Glomerular Mesangial Cell-specific Transactivation of Matrix Metalloproteinase 2 Transcription Is Mediated by YB-1*

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Mesangial cell (MC) activation plays a pivotal role in the development of the end stage sclerotic lesion characteristic of most forms of chronic glomerular disease. We have previously demonstrated that MC activation is directly linked to high level expression of the matrix metalloproteinase-2 (MMP-2) enzyme (Turck, J., Pollock, A. S., Lee, L., Martí, H.-P., and Lovett, D. H. (1996) J. Biol. Chem. 271, 15074–15083), the transcription of which is regulated in a tissue-specific fashion. Recent studies (Harendza, S., Pollock, A., Mertens, P. R., and Lovett, D. H. (1995) J. Biol. Chem. 270, 18786–18796) delineated a strong cis-acting enhancer element, designated MMP-2 RE1, within the 5′-flanking region of the rat MMP-2 gene. Gel shift, DNA footprint, and transcriptional analyses mapped the enhancer element to a unique 40-base pair (bp) sequence located at −1232 to −1282 bp relative to the translational start site. Bromodeoxyuridine-substituted UV cross-linking of the 40-bp enhancer element with MC nuclear extracts yielded a single protein of 52 kDa, while Southwestern blot analysis with MMP-2 RE1 demonstrated three hybridizing nuclear proteins of 52, 62, and 86 kDa size. Screening of a human MC cDNA expression library with MMP-2 RE1 exclusively yielded clones with the identical sequence of the transcription factor YB-1. Western blot and supershift gel analysis of MC nuclear extracts with an anti-YB-1 antibody confirmed the presence of YB-1 within the shifted complex. Examination of the MMP-2 RE1 sequence revealed an incomplete Y-box sequence (CTGCTGGGCAAG), which specifically interacted with recombinant YB-1 on DMS protection footprinting analysis. YB-1 protein preferentially bound the single-stranded components of the 40-bp MMP-2 RE1 and, with increasing concentrations, formed multimeric complexes. Co-transfection of YB-1 in MC increased the enhancer activity within the context of the native MMP-2 promoter, while transfection of non-MMP-2-synthesizing glomerular epithelial cells with YB-1 led to transcriptional suppression. This study indicates that YB-1 is a major, cell type-specific transactivator of MMP-2 transcription by glomerular mesangial cells.

The matrix metalloproteinases (MMPs),¹ which include the interstitial collagenases, stromelysins 1–3, matrilysin, 92-kDa gelatinase B (MMP-9), and the 72-kDa gelatinase A (MMP-2), constitute a family of matrix-degrading enzymes with distinctive extracellular matrix substrate specificities. These enzymes are characterized by activity at neutral pH, secretion in latent, proenzyme forms, dependence upon zinc for catalytic activity, and inhibition by tissue inhibitors of metalloproteinases 1–3 (1). While MMP-2 and MMP-9 share similar substrate specificities, encompassing type IV and V collagen, fibronectin, and laminin (2, 3), their patterns of expression are distinct. MMP-2 has been the focus of considerable interest, since high level expression of this enzyme in metastatic tumor cells correlates with overall metastatic potential (4, 5), presumably due to enhanced degradation of the major basal membrane component, Type IV collagen.

Furthermore, MMP-2 is of importance in inflammatory reactions found in numerous tissues, including the kidney. The development of the sclerotic lesion in chronic glomerulosclerosis is temporally correlated with high level expression of MMP-2 (6). Remarkably, MMP-2 acts not only as a matrix-degrading enzyme but also directly stimulates growth by glomerular mesangial cells (MC) (7) with assumption of an inflammatory phenotype characterized by increased synthesis of prosclerotic interstitial collagens.

Although in most cell culture systems MMP-2 expression is constitutive in nature, there are cells described with a differentially regulated expression of MMP-2 both in vivo and in vitro. For example, MMP-2 is highly expressed in a temporally limited pattern in the developing murine lung and kidney (8). In vitro, MMP-2 expression by MCs can be stimulated with interleukin-1β, tumor necrosis factor-α, and transforming growth factor-β (9). The 5′-flanking region up to −1686 bp relative to the translation start site of the rat MMP-2 gene was recently evaluated for the existence of MC-specific regulatory elements (10), with the resultant identification of an apparently unique, 40-bp cis-acting enhancer element located at −1282 to −1322 bp relative to the translation start site, designated MMP-2 RE1. The MMP-2 RE1 confers cell-specific transactivation of luciferase reporter constructs containing MMP-2 RE1 in conjunction with either the homologous MMP-2 or a heterologous SV40 promoter. Furthermore, specific DNA binding activity to this element was demonstrated in MC nuclear extracts (10). Although nuclear protein binding activity to this enhancer element was also detected in other cell types, positive transactivation in conjunction with the homologous MMP-2 promoter was confined to MCs.

¹The abbreviations used are: MMP, matrix metalloproteinase; MC, mesangial cell; GEC, glomerular epithelial cell; IRE, interferon response element; SV40, simian virus 40; DMS, dimethyl sulfate; EMSA, electrophoretic mobility shift; bp, base pair(s); PBS, phosphate-buffered saline; DTT, dithiothreitol; BrdUrd, bromodeoxyuridine.
Given these findings, we set out to identify the MC nuclear protein binding activity that interacts with the MMP-2 RE1 by expression screening of a MC cDNA library. These studies have identified the transcription factor YB-1 as a component of the MMP-2 transactivating complex. YB-1 was found to preferentially bind both the sense and antisense single-stranded components of the MMP-2 RE1, as compared with double-stranded DNA. Co-transfection studies using a YB-1 expression vector and reporter constructs containing the MMP-2 RE1 confirmed the role of YB-1 as a regulator of MMP-2 transcription, with cell-specific transactivation or repression.

**MATERIALS AND METHODS**

**Cells and Culture Conditions**—The isolation and maintenance of rat glomerular mesangial and epithelial cells have been described in detail (9, 11–13). Mesangial cells were maintained in RPMI 1640 medium supplemented with 1% nonessential amino acids, 2 mM glutamine, 100 µg of streptomycin, 100 units/ml penicillin, and 10% fetal bovine serum. Glomerular epithelial cells were grown in K1 medium (DMEH-16/F12 medium (50:50) supplemented with 2 µM glutamine, 10 mM HEPES, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenous acid, 100 µg of streptomycin, 100 units/ml penicillin, and 2% N-Serum (Collaborative Biomedical Products). YB-1 and MMP-2 Transcription

**Plasmids**—The upstream 1686 bp relative to the translation start site of the MMP-2 gene, which includes the MMP-2 RE-1 and the homologous MMP-2 promoter (10), were subcloned into the promoterless luciferase expression vector pGL2-Basic (Promega) and designated as pT4-Luc 13686. An 80-µmol sequence, 3′CTCGCTGGGAAAATTTGTCTCATCCCCGCTGGCACTTGAAGTTGCAAAACACCATAGACTCAAGGCGCGACTAAGCTT 5′, which includes the MMP-2 RE1 sequence (underlined) between −1322 and −1262 bp, was subcloned into pGL2-Promoter which includes the heterologous SV40 promoter, and designated as pT4-Luc 13424. The 40-µmol sequence between −1322 and −1262 bp was also subcloned into pGL2-Promoter and was designated pT4-Luc 13422B-P. A YB-1 expression vector (PGS5-YB-1) was kindly provided by Dr. Jenny F.-Y. Ting (University of North Carolina) and consisted of the complete YB1 open reading frame cloned into the expression vector pSG5 (Stratagene). The β-galactosidase expression vector pSV-β-galactosidase (Promega), was included as a standardization control.

**Nuclear Cell Extracts**—Cells of each studied type were grown to 90% confluency in 150-mm tissue culture dishes, washed twice with ice-cold phosphate-buffered saline (PBS) without calcium and magnesium, and confluency in 150-mm tissue culture dishes, washed twice with ice-cold PBS, and extracted with 400 µg of poly(dI-dC), and 12 µg of acetylated bovine serum albumin in a 2 µM NaCl solution (PBS) and nuclei were extracted by centrifugation at 14,000 g at 4 °C overnight in binding buffer. YB3 is a member of the Y-box tran-f acetion factor family with an overall amino acid homology between the proteins of 79 and 100% for the most conserved DNA binding domain (16). The radiolabeled oligonucleotides were added 60 min prior to electrophoresis. For competition experiments, nonlabeled oligonucleotides were added in a 500 µM excess or as indicated in the figure legends.

**Southern Blot Analysis**—Nuclear cell extracts from MCs and GECs (30 µg) were electrophorased on 10% SDS-polyacrylamide gels and transferred by electroblotting onto nitrocellulose membranes. The nitrocellulose membranes were blocked in 25 mM HEPES, pH 8.0, 10% glyceral, 50 mM NaCl, 1 mM EDTA, 2.5% dried milk powder for 12 h at 4 °C, washed for 5 min in TNE-50 buffer (10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT), and probed for 4 h at 30 °C in TNE-50 containing 10 µg/ml poly(dI-dC) and radiolabeled oligonucleotides (10 fmoi/ml). Double-stranded oligonucleotides were end-labeled with γ-32P ATP using T4 kinase (Promega), 10 µC/mol and purified on Sephadex G25 spin columns. The probes were washed three times in TNE-50 for 1 min each at 4 °C and autoradiographed.

**Expression Cloning**—A 4711 human MC cDNA library was prepared according to a standard methodology. The library was screened with the radiolabeled MMP-2 RE1 oligonucleotide according to Singh et al. (17). Nitrocellulose membranes (Hybond-C, Amersham Corp.) were washed in TNE-50 for 10 min at room temperature and blocked for 12 h at 4 °C with blocking solution as above. Hybridization with the radiolabeled probe and washing were as described under “Southern Blot Analysis.” Corresponding positive signals in duplicate lifts were purified to homogeneity by secondary and tertiary screens. λ phage DNA was isolated, and the cDNA was subcloned into the EcoRI site of pGEM7+ (Promega) and sequenced with the TaqTrack sequencing system (Promega).

**Western Blot Analysis of Nuclear Extracts**—Parallel lanes of the nitrocellulose membranes used for Southern blot analysis were washed in TNE-50 until radioactivity was no longer detectable and blocked in TTBS (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) and incubated for 1 h at room temperature with 5% dried milk powder for 1 h at room temperature. The strips were rinsed with TTBS and incubated for 12 h at 4 °C with the immune or preimmune anti-YB3 serum (1:250 dilution). Membranes were washed three times with TTBS for 5 min each and incubated for 2 h at room temperature with 2 µg/ml goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) in TTBS. After another three washes with TTBS, Vectastain (Vector Laboratories, Burlingame, CA) was added for 1 h at room temperature, and the membranes were washed three times with TTBS and stained in TTBS containing 0.8 mg/ml diaminobenzidine (Sigma), 0.04% NiCl2, and 0.01% H2O2.

**Transient Transfections**—Transient transfections of MCs were performed with Lipofectin (Life Technologies, Inc.) according to Felgen et al. (18). Lipofectin (39 µl) was diluted in 180 µl of RPMI 1640 medium containing 10% NU-serum and 2 mM glutamine and preincubated for 45 min at room temperature. For each transfection, 1 µg of the corresponding luciferase construct (pGL2-Basic, pGL2-Promoter, pT4-Luc 1686, pT4-Luc 1342B-P), 2 µg of a control expression vector or pSG5-YB1 was used. To standardize for transfection efficiency, 2.5 µg of pSV-β-galactosidase vector DNA was included. Plasmid DNA was dissolved in DNA-H2O and diluted in 100 µl of RPMI 1640 medium, 73 µl of the preincubated Lipofectin mixture was added, and the combined solutions were incubated for 15 min at room temperature, after which they were diluted with 800 µl of NU-serum containing medium. Cells at 70–80% confluency in six-well culture plates were washed twice with 2 ml of PBS, and the DNA/Lipofectin mixture was added. Cells were incubated for 18 h at 37 °C at 5% CO2, after which the medium was removed and replaced with normal culture medium. Cells were grown for an additional 24 h, washed once with ice-cold PBS, and extracted with 400 µl of Triton lysis buffer (1% Triton X-100, 1 mM DTT, 25 mM glycy- cine, pH 7.8, 15 mM MgSO4) on ice for 30 min.

Glomerular epithelial cells were transfected according to McMillan et al. (19) with DEAE-dextran. For each transfection, 500 µg of the corresponding luciferase construct (pGL2-Basic, pGL2-Promoter, pT4-Luc 1686, pT4-Luc 1342B-P), 1 µg of the control expression vector pSG5 or

fragment of DNA polymerase I, followed by digestion with Bgl II and Asp718, respectively. Single- and double-stranded probes were purified on acrylamide gels and eluted, and 2 x 106 cpm of labeled probe were included in each binding reaction. The samples were electrophoresed on 4% polyacrylamide, 15% glyceral gels in a buffer containing 1 x Tris borate/EDTA, followed by autoradiography. Electrophoretic shift assays, 2 µl of a rabbit polyclonal antibody to the YB3 protein (a kind gift from Wanda F. Reynolds, La Jolla, CA), which cross-reacts to YB-1 (15), or preimmune serum were preincubated with nuclear extracts at 4 °C overnight in binding buffer. YB3 is a member of the Y-box transcription factor family with an overall amino acid homology between the proteins of 79 and 100% for the most conserved DNA binding domain (16). The radiolabeled oligonucleotides were added 60 min prior to electrophoresis. For competition experiments, nonlabeled oligonucleotides were added in a 500 µM excess or as indicated in the figure legends.
pSG5-YB-1, and 500 ng of the pSV-β-galactosidase vector were mixed with 50 μl of Tris-buffered saline and 50 μl of DEAE-dextran (1 mg/ml H2O). Cells were grown to 60% confluence and were washed sequentially with PBS and Tris-buffered saline. The DNA/DEAE-dextran solution was added and incubated for 60 min at 37 °C. Subsequently, the cells were washed with Tris-buffered saline, treated with 10% Me2SO in PBS for 1 min at room temperature, and washed again with 2 ml of PBS. After the PBS was removed, cells were incubated with K1 medium containing 100 μg chloroquine for 4 h at 37 °C. Another medium exchange was performed, and cells were harvested after 36 h for luciferase and β-galactosidase assays.

Luciferase assays were performed with 100 μl of the lysates as described previously by Brazier et al. (20). β-Galactosidase activity was measured as reported (21). All transfections were performed in triplicate and were repeated at least three times. Transfection results were averaged and are expressed as the means (S.D. less than 15%).

**Protein Expression and Purification**—A pRSET vector (Invitrogen) containing an insert coding for a hexahistidine-T7 epitope-YB-1 fusion protein was a kind gift from Dr. K. Chien (University of California San Diego). For expression in *E. coli*, bacteria were grown in SOB-ampicillin medium to an optical density of 0.6 before induction with isopropyl-1-thio-β-D-galactopyranoside. After 1 h, M13/T7 helper phage encoding T7 polymerase were added in a ratio of 5:1. The bacteria were grown for another hour before collection and lysis by three freeze/thaw/sonication cycles. Purification of recombinant YB-1 was performed with Ni²⁺ affinity columns as outlined by the manufacturer. Purity of the expressed YB-1 fusion protein was ascertained by analytic SDS-polyacrylamide gel electrophoresis.

**DMS Protection Footprinting Analysis**—For footprinting analysis of the individual single strands encompassing the MMP-2 RE1, oligonucleotides were end-labeled with polynucleotide kinase and [γ-32P]ATP, followed by purification on 15% polyacrylamide gels. The isolated probes were extracted from the gels with 0.5 M ammonium acetate overnight and ethanol-precipitated. The DNA/YB-1 reaction was prepared as described for electrophoretic mobility shift assays except that 2 × 10⁵ cpm of the end-labeled oligonucleotides were included. After incubation for 20 min at 4 °C, 4 μl of 1:10 DMS solution were added for 45 s, and the reaction was terminated by adding 4 μl of 10 mM DTT (22). The samples were loaded on a 4% polyacrylamide gel to separate the free probes from YB-1-bound oligonucleotides, which were excised from the gel and eluted in Tris/EDTA. The methylated DNA was cleaved with 150 μl of 1 M piperidine (30 min at 90 °C) and dried under vacuum. The pellets were dissolved in 200 μl of TE and phenol/chloroform-extracted, followed by ethanol precipitation in the presence of 3 μg of yeast tRNA as carrier. The cleaved oligonucleotide probes were separated on 12.5% sequencing gels with equivalent amounts of free and bound probe loaded. G + A (Maxam-Gilbert) reactions were included as well.

**RESULTS**

**Physicochemical Characteristics of Nuclear Protein Binding Activities to MMP-2 RE1 by BrdUrd-substituted UV Cross-linking and Southwestern Blot Analysis**—BrdUrd-substituted double-stranded 40-bp MMP-2 RE1 as probe was incubated in the presence of mesangial cell nuclear extract and exposed for increasing time periods to short-wave UV light (254 nm). There was a time-dependent increase in the relative abundance of a single labeled protein with molecular mass of 52 kDa (Fig. 1A). Specificity of this reaction was confirmed by inclusion of a 100-fold molar excess of unlabeled probe, which resulted in the elimination of the band. Using Southwestern blot analysis with the radiolabeled 40-bp MMP-2 RE1 as probe, binding conditions (see “Materials and Methods”) were optimized. Three distinct proteins with molecular masses of 52, 62, and 86 kDa were identified (Fig. 1B) and could be competed by inclusion of a 100-fold molar excess of unlabeled probe. Thus, both forms of independent physicochemical analysis identified a 52-kDa MC nuclear protein that specifically interacts with the 40-bp MMP-2 RE1, as well as two additional proteins of 62 and 86 kDa on the more sensitive Southwestern blots.

**Isolation of YB-1 by Southwestern Screening of a Human Mesangial Cell cDNA Expression Library**—The expression cloning of MC nuclear proteins that interact with the MMP-2 RE1 was performed as described by Singh et al. (17). The binding conditions were determined by the initial Southwestern blot analysis, and the radiolabeled 40-bp MMP-2 RE1 was used as a probe. Screening of 1.5 million colonies yielded six positive cDNA clones, all of which encoded overlapping regions of the cDNA of YB-1, a member of the Y-box transcription factor family (23, 24). All six clones included the cold shock domain, which confers DNA binding activity (25), and the largest clone included the complete sequence encompassed by known internal EcoRI restriction sites. Full-length sequencing of both strands revealed 100% sequence identity with YB-1. YB-1 was originally identified as transcription factor with binding specificity for the inverted CCAAT box (23) and for an interferon response element (IRE) (26). Comparison of the sequence of the 40-bp MMP-2 RE1 with the Y-box consensus sequence revealed a match of 8 out of 12 bases (Fig. 2), with two nonaligning bases present within the inverted CCAAT consensus core. Furthermore, a complete consensus sequence for an IRE is present (GAAAC) within the MMP-2 RE1, which can also bind YB-1 (26).

Experiments were performed to further characterize the binding properties of YB-1 to the MMP-2 RE1 using recombinant protein. YB-1 was expressed as a fusion protein with a 33-aminoc acid fusion peptide containing a hexahistidine sequence, which can bind to a Ni²⁺ column, and a 12-aminoc acid T7 epitope tag at the N terminus. *E. coli* were transformed with the expression vector, induced with isopropyl-1-thio-β-D-galac-
YB-1 and MMP-2 Transcription

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YB-1 Prefersentially Binds the Single-stranded Components of the MMP-2 RE1 and Exhibits Multimerization Properties—Previous studies of YB-1 interactions have revealed the ability of this transcription factor to bind not only to double-stranded DNA, but also with even higher affinity to single-stranded DNA (28, 29). To evaluate this within the structural context of the MMP-2 RE1, double- and single-stranded sense and antisense radiolabeled probes were incubated with recombinant YB-1 for gel shift analysis. All possible precautions were taken to prevent the generation of single strandedness within the double-stranded probe; the probe was stored at 4 °C after purification on acrylamide gels, and no further ethanol precipitation was performed prior to gel shift analysis.

As shown in Fig. 5, recombinant YB-1 protein binds to the single-stranded MMP-2 RE1 components (both sense and antisense) with approximately 100 times greater affinity than with the double-stranded element. Furthermore, inclusion of increasing amounts of recombinant YB-1 protein in the binding reaction resulted in an ascending series of shifted single-stranded oligonucleotides, consistent with multimerization (Fig. 6). In contrast, the double-stranded probe did not exhibit multimerization properties under the same binding conditions.

Additional studies compared the EMSA patterns of the double-stranded probe when incubated with recombinant YB-1 protein, mesangial cell nuclear extracts, or glomerular epithelial cell nuclear extracts. These studies are summarized in Fig. 7, which demonstrates that the electrophoretic mobility of the double-stranded oligonucleotide was considerably slower with the mesangial cell nuclear extracts as compared with recombinant YB-1, which is consistent with the binding of an additional partner protein(s). In contrast, the dominant shifted oligonucleotide in the GEC extracts has the same relative mobility as recombinant YB-1.

topyranoside, and transformed with M13/T7 phage expressing T7 RNA polymerase. Using this system, the previously reported repression of bacterial growth by YB-1 (15) can be circumvented by induction of protein synthesis after the host cells have grown. Western blot analysis of the purified recombinant protein using the anti-T7 epitope tag antibody revealed two major bands of sizes 54 and 58 kDa (not shown). Recombinant YB-1 migrates with a molecular size of approximately 52–56 kDa on SDS-polyacrylamide gels, although the calculated molecular mass based on the cDNA sequence is 35.6 kDa. The difference has been attributed to an increased Stokes' radius due to the highly charged C terminus of YB-1 (27). Comparison of the Western blotting patterns of MC nuclear extracts and those of non-MMP-2-synthesizing GECs revealed an identical pattern of three hybridizing proteins of 52, 62, and 82 kDa (Fig. 3, lanes A and E). Western blots of the MC and GEC nuclear extracts were performed on parallel lanes using polyclonal anti-YB3 antibody, which cross-reacts with YB-1. Two apparent isoforms with molecular sizes of 52 and 56 kDa of YB-1 protein were detected in the nuclear extracts for both MCs and GECs (Fig. 3, lanes B and D). Thus, one of the MMP-2 RE1-hybridizing nuclear proteins has an electrophoretic mobility similar to the smaller, 52-kDa isoform of the YB-1 protein detected by Western blot analysis.

Further experiments were conducted to confirm that YB-1 is indeed a component of the shifted complexes obtained with mesangial cell nuclear extracts and the MMP-2 RE1. When the preimmune serum was included in the binding reaction, no difference of the relative mobility of the shifted complex could be seen. In contrast, inclusion of the polyclonal antibody that recognizes YB-1 in the binding reaction of the electrophoretic mobility shift assay (EMSA) caused a supershift (Fig. 4, compare lanes C and D), confirming the presence of YB-1 in the complex.
DMS protection footprinting was performed to determine the sites of YB-1/DNA interaction on the sense and antisense single-stranded MMP-2 RE1 components. The DNA binding pattern of YB-1 is complex and extends over at least 10 nucleotides on the sense and 35 nucleotides on the antisense strand (Fig. 8). Protected sites are present within the inverted CCAAT Y-box motif on both strands as well as the IRE on the antisense strand. In contrast to the relatively straightforward DMS protection obtained with single-stranded templates, we were unable to consistently determine sites of YB-1 interaction with double-stranded template, presumably due to the relative low affinity of YB-1 binding under these conditions.

Cell-specific Transactivation of the MMP-2 Enhancer Element by YB-1—The influence of YB-1 on MMP-2 gene transcription was assessed in transient transfection studies using mesangial cells, which constitutively synthesize MMP-2, and glomerular epithelial cells, which do not (19). Transfections were performed with reporter constructs containing the whole 5′ regulatory sequence up to −1686 bp (pT4-Luc1686). pT4-Luc1686 includes the homologous MMP-2 promoter and the strong cis-acting MMP-2 RE1. In addition, the 40-bp MMP-2 RE1 was analyzed in the context of a heterologous SV40 promoter (pT4-Luc1342B-P). Transfections were performed in the absence and presence of pSG5-YB-1, a eukaryotic expression vector. The influence of YB-1 was assessed in transient transfection studies using mesangial cells, which constitutively synthesize MMP-2, and glomerular epithelial cells, which do not (19). Transfections were performed with reporter constructs containing the whole 5′ regulatory sequence up to −1686 bp (pT4-Luc1686). pT4-Luc1686 includes the homologous MMP-2 promoter and the strong cis-acting MMP-2 RE1. In addition, the 40-bp MMP-2 RE1 was analyzed in the context of a heterologous SV40 promoter (pT4-Luc1342B-P). Transfections were performed in the absence and presence of pSG5-YB-1, a eukaryotic expression vector.

**FIG. 5.** Recombinant YB-1 preferentially binds the single-stranded components (SS1, SS2) of the MMP-2 RE1 as compared with the double-stranded form (DS). Controls (con) consist of the respective oligonucleotide probes alone. Lanes labeled YB-1 represent incubation of 50 ng of recombinant YB-1 with the respective double-stranded and single-stranded probe. Lanes labeled comp include a 100-fold excess of unlabeled oligonucleotide as a competitor.

**FIG. 6.** Concentration-dependent multimerization of recombinant YB-1 on the single-stranded (SS1, SS2) as opposed to the double-stranded (DS) forms of the MMP-2 RE1 using concentrations of YB-1 ranging from 1 to 10, 50, and 100 ng/binding reaction.

**FIG. 7.** EMSA performed with the MMP-2 RE1 comparing relative mobilities of control (lane A), MC nuclear extracts (lane B), GEC nuclear extracts (lane C), and recombinant YB-1 (lane D).

**FIG. 8.** DMS protection footprinting analysis performed with the sense (S1) and antisense (S2) strands of the MMP-2 RE1 and recombinant YB-1 (200 ng/binding reaction). YB-1 interacts with multiple residues within the Y-box motif of both strands (arrows) and with only the IRE of S2. The binding of YB-1 extends over 35 nucleotides on the antisense strand.
vector for YB-1. Luciferase activity in all experiments was normalized for transfection efficiency by co-transfection with a pSV-β-galactosidase control vector. Comparison of the relative luciferase activities shows that overexpression of YB-1 does not affect the activities of the control luciferase vectors (pGL2-Basic and pGL2-Promoter). As previously shown in mesangial cells, a significant increase in transcription activity can be detected with construct pT4-Luc1686, which includes the MMP-2 RE1 in the context of the homologous MMP-2 promoter, while this construct exhibits only modest activity in GEC.

Co-transfection of MCs with the YB-1 expression vector further stimulated this activity 3-fold (Fig. 9) but had no effect on GEC activity.

To confirm the specific nature of YB-1 interaction with the MMP-2 RE1 alone, additional transfections were performed with construct pT4-Luc1342B-P, which contains the 40-bp enhancer element within the context of the heterologous SV40 promoter. As reported previously, (10), this construct demonstrates positive transactivation within both mesangial and glomerular visceral epithelial cells (Fig. 10). Co-transfection of mesangial cells with the YB-1 expression vector led to a further 3-fold increase in transcriptional activity, while co-transfection of GEC with this vector resulted in a repression of transcriptional activity. Thus, the effects of YB-1 overexpression on the activity of the MMP-2 RE1 are dependent upon the nature of the transfected cell type.

**DISCUSSION**

In this study, we have identified the transcription factor, YB-1, as a major regulator of the cell-specific regulation of
MMP-2 gene transcription. YB-1 was obtained by expression screening of a mesangial cell λgt11 cDNA library using the MMP-2 RE1 as a probe (10). The electrophoretic mobility of recombinant YB-1 closely matches that of the 52-kDa nuclear protein obtained with UV cross-linking of mesangial cell nuclear extracts with the MMP-2 RE1. A protein with a similar molecular mass of 52 kDa was also observed following Southern blot analysis of both mesangial and glomerular epithelial cell nuclear extracts. In addition, Western blots of nuclear extracts from both cell types probed with anti-YB-1 antibody also demonstrated proteins of 56–52 kDa. The specific interaction of YB-1 with the MMP-2 RE-1 was confirmed by gel shift analysis with recombinant YB-1 protein and by supershift analysis with specific antibody. We note that the sensitive Southern blot analysis of mesangial and glomerular epithelial cell nuclear extracts also detected proteins with molecular masses of 62 and 86 kDa, which were not recovered from our mesangial cell expression library. Future studies are planned using alternative screening strategies to identify these potentially significant MMP-2 RE-1 binding proteins.

YB-1 belongs to a family of evolutionarily highly conserved transcription factors, the Y-box family (25, 30). Members of this family derived from bacteria and extending to mammals all include a homologous 80-amino acid DNA binding domain, termed the cold shock domain (31). The DNA binding region of the YB-1 protein recognizes a relatively conserved element, the Y-box, which contains a core inverted CCAAT sequence. Upon reinpection of the MMP-2 RE1 sequence following the expression cloning of YB-1, a significant homology between a Y-box consensus sequence and the MMP-2 RE1 sequence was evident (Fig. 2).

Functional characterization of a related member of the Y-box transcription factor family, FRQY, has revealed a domain at the C-terminal region of the protein that facilitates both homomultimerization and interaction with other transcription factors (32). Our results with recombinant YB-1 protein indicate that concentration-dependent multimerization does occur upon binding to the MMP-2 RE1 sequence, particularly within the single-stranded context. The propensity of YB-1 to multimerize on this element is the most probable explanation for the very extended DNA footprint observed in this study. The actual functional significance of YB-1 protein multimerization along a recognition element remains conjectural at this time, but it may facilitate interactions with other transactivating factors.

YB-1 bind with a much higher affinity to the single-stranded components of the MMP-2 RE-1. In this regard, it is of interest that YB-1 may convert double-stranded DNA into single strands. MacDonald et al. (29) described the ability of YB-1 to promote a single-stranded DNA conformation in the 5′ regulatory region of MHC class II genes, which are repressed by YB-1. A model has been proposed whereby the YB-1-mediated induction of single-stranded regions in critical regulatory regions may prevent the binding or activity of positive transactivating factors. This model has the drawback that positive transactivation mediated by YB-1, as shown in our present study with mesangial cells, cannot be explained.

Interactions of YB-1 or related proteins with the regulatory regions of a rather diverse set of genes have been reported. These include the above mentioned class II MHC genes, the multidrug resistance 1 gene, the myosin light chain 2v gene, and the genes for the epidermal growth factor receptor and γ-globin, among others (23, 33–36). These studies have demonstrated that within the individual gene contexts, YB-1 can act either in a positive or repressive manner to mediate transcription. In our current analysis, we demonstrated the unexpected finding that YB-1 can act on a single gene, the MMP-2 gene, in a positive or negative manner that is dependent on the cellular context.

Given the fact that nuclear extracts from both mesangial cells (positive transactivation) and GECs (repression) contain the YB-1 protein, it is likely that its mechanism of action is dependent upon either cell-specific interactions with the intrinsic MMP-2 promoter or upon the interaction of YB-1 with distinct transcriptional partner proteins. YB-1 interaction with transcriptional regulators has been described in several systems. For example, Zou and Chien (15) have recently demonstrated the cell-specific activation of the myosin light chain 2v gene by YB-1 and have documented through immunoprecipitation techniques the interaction of YB-1 with a 30-kDa protein in cardiac muscle cells. Chen and colleagues (37) have extensively analyzed the complex pattern of interaction of YB-1 with the single-stranded DNA binding factor, Pur α, in the control of human JC polyomavirus promoters. The decreased electrophoretic mobility of the MMP-2 RE1 oligonucleotide when incubated with mesangial cell nuclear extracts, as compared with either recombinant YB-1 protein or GEC extracts, strongly suggests the presence of an additional partner protein(s). While immune precipitation studies of radiolabeled nuclear extracts of mesangial cells using the anti-YB-1 antibody do not indicate the presence of preformed YB-1-partner protein complexes, we have recently recovered two nuclear proteins of 34 and 67 kDa size when the incubation includes the MMP-2 RE1 oligonucleotide.2

In summary, the current study has documented the transcriptional regulation of yet another critical growth regulatory factor, MMP-2, by YB-1. Future studies aimed at the characterization of mesangial cell-specific YB-1 transcriptional partner proteins may be expected to provide additional insights into the regulation of MMP-2 transcription and may offer alternative strategies for the pharmacologic regulation of this growth and differentiation determinant.

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