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Transcriptional Induction of Matrix Metalloproteinase-9 in the Chondrocyte and Synoviocyte Cells Is Regulated via a Novel Mechanism: Evidence for Functional Cooperation between Serum Amyloid A-Activating Factor-1 and AP-1[1]

Alpana Ray,[2]*† B. Sonny Bal,† and Bimal K. Ray[2]*†

Increased expression of matrix metalloproteinase-9 (MMP-9) by IL-1β and TNF-α is regarded as a key factor in the degradation of cartilage during arthritis. However, the underlying molecular mechanism of this induction process especially in the cells of the joint capsule remains elusive. Chondrocytes and synoviocytes, the resident cells of joint capsule, markedly increase transcription of *MMP-9* in response to IL-1β- and TNF-α-mediated stimulation. Using progressively deleted and mutant promoter constructs of *MMP-9*, we show that serum amyloid A-activating factor (SAF)-1, a novel transcription factor, and the AP-1 family of proteins cooperatively regulate cytokine-mediated induction of *MMP-9* in the resident cells of the joint capsule. In the *MMP-9* promoter, SAF-1 and AP-1 DNA-binding elements are present in close proximity with only 14 nucleotides apart. SAF-1 DNA-binding activity is increased in both cytokine-stimulated cells as well as in osteoarthritic cartilage tissues. Although overexpression of SAF-1 could increase expression of the *MMP-9* promoter and endogenous MMP-9 gelatinolytic activity, for maximal induction of *MMP-9* gene concurrent participation of SAF-1 and AP-1 is required. Mutation of either one of these two elements resulted in a severe reduction in cytokine responsiveness of *MMP-9* promoter and compromised the transactivation potential of both SAF-1 and AP-1. Simultaneous requirement for two distinct DNA-binding elements suggests that SAF-1 and AP-1 function in a mutually beneficial manner acting as essential coactivators to drive cytokine-mediated transcriptional activation of *MMP-9*. *The Journal of Immunology,* 2005, 175: 4039–4048.

Matrix metalloproteinase (MMP)[3]-9 is a member of the degrading MMP family. Overexpression of these proteins is linked with various pathological diseases that involve connective tissue destruction and breakdown of the extracellular matrix (ECM) (1, 2). MMP also plays a critical role in a number of physiological processes such as embryonic development, wound repair, endometriosis, and bone growth. There are over 20 members of this family, which are further grouped in five subfamilies according to their properties and substrate specificities. Because of powerful proteolytic ability, the activity of the MMP is regulated by several mechanisms among which control of gene expression is the primary mode of regulation. This action is followed by activation of proenzymes by proteolytic processing of the N-terminal inhibitory domain and inhibition of enzyme activity by naturally occurring tissue inhibitor of metalloproteinases. Under pathogenic condition dysregulation in the gene expression is shown to be the primary cause of excessive synthesis of this family of proteins.

MMP-9 has the ability to cleave a variety of ECM components including denatured collagen (gelatin) generated by thermal denaturation at body temperature after specific cleavage of triple helix region of fibrillar collagen molecules by collagenases, types I, IV, V, and XI collagen, aggrecan, elastin, and fibronectin (3, 4). This protein is shown to be present in excessive amounts in a number of pathologic conditions including metastasis of cancer, arthritis, atherosclerosis, and ulcerating corneas (5–9). In all of these conditions the cellular level of MMP-9 is significantly increased primarily via increased gene expression. Thus considerable focus is given to shed light on the mechanism of MMP-9 synthesis in the diseased state.

MMP-9 is synthesized by a variety of cell types, but due to its extensive proteolytic ability, normal expression of MMP-9 is very low and tightly regulated. The expression of pro-MMP-9 in most cells can be induced by cytokines like IL-1β, TNF-α, tumor promoting agent, TGF-β growth factor, LPS, oncogenes, and the agents that stimulate cell migration (10–14). After secretion into the ECM, the activity of MMP-9 is further regulated by its conversion to the processed active form and inactivation by specific tissue inhibitor of metalloproteinase. Most of the mechanistic studies available on understanding induction of MMP-9 are concentrated on its activation mechanism under oncogenic condition and oncoprotein mediated malignant transformation (15–17). The proximal promoter of *MMP-9* is highly conserved and shown to contain multiple functional elements including a proximal AP-1 element, which is a hallmark of many MMP promoters, and NF-κB, PEA3/ets, Sp1, and a GT box element (15, 16). Three motifs, AP-1, NF-κB, and Sp1 are shown to contribute positively to the induction of *MMP-9* expression in response to tumor promoting

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[3] Abbreviations used in this paper: MMP, matrix metalloproteinase; SAF, serum amyloid A-activating factor; ECM, extracellular matrix; CAT, chloramphenicol acetyltransferase; OA, osteoarthritis.

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agent and TNF-α cytokine in osteosarcoma and HT1080 fibrosarcoma cells (15). In contrast, AP-1 and the GT box element were shown to be necessary for v-Src mediated activation of MMP-9 in HT1080 fibrosarcoma cells (16). The stimulation of *MMP-9* by *ras* oncogene in OVCAR-3 malignant ovarian cells is shown to be mediated through a MEK1-independent signaling pathway and require multiple transcription factor binding elements (17). Transcriptional up-regulation of *MMP-9* during spontaneous epithelial (S) to neuroblast (N) conversion by SK-N-SH neuroblastoma cells involved in enhanced invasivity is shown to be dependent on GT box and NF-κB elements (18). In a different note, studies with transgenic mice indicated that three transcription factors NF-κB, AP-2, and Sp1 are involved in regulating *MMP-9* expression during developmental and injury-related tissue remodeling (19).

Although MMP-9 has been implicated in the degradation and damage of articular cartilage linked with the pathology of rheumatoid arthritis, osteoarthritis (OA), rapidly destructive hip OA (20–24), how its induction is regulated in the cells of joint capsule in response to cytokines overexpressed during disease conditions is still unknown. Limited investigations on cytokine-mediated *MMP-9* expression that are available have used either tumor or transformed cells (15, 25). Because TNF-α and IL-1β are regarded as master cytokines and a synergy between TNF-α and IL-1β has been demonstrated in mediating arthritis in vivo (26), we investigated the regulation of MMP-9 in chondrocyte and synovial cells in response to TNF-α and IL-1β cytokines. We report that SAF-1 (27), a novel inflammation responsive transcription factor, is involved in mediating MMP-9 induction in osteoarthritic condition and in the resident cells of joint capsule by TNF-α and IL-1β. The SAF-1 binding element resides within the GT box sequence of MMP-9, which was previously characterized as an important element, but the identity of proteins that interact at this region was never revealed. The GT box sequence is highly conserved and present adjacent to the proximal AP-1 element (−79/73) of *MMP-9*. We also show that SAF-1 and AP-1 support and synergize the function of each other, which explains many earlier findings that demonstrated strict requirement of both the GT box and AP-1 binding elements in the induction of *MMP-9* expression. Together, current study provides mechanistic information on the induction mechanism of MMP-9 in chondrocyte and synovial cells.

Materials and Methods

Cartilage specimens

Articular cartilage specimens were collected from the hip and knee joints of five dogs (one each of Australian shepherd, Labrador retriever, hound mix, Labrador, and a mixed breed), whose age and body weight ranged between 2 and 7 years and 23 and 31 kg, respectively. All of these animals developed spontaneous OA, and were undergoing orthopaedic surgery at the University of Missouri Veterinary Medical Teaching Hospital (Columbia, MO). Normal cartilage specimens were obtained from three cadavers. All procedures were approved by the Animal Care and Use Committee. Severity of the osteoarthritic changes in all cartilage specimens was graded using the Outerbridge System (28) and verified by histologic examination.

Cell lines

Chondrocyte cell HTB-94 (SW1353) was derived from a primary grade II chondrosarcoma of the right humerus of a patient. Rabbit synovial fibroblast cell, HIG-82 (CRL 1832) was derived from rabbit joint tissue. These cells were obtained from American Type Culture Collection and grown in DMEM containing a high concentration of glucose (4.5 g/L) and supplemented with 7% FBS. For the collection of conditioned medium, cells were changed to serum-free medium and 48 h later the conditioned medium was collected.

Transfection analysis

HTB-94 and HIG-82 cells were transfected by a calcium phosphate method as described (29). Briefly, cells were seeded at 50% confluency and 24 h later, the cells were transfected by adding chloramphenicol acetyltransferase (CAT) reporter plasmid DNA together with pSVβ-galactosidase (Promega) plasmid DNA. The pSVβ-galactosidase DNA was used to monitor the efficiency of transfection and to normalize the cell extracts used for CAT assay. In some cotransfection reactions, expression plasmid DNA for SAF-1, c-Fos, c-Jun, NF-κB, Sp1, or revSAF-1 was included. After 16 h incubation with DNA, cells were glycerol shocked and fresh growth medium was added. In some reactions, the cells were stimulated with human IL-1β (500 U/ml, which is equivalent to 5 ng/ml) or TNF-α (40 μg/ml). The cells were harvested 24 h later and CAT activity was measured as described (29). β-Galactosidase activity was assayed with the substrate ONPG (*o*-nitrophenyl-β-D-galactopyranoside) as described (29). Cell extracts containing equivalent amount of β-galactosidase activity were used for CAT assay. Before the CAT assay, each cell extract was heated at 60°C for 10 min to inactivate endogenous acetylase activity. Different agents used in the transfection assay had no effect on β-galactosidase expression. All transfections were performed in duplicate and the experiments were repeated at least three times. The data for CAT activity assays represent the mean and SE of the mean derived from these three independent experiments.

Plasmid constructs

The 670wtMMP-9CAT, 670Sp1mutMMP-9CAT, 670NF-κBmutMMP-9CAT, 670AP1mutMMP-9CAT, and 670GTmutMMP-9CAT constructs have been previously described (15). These plasmids were a gift of Dr. M. Seiki (Institute of Medical Science, University of Tokyo, Tokyo, Japan) and obtained from Dr. D. Boyd (MD Anderson Cancer Center, Houston, TX). Progressive deletion constructs of *670wtMMP-9* promoter were synthesized by PCR amplification using different upstream primers starting at positions −570, −400, −241, −90, and −20 and a single downstream primer ending at position +54 of *MMP-9*. Amplified DNA molecules were ligated to pBLCAT3 vector DNA. The pcDSAF-1 (27), pcDc-Fos, pcDc-Jun, pcDNF-κB, and pcDSp1 expression plasmids were prepared by inserting a full length cDNAs of each transcription factor under the control of CMV promoter in pcDNA3 vector (Invitrogen Life Technologies). The mutant SAF-1 expression plasmid pcDSAF-1(mt) was constructed by inserting an out-of-frame SAF-1 cDNA that does not produce any SAF-1 protein.

Nuclear extracts and EMSA

Nuclear extract from normal and osteoarthritic cartilage tissues, HTB-94, and HIG-82 cells were prepared as described earlier (27). For EMSA, MMP-9 probe was prepared by radiolabeling a double-stranded *MMP-9* oligonucleotide containing nucleotides from position −71 to −39, which has the following sequence: 5′-CACTTGCCTGTCAAGGAGGGGT GGGGTCACAGG-3′. The labeling of DNA was performed by filling in the overhangs at the termini with Klenow fragment of DNA polymerase, incorporating [α-^{32}P]dCTP as a probe. EMSAs were performed following a standard protocol described earlier (27). In some binding assays, competitor oligonucleotides were included in the reaction mixtures. Competitor oligonucleotides used were: SAF-1 oligonucleotide 5′-CCCTTCCTCTC CACCCACAGCCCCCATGG-3′; Sp1 oligonucleotide 5′-TCGACTGG GCGGAGTCTGGA-3′; GT box oligonucleotide 5′-GGAGGGGTGGG GTC-3′; and nonspecific oligonucleotide 5′-TCGAACTGAACTT GAGCAGCTAGTT-3′. For Ab interaction studies, anti-SAF-1 or anti-Sp1 Ab (1 μl of a 10-fold diluted Ab) was added to the binding reaction mixture during a preincubation for 30 min on ice. Anti-Sp1 Ab was obtained from Santa Cruz Biotechnology, and a polyclonal anti-SAF-1 Ab was developed in rabbit using purified mouse SAF-1, which was further purified by ammonium sulfate fractionation and affinity chromatography using protein G-agarose. This anti-SAF-1 Ab recognizes multiple epitopes of full-length SAF-1 and does not cross-react with other known proteins.

Quantitation of MMP-9 mRNA

Total RNA prepared from normal and osteoarthritic canine cartilage tissues was amplified by RT-PCR using a kit from Invitrogen Life Technologies. MMP-9 primers used for PCR amplification were 5′-GGCTCACCAA GAAGACTTTCTTC-3′ (forward) and 5′-CAAAGGACACGTAGC CCACTTCG-3′ (reverse). MMP-9 forward primer is present in exon 10 whereas MMP-9 reverse primer is present in exon 13. Primer sequences for β-actin were 5′-GGGCATGGGCCAGAAGGACTCC-3′ (forward) and 5′-AGGACAGCCTGGATGGCCAC-3′ (reverse). The amplified products using MMP-9 and β-actin primers were 380 and 285 nucleotides, respectively. Reaction mixtures were first denatured at 94°C for 1 min. The PCR condition was 94°C for 30 s, 58°C 30 s, 72°C 45 s for 20 cycles. To ensure that amplification was in the linear range, PCR was terminated at 20 cycles. PCR products were resolved by 1.4% agarose gel electrophoresis.

Gelatin zymography

Equal protein (20 μg) of synovial fluid collected from normal and osteoarthritic dogs or conditioned medium from cultured cells were fractionated in a 11% SDS-polyacrylamide gels containing 0.1% gelatin. After electrophoresis, SDS was removed by dialysis with 2.5% Triton X-100 buffer and incubated in 50 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, 1.25% Triton X-100 buffer for 16 h at 37°C. The gels were stained with Coomassie blue.

Immunohistochemical analysis

Immunohistochemical staining was performed using anti-MMP-9 Ab (Sigma-Aldrich) and anti-SAF-1 rabbit IgG, prepared as described above, as primary Ab. HRP-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) was used as the secondary Ab. As control preimmune rabbit IgG was used. Tissue sections were cut 5 μm thick, deparaffinized in xylene, and rehydrated in graded ethanol solutions followed by washing with a buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl). Endogenous peroxidase activity was quenched by immersion in 3% H_2O_2 in methanol for 20 min followed by rinses in buffer. The slides were then incubated in 0.1% trypsin solution with 0.1% $CaCl_2$ for 60 min at 37°C to unmask Ags. Nonspecific binding was blocked for 30 min at 37°C with 10% normal goat serum. Slides were incubated overnight at 4°C with either anti-SAF-1 IgG or preimmune IgG as a control at a concentration of 1.0 μg/ml. The slides were rinsed twice in washing buffer (50 mM Tris-HCl, (pH 7.5), 0.15 M NaCl, 0.05% Tween 20), and then incubated with secondary Ab. Bound primary Ab was detected using a HRP method with substrate-chromogen solution. Sections were counterstained with Mayer's hematoxylin solution.

Western immunoblot analysis

Cell extracts (50 μg protein) or conditioned mediums were fractionated in a 5/11% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. For evaluation of the relative amount of proteins in each lane, proteins were stained with Ponceau S solution (Sigma-Aldrich). Immunoblotting was performed using 1/1000 dilution of anti-MMP-9 (Sigma-Aldrich), or anti-c-Fos or anti-c-Jun (both from Santa Cruz Biotechnology) and anti-SAF-1 Ab (prepared as described above). Bands were detected by using a chemiluminescence detection kit (Amersham Biosciences).

Results

MMP-9 level in cartilage and synovial fluids of osteoarthritic joints

Immunohistochemical analysis of articular cartilage tissue from knee joint of dogs with and without OA indicated that MMP-9 protein level is almost undetectable in the normal cartilage tissue but is highly increased in osteoarthritic cartilage (Fig. 1*A*). To determine the level of MMP-9 proteolytic activity we used gelatin zymography. In this assay we compared the gelatin breakdown ability of MMP-9 in the synovial fluid of normal and osteoarthritic dogs. Compared with normal joint synovial fluid (Fig. 1*B*, *lane 1*), synovial fluid of dogs with OA (Fig. 1*B*, *lane 2*) contains much higher levels of 92- and 88-kDa MMP-9 gelatinases. In contrast, there was no change in the MMP-2 gelatinase activity migrating at 72 kDa position. These findings indicated that MMP-9 is differentially expressed during the disease. To examine whether increased gelatinolytic activity of MMP-9 is due to a corresponding increase in the MMP-9 protein level, Western blot analysis was performed. Results shown in Fig. 1*C* revealed that although normal canine synovial fluid contains almost undetectable levels of MMP-9 protein, synovial fluid of dogs with OA contains this protein at an appreciable level. Densitometric analysis of the Western blot data indicated that MMP-9 protein level is significantly higher in dogs with OA compared with levels of normal animals (Fig. 1*D*). Next, limited-cycle RT-PCR using RNA isolated from canine normal and osteoarthritic cartilage tissues was performed to determine whether increase of MMP-9 protein is a consequence of increased MMP-9 mRNA level (Fig. 1*E*). Absence of any amplified product (Fig. 1*E*, *lane 1*) indicated that MMP-9 mRNA level is very low in normal cartilage. However, its level is substantially increased in the osteoarthritic cartilage (Fig. 1*E*, *lane 2*).

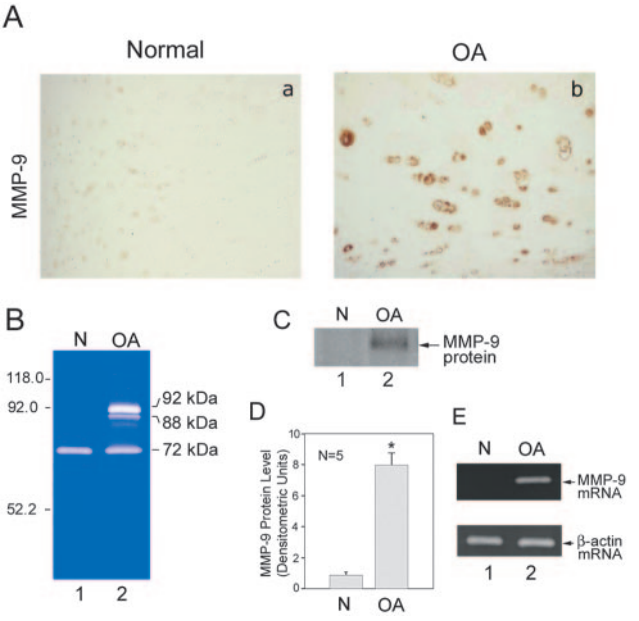

FIGURE 1. Osteoarthritic cartilage tissue contains higher level of MMP-9. *A*, Immunohistochemical analysis of canine normal (N) and osteoarthritic cartilage tissue sections was performed with anti-MMP-9 Ab as described in *Materials and Methods*. *B*, Increased gelatinolytic activity in joint fluids in dogs with OA. Equal protein amounts of joint fluid from normal (*lane 1*) and osteoarthritic (*lane 2*) dogs were subjected to gelatin zymography. *C*, Osteoarthritic cartilage tissue contains higher levels of MMP-9 protein. Equal protein amounts of cartilage tissue extracts (50 μg of protein per lane) from normal (*lane 1*) and osteoarthritic (*lane 2*) dogs were fractionated in a SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted using anti-MMP-9 Ab. *D*, Densitometric analysis of the immunoblot. Results represent the mean ± SE of three separate experiments. *, $p < 0.05$ compared with normal tissue. *E*, Total RNA (1.5 μg) extracted from normal or osteoarthritic cartilage tissues was reverse transcribed, and cDNAs were subjected to multiplex PCR using primers for canine MMP-9 and β-actin. PCR products were resolved in 1.4% agarose gel.

IL-1β and TNF-α responsive elements of MMP-9 in chondrocyte and synoviocyte cells

TNF-α and IL-1β, which are present at high levels in arthritic joints, are regarded as major cytokines that mediate increased gene expression in arthritis. At present little is known on the induction mechanism of TNF-α- and IL-1β-mediated *MMP-9* expression in the resident cells of joint capsule. We used a series of CAT reporter constructs containing progressively deleted *MMP-9* promoter DNA (Fig. 2*A*) to understand the regulatory mechanisms governing TNF-α- and IL-1β-mediated induction of *MMP-9* in HTB-94 chondrocyte and HIG-82 synovial cells. The longest reporter, containing *MMP-9* promoter sequence from nucleotide position −670 to +54, was strongly stimulated by both IL-1β and TNF-α (Fig. 2*B*). The promoter containing sequences from −570 to +54 of *MMP-9* exhibited moderate decrease in both basal level of transcription as well as in the level of cytokine stimulation. Deletion of sequences up to nucleotide position −400 showed small but noticeable further reduction of cytokine responsiveness compared with that of −570/+54 reporter. With additional deletion of sequences up to nucleotide position −90, 2- to 3-fold stimulatory response to both cytokines was consistently observed. However, when the promoter sequences were deleted up to nucleotide position −20, there was a precipitous drop in the cytokine responsiveness, suggesting the presence of critical cytokine-responsive elements within nucleotide position −90 and −20. A

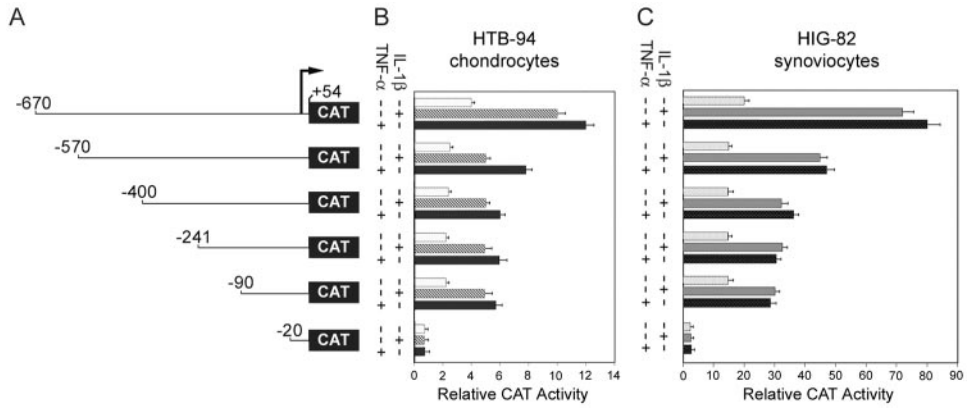

FIGURE 2. Functional analysis of the promoter region of human MMP-9 gene. *A*, Schematic showing various progressively deleted reporter constructs of human *MMP-9* promoter. *B*, HTB-94 chondrocyte cells were transiently transfected in duplicate with CAT reporter plasmids (1.0 μg each) containing progressively deleted fragments of the human *MMP-9* promoter. Transfected cells were incubated without (−) or with (+) either IL-1β (500 U/ml) or TNF-α (40 μg/ml) for 24 h as indicated. Fold induction of the CAT activity in the transfected cells relative to that of empty plasmid vector (pBLCAT3)-transfected uninduced cells was determined and plotted as relative CAT activity. Transfection experiments were performed in duplicate and the experiments were repeated at least three times. The data represent the mean and SE of the mean derived from three independent experiments. *C*, Synovial fibroblast cells, HIG-82, were transiently transfected and CAT activity was determined following a method similar to that described in *B*.

similar response of the cytokines was also observed when HIG-82 synoviocyte cells were used, indicating that both cell types use the same promoter elements for cytokine induction of *MMP-9* (Fig. 2*C*). It was, however, noticeable that the basal transcriptional activity of HIG-82 cells is substantially higher than that of HTB-94 cells. This effect could be due to a potential difference in the endogenous pool of transcriptional machinery among these two cell types. Together, these results indicated the presence of multiple regulatory elements, mapping to positions −670 to −570, −570 to −400, and −90 to −20 of the *MMP-9* promoter, are involved in regulating IL-1β-and TNF-α-mediated induction in chondrocyte and synoviocyte cells. Comparison of sequences at these three regions indicated the presence of one consensus NF-κB binding site between −600 and −590 (16), one Sp1 binding site between −562 and −555 (16), one AP-1 binding site between −79 and −73 (16), and a GT box (15) homologous to the so-called retinoblastoma control element (30) between −57 and −45.

Mutational analysis revealed that AP-1, GT box, and NF-κB elements mediate IL-1β and TNF-α response

To determine the contribution of NF-κB, Sp1, AP-1, and GT box elements in the context of surrounding sequences, we constructed

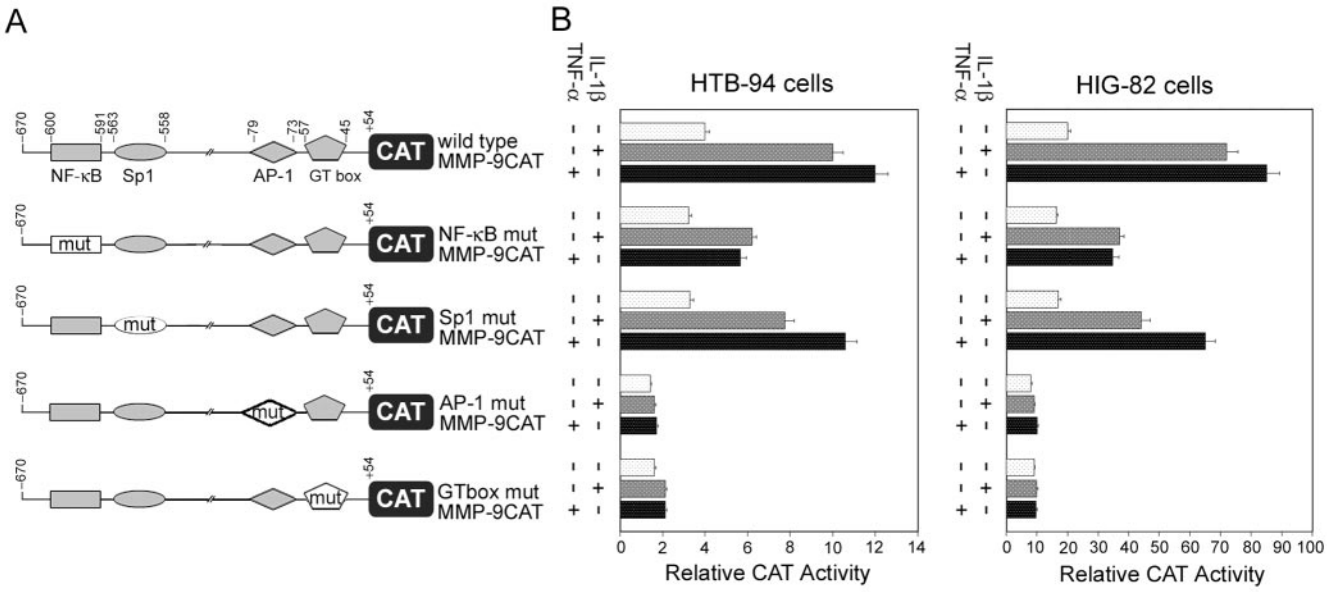

FIGURE 3. Mutation of the AP-1 or GT box sequence abrogates responsiveness to IL-1β and TNF-α. *A*, Physical maps of five reporter plasmids carrying either wild-type *MMP-9* promoter sequences from −670 to +54 (wild-type MMP-9CAT) or specific mutation at NF-κB site (NF-κB mutMMP-9CAT), specific mutation at Sp1 site (Sp1 mutMMP-9CAT), specific mutation at AP-1 site (AP-1 mutMMP-9CAT), or specific mutation at GT box (GTbox mutMMP-9CAT) are shown. Wild-type and mutant (mut, underlined) sequences of these sites are: NF-κB (wild-type) GGAATTCCCC, NF-κB (mutant) TTAATTCCCC; Sp1 (wild-type) GGGCCGG, Sp1 (mutant) GGGTTGG; AP-1 (wild-type) TGAGTCA, AP-1 (mutant) TTTGTCA; GT box (wild-type) GGAGGGGTGGGGT, GT box (mutant) GGAGGGGTAAGGT. *B*, The reporter plasmids (1.0 μg each) were transfected in duplicate in HTB-94 or HIG-82 cells, as indicated. Following transfection, cells were incubated without (−) or with (+) IL-1β (500 U/ml) or TNF-α (40 μg/ml) for an additional 24 h, as indicated. Fold induction of the CAT activity in the transfected cells relative to that of empty plasmid vector (pBLCAT3)-transfected uninduced cells was determined and plotted. The result represents mean and SE of the mean derived from three independent experiments.

single site mutant reporters containing specific mutation in the designated region. The response of these mutant reporters to TNF-α and IL-1β stimulation was monitored by transfection in HTB-94 and HIG-82 cells (Fig. 3). Mutation of the NF-κB element considerably reduced both the basal transcription and the inductive effect of IL-1β and TNF-α. However, the stimulatory response was not totally abrogated suggesting that downstream elements could also be involved in promoter activation. In contrast, mutation of either AP-1 or GT box element virtually eliminated response to both cytokines and to the basal transcriptional activity. The response of Sp1 site mutation showed considerably less effect on *MMP-9* transcription. These results suggested that IL-1β- or TNF-α-mediated stimulation of MMP-9 is primarily regulated by AP-1, GT box, and NF-κB elements.

Identification of a GT box binding protein in cytokine-stimulated cells and osteoarthritic cartilage extracts

The GT box sequence in the *MMP-9* promoter is highly conserved, except that GGGGTGGGG sequence in the mouse and rat is replaced by GGGGAGGGG in the human. As our studies indicated a regulatory role of the GT box sequence in mediating cytokine stimulation, we examined whether any nuclear protein interacts with this region of *MMP-9*. To determine this effect, DNA-binding assay was performed using *MMP-9* promoter region spanning nucleotide position −71 to −39 that encompasses the GT box element (−57/−45) as a probe. Nuclear extracts prepared from untreated, TNF-α- and IL-1β-stimulated HIG-82 cells were used. As seen in Fig. 4*A*, *lane 1*, proteins of untreated cell nuclear extract formed two faint DNA-protein complexes, termed as complex "b" and complex "c". There was marked increase in the intensity of these two complexes when equal protein amounts of nuclear extract of IL-1β-stimulated (Fig. 4*A*, *lane 2*) or TNF-α-stimulated (Fig. 4*A*, *lane 3*) cells were used. In addition another faint DNA-protein complex termed as "a" was seen to be formed by cytokine-treated cells. Formation of these complexes was inhibited by the GT box binding oligonucleotide (Fig. 4*A*, *lanes 6* and *7*) but not by the nonspecific oligonucleotide (Fig. 4*A*, *lanes 4* and *5*). These data indicated that cytokine-stimulated synoviocyte cells contain GT box binding proteins. Nuclear extracts prepared from cytokine-stimulated HTB-94 chondrocyte cells also exhibited the presence of similar proteins (data not shown).

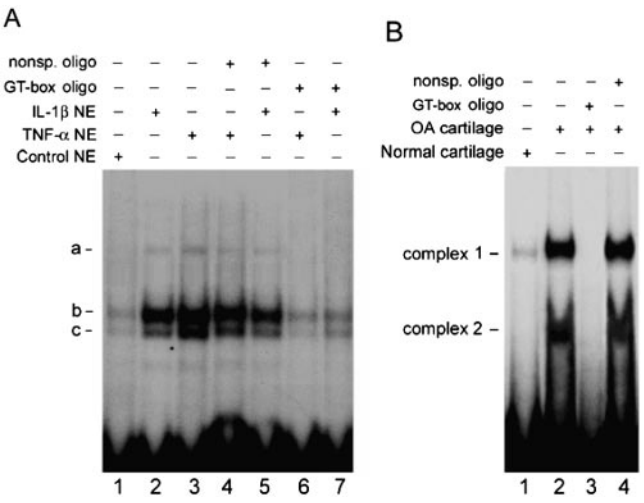

FIGURE 4. Identification of a transcription factor interacting with GT box element of *MMP-9* in cytokine-induced cells and osteoarthritic cartilage. *A*, Nuclear extracts (NE), 10 μg, from normal (*lane 1*), IL-1β-treated (*lanes 2*, *5*, and *7*), or TNF-α-treated (*lanes 3*, *4*, and *6*) HIG-82 cells were incubated with a ^{32}P-labeled *MMP-9* (−70/−10) DNA. In addition, 100-fold molar excess of nonspecific (nonsp.) oligonucleotide (*lanes 4* and *5*) and GT box oligonucleotide (*lanes 6* and *7*) were included. Three DNA-protein complexes are designated as "a," "b," and "c". *B*, Nuclear extracts (10 μg) from normal (*lane 1*) or osteoarthritic (*lanes 2–4*) canine cartilage tissues were incubated with a ^{32}P-labeled *MMP-9* (−70/−10) DNA. In addition, a 100-fold molar excess of GT box oligonucleotide (*lane 3*) and nonspecific oligonucleotide (*lane 4*) were included. Two DNA-protein complexes are designated as complex 1 and complex 2. DNA-protein complexes were resolved in a 6% nondenaturing polyacrylamide gel.

We next investigated whether osteoarthritic cartilage contains GT box binding nuclear protein. One faint DNA-protein complex, complex 1 as seen in Fig. 4*B*, *lane 1*, was formed with normal cartilage nuclear extract but osteoarthritic cartilage nuclear extract formed two highly prominent DNA-protein complexes, complex 1 and complex 2 (Fig. 4*B*, *lane 2*). Both complexes 1 and 2 were inhibited by the addition of GT box oligonucleotide (Fig. 4*B*, *lane 3*) but not by nonspecific oligonucleotide (Fig. 4*A*, *lane 4*). Comparison of the migration pattern of DNA-protein complexes formed by HIG-82 cells and cartilage tissue extract indicated that complex 2 comigrates with the complexes b and c, whereas complex 1 comigrates with complex a. Occasionally, complex 2 is seen to be migrated as two very closely spaced complexes (data not shown). These results clearly indicated that stimulation of a DNA-binding activity interacting with GT box regulatory element of *MMP-9* occurs during cytokine stimulation and under osteoarthritic condition in the cartilage.

Characterization of the GT box binding protein

Because the GGGGTGGGG sequence could be potential binding element of at least two transcription factors, SAF-1 (27) and Sp1 (31), we examined which of these proteins might interact with the GT box. Competitor oligonucleotides containing SAF-1 and Sp1 binding sequences and specific Abs to SAF-1 and Sp1 were used in the DNA-binding assay using TNF-α-stimulated HIG-82 (Fig. 5, *lanes 1–4*) and osteoarthritic cartilage (Fig. 5, *lanes 5–8*) nuclear extracts. No change in the complex formation was seen with Sp1 oligonucleotide (Fig. 5, *lanes 1* and *5*) or Sp1 Ab (Fig. 5, *lanes 2* and *6*), which indicated that Sp1 is not involved in the formation of any of these complexes. SAF-1 oligonucleotide inhibited complex b, complex c (Fig. 5, *lane 3*), and complex 2 (Fig. 5, *lane 7*). Further, complexes b and c (Fig. 5, *lane 4*) and complex 2 (Fig. 5, *lane 8*) were supershifted by SAF-1 Ab. Complex a (Fig. 5, *lanes 1–4*) and complex 1 (Fig. 5, *lanes 5–8*) remained unaffected by all of these agents suggesting that their formation involves neither Sp1 nor SAF-1 binding. Using Abs to a number of different transcription factors in the DNA-binding assay, we were unable to detect any ablation/supershift of this DNA-protein complex (data not shown). Thus the identity of the protein involved in the formation of complex a and complex 1 still remains unknown. Similar results were obtained when IL-1β-stimulated nuclear extract was used in the DNA-binding assay (data not shown). Together these results suggested that induction of SAF-1 activity in cytokine-treated cells and in osteoarthritic cartilages results in its enhanced interaction with the GT box element of *MMP-9*. This finding raises the question whether increased binding of SAF-1 is responsible for the cytokine-mediated induction of *MMP-9* expression because GT box has been found to be an essential promoter element for *MMP-9* expression in response to cytokine action (Fig. 3).

SAF-1 induces MMP-9 *expression in chondrocyte and synoviocyte cells*

To assess the role of SAF-1 in *MMP-9* expression, this transcription factor was ectopically expressed in both HTB-94 and HIG-82

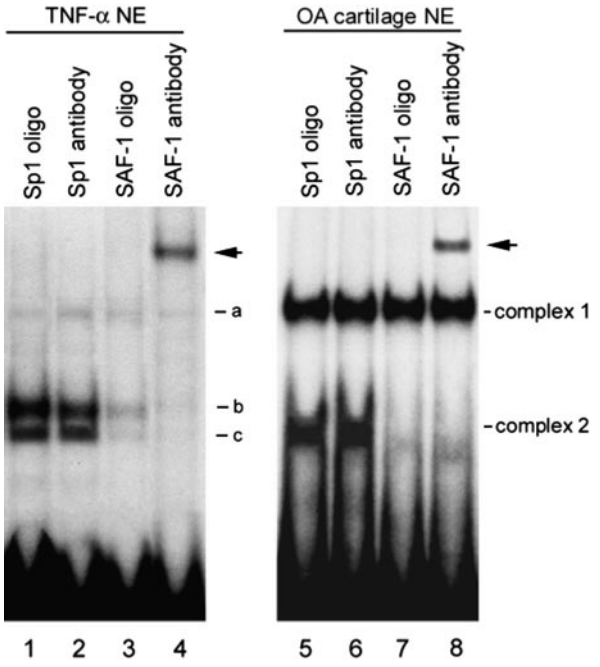

FIGURE 5. SAF-1 transcription factor interacts with GT box element of *MMP-9* promoter. Nuclear extracts (NE), 10 μg, from TNF-α-treated HIG-82 cells (*lanes 1–4*) and osteoarthritic canine cartilage tissues (*lanes 5–8*) were incubated with a ^{32}P-labeled *MMP-9* (−70/−10) DNA. In addition, 100-fold molar excess of Sp1 oligonucleotide (*lanes 1* and *5*), 100-fold molar excess of SAF-1 oligonucleotide (*lanes 3* and *7*), anti-Sp1 Ab (*lanes 2* and *6*), and anti-SAF-1 Ab (*lanes 4* and *8*) were included as indicated. DNA-protein complexes were resolved in a 6% nondenaturing polyacrylamide gel. Arrow indicates supershifted DNA-protein complex.

cells. Cotransfection of 670MMP-9CAT reporter DNA with increasing concentrations of SAF-1 expression plasmid, pcDSAF-1, resulted in a dose-dependent increase of reporter gene expression in both cell types (Fig. 6). The effect was specific because mutant SAF-1 expression plasmid, pcDSAF-1(mt), was unable to induce *MMP-9* promoter activity. To test whether the effect of SAF-1 is mediated through its interaction with the GT box sequence, we used mutant reporter plasmid 670GTmutMMP-9CAT, containing mutation at this region. This mutant reporter remained virtually unresponsive to SAF-1 compared with the wild-type 670MMP-9CAT reporter, suggesting that SAF-1-mediated induction of *MMP-9* promoter involves interaction with the GT box.

SAF-1 is responsible for cytokine-mediated activation of MMP-9

Activation of *MMP-9* promoter by SAF-1 suggested that SAF-1 might also activate endogenous MMP-9 expression. To test, conditioned medium of SAF-1 and mutant SAF-1 overexpressing cells were examined for MMP-9-specific gelatinolytic activity (Fig. 7). Untreated HTB-94 cells showed virtually no MMP-9 but high level of MMP-2-specific gelatinolytic activity (Fig. 7*A*, *lane 1*). However, in the SAF-1-transfected cells, marked increase of 92-kDa MMP-9 gelatinase activity was observed (Fig. 7*A*, *lane 2*). Similar increase in the MMP-9 activity was also evident in the SAF-1-transfected HIG-82 cells (Fig. 7*A*, compare *lanes 8* and *9*). In HIG-82 cells, the active MMP-9 band migrating at 88 kDa position was also quite apparent (Fig. 7*A*, *lane 9*). In contrast to this finding, overexpression of mutant SAF-1 did not stimulate MMP-9 expression (Fig. 7*A*, *lanes 3* and *10*), indicating the specificity of SAF-1 action. As a positive control, equivalent amount of conditioned medium of TNF-α (Fig. 7*A*, *lanes 4* and *11*) and IL-1β (Fig. 7*A*, *lanes 5* and *12*) cells were tested. Considering that only ~30% cells are usually transfected, it was assuring to note that SAF-1 transfected cells produced almost same level of induction as that obtained with these two proinflammatory cytokines. When the cells were transfected with pCMV-revSAF-1 plasmid there was considerable loss of the stimulatory effects of the cytokines (Fig. 7*A*, compare *lanes 6*, *7*, *13*, and *14* with *lanes 4*, *5*, *11*, and *12*, respectively). Western immunoblot analysis (Fig. 7*B*) further indicated that the protein level of MMP-9 in both chondrocyte (HTB-94) and synoviocyte (HIG-82) cells is increased by SAF-1 whereas MMP-2 protein level remains the same. Together, these results suggested that SAF-1 stimulates MMP-9 expression in both chondrocyte and synoviocyte cells.

Synergistic transactivation of MMP-9 *promoter by SAF-1 and AP-1*

Results of Fig. 3 have shown that mutation of either the SAF-1 binding GT box element (−57/−45) or the adjacently located AP-1 (−79/−73) element severely inhibits cytokine-mediated stimulation of *MMP-9* expression. This finding suggested that these two transcription factors may function in a mutually beneficial manner. Increasing evidence suggests that control of transcription of mammalian gene is complex and involves participation of a number of transcription factors. Under diseased condition, multiple inflammation responsive transcription factors are activated and exert their combined effect in modulating expression of the candidate gene. NF-κB (−599/−590) and Sp1 (−562/−557) elements, which are present distally from AP-1 and SAF-1,

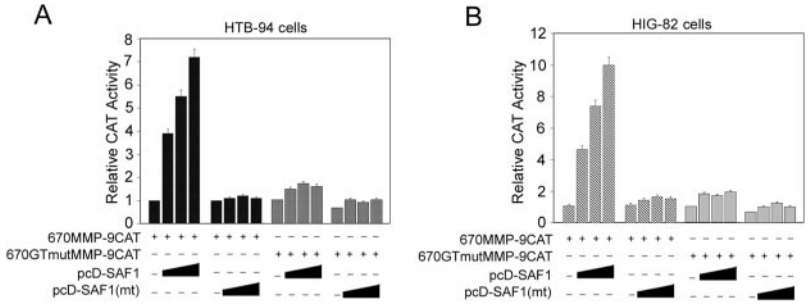

FIGURE 6. Dose-dependent stimulation of *MMP-9* promoter by ectopic expression of SAF-1. *A*, HTB-94 chondrocyte cells were transfected with 1.0 μg of 670MMP-9CAT or 670GTmutMMP-9CAT reporter plasmid either alone or with increasing concentrations (0.1, 0.2, and 0.4 μg) of pcDSAF-1 or pcDSAF-1(mt) expression plasmid DNA. *B*, HIG-82 synoviocyte cells were cotransfected with 1.0 μg of either 670MMP-9CAT or 670GTmutMMP-9CAT reporter plasmid alone or with increasing concentrations (0.1, 0.2, and 0.4 μg) of pcDSAF-1 or pcDSAF-1(mt) expression plasmid DNA. Fold induction of the CAT activity in the cotransfected cells relative to that of reporter plasmid alone was determined and plotted as relative CAT activity. The result represents mean and SE of the mean derived from three independent experiments. Presence and absence of a specific plasmid are denoted by "+" and "−", respectively.

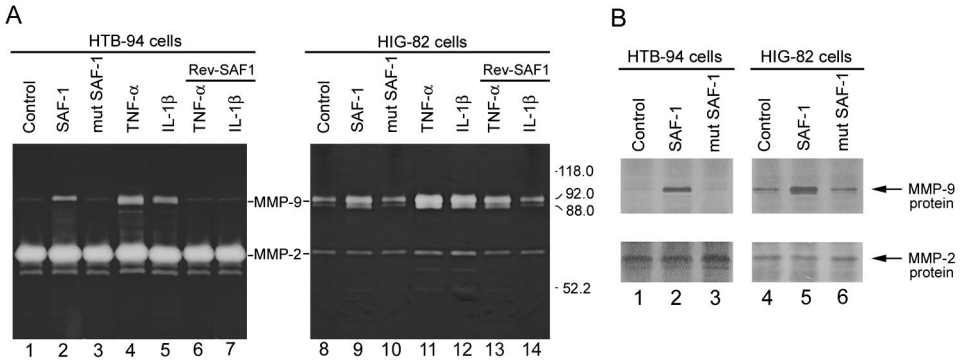

FIGURE 7. Increase of MMP-9 gelatinolytic activity and MMP-9 protein level in SAF-1-transfected chondrocyte and synoviocyte cells. *A*, HTB-94 chondrocyte and HIG-82 synoviocyte cells were grown in 60-mm dishes and stimulated with IL-1β (500 U/ml) or TNF-α (400 μg/ml) or transfected with 5.0 μg of expression plasmid DNA, pcDSAF-1 (SAF-1), or pcDSAF-1(mt) (mutSAF-1), as indicated. In some assays, cells were transfected with pcDRev-SAF-1 (Rev-SAF1) and then stimulated with IL-1β (500 U/ml) or TNF-α (400 μg/ml). Aliquots of conditioned medium were normalized for cell number differences and equal protein amounts of conditioned medium were subjected to zymography using a 9% SDS-PAGE containing 1 mg/ml gelatin. *B*, Western immunoblot analysis for MMP-9 protein level in SAF-1-transfected cells. Equal protein amounts of conditioned medium were concentrated by freeze drying (50 μg of protein per lane) from control (*lanes 1* and *4*), pcDSAF-1-transfected (*lanes 2* and *5*), and pcDmutSAF-1-transfected (*lanes 3* and *6*) cells were fractionated in a SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted using anti-MMP-9 Ab.

showed moderate effect on MMP-9 stimulation. Although *MMP-9* promoter contains two additional distally located AP-1 elements, only the proximal AP-1 site (−79/−73) was shown to be regulatory (16). This earlier finding suggests potential cross-talk between this AP-1 site and the neighboring GT box element, which is now found to be the binding site of SAF-1. To test whether simultaneous presence of these transcription factors modulates *MMP-9* transcription, we examined the effect of coexpression of these proteins in the cells (Fig. 8). Interestingly, coexpression of SAF-1 and AP-1 family members demonstrated a synergistic level of induction that was much higher than the additive value when these two groups of proteins were expressed separately. In contrast, coexpression of SAF-1 and NF-κB or AP-1 and NF-κB showed a less than additive level of activation, and coexpression of SAF-1 and Sp1 showed slight but consistent antagonistic effect (Fig. 8*B*).

Mutation of either AP-1 or SAF-1 element in the MMP-9 *promoter severely abrogates mutual responsiveness*

The synergy between SAF-1 and AP-1 family members on *MMP-9* transcription was further studied by two independent sets of experiments. In the first set, HTB-94 cells were cotransfected with the CAT reporter and increasing amounts of either SAF-1 or c-Fos plus c-Jun expression plasmids or a combination of both SAF-1 and c-Fos plus c-Jun expression plasmids at different concentrations (Fig. 9*A*). Interestingly, simultaneous presence of SAF-1 and AP-1 proteins stimulated *MMP-9* promoter in a synergistic dose-dependent manner. Western blot analysis indicated dose-dependent expression of SAF-1, c-Jun, and c-Fos proteins in transfected cells (Fig. 9*B*). In the second set of experiments, we used three different CAT reporter DNA (wild-type MMP-9CAT, AP-1 mut-MMP-9CAT, and GT box mutMMP-9CAT as described in Fig. 3) to transfect HTB-94 cells alone or with in combination of equal amounts of SAF-1 and c-Fos plus c-Jun expression plasmids (Fig. 9*C*). Although SAF-1 and c-Fos plus c-Jun plasmids were individually able to activate wild-type MMP-9 promoter and together they acted in a synergistic fashion, mutation of any one of these two elements not only inhibited transactivation potential of the respective transcription factor but also inhibited the stimulatory effect of the other protein whose binding site was not mutated. Western blot analysis was performed, which verified that none of these results were due to any variation of the expression of SAF-1 and c-Jun plus c-Fos proteins in transfected cells (data not shown).

Together, these results suggested that participation of both SAF-1 and AP-1 and their respective binding elements are necessary for induction of MMP-9. Transcriptional induction of MMP-9 from ectopic expression of only SAF-1 or c-Jun plus c-Fos proteins is most likely due to the use of the limited endogenous pool of protein that acts in synergy with the ectopically expressed partner. In the event of mutation of either the SAF-1 or AP-1 element, the

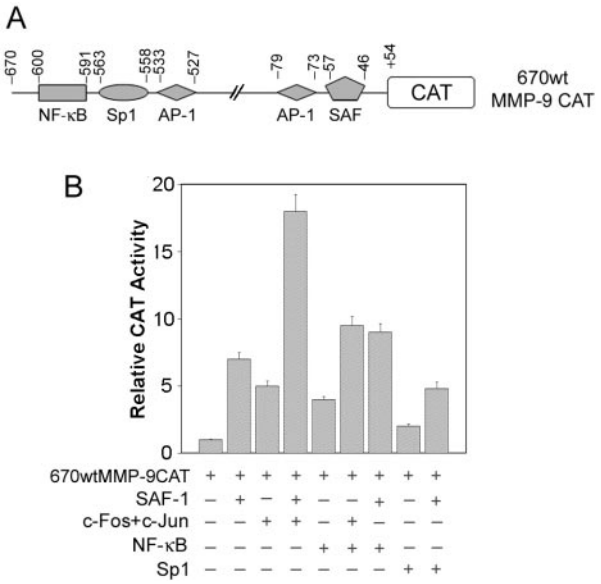

FIGURE 8. SAF-1 and AP-1 synergistically activates *MMP-9* promoter activity. *A*, A physical map of CAT reporter gene containing *MMP-9* promoter sequence from −670 to +54 is shown. Location of a distal NF-κB, Sp1, and AP-1 binding sites and a proximal AP-1 and SAF binding sites are indicated with nucleotide positions. *B*, HTB-94 chondrocyte cells were transfected with 1.0 μg of 670MMP-9CAT reporter DNA either alone or with 0.5 μg of pcDSAF-1, pcDc-Fos plus pcDc-Jun (c-Fos+c-Jun), pcDp65NF-κB, or pcDSp1 expression plasmid DNAs alone or in combination, as indicated. CAT activity in the transfected cells was measured as described in *Materials and Methods*. Fold induction of the CAT activity in the cotransfected cells relative to that of reporter plasmid alone was determined and plotted as relative CAT activity. The result represents mean and SE of the mean derived from three independent experiments. Presence and absence of a specific plasmid are denoted by "+" and "−," respectively.

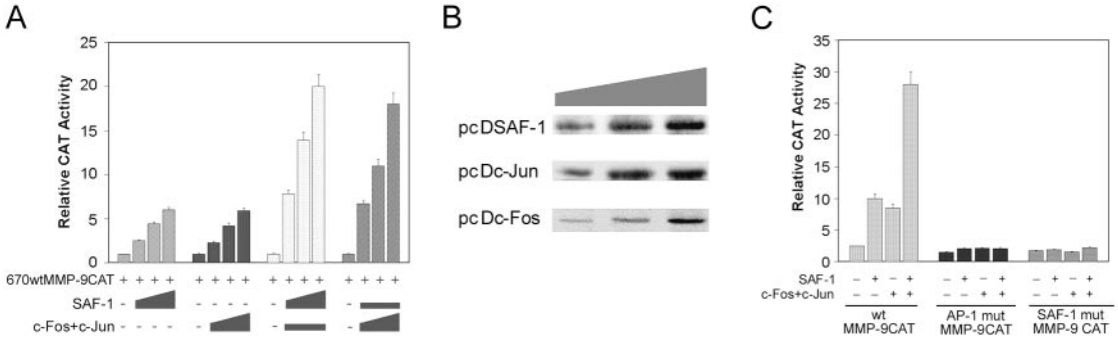

FIGURE 9. SAF-1- or AP-1-mediated transactivation of MMP-9 requires both elements. *A*, HTB-94 chondrocyte cells were transfected with (+) 1.0 μg of 670MMP-9CAT reporter DNA and increasing concentrations (0.1, 0.2, and 0.4 μg) of pcDSAF-1 or pcDc-Fos plus pcDc-Jun (c-Fos+c-Jun) expression plasmids, as indicated. In addition, some transfection assays contained constant amounts (0.5 μg) of either pcDSAF-1 or pcDc-Fos plus pcDc-Jun expression plasmids, as indicated. CAT activity was measured as described in *Materials and Methods* and fold induction of the CAT activity in the cotransfected cells relative to that of reporter plasmid alone was determined and plotted as relative CAT activity. The result represents mean and SE of the mean derived from three independent experiments. *B*, Fifty micrograms of protein per lane from the cells transfected with increasing concentrations (0.1, 0.2, and 0.4 μg) of pcDSAF-1 or pcDc-Fos plus pcDc-Jun expression plasmids were subjected to Western blot analysis using anti-SAF-1, anti-c-Jun, and anti-c-Fos Abs, as indicated. *C*, Three reporter plasmids carrying either 670 bp of wild-type MMP-9 promoter sequences (wt MMP-9CAT), or specific mutation at AP-1 site (AP-1 mutMMP-9CAT) or specific mutation at SAF-1 site (SAF-1 mutMMP-9CAT) were cotransfected with 0.5 μg of either pcDSAF-1 or pcDc-Fos plus pcDc-Jun (c-Fos+c-Jun) expression plasmids, alone, or in combination, as indicated. Relative CAT activity was determined and plotted as described in *A*. The result represents mean and SE of the mean derived from three independent experiments. Presence and absence of a specific plasmid are denoted by "+" and "−," respectively.

inability of endogenous protein to interact with the *MMP-9* promoter results in the loss of synergy.

MMP-9, SAF-1, c-Jun, and c-Fos are present at high levels in osteoarthritic cartilage

In light of the involvement of SAF-1 and AP-1 transcription factors in the induction of MMP-9 in osteoarthritic cartilage, one begs the question about the level of these proteins in the diseased tissue. Therefore to assess these levels, immunohistochemical analysis of cartilage tissue was performed (Fig. 10). This experiment indicated that expression pattern of SAF-1, c-Jun, and c-Fos is similar to that of MMP-9. The levels of these proteins are much higher in osteoarthritic cartilage compared with normal cartilage. Together these results indicate that increased activity of SAF-1 and AP-1 and their cooperative action gives rise to the induction of MMP-9 in the osteoarthritic cartilage.

Discussion

We have identified a novel mechanism that delineates induction of MMP-9 by cytokines in the resident cells of joint capsule and show that MMP-9 is a target of and regulated by transcription factor SAF-1. This is evident by 1) demonstration of SAF-1 binding element at the conserved proximal promoter region of *MMP-9* and specific interaction of SAF-1 to the site, 2) increased levels of SAF-1 DNA-binding activity in osteoarthritic nuclear extract, 3) SAF-1-dependent activation of *MMP-9* promoter and endogenous MMP-9 gene, and 4) increase of SAF-1 in osteoarthritic cartilage. Further, we provide evidence of transcriptional cooperativity between AP-1 and SAF-1 in maximal stimulation of *MMP-9* expression. Mutation of either element alone in the *MMP-9* promoter severely diminished cytokine responsiveness, suggesting that the two elements have a mutually

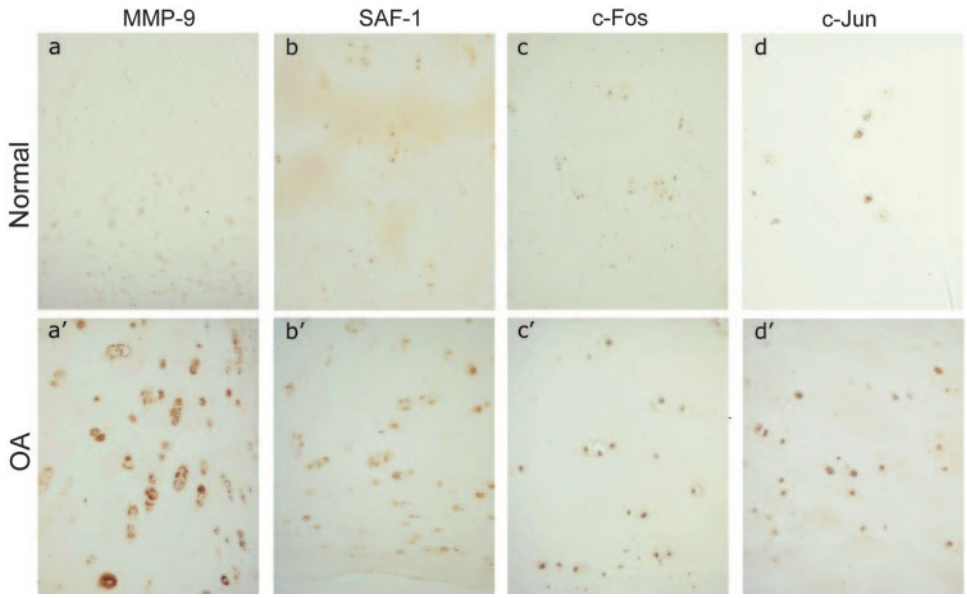

FIGURE 10. Increased levels of MMP-9, SAF-1, and AP-1 in osteoarthritic cartilage tissue. Immunohistochemical analysis of age-matched canine normal and osteoarthritic cartilage tissue sections was performed as described in *Materials and Methods*. Serial sections were immunostained with anti-MMP-9 Ab (*a* and *a′*) at magnification ×100; anti-SAF-1 Ab (*b* and *b′*) at magnification ×100; anti-c-Fos Ab (*c* and *c′*) at magnification ×100; and anti-c-Jun Ab (*d* and *d′*) at magnification ×100.

dependent and a cooperative relationship. Interdependency of these regulatory transcription factors in the expression of *MMP-9* could have pathophysiological significance in the manifestation of arthritis.

Although increased MMP-9 proteolytic activity due to induction of this protein in chondrocyte cells and synovial lining cells by specific cytokines is implicated in the degradation and damage of cartilage, specific mechanisms controlling induction of *MMP-9* are still sketchy. The systematic analysis of serially truncated and specifically mutated reporter constructs identified the requirement of multiple regulatory elements for IL-1β- and TNF-α-mediated induction of MMP-9 (Figs. 2–5), in which the role of SAF-1 was earlier unknown. The SAF-1 DNA binding site positioned between -57 and -46 in human *MMP-9* promoter is highly conserved and located within a region that was previously identified as the GT box element (16). The GT box element, in several previous reports, was shown to be critical in mediating MMP-9 induction during oncogenic and oncoprotein-mediated malignant transformation (16–18), but the protein interacting at this region was never identified. This report is the first revealing a nuclear protein in cytokine-induced chondrocyte and synoviocyte cells as well as in osteoarthritic cartilage, which interacts with the GT box element of the human *MMP-9*. The GGGGA/TGGGG sequence of the GT box, which could potentially act as a Sp1 binding element, does not interact with Sp1 that is present in the chondrocyte, synoviocyte, or osteoarthritic cartilage nuclear extracts. This was evident by the failure of Sp1 oligonucleotide or anti-Sp1 Ab to inhibit the formation of specific complexes (Fig. 5).

In addition to the identification of MMP-9 as a target of the inflammation-responsive novel transcription factor, SAF-1, this report provides the first evidence of functional cooperation between the SAF-1 and AP-1 family of proteins in achieving maximal *MMP-9* induction. AP-1 transcription factors belong to a family whose members contain a basic leucine zipper DNA-binding domain. Members of this family include c-Fos, Fra-1, FosB, c-Jun, JunB, and JunD. AP-1 family members form homo- or heterodimeric complexes to bind to specific DNA elements in the gene promoter. Protein kinases such as JNK and p38 MAPK are known to phosphorylate and activate AP-1 family members (32). In this study we show that transcriptional induction of *MMP-9* involves mutually dependent and functionally synergistic actions of AP-1 and SAF-1. We have demonstrated this effect by cotransfection of wild-type *MMP-9* promoter with expression plasmids and with mutant *MMP-9* promoter constructs (Figs. 8 and 9) and also by loss of cytokine responsiveness (Fig. 3). Mutation of either transcription factor binding site in the context of surrounding sequences diminishes the ability of SAF-1 or AP-1 to drive *MMP-9* promoter function. These results clearly show that both factors require cooperation from the other in regulating *MMP-9* expression. Transcriptional induction of *MMP-9* by only SAF-1 or AP-1 family of proteins is most likely due to the use of the limited endogenous pool of protein that acts in synergy with the ectopically expressed partner. In the event of mutation of any of these elements, endogenous protein being unable to interact with the *MMP-9* promoter cannot facilitate other transcription factor action. Such a symbiotic relationship was not seen between NF-κB and AP-1 or NF-κB and SAF-1 or SAF-1 and Sp1 (Fig. 8). In fact, SAF-1 and Sp1 modestly antagonized the function of the other. Incidentally, SAF-1 and Sp1 exhibited cooperative relationship in augmenting serum amyloid A gene expression (33). These apparently opposing effects suggest that functional relationship between SAF-1 and Sp1 is gene-specific and depends on the surrounding sequences as well. Overall, our findings introduce an important step into the developing model of *MMP-9* regulation during cytokine-mediated induction in chondrocyte and synoviocyte cells.

The synergistic relationship between SAF-1 and AP-1 suggests that even under mild inflammatory conditions, the outcome of a relatively low level of induction of these two transcription factors would result in a much higher level of MMP-9 protein. In contrast, inhibition of any single transcription factor activity would significantly lower the level of *MMP-9* expression. There are several possible mechanisms by which synergy between SAF-1 and AP-1 may be achieved, and elucidating the mechanism for such symbiotic relationship is an important area for future investigations. First possible mechanism is that close proximity of the AP-1 and SAF-1 element is responsible for mediating the functional cooperation between these two groups of proteins. For optimal interaction with the *MMP-9* promoter, it is possible that interaction of each transcription factor with DNA in vivo may be enhanced by cooperative recruitment and stabilization or by an alteration in local DNA structure, which is fostered by the simultaneous interaction of both transcription factors. A second possible mechanism is that a physical, protein-protein interaction between SAF-1 and AP-1 family members is responsible for mediating functional cooperation. The accounts of protein-protein interaction between transcription factors in the regulation of gene expression are continuously growing. The AP-1 transcription factor has been reported to interact with a wide variety of transcription factors, including NF-κB (34), Smads (35), and NFAT (36), but no report on interaction between SAF-1 and AP-1 is available.

In summary, identification of SAF-1 as the transcription factor interacting with the GT box element, which was earlier known to play a crucial role in *MMP-9* expression, provides a new insight into the induction mechanism of this gene in the pathogenesis of OA. Furthermore, the functional cooperation between SAF-1 and AP-1 not only solidifies the role of SAF-1 as a regulator of MMP-9 but also supports a more complex model of SAF-1-mediated regulation, one which involves cooperation with neighboring response elements.

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Disclosures

The authors have no financial conflict of interest.

References

1. Woessner, J. F., Jr. 2002. MMPs and TIMPs: an historical perspective. *Mol. Biotechnol.* 22: 33–49.
2. McCawley, L. J., and L. M. Matrisian. 2001. Matrix metalloproteinases: they're not just for matrix anymore. *Curr. Opin. Cell Biol.* 13: 534–540.
3. Shipley, J. M., G. A. Doyle, C. J. Fliszar, Q. Z. Ye, L. L. Johnson, S. D. Shapiro, H. G. Welgus, and R. M. Senior. 1996. The structural basis for the elastolytic activity of the 92-kDa and 72-kDa gelatinases: role of the fibronectin type II-like repeats. *J. Biol. Chem.* 271: 4335–4341.
4. Nguyen, Q., G. Murphy, C. E. Hughes, J. S. Mort, and P. J. Roughley. 1993. Matrix metalloproteinases cleave at two distinct sites on human cartilage link protein. *Biochem. J.* 295: 595–598.
5. Duffy, M. J., T. M. Maguire, A. Hill, E. McDermott, and N. O'Higgins. 2000. Metalloproteinases: role in breast carcinogenesis, invasion and metastasis. *Breast Cancer Res.* 2: 252–257.
6. Crowe, D. L., and T. N. Brown. 1999. Transcriptional inhibition of matrix metalloproteinase 9 (MMP-9) activity by a c-*fos*/estrogen receptor fusion protein is mediated by the proximal AP-1 site of the *MMP-9* promoter and correlates with reduced tumor cell invasion. *Neoplasia* 1: 368–372.
7. Martel-Pelletier, J., D. J. Welsch, and J. P. Pelletier. 2001. Metalloproteases and inhibitors in arthritic diseases. *Best Practice & Research in Clinical Rheumatology* 15: 805–829.
8. Galis, Z. S., and J. J. Khatri. 2002. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. *Circ. Res.* 90: 251–262.

9. Fini, M. E., W. C. Parks, W. B. Rinehart, M. T. Girard, M. Matsubara, J. R. Cook, J. A. West-Mays, P. M. Sadow, R. E. Burgeson, J. J. Jeffrey, et al. 1996. Role of matrix metalloproteinases in failure to re-epithelialize after corneal injury. *Am. J. Pathol.* 149: 1287–1302.
10. Okada, Y., H. Tsuchiya, H. Shimizu, K. Tomita, I. Nakanishi, H. Sato, M. Seiki, K. Yamashita, and T. Hayakawa. 1990. Induction and stimulation of 92-kDa gelatinase/type IV collagenase production in osteosarcoma and fibrosarcoma cell lines by tumor necrosis factor α. *Biochem. Biophys. Res. Commun.* 171: 610–617.
11. Watanabe, H., I. Nakanishi, K. Yamashita, T. Hayakawa, and Y. Okada. 1993. Matrix metalloproteinase-9 (92 kDa gelatinase/type IV collagenase) from U937 monoblastoid cells: correlation with cellular invasion. *J. Cell Sci.* 104: 991–999.
12. Wahl, S. M., J. B. Allen, B. S. Weeks, H. L. Wong, and P. E. Klotman. 1993. Transforming growth factor β enhances integrin expression and type IV collagenase secretion in human monocytes. *Proc. Natl. Acad. Sci. USA* 90: 4577–4581.
13. Saarialho-Kere, U. K., H. G. Welgus, and W. C. Parks. 1993. Distinct mechanisms regulate interstitial collagenase and 92-kDa gelatinase expression in human monocytic-like cells exposed to bacterial endotoxin. *J. Biol. Chem.* 268: 17354–17361.
14. Mauviel, A. 1993. Cytokine regulation of metalloproteinase gene expression. *J. Cell. Biochem.* 53: 288–295.
15. Sato, H., and M. Seiki. 1993. Regulatory mechanism of 92 kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells. *Oncogene* 8: 395–405.
16. Sato, H., M. Kita, and M. Seiki. 1993. v-Src activates the expression of 92-kDa type IV collagenase gene through the AP-1 site and the GT box homologous to retinoblastoma control elements: a mechanism regulating gene expression independent of that by inflammatory cytokines. *J. Biol. Chem.* 268: 23460–23468.
17. Gum, R., E. Lengyel, J. Juarez, J. H. Chen, H. Sato, M. Seiki, and D. Boyd. 1996. Stimulation of 92-kDa gelatinase B promoter activity by *ras* is mitogen-activated protein kinase kinase 1-independent and requires multiple transcription factor binding sites including closely spaced PEA3/ets and AP-1 sequences. *J. Biol. Chem.* 271: 10672–10680.
18. Farina, A. R., A. Tacconelli, A. Vacca, M. Maroder, A. Gulino, and A. R. Mackay. 1999. Transcriptional up-regulation of matrix metalloproteinase-9 expression during spontaneous epithelial to neuroblast phenotype conversion by SK-N-SH neuroblastoma cells, involved in enhanced invasivity, depends upon GT-box and nuclear factor κB elements. *Cell Growth Differ.* 10: 353–367.
19. Mohan, R., W. B. Rinehart, P. Bargagna-Mohan, and M. E. Fini. 1998. Gelatinase B/*lacZ* transgenic mice, a model for mapping gelatinase B expression during developmental and injury-related tissue remodeling. *J. Biol. Chem.* 273: 25903–25914.
20. Kaneko, M., T. Tomita, T. Nakase, Y. Ohsawa, H. Seki, E. Takeuchi, H. Takano, K. Shi, K. Takahi, E. Kominami, et al. 2001. Expression of proteinases and inflammatory cytokines in subchondral bone regions in the destructive joint of rheumatoid arthritis. *Rheumatology* 40: 247–255.
21. Ahrens, D., A. E. Koch, R. M. Pope, M. Stein-Picarella, and M. J. Niedbala. 1996. Expression of matrix metalloproteinase 9 (96-kd gelatinase B) in human rheumatoid arthritis. *Arthritis Rheum.* 39: 1576–1587.
22. Okada, Y., K. Naka, K. Kawamura, T. Matsumoto, I. Nakanishi, N. Fujimoto, H. Sato, and M. Seiki. 1995. Localization of matrix metalloproteinase 9 (92-kilodalton gelatinase/type IV collagenase = gelatinase B) in osteoclasts: implications for bone resorption. *Lab. Invest.* 72: 311–322.
23. Masuhara, K., T. Nakai, K. Yamaguchi, S. Yamasaki, and Y. Sasaguri. 2002. Significant increases in serum and plasma concentrations of matrix metalloproteinases 3 and 9 in patients with rapidly destructive osteoarthritis of the hip. *Arthritis Rheum.* 46: 2625–2631.
24. Masuhara, K., S. Bak Lee, T. Nakai, N. Sugano, T. Ochi, and Y. Sasaguri. 2000. Matrix metalloproteinases in patients with osteoarthritis of the hip. *Int. Orthop.* 24: 92–96.
25. Sanceau, J., D. D. Boyd, M. Seiki, and B. Bauvois. 2002. Interferons inhibit tumor necrosis factor-α-mediated matrix metalloproteinase-9 activation via interferon regulatory factor-1 binding competition with NF-κB. *J. Biol. Chem.* 277: 35766–35775.
26. Henderson, B., and E. R. Pettipher. 1989. Arthritogenic actions of recombinant IL-1 and tumour necrosis factor α in the rabbit: evidence for synergistic interactions between cytokines in vivo. *Clin. Exp. Immunol.* 75: 306–310.
27. Ray, A., and B. K. Ray. 1998. Isolation and functional characterization of cDNA of serum amyloid A-activating factor that binds to the serum amyloid A promoter. *Mol. Cell. Biol.* 18: 7327–7335.
28. Outerbridge, R. E. 1961. The etiology of chondromalacia patellae. *J. Bone Jt. Surg. Br.* 43: 752–757.
29. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual,* 2nd Ed. Cold Spring Harbor Laboratory Press, Plainview, NY.
30. Kim, S. J., H. D. Lee, P. D. Robbins, K. Busam, M. B. Sporn, and A. B. Roberts. 1991. Regulation of transforming growth factor β1 gene expression by the product of the retinoblastoma-susceptibility gene. *Proc. Natl. Acad. Sci. USA* 88: 3052–3056.
31. Dynan, W. S., and R. Tjian. 1983. The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. *Cell* 35: 79–87.
32. Karin, M., Z. Liu, and E. Zandi. 1997. AP-1 function and regulation. *Curr. Opin. Cell Biol.* 9: 240–246.
33. Ray, A., H. Schatten, and B. K. Ray. 1999. Activation of Sp1 and its functional co-operation with serum amyloid A-activating sequence binding factor in synoviocyte cells trigger synergistic action of interleukin-1 and interleukin-6 in serum amyloid A gene expression. *J. Biol. Chem.* 274: 4300–4308.
34. Perkins, N. D. 1997. Achieving transcriptional specificity with NF-κB. *Int. J. Biochem. Cell Biol.* 29: 1433–1448.
35. Liberati, N. T., M. B. Datto, J. P. Frederick, X. Shen, C. Wong, E. M. Rougier-Chapman, and X. F. Wang. 1999. Smads bind directly to the Jun family of AP-1 transcription factors. *Proc. Natl. Acad. Sci. USA* 96: 4844–4849.
36. Ramirez-Carrozzi, V. R., and T. K. Kerppola. 2001. Control of the orientation of Fos-Jun binding and the transcriptional cooperativity of Fos-Jun-NFAT1 complexes. *J. Biol. Chem.* 276: 21797–21808.