The Y-box Binding Protein YB-1 Suppresses Collagen α1(I) Gene Transcription via an Evolutionarily Conserved Regulatory Element in the Proximal Promoter*

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Received for publication, April 9, 2001, and in revised form, May 25, 2001
Published, JBC Papers in Press, June 6, 2001, DOI 10.1074/jbc.M103145200

Appropriate expression of collagen type I, a major component of connective tissue matrices, is dependent on tight transcriptional control and a number of trans-activating and repressing factors have been characterized. Here we identify the Y-box binding protein-1 (YB-1) as a novel repressor of the collagen type α1(I) (COL1A1) gene. Collagen type I mRNA and protein levels decreased upon overexpression of YB-1 by transfection in NRK fibroblasts. The human, rat, and mouse COL1A1 promoter -220/+115 contains three putative Y-boxes, one of these sites, designated collagen Y-box element (CYE), includes a Y-box plus an adjacent 3′ inverted repeat. DNase-I footprinting and Southwestern blotting with fibroblast nuclear extract demonstrated binding of several nuclear proteins across the CYE, one of which was identified as YB-1. Recombinant YB-1 bound the CYE sequence in gel shift assays with a preference for single-stranded templates. The entire sequence (–88/–48) was required for high affinity binding. Complex formation of endogenous YB-1 with the CYE was established by supershift studies. COL1A1 promoter-reporter constructs were suppressed up to 80% by cotransfection with YB-1 in a variety of cell types. In addition, CYE conferred YB-1 responsiveness on two heterologous promoters further demonstrating the importance of this repressor region. Mung bean nuclease sensitivity analysis suggested that repression is most likely exerted through changes in DNA conformation.

Collagens are major components of the extracellular matrix, where they not only serve as structural proteins but, via interactions with specific integrins, can modulate cell proliferation, differentiation, and function (1). Physiological processes such as wound healing are dependent on tight spatial and temporal expression of collagen genes by mesenchymal cells, and a broad spectrum of fibrotic conditions is associated with collagen accumulation (2). Interstitial collagen type I is a heterotrimetric protein composed of two α1 and one α2 chains encoded by separate genes, collagen α1(I) (COL1A1) and α2(I) (COL1A2), that are tightly and coordinately regulated in a tissue- and cell-type-specific manner (2, 3).

Regulation of collagen gene expression occurs primarily at the level of transcription (2–4). A number of regulatory elements required for constitutive and inducible expression have been identified in the promoter and first intron of both the COL1A1 and COL1A2 genes (2, 5, 6) with gene transcription controlled by the complex interplay of positive and negative regulatory factors. Much of the information on cis-acting elements and their respective binding activities is derived from work on the mouse COL1A1 gene and, in particular, analysis of sequences between −220 and +115. The proximal promoter region of the COL1A1 gene is highly conserved between species (7). Known regulatory elements are summarized in Fig. 1A and include a TATA-box, two regions containing inverted CCAAT sequences, one of which binds CBF (8), as well as binding sites for Sp1 and NF-1/CTF (9), c-Krox (10), and BFCOL1 (11). The CBF binding site between −100 and −96 has been shown to be required for basal activity, and an inhibitory factor, IF2, binds to sequences flanking this site (1). Sp1 and NF-1 have been shown to bind in a mutually exclusive manner to the two sites located between −105 to −78 and −129 to −110. c-Krox binds preferentially to the purine-rich region (−190 to −170), whereas BFCOL1 shows stronger affinity for the pyrimidine-rich sequences between −160 and −130 (11).

Inverted CCAAT-boxes can form the core of binding sites for Y-box binding proteins. The presence of such regions in the COL1A1 promoter together with the previously reported finding that YB-1 (also known as dbP), a prominent member of this family of evolutionarily conserved DNA/RNA binding factors, can up-regulate expression of the matrix metalloproteinase-2 (MMP-2) gene in activated mesangial cells (12, 13), raised the question whether YB-1 might also play a role in regulating COL1A1 expression. YB-1 has been implicated in the regulation of a variety of genes (14) and may, depending on the

* This work was supported by Deutsche Forschungsgemeinschaft SFB542, Project C4 (to P. R. M.), Interdisziplinäres Zentrum für Klinische Forschung “BIOMAT”; Bundesministerium für Bildung und Forschung SFB542, Project C4 (to P. R. M.); National Institutes of Health Grant 5 R01 K24602-02 (to J. T. N.); the Wellcome Trust, UK, Grant 044502Z/95Z/040 (to G. E. L.); and a research fellowship from the Royal College of Surgeons, London (to K. S.). 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.

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§§ The abbreviations used are: COL1A1, collagen α1(I); YB-1, Y-box binding protein-1; rYB-1, recombinant YB-1 protein; CYE, collagen Y-box element; IR, inverted repeat; CMV, cytomegalovirus; MMP-2, matrix metalloproteinase-2; RE-1, response element-1 (YB-1 binding element in the rat MMP-2 promoter); CBF, CAAT binding factor; NF-1, nuclear factor-1; BFCOL1, binding factor of a type-I collagen promoter protein; NRK, NRK-42F rat kidney fibroblasts; MBN, mung bean nuclease; T4-PNK, T4 polynucleotide kinase; CAT, chloramphenicol acetyltransferase; HPLC, high pressure liquid chromatography; EMSA, electrophoretic mobility shift assay; kb, kilobase(s); bp, base pair(s).
cellular context, act as either a transcriptional activator or repressor even of the same gene (15). Closer inspection of the COL1A1 gene sequences between −220 and +115 revealed a third putative Y-box protein binding site with striking similarity to the YB-1 binding element-1 (RE-1) in the rat MMP-2 promoter (12, 13) (Fig. 1B). Notably, the homology extended to a 3′ inverted repeat also present in the RE-1 (Fig. 1C).

Based on these homologies, the present study focused on the functionality of this site, which we have designated the collagen Y-box element (CYE). The data show that YB-1 is expressed in the nuclei of collagen-producing fibroblasts and that both recombiant and endogenous YB-1 bind specifically to the sense and antisense strands of the CYE. Furthermore, YB-1 suppresses the activity of the COL1A1 promoter through this element. A potential insight into the functional activity of this protein is provided by the observation that YB-1 induced strand separation in the inverted repeat region of CYE, which may prevent or disrupt binding of positive regulatory factors and thereby repress transcriptional activity. Overexpression of YB-1 suppresses endogenous collagen gene expression and protein production. These data identify YB-1 as a novel transcriptional repressor of COL1A1 gene expression.

**EXPERIMENTAL PROCEDURES**

**Cells and Culture Conditions**

The rat renal fibroblast cell line NRK-49F (European Collection of Animal Cell Culture, Porton Down, UK) was maintained in Dulbecco’s modified Eagle’s medium/Ham’s F12 medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and amphotericin-B (Life Technologies, Inc.). Confluent cells were made quiescent by incubation in 0.5% fetal calf serum for 48 h. A variety of other cell types were used, which, unless otherwise indicated, were maintained as described above: Chinese hamster ovary cells, rat mesangial cells (15), mouse 3T3 fibroblasts, and fetal Harlan Sprague-Dawley rat cardiac fibroblasts (passages 6–9 (16)).

**Plasmids**

**pSg5-YB-1**—A YB-1 expression vector (pSG5-YB-1) containing the complete human YB-1 open reading frame cloned into the expression vector pSG5 (Stratagene) was kindly provided by J.-P. Ying (University of North Carolina).

**Mouse Collagen a1(I) Promoter Constructs**—pGLα1-2.3 was constructed by subcloning a fragment containing sequences between −2310 and +115 of the mouse COL1A1 gene originating from the genomic clone pG7o (a gift from J. Rossert, INSERM, Hopital Tenon, Paris, France (1)) into the HindIII site of pGL3Basic (Promega). The original 3′ end XbaI site was converted to a HindIII site by ligation to a linker, pCAAGCTTG, and digested with HindIII prior to ligation into pGL3Basic. To create the pGLα1–220 construct containing sequences between −220 and +115, a BglII fragment was excised from pGLα1–2.3, and the plasmid was religated. Sequencing across the cloning sites revealed a discrepancy with the published sequence with an extra G between positions 11 and 12; however, this is not within or close to any putative transcription factor binding site. To avoid confusion with the numbering of transcription factor binding sites described by other investigators, we have used the published base pair numbering to identify regions of interest. The transcriptional start site is from GenBank accession number X45876. The sequence up to −1627 is available from GenBank and the location of the 5′-end of pGLα1–2.3 is from an unpublished sequence.

**Rat Collagen a1(I) Promoter Constructs**—pColCAT3.6/1.6 (also known as B16) was a gift from A. Lichtler and D. Rowe (University of Connecticut Health Center). The plasmid contains rat COL1A1 gene sequences between −3518 and +1584. It is identical to B15 (17) except for an EcoRV to ClaI conversion in the polylinker. To create the promoter deletion constructs pColCAT–1.3 or −1.8 and −0.4–1.6, B16 was digested with NheI (−3382) together with Thh111 (−1282) or MunI (−390), respectively, and religated. The intron-less pColCAT–2.3/0 is identical to B47 (17) except that it lacks the 5′ HindIII fragment between −3544 and −2369 present in B16.

**pGL3P-CYE and pGL3-CMV850-CYE**—A double-stranded oligonucleotide homologous to the −95 to −45 sequence of the mouse COL1A1 promoter, encompassing the entire CYE, was subcloned into the KpnI and BglII sites of pGL2Promoter (Promega) and designated pGL2P-CYE. The oligonucleotide was also cloned between the KpnI and SmaI sites of the pGL3B promoter portion of pGL3-CMV850 (a gift from O. Schick, Erath, University College London, UK) after blunting of the BglII site (designated pGL3-CMV850-CYE). This vector exhibits high basal activity in NRK fibroblasts.

**Electrophoretic Mobility Shift Assay (EMSA)**

Nuclear extracts were prepared as described in (18). Protein concentration was determined by the BCA assay (Pierce). Oligonucleotides were end-labeled with T4 polynucleotide kinase (T4-PNK; Promega) and γ-[32P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech) and gel-purified. EMSAs were performed exactly as described (13) using recombinant YB-1 (rYB-1) protein (5 ng/binding reaction) or nuclear extracts (5 μg/binding reaction). Recombinant YB-1 was prepared from a pRSET vector (Invitrogen) containing an insert coding for a hexahistidine-T7 epitope-YB-1 fusion protein (a gift from Dr. Chien, University of California, San Diego) as described by Mertens et al. (12). For competition experiments, unlabeled oligonucleotides or nonspecific DNA (500× oligo) were added at a 50-fold molar excess over the binding reaction. 15 min prior to the addition of labeled oligonucleotides followed by a 30 min incubation period and subsequent separation on polyacrylamide gels. Titration experiments demonstrated that at lower competitor concentrations (100- and 250-fold molar excess) specific competition was incomplete under the chosen conditions. Relative binding affinities were determined by quantitation of shifted bands using a Phosphorimager system (Bio-Rad). For supershift assays, affinity-purified rabbit anti-YB-1 antibody raised against a C-terminal epitope (13) was added to the nuclear extracts 30 min prior to the addition of labeled oligonucleotides, and the binding reaction was incubated overnight at 4 °C. In controls non-immune IgG was added.

**DNase I Footprinting**

A 32P-labeled probe corresponding to −220/+115 of mouse COL1A1 was generated by polynucleotide chain reaction amplification. Briefly, 4 pmol of a forward primer (5′-CCGGGCGTGAATCGTG-3′), corresponding to vector sequence plus the 5′ 8 bases of the promoter sequence in pGLα1–220, was end-labeled using T4-PNK and γ-[32P]ATP. pGLα1–220 was used as template for a standard polynucleotide chain reaction using the 32P-labeled forward primer, the reverse GLprim2 (Promega), 1 unit of Red Hot DNA polymerase (Abgene), and 1 mM MgCl2. The DNA probe was gel-purified (QIAquick kit, Qiagen). A 10-bp adder (Life Technologies, Inc.), end-labeled with [γ-32P]ATP by T4-PNK, was used as a molecular weight standard. Binding reactions were performed in a final volume of 50 μL 32P-labeled DNA probe (−0.1 ng of labeled DNA) was incubated for 30 min at ambient temperature in 20 μL of binding buffer (10% glycerol, 50 mM KCl, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1 μg of poly(dI-dC) (Sigma)). Nuclear extract, prepared as above, was added, and the reaction was incubated at room temperature for 25 min. The binding reaction was transferred to a tube containing 2 μL of RQ1 DNase I (0.5–1 unit/reaction, Promega) and 1 μL of 250 mM MgCl2, 250 mM CaCl2 (final concentrations 5 mM). After 3 min at room temperature, the reaction was terminated with 100 μL of stop buffer (200 mM NaCl, 30 mM EDTA, 1% SDS), phenol-chloroform extracted, ethanol-precipitated, and resuspended in 95% formamide buffer (Amersham Pharmacia Biotech). Samples were electrophoresed on 6% acrylamide, 7 M urea gels in 1× Tris borate-EDTA buffer, dried, and autoradiographed.

**Mung Bean Nuclease Sensitivity Analysis**

A mung bean nuclease (MBN) sensitivity analysis was performed as described previously (12). The strictly double-stranded, asymmetrically end-labeled probe was prepared by digesting pT4-Luc-CYE Site I with BglII or KpnI, and the resultant overhanging 5′-ends were dephosphorylated with calf intestinal alkaline phosphatase and end-labeled with γ-[32P]ATP using T4-PNK. The DNA fragment was released by BglII/KpnI digestion and gel-purified. About 104 cpm of probe was included in the binding reaction with either rYB-1 (10 ng) or mesangial cell nuclear proteins (10 μg) at saturating concentrations of MBN enzyme (50 units, Promega) as determined by titration.
Southwestern Blot Analysis

Southwestern blot analysis was performed as described (15) using NRK nuclear proteins (50 µg) and 32P-end-labeled CYE oligonucleotide probes (106 cpm/ml).

Western Blot Analysis

Western blotting with affinity-purified rabbit anti-YB-1 antibody (1:1000) was performed as described by Mertens et al. (15) using NRK nuclear proteins (30 µg). For Western blot analysis of Southwestern blots, radioactivity was removed by repeated washing in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol prior to blocking and incubation with anti-YB-1 antibody. For Western blot analysis of collagen of type I protein, lysates were prepared from NRK cells 72 h after transfection with pSG5-YB-1 or pSG5. Proteins (30 µg/sample) were separated on a 10% SDS-polyacrylamide gel, electroblotted, and probed with an anti-collagen type I antibody (Southern Biotechnology Associates Ltd.), diluted 1:1000 in phosphate-buffered saline containing 0.2% Tween 20, followed by ECL detection.

Transient Transfections

NRK fibroblasts, Chinese hamster ovary cells, and NIH3T3 fibroblasts (~70% confluent) were transfected with purified plasmid DNA (Endotoxin-Free Qiagen Maxi-Prep Kit, Qiagen) using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. A mixture of collagen promoter-reporter constructs (0.5–2 µg) and the YB-1 (1.0–1.5 µg) expression vector or the pSG5 control vector was co-transfected using 6 µl of LipofectAMINE. The total amount of transfected DNA was equalized by the addition of pSG5 control plasmid. All transfections were carried out in triplicate. At least two different DNA preparations were tested to eliminate potential preparation artifacts.

After 12 h, transfection medium was replaced with medium containing 60% confluence, and mesangial cells were transfected according to the manufacturer’s instructions using the calcium phosphate precipitation method (21). Luciferase activity was measured using the Hirt lysis buffer (Roche Molecular Biochemicals) for CAT reporter constructs. Luciferase activity was measured using 

RNA Extraction and Northern Blot Analysis

Total RNA was extracted using Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. Northern blotting using an [32P]dCTP-labeled vector DNA (20) or pEGFP-YB-1 (CLONTECH) and was determined at 50–60% (20). Rat cardiac fibroblasts (~90% confluent) and mesangial cells were transfected according to the manufacturer’s instructions using the calcium phosphate precipitation mammalian transfection system (Promega) and T6-50 (Promega), respectively.

RESULTS

Identification of Putative YB-1 Binding Sites in the Mouse COL1A1 Gene Promoter—Sequence analysis of the region ~220 to ~115 mouse COL1A1 gene showing the inverted CAATT-boxes (bold), putative YB-1 binding sites, and the CYE, including the Y-box and the adjacent 3′ inverted repeat (bold italics). The CYE oligonucleotide (Table I) is indicated by a solid line. Known regulatory elements and transcription factor binding sites are illustrated: TATA box, AP-1 (27), NF-1, Sp-1 (9), CBF (8), IF-2 (1), BFCOL1 (11), and e-krox (10). The 12-bp repeat that brackets the proximal CAAT motif (1) is also shown (bold dashed line). The right-angle arrow indicates the start of transcription. B, sequence homology between the Y-box consensus sequence, MMP-2 RE-1, and the putative YB-1 binding regions in the proximal promoter of the mouse COL1A1 gene (Sites I, II, and III). C, comparison of the rat MMP-2 RE1 with the mouse COL1A1 Site I and adjacent sequence showed homologies that include a 3′ inverted repeat (inverted repeats are marked in bold). The region encompassing the Y-box and 3′ inverted repeat (~83 to ~59) was designated the collagen Y-box element (CYE). D, sequence similarities of the CYE in the mouse, rat, and human COL1A1 promoters.

Site I included an inverted repeat (RI) similar to the one present in the RE-1 (Fig. 1C). Based on these homologies the present study focused on this region (~83 to ~59), which we designated the collagen Y-box element. Comparison of CYE in mouse, rat, and human COL1A1 genes showed strong sequence similarity across this element (Fig. 1D).
Nuclear Protein Binding Regions within the −220 to +115 Regulatory Sequence—DNase I footprinting of −220 to +115 of the mouse COL1A1 gene with nuclear extracts from NRK fibroblasts was performed to determine the sites of protein interaction with the promoter. Six protected regions (A–F) were identified (Fig. 2) that lie between −190 and −177 (region A), −177 and −155 (region B), −145 and −135 (region C), −130 and −105 (region D), −95 and −65 (region E), and −65 and −40 (region F). The Y-box of CYE is located within region E, and the IR lies at the boundary between E and F and coincides with a hypersensitive site between −70 and −60 (Fig. 2).

Karsenty and de Crombrugghe (1) previously described 4 protected regions (which they also designated A–D) in DNase I footprinting of the same region of mouse COL1A1 with nuclear extracts from mouse NIH3T3 fibroblasts. Region A, protected by NRK fibroblast nuclear extract, is identical to the synonymous region reported by these investigators. Our regions C, D, and E all lie within the protected regions previously identified; however, the regions we have designated B and F have not been described previously. For comparison, DNase I footprinting of the −220 to +115 segment with NIH3T3 extracts was also performed (data not shown). When 20–30 μg of NIH3T3 or NRK nuclear proteins were used, the footprints obtained were identical to the published ones (1). However the addition of higher protein concentrations (48 μg) revealed the additional protected regions described herein.

Recombinant YB-1 Binds to CYE Oligonucleotides—First, a series of DNA binding studies were performed with rYB-1 and oligonucleotides containing the complete CYE sequence (−88 to −48; Table I), both sense (SS1) and antisense (SS2) strands. As has been reported previously, double-stranded probes demonstrated only weak rYB-1 binding activity (data not shown), and therefore these studies focused on single-stranded binding assays. rYB-1 formed several specific complexes with both SS1 and SS2 consisting of closely migrating bands (SS1, lane 1, and SS2, lane 5 in Fig. 3A). Competition experiments were performed with truncated CYE oligonucleotides encompassing either the Y-box (Y, Table I) or the inverted repeat (IR, Table I). Homologous competitor DNA completely blocked rYB-1 binding to the CYE (SS1, lane 4, and SS2, lane 8), whereas competitor oligonucleotides Y and IR only partially inhibited binding. The Y oligonucleotide diminished the fastest migrating bands formed with SS1 and SS2 (lanes 2 and 6), whereas the other complexes were unaffected. The IR oligonucleotide did not compete for rYB-1 binding to SS2 (lane 7). Thus, maximal rYB-1 binding to the CYE is dependent on the whole sequence including the Y-box and 3′ adjacent IR. To substantiate this finding, a direct comparison of rYB-1 binding to the SS1 of CYE, Y, and IR was performed (Fig. 3B). Specific complex formation was observed with Y (compare lane 1 with lanes 4 and 5), whereas IR was not bound by rYB-1 (lane 2). A comparison of the relative binding affinities revealed an −10-fold higher affinity of rYB-1 for the complete CYE (lane 3) compared with the Y-box alone (lane 1). These results emphasize the importance of the whole sequence context for high affinity YB-1 binding. To extend this finding and further define the sequence requirements for binding, specific mutations (G to T substitutions) were introduced into the CYE sense strand in either the Y-box (CYEmut1, Table I) or the IR (CYEmut2, Table I). As seen in Fig. 3C, rYB-1 binding was not, as expected, diminished with the introduced mutations in the Y-box but was even increased. In contrast, binding of rYB-1 to the CYEmut2 oligonucleotide was somewhat weaker than binding to the wild type oligonucleotide. These results emphasize the importance of the whole sequence context for high affinity YB-1 binding and indicate that simple base substitutions in the Y-box sequence do not
necessarily result in weaker binding. An oligonucleotide spanning the region −112 to −83 encompassing the Site II Y-box at −100 to +96 (Fig. 1A) failed to bind rYB-1 (lane 4 in Fig. 3C). This finding again supports the notion that YB-1 binding not only requires a Y-box but is dependent on more complex sequence determinants, and although Site II displays high homology to the YB-1 binding consensus sequence, it is not a functional binding site for the recombinant protein.

Sizing of the CYE Binding Activities—Western blot analysis showed that YB-1, a protein with a calculated molecular size of 35 kDa and apparent size of 52 kDa in SDS-polyacrylamide gel (23), is expressed in nuclear extracts from quiescent and proliferating NRK cells (Fig. 4A). Anti-YB-1 antibody also cross-reacted with two other proteins (−200 and 35 kDa, indicated by asterisks).

Southwestern blotting of nuclear proteins was used to determine the sizes of the CYE-binding proteins (Fig. 4B). Single-stranded sense (SS1) and antisense (SS2) CYE oligonucleotides bound several proteins from NRK fibroblasts with estimated molecular sizes of 90–200, 54, and 35 kDa. Two of these activities were of the same size as those detected by the antibody (see above). Some differences in the binding were observed with SS1 compared with SS2, with additional SS2 binding activities of −69 and 14 kDa. A comparison of extracts from quiescent and proliferating cells showed differential regulation of some CYE-binding proteins with the appearance of a novel high molecular weight protein binding to SS2 in proliferating cells. Subsequent Western blotting of the same membrane with anti-YB-1 antibody confirmed the presence of endogenous YB-1 (52 kDa; arrow in Fig. 4B) with similar levels of the protein in quiescent and proliferating cells. The identity of the other CYE-binding proteins remains to be established. Data base searches of this region for transcription factor binding sites showed potential binding sites for Sp1, NF-1, LBP-1, and Ets family members.

To elucidate protein-DNA complex formation under nonde-naturing conditions, EMSAs were performed with mesangial cell (a cell type known to constitutively express YB-1 (15)) or NRK nuclear proteins and SS1 and SS2 CYE oligonucleotide probes. As shown in Fig. 5A, several distinct complexes formed with SS1 and NRK nuclear proteins (lane 3), with complexes 2 and 3 exhibiting the same mobilities as those formed with mesangial cell nuclear extract (lane 2). Incomplete competition was observed with specific competitor, i.e. complex 3 was lost, whereas complex 2 was diminished (lane 4). Nonspecific competitor (500×) did not affect complex formation (lane 5). When the IR and Y oligonucleotides were used as competitors, IR did not compete for binding, whereas Y partially competed for complex formation (lanes 6 and 7). In parallel experiments using SS2 as probe (Fig. 5B), three distinct complexes of similar mobilities could be detected with mesangial cell (lane 2) and NRK (lane 3) nuclear proteins. Homologous competitor DNA diminished all bands with NRK proteins (lane 4), whereas heterologous DNA had no effect (lane 5). With IR or Y oligonucleotides as competitors (500×), complexes 2 and 3 were diminished but not completely inhibited (lanes 6 and 7). Taken together, these results demonstrate the formation of several closely migrating complexes with both strands of the CYE. Major complex formation is dependent on the whole sequence, as the Y and IR sequences only partially diminish complex formation.

To test for YB-1 participation in this regard, supershift studies were performed using a specific anti-YB-1 antibody (Fig. 5C). The anti-YB-1 antibody used in this study is directed against epitopes in the C terminus of the protein (13), which also contributes to DNA binding (26). As a result this antibody mainly disrupts DNA binding, leading to diminished bands (bands designated 4 and 5 in lane 2 with SS1 and 9, 10, and 11 in lane 8 with SS2), whereas supershifts are rather weak and are visualized only with prolonged autoradiography (indicated by asterisks in lanes 5 and 11). The supershift with SS1 in lane 5 nearly coincided with preformed complexes (2>) and exhibited a similar mobility as that observed with SS2 in lane 11. In addition, a high mobility band (designated 12>) appeared with SS2, most likely representing a protein that interacts with YB-1 and by itself binds the probe. Unrelated rabbit IgG had no effect on complex formation. These findings indicate that endogenous YB-1 binds to both CYE strands forming several distinct complexes.

### Table I

| CYE Oligonucleotides | Y-box | Inverted repeat |
|----------------------|-------|-----------------|
| CYE SS1              | GGCCGCTGCTGCTCCCTCCCTCCAGAGGAGGGTTCTCCCT | |
| CYE SS2              | GCCCGAGGACGAGGAGGGAGGCTCTGCTCCCTCCACAGAGGAGGGTTCTCCCT | |
| Y                    | GGCCGCTGCTGCTCCCTCCCTCCAGAGGAGGGTTCTCCCT | |
| IR                   | CCTCCCTCCAGAGGAGGGTTCTCCCT | |
| Cytomt1              | CTGGG GCCGCTGCTGCTGCTCCTCCCTCCCTCCAGAGGAGGGTTCTCCCT | |
| Cytomt2              | CAGGGGCCGCTGCTGCTGCTCCTCCCTCCCTCCAGAGGAGGGTTCTCCCT | |

YB-1 Repression of Collagen Gene Transcription

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Previous studies have suggested that YB-1 can promote strand separation in a sequence-specific fashion (22). MBN sensitivity analysis, which specifically detects single-stranded DNA regions, was used to test for strand separation within the CYE (Fig. 6). A strictly double-stranded probe containing the entire CYE was generated and labeled asymmetrically at the 5′-end. The absence of banding upon addition of MBN to the probe alone (lane 2) confirmed the double-stranded nature of the probe. In the presence of rYB-1, MBN produced a distinct banding pattern that was confined to the 3′ IR (lane 3) with no banding in the Y-box motif. Nuclear extracts from quiescent and serum-stimulated mesangial cells induced a similar banding pattern to that observed with rYB-1, which was enhanced with prolonged serum exposure (lane 4-6). Strong bands at the 3′-end indicated the presence of exonuclease activity. To confirm the specificity of the DNA structural changes and exclude phasing of the enzyme to the ends of the probe, experiments were repeated with probes labeled at the 5′-end of the opposite strand. A similar pattern was obtained with these probes, i.e. single-stranded regions appeared in the IR region (lane 7),
confirming that YB-1 induces DNA strand separation in the inverted repeat region of CYE.

YB-1 Suppresses Collagen \( \alpha(1) \) Promoter Activity—In NRK cells co-transfected with mouse COL1A1 promoter-reporter constructs and a eukaryotic YB-1 expression vector, YB-1 suppressed the activity (2–3-fold) of both pGLa1–2.3 (containing 2.3 kb of 5’ sequence) and pGLa1–220 (containing 220 bp of 5’ sequence) (Fig. 7A). Suppression of pGLa1–220 by YB-1 was concentration-dependent (Fig. 7B). Although co-transfection of the empty vector, pSG5, caused some decrease in mouse COL1A1 promoter activity, the YB-1-dependent effect was more marked and concentration-dependent. YB-1 overexpression suppressed pGLa1–220 activity in a variety of cell types including rat mesangial cells (13.1% of control cells co-transfected with the empty pSG5 vector and the collagen promoter construct), primary cultures of rat cardiac fibroblasts (27.6% of control), mouse 3T3 fibroblasts (21% of control), and Chinese hamster ovary cells (49% of control) (Fig. 7C).

The proximal COL1A1 promoter sequence is highly conserved between species (27). To determine whether YB-1 spe-
An elucidation of the molecular mechanisms governing collagen production is essential to understanding not only normal tissue morphogenesis and homeostasis but also disease processes associated with under- or over-expression of this protein. The present study focused on the regulation of the collagen α1(I) chain in renal fibroblasts (NRK-49F) as a model collagen-producing connective tissue fibroblast. Previous studies on the COL1A1 gene sequence between −220 and +115 have demonstrated its importance in basal and inducible gene regulation, and a number of cis-regulatory elements and their cognate binding proteins have been identified (Fig. 1A). The presence of two inverted CCAAT-boxes in this region, which can form the core for the binding sites of Y-box binding factor-1 (YB-1), together with the previously reported finding that YB-1 can up-regulate MMP-2 gene expression (13), raised the question of whether YB-1 might also play a role in regulating COL1A1 expression.

Previously, binding of NF-1 and CBF/NF-Y to the inverted CCAAT-boxes at −100 to −96 (Site II; Fig. 1B) and −126 to −122 (Site III) in the COL1A1 promoter has been demonstrated (8, 9) but, apart from one report (6), the potential role of Y-box binding factors in regulating collagen gene expression has not been well studied. This prompted a closer inspection of the −220 to +115 region for potential YB-1 binding sites, and revealed a third putative site (−83 to −72) with striking similarity to the YB-1-binding element in the MMP-2 gene (13). The present study focused on the functionality of this region (−83 to −59), which we designated the collagen Y-box element.

DNase I footprinting of the −220 to +115 sequence of COL1A1 with NRK nuclear proteins generated a closely arranged pattern of footprints extending from −190 to −40 similar to that described by other investigators using nuclear proteins from mouse NIH3T3 fibroblasts (1). Titration of NRK nuclear proteins to saturate the binding sites revealed two additional, novel footprints between −177 and −155 and between −65 and −40. A strong hypersensitive site at the boundary of footprints E and F coincided with CYE and implied the presence of potentially important regulatory elements in this region. Notably, the Y-box of CYE is located in region E and the more 3′ portion of the inverted repeat in region F. In further support of the functional importance of this site, the CYE region shows strong inter-species homology (7) suggesting evolutionary conservation of this element.

YB-1 is a prominent member of the highly conserved Y-box binding factor family with an ever-increasing number of target genes (19). Previously, trans-activation of the rat COL1A1 gene expression by another member of the Y-box transcription factor family, chkYB-1b (6), has been demonstrated. However this protein binds a more 5′ region between −200 and −133, and the relatedness of chkYB-1b to YB-1 is still unclear. Our data suggest different modes of action of the two proteins in the context of the COL1A1 promoter.

To elucidate the functionality of CYE, we first examined binding of rYB-1 to this region. In EMSA, rYB-1 bound to CYE oligonucleotides with a preference for single-stranded sequences of both coding and non-coding strands. Sequence specific binding of YB-1 and other Y-box proteins to single-stranded DNA has been demonstrated previously (14, 19, 28–31). Although YB-1 was originally cloned as a CCAAT binding factor (32), there does not seem to be an absolute requirement for this motif, and many YB-1 binding sites contain either an imperfect CCAAT-box or lack one altogether, suggesting an important role for flanking sequences in DNA recognition by this protein. Interestingly, an oligo-

**FIG. 4. YB-1 binding activity in NRK fibroblast nuclear extracts.** A, Western blot analysis of nuclear extracts (30 μg) from quiescent (lane 1) and proliferating (lane 2) NRK fibroblasts with anti-YB-1 antibody showed the presence of YB-1 protein (52 kDa; arrow). The antibody detected two additional bands (*) with apparent molecular size of >200 and 35 kDa. Lane 3, control without primary antibody. The position of the molecular size markers, 14–200 kDa, is shown. B, Southern blotting of nuclear extracts (50 μg) from quiescent (lanes 1 and 3) and proliferating (lanes 2 and 4) NRK cells with SS1 and SS2 CYE oligonucleotides showed binding of several nuclear proteins. The presence of YB-1 (54 kDa; arrow) was confirmed by Western blotting of the same membrane with anti-YB-1 antibody. The position of the molecular size markers, 14–200 kDa, is indicated.

**Over-expression of YB-1 Suppresses Endogenous Collagen Gene Expression and Collagen Production—Northern blot analysis of NRK cells transfected with the YB-1 expression vector showed that overexpression of YB-1 suppressed endogenous COL1A1 mRNA levels in a concentration- and time-dependent manner (Fig. 8A). The decrease in collagen gene expression was reflected in decreased collagen production as measured by HPLC (Fig. 8B). Western blotting with an antibody to collagen I antibody also showed a trend toward decreased protein levels with a decrease of 17.8 ± 1.25% (mean ± S.D. of two experiments with triplicate wells in each experiment) in pSG5-YB-1 transfected cells compared with controls.

**DISCUSSION**
nucleotide (−112 to −83) encompassing a bona fide inverted CCAAT-box (−100 to −96) in the COL1A1 promoter (Site II) failed to bind rYB-1 despite the fact that sequence comparison showed a near perfect match to the Y-box consensus. The importance of flanking sequences in the CYE is reinforced by the diminished, or absent, binding of truncated oligonucleotides Y and IR. The complexity of the sequence requirement for binding is further emphasized by mutational analysis. Previous studies have suggested that guanine bases are important for YB-1 binding (22); however, G to T substitutions in either the Y-box or the inverted repeat of CYE did not abolish rYB-1 binding to CYE. These data suggest that YB-1 may contact the promoter over a number of base pairs, making mutational analysis using limited base substitutions difficult to interpret.

Endogenous YB-1 also bound CYE oligonucleotide probes, forming several distinct complexes on both sense and antisense strands. The nature of the multiple complexes is not clear but may represent the formation of multimers, as has previously been shown to occur with rYB-1 (15, 22). Moreover, on Western blots of nuclear proteins the anti-YB-1 antibody cross-reacted with three bands, one with the expected molecular size for YB-1.

**Fig. 5.** Endogenous YB-1 participates in specific complex formation with CYE. A, EMSA of mesangial cell (MC NE, lane 2) or NRK (lane 3) nuclear extracts with CYE SS1 oligonucleotide showed the formation of several complexes (complexes 1–4) that were partially competed by specific DNA (500× SS1, lane 4) and Y oligonucleotide (lane 7) but not with nonspecific or IR oligonucleotides (lanes 5 and 6). B, EMSA of mesangial cells (lane 2) or NRK (lane 3) nuclear extracts with CYE SS2 oligonucleotide showed the formation of three complexes (complexes 1–3) that were competed by specific competitor (lane 4) but not by nonspecific DNA (lane 5). The IR and Y oligonucleotides diminished the intensity of complex 2 and 3 (lanes 6 and 7). C, supershift analyses were performed using an anti-YB-1 antibody. Visualization of supershifts required a prolonged exposure time of 36 h (SS1, lanes 4–6; SS2, lanes 10–12). Complexes formed are indicated by numbers to the left of each panel (SS1, lanes 1 and 4, complexes 1–6; SS2, lanes 7 and 11, complexes 7–11). Inclusion of the antibody led to additional low mobility complexes (*, lanes 5 and 11), whereas several complexes (complexes 4, 5, and 9–11) were diminished. With SS2, inclusion of the antibody led to the formation of a high mobility complex designated as 12. Unrelated rabbit IgG was added to control reactions (lanes 3, 6, 9, and 12).
as well as a smaller protein, potentially a proteolytic fragment of YB-1 (33, 34), and a third, larger protein that has not been characterized. Alternatively, the multiple bands may be produced by the interaction of as yet undefined proteins with the oligonucleotide or complex formation between YB-1 and other factors. Southwestern blotting suggested that there are a number of single-stranded DNA-binding proteins of different molecular sizes that can interact with this region. Data base searches of the CYE region for transcription factor binding sites (35) showed potential binding sites for Sp1, NF-1, LBP-1, and Ets family members. Analysis of YB-1 interactions with other transcription factors was not performed in the present study. However, in other systems, YB-1 has been shown to act indirectly by affecting the activity of other regulatory proteins including Sp1 (36–39). Sp1 is known to activate COLIA1 transcription (3), and an Sp1 binding site lies in close proximity to CYE raising the possibility of reciprocal regulation by Sp1 and YB-1.

In transient co-transfections, YB-1 dose-dependently suppressed COLIA1 promoter activity via sequences within the −220 to −115 region. A similar suppression of activity by YB-1 was observed with constructs extending 2.3 kb 5′ of the transcription start-site, suggesting that the dominant repressive site lies in the proximal region and arguing against upstream regulation.

**Fig. 6.** YB-1 induces single-stranded regions in CYE. MBN sensitivity analysis was performed to identify regions of single-strandedness. Incubation of the double-stranded γ32P end-labeled CYE oligonucleotide probe alone (lane 1) or with MBN (lane 2) had no effect. rYB-1 (10 ng) induced banding in the 3′ inverted repeat sequence (arrows) but not in the Y-box (lane 3). A similar pattern was detected with nuclear proteins from serum-synchronized mesangial cells (MC-NE, lanes 4–6). Banding in the IR region was also observed when mesangial cell nuclear extract was added to probes labeled at the 5′-end of the opposite strand (lane 7).

(52 kDa) as well as a smaller protein, potentially a proteolytic fragment of YB-1 (33, 34), and a third, larger protein that has not been characterized. Alternatively, the multiple bands may be produced by the interaction of as yet undefined proteins with the oligonucleotide or complex formation between YB-1 and other factors. Southwestern blotting suggested that there are a number of single-stranded DNA-binding proteins of different molecular sizes that can interact with this region. Data base searches of the CYE region for transcription factor binding sites (35) showed potential binding sites for Sp1, NF-1, LBP-1, and Ets family members. Analysis of YB-1 interactions with other transcription factors was not performed in the present study. However, in other systems, YB-1 has been shown to act indirectly by affecting the activity of other regulatory proteins including Sp1 (36–39). Sp1 is known to activate COLIA1 transcription (3), and an Sp1 binding site lies in close proximity to CYE raising the possibility of reciprocal regulation by Sp1 and YB-1.

**Fig. 7.** Overexpression of YB-1 suppresses the COLIA1 promoter via CYE. A, NRK fibroblasts were co-transfected with mouse COLIA1 promoter-reporter constructs (pGLA1–2.3 and pGLA1–220, 2 µg/well) and either pSG5 or pSG5-YB-1 (0.5 µg/well). Luciferase activity was measured 24 h after transfection. B, NRK cells were transfected with pGLA1–220 (2 µg/well) and increasing concentrations of pSG5-YB-1 were transfected into different cell types: 3T3 fibroblasts (3T3), rat cardiac fibroblasts (RCF), rat mesangial cells (MC), Chinese hamster ovary cells (CHO). Luciferase activity was measured after 24 h. The activity in cells transfected with pSG5-YB-1 was calculated relative to cells transfected with pSG5. Because absolute luciferase activity varied in different cell types, the data are presented as % activity relative to control values set as 100%. Data are from a representative experiment with triplicate determinations.
sites capable of over-riding this repression. Repression of mouse COL1A1 promoter-reporter constructs by YB-1 in a number of different cell types implies a conserved regulatory mechanism. Furthermore, YB-1 also repressed activity of the rat proximal promoter. Unlike the mouse COL1A1 promoter, the rat promoter appears to be further repressed by YB-1 when the sequence is extended up to −3.6 kb, suggesting additional responsive upstream sites in the rat gene. In support of this idea is a recent report by Stoddart et al. (40) in which they describe (as unpublished observations) the cloning and characterization of an inhibitory protein identified as YB-1, which binds to the TGF-β activation element at −1624 bp in the rat COL1A1 promoter (40). However, sequences in this region show little homology in the rat and mouse genes. The CYE also confers YB-1 responsiveness on two different viral promoter contexts.

Numerous single-stranded DNA-binding proteins have been identified (41–45), raising the question of how these proteins approach their binding motifs within the DNA. YB-1 has both double- and single-stranded DNA binding properties and, most importantly, can induce DNA strand separation (12, 22, 46). This observation led to a model in which the repressive YB-1 effect is explained by this protein opening up the DNA and thereby preventing binding of other transcription factors to the double-stranded sequence (22). In the COL1A1 promoter, MBN sensitivity analysis showed that YB-1 specifically induced strand separation in the inverted repeat sequence of CYE, suggesting that this model of transcriptional repression by YB-1 may also apply to the COL1A1 gene.

In conclusion, our data identify YB-1 as a novel repressor of COL1A1 gene transcription acting via an evolutionarily conserved element, the CYE, in the proximal promoter. In normal intact tissues, collagen type I is generally expressed at low levels, and it is likely that repressor elements, particularly in the minimal promoter, play an important role in maintaining tight transcriptional control. Further, we speculate that lifting of YB-1 repression may be important in the activation of gene transcription. Future studies aimed at characterizing the mechanism by which YB-1 regulates collagen gene transcription should provide additional insights into the regulation of this major constituent of the extracellular matrix and provide new strategies for modulating its expression.

Acknowledgments—We are grateful to those individuals cited in the text who kindly provided reagents. We are indebted to J. Palmen (Cardiovascular Genetics, Department of Medicine, Royal Free and University College Medical School) for help with DNA sequencing and to L. Reynolds (Richard Dimbleby Imperial Cancer Research Fund, Department of Cancer Research, St. Thomas’s Hospital) for help with HPLC analysis.

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The Y-box Binding Protein YB-1 Suppresses Collagen \(\alpha_1(1)\) Gene Transcription via an Evolutionarily Conserved Regulatory Element in the Proximal Promoter

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*J. Biol. Chem.* 2001, 276:29880-29890.
doi: 10.1074/jbc.M103145200 originally published online June 6, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103145200

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