Transcription Regulatory Complex Including YB-1 Controls Expression of Mouse Matrix Metalloproteinase-2 Gene in NIH3T3 Cells

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Matrix metalloproteinase 2 (MMP-2) is a metalloproteinase belonging to a family of structurally related zinc-dependent endopeptidases capable of degrading extracellular matrix components. To elucidate the functional promoter of the mouse MMP-2 gene, systematic transient expression analysis of the 5′-flanking region of the MMP-2 gene was performed using serially nested deletions. The deletion analysis indicated that the proximal 327-bp sequence from nucleotide positions −313 to +14 relative to the transcription start site is essential for minimal promoter activity and that a 10-bp sequence of the promoter at positions −939 to −930 is required for high expression level of the MMP-2 gene. The 10-bp fragment functioned as a potent stimulator of heterologous SV40 promoter activity. This element is identical to the YB-1 binding motif (Y-box) present within the responsive element-1 (RE-1), which has been shown to act as a potent *cis*-activator of transcription of the rat MMP-2 gene. The binding of a nuclear factor(s) to the 10-bp fragment was also revealed by electrophoretic mobility shift assays (EMSAs). Antibody-supershift EMSAs of nuclear extracts from NIH 3T3 cells demonstrated YB-1 binding to the RE-1 sequence. It was concluded that the RE-1 is the conserved element for potent expression of MMP-2 gene among rodents.

Key words matrix metalloproteinase 2 (MMP-2); transcription; promoter; luciferase assay; electrophoretic mobility shift assay

During metastasis, invasive cells must transverse tissue barriers comprised by a variety of extracellular matrices (ECM). This process depends on the ability of tumor cells to degrade the surrounding matrices and then migrate through the matrix defects.[1] Matrix metalloproteinase (MMP)-2 (type IV collagenase; gelatinase A) has been suggested to play a role in tumor metastasis.[2] The activity of MMP-2 is regulated at three steps: gene expression by several transcription factors, proenzyme processing by tissue inhibitors of metalloproteinase-2 and membrane-type metalloproteinases, and inhibition of enzymatic activity by naturally occurring tissue inhibitors of metalloproteinases.[3] Recently, the mechanisms of human MMP-2 gene expression in cancer cells have become clear. Qin *et al.*[4] demonstrated that several transcription factors, including Sp1, Sp3 and AP-2, participate in the control of constitutive MMP-2 gene expression. The same group also demonstrated that Brg-1 plays a role in the regulation of constitutive expression of the MMP-2 gene by recruitment of Sp1, AP-2 and Pol II to the MMP-2 promoter.[5] Furthermore, p53 has been also shown to transcriptionally activate the expression of the human MMP-2 gene.[6] Interestingly, it was reported that ATF3 interferes with human MMP-2 expression by antagonizing p53-dependent activation of the MMP-2 gene promoter.[7]

On the other hand, four regulatory *cis*-elements for the gene expression of rat MMP-2 have been identified. Analyses of rat MMP-2 gene transcription have demonstrated that specific interaction of the nuclear factor YB-1, a member of the Y-box transcription factor family, with a 40-bp enhancer element, denoted as RE-1, located at nucleotide positions −1322 to −1282 relative to the translational start site is necessary for potent expression of the rat MMP-2 gene.[8,9] The same authors have also shown that combinatorial interaction of AP-2 and p53 with YB-1 in RE-1 yields more increased MMP-2 expression.[10] Another regulator of rat MMP-2 transcription, nm23-β, that acts by competitive interference with the transactivator YB-1, leading to suppression of MMP-2 transcription, has also been identified in RE-1.[11] Moreover, under hypoxic conditions, MMP-2 transcription has been reported to be regulated in an AP-1-binding site located at positions −1394 to −1385 through JunB/Fra1 and JunB/FosB heterodimers.[12] An additional silencer element in which PU.1 transcription factor binds was isolated in a further upstream region (positions −1854 to −1849) of the rat MMP-2 gene.[13] In addition to the PU.1 binding element, AP-1 binding element and RE-1, a fourth region at positions −1021 to −972, designated RE-2, was identified as an Ets-1-binding site in which rat MMP-2 transcription was increased through Ets-1 transcription factor.[14]

We previously showed that extracellular matrix tenascin-X-deficient (TNX-/-) mice exhibit promotion of tumor invasion and metastasis due to increased levels of MMP-2 and MMP-9 activities.[15] It has also been revealed that the induction of MMP-2 expression by TNX deficiency is mediated through c-Jun N-terminal kinase and protein tyrosine kinase phosphorylation pathways.[16] However, there have been no reports on expression of the mouse MMP-2 gene. Sequence comparison of the rat and mouse 5′-flanking 2-kb regions of the MMP-2 gene shows a high degree of conservation with more than 80% similarity. Especially among the 5′-flanking regions the important elements that bind the same set of transcription factors are conserved evolutionally. In this study, to clarify the transcriptional regulation of the mouse MMP-2 gene, the promoter of the mouse MMP-2 was functionally characterized.

MATERIALS AND METHODS

Cells Mouse NIH3T3 cells were grown at 37 °C in a 5% CO_2 humidified atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) (Nissui) supplemented with 10% fetal bovine serum (FBS), penicillin (10 units/ml) and streptomycin (10 μg/ml).

Plasmids The upstream 1692 bp relative to the transcrip-

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tion start site of the mouse MMP-2 gene, which include the mouse MMP-2 RE-1 (mRE-1) and the homologous MMP-2 promoter, were subcloned into the promoter-less luciferase expression vector pGL3-Basic (Promega, Tokyo, Japan) and designated as p(−1692)Luc. The position (+1) of the transcription start site corresponded to nucleotide position 4641 in accession number AB125668 [DNA Data Base of Japan (DDBJ, Mishima)].[16] Luciferase constructs carrying a 5′-deleted promoter of the mouse MMP-2 gene were created as follows. The following oligonucleotide primers were prepared [forward primers 5′-GGACGCGTCCTACTCCCCAAAGCCAGA-3′ for p(−1048)Luc, 5′-GGACGCGTTGCTGGGCAAGTCTGAACTT-3′ for p(−939)Luc, 5′-GGACGCGTGTCTGAACTTGTCAGAAGA-3′ for p(−929)Luc, 5′-GGACGCGTATTAGTTGTGTGTGT-3′ for p(−585)Luc, 5′-GGACGCGTGGACCCTCTTGTTTCT-3′ for p(−313)Luc, 5′-GGACGCGTCACCCGGTGCTGCCAGCAC-3′ for p(+14)Luc and reverse GV4 primer 5′-GGAGATCTCTCCGCGTGTCACCCT-3′]. Restriction sites *Mlu* I and *Bgl* II were incorporated into forward and reverse primers, respectively. Thirty cycles of polymerase chain reaction (PCR) amplification consisting of denaturation at 98 °C for 20 s and annealing and extension at 68 °C for 5 min each were allowed to proceed in a MiniCycler™ (Funakoshi, Tokyo, Japan). A single band was obtained by agarose gel electrophoretic analysis. The PCR products were digested with *Mlu* I and *Bgl* II restriction enzymes and cloned into *Mlu* I-*Bgl* II sites of the pGL3-Basic vector. The construct pΔmRE-1 with deletion of a part (positions −939 to −930) of mouse RE-1 (10-bp mRE-1) was created as follows. The first PCR with forward MluGV5 primer 5′-GGACGCGTTTCTGGGTAAGGCAATG-3′ and reverse primer 5′-CAGACGGCTGTAGAGTGGGTTAGC-3′ on p(−1692)Luc template was performed, and a 0.7-kb fragment was obtained. Another PCR with forward primer 5′-CAGCCGTCTGAACTTGTCAGAAGACC-3′ and reverse GV4 primer was done, and a 1.3-kb fragment was obtained. Then the second PCR with forward MluGV5 and reverse GV4 primers on a DNA mixture of 0.7-kb and 1.3-kb fragments as a DNA template was performed. The 2.0-kb PCR products were digested with *Mlu* I and *Bgl* II restriction enzymes and cloned into *Mlu* I-*Bgl* II sites of the pGL3-Basic vector. The construction of pmRE-1ovec, which encodes the 10-bp mRE-1 from positions −939 to −930, TGCTGGGCAA, linked two times in tandem in front of the heterologous SV40 promoter, was performed as follows. Briefly, oligonucleotides corresponding to a part of the mRE-1 sequence, 5′-CGAG**TGCTGGGCAA**G-3′ and 5′-TCGAC**TTGCCCAGCA**CTCGAGCT-3′, were synthesized and phosphorylated with ATP and polynucleotide kinase. The bold letters indicate the 10-bp mRE-1 sequence. The complementary strands of oligonucleotides were annealed and inserted into *Sac* I-*Xho* I sites of pUC19 (Takara, Tokyo, Japan). The oligonucleotides linked two times in tandem was prepared in pUC19 as in the OVEC system,[17] and the sequences were inserted into the *Sac* I-*Xho* I sites of pSVpLuc,[18] which carries the luciferase gene under the control of the SV40 promoter. All cloned DNAs were confirmed by sequencing.

Transfection and Luciferase Assays Two micrograms of the respective reporter plasmid and 1.5 μg of the β-galactosidase expression vector (pCMV-β-gal) in addition to 0.5 μg of pAdVAntage Vector (Promega) were cotransfected into NIH3T3 cells (60% confluent) by the calcium phosphate coprecipitation method,[19] and the NIH3T3 cells were then incubated for 48 h. Four hours after transfection, NIH3T3 cells were boosted with 20% DMSO (dimethylsulfoxide) for 1 min at room temperature. Luciferase assays were performed as described previously.[19]

Preparation of Nuclear Extracts NIH3T3 cells were grown to 90% confluence in tissue culture dishes, washed twice with phosphate-buffered saline (PBS), and scraped in 10 ml of cold PBS. Nuclear extracts were prepared according to the method of Dignam *et al*.[20] The cells was resuspended in 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol (DTT) and incubated on ice for 15 min. The suspension was passed through a 24 G needle 6 times. The suspension was then subjected to centrifugation at 12000 rpm for 5 min at 4 °C. The pellet was suspended in 20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.5 mM DTT and incubated on ice for 30 min. The nuclear lysate was then subjected to centrifugation at 12000 rpm for 5 min at 4 °C, and the nuclear extract was collected. The nuclear extract was dialyzed for 1 h against 20 mM HEPES, pH 7.9, 20% glycerol, 20 mM KCl, 2 mM $MgCl_2$, 0.2 mM EDTA, and 0.5 mM DTT at 4 °C. Protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL, U.S.A.) using bovine serum albumin (BSA) as a standard. Nuclear cell extracts were stored at −80 °C until use in electrophoretic mobility shift assays (EMSA).

Electrophoretic Mobility Shift Assays (EMSAs) EMSAs were carried out as described previously.[19] Briefly, a reaction mixture containing 20 μg of NIH3T3 cell nuclear extract was incubated for 30 min at room temperature in a final volume of 15 μl with a binding mixture containing 100 mM KCl, 1 mM EDTA, 1 mM DTT, 4 μg of BSA, 2 μg of poly(dI-dC), 4% Ficoll 400 and an infrared fluorescent molecule IRDye800-conjugated probe, with or without non-labeled competitor oligonucleotides at a 10- or 100-fold molar ratio against the probe. DNA-protein complexes formed in the mixture were separated in a 4% polyacrylamide gel containing 0.25 X TBE buffer (1 X TBE: 90 mM Tris, 90 mM boric acid, and 2.5 mM EDTA) and visualized by an infrared imaging system (Odyssey, LI-COR, Lincoln, Nebraska, U.S.A.). The nucleotide sequence of the sense strand of the 5′-terminal IRDye800-conjugated oligonucleotide is as follows: IRD/mRE-1, 5′-CCACTCTACAGCC**TGCTGGGCAA**GTCTGA-3′. This IRDye800-labeled oligonucleotides was purchased from Aloka (Mitaka, Tokyo, Japan). Nucleotide sequences of the sense strand of competitor mRE-1 and mutated mRE-1-mu oligonucleotides were 5′-CTACAGCC**TGCTGGGCAA**GTCTGA-3′ and 5′-CTACAGCCTACTAACGGGTCTGA-3′, respectively. Complementary strands of oligonucleotides were also prepared and preannealed with their sense oligonucleotides prior to EMSA analysis. The bold and underlined letters indicate the 10-bp mRE-1 sequence and mutated sequence, respectively. For supershift assays, 2 μg of anti-YB-1 antibody (Santa Cruz Biotechnology, Delaware, CA, U.S.A.) or normal IgG was incubated on ice for 1 h with nuclear extracts after the addition of the probe.

RESULTS AND DISCUSSION

Comparison of the 5′-Flanking Regions of the Mouse and Rat MMP-2 Genes To elucidate the general map of the *cis*-element in the 5′-flanking region of the mouse MMP-2 gene, we have cloned the gene.[16] An approximately 5-kb region of the 5′-flanking region of the MMP-2 gene was sequenced (DDBJ accession number: AB125668). The transcription start site (+1) was identified by comparison with a sequence from an EST clone with the most extended 5′ sequence (DDBJ accession number: BY153123). The sequence, beginning −1700 bp upstream of the transcription start site to nucleotide position +400, is shown in Fig. 1A. A comparison of the mouse MMP-2 5′-flanking region between position −1309 and the translation initiation codon (+338) with the rat orthologous region between positions −1606 and +1[8] using DNASIS maximum matching computer program (Hitachi software engineering) revealed a high degree of homology with approximately 83% nucleotide identity. In the case of rat MMP-2 gene, +1 was positioned at the translation initiation codon,[8] thus in this paper the numbering relative to the translation initiation codon in the sequence of rat MMP-2 gene was also applicable. A comparison of the 5′-untranslated regions of mouse and rat MMP-2 mRNAs revealed that the mouse mRNA is 72 nucleotides longer.

In the promoter region of the rat MMP-2 gene, four regulatory *cis*-acting elements, at −1854/−1849 (PU.1-binding site),[13] −1394/−1385 (AP-1-binding site),[12] −1322/−1282 (designated RE-1, YB-1·AP-2·p53 ternary complex-binding site)[10] and −1021/−972 (designated RE-2, Ets-1-binding site),[14] have so far been identified. Next, it was examined whether these four regulatory elements identified in the 5′-flanking region of the rat MMP-2 gene are also conserved in the orthologous region of the mouse MMP-2 gene. As shown in Fig. 1B, comparison of the sequences of the PU.1-binding site, AP-1-binding site, RE-1 and RE-2 in the rat MMP-2 gene with the mouse counterpart revealed the agreement of 4 out of 6 bases (66.7% identity), 7 out of 10 bases (70% identity), 39 out of 41 bases (95.1% identity) and 41 out of 50 bases (82% identity), respectively. Among them, the RE-1 sequence has shown the highest predictive significance. The Y-box motif 5′-CTGGGCAAG-3′ in the mouse RE-1 is completely identical to that in the rat RE-1.

Transcriptional Activity of the Mouse MMP-2 Regulatory Region in NIH3T3 Cells MMP-2 is abundantly secreted in conditioned media from mouse NIH3T3 cells (data not shown), suggesting constitutive expression of the MMP-2 gene in NIH3T3 cells. To identify the important elements in the activity of the mouse MMP-2 promoter, we created luciferase constructs carrying a 5′-deleted mouse MMP-2 promoter, and then NIH3T3 cells were transiently transfected with these 5′ deletion constructs (Fig. 2A). Transfection efficiency was monitored by the cotransfection of a β-galactosidase-expressing plasmid. As shown in Fig. 2A, minimal promoter activity with a level of 30 fold above the background was observed in the cellls transfected with construct containing the 5′-flanking sequence up to position −313 [construct p(−313)Luc]. The relative promoter activity was not changed with constructs increasing in length up to 929 bp [construct p(−929)Luc]. However, a further 10-bp inclusion spanning positions −939 to −930 [construct p(−939)Luc] caused a dramatic increment in transcriptional activity (level of 394 fold above the background). The sequence from positions −939 to −930 (5′-TGCTGGGCAA-3′) corresponds to

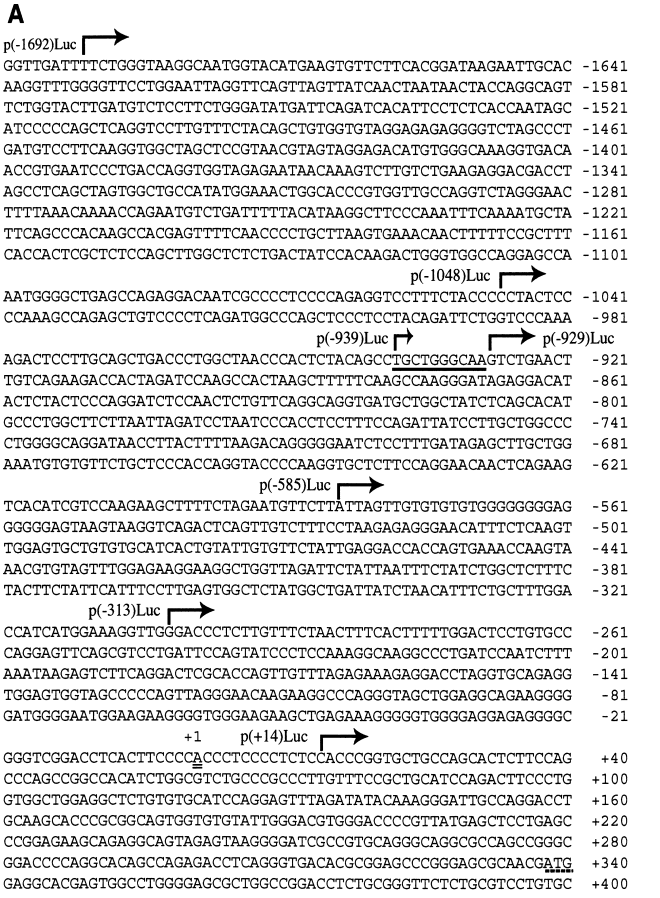

Fig. 1. (A) Nucleotide Sequence of the 5′-Flanking Region of the Mouse MMP-2 Gene

The sequence is numbered relative to the transcription start site A (a letter with a double underline) indicated as +1. The ATG translation initiation codon (position +338) is marked with a broken line. The 5′ terminus for each deletion construct is indicated by a bent arrow. The 10-bp mRE-1 sequence from positions −939 to −930 is underlined.

(B) Sequence Comparison between the Transcription Factor Binding Sites Experimentally Identified in the Rat MMP-2 Promoter Regions (rMMP2) and the Orthologous Regions in the Mouse MMP-2 Promoter (mMMP-2)

(a) PU.1-binding site. (b) AP-1-binding site. (c) RE-1. (d) RE-2. The rat sequence number is relative to the translation initiation codon according to Harendza *et al.*,[8] whereas that of the mouse is relative to the transcription start site as indicated in Fig. 1A.

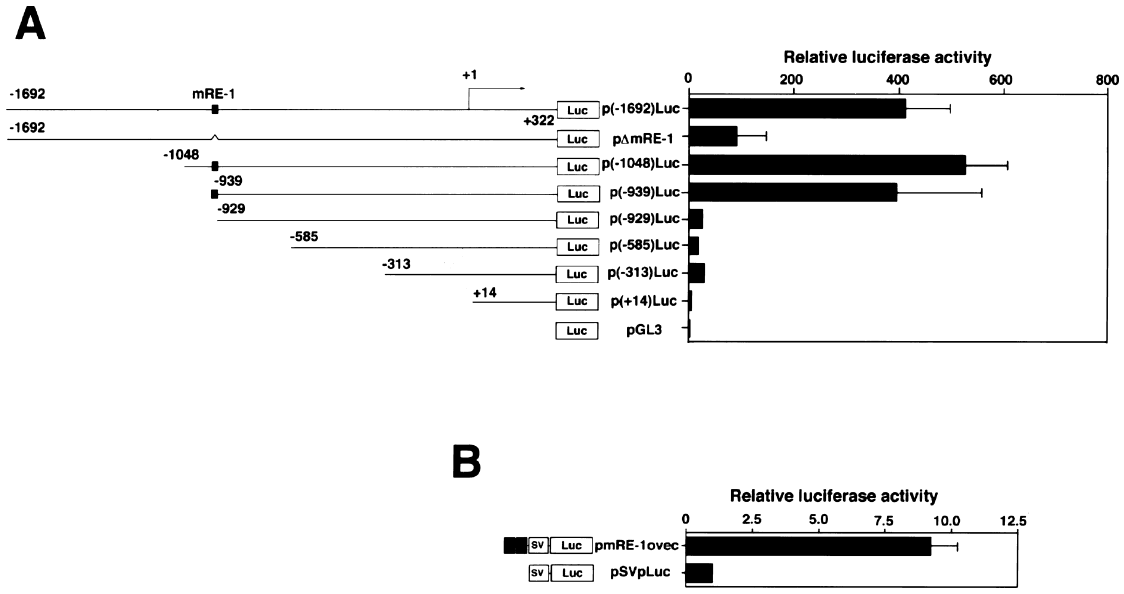

Fig. 2. Functional Analysis of the 5′-flanking Region of the Mouse MMP-2 Gene

(A) Promoter Activity of the 5′-Flanking Region of the Mouse MMP-2 Gene

Luciferase gene constructs with various 5′-deleted sequences and a deleted construct (pΔmRE-1) in which the 10-bp mRE-1 sequence was removed are shown schematically. The transcription start site (+1) is marked by a bent arrow. Black boxes indicate 10-bp mRE-1. ^ indicates deleted 10-bp mRE-1 in pΔmRE-1 construct. All plasmids share a common 3′-end at position +322. Denomination of the plasmids corresponds to the most 5′ nucleotide position included in the fragment. A series of 5′-deletion constructs in the 5′-flanking region of the mouse MMP-2 gene and the 10-bp mRE-1-deleted construct were linked to the luciferase reporter gene and transiently transfected into mouse NIH3T3 cells together with pCMV-β-gal. The transfection efficiency was monitored by determination of β-galactosidase activity and used for correction of luciferase activity. The promoter activity is shown as the luciferase activity of the various mutants relative to that of pGL3-basic (set at 1).

(B) Transcriptional Activity of the 10-bp mRE-1 Sequence in NIH3T3 Cells

The 10-bp mRE-1 sequence of the mouse MMP-2 gene was subcloned into pSVpLuc upstream of the heterologous SV40 promoter, yielding pmRE-1ovec construct. pmRE-1ovec contains mRE-1 elements linked two times in tandem. Transient transfections were carried out using NIH3T3 cells. Black boxes indicate two mRE-1 elements. SV indicates SV40 promoter. The promoter activity is shown as the luciferase activity of pmRE-1ovec relative t o that of pSVpLuc (set at 1). Results represent averages from at least three independent experiments, and bars indicate the standard error.

the YB-1 binding motif (Y-box).[9] Thus, this 10-bp sequence with potent transcriptional enhancing activity was designated as 10-bp mouse RE-1 (mRE-1) in this paper. The constructs containing 5′-flanking sequences up to position −1692 [p(−1692)Luc] showed similar high levels of luciferase activity in the range of 238 to 527 fold above the background. Furthermore, in order to confirm that the 10-bp sequence is of principal importance for the transcriptional enhancing activity, a deleted construct (pΔmRE-1) in which the 10-bp mRE-1 sequence was removed was transfected into NIH3T3 cells and the luciferase activity was measured. Deletion of the 10-bp mRE-1 had a dramatic effect on the activity of the MMP-2 promoter. Deletion of the 10-bp mRE-1 reduced the activity of the promoter to 21.3% compared with original p(−1692)Luc. When this 10-bp region was cloned into the luciferase vector pSVpLuc upstream of the heterologous SV40 promoter (pmRE-1ovec), it was found to enhance transcription to a level of 9.2 fold above the background (Fig. 2B). These results indicate that the 10-bp mRE-1 region between positions −939 to −930 with a YB-1 binding motif has potent transcriptional enhancing activity, as observed in the rat RE-1.[8]

Binding of Transcription Factor YB-1 to 10-bp mRE-1 As indicated above, transcriptional enhancing activity was localized to the 10-bp mRE-1 sequence with a YB-1 binding motif between positions −939 and −930. The activity is likely to be derived from the interaction between this sequence and nuclear factor(s). To identify the interaction of nuclear protein(s) with the 10-bp mRE-1 sequence, electrophoretic mobility shift assays (EMSAs) were carried out with nuclear extracts from NIH3T3 cells using an IRDye800-conjugated double-stranded oligo probe, IRD/mRE-1, that contains the 10-bp mRE-1 sequence. The mobility of the IRDye800-conjugated oligo probe was significantly retarded in the presence of nuclear extracts from NIH3T3 cells, due apparently to the formation of a major DNA-protein complex (Fig. 3A). Specificity of this interaction was demonstrated in lane 4, where addition of a 100-fold molar excess of double-stranded unlabeled 10-bp mRE-1 oligonucleotides (mRE-1) to the DNA-nuclear extract mixture resulted in disappearance of the shifted bands. The specificity of the DNA-nuclear protein was further confirmed by the fact that mutated unlabeled oligonucleotides (mRE-1-mu) failed to compete for nuclear protein binding (lane 6). The formation of the oligonucleotide-protein complex was significantly inhibited when the NIH3T3 cell nuclear extracts were preincubated with a specific anti-YB-1 antibody, whereas preincubation with a control normal IgG had no significant effect on the formation of the oligonucleotide-protein complex (Fig. 3B). These results suggest that a nuclear protein(s) with YB-1 interacts with this 10-bp mRE-1 sequence, leading to potent transcription of the mouse MMP-2 gene.

In summary, we identified the region with basal promoter activity and identified a potent *cis*-acting regulatory element that critically affects its transcriptional activation in mouse NIH3T3 cells. This element, designated 10-bp mRE-1, is localized at positions −939 to −930 and contains a YB-1 binding motif. Using EMSA, we demonstrated the binding of

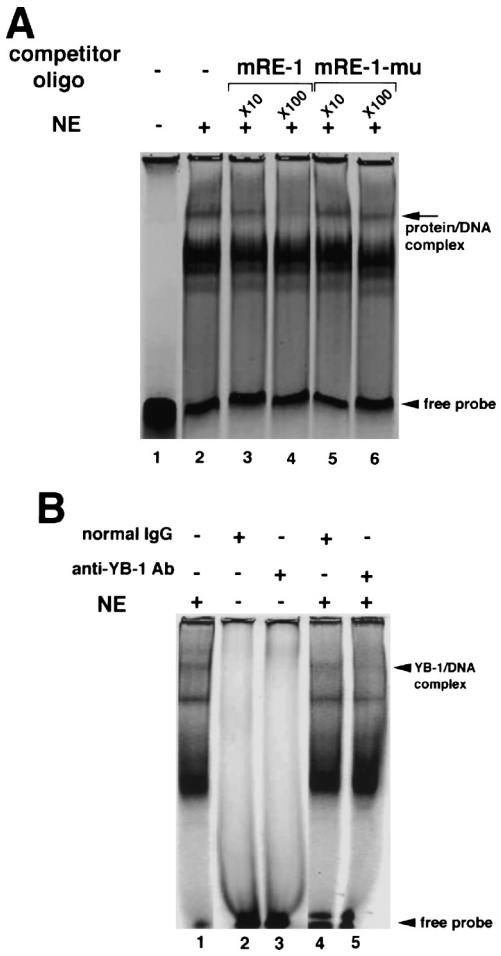

Fig. 3. Binding of YB-1 to mRE-1

(A) Electrophoretic Mobility Shift Assays (EMSAs) with a double-stranded mRE-1 Oligo Probe and Nuclear Extract from NIH3T3 Cells

The IRDye800-conjugated oligo probe IRD/mRE-1was incubated in the absence (lane 1) or presence of nuclear extracts (NE) prepared from NIH3T3 cells (lanes 2—6). The following unlabeled double-stranded competitor oligonucleotides were added at a 10-fold or 100-fold molar excess: lane 3, 10-fold excess mRE-1; lane 4, 100-fold excess mRE-1; lane 5, 10-fold excess mRE-1-mu; lane 6, 100-fold mRE-1-mu. Nuclear protein-DNA complexes were resolved by native 4% polyacrylamide gel electrophoresis and visualized by the infrared imaging system Odyssey. The protein·DNA complex is indicated with an arrow.

(B) Supershift Study Using a Specific Anti-YB-1 Antibody

The IRDye800-conjugated oligo probe IRD/mRE-1was incubated with the nuclear extract in the absence of antibodies (lane 1). The probe was also incubated with normal IgG (lane 2) or anti-YB-1 antibody (lane 3) in the absence of the nuclear extract. Furthermore, normal IgG (lane 4) or anti-YB-1 antibody (lane 5) was added by means of a 1-h incubation with the probe and nuclear extract. The protein-DNA complex was resolved by 4% polyacrylamide gel. The YB-1·DNA complex is indicated with an arrowhead. Free probe is also shown.

transcription factor YB-1 to this element. We also speculate that in mouse MMP-2 expression, a YB-1·AP-2·p53 ternary complex interacts with the 10-bp mRE-1 by the same molecular mechanism as that observed in the increment of expression level of the rat MMP-2 gene.

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