A Synergistic Interaction of Transcription Factors AP2 and YB-1 Regulates Gelatinase A Enhancer-dependent Transcription*

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The matrix metalloproteinase gelatinase A plays a central role in several critical physiologic processes, including angiogenesis, tumor invasion/metastasis, and chronic inflammation. We demonstrate that high level gelatinase A expression is mediated by a unique interaction of two developmentally regulated transcription factors, AP2 and YB-1, within a discrete 40-base pair enhancer element (RE-1) located in the 5′-flanking region of the gelatinase A gene. Electrophoretic mobility shift assays and immunoprecipitation experiments confirmed a direct interaction of AP2 with this binding sequence in the form of AP2-YB-1 heteromeric complexes. Binding of AP2-YB-1 complexes to the RE-1 sequence results in the formation of extended single-stranded DNA regions and may stabilize DNA conformational changes. Overexpression of YB-1 and AP2 proteins by gelatinase A synthesizing hepatoma HepG2 cells induced a synergistic increase in the RE-1-mediated transcription of nearly 160-fold. Thus, the transcription of gelatinase A is subject to a previously unrecognized interplay of double (AP2) and single-stranded (YB-1) DNA binding transcription factors to yield a highly regulated pattern of gene expression.

Cellular migration and invasion are fundamental aspects of many critical biological processes including development, angiogenesis, wound repair, and tumor metastasis. Whereas several distinctive proteolytic systems have been implicated in these processes, the matrix metalloproteinase (MMP) gene family, which currently includes 19 members, has been the focus of intense investigational scrutiny. As a group, these enzymes are characterized by activity in the extra- (or peri-) cellular space, secretion as latent zymogens, dependence on zinc for catalytic activity, and inhibition by the tissue inhibitors of metalloproteinases (1). Individual members of the MMP family exhibit distinctive patterns of proteolytic activity against extracellular matrix proteins, thereby permitting a highly regulated mechanism for matrix turnover.

The 72-kDa gelatinase A (also denoted MMP-2) displays a number of features that distinguish it from other members of the MMP gene family. Although secreted as a soluble proenzyme, gelatinase A has been recently shown to bind to the α,β3 vitronectin receptor on the surfaces of invasive cells (2). This surface localization is presumably a major determinant of the ability of gelatinase A to act as a growth and differentiation factor for cells in vitro (3). In addition to the unique pattern of surface expression and action as a growth and differentiation factor, the transcriptional regulation of gelatinase A is noteworthy. In contrast to other MMPs, the 5′-flanking region of this gene lacks TATA or CAAT boxes or functional AP-1- or NFκB-binding sites (4, 5). Although both the human and rat genes lack functional AP-1 or NFκB elements, exposure of glomerular mesangial cells to phorbol ester, prostaglandin E2, or cAMP analogs resulted in enhanced gelatinase A transcription and synthesis, suggesting that a functional AP2 element may regulate the responses to these stimulatory factors (6, 7).

Recent studies from our laboratory, using the glomerular mesangial cell as a model cell capable of high level gelatinase A synthesis, have defined a potent 40-bp cis-acting response element (gelatinase A RE-1) in the near 5′-flanking region of the rat gelatinase A gene which, in conjunction with the proximal promoter, confers cell-specific transcriptional regulation (4). To identify transacting factors, which may regulate gelatinase A transcription, we used the RE-1 as a probe in expression screening and identified the transcription factor YB-1 as a specific nuclear protein component that regulates RE-1 activity (8). YB-1 is a member of a family of highly conserved DNA-binding proteins that has been shown to regulate the transcription of several central growth or differentiation genes (9–11), and its association with the transcriptional control of the growth regulatory enzyme, gelatinase A, is consistent with a postulated role for YB-1 as a key developmental transcription factor (9).

Although our transfection data indicated that YB-1 did mediate, through the RE-1, gelatinase A transactivation, further analysis of nuclear gel shift experiments suggested that additional transcriptional elements were involved. In this study we demonstrate that a second, differentiation-dependent transcription factor, AP2, can directly interact with YB-1 to induce a synergistic activation of RE-1 transcriptional activity. These effects of AP2/YB-1 are associated with DNA structural alteration, including induction of single-stranded DNA, which may provide a structural template for additional partner protein binding.

MATERIALS AND METHODS

Cells and Culture Conditions—HepG2 hepatoma cells were obtained from ATCC and grown in DME H-16 medium containing 100 μg of streptomycin, 100 units/ml penicillin, and 10% fetal bovine serum. Rat glomerular mesangial cells were grown and characterized as reported in detail (4).

Plasmids Used in This Study—An 80-bp sequence 1342CCCTGGCGAAATTTGCTATAGCCGGCAAGTCTGAACTTGTCAGAAA-3132CTTCATACCTCGCCGACTAGCTTTTTTTTTT, which includes the RE-1 sequence (underlined) between 1322 and 1282 bp relative to...
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the translation start site of the rat gelatinase A gene (4), was subcloned into the pG2L2-Promoter vector (Promega), which contains a heterologous SV40 promoter, and was designated pT4-Luc 1342P. The 40-bp sequence between −1322 and −1282 was also subcloned into pG2L2-
Promoter and was designated pT4-Luc RE-1. A second luciferase re-
porter, pT4-Luc1342-B, was subcloned between −1386 and −1286 bp in order to separate the free probe from AP2-
bound oligonucleotides, which were excised from the gel and eluted in 0.3 M sodium acetate, pH 5.0. The methylated DNA was cleaved with piperidine and separated on 12.5% sequencing gels with equivalent amounts of free and bound probe loaded. G and G + A (Maxam-
Gilbert) reactions were included as well.

Mesangial cell nuclear extracts were prepared as described by Brasier
et al. (12), with minor modifications. The asymmetrically end-
labeled probe generated with pT4-1342-P (10^5 cpm) was incubated in the presence of 1 μg of poly(dI-dC)-poly(dI-dC) for 30 min at 22 °C, followed by addition of 2 μg of poly(dI-dC) and proteins in binding buffer A (without EDTA, supplemented with 4 mM MgCl2) in a total volume of 15 μL at 25 °C for 20 min. The reaction volume was diluted 5-fold, and a
volume of mung bean nuclease buffer was added. Mung bean nuclease treatments were performed at saturating concentrations of enzyme, as previously determined by titration. 50 units of mung bean nuclease (100 units/μL; Promega) were added to each reaction and incubated for 20 min at 37 °C, followed by termination with 240 μL of stop buffer (100 mM Tris/HCl, pH 8.0, 100 mM NaCl, 20 mM EDTA, 0.1% SDS, 100 μg/mL proteinase K). After incubation in stop buffer at 37 °C for 15 min, reactions were phenol/chloroform extracted once and precipitated. Sam-
ple purifications on a 12.5% polyacrylamide urea gel with parallel lanes containing chemical sequencing reactions.

Transient Transfections—HepG2 cells were transfected using CaPO4, essentially as described (13). For each triplicate transfection, 3 μg of the corresponding luciferase construct (pGL2-Promoter, pT4-Luc RE-1) and 3 μg of pSVβ-gal were used. Titration experiments were performed with a fixed amount of the pG5S-YP1 expression plasmid and variable amounts of pGS-AP2 plasmid, as detailed in the figure legends. The amount of total DNA was equalized in each reaction by inclusion of pGS5 plasmid DNA. The luciferase activity was normalized to β-galactosidase activity to account for variations in the efficiency of transfection, as described previously (4). Cells at 50–60% confluence in 6-well culture plates were washed twice with 2 mL of phosphate-buffered saline, and the calcium phosphate precipitates were added. Mesangial cell nuclear extracts were prepared as described above. Cells were grown for another 42 h, washed twice with ice-cold phosphate-buffered saline, and extracted with 400 μL of Triton lysis buffer (1% Triton X-100, 1 mM dithiothreitol, 25 mM glycyglycine, pH 7.8, 15 mM MgSO4) on ice for 30 min, followed by measurement of luciferase and β-galactosidase activity. Luciferase assays were performed with the lysates as described previously by Brasier et al. (14). β-Galactosidase activity was measured as reported (15). All transfec-
tions were performed in triplicate and were repeated at least three times. Transfection results were averaged and are expressed as the mean (S.D. less than 15%).

For stimulation experiments, cultured mesangial cells were tran-
siently transfected with constructs pT4-Luc18668, pT4-LucRE-1, or the promoterless pG5L2Basic (Promega) using Lipofectin (Life Technologies, Inc.), as previously reported (4). Cells at 60% confluence on 6-well

RESULTS

AP2 and YB-1 Bind to the MMP-2 RE-1—Recent studies have de-
scribed a 40-bp enhancer element (RE-1) in the rat gelatinase A gene 5′ region that mediates its cell type-specific expression (4). These findings were extended by the observation that a developmentally regulated transcription factor, YB-1, specifically bound to the RE-1 through a Y box sequence (CAGCTGTTGGCGAGAAG) in the RE-1 5′ region (8). Comparisons of gel shifts using mesangial cell nuclear extracts to those per-
formed with recombinant YB-1 protein suggested that addi-
tional nuclear proteins were involved in the RE-1 sequence binding. Inspection of the RE-1 sequence revealed the sequence AGCTGTCTGGGCA (Fig. 1A) which is homologous to the con-
sensus sequence proposed for AP2 by Inagawa et al. (16) and

DMS Protection Footprinting and Mung Bean Nuclease Analysis— Footprinting reactions were performed as described previously (4) using the double-stranded RE-1 [32P]ATP end-labeled probes pre-
pared with pT4-Luc 1342-P. The DNA/AP2 binding reaction included 10^5 cpm of the end-labeled oligonucleotides and about 20 ng of recom-
binant AP2. After incubation for 20 min at 22 °C, 4 μL of 1:10 DMS solution were added for 45 s, and the reaction was terminated by adding 4 μL of 1 M dithiothreitol. The samples were loaded on 4%
Gel shift experiments using recombinant AP2 and the gelatinase A RE-1 sequence demonstrated a specific interaction of AP2 protein with this element (Fig. 1B, panels I–III). Competition studies indicated that AP2 preferentially binds the RE-1 element in the double-stranded form, with an approximately 100-fold lesser affinity for the single-stranded homolog. This observation contrasts with our earlier observations of preferential binding of recombinant YB-1 to the RE-1 sequence.

**Fig. 1. AP2 binding to the RE-1 sequence.** A, the 40-bp sequence encompassing gelatinase A response element-1 (RE-1) is located at −1322 bp relative to the translational start site of the rat gelatinase A gene. This sequence contains an incomplete Y box motif (CTGATTGGCTAA). Comparison of the RE-1 sequence with the transcription factor AP2-binding motif, as determined by Imagawa et al. (16) and Mitchell et al. (17), demonstrates a high degree of sequence homology. B, electrophoretic mobility shift assays were performed with recombinant AP2 protein and the radiolabeled RE-1 element as a probe. Incubation of 2 ng of recombinant AP2 protein with the double-stranded RE-1 probe (panel I) leads to the formation of two closely migrating DNA-protein complexes. Homologous, but not heterologous, unlabeled DNA competed for complex formation when included in excess in the binding reaction (fold excess as indicated). In addition, both single strands (sense, panel II; antisense, panel III) were specifically bound by AP2 protein, albeit with a much lower affinity as indicated by the complete competition obtained with only a 10-fold excess of homologous competitor DNA. C, DMS protection footprinting analysis was performed with 20 ng of AP2 recombinant protein and the asymmetrically end-labeled double-stranded RE-1 probe (10 fmol). After separation of bound and unbound fractions by gel shift, piperidine-cleaved fragments were separated on 12.5% polyacrylamide gels. Comparison of control reactions (inner lanes) with the AP2-bound RE-1 probe (outer lanes) revealed Gs protected by AP2 binding, as indicated by arrows.

Mitchell et al. (17). Gel shift experiments using recombinant AP2 and the gelatinase A RE-1 sequence demonstrated a specific interaction of AP2 protein with this element (Fig. 1B, panels I–III). Competition studies indicated that AP2 preferentially binds the RE-1 element in the double-stranded form, with an approximately 100-fold lesser affinity for the single-stranded homolog. This observation contrasts with our earlier observations of preferential binding of recombinant YB-1 to the
FIG. 2. AP2-YB-1 heteromeric complex formation. A, DNA binding studies were performed to evaluate AP2-YB-1 complex formation with the RE-1 probe (2 fmol). When recombinant AP2 protein (2 ng) or T7-tagged YB-1 (2 ng) were included in the binding reactions (lanes B and C), distinct DNA-protein complexes with differing mobilities were obtained. The combination of AP2 and YB-1 (2 ng each) in the binding reaction yielded additional bands with distinctly lower electrophoretic mobilities (lane D). The inclusion of anti-AP2 antibody (lane E) or anti-T7 antibody (lane F),
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or both antibodies together (lane G) in the binding reactions yielded extensive supershifts and diminished bands. B, panel I, DNA binding studies conducted with a double-stranded oligonucleotide (2 fmol) containing the AP2 consensus motif (GGCCCGCCG) but without a Y box motif. The gel shift analysis demonstrates binding of recombinant AP2 (2 ng) in lane B but not YB-1 (2 ng) to the oligonucleotide (lane C). The combination of both proteins yielded one major DNA complex with diminished mobility consistent with heteromer formation (lane D). Homologous unlabeled DNA competed successfully for complex formation and confirmed binding specificity. Panel II, protein-DNA complex binding affinities were evaluated with gel shift analyses as above. Increasing amounts of AP2 protein (range 0–2 ng) were included in the binding reaction in the absence (filled squares) and presence (filled circles) of a constant amount of YB-1 protein (2 ng). The inclusion of both YB-1 and AP2 in the reaction mixture led to the formation of additional, more slowly migrating bands (lane D), which were shown using supershift analysis to be composed of YB-1/AP2 heteromeric complexes (lanes E–G). These results are consistent with heteromeric AP2/YB-1 complex binding to the RE-1 sequence or with the independent binding of both proteins to the element.

To investigate whether heteromeric YB-1/AP2 binding occurred with other known AP2 binding sequences, we performed similar gel shift analyses with a double-stranded AP2 consensus sequence GATCGAAGCTACGGCCCGT, which does not include a Y box sequence. As expected, recombinant AP2 (2 ng) could shift this double-stranded consensus sequence (Fig. 2B, panel I, lane B), whereas recombinant YB-1 (2 ng) alone did not (lane C). The addition of both YB-1 and AP2 to the reaction mixture led to the formation of a heteromic complex (lane D) with a nearly 20-fold increase in DNA binding activity as compared with AP2 alone. It should be noted that the AP2 consensus sequence in the single-stranded form did not bind YB-1 or AP2. This experiment is notable for the fact that YB-1 appears to facilitate the interaction of the AP2 protein with this DNA sequence even in the absence of a canonical Y box sequence. The cooperative nature of the YB-1/AP2 interaction with the consensus sequence is further defined in Fig. 2B, panel II, which demonstrates that the presence of a fixed concentration of YB-1 protein (2 ng) greatly facilitates complex formation and DNA binding activity for very low AP2 concentrations.

Competition studies were next performed to determine differences between the stability of AP2-DNA and the AP2-YB-1-DNA complexes (Fig. 2C). Homologous competitor double-stranded DNA was added for the indicated periods after specific protein-DNA complexes had been formed, and the reactions were subjected to gel shift analysis. Differences in complex persistence were quantified by densitometry and demonstrated a dissociation half-life for AP2-DNA complexes of 5 min, whereas AP2-YB-1-DNA complexes showed an increased stability with a dissociation half-life estimated at 60 min.

DMS footprinting studies using both AP2 and YB-1 proteins were performed with the double-stranded RE-1 element to determine the heteromic contact points within this sequence (Fig. 3). In addition to sites protected in the Y box when using AP2 alone (cf. Fig. 1C), inclusion of YB-1 protein resulted in protected guanines within the distal regions of the RE-1 element.

The above studies indicate YB-1 and AP2 interact within the context of a double-stranded oligonucleotide template. To determine whether AP2 and YB-1 associate within solution phase, immunoprecipitation studies were performed in the absence of a DNA template. For these experiments recombinant AP2 protein was incubated in the gel shift binding buffer with T7 epitope-tagged recombinant YB-1 protein. After incubation with anti-AP2 antiserum, immune complexes were recovered with Pansorbin, electrophoresed, and transferred to nitrocellulose membranes followed by probing with an anti-T7 monoclonal antibody. As shown in Fig. 4, YB-1 is co-precipitated by the anti-AP2 antiserum, consistent with association of AP2 and...
Fig. 4. AP2/YB-1 co-immunoprecipitation studies. Recombinant AP2 (20 ng) and T7 epitope-tagged YB-1 (20 ng) were incubated with polyclonal anti-AP2 and recovered on Pansorbin beads as detailed under “Materials and Methods.” After transfer to nitrocellulose membranes, the blots were probed with anti-T7 monoclonal antibody. Lanes A and B are controls; lane C is a positive control for T7-tagged YB-1 protein; lane D represents the immunoprecipitation experiment with all components present in the incubation mixture.

YB-1 proteins in solution.

AP2/YB-1 Interactions Alter DNA Conformation—MacDonald et al. (12) proposed a model for YB-1 inhibitory action on MHC class II DR gene expression by induction or stabilization of single-stranded regions within the regulatory X and Y boxes. By using a similar approach, we utilized the ability of mung bean nuclease to cleave single-stranded regions of DNA in the presence of divalent cation cofactors. The results of these studies are detailed in Fig. 5. Incubation of the gelatinase A RE1 element with YB-1 (2 ng) alone followed by nuclease digestion produced cleavages at single-stranded regions within the oligonucleotide which extended from the Y box motif to the 3’ probe terminus. In contrast, equivalent concentrations (2 ng) of recombinant AP2 did not induce single-stranded cleavage in the oligonucleotide probe. The mung bean nuclease cleavage pattern was considerably altered, however, when the incubation included both YB-1 and AP2 proteins and was dependent upon the amount of YB-1 protein added. Inclusion of AP2 protein resulted in a protein steric effect, with “footprinting” of the AP2-binding motif and protection against development of single-strandedness. Furthermore, a distinct cleavage pattern was observed at the ends of an inverted repeat sequence, GT-TCAGTCTGAAC (Fig. 5, arrows), which was most pronounced at equimolar YB-1 and AP2 concentrations. The mung bean nuclease-sensitive sites coincided with a nonprotected site observed in prior DMS protection footprinting analysis using either nuclear extracts (4) or recombinant YB-1 protein (8). To rule out that the distinct nuclease cleavage pattern was produced by transcription factor phasing to the ends of the oligonucleotide, we synthesized a 6-bp extended 3’ end-labeled probe and repeated the mung bean nuclease sensitivity assay. Again, the pattern coincided with cleavage at the ends of the inverted repeat sequence (data not shown).

AP2 and YB-1 Synergistically Transactivate the Gelatinase A 40-bp RE-1 in HepG2 Cells—Transient transfection studies were conducted to examine the activities of AP2 and YB-1 proteins on gelatinase A RE-1 transcription within a cellular context. For these studies we utilized the properties of the HepG2 human hepatoma cell line. These cells express gelatinase A and YB-1 but are devoid of endogenous AP2 (16). For these studies HepG2 cells were transfected with the reporter construct pT4-Luc RE-1 that contains the gelatinase A RE-1 enhancer element fused to the SV40 early promoter. Co-transfections were performed with the eukaryotic expression vectors, pGS5-AP2 and pGS5-YB-1, at varying plasmid DNA concentrations. Correction for the varying plasmid DNA amounts was performed by inclusion of the appropriate quantity of pGS5 vector DNA, whereas normalization for transfection efficiency was achieved by inclusion of a pSVβ-gal vector. The results of these experiments are summarized in Fig. 6. Transfection of HepG2 cells with the RE-1 enhancer construct alone stimulated luciferase activity by approximately 17-fold as compared with the control luciferase vector, pGL2-Promoter. Co-transfection with the YB-1 expression vector led to a further 3-fold induction in RE-1-dependent luciferase activity, a level of positive YB-1-mediated transactivation similar to our previous findings with transfected glomerular mesangial cells (8). In contrast to this result, co-transfection of HepG2 cells with the AP2 expression vector alone led to a 90% decline of RE-1 enhancer luciferase activity in a concentration-dependent manner. To determine the combined effects of AP2 and YB-1 overexpression, we used a fixed concentration of the YB-1 vector (1 μg/ml) along with concentrations of the AP2 vector ranging from 0.1 to 2 μg/ml. Inclusion of even the smallest amount of AP2 vector DNA (0.1 μg/ml) in the transfection resulted in a large increase in reporter activity as compared with YB-1 alone. As shown in Fig. 6, maximal reporter activity was obtained at equal concentrations of the AP2/YB-1 plasmids, which induced a 4–5-fold increase in pT4-Luc RE-1 enhancer activity as compared with YB-1 transfections alone. When compared with the inhibitory effect of AP2 transfection alone, this stimulation in RE-1 activity by the AP2/YB-1 combination is nearly 160-fold. Thus, maximal reporter activity was dependent upon the AP2/YB-1 molar ratio.

Fig. 5. Mung bean nuclease sensitivity analysis. Mung bean nuclease (MBN) analysis was performed to identify regions of single strandedness induced by AP2 or YB-1 binding to the end-labeled RE-1 oligonucleotide. Incubation of the oligonucleotide with mung bean nuclease alone had no effect. Inclusion of recombinant YB-1 protein (2 ng) in the reaction mixture, followed by treatment with mung bean nuclease, yielded cleavage consistent with formation of an extended region of single strandedness. Inclusion of an equivalent concentration of AP2 protein did not result in the formation of single-stranded regions. Incubation of a fixed concentration of AP2 protein (2 ng) with increasing concentrations of YB-1 (0.2, 1.0, and 2.0 ng) resulted in a distinctive cleavage pattern. The ends of an inverted repeat sequence are indicated by arrows. IRE denotes the interferon response element GAAAC in the 3’ region of the sequence.
Transient transfection studies. Transient transfections of hepatoma HepG2 cells evaluated the transactivation of luciferase reporter constructs which include the RE-1 sequence in the context of the heterologous SV40 promoter (pT4-Luc RE-1). Transfection of this construct alone led to a 17-fold increase in luciferase activity as compared with the control pGL2-Promoter plasmid. Inclusion of a YB-1 expression plasmid led to a further 3-fold increase in luciferase expression. The potential cooperative interactions of AP2 and YB-1 on the activity of the pT4-Luc RE-1 plasmid was determined by titrating the amount of AP2 expression plasmid (0, 0.1, 0.5, 1.0, and 2.0 μg) with a constant amount of YB-1 expression plasmid (1.0 μg). The total amount of DNA was equalized in each transfection procedure with expression plasmid pGS5 DNA. Co-transfection of the AP2 vector alone resulted in nearly complete inhibition of RE-1-dependent luciferase activity, whereas inclusion of the YB-1 vector yielded a very large increase in RE-1-dependent luciferase activity.

The final series of studies examined the interactions of the YB-1 and AP2 proteins within the cellular context of cultured glomerular mesangial cells. To determine whether the YB-1 and AP2 proteins can specifically interact intracellularly, nuclear extracts from mesangial cells were analyzed by gel shift methods using radiolabeled RE-1 in the presence or absence of antibodies to YB-1 and AP2. As detailed in Fig. 7, incubation of mesangial cell nuclear extracts with radiolabeled double-stranded RE-1 yielded a prominent shifted band, which was supershifted upon inclusion of YB-1 antibody. Similarly, a supershift was detected when anti-AP2 antibody was included in the incubation mixture. Addition of both YB-1 and AP2 antibodies resulted in a further decrease in electrophoretic mobility of the RE-1 oligonucleotide. These results indicate that native YB-1 and AP2 proteins present in mesangial cell nuclear extracts are capable of forming a heteromeric complex in the presence of the RE-1 oligonucleotide template.

To confirm further the significance of AP2/YB-1 interactions within a cellular context, point mutation of two bases at the extreme 5’ end of the AP2-binding site in the pT4-Luc RE-1 construct was performed (G$^{393}$C$^{38}$ to T$^{39}$A$^{38}$ from Fig. 1). Transient transfection of the control pT4-Luc RE-1 and the mutated construct (denoted pT4-Luc RE-1*+) was performed with mesangial cells (Fig. 8). Although co-transfection with YB-1 and AP2 expression plasmids yielded significant increases in luciferase reporter activity (either alone or in combination), the mutated pT4-Luc RE-1*+ expressed little luciferase activity, which was not augmented by co-transfection with the AP2 or YB-1 expression plasmids. Thus, optimal expression of the RE-1 element within the mesangial cell context is dependent upon an intact AP2-binding motif.

As previous studies have demonstrated that maneuvers that elevate cAMP levels in mesangial cells stimulate MMP-2 transcription and secretion (7), we determined the effects of the pathophysiologically relevant mediator, prostaglandin E$_2$, on the activity of the MMP-2 reporter constructs. As summarized in Fig. 9, a 2-h incubation with 2 μM prostaglandin E$_2$ increased luciferase expression by the pT4-Luc1686 construct (containing the RE-1 element within the context of the intrinsic MMP-2 promoter) by 1.6-fold. The activity of the RE-1 element within the context of the heterologous SV-40 promoter (pT4-LucRE-1) was also significantly increased (2.3-fold) by a 2-h incubation with prostaglandin E$_2$. These experiments indicate that the previously reported increases in MMP-2 transcription induced by exposure of mesangial cell to prostaglandin E$_2$ are mediated, at least in part, through the positive transcriptional action of the RE-1 element.

**DISCUSSION**

The studies outlined in this report extend our previous observations on the transcriptional control of the gelatinase A gene and the critical role of the 40-bp RE-1 enhancer element in this process. An expression screening strategy using this element as a probe yielded the transcriptional regulatory protein, YB-1, which is a major determinant of the transcriptional activities of several growth-related genes (8). The strong preference of YB-1 for binding to single-stranded, as opposed to double-stranded, DNA, coupled with the complex patterns observed with nuclear extract gel shift analyses, strongly suggested that additional transcriptional factors associate with the RE-1. Given the fact that treatment of glomerular mesangial cells with phorbol ester, prostaglandin E$_2$, or cAMP analogs induces gelatinase A transcription and synthesis (6, 7), we considered that AP2 was a likely candidate for this role. Inspection of the RE-1 sequence revealed the sequence, GCCT-GCTGGGG, which is homologous to the AP2 consensus sequence, GCCCCG-GGC (16).

The ability of recombinant AP2 protein to bind to the RE-1 sequence was demonstrated by gel shift analyses and shown by DNA footprinting methodology to occur within the putative...
partial consensus sequence. Interestingly, this partial consensus sequence is contiguous with the incomplete Y box sequence which interacts with recombinant YB-1 protein (8). Whereas YB-1 alone has little affinity for the RE-1 element in the double-stranded form (8), addition of YB-1 to the AP2/oligonucleotide incubation mixture led to extensive complex formation consisting of AP2-YB-1 heteromeric complexes. These studies suggested that AP2, by interaction with the RE-1 element in the double-stranded form, facilitates subsequent YB-1 binding and heteromer formation. Experiments with an AP2 consensus sequence oligonucleotide lacking a Y box confirmed this impression. Whereas recombinant YB-1 protein had no detectable binding activity for this sequence in the double-stranded form, inclusion of AP2 resulted in a dramatic increase in the amount of shifted oligonucleotide as compared with AP2 alone. Not only was complex formation facilitated, there was a dramatic increase in the half-life \( (\text{i.e. stability}) \) of these complexes. The physiological significance of these interactions was confirmed by the transfection experiments with the mutated AP2 consensus-binding site and by the increase in RE-1 transactivating function following exposure of mesangial cells to prostaglandin E\(_2\).

The immunoprecipitation studies indicated that the interaction of AP2 with YB-1 is not dependent upon the presence of an appropriate oligonucleotide template, as we were able to recover AP2-YB-1 complexes from solution phase. That AP2 and YB-1 proteins are associated in the \textit{in vivo} context was demonstrated by the sequential decreases in RE-1 electrophoretic mobility following sequential addition of anti-AP2 and YB-1 antibodies. Several recent reports have also demonstrated interactions of YB-1 with different partner proteins, including YY1, Pur-\(\alpha\), NF-\(\kappa\)B/relA subunit, DNA-binding protein A, and cardiac ankyrin repeat protein (18–23). In most of these studies, YB-1 partnering was demonstrated by differences in binding affinities for the respective regulatory elements in gel shift analysis; a successful co-immunoprecipitation has only been reported for the cardiac ankyrin repeat protein (23).

DMS protection footprinting analyses of the RE-1 element probed with both YB-1 and AP2 were informative in that AP2 binding to the partial consensus sequence overlapped the YB-1-binding sites within the partial Y box sequence. Composite and competitive binding of AP2 and other transcription factors has been described for several promoters, including prothymosin-\(\alpha\), ornithine decarboxylase, and the AP2\(\alpha\) genes (24, 25).
such a placement of transcriptional binding sites can facilitate either cooperative or competitive interactions and permit a finer level of transcriptional control than that achieved by binding of single factors to promoter elements (26).

The footprinting analysis of the AP2/ YB-1 combination also revealed a more extensive protection of guanines at the 3' end of the oligonucleotide sequence as compared with footprinting with AP2 alone. These studies suggest that following the AP2-facilitated binding of YB-1 to the RE-1 sequence, there may be assembly of YB-1 multimers along the length of the element. The ability of high concentrations of YB-1 to form multimeric complexes on the single-stranded components of the RE-1 has been recently demonstrated (8).

AP2/YB-1 binding to the RE-1 element induced pronounced changes in DNA structure. Single-stranded DNA was formed immediately distal to the AP2-binding site and extended to nearly the 3' end of the oligonucleotide probe. It is conceivable that similar changes are of importance when cruciform DNA extrusions with intrastrand base pairing occur as they have been described for the cAMP response element of the human enkephalin gene promoter and the N4 virion RNA polymerase promoter (27–30). Interestingly, the 3' sequence of the RE-1 contains an inverted repeat sequence that may also participate in intrasubstrate base pairing. These major changes in DNA structure induced by the combination of AP2/YB-1 may help to explain the synergistic effects of the combination on the levels of RE-1-dependent transcription rates observed with HepG2 cells.

The number of identified single strand-specific DNA-binding proteins involved in the regulation of transcription is steadily increasing and includes, among others, the far upstream element-binding proteins of the c-myc gene (31, 32), Pur α (19), and the heterogeneous nuclear ribonucleoprotein K (33). Major questions remain as to how these proteins, with specific single-stranded DNA recognition motifs, locate their cognate target sequences and how DNA melting and strand separation is mediated to permit access. In the current study, we propose a model whereby AP2 binding to the double strand RE-1 element permits subsequent attachment of the lower affinity YB-1 protein, which then mediates DNA helical separation. These effects could permit the recruitment of additional transactivating single strand binding factors to this region and thus converge the structural and functional data obtained in this study. In support of this concept, initial studies using Southwestern blot analysis and oligonucleotide affinity techniques have revealed a 17-kDa RE-1 single strand-specific DNA-binding protein in HepG2 nuclear extracts.

Our present studies with the rat gelatinase A promoter region and the identification of YB-1 and AP2 as important regulatory proteins represent a convergence with the analyses of the human gelatinase A promoter by Frisch and colleagues (34, 35). These workers identified, within the context of the human fibrosarcoma HT1080 cell line, a strong enhancer element (denoted r2) located approximately 1650 bp from the transcriptional start site, a location not dissimilar to the rat RE-1 found at 1322 bp. Gel shift analyses and DNA footprinting, as well as complex formation with the adenovirus E1A protein, all indicated a specific interaction of the r2 sequence with the AP2 protein. Alignments of the two respective elements demonstrate a significant degree of homology within the region of the partial AP2 consensus sequences, and gel shift analyses with recombinant YB-1 and the r2 element indicate that binding, particularly to the single-stranded component, does occur.

We conclude that the rat gelatinase A RE-1 element is the structural homolog of the human gelatinase A r2 enhancer element, and we propose that a common feature of these elements is the initial binding of AP2 to the double-stranded components, recruitment of YB-1 with DNA helical unwinding, and subsequent potential binding of additional single strand-specific transcription factors. The identification and molecular characterization of these additional single strand-specific transcription factors may be expected to provide further insights into the regulation of this element, which appears to play such a central role in the control of gelatinase A gene transcription.

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A Synergistic Interaction of Transcription Factors AP2 and YB-1 Regulates Gelatinase A Enhancer-dependent Transcription

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