YY1 Is a Positive Regulator of Transcription of the \textit{Col1a1} Gene*

Received for publication, October 30, 2000, and as revised form, July 25, 2001
Published, JBC Papers in Press, August 20, 2001, DOI 10.1074/jbc.M009881200

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Both cell-specific and ubiquitous transcription factors in fibroblasts have been identified as critical for expression of the \textit{Col1a1} gene, which encodes the \(\alpha1\) chain of type I collagen. Here, we report that Yin Yang 1 (YY1) binds to the \textit{Col1a1} promoter immediately upstream of the TATA box, and we examine the functional implications of YY1 binding for regulation of \textit{Col1a1} gene expression in BALBc/3T3 fibroblasts. The \textit{Col1a1} promoter region spanning base pairs (bp) \(-56\) to \(-9\) bound purified recombinant YY1 and the corresponding binding activity in nuclear extracts was supershifted using a YY1-specific antibody. Mutation of the TATA box to TgTA enhanced YY1 complex formation. Mutation analysis revealed two YY1 core binding sites at \(-40/-37\) bp (YY1A) and, on the reverse strand, at \(-32/-29\) bp (YY1B) immediately adjacent to the TATA box. In transfections using \textit{Col1a1}-luciferase constructs, mutation of YY1A decreased activity completely (wild-type p350 (p350wt), \(-222/+113\) bp) or partially (p130wt, \(-84\) bp/+13 bp), whereas mutation of YY1B blocked the expression of both promoter constructs. Cotransfection with pCMV-YY1 increased p350wt and p130wt activities by as much as 10-fold, whereas antisense YY1 decreased constitutive expression and blocked the increased activity due to pCMV-YY1 overexpression. The mTgTA constructs were devoid of activity, arguing for a requirement for cognate binding of the TATA box-binding protein (TBP). Electrophoretic mobility shift assays performed under conditions permitting TBP binding showed that recombinant TBP/TFIID and YY1 could bind to the \(-56/-9\) bp fragment and that YY1B was the preferred site for YY1 binding. Our results indicate that YY1 binds to the \textit{Col1a1} proximal promoter and functions as a positive regulator of constitutive activity in fibroblasts. Although YY1 is not sufficient for transcriptional initiation, it is a required component of the transcription machinery in this promoter.

Type I collagen is the most abundant and well characterized collagen in vertebrates. It forms 90% of the organic mass of bone and tendon and is the major collagen of skin, ligaments, cornea, and many interstitial connective tissues. The helical type I collagen molecule is a heterotrimer composed of two \(\alpha1\) chains and one \(\alpha2\) chain, which are encoded by the \textit{Col1a1} and \textit{Col1a2} genes, respectively. It is synthesized in large quantities by fibroblasts and osteoblasts and to a lesser extent by nearly all other cell types. It is present in reticular fibers of most parenchymal tissues, such as lung, kidney, liver, muscle, and spleen, with the exception of hyaline cartilage, brain and vitreous humor (3). Besides its biomechanical properties, type I collagen is important as an adhesive substrate for many cells and plays a major role in tissue and organ development, cell migration, proliferation and differentiation, wound healing, tissue remodeling, and homeostasis (4). Because type I collagen is a ubiquitous molecule with many biological functions, the regulation of its expression has been well studied in the past twenty years. Several \textit{cis}-acting elements within the \textit{Col1a1} and \textit{Col1a2} promoters have been identified that interact with transcription factors such as CCAAT-binding factor (CBF), Sp1, NF-1, and c-Krox, which are critical for regulation of constitutive expression (5–9). Although knowledge about the regulation of type I collagen synthesis during development and in pathological states, such as lung fibrosis and rheumatoid arthritis, remains a major interest, the understanding of its regulation under normal physiological conditions is crucial.

YY1 (10), also termed NF-E1 (11, 12), or upstream conserved region-binding protein (13), is a transcription factor containing four \(\text{Cys}_2\text{-His}_2\) zinc fingers that can either activate or repress transcription depending upon promoter context. YY1 binds to the \((\text{C/t/a})\text{CAT}(\text{T/a})(\text{T/g/c})\) consensus sequence located in the promoters of many viral and cellular genes (14). Moreover, the YY1 binding site can also serve as a transcriptional initiator (15, 16), because it can direct specific transcription in the absence of binding sites for other factors (17) including TATA-binding proteins. It has been shown that YY1 may be required for function of the initiator element, because antibodies to YY1 can block transcription (17). The critical importance of YY1 in growth and differentiation has been demonstrated in mice in which targeted disruption of YY1 results in peri-implantation lethality (18).

Studies in our laboratory have focused on the regulation of collagen gene expression by cytokines and prostaglandins. Recently, we identified a region within the proximal \textit{Col1a1} promoter, which was down-regulated by E-series prostaglandins added to transiently transfected fibroblasts or induced by in-

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*This work was supported in part by National Institutes of Health Grants P01-AR05564 and R01-AR45378 (to M. B. G.) and CA68544 (to P. E. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ This work was completed in partial fulfillment of requirements for Dr. Riquet’s Ph.D. obtained at the Universite Paris 7-Denis Diderot, UFR Lariboisiere Saint Louis. We are grateful to the members of his committee, Drs. M.-C. deVernejoul, P. Orcel, M. Corvol, and F. Berenbaum.

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to tumor necrosis factor (TNF)-
chased from Operon Technologies, Inc. (Alameda, CA). Double-stranded
probes and competitors are listed in Fig. 1 and were pur-
expressed and purified according to Shi
binding reaction mixture. The His-tagged YY1 protein (His-YY1) was
preincubated for 30 min with the nuclear extract prior to addition to the
specific YY1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was
amid gels using Tris borate-EDTA buffer (TBE) (45 mM Tris borate, pH
12% glycerol, and 0.5 mg/ml bovine serum albumin (19). The protein-
ery thesis was assessed by DNA sequencing at the Beth
Israel Deaconess Medical Center DNA sequencing facility using the
ABI PRISM® BigDye™ primer cycle sequencing kit (Applied Biosys-
tems, Foster City, CA) and the automatic DNA sequencer model 373A (Applied Biosystems). The pCMV-YY1 plasmid used in cotransfections was described previously (23). The antisense YY1 plasmid (pCMV-
sciency. The presence of binding to the USE, YY1A, YY1B, or TATA is marked with a
each sequence. The potential YY1 binding
regions are indicated by small circles above (YY1A) and below (YY1B) the wild-type sequence.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Nuclear Extract Preparation—**BALB/c3T3 fibro-
basts were grown to subconfluence, as described previously (19), and
left untreated or stimulated for 2–4 h with recombinant murine interleu-
kin-1 (IL-1β (19). Mori et al. (20) identified a correspond-
ing region in the human COLLA1 promoter that was sensitive
to tumor necrosis factor (TNFα)-induced prostaglandin E2. A
close study of the DNA sequence of the minimal promoter of the
mouse Col1a1 gene and preliminary studies of DNA-binding
protein interactions with the sequence spanning –84 bp to –29
bp of the Col1a1 minimal promoter permitted us to identify
DNA sequences upstream of the TATA box that presented
features of potential Yin Yang 1 (YY1) DNA binding sites.

In the study presented here, we show that the YY1 transcription
factor binds to two sites in the mouse Col1a1 minimal core
promoter one located 11 bp upstream from the TATA box, and
the other immediately adjacent to the TATA box on the com-
plementary strand. Mutation analysis of these sites in trans-
ient transfections and overexpression of YY1 and antisense-
YY1 in cotransfections demonstrate that YY1 functions as a
transcriptional activator of the Col1a1 promoter in fibroblasts.

**RESULTS**

**Electrophoretic Mobility Shift Assay (EMSA) and Supershift Anal-
ysis—**The synthetic oligonucleotides that were used for EMSA analysis as
labeled probes and competitors are listed in Fig. 1 and were pur-
chased from Operon Technologies, Inc. (Alameda, CA). Double-stranded
DNA oligonucleotides were end-labeled using T4 polynucleotide kinase and [α-32P]dATP. Binding reactions were carried out for 30 min at room
temperature using 3 μg of nuclear extract in a final volume of 20 μl
containing 12 mM HEPES-KOH (pH 7.9), 0.94 mM EDTA, 1 mM dithi-
othreitol, 0.45 mM MgCl2, 0.2 mM ZnCl2, 90 mM KCl, 1.5 mM poly(dI-dC),
12% glycerol, and 0.5 mg/ml bovine serum albumin (19). The protein-
DNA complexes were separated in low ionic strength 4% polyacryl-
amide gels using Tris borate-EDTA buffer (TBE) (45 mM Tris borate, pH
8.3, and 1 mM EDTA) and autoradiographed. For supershift analysis, a
specific YY1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was
preincubated for 30 min with the nuclear extract prior to addition to the
binding reaction mixture. The His-tagged YY1 protein (His-YY1) was
expressed and purified according to Shi et al. (10) and added in place of
nuclear extracts, as described (23). The YY1 consensus oligonucleotide
(Santa Cruz Biotechnology) was used both as labeled probe and as
unlabeled competitor in EMSAs.

To analyze the TBP binding to Col1a1 promoter sequences, the
reactions were carried out for 45 min at 30 °C, using 150 ng of recom-
binant TFIID (TBP; sc-4000 from Santa Cruz), in a final volume of 20 μl
containing 12 mM HEPES-KOH (pH 7.9), 0.15 mM EDTA, 6 mM MgCl2,
90 mM KCl, 1 mM dithiothreitol, 10% glycerol, 0.5 mg/ml bovine serum
albumin, and 0.4 μg poly(dG-dC). A double-stranded oligonucleotide
containing the TBP/TFIID consensus, 5′-CCAGAGCATATAAT-3′ (forward) and 5′-TCCCTACCTATTTATGCTCT-
GC-3′ (reverse), purchased from Operon, was used as labeled probe and
unlabeled competitor.

**DNA Constructs and Site-directed Mutagenesis—**Col1a1 promoter
fragments from plasmids pG60 and pK1 containing the fragments span-
ning –222 to +113 bp and –84 to +13 bp (7) were subcloned in the pGL2
basic vector (Promega, Madison, WI) and named p350wt and p130wt,
respectively. These reporter constructs were used as templates to gene-
rate the point mutations listed in Fig. 1 by a four-primer polymerase
chain reaction mutagenesis technique (24). DNA fragments containing
point mutations were then cloned into the pGL2 basic vector. Success of
site-directed mutagenesis was assessed by DNA sequencing at the Beth
Israel Deaconess Medical Center DNA sequencing facility using the
ABI PRISM® BigDye™ primer cycle sequencing kit (Applied Biosys-
tems, Foster City, CA) and the automatic DNA sequencer model 373A (Applied Biosystems). The pcMV-YY1 plasmid used in cotransfections was described previously (23). The antisense YY1 plasmid (pCMV-
YY1) was prepared by inserting the YY1 region encoding amino acids
84–854 in the antisense orientation downstream from the CMV prom-
er in the CMV5 vector. Antisense Gal4 inserted in the CMV5 vector
(pCMV-asGal4) served as a negative control.

**Transient Transfection and Luciferase Assays—**Transient trans-
fection experiments were carried out in BALB/c3T3 cells using the Lipo-
fectAMINE PLUS™ reagent system (Life Technologies, Inc.). Cells
were seeded in 6-well tissue culture plates at 2 × 105 cells/well in
Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum
24 h prior to transfection. For each well, 1 μg of Col1a1-luciferase
construct, 5 μg of SV40-β-galactosidase vector, 6 μl of LipofectAMINE
reagent, and 92 μl of Opti-MEM I medium (Life Technologies, Inc.) were
mixed and incubated for 15 min at room temperature. PLUS reagent (4
μl) in 100 μl of Opti-MEM I medium was then added to each reaction
mixture and the incubation was continued for an additional 30 min at
room temperature. Finally, the transfection mixture was combined with 800
μl of Opti-MEM I, and the lipid-nucleic acid complex was transferred to
the washed cell monolayer in each well. After incubation for 5 h at
37 °C, the transfection mix was replaced by Dulbecco’s modified Eagle’s
medium containing 10% fetal calf serum, and incubation was continued
for 18 h. In cotransfection experiments, incubations were continued for
30 h after lipofection prior to harvest of the cells. Cell lysates were
prepared by extraction with 200 μl of reporter lysis buffer (Promega),
and the protein content was determined using the Coomassie Plus
protein assay reagent (Pierce). Luciferase activities were determined by
chemiluminescence assays using the Autolumat LB953 luminometer
(EG&G Berthold, Oak Ridge, TN) and normalized to total protein.

**Binding of Nuclear Factors to the Col1a1 Proximal Promoter—**Previous work in our laboratory showed that constitutive
or IL-1-induced prostaglandins could down-regulate type I coll-
lagen gene expression in fibroblasts and demonstrated the existence of a potential prostaglandin E2-responsive element
within the Col1a1 proximal promoter (19), which overlapped
with a previously described region responsive to TNFα (20).

To further investigate the binding of nuclear proteins to the prox-
imal Col1a1 promoter, we performed EMSA experiments using
Col1a1 sequences spanning –56 to –9 bp and –56 to –25 bp and

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1 The abbreviations used are: IL, interleukin; TNF, tumor necrosis factor; YY1, Yin Yang 1; EMSA, electrophoretic mobility shift assay; TBP/TFIID, TATA box-binding protein; wt, wild-type; USE, upstream element; bp, base pairs; CMV, cytomegalovirus; as, antisense.
compared binding to a probe containing the well known TgTA (25) single-point mutation (mTgTA), as listed in Fig. 1. As shown in Fig. 2, the −56/−9 bp probe bound a specific complex. Surprisingly, the TgTA mutation increased the intensity of the complex by at least 3-fold compared with the wild-type −56/−9 bp probe. In contrast, binding of the complex to the −56/−25 bp probe, which terminated with TATA at the 3’-end, was very weak. These differences in the strength of binding indicated that the presence and position of the TATA box within the oligonucleotide could markedly affect binding activity.

We also introduced mutations into the −56/−25 bp probe at sites homologous with those determined previously to be involved in the TNF-α response of the human COL1A1 promoter (20). Mutation of one of these sites (CCATCAA to CCgTCAA), with (m1) or without (m2) a mutation at the other site (CCAGCT to CCAaCT), located 7 base pairs upstream (upstream element (USE); see Fig. 1), completely disrupted the formation of the specific complex (Fig. 2). Close examination of possible consensus sequences for known factors revealed a potential YY1 binding site (CCAT) that could account for the lack of binding to the m1 and m2 mutants due to the mutation, CCgT, of the core YY1 binding site. Because the nuclear extracts from cells treated with TNF-α did not show any significant or consistent difference in binding to these probes in replicate experiments, nuclear extracts from untreated BALBc/3T3 cells were used for all further EMSA experiments.

**EMSA Analysis of YY1 Binding to the Col1a1 Promoter**—To determine whether the formation of the specific complex to the Col1a1 promoter sequence shown in Fig. 2 was indeed due to binding of YY1, we performed competition assays using the YY1 consensus sequence and Col1a1 probes containing the wild-type sequence −56/−9 bp and the TgTA mutation. In the experiment presented in Fig. 3, the labeled −56/−9 bp and mTgTA probes were incubated with BALBc/3T3 nuclear extracts in the absence or presence of the excess unlabeled YY1 consensus oligonucleotide. Similar to the results shown in Fig. 2, the intensity of binding of the specific complex to the mTgTA probe was increased by 3-fold compared with the −56/−9 bp probe. Binding of this complex to either probe disappeared when the reaction mixture was incubated in the presence of YY1 consensus at 20× or greater molar excess (Fig. 3). Thus, we tentatively identified the binding activity in nuclear extracts as YY1.

**Supershift Analysis of YY1 Binding to the Col1a1 Promoter**—To further characterize the YY1 binding to the Col1a1 promoter, we performed supershift analysis using the YY1-specific antibody and compared binding of proteins in nuclear extracts with binding of purified recombinant His-YY1. As shown in Fig. 4, end-labeled oligonucleotides containing the YY1 consensus binding site and the Col1a1 sequence −56/−9 bp without and with the mutation in the TATA box (mTgTA) were incubated either with nuclear extracts from BALBc/3T3 cells or with the recombinant YY1 protein (YY1) in the absence (−) or presence (+) of the YY1-specific antibody. All three probes were observed to bind a factor in nuclear extracts with mobility similar to bound YY1. Further- more, the YY1 complex produced by either nuclear extract or His-YY1 was clearly supershifted by the YY1 antibody in the presence of either nuclear extract or purified His-YY1. Note that the decreased mobility of the YY1 complex formed with the recombinant protein was ascribable to the N-terminal His tag of the recombinant YY1.
EMSA Competition Analysis Reveals Two YY1 Binding Sites—To further investigate the binding of YY1 and the influence of the TATA box, competition EMSA analyses were performed using the –56/9 bp wild-type sequence and mTgTA as probes. End-labeled probes were incubated with nuclear extracts in the absence or presence of excess competitors containing the wild-type sequences –56/9 bp (wt1) and –56/25 bp (wt2) and mutants thereof. As shown in Fig. 5, the wild-type and mutant sequences spanning –56/9 bp (wt1, mTgTA, m3) were more effective competitors than the shorter sequences derived from –56/25 bp (wt2, m1, m2, m4, and m5). Both wt1 and mTgTA completely blocked binding of YY1 to the wild-type Col1a1 probe, whereas wt1 was a slightly less effective competitor than mTgTA against the binding to the mTgTA probe. On the other hand, the shorter wt2 oligonucleotide competed only partially for binding to both probes. The m1 mutant containing mutations in both the putative YY1 site and the upstream CCAGCT site (the USE) and the m2 mutant containing only the YY1 site mutation did not compete for binding to the –56/9 bp probe and even appeared to enhance binding on the mTgTA probe. However, the m3 oligonucleotide containing the USE and YY1 mutations in the –56/9 bp sequence produced competition similar to wt1 on both probes. This was surprising, because m3 contains the same mutations as those in m1 but in the longer sequence. Nevertheless, the m4 mutant containing the mutation of the USE site or the m5 mutant with the TgTA mutation in the shorter –56/25 bp sequence competed for YY1 binding on the –56/9 bp probe but not on the mTgTA probe (Fig. 5). Thus, the proximity of the TATA box and the YY1 site to the end of the –56/25 bp may explain the lack of effective competition by the wt2, m1, and m2 oligonucleotides, particularly when mTgTA is used as probe.

Upon closer examination, we discovered another potential YY1 site on the reverse strand (5’-CCAT-3’) immediately adjacent to the TATA box. Mutation of this new site (YY1B) in the m6 oligonucleotide, also containing mutations in the USE and the original YY1 site, now designated YY1A, completely prevented competition for YY1 binding (Fig. 6A). Mutation of only the downstream YY1B site (m7) produced a partial competition for binding, thereby indicating that the upstream YY1A site is capable of binding YY1 when the downstream YY1B site is unavailable (Fig. 6A). To determine whether YY1 is capable of utilizing both binding sites, the m3, m6, and m7 oligonucleotides were labeled and compared with wt1 and mTgTA probes for binding to His-YY1. The m3 probe, containing the YY1A site mutation leaving YY1B intact, showed reduced binding to His-YY1 compared with the wt1 and mTgTA compared with the wt1 and mTgTA probes (Fig. 6B). In contrast, the m6 probe with mutations in both YY1 sites did not bind detectable His-YY1 (Fig. 6B), whereas binding to the m7 probe, with only the YY1B site mutated, was not evident on the exposure shown here but was just detectable on overexposed blots not shown. These results suggest that both sites are capable of binding YY1 and that the downstream YY1B site is integrally involved in YY1 binding.

Functional Analysis of the Role of the YY1 Sites in Col1a1 Expression—In order to determine the functional role of the YY1 binding sites in the Col1a1 promoter in fibroblasts, we carried out transient transfection experiments with the –84/+13 core promoter construct (p130wt), which contains the YY1 elements and can support minimal constitutive expression, as well as with
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The luciferase reporter vectors, named for the corresponding mutations or point mutations were transiently transfected into BALBc/3T3 cells. Constructes containing Col1a1 sequences spanning –84 to +13 bp (p130wt), –222 to +113 bp (p350wt), or point mutations were transiently transfected into BALBc/3T3 cells. The luciferase reporter vectors, named for the corresponding mutations listed in Fig. 1, are indicated below each graph: A, pGL2b (empty vector), p130wt, and p130 mutants; B, pGL2b (empty vector), p350wt, and the p350 mutants. Promoter activities are normalized to total protein and expressed as fold expression of p130wt. Each value represents the mean ± S.D. for three independently transfected cultures from one representative experiment. Note that the luciferase activity of the p350wt construct is an order of magnitude greater than that of the p130wt construct.

To compare the functional roles of the YY1A and YY1B sites, we compared the expression of mutant p130 and p350 constructs containing the YY1B mutation in the presence (p130m6 and p350m6) or absence (p130m7 and p350m7) of the YY1A mutation. The expression of the constructs containing the YY1B mutation in both p130 and p350 expressed at dramatically reduced levels compared with the respective wild-type sequences (Fig. 7). The m6 and m7 mutations more effectively decreased expression than the m1/3 and m2 mutations in the p130 promoter. However, among the p130 constructs, only the p130m6 construct with mutations in both YY1 sites expressed at the level of the empty vector (Fig. 7A). These results indicate that YY1B is more important than YY1A for expression of the core p130wt promoter, whereas both sites are required for constitutive expression of the p350wt Col1a1 promoter.

Col1a1 Expression Is Stimulated by Overexpression of YY1 and Inhibited by Antisense YY1—To determine whether overexpression of YY1 could modify Col1a1 expression, cotransfection experiments were performed. Cotransfection of pCMV-YY1 did not significantly affect the low levels of expression of p130wt, except possibly at a higher level (100 ng) of the vector. In contrast, pCMV-YY1 overexpression produced a dose-dependent increase in the expression of p350wt (Fig. 8A). Overexpression of antisense YY1 decreased the activities of both p130wt and p350wt, compared with activities after cotransfection with the antisense Gal4 control vector (Fig. 8, A and B). High levels (200 ng) of pCMV-asYY1 were required to effectively block endogenous YY1 and the low levels of expression of the p130wt construct were more strongly reduced than the higher levels of p350wt expression. Furthermore, overexpression of pCMV-asYY1 together with pCMV-YY1 at equivalent levels (50 ng) prevented the increased p350wt expression due to overexpressed YY1 (Fig. 8A). Finally, cotransfection of pCMV-YY1 increased and asYY1 decreased the activities of p130m1/3 and p130m7 (Fig. 8B), whereas expression of the inactive p130m6 was unaffected (data not shown). These results indicate that both YY1A and YY1B sites can respond to YY1 overexpression but that at least one site is required in the presence of the intact TATAAA motif. These results further confirm that YY1 is a positive regulator of the Col1a1 minimal promoter and that YY1B is the preferred site.

Binding of YY1 and TBP/TFIID to the Col1a1 Promoter—Because YY1 appeared to bind more strongly to the mTgTA probe, whereas the p350mTgTA reporter lacked activity, it was of interest to determine whether TBP/TFIID binding to the TATA box could occur in the presence of YY1. Because TBP binding requirements differ somewhat from those for the standard EMSA, we compared binding of recombinant preparations of YY1 and TBP to the –59/–9 bp (wt1) probe under the different binding conditions. Binding of recombinant TBP to wt1 was observed clearly when the TBP binding buffer (TBP-bb) was used but not in the usual EMSA binding buffer (designated YY1-bb in Fig. 9A) in which binding of YY1 was seen. In TBP-bb, 3-fold YY1 compared with TBP was insufficient to observe YY1 binding, whereas at 15-fold excess YY1 binding occurred and TBP binding was attenuated (Fig. 9A). YY1 and TBP also bound, but at decreased intensities, to the m3 mutant containing the mutation in the YY1A site. In contrast, TBP bound strongly to the m7 mutant with the YY1B mutation. Binding of YY1 added alone appeared to produce a shifted band of lower mobility on the m3 probe, possibly due to decreased DNA bending by YY1 binding to the YY1A site. Nevertheless,
TBP binding to both mutants was attenuated in the presence of 15-fold excess YY1 (Fig. 9A).

To further characterize the YY1 and TBP binding interactions with the Col1a1 promoter, the −59/−9 (wt1) and −59/−25 (wt2) probes were compared using conditions for TBP binding (Fig. 9B). Again, increasing amounts of YY1 were able to attenuate, but not block completely, TBP binding to wt1. Competition analysis showed that excess unlabeled wt1, TBP/TFIID consensus (TA), m3, and m7 were all able to block TBP binding, whereas mTgTA (Tg) was not. Furthermore, the −59/−25 bp (wt2) sequence bound poorly if at all to recombinant YY1 binding occurred even in the presence of TBP (Fig. 9B, right panel). These results indicate that both YY1 and TBP can bind to the longer wt1 probe containing the complete TATAAA motif, whereas TBP binding is absent on the shorter wt2 probe, which ends at TATA.

**DISCUSSION**

In this paper we report the presence of previously unidentified cis-acting elements within the Col1a1 minimal promoter that interact with the YY1 transcription factor. Our results indicate that YY1 binds to either of two sites, one upstream from (YY1A; CCATCA at −40/−34 bp) and the other adjacent to (YY1B; AAGATGGA at −35/−29 bp) the TATA box. Furthermore, we demonstrate using reporter gene studies that the integrity of both the YY1 sites is required for strong transcription directed by the Col1a1 core promoter in fibroblasts. Using probes containing mutations similar to those made in the human COL1A1 promoter by Mori et al. (20), we did not observe any differences in binding due to either IL-1 (data not shown) or TNF-α treatment. However, constitutive expression was significantly altered by mutations in the YY1A and YY1B sites, the latter of which is not present in the human promoter. Nevertheless, treatment with either cytokine did inhibit Col1a1 promoter expression in transient transfections (data not shown). Our results are reminiscent of the findings of Higashi et al. (26), who showed that IFN-γ inhibited expression of the human COL1A2 promoter in transient transfections but did not modify binding of nuclear factors in EMSA experiments. Furthermore, Greenwel et al. (27) have reported that TNF-α inhibits COL1A2 promoter expression via interaction of C/EBPβ and C/EBPδ with elements upstream of the proximal promoter.

Others have shown that only three factors, YY1, TFIIB, and RNA polymerase II, are minimal requirements for accurate initiation of constitutive transcription on a supercoiled adenovirus P5 promoter template DNA, thereby providing an example of accurate and efficient transcriptional initiation by polymerase II in the absence of TBP/TFIID (28). In our transient transfection studies, mutation of the TATA box to TgTA, known to eliminate binding of the TATA box binding protein, TBP/TFIID (25), severely diminished the activity of the Col1a1 −84/−13 core promoter (p130 construct) and completely eliminated expression directed by the strong Col1a1 −222/+113 promoter (p350 construct). This result supports an argument for an involvement of direct interaction of TBP with the Col1a1 TATA box. The involvement of YY1 binding appears to depend upon the promoter context. Disruption of the YY1A site in the p130 core promoter construct results in a modest decrease in activity of ~40%, whereas the same mutation in the p350 construct results in an almost complete (99%) loss of function. Similarly, mutation of the YY1B site adjacent to the TATA box completely reduces p350 activity, but the p130 activity is reduced to the level of activity of p130mTgTA. In contrast, mutations in both YY1 sites are required to completely block the activity of either promoter construct. Therefore, in the case of the Col1a1 promoter, YY1 appears not to function according to the model of Usheva and Shenk (28), where it serves to functionally replace TBP on the

**Fig. 8. Overexpression of YY1 stimulates and antisense YY1 inhibits Col1a1 promoter expression.** The BALB/c3T3 cells were cotransfected with the p130wt or p350wt promoter construct, as indicated, and the expression vectors pCMV-YY1, pCMV-asYY1, and pCMV-asGal4 as negative control. A, the p130wt (open bars) or the p350wt (closed bars) promoter construct was cotransfected with 50 or 100 ng of pCMV-YY1 or 50, 100, or 200 ng of pCMV-asYY1. B, the p130wt (open bars) or the p350wt (closed bars) construct was cotransfected with 50 ng of pCMV-YY1 alone or together with 50 ng of pCMV-asYY1. C, the p130m1/3 or p130m7 construct was cotransfected with 50 ng of pCMV-YY1 or pCMV-asYY1. After transfection, the cells were incubated for 30 h prior to harvest for luciferase activity. Each value represents the mean ± S.D. for three independently transfected cultures from one representative experiment and is expressed relative to that of the p130wt or p350wt promoter construct, respectively.
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FIG. 9. Binding of YY1 and TBP/TFIID to the Col1a1 promoter. A, the end-labeled oligonucleotides, −56/−9 bp (wt1), m3, and m7, were incubated either alone (0) or with recombinant TBP/TFIID or YY1, alone or together, in TBP binding buffer (TBP-bb), except where indicated (YY1-bb, standard EMSA buffer). B, the −56/−9 bp (wt1) and −56/−25 bp (wt2) probes were labeled and incubated either alone (0) or with recombinant TBP (TFIID) or YY1, alone or together, in TBP binding buffer (TBP-bb). Competitor oligonucleotides, wt1, mTgTA (Tg), TBP/TFIID consensus (TA), m3, and m7 were added at 100× molar excess. Note that the same amount of TBP, where added, was used in each reaction, and YY1 was added at concentrations of 3-fold (+), 9-fold (++), or 15-fold (+++) that of TBP.

TATA-less adeno-associated virus P5 promoter by recruiting TFIIB and RNA polymerase II. Rather, because the YY1B site occupies the position of the TFIIB recognition element, it may either stabilize TFIIB interaction with TBP/TFIID (29) or interact with TAFII55 (30).

YY1 is constitutively expressed and is known to interact with other proteins involved in transcriptional regulation, such as c-Myc (31), Sp1 (32, 33), CREB/ATF (34), and NF-κB (35). YY1 has been shown to be involved in suppression of the interferon-γ (IFN-γ) promoter in T cells through binding to an AP-2-like repressor protein and competition with AP-1 for DNA binding (36). On the other hand, YY1 may play a positive role in activation-induced IFN-γ transcription through interaction with NFAT (37). However, YY1 may also repress Sp1- or CREB-mediated transcription by interfering with communica-

tion between coactivators and targets within the transcription machinery (38). For example, repression by YY1 of activation of the low density lipoprotein receptor gene mediated by the sterol regulatory element-binding protein is independent of YY1 binding directly to the low density lipoprotein receptor promoter, because YY1 interacts in solution with Sp1 and sterol regulatory element-binding protein (23). YY1 has been shown to bind to the upstream Col1a2 promoter region at −690/−613 bp and to modify Sp1 binding and repress Sp1-stimulated activity in hepatic stellate cells (39). Compared with the proximal Col1a2 promoter, which binds many of the same factors, the position of YY1 binding sites immediately upstream of the TATA box seems to be unique to the Col1a1 promoter, where our studies show activation rather than repression of transcription by YY1. The observation that YY1 binding is critical for strong transcription by the Col1a1 promoter also suggests that its function is distinct from the inhibitory role played by this factor in vitamin D activation of the osteocalcin gene in osteoblasts (40) or in IL-1β-mediated suppression of the skeletal α-actin gene in myocytes, which is associated with increased abundance and activity of YY1 via a site within a serum response element (41, 42).

The observation that YY1 appears more important for p350 than for p130 activity suggests that YY1 may serve to couple the activities of transcription factors that bind upstream of the −84 bp position to the TBP-dependent core promoter. In this regard YY1 may serve as a facilitator protein, linking an enhancer to a core promoter (43). A somewhat similar situation has been reported recently for the IL1B core promoter, in which binding of the Spi-1/PU.1 transcription factor to the core promoter facilitates the activation by a C/EBPβ factor that binds to a distant enhancer via a protein–protein interaction (44). It is possible that YY1 may serve a similar function in the Col1a1 gene through protein-protein interactions with one or more of the factors that bind to the sequences upstream of −84 bp and drive strong constitutive expression. These factors include CBF, Sp1, and NF-1, which bind to the region spanning −222 to −84 bp (5–9, 45) and could conceivably cooperate with YY1.

CBF appears to play a critical role in the upstream Col1a1 sequence (7). The factors of the CBF/NF-Y family and YY1 have often been observed to bind to overlapping sites in genes and to play a role in mutual modulation of activity (46). In the context of the Col1a1 −84/−13 bp core promoter, where the DNA sequences required for the binding of these factors have been deleted, disruption of YY1 interaction with either of its binding sites in the promoter affects the level of constitutive expression, but to a lesser extent than in the longer p350wt construct. Nevertheless, mutation of both YY1 sites abolishes the activities of both promoter constructs. Thus, YY1 is able to activate Col1a1 transcription in the absence of binding of factors, such as CBF and NF-1, to upstream sites, although at a significantly lower level. However, it is possible that YY1 could also interact with transcription factors present in nuclear extracts but not bound to DNA. Interestingly, binding activities of both CBF/NF-Y and YY1 may be inhibited independently by direct interaction with the Y-Box transcription factor, YB-1 (47). During terminal differentiation of myeloid cells, YY1 transactivates the p91

promoter by competing with the CCAAT displacement protein (48). These findings suggest possible mechanisms by which YY1 may function to release the promoter from inhibition by the upstream factor. YY1 has also been shown to interact with p300 and CBP, both of which have associated histone deacetylase activity and thereby cause chromatin to form a looser conformation permitting other factors to interact more efficiently with DNA (49, 50). In the context of the endogenous Col1a1 promoter, this may allow upstream factors to more easily access and interact with the general
transcription factors. Furthermore, it has been proposed that YY1, via multiple regulatory elements, may support association of the histone H4 gene with the nuclear matrix in a transient open chromatin state (51).

In our experiments, the p350m4 construct, containing the USE mutation (CCAGCT to CCAaCT) at position −45 bp upstream from the YY1A site, showed 30% decreased activity compared with the wild-type construct (p350wt), although the p130m4 construct expressed at a level the same as or higher than the p130wt. Our three-dimensional modeling of the Col1a1 gene promoter, using data derived from the crystal structures of TBP/TFIIB (52) and YY1 (53), did not show any striking evidence of an interaction between the USE at −45 bp and any amino acid residue of the YY1 protein. The oligonucleotide containing the USE mutation (m4) produced the same partial competition as wt2 for binding of YY1 to the wild-type (wt1) and the mTGTA probes (Fig. 5). These results, combined with the transfection data, indicate that the USE probably does not influence the interaction between YY1 and its binding sites in this promoter context. However, the YY1A site appeared to be critical for binding only to the short −56/−25 bp wt2 sequence (m1 and m2), possibly because the location of the YY1B site at close proximity to proteins bound to the TATA region, thus brings YY1-induced DNA bending via the YY1A site could bring the interactions between TBP/TFIID and TFIIB or other factors, such as NF1, that are bound to upstream sites into even, YY1-induced DNA bending via the YY1A site could bring the complexes. To address this issue, we performed EMSA analysis with TFIIB via YY1 domains distinct from its transactivator function suggest that YY1 via the YY1B site may serve to stabilize the interactions between TBP/TFIIB and TFIIB or other members of the transcription initiation complex (29). Although further work will be required to define these interactions, our functional analysis of the YY1A and YY1B sites and cotransfection studies show clearly that YY1 is required for Col1a1 promoter activity and that both sites are important in the context of the intact promoter and upstream constitutive regulatory sites. We conclude that YY1 is necessary but not sufficient for transcriptional initiation of the constitutively expressed Col1a1 promoter and likely functions to integrate an upstream enhancer function with the core promoter.

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