A functional activating protein 1 (AP-1) site regulates matrix metalloproteinase 2 (MMP-2) transcription by cardiac cells through interactions with JunB–Fra1 and JunB–FosB heterodimers

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Enhanced synthesis of a specific matrix metalloproteinase, MMP-2, has been demonstrated in experimental models of ventricular failure and in cardiac extracts from patients with ischaemic cardiomyopathy. Cultured neonatal rat cardiac fibroblasts and myocytes were used to analyse the determinants of MMP-2 synthesis, including the effects of hypoxia. Culture of rat cardiac fibroblasts for 24 h in 1 % oxygen enhanced MMP-2 synthesis by more than 5-fold and augmented the MMP-2 synthetic responses of these cells to endothelin-1, angiotensin II and interleukin 1β. A series of MMP-2 promoter–luciferase constructs were used to map the specific enhancer element(s) that drive MMP-2 transcription in cardiac cells. Deletion studies mapped a region of potent transactivating function within the 91 bp region from -1433 to -1342 bp, the activity of which was increased by hypoxia. Oligonucleotides from this region were cloned in front of a heterologous simian-virus-40 (SV40) promoter and mapped the enhancer activity to a region between -1410 and -1362 bp that included a potential activating protein 1 (AP-1)-binding sequence, C^{-1394}**CTGAC**CTCC. Site-specific mutagenesis of the core TGAC sequence (indicated in bold) eliminated the transactivating activity within the -1410 to -1362 bp sequence. Electrophoretic mobility shift assays (EMSAs) using the -1410 to -1362 bp oligonucleotide and rat cardiac fibroblast nuclear extracts demonstrated specific nuclear-protein binding that was eliminated by cold competitor oligonucleotide, but not by the AP-1-mutated oligonucleotide. Antibody-supershift EMSAs of nuclear extracts from normoxic rat cardiac fibroblasts demonstrated Fra1 and JunB binding to the -1410 to -1362 bp oligonucleotide. Nuclear extracts isolated from hypoxic rat cardiac fibroblasts contained Fra1, JunB and also included FosB. Co-transfection of cardiac fibroblasts with Fra1–JunB and FosB–JunB expression plasmids led to significant increases in transcriptional activity. These studies demonstrate that a functional AP-1 site mediates MMP-2 transcription in cardiac cells through the binding of distinctive Fra1–JunB and FosB–JunB heterodimers. The synthesis of MMP-2 is widely considered, in contrast with many members of the MMP gene family, to be independent of the AP-1 transcriptional complex. This report is the first demonstration that defined members of the Fos and Jun transcription-factor families specifically regulate this gene under conditions relevant to critical pathophysiological processes.

Key words: gelatinase A, hypoxia, MT1-MMP, protein kinase C, ventricular remodelling.

INTRODUCTION

Dysfunctional remodelling of the heart in response to injury represents a final common pathway in ventricular failure. Changes in the capacity of cardiac fibroblasts to proliferate and to synthesize extracellular matrix (ECM) proteins, particularly the scar types I and III collagens, are primarily responsible for these events [1]. These changes in the cardiac fibroblast phenotype are driven by conditions and factors commonly present within the failing heart, including hypoxia and enhanced release of fibrosis-inducing peptides, growth factors, and cytokines [2,3]. Ventricular remodelling is also associated with enhanced synthesis of several matrix metalloproteinases (MMPs), of which MMP-2 may be of central importance [4]. MMP-2, also known as gelatinase A, is a member of a large family of zinc-binding proteinases. This 72 kDa enzyme is active at neutral pH and degrades multiple ECM components, including gelatins, type IV and V collagens, fibronectin and laminin. MMP-2 has an extensive tissue distribution and has important roles in wound healing, angiogenesis, platelet aggregation and tumour metastasis [5–7]. MMP-2 is secreted as an inactive proenzyme that is subsequently proteolytically processed to the active form by formation on the cell surface of a ternary MMP-2–TIMP2–MT1-MMP complex (where TIMP2 is tissue inhibitor of MMP2 and MT1-MMP is membrane-type-1 MMP, also known as MMP-14) [8]. This level of control permits finely co-ordinated regulation of proteolytic activity within the pericellular space.

Transcriptional regulation has an important role in the synthesis of the MMP enzymes. The promoter regions of many MMP genes, including MMP-1, -3, -7, -9, -10, -12 and -13, are highly conserved, with the common presence of a proximal (approx. -70 bp) activating-protein-1 (AP-1) binding site [9,10]. These conserved proximal AP-1 binding sites mediate many of the enhanced transcriptional responses of these genes to a variety of cellular stimuli. In marked contrast, the promoter of the MMP-2 gene is notable for the absence of the conserved proximal

Abbreviations used: AP-1, activating protein 1; AP-2, activating protein 2; BCA, bicinchoninic acid; BCS, bovine calf serum; ECM, extracellular matrix; EMSA, electrophoretic mobility shift assay; MEM, minimal essential medium; MMP, matrix metalloproteinase; MT1-MMP, membrane-type-1 MMP; PKC, protein kinase C; RE1, response element 1; Sp1, selective promoter factor 1; SV40, simian virus 40; TI medium, serum-free medium containing transferrin and insulin.

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AP-1 site. This observation, coupled with the inability of phorbol ester to stimulate MMP-2 synthesis, has led to the general conclusion that MMP-2, in contrast with most members of the MMP gene family, is not regulated by AP-1 [9,10].

The potential function of specific MMPs in cardiac disease is an area of active investigation. Several groups have demonstrated that cardiac tissue and cells synthesize specific members of the MMP gene family, including the interstitial collagenases, MMP-2 and MMP-9 [11–17]. Ischaemia/reperfusion of the isolated rat heart induced rapid increases in MMP-2 protein in the coronary effluent that directly correlated with the duration of ischaemia [18]. Patients with end-stage cardiomyopathy have a higher left ventricular MMP zymographic activity as compared with controls [19]. A study by Li et al. [20] showed an increase in MMP-9 gelatinolytic activity in patients with ischaemic cardiomyopathy and dilated cardiomyopathy. These studies suggest that individual MMP proteins may be major mediators of ventricular dysfunction or fibrosis.

Ventricular failure that results from ischaemic myocardial injury is not solely the consequence of passive ECM accumulation, and may be more accurately defined as a dysfunctional ECM remodelling process that involves both enhanced synthesis and degradation of the matrix [4]. The activity of this process is driven by the production of factors such as angiotensin II, endothelins and cytokines [3], and an increased understanding of the mechanisms whereby ischaemia contributes to scar tissue formation will provide insights into future targets for pharmacological intervention [4].

In the present study, primary cultures of neonatal rat cardiac fibroblasts and myocytes were used to identify how the major MMP synthetic product, MMP-2, is regulated. Synthesis of MMP-2 was enhanced by culture under hypoxic conditions and was further increased by exposure to angiotensin II, endothelin I and interleukin 1β. Transcription-regulation studies using a series of MMP-2 promoter–luciferase reporter constructs localized a cardiac-cell-specific enhancer element that contains a nearly complete AP-1-consensus-binding sequence. The functional significance of the AP-1 site for MMP-2 transcription in cardiac cells was confirmed by mutagenesis and electrophoretic mobility shift assay (EMSA) studies. The AP-1 site is occupied by JunB–Fra1 heterodimers under normoxic conditions, while culture under hypoxic conditions resulted in formation of JunB–FosB heterodimers. This is the first demonstration of MMP-2 transcriptional regulation by a functional AP-1 binding site, and may be directly related to enhanced cardiac MMP-2 synthesis during ischaemia and ventricular injury.

MATERIALS AND METHODS

Cardiac fibroblast isolation

Primary cultures of neonatal rat cardiac fibroblasts were prepared as described in [3,21]. Briefly, 1- or 2-day-old Sprague–Dawley neonatal rat pups were killed and the hearts were removed under sterile conditions. Ventricular tissue was finely minced and subjected to a series of incubations with 1% (w/v) trypsin in calcium-free buffered Hepes containing deoxyribonuclease II. Trypsinization was stopped with 10% (v/v) bovine calf serum (BCS) at 4 °C. After collection by centrifugation (400 ***g*** for 10 min at 4 °C), non-myocytes were separated from myocytes by a 30 min period of pre-plating in minimal essential medium (MEM) with 5% (v/v) BCS at 37 °C in air/CO_2 (99:1). The unattached cells (mainly myocytes) were removed and the plates were washed with MEM several times. Under these conditions, 95% of the attached cells are fibroblasts [3]. After overnight incubation, the fibroblasts were re-plated in 60 mm diameter culture dishes for transfection experiments at a density of $(6–8) \times 10^6$ cells/dish. For experiments requiring nuclear extracts, fibroblasts were allowed to grow to confluence in 100 mm-diameter dishes in normoxic conditions. For the hypoxia experiments, cells were grown in normoxic conditions until they reached 80% confluence. The medium was replaced with serum-free medium containing transferrin (10 μg/ml) and insulin (10 μg/ml) for 24 h. Before harvesting, the cells were incubated in 5% O_2 for 1 h. Nuclear extracts were prepared according to Dignam et al. [22]. Potassium chloride (1 M) solution was used for extraction, followed by overnight dialysis at 4 °C in 20 mM Hepes, pH 7.9, 20% (v/v) glycerol, 100 mM potassium chloride, 0.2 mM EDTA, 0.5 mM dithiothreitol and 0.2 mM PMSF. Protein concentrations were determined with the bicinchoninic acid (BCA) assay (Pierce) using BSA as a standard.

Cardiac myocyte isolation

Primary myocyte cultures were prepared from the hearts of 1- to 2-day-old rat pups as described in [23]. Cells were plated on to 35 mm-diameter dishes at a final density of 200–250 cells/mm^2. Cells were then incubated overnight in 5% (v/v) BCS in 99% air/1% CO_2 at 37 °C. The medium was supplemented with 1.5 μmol/l vitamin B_{12}, 50 units/ml penicillin, and 0.1 mM bromodeoxyuridine to prevent non-myocyte proliferation, as described in [23]. The medium was changed to serum-free medium containing transferrin (10 μg/ml) and insulin (10 μg/ml). Transfections were then carried out as described below.

Luciferase reporter constructs

The constructs were prepared as described in [24]. Briefly, a subcloned 6 kb *Kpn*I/*Not*I rat genomic MMP-2 promoter fragment was used as a template to prepare a series of 5′ flanking region reporter constructs in the promoterless luciferase expression vector pGL2-Basic (Promega). The 5′ PCR primer included a *Kpn*I site: the 3′ PCR primers included a *Bgl*II site to permit directional subcloning into the polylinker region of pGL2-Basic. Initial constructs included sequences extending 1686, 1007, 573, 383, 321, 267 and 293 bp 5′ relative to the translational start site, thereby generating constructs pT4-Luc1686, pT4-Luc1007, pT4-Luc573, pT4-Luc383, pT4-Luc321, pT4-Luc267 and pT4-Luc293 respectively. Further fine mapping of enhancer activity was performed by the preparation of sequential deletion constructs extending from −1686 to −1181 bp. To measure enhancer activity in the context of a heterologous promoter, fragment −1433 to −1345 bp was subcloned in a normal orientation into the expression vector pGL2-Promoter (Promega), which contains a heterologous simian-virus-40 (SV40) core promoter, thereby generating construct pT4-Luc1433P. A series of overlapping deletions of the region were constructed using nucleotides 1410–1362, 1377–1392, 1433–1396, and were designated pT4-Luc 1410P, pT4-Luc1377P and pT4-Luc1433P respectively. Site-specific mutagenesis to convert the nucleotide sequence $T^{-1392}GAC$ into $A^{-1392}CAC$ in the construct pT4-Luc1433 was performed using a commercial mutagenesis kit (Stratagene), and the mutated construct was designated pT4-Luc1433mut.

Transient transfection

Plasmid DNA (0.5 μg/dish) was diluted in serum-free medium and Fugene (Boehringer Mannheim) reagent (2 μl/dish) was added. The DNA/Fugene mixture was incubated for 10–15 min before being added to the cells. After I h, the mixture was removed and complete growth medium containing 5% BCS

was added. The cells were placed in an incubator gassed with 99 % air/1 % CO_2 at 37 °C and were harvested after 48 h. The luciferase assay, as a measure of promoter activity, was performed according to Brasier et al. [25], using a Monolight 2010 luminometer. A β-galactosidase expression plasmid (pCMV-β-gal, 0.5 μg/dish) was used to normalize for transfection efficiency using a commercial β-galactosidase chemiluminescence assay kit (Clontech). To assess the effects of hypoxia on transcription rates, the cells were transfected as detailed above, but, for the final hour of culture, were placed in 5 % O_2 prior to harvesting and analysis. All transfections were performed in triplicate or quadruplicate, and transfection experiments were repeated at least four times. Transfection results were averaged and are expressed as means $\pm$ 1 S.D.

Co-transfection studies

Plasmids containing the complete coding sequences for human FosB and JunB were obtained from the American Type Culture Collection (ATCC) and the cDNA inserts were subcloned into the *Eco*RI/*Bam*HI cloning site of the expression plasmid pSG5, in which transcription is driven by the SV40 early promoter (Stratagene), yielding pSG5-FosB and pSG5-JunB. To obtain the Fra1 cDNA, reverse transcriptase-PCR was employed using mRNA templates from cultured neonatal rat fibroblasts with the forward primer 5′-ACCGGTACCGGTCCACCATGTACCG-AGACT-3′ and the reverse primer 5′-CCACGTACGCTTCA-CAAGCCAGGAGTGT-3′. The resultant PCR product was subcloned into pSG5 as detailed above and was designated pSG5-Fra1.

For the co-transfection studies, cardiac fibroblasts were transfected with the reporter construct pT4-Luc1433, as detailed above, in the presence or absence of 50 ng of pSG5-FosB, pSG5-Fra1 and pSG5-JunB, or designated combinations of the expression plasmids. Results were normalized by transfection with the pCMV-β-gal expression plasmid and are expressed as means $\pm$ 1 S.D. Levels of significance were determined by either Student's *t* test or ANOVA where appropriate.

Western blot

Cardiac fibroblasts and myocytes were plated at a density of 1×10^4 cells/16 mm-diameter well. After overnight attachment, the cells were washed and placed in serum-free medium containing transferrin and insulin (TI medium), supplemented with 0.1 % (w/v) BSA for 24 h. For the experiments summarized in Figure 2, the cells were cultured for 24 h in TI medium supplemented with 0.1 % BSA in the presence or absence of the indicated concentrations of angiotensin II, endothelin-1, interleukin 1β (R&D Systems) or combinations as specified in Figure 2, under normoxic or hypoxic conditions as detailed above. Conditioned medium was harvested, cleared by centrifugation at 400 ***g*** for 10 min, and aliquots of 50 μl were stored at -80 °C prior to Western blot analysis. Cell layers were washed with PBS and lysed in a homogenization buffer composed of 20 mM Hepes, pH 8, 0.1 % Triton X-100, 1 mM PMSF and 1 mM EDTA. The extracts were cleared of debris by centrifugation at 400 ***g*** for 10 min. Cleared extracts were then centrifuged at 100000 ***g*** for 30 min at 4 °C. Protein concentrations were determined using the BCA method (Pierce). The individual supernatant fractions and particulate fractions in the protein concentrations specified in Figure 2 were incubated in either reducing or non-reducing sample buffer as denoted and electrophoresed by SDS/PAGE (7.5 % gels), followed by transfer to nitrocellulose membranes (0.2 μm). These membranes were incubated with murine monoclonal anti-(MMP-2) and anti-(MT-MMP1) antibodies (Oncogene Sciences). The monoclonal anti-(MMP-2) antibody is a purified IgG1κ directed against the synthetic peptide sequence corresponding to residues 524 to 539 of human MMP-2 and has no cross-reactivity with MMP-1, -3, -9, -13 or -14. The monoclonal anti-(MT1-MMP) antibody is a purified IgG3κ directed against residues 319 to 333 in the haemopexin domain of human MT1-MMMP and has no significant cross-reactivities. Washed membranes were incubated with affinity-purified horseradish-peroxidase-conjugated goat anti-mouse IgG (Vector), followed by development with a chemiluminescence kit (Amersham Biosciences). Conditioned medium and particulate fractions from the human fibrosarcoma cell line HT1080 (ATCC), which synthesizes both MMP-2 and MT1-MMP, were used as positive controls. Laser densitometry was used to determine the relative ratios of cellular synthesis of the respective MMP enzymes, using calibration with serial dilutions of recombinant enzymes (Chemicon).

EMSAs

These experiments were carried out according to Carthew et al. [26]. Synthetic or PCR-derived oligonucleotides were digested with *Bgl*II and the resultant overhanging 5′ ends filled in with [α-^{32}P]dCTP (3000 Ci/mmol) using the Klenow fragment of DNA polymerase I. Binding reactions included 1×10^4 c.p.m. of labelled DNA in a 12.5 μl reaction mixture containing 2 μg of poly(dI-dC) · (dI-dC), 300 μg/ml acetylated BSA, 20 % (v/v) glycerol and 5 μg of nuclear extract in dialysis buffer. After a 15 min incubation at 25 °C, the samples were loaded on to pre-electrophoresed 4 % (v/v) polyacrylamide (40:1 acrylamide/bisacrylamide)/15 % (v/v) glycerol gels. The gels were electrophoresed at 35 mM for 2.5 h in a buffer containing 1 × TBE (45 mM Tris/borate/1 mM EDTA). The gels were dried and autoradiographed. For competition experiments, unlabelled oligonucleotides were added to the incubation mixture in the specified concentrations.

RESULTS

Initial studies focused on the characterization of the types of MMPs that are synthesized by cultured rat neonatal cardiac fibroblasts and myocytes. As shown in Figure 1, Western blot analysis for MMP-2 examined the relative amounts of enzyme protein present in particulate fractions and in the conditioned media from fibroblasts and myocytes (Figure 1A). The conditioned medium from fibroblasts contained significantly more MMP-2 (approx. 10-fold), including the active 62 kDa form, than did conditioned media from myocytes that were cultured at an identical cell density. Examination of the particulate fractions demonstrated significant amounts of cell-associated MMP-2 protein in the fibroblast preparations, including the 62 kDa active enzyme, consistent with cell-surface-dependent activation. The activator of MMP-2, MT1-MMP, is cell-membrane-associated, and Western blots of particulate fractions from cultured cardiac fibroblasts and myocytes were examined (Figure 1B). When the particulate fractions were examined under reducing conditions, both cell types were found to express MT1-MMP, which was detected in the latent (62 kDa) and active (58 kDa) forms. Western blots of fibroblast and myocyte particulate fractions electrophoresed under non-reducing conditions detected the 62 and 58 kDa forms of MT1-MMP, as well as 124 kDa dimers.

Dimerization has been proposed as one possible mechanism for the activation of the latent MT1-MMP enzyme on the cell

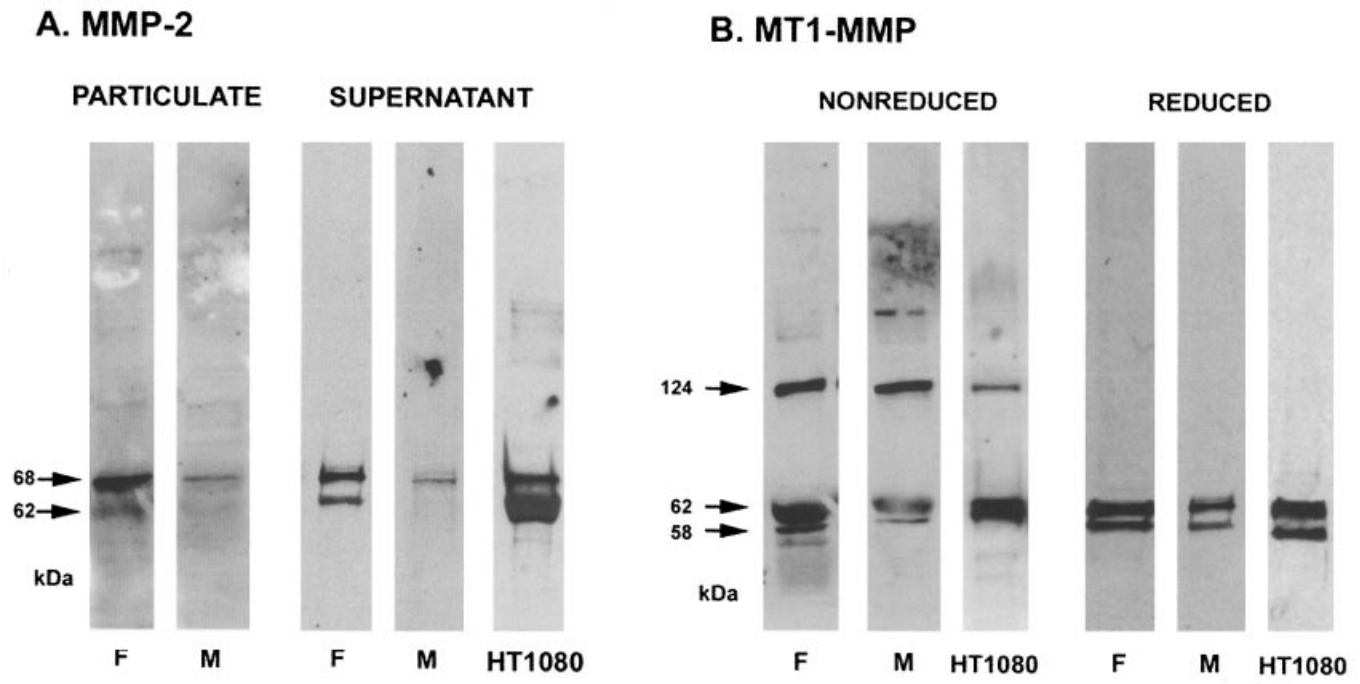

Figure 1 MMP-2 and MT1-MMP synthesis by cardiac cells

(**A**) Western blot analysis of cardiac fibroblast (F) and myocyte (M) particulate and supernatant fractions (25 μg of protein/lane) probed with a monoclonal antibody against MMP-2. The arrows indicate the latent 68 kDa and active 62 kDa forms of MMP-2. Supernatant from the fibrosarcoma cell line, HT1080, was used as a positive control. (**B**) Western blot analysis of cardiac fibroblast (F) and myocyte (M) particulate fractions (25 μg of protein/lane) probed with a monoclonal antibody to MT1-MMP under non-reduced and reduced conditions. MT1-MMP is found in the latent 62 kDa and active 58 kDa forms, as well as in 124 kDa dimers under non-reduced conditions. The 124 kDa dimers are absent when β-mercaptoethanol is included in the sample buffer. HT1080 particulate fractions were used as a positive control.

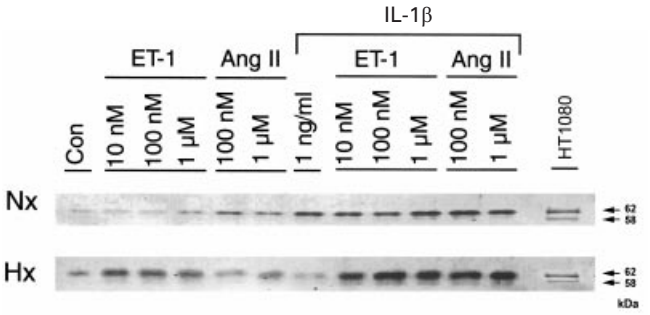

Figure 2 Western blot for MMP-2 protein in conditioned medium from cultured cardiac fibroblasts incubated for 24 h under conditions of normoxia (Nx) or hypoxia (Hx)

Cultures containing 1×10^4 cells/well were incubated either with serum-free medium alone or with the added peptides in the indicated concentrations. Samples (20 μl) were electrophoresed and probed with a monoclonal anti-(MMP-2) antibody. Supernatant from cultured HT1080 cells was used as a positive control. ET-1, endothelin-1; Ang II, angiotensin II; IL-1β, interleukin Iβ.

surface [27], and the current results are consistent with this hypothesis.

Western blot analysis was performed on the conditioned media from cultured cardiac fibroblasts to assess the effects of hypoxia or exposure to endothelin-1, angiotensin II or interleukin 1β on levels of secreted MMP-2. The results of these experiments are summarized in Figure 2. Culture under hypoxic conditions alone led to a 4–5-fold increase in MMP-2 secretion. Cells cultured under normoxic conditions showed only minimal increases in MMP-2 secretion when incubated with endothelin-1, whereas angiotensin II moderately increased MMP-2 synthesis. The combination of interleukin 1β with either endothelin-1 or angiotensin II resulted in additive increases in MMP-2 secretion under normoxic conditions. Culture under hypoxic conditions enhanced the MMP-2 synthetic response of cardiac fibroblasts to endothelin-1 and to combinations of endothelin-1 and angiotensin II with interleukin 1β.

To examine the transcriptional regulation of the MMP-2 gene within the context of cardiac fibroblasts and myocytes, transient transfections were performed with a series of rat genomic MMP-2 5′-flanking region deletion constructs, using luciferase activity as the transcriptional reporter. All results were normalized by co-transfection with a β-galactosidase expression plasmid. As shown in Figure 3(A), transient transfections of fibroblasts and myocytes demonstrated little activity within the proximal promoter (up to -383 bp), whereas considerable increases in luciferase activity were obtained in both cell types between -1007 and -1686 bp, relative to the translational start site. A second series of transient transfections was performed with a set of smaller deletions that spanned this region, the results of which are summarized in Figure 3(B). For both fibroblasts and myocytes, a highly significant degree of transactivating activity is located between -1433 and -1342 bp. Notably, the construct ending at -1342 bp, which includes the original response element 1 (RE1) enhancer (-1322 to -1282 bp) that was identified in renal and hepatic cells, had relatively weak transactivating function.

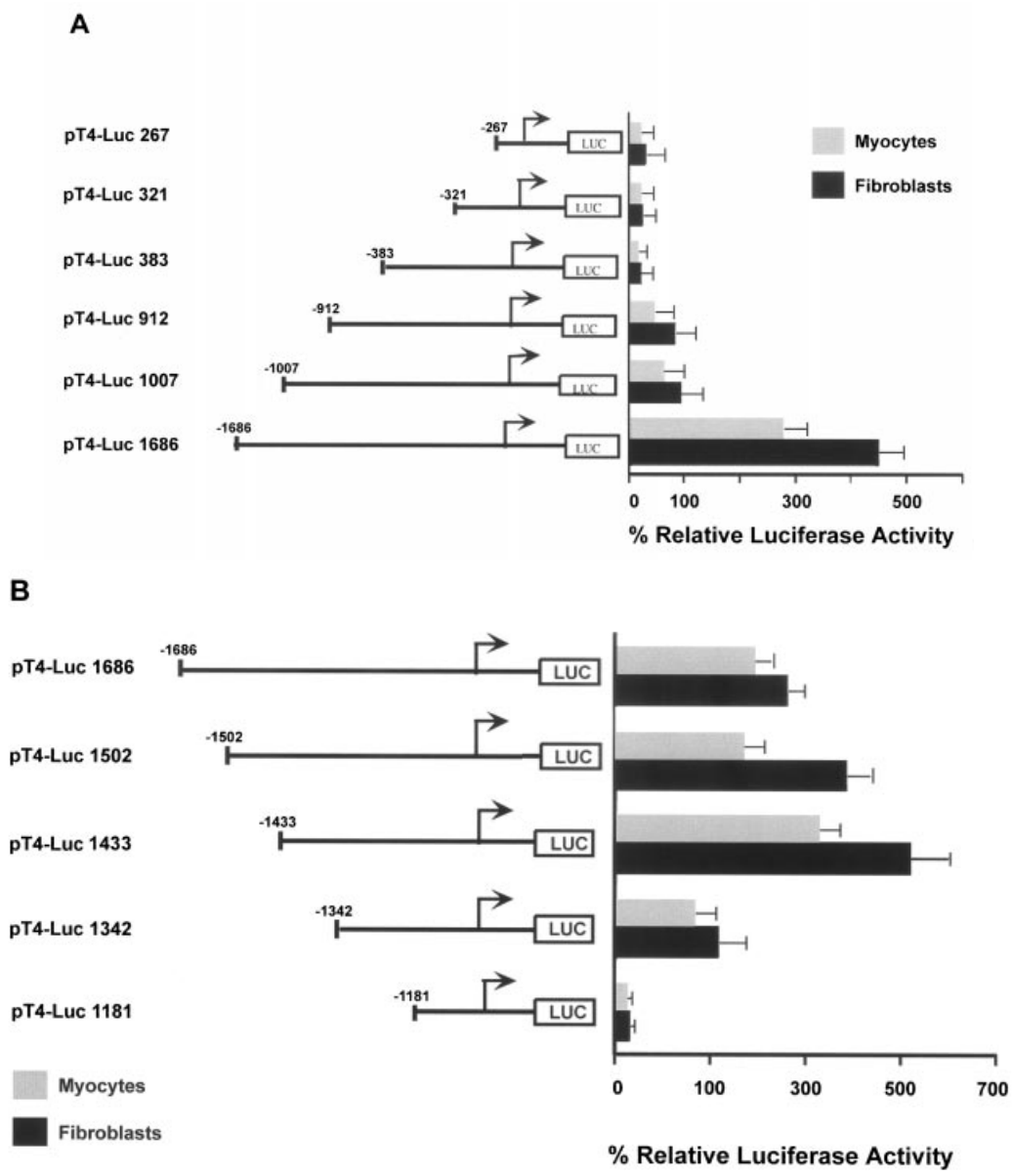

Figure 3 Transient transfection of cardiac cells with MMP-2 luciferase reporter constructs

(**A**) Transient transfections of cardiac fibroblasts and myocytes with a series of MMP-2–luciferase deletion constructs. Nucleotide positions relative to the translational start are denoted. Results are ratios of luciferase compared with β-galactosidase activity, with a relative luciferase value of 1 assigned to an enhancer-less SV40–pGL2–luciferase reporter plasmid (results are means $\pm$ S.D. of quadruplicate determinations). (**B**) Transient transfections of cardiac fibroblasts and myocytes using a series of MMP-2–luciferase deletion constructs spanning the region from -1686 to -1181 bp relative to the MMP-2 translational start site. Methods of analysis are as in (**A**).

These experiments are consistent with the existence of a second, tissue-specific, enhancer element located between -1433 and -1342 bp that drives MMP-2 transcription in cardiac cells. This region does not mediate the enhanced MMP-2 synthetic responses to endothelin-1, angiotensin II or interleukin 1β, as transient transfections localized the responsive region to these peptides between -1686 and -1502 bp (Figure 4).

Further transfection-mapping studies localized the region of transactivating function within the 91 bp sequence located from -1433 to -1342 bp. A series of overlapping oligonucleotides was synthesized and cloned in front of the heterologous SV40 promoter. These experiments were designed to fine-map further the location of the putative cardiac-specific enhancer element and to determine if its function was dependent upon the intrinsic MMP-2 promoter. As summarized in Figure 5, transactivating function could be refined to a minimal 48 bp oligonucleotide sequence that extends from -1410 to -1362 bp. Examination of this sequence revealed a potential AP-1 binding sequence, C^{-1394}CTGACCTCC, that closely resembles the consensus AP-1-binding sequence, RSTGACTN[A/C]C. No other significant transcription-factor-binding sites were identified using a variety of search algorithms. To determine whether or not the identified transactivating function resided with this AP-1 site, the transient transfections were repeated following site-specific mutagenesis of

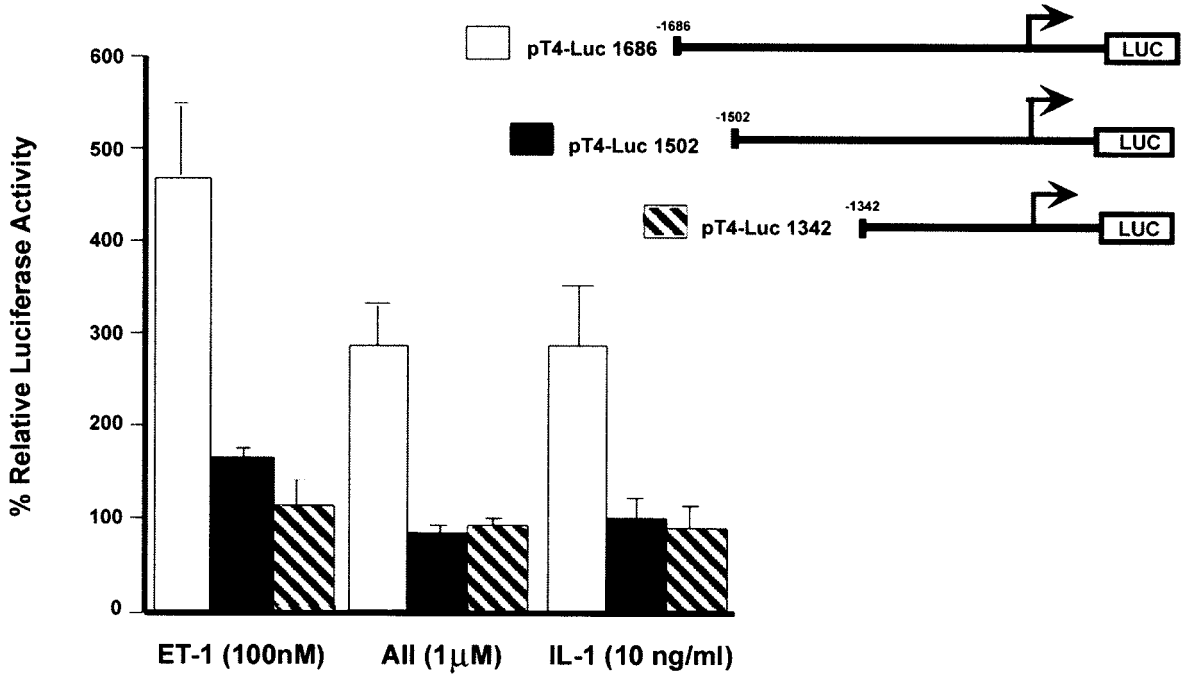

Figure 4 Transient transfections of cardiac fibroblasts and myocytes using the MMP-2–luciferase deletion constructs to assess the effect of incubation with endothelin-1 (ET-1), angiotensin II (AII) and interleukin 1β (IL-1), in the concentrations specified, on MMP-2 transcriptional activity within the context of the respective constructs

Methods of analysis are as given in Figure 3(A).

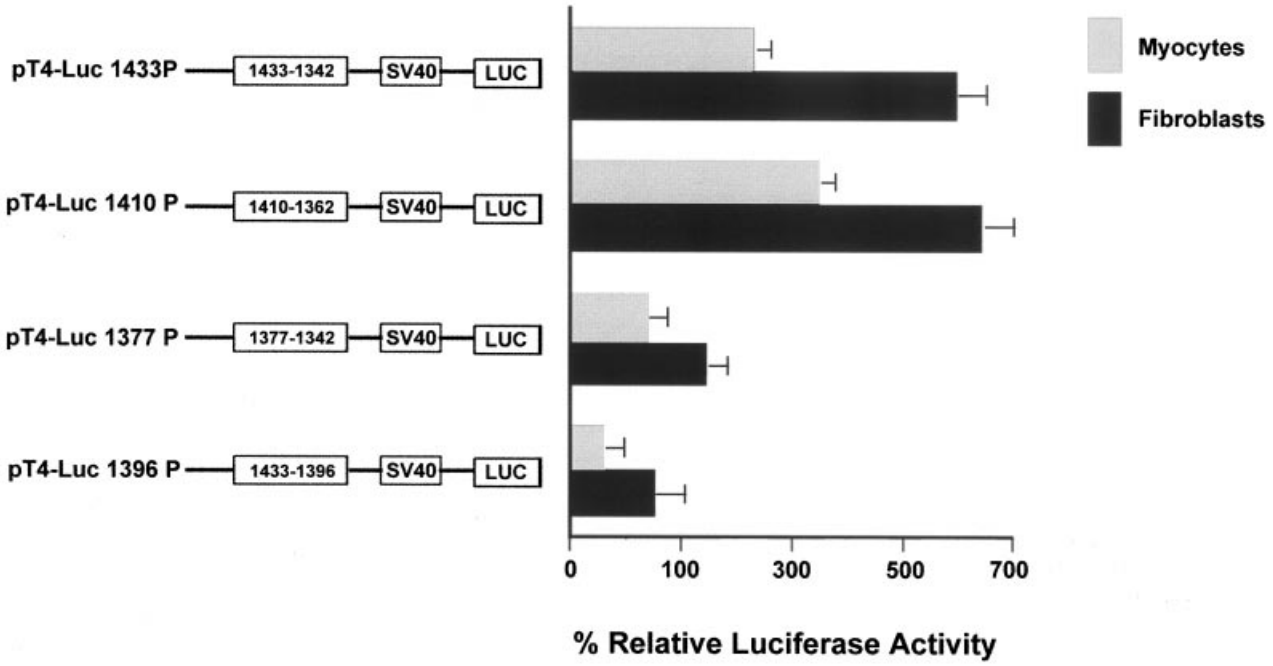

Figure 5 Transient transfections of cardiac fibroblasts and myocytes with luciferase reporter constructs containing overlapping oligonucleotides spanning the −1433 to −1342 bp region cloned in front of the heterologous SV40 promoter

Data analysis is as detailed in Figure 3(A).

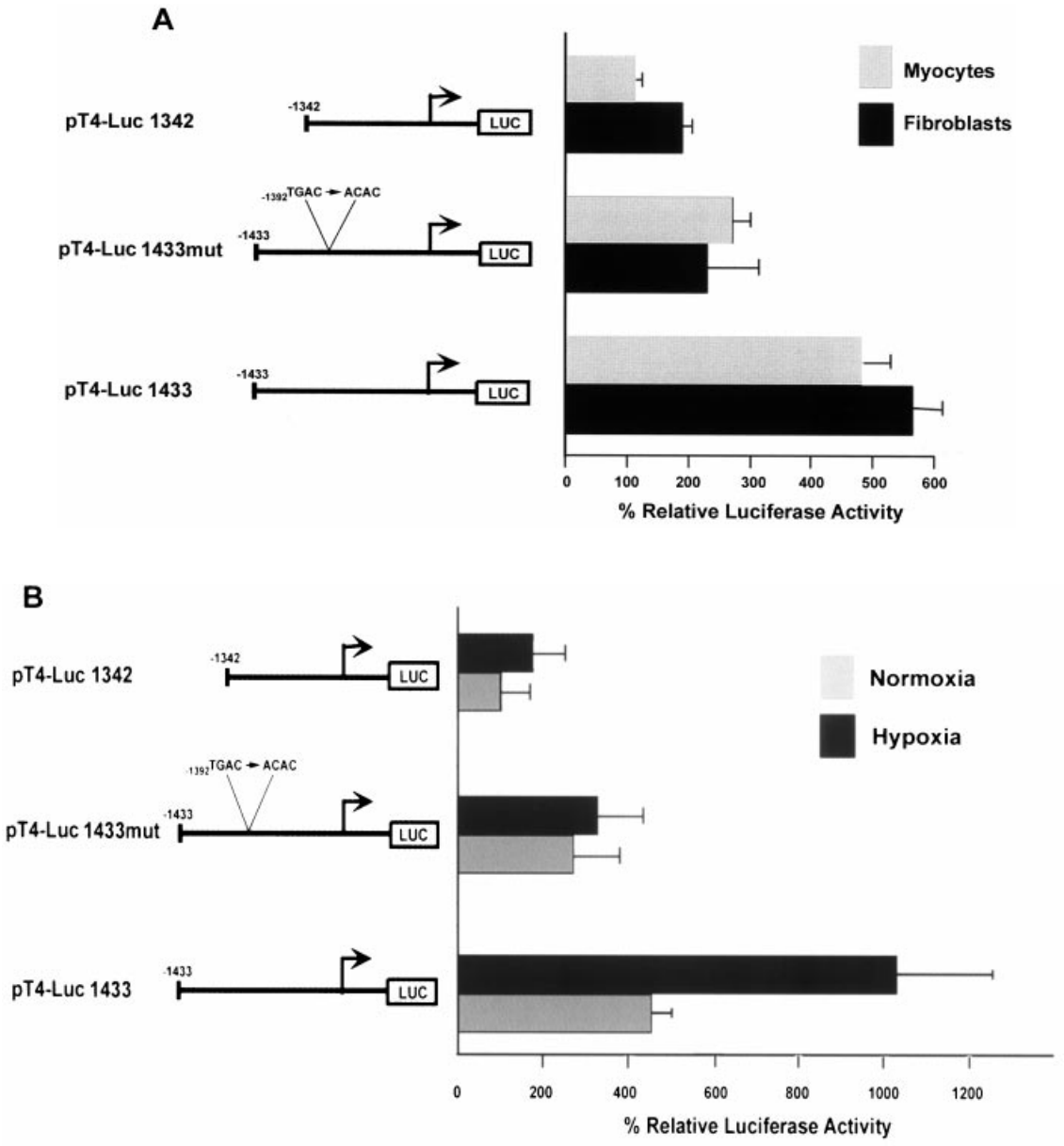

Figure 6 A functional AP-1 site regulates MMP-2 transactivating function

(**A**) Transient transfection of cardiac fibroblasts and myocytes with MMP-2–luciferase reporter constructs: effects of site-specific mutation of the AP-1 site on transcriptional activity. Cells were transfected with pT4-Luc1342, pT4-Luc1433 and pT4-Luc1433mut (containing core AP-1-binding-site mutation). Mutation of the AP-1-binding site results in a reduction of transactivation function to a level observed with the relatively inactive pT4-Luc1342 construct. (**B**) Transient transfection of cardiac fibroblasts with MMP-2–luciferase reporter constructs: effects of hypoxia on AP-1-binding-site-dependent transcriptional activity. Cells were transfected with pT4-Luc1342, pT4-Luc1433 and pT4-Luc1433mut. Cells were maintained either under normoxic conditions or placed in 5% O_2 for 1 h prior to harvest (hypoxia). Hypoxia stimulates a 2-fold increase in pT4-Luc1433 reporter activity that is completely absent in the AP-1-mutated construct. Hypoxia has no specific effect on the reporter activity of the truncated pT4-Luc1342 construct that lacks the AP-1 binding site.

the core TGAC sequence to ACAC, thereby creating the pT4-Luc1433mut construct. As summarized in Figure 6(A), transient transfection of the mutated construct in both fibroblasts and myocytes resulted in a reduction of luciferase activity to the level obtained with the shorter pT4-Luc1342 reporter construct. This experiment demonstrated that virtually all of the enhancer activity found within the -1410 to -1362 bp region can be ascribed to the AP-1 site located at -1394 bp.

These same constructs were used to determine if hypoxia enhances MMP-2 transcription via action on the AP-1 binding site. Cardiac fibroblasts were transiently transfected with pT4-Luc1342, pT4-Luc1433 or pT4-Luc1433mut. One group of cells was placed in 5% O_2 for the last hour of culture prior to harvest, and the reporter activities were compared with cells that were maintained under normoxic conditions (results summarized in Figure 6B). Cardiac fibroblasts that were cultured under hypoxic conditions exhibited a >2-fold increase in pT4-Luc1433 luciferase activity, whereas no significant effect was observed on the reporter activity of the AP-1 mutated construct, pT4-Luc1433mut, or the truncated pT4-Luc1342 construct. These

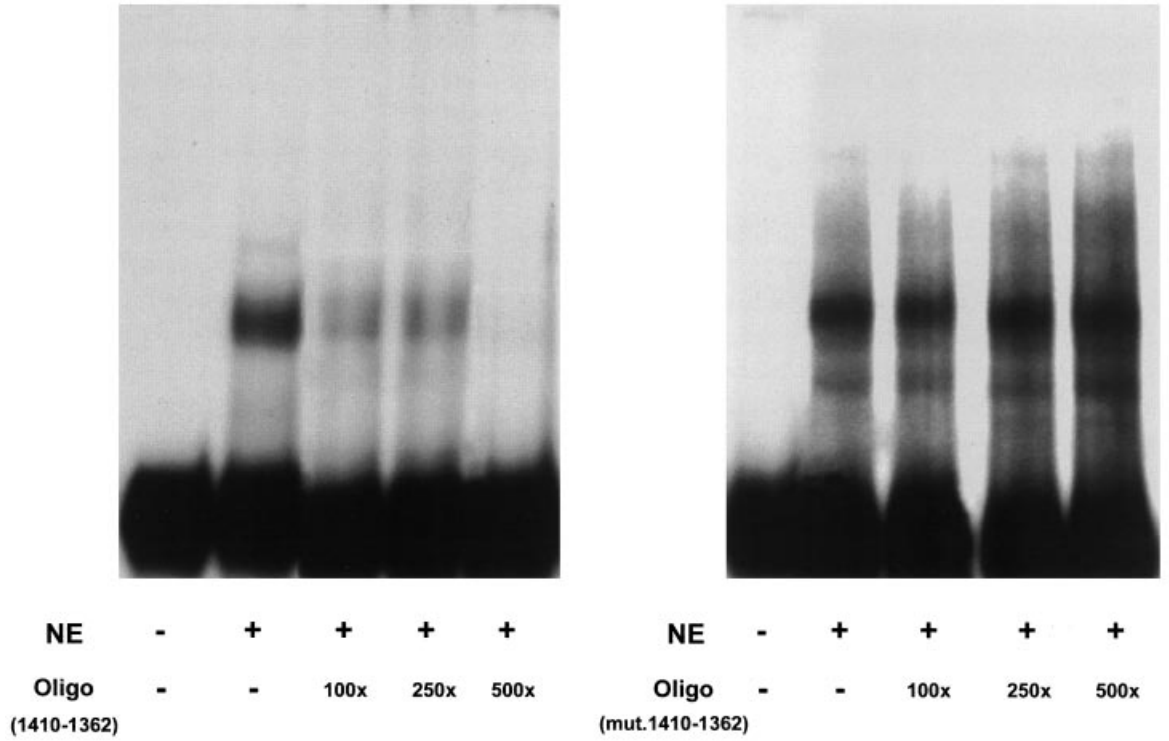

Figure 7 EMSA of cardiac fibroblast nuclear extracts demonstrates specific nuclear protein–DNA interactions with the 1410–1362 bp oligonucleotide containing the AP-1 site

In the left panel, protein–DNA binding is specifically inhibited by the inclusion of increasing concentrations of cold competitor oligonucleotide into the incubation mixture. In the right panel, the specificity of binding to the AP-1-binding site is demonstrating by the failure of cold mutated competitor oligonucleotide to reduce protein–DNA binding. NE, nuclear extract.

results are consistent with an absolute requirement for an intact AP-1-binding site at −1394 bp for hypoxia-dependent increases in MMP-2 transcription.

EMSAs were performed to determine if protein binding to the AP-1 site could be demonstrated using nuclear extracts from cardiac fibroblasts that were cultured under normoxic and hypoxic conditions. As AP-1-binding activity has been ascribed to the Fos proteins, c-fos, FosB, Fra1 and Fra2, and the Jun proteins, c-jun JunB and JunD [28,29], we used antibody-supershift or depletion studies to determine the identity of the nuclear proteins that are involved. Inclusion of fibroblast nuclear extracts from cells that were cultured under normoxic conditions with the radiolabelled −1410 to −1362 bp oligonuclotide yielded a clear-cut mobility shift with consistent protein binding (Figure 7). The specificity of the binding to the AP-1 site within the −1410 to −1362 bp oligonucleotide was confirmed by elimination of binding by increasing concentrations of cold competitor oligonucleotide and by the fact that competition with cold competitor oligonucleotide with the mutated AP-1 site did not compete for protein binding.

As seen in Figure 8(A), inclusion of antibodies against the specific members of the Fos and Jun protein families with nuclear extracts from fibroblasts that were cultured under normoxic conditions yielded supershifted bands that were specific for Fra1 and JunB. When nuclear extracts were prepared from fibroblasts under hypoxic conditions, antibody-inclusion experiments indicated that FosB, in addition to Fra1 and JunB, was now a component of the shifted complexes.

Double antibody gel-shift experiments were performed to determine whether JunB bound to the −1410 to −1362 bp oligonucleotide in the homodimeric form or as a heterodimer with either Fra1 or FosB (Figure 8B). When the antibody against JunB was used alone, a supershifted complex was observed. The simultaneous inclusion of Fra1 and JunB antibodies resulted in a loss of the shifted complex using extracts from normoxic fibroblasts. These studies indicate that JunB interacts with the oligonucleotide in the heteromeric form with Fra1. A similar depletion of Fra1–JunB complexes was observed in the double antibody studies using hypoxic fibroblast nuclear extracts, while double antibody experiments with anti-FosB and anti-JunB antibodies generated a new supershifted band, consistent with the presence of FosB–JunB heterodimers. The double antibody experiments also indicate that JunB–JunB homodimers do not significantly interact with the AP-1 site within this sequence.

The EMSA studies indicate that the MMP-2 AP-1 site is occupied by Fra1–JunB and FosB–JunB heterodimers. To confirm the functional significance of these observations for MMP-2 transcription, a final series of experiments used transient transfection of cardiac fibroblasts with construct pT4-Luc1433 and low concentrations of expression plasmids that encode Fra1, JunB or FosB. As summarized in Figure 9, co-transfection with the low concentrations of the expression plasmids for FosB, JunB or Fra1 alone did not significantly increase pT4-Luc1433 reporter activity. However, the combination of either FosB–JunB or Fra1–JunB led to 2- to 3-fold increases in reporter activity. Co-transfection of these constructs with the pT4-Luc1433mut construct had no effect on transcription rates, as expected (results not shown). These results confirm the functional significance of the hypoxia-mediated increases in AP-1-binding activities for enhanced MMP-2 transcription.

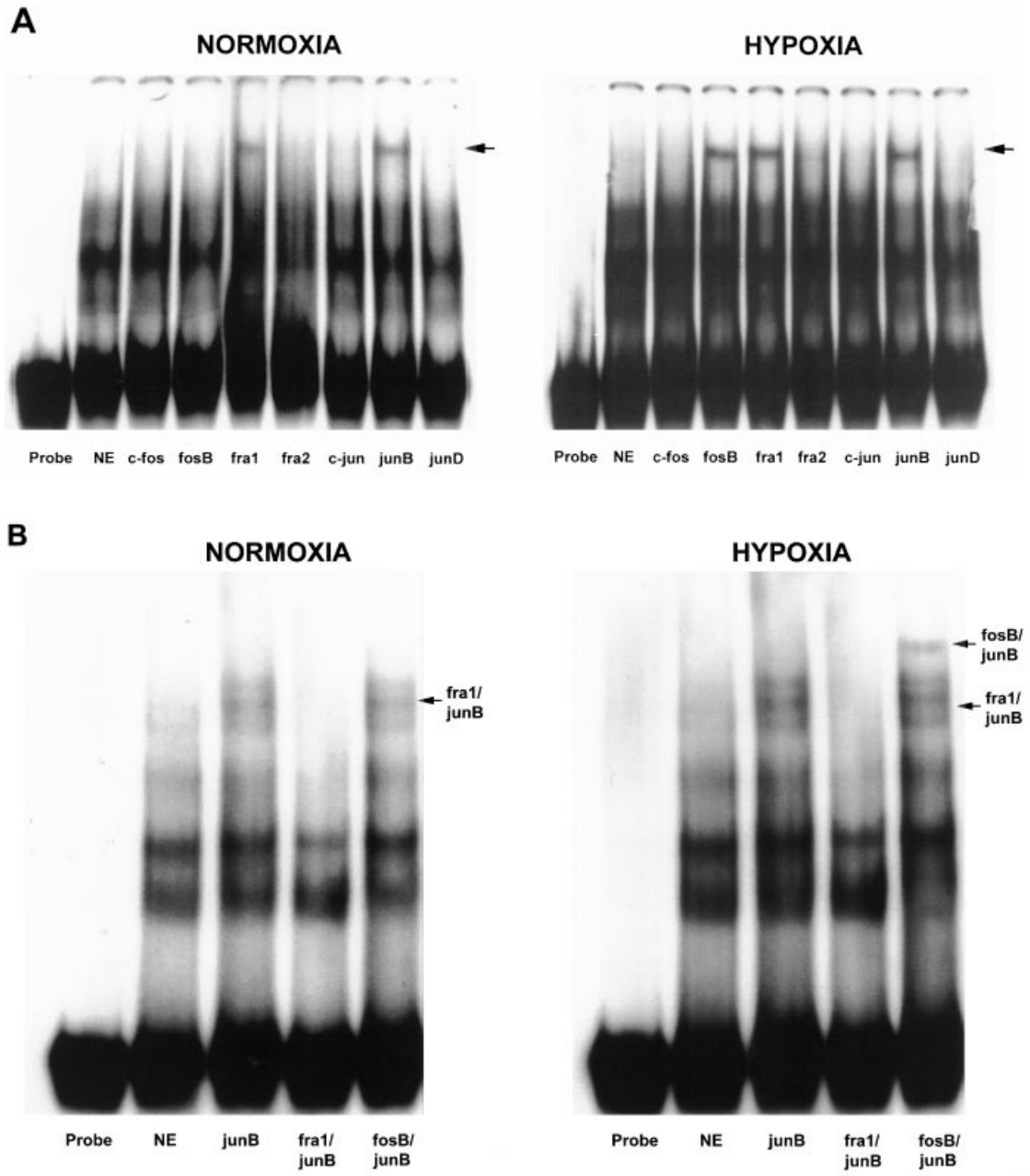

Figure 8 Identification of AP-2 site nuclear binding proteins

(**A**) EMSA of cardiac fibroblast nuclear extracts using antibodies to specific members of the Fos and Jun transcription factor families. Nuclear extracts from cells that were cultured under normoxic conditions demonstrate antibody supershifts to Fra1 and JunB. Nuclear extracts from cells that were cultured under hypoxic conditions also contained binding activities to Fra1 and JunB and the additional expression of FosB. (**B**) Double antibody EMSA of cardiac fibroblast nuclear extracts. In the extracts from normoxic cells, use of the JunB antibody alone yields a supershifted band, as seen in Figure 7.The combination of JunB and Fra1 antibodies results in depletion of the shifted complex, while the combination of JunB and FosB antibodies has no effect on mobility. These findings are compatible with the existence of Fra1–JunB heterodimers in the nuclear extracts. A similar pattern was present in the double antibody Fra1–JunB experiments with hypoxic cell nuclear extracts. In addition, the combination of FosB–JunB antibodies yielded an additional supershifted band consistent with the formation of FosB–JunB heterodimers. NE, nuclear extract.

DISCUSSION

In the present study, we examined the transcriptional regulation of MMP-2 within the context of cultured rat cardiac myocytes and fibroblasts. Although both cell types produce relatively equal amounts of the activator MT1-MMP, cardiac fibroblasts synthesized nearly 10 times more MMP-2 under the conditions used in this study. Culture under hypoxic conditions resulted in further increases in cardiac fibroblast MMP-2 synthesis and inclusion of endothelin-1, angiotensin II or interleukin 1β augmented the synthetic responses to hypoxic conditions. Promoter-mapping studies using a series of MMP-2 genomic deletion constructs localized an enhancer element within a 48 bp sequence located between -1410 and -1362 bp relative to the MMP-2 translational start site. This element is operative within the context of both cardiac fibroblasts and myocytes; however, transcriptional activity was much greater with fibroblasts, a finding consistent with the relative levels of MMP-2 protein that

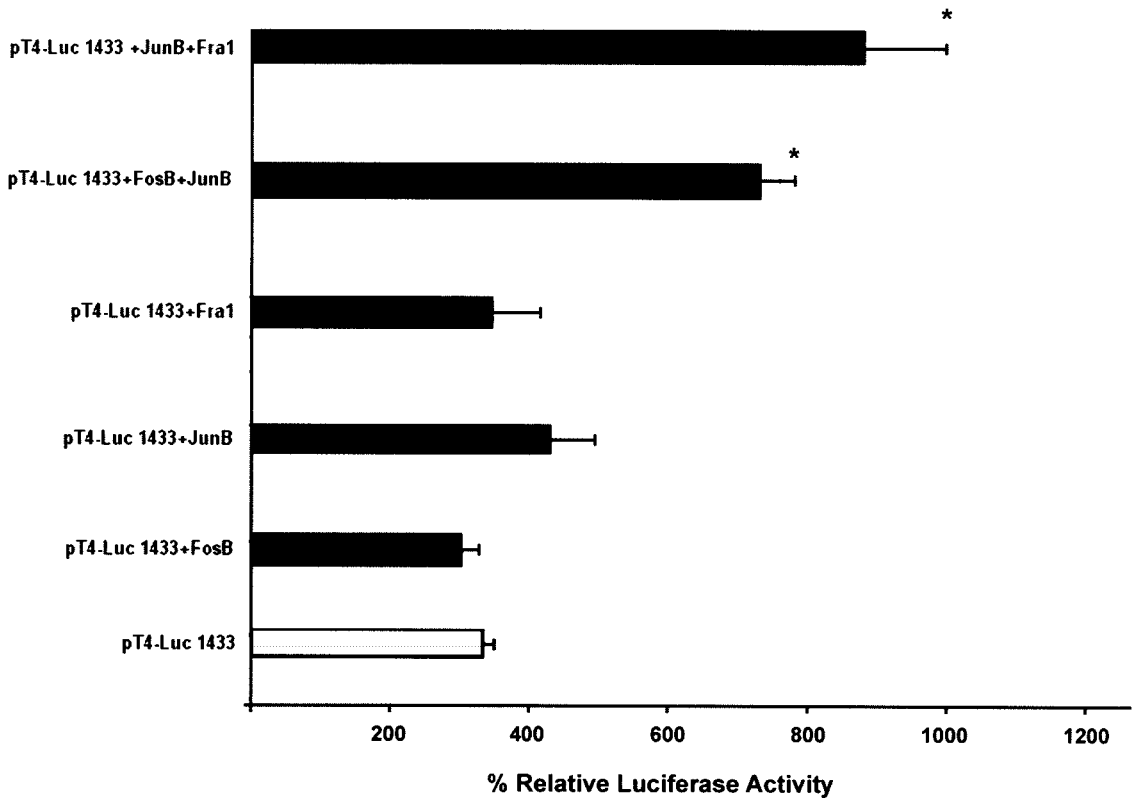

Figure 9 Co-transfection of FosB–JunB or Fra1–JunB expression plasmids stimulates pT4-Luc1433 reporter activity

Cardiac fibroblasts were transfected with the pT4-Luc1433 MMP-2 reporter construct. Expression plasmids encoding FosB, JunB or Fra1 were co-transfected individually (50 ng of plasmid DNA) or as combinations of FosB–JunB or Fra1–JunB (50 ng of plasmid DNA each). $^{*}P < 0.05$.

are synthesized by these cell types. The deletion studies also demonstrated that the increased transactivation induced by exposure to endothelin-1, angiotensin II and interleukin 1β is not dependent upon the -1410 to -1362 bp enhancer element, but is found between -1686 to -1502 bp relative to the translational start site. We are currently fine-mapping the discrete elements in this region that mediate enhanced MMP-2 transcription and believe that the increase in MMP-2 synthesis induced by the combination of hypoxia and peptide factors is mediated by additive effects upon physically discrete enhancer elements in the MMP-2 promoter.

Examination of the -1410 to -1362 bp sequence revealed a potential AP-1 complex binding site at -1394 to -1384 bp. Mutagenesis of the core AP-1 recognition sequence, $T^{-1392}GAC$, completely eliminated enhancer-dependent transactivation function as well as the enhanced transcriptional response to hypoxia. EMSA studies, combined with single and double antibody studies, indicate the specific binding of Fra1/JunB heterodimers to the AP-1 site using nuclear extracts from fibroblasts cultured under normoxic conditions. Nuclear extracts from cells cultured under hypoxic conditions demonstrated the specific interaction of FosB–JunB heterodimers with the -1410 to -1362 bp oligonucleotide. Furthermore, transfection of combinations of either Fra1–JunB or FosB–JunB expression plasmids yielded significant increases in transcriptional activity and confirm the functional significance of the EMSA studies.

Inducers of MMP expression are generally thought to act at the level of transcriptional activation of the genes [9,10]. The promoter region of MMP-2 exhibits marked differences from the promoter region of other MMPs [9,10,30]. The MMP-2 promoter is distinguished by the lack of common transactivating sequences such as TATA or CAAT boxes, or nuclear-factor-κB-binding sites that are commonly found in other MMP genes. However, the MMP-2 promoter does contain functional selective promoter factor 1 (Sp1) and activating protein 2 (AP-2) elements [31]. Extensive analyses of the transcriptional regulation of MMP-2 within the context of glomerular mesangial cells and hepatoma cell lines has delineated a strong enhancer element (termed RE1), located between -1322 and -1282 bp [24]. Expression cloning has revealed that RE1 specifically interacts with two key transcription factors: AP-2 and Y-box transcription factor 1 (YB-1) [32,33]. The RE1 site was originally localized by serial fine-mapping of MMP-2 genomic reporter constructs, and the AP-1 site found within this study did not demonstrate positive transactivation function within the context of mesangial cells or hepatoma cell lines [32,33]. Conversely, the RE-1 site exhibits no significant activity within the context of the cardiac cell types that were investigated in this study, suggesting that there are cell- (or tissue-) specific differences in the expression patterns of the cognate transcription factors that drive the transactivation process. Although the current study focused on the identification of a functional AP-1-binding site in the rat MMP-2 5′-flanking region, we note that the human MMP-2 promoter contains an AP-1-binding site located in a similar relative position (-1271 to -1265 bp; [31]).

MMP-2 has been widely considered to be an AP-1-unresponsive gene, primarily based on the absence of gene induction following exposure to PMA [34–36]. The promoters of

the MMP-1 (interstitial collagenase) and MMP-3 (stromelysin) genes served as the original model systems for the delineation of PMA-responsive elements located in the proximal promoters of these genes [37,38]. These elements specifically bind c-fos–c-jun heterodimers, a process induced by exposure to PMA and subsequent protein kinase C (PKC) activation. In contrast with PKC-mediated activation of c-fos and c-jun transcription or phosphorylation, FosB expression can be independent of PKC activation [39]. Additionally, JunB N-terminal regulatory domain phosphorylation is mediated by the $p34^{cdc2}$-cyclin B kinase and is not dependent upon PKC activation [40]. Thus the functional AP-1-binding site identified in the present study is occupied by Fos/Jun transcription family members that, in large part, operate in a PKC-independent manner. Although the core TGAC sequence identified in this site is characteristic of other defined AP-1-binding sites, the immediate flanking sequences diverge significantly from the well-studied c-fos/c-jun AP-1-binding sites in the MMP-1 and MMP-3 promoters. This observation suggests that the restricted pattern of Fra1, JunB and FosB binding to the MMP-2 AP-1 site is mediated by the nucleotide sequences that surround the core binding matrix, as has been previously suggested [41].

The signal transduction mechanism(s) by which hypoxia regulates genes has not been clearly determined [29]. Increased activity of the AP-1 complex in response to hypoxia is in substantial part due to increased abundance of its components mediated by enhanced RNA transcription [29]. Thus hypoxia induces expression of various proto-oncogenes, including those that encode Jun and Fos proteins [42–45]. In rat cardiac myocytes, increased expression of mRNA for c-fos, c-jun, JunD and JunB occurs within 1 h of hypoxia [46]. This induction of mRNA expression is independent of metabolic switching from aerobic to anaerobic metabolism [47], precedes the hypoxia-induced drop in cellular ATP concentrations [47], and correlates with increased levels of Fos and Jun proteins [46]. Increased levels of mRNA for c-fos and c-jun have also been observed during brief regional ischaemia [48]. In addition, hypoxia also increases the expression of redox factor-1 [49], which is involved in enhancing the DNA-binding activity of AP-1 complexes [29].

In summary, the results of this study are of importance because they indicate that, in rat cardiac cells, the MMP-2 promoter is subjected to a novel form of transcriptional regulation compared with other tissues. The involvement of AP-1 in the transcriptional regulation of MMP-2 in the heart suggests that MMP-2 activation is part of the cardiac cellular response to damaging stress, such as ischaemic injury leading to ventricular remodelling. A recent study indicates that up-regulation of MMPs in ischaemic and failing hearts contributes to the ventricular remodelling process [4]. Our results also indicate that MMP-2 synthesis is increased in response to molecules, such as interleukin 1β, angiotensin II and endothelin-1, that are released during cardiac ischaemia and myocardial infarction [3,29]. To better understand the complex mechanism by which MMP-2 contributes to the alterations in cardiac structure and function that constitute the remodelling process, future studies of transcriptional regulation of this gene in response to these molecules and others that contribute to ventricular hypertrophy and failure after myocardial infarction, including catecholamines and naturetic peptides [3,29], will be necessary.

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