the more proximal element (FP1) and Sp1 interacted with the more distal element (FP2) in vitro. The functional importance of NF-I

TABLE 3. Effects of NF-I and Sp1 overexpression on collagen $\alpha 1(I)$ and herpes simplex virus *tk* promoter activities in NIH 3T3 fibroblasts: effect of cotransfection of NF-I and Sp1

Plasmid	Mean fold induction $\pm$ SEM with ^a :			
	pRSV control, pSV control	pRSV control, pSVSp1-F	pRSV-NF-I, pSV control	pRSV-NF-I, pSVSp1-F
Col3luc	1.00 $\pm$ 0.08	0.60 $\pm$ 0.08	11.95 $\pm$ 2.02	7.29 $\pm$ 0.4
ptkluc	1.00 $\pm$ 0.07	1.93 $\pm$ 0.11	8.07 $\pm$ 0.88	11.98 $\pm$ 1.73

^a Results were calculated as described in Materials and Methods. Luciferase activity is expressed as fold induction with respect to cotransfection with both control expression plasmids.

was demonstrated by overexpression of NF-I in NIH 3T3 cells, which led to an increase of $\alpha 1(I)$ promoter activity. In contrast, overexpression of Sp1 reduced basal promoter activity and mutation of the distal G+C-rich region had no effect on $\alpha 1(I)$ promoter expression.

Several families of CCAAT-binding transcription factors have been defined by their differential affinities for promoter elements (9). When distinct DNA-binding sites for NF-I (from the adenovirus origin of replication), CP-2 (from the γ -fibrinogen promoter), CBF [from the murine $\alpha 2(I)$ promoter], CP-1 (from the adenovirus major late promoter) were used, gel retardation assays demonstrated that only the NF-I-binding site competed with the lower-mobility complexes formed with NIH 3T3 nuclear extracts and oligonucleotides FP1 and FP2. NF-I is actually a family of transcriptional factors generated by alternative splicing of a single gene (40). The multiple lower-mobility complexes might represent binding by different NF-I family members. An anti-NF-I serum supershifted this complex, providing further evidence that NF-I is the binding protein responsible for B complex formation.

Previous studies demonstrated that purified CBF can bind to the first footprint and transactivate the murine $\alpha 1(I)$ collagen promoter (32). However, most CCAAT-binding proteins interact with any given CCAAT site with a certain affinity because of sequences flanking the CCAAT motif (9). Gel retardation assays demonstrated that CBF was present in the NIH 3T3 nuclear extracts and recognized the binding site for CP-1, as was previously suggested (9, 31). Although purified CBF may be able to bind, our results indicate that NF-I is the major CCAAT-box-binding factor that interacts with the $\alpha 1(I)$ promoter. Jones et al. (22) compared several NF-I-binding sites of viral and eukaryotic promoters for the ability to bind this factor. There is a striking similarity between FP1 and, to a lesser degree, FP2 and the high-affinity NF-I-binding sites (Fig. 6).

TAACCGA NNN N A/CGGT	consensus NF-I
TAACCGA agt t AGGT	Ad-2/5 origin
TAACCGA cgc g CGGT	ras (-300)
TAACCGA ccc c CGGc	FP1 $\alpha 1(I)$
TAACCCc cgg c CcGg	FP2 $\alpha 1(I)$

FIG. 6. Comparison of high-affinity NF-I-binding sequences aligned with respect to the CCAAT motif. The consensus sequence is derived from Jones et al. (22) and de Vries et al. (12). The noncoding strand sequences of FP1 and FP2 are shown. Matches with the consensus sequences are designated by capital letters. Ad-2/5, adenovirus types 2 and 5.

The closer resemblance of the FP1-binding site to strong NF-I recognition sequences might explain the higher binding affinity of FP1 for NF-I, compared with that of FP2, in the *in vitro* assays. Mutation of the CCAAT box motif of FP1 affected the promoter activity to a greater extent than did a similar mutation in the more distal FP2, indicating that the higher-affinity binding site for NF-I has more importance in transactivation. The close proximity of FP1 to the TATA box may also increase the transactivating potential of NF-I, as demonstrated for other promoters, including human β -globin (22).

The second motif in FP1 and FP2 is a G+C-rich 12-bp repeat resembling the binding site for Sp1 or MLTF (a transactivating factor in the adenovirus major late and γ -fibrinogen promoters). Mobility shift assays with the corresponding oligonucleotides as competitors demonstrated that the Sp1-binding site competed for the higher-shifted complex. Further evidence that Sp1 binds to the 12-bp repeat was shown when a specific Sp1 antiserum inhibited the retarded complex. In addition, these sites were protected from DNase I digestion by purified Sp1. Previous experiments using different binding conditions failed to detect binding of Sp1 to FP1 (5, 24). Under low magnesium concentrations, interaction of Sp1 with both FP1 and FP2 was greatly increased while the affinity of Sp1 for the consensus Sp1 recognition site was reduced (data not shown). The central sequence of the 12-bp repeats (GGGGC CGGG), which differs from the Sp1 consensus sequence (GGGGCGGGG) by a central G-to-C transversion, may account for this phenomenon. A similar Mg^{2+} dependency was reported (10) in which MLTF binds preferentially to the γ -fibrinogen promoter at higher Mg^{2+} concentrations compared with the adenovirus major-late-promoter-binding site. Also, a G-to-C transversion in the Sp1 consensus binding site reduces the binding affinity of Sp1 (21). Such a change may render the interaction of Sp1 with this binding site more susceptible to changes in ion concentrations, a possible means of gene regulation. Recently, an Sp1-binding site with such a deviation from the Sp1 consensus sequence was shown to be important in the regulation of a human promoter (1).

The consensus sequences for NF-I- and Sp1-binding sites in FP1 and FP2 overlap (Fig. 1). Since both factors make contact with the major groove (12), it seems unlikely that both factors bind to each footprint simultaneously. Furthermore, there were no gel-retarded complexes with NIH 3T3 nuclear extracts which contain both NF-I and Sp1. There are now many examples of overlapping DNA-binding sites for transcriptional factors, including NF-I and AP-2 in the human growth hormone promoter (11) and AP-2 and AP-3 in the simian virus 40 enhancer (33).

Previous studies have demonstrated that both NF-I and Sp1 stimulate transcriptional activity (16, 22). For example,

both factors stimulate transcription from the herpes simplex virus *tk* promoter, which has nonoverlapping NF-I- and Sp1-binding sites (reference 23 and Table 2). However, while overexpression of NF-I stimulates $\alpha 1(I)$ promoter activity in NIH 3T3 cells, overexpression of Sp1 inhibits basal and NF-I-stimulated $\alpha 1(I)$ activities (Table 2). Because of the overlapping NF-I- and Sp1-binding sites in the $\alpha 1(I)$ promoter, Sp1 might interfere with NF-I binding and transactivation. In *Drosophila* Schneider cells, where neither factor is expressed, NF-I and Sp1 transactivate the $\alpha 1(I)$ promoter (34a). Therefore, competition of NF-I and Sp1 for overlapping binding sites may represent a novel mechanism to regulate $\alpha 1(I)$ transcription.

This analysis revealed complex relationships between DNA-binding proteins and the collagen $\alpha 1(I)$ promoter and suggests several mechanisms for modulating $\alpha 1(I)$ gene expression. For example, since Sp1 expression is highly regulated during development in different tissues (38), varying Sp1 concentrations can regulate $\alpha 1(I)$ transcription. Another potential regulatory mechanism is methylation inactivation, since FP1- and FP2-binding sites contain CpG nucleotides which display differential methylation patterns in cells, reflective of their $\alpha 1(I)$ gene expression (6). Although direct Sp1 binding is unaffected by methylation (19), methylation can inhibit gene expression by indirect mechanisms (3). Finally, Sp1 and, probably, NF-I can be phosphorylated, which may modulate their transactivating activity (20).

In conclusion, *trans*-acting factors NF-I and Sp1 interact with the two proximal DNase I-protected elements of the collagen $\alpha 1(I)$ promoter and lead to high transcriptional activity in fibroblasts. We believe that differential binding of these factors, in concert with other regulatory elements, will elicit distinct patterns of activity of the collagen $\alpha 1(I)$ gene.

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