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*

(Received for publication, May 3, 1993, and in revised form, July 9, 1993)

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The 92-kDa type IV collagenase (matrix metalloproteinase-9; MMP-9) is frequently expressed in cells showing an invasive nature during physiological and pathological processes, and the expression is strictly controlled by a variety of trans-membrane signals. Binding sites for NF-κB, Sp-1, and AP-1 are reportedly required for induction of MMP-9 gene expression by tumor necrosis factor-α or 12-O-tetradecanoylphorbol-13-acetate. Comparison of the sequence of the newly cloned mouse MMP-9 promoter region with our previous human isolate revealed that, in addition to the above mentioned elements, four units of GGGG(T/A)GGGG sequence (GT box) were conserved between the two species. In this study, we have demonstrated that one of the GT boxes located downstream of the AP-1 site is essential along with the AP-1 site for the activation of the promoter by v-Src but not by tumor necrosis factor-α or 12-O-tetradecanoylphorbol-13-acetate. Gel mobility-shift assays revealed that binding proteins for retinoblastoma control element, including Sp-1 family protein, can bind specifically to GT boxes. Thus, the v-Src signals to the AP-1 site and to the GT box homologous to retinoblastoma control element acted synergistically in transcriptional activation. These results suggest that certain v-Src-mediated signals are propagated along pathways that are independent of inflammatory cytokines.

Matrix metalloproteinases (MMP) are a family of zinc-containing endopeptidases that are collectively capable of degrading most or all of the constituent macromolecules of the extracellular matrix. This enzyme family includes the two genetically distinct 92- and 72-kDa type IV collagenases (MMP-9 and MMP-2, respectively (1, 2)), two separate interstitial collagenases (3, 4), three types of stromelysin (5-7), and pump-1 (8). Thus, these enzymes are involved in both physiological and pathological processes including wound healing, inflammation, differentiation, development, rheumatoid arthritis, tumor invasion, and other fibrotic manifestations (9-14).

Expression of some MMPs are regulated by cytokines and tumor promoters, namely tumor necrosis factor-α (TNF-α), epidermal growth factor, interleukin-1, and 12-O-tetradecanoylphorbol-13-acetate (TPA) (15-20). The TPA responsive element (TRE) can be bound by AP-1 proteins and is thought to be the common element that mediates the induction of gene promoter activity (21). The TREs in the promoter regions of the stromelysin-1 and interstitial collagenase genes have been mapped as the responsive elements in this induction (21-25).

We previously examined the mechanism of MMP-9 gene activation by TNF-α as a model system for signal transduction involved in the gene regulation of MMPs during inflammation (26). Unlike interstitial collagenase and stromelysin-1, our data showed that transcription of MMP-9 promoter was stimulated by TNF-α through binding to sites for not only AP-1 but also NF-κB and Sp-1.

MMP-9 is often expressed by malignant tumor cells, but its expression is not necessarily coordinated with that of interstitial collagenase and stromelysin-1 (27). The expression of MMP-9 is thought to potentiate the invasive character of the producer cells through its capacity to degrade type IV collagen, a major component of basement membrane. In fact expression of MMP-9 mRNA correlates well with tumorigenicity and metastatic ability of tumor cells (28). In this context, it is important to understand the regulatory mechanism of the expression of this gene in malignant tumor cells. Thus, we examined the effect on MMP-9 gene expression of oncogene products which are potent inducers of malignant tumors.

We show here that expression of v-Src induces the synthesis of MMP-9, which is mediated by alterations in activity of binding factors for the AP-1 site and the sequence motif GGGG(T/A)GGGG (GT box). This GT box is homologous to the so-called retinoblastoma (Rb) control element (RCE) (29, 30), and Rb can produce an anti-oncogene or tumor suppressor gene product (31-38) which is involved in regulating transcription of certain genes. However, the mechanism through which Rb functions to suppress tumorigenesis is unknown,
although Rb also regulates transcription of the c-fos, c-myc, and transforming growth factor β1 (TGF-β1) promoters in either a positive or negative manner depending on cell type (30). This action is mediated through a common motif termed RCE (29, 30).

Although the AP-1 site is essential for response to TNF-α and TPA, transactivation of human MMP-9 promoter by these mediators can only be attained with the cooperation of the binding site for either NF-kB or Sp-1 but not the GT box. Based on a comparative sequence analysis of the human and murine promoter regions of the MMP-9 gene, the above mentioned motifs are all well conserved in both genes. These findings suggest the existence of distinct mechanisms by which the transactivation of the MMP-9 gene is activated during both oncoprotein-mediated malignant transformation and inflammation.

MATERIALS AND METHODS

Determination of the Nucleotide Sequence of the Mouse MMP-9 Gene—A genomic DNA clone for the mouse MMP-9 gene was isolated from a Charon28 phage genomic library of BALB/C mouse obtained from the Japanese Cancer Research Resources Bank by using human MMP-9 eDNA as a probe. The DNA sequence was determined by the dideoxy chain termination method (39). Human and mouse MMP-9 Genes—To approach to identification of important regulatory sequences in the human MMP-9 gene, we compared human and mouse MMP-9 gene sequences (Fig. 1). Binding sites for AP-1, Sp-1, and NF-κB, which had previously been identified as essential elements for the induction of human MMP-9 gene by TPA and TNF-α (26), were conserved in the 5′-flanking regions of the human and mouse genomes. In addition to the elements described above, a CA repeat region lying upstream of the TRE, several copies of GGGAGGG and related sequences were conserved in the mouse genome.

Induction of MMP-9 by Oncoproteins—We initially examined whether MMP-9 gene promoter was activated by the following oncoproteins: c-Jun, JunB, c-Fos, v-Src, Ha-Ras, and inflammation. The transcription of the MMP-9 gene is activated by v-Src but not by Ha-Ras or both forms of ErbB-2. To confirm the stimulatory effect of v-Src on MMP-9 gene expression, the effect of v-Src expression on the synthesis of the enzyme was examined by a gelatin-substrate gel analysis (Fig. 2B). Culture fluids from HT1080 cells transfected with v-Src expression plasmid and control plasmid were harvested and analyzed for the synthesis of MMP-9. Transfection of v-Src expression plasmid enhanced the synthesis of MMP-9 by 2–3-fold (lane 2), which is a significant induction considering the inherent efficiency of transfection by the calcium phosphate co-precipitation method.

Mapping of the v-Src-responsive Element in the MMP-9 Promoter—To determine the basis of enhanced MMP-9 transcription caused by v-Src, a series of plasmids containing MMP-9 promoter mutants linked to the CAT gene were transfected with v-Src expression or control plasmids, and the effect

RESULTS

Sequence Comparison of the 5′-End of the Mouse and Human MMP-9 Genes—To approach to identification of important regulatory sequences in the human MMP-9 gene, we compared human and mouse MMP-9 gene sequences (Fig. 1). Binding sites for AP-1, Sp-1, and NF-κB, which had previously been identified as essential elements for the induction of human MMP-9 gene by TPA and TNF-α (26), were conserved in the 5′-flanking regions of the human and mouse genomes. In addition to the elements described above, a CA repeat region lying upstream of the TRE, several copies of GGGAGG and related sequences were conserved in the mouse genome.

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Fig. 2. A, activation of MMP-9 promoter by various oncogene products. One μg of reporter plasmid (−634-CAT) was co-transfected with 4 μg of expression plasmid for each oncogene product into HT1080 cells. CAT activity expressed in the cells 24 h after transfection was measured. B, induction of MMP-9 by v-Src. Culture fluids from HT1080 cells transfected with control plasmid and v-Src expression plasmid (lanes 1 and 2, respectively) were harvested and analyzed for the synthesis of MMP-9 on 7.5% polyacrylamide gels containing 0.5 mg/ml gelatin.

v-Src was compared to that of c-Jun and TPA (Fig. 3). Stimulation of transcription from the promoter of MMP-9 gene by TPA is mediated through binding sites for not only AP-1 but also for either Sp-1 or NF-xB (26). Transcription from the promoter was markedly stimulated by c-Jun and v-Src until the deletion extended to nucleotide position −90 (−90-CAT). In contrast, transcription from the promoter was drastically lost by deleting the AP-1 site (−73-CAT); however, only v-Src still significantly stimulated the promoter lacking the AP-1 site. To confirm the role of the AP-1 site, a double point mutation was introduced into the sequence (TGAGTCA to TTGTGACA) in the −634-CAT construct. The mutant promoter did not significantly respond to either c-Jun or TPA but still had a residual response to v-Src. Mutation or deletion of the possible binding sites for Sp-1 and NF-xB did not significantly alter the response to either c-Jun or v-Src. These results suggest that the AP-1 site is cis-element essential for the MMP-9 promoter response to stimuli. In addition to the AP-1 site, another sequence within nucleotide position −73 is essential for v-Src inducibility.

Apart from the elements described above, three copies of GA box and a CACA box located upstream of AP-1 site are conserved in the human and mouse promoter regions. Deletion of the −531 to −91 region from the −634-CAT reporter plasmid containing three copies of GA box and a CACA box did not have any significant effect on basal level or stimulated expression (HinfI−90-CAT).

v-Src-responsive Element Includes the GT Box—Based on comparative sequence analysis of the human and murine genes, the GGGGTGGGG sequence located between the AP-1 site and TATA box in the human gene corresponds to a homologous GC-rich motif (GGGGAGGGGCGGGG) in the mouse gene. We introduced mutations in this element of the −90-CAT construct to determine whether this element contributes to the regulation of MMP-9 gene expression (Fig. 4A). Since the binding site for AP-1 is included in this promoter region, the effects of AP-1 family members c-Jun and JunB were compared with that of v-Src. Mutating the GGGGTGGGG sequence to GAAGTGGGG (−90gtmut.1-CAT) or GGGTAAGG (−90gtmut.2-CAT) completely abolished transcriptional response to v-Src, whereas only a slightly decreased basal level and response to c-Jun and JunB was observed. When the GGGGTGGGG sequence was changed to GGGGGCGGGG (a possible binding sequence for Sp-1 protein, −90gtmut.3-CAT), the promoter was activated by c-Jun and JunB, but less effectively by v-Src than for the wild type promoter. Mutation of the GGGGTGGGG sequence to GGGGAGGGG (GA box) (−90gtmut.4-CAT), which is the same as in the mouse promoter, did not have any significant effect on the response to v-Src.

Next we examined the role of the GT box in the promoter lacking the AP-1 site (−73-CAT) (Fig. 4B). Mutation of the wild type sequence to GAAGTGGGG (−73gtmut.1-CAT) or GGGTAAGG (−73gtmut.2-CAT) abolished the response to v-Src, suggesting that the GT box on its own confers v-Src inducibility, which is synergistically enhanced by AP-1-mediated transactivation.

GT Box Is Not Essential for TPA Inducibility—To compare the mechanism of induction by TPA and v-Src, the effect of TPA on induction of the transcription from the MMP-9 promoter by v-Src was examined (Fig. 5). The promoter containing the GT box and binding sites for AP-1, NF-xB, and Sp-1 was activated by v-Src and TPA, but simultaneous induction by v-Src and TPA did not show either synergistic or additive effects. Mutation of the GT box, which abolished the response to v-Src, did not have a significant effect on TPA inducibility (−634gtmut.2-CAT), and v-Src did not affect TPA inducibility of the promoter. TPA not only failed to activate transcription from the reporter plasmid lacking binding sites for NF-xB and Sp-1 (−90-CAT) but also repressed the transcriptional activation induced by v-Src.

Augmentation of AP-1-mediated Transactivation of MMP-9 Promoter by v-Src—Transforming oncogene products Ha-Ras and v-Src reportedly activate c-Jun by phosphorylating its transcription-activation domain (42, 43). This led us to examine the contribution of this pathway to the transactivation of the MMP-9 promoter by v-Src. As shown above, overexpression of c-Jun and JunB stimulated the promoter regardless of the GT box, and transactivation by c-Jun and JunB was further augmented by v-Src (Fig. 6). Thus, the GT box was not essential for the augmentation of c-Jun- and JunB-mediated transactivation by v-Src. However, a stimulatory effect of v-Src on the promoter lacking a GT box was not observed in the absence of exogeneous c-Jun and JunB expression as mentioned above. Augmentation of c-Jun- and JunB-mediated activation by v-Src was not due to increased expression of these proteins by v-Src, because the SV40 early promoter used to control these genes was not significantly affected by v-Src. Moreover, v-Src could stimulate the activity of c-Jun and JunB expressed at saturated levels (data not
Fig. 3. Mapping of v-Src-responsive elements. One μg of the various reporter plasmids were transfected into HT1080 cells together with 4 μg of control, c-Jun, or v-Src expression plasmid. TPA (50 ng/ml) was included in the culture medium after transfection of control plasmid (+TPA). Apmu, SPrnu, and kBmu are the mutations in the TRE, Sp-1, and xB consensus sequences, respectively, as described previously (26).

As shown. As described above Ha-Ras on its own could not induce the transcription from MMP-9 promoter, but it did cooperate with c-Jun and JunB to activate the promoter. These results suggest that augmentation of the activity of c-Jun and JunB may not be the main pathway for the activation of the MMP-9 promoter by v-Src. TPA which is also known to activate c-Jun failed to augment transactivation by c-Jun and JunB (data not shown). Reportedly v-Src also induces expression of c-fos which forms heterodimers with members of the Jun family and enhances AP-1-mediated transactivation (44). Expression of c-fos enhanced c-Jun and JunB activity, but this was less extensive than that observed with v-Src.

Characterization of Nuclear Factors Which Bind to the GGGTGGGG Sequence—As demonstrated above, v-Src-responsive elements in the human MMP-9 promoter were delimited to the AP-1 site and the GT box located between the AP-1 site and TATA box. To characterize the nuclear factors which bind to the GT box, nuclear extracts were prepared from HT1080 cells which constitutively synthesize the enzyme and non-producer HepG2 cells. DNA binding activity was analyzed by gel retardation assays using a 32P-labeled double-stranded oligonucleotide DNA encompassing the GGGTGGGG sequence (nucleotide positions -64 to -39) as a probe (Fig. 7). Two major retarded bands, A and B, were observed with the extract from HT1080 cells (lane 1). Addition of unlabeled oligonucleotide to the binding reaction competed for the formation of complex A and B. A new band, C, migrating faster than A and B appeared at lower concentration of competitor (lane 2) but abolished at a higher concentration (lane 3). Oligonucleotides gtm.1 (GAAGTGGGGG) and gtm.2 (GGGGTGAGG) failed to compete for complex formation (lanes 4 and 5, respectively). The oligonucleotide gtm.3, which contains the GGGGCTGG sequence, competed for the formation of complex A only and caused band B to become even stronger (lane 6). Since the GGGGCTGG sequence is identical with that of the binding site for Sp-1 protein, an oligonucleotide (SVSPl) containing the Sp-1 binding sequence of the SV40 promoter was tested. SVSPl also blocked the formation of complex A only but not complex B (lane 7). The oligonucleotide gtm.4 (GGGGAGGGG) showed the same competition pattern as the wild type gt oligonucleotide, and a faster migrating band C was observed at the lower competitor concentration (lanes 8 and 9).

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A nuclear extract from HepG2 cells also generated the two major retarded bands A and B, but the faster migrating band B was much weaker than that seen with HT1080 cells (lane 10). Formation of complexes A and B was interfered with by unlabeled gt oligonucleotide, and in this case a faster migrating band C was detected (lanes 11 and 12). Oligonucleotides gtm.3 and SVSPl competed for the formation of band A (lanes 13 and 14, respectively).

GT Box Is Homologous to RCE—A GT box or closely related sequence has been identified in the promoter regions of the c-myc, c-fos, TGF-β, and insulin-like growth factor II genes and has been mapped as a RCE (29, 30). We therefore compared c-fos RCE binding factors with those of the GT box. As shown in Fig. 8, a c-fos RCE oligonucleotide formed similar complexes with nuclear factors as the gt oligonucleotide probe (lane 1), which were specifically abolished by excess gt oligonucleotide (lane 4). Oligonucleotides gtm.1 and gtm.2,
and that one of them is Sp-1 or a closely related protein. Above, the nuclear extract from HepG2 cells showed a binding complex B (lane 3), which failed to compete for complex formation by the SVSp1 oligonucleotide (data not shown). The SVSp1 oligonucleotide competed for complex formation with the RCE probe, did not affect complex formation with the GT box mutant reporter plasmid, did not affect complex formation with the RCE probe (-90-CAT reporter plasmid or its GT box mutants was co-transfected into HT1080 cells with 4 μg of control, v-Src, Ha-Ras, or c-Fos expression plasmid in the presence or absence of 0.5 μg of c-Jun or JunB expression plasmid.

![Diagram](image)

**Fig. 4. Requirement for the GT box for induction by v-Src.** A, one μg of the −90-CAT reporter plasmid or its GT box mutants was co-transfected into HT1080 cells with 4 μg of control, c-Jun, JunB, or v-Src expression plasmid. B, one μg of −73-CAT reporter plasmid or its GT box mutants was co-transfected into HT1080 cells with 4 μg of control or v-Src expression plasmid.

which failed to compete for complex formation by the RCE probe (data not shown). The SVSp1 oligonucleotide competed for the formation of the slower migrating complex A but not of complex B (lane 3). These results indicate that binding factors for c-fos RCE are similar or identical to those of the GT box and that one of them is Sp-1 or a closely related protein.

**Inducibility by v-Src in HepG2 Cells—**As demonstrated above, the nuclear extract from HepG2 cells showed a binding pattern to the GT box distinct from that of HT1080 cells, which led us to examine the effect of v-Src in HepG2 cells. Transcription from the MMP-9 promoter (−634-CAT), which contains binding sites for NF-κB, Sp-1, and AP-1, was stimulated by Jun and TPA in both HT1080 and HepG2 cells. However, the promoter was transactivated by v-Src only in HT1080 cells and not in HepG2 cells (Fig. 9). In contrast, the collagenase promoter was activated by these effectors including v-Src in both cells.

**DISCUSSION**

**v-Src Can Induce Expression of MMP-9—Oncoprotein-mediated malignant transformation affects gene expression, the products of which can directly modulate the malignant phenotype of tumor cells, e.g. potentiating their invasive ability. Since the expression of MMP-9 is thought to potentiate the invasive character of the producer cells, it was of interest to examine the effect of oncogene products on the expression of this gene. In this communication we have demonstrated that the v-Src gene product stimulates the expression of the MMP-9 gene and determined which cis-acting elements respond to v-Src induction. These elements are distinct from those responsible for induction by TNF-α, an inflammatory cytokine, and TPA (26). Transactivation of MMP-9 Promoter by v-Src Is Mediated via an AP-1 Site and a GT Box—**A number of studies have shown that v-Src can affect expression of various genes such as c-myc, c-fos, c-jun, junB, EGR-1, TGF-β, collagenase, and stromelysin-1 (25, 45–49). The v-Src protein was reported to activate the collagenase, stromelysin-1 and TGF-β genes through an AP-1 element in their promoters (25, 45, 49). Transfection with the v-src oncogene enhances not only gene expression of c-jun but also the transcriptional activity of the c-jun protein (43). v-Src was also reported to transactivate promoters of the c-fos and junB genes, although cis-acting elements responding to v-Src in these promoters were different not only from AP-1 but also from each other (44, 47).

Transforming oncoproteins such as v-Src, Ha-Ras, and Raf-1 stimulate AP-1 activity by phosphorylating the activation domain of c-Jun in a signaling cascade where Ha-Ras acts downstream to v-Src and upstream to Raf-1 (43). Thus, TPA-inducible MMP genes such as interstitial collagenase and stromelysin-1 are induced by these oncoproteins (25, 43, 45). In contrast, the MMP-9 promoter was transactivated by v-Src but not by Ha-Ras, although both v-Src and Ha-Ras...
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competition

FIG. 7. Identification of factors binding to the GT box. Gel-mobility shift assays were performed using a 32P-labeled double-stranded oligonucleotide (indicated as gt, nucleotide positions -63 to -37 in the human MMP-9 promoter) encompassing the GT box as a probe. Nuclear extracts from HT1080 (lanes 1–9) and HepG2 (lanes 10–14) were incubated with the probe (approximately 50 pg) in the presence or absence of a competitor oligonucleotide as indicated. Competitor oligonucleotides gtmut.1 to gtmut.4 have nucleotide substitutions in the GT box as indicated. SVSPl corresponds to the Sp-1 binding sites in the SV40 promoter.

FIG. 8. Comparison of the GT box and RCE. Gel-mobility shift assays using a 32P-labeled double-stranded oligonucleotide probe representing the c-fos RCE (nucleotide positions -102 to -84 in the human c-fos promoter, indicated as RCE) were performed. Nuclear extract from HT1080 cells was incubated with RCE probe (approximately 50 ng) in the presence or absence of a competitor oligonucleotide (10 ng) as indicated, cf. Fig. 7.

c-fos: AGAGGGGTTCCGGGCGGG

gt: CTCTCAAGGAAGGTTCCCTACACAGG

SVSPl: GGGAAATGGCCGAACTGGCGGAATAGG

c-fos: AGAGGGGTTCCGGGCGGG

gt: CTCTCAAGGAAGGTTCCCTACACAGG

SVSPl: GGGAAATGGCCGAACTGGCGGAATAGG

FIG. 9. Comparison of v-Src inducibility in HT1080 and HepG2 cells. One μg of MMP-9 or collagenase promoter reporter plasmid (−634-CAT and collagenase-CAT, respectively) was co-transfected with 4 μg of control, Jun, or v-Src expression plasmid into HT1080 or HepG2 cells. TPA (50 μg/ml) was included in the culture medium after transfection with control plasmid (TPA).

cells
cat activity

| Cells   | Promoter    | CAT Activity | % Conversion |
|---------|-------------|--------------|--------------|
| HT1080  | -634-CAT    |              |              |
|         | collagenase-CAT |            |              |
| HepG2   | -634-CAT    |              |              |
|         | collagenase-CAT |            |              |

To v-Src. Thus, the effect of v-Src may involve transcriptional and/or post-transcriptional augmentation of AP-1 activity which alone may not be strong enough to stimulate the promoter and thus require synergistic cooperation with GT box-mediated activation. The particular mechanism by which the v-Src signal stimulates GT box-mediated activation remains to be determined. Interestingly, the location of the GT box is important, because the G(A/C) box located upstream of the AP-1 site does not contribute to activation by v-Src.

v-Src reportedly alleviates repression of c-Jun by a cell-specific inhibitor (50); however, this is not the case during transactivation of the MMP-9 promoter by v-Src, because v-Src can activate the promoter in NIH3T3 cells which lack an inhibitor (data not shown).
### Table: Gene Sequences Homologous to the G(A/T) Box of the MMP-9 Promoter

| Gene                  | Sequence                        |
|-----------------------|---------------------------------|
| human 92-kDa type IV collagenase GT or GA motif | -53GGGGTGGGG-45 -243GGGGAGGGG-251 -294GGGGAGGGAGGGG-306 -480GGGGAGGGG-472 |
| mouse 92-kDa type IV collagenase GA motif         | -68GGGGAGGGGCGGGG-55 -149GGGAGGGG-141 -395CAGGGAGGGG-385 -423GGGGAGGGG-415 |
| Histone H4 GA motif                                    | -99GGGGAGGGG-108 |
| c-myc GA motif                                      | -144GGGGAGAG-151 -135GGGGAAGG-142 -126GGGGAGGG-133 -117GGGGAGGGG-124 -101CGGGAGGGG-108 |
| c-myc RCE                                           | -126GAGGTTGGGGGT-136 -117GAGGTTGGGGA-127 -82GGGCGTGGGGG-72 |
| c-fos RCE                                           | -87AGGGGTGGGG-97 -72CACGGGGGCGC-82 |
| junB RCE                                            | -86GGGGCGCGGGG-76 |
| TGFβ1 RCE                                           | -165TCGGCTGGGGG-175 -158GACGGTGCGCC-148 -92CTGGTTGGGGG-82 |
| TGFβ2 RCE                                           | +245TAGGTTGGGTT+255 +314AAGGTTGGGAG+324 +375CTGGCTGGGGG+365 +440GTGGCTGGTGT+450 +685CGCGTTGGCTG+695 |
| TGFβ3 RCE                                           | -678CGGGGTGGGGG-688 -664GAGGGCTGGGGG-674 -382GGGGCTGGGGG-372 -52GGGGTGGGGG-63 |
| IGF-II RCE                                          | -55AGGGGTGGGGG-44 -43TAGGGTTGGGAC-33 |

**FIG. 10.** Sequences homologous to the G(A/T) box of the MMP-9 promoter. Note that certain sequences are in the inverse (3' to 5') orientation.

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v-Src and TPA Stimulate the MMP-9 Promoter by Different Mechanisms—The AP-1 site is also essential to confer inducibility by TPA and TNF-α, but transactivation of MMP-9 promoter by TPA and TNF-α can only be attained with the cooperation of the binding site for either NF-κB or Sp-1, but not the GT box. Expression of anti-sense RNA for c-jun mRNA, rather than junB mRNA, repressed the response to both v-Src and TPA (data not shown). This suggests that c-Jun is involved in the activation by v-Src and TPA. Induction of c-jun by the protein kinase C agonist TPA is thought to result from a positive autoregulatory mechanism (21) and to involve dephosphorylation of preexisting c-Jun at inhibitory
phosphorylation sites next to its DNA binding domain (51). The fact that v-Src cooperates with c-Jun but not with TPA suggests that v-Src can not stimulate c-Jun which is induced and activated by TPA, i.e., c-Jun activated by signals from protein kinase C requires different factors for transactivation of the MMP-9 promoter than from v-Src. In contrast, transcription of interstitial collagenase and stromelysin-1 promoters is activated by both TPA and oncogenes via binding sites for AP-1 and Ets (25, 45).

**GT Box Is Homologous to RCE**—The sequence motif ‘GGGGTGGGG’ or closely associated sequence has been identified in the promoter regions of c-myc, c-fos, TGF-β, and insulin-like growth factor II (30, 52). Interestingly, these sequences have been mapped as RCE in these promoters (Fig. 10), and GC-rich sequences in junB and EGR-1 gene promoters were also identified as RCE. Oligonucleotides encompassing the GT box competed specifically for the formation of c-fos RCE complex, indicating that the GT box of the MMP-9 promoter is homologous to RCE. Kim et al. (52) have shown that Sp-1 can bind to, and stimulate transcription from, these promoters via RCE and that Rb can positively regulate RCE-mediated transcriptional activity by Sp-1. Recently, Kingsley and Winoto (53) have cloned new members of the Sp-1 family which bind to both GC/T boxes. Expression of the c-myc, c-fos, TGF-β, junB, and EGR-1 genes, all of whose promoters contain RCE, has been reported to be induced by v-Src; however, a contribution of RCE has not yet been demonstrated (44, 46-49). An attempt to detect a specific effect of expression of the Rb gene or anti-sense RNA for the Rb gene on the MMP-9 promoter was unsuccessful. However, the possibility cannot be ruled out that anti-oncogene product Rb can regulate the expression of the MMP-9 gene either negatively or positively through RCE with consequent modulation of the metastatic potential of tumor cells.

Daily et al. (54) purified a nuclear factor, H4TF1, which binds to the GGGGAGGG sequence motif but not to the GC box, binding site for Sp-1, and stimulates transcription from the histone H4 promoter. A GA box or closely related motif has also been identified in the promoter region of c-myc, type IV collagen, laminin B1, laminin B2, and fibronectin (55, 56). One of GT box-binding factors we detected here by gel mobility shift assays, which specifically bound to GT/A but not to GC box, binding site for Sp-1, and stimulates transcription from the Sp-1 family protein exists at a comparable level in both HT1080 cells than in non-producing HepG2 cells, in which v-Src failed to transactivate MMP-9 promoter. In contrast, the Sp-1 family protein exists at a comparable level in both cell types. This suggests that a GT/A box-specific binding factor may participate in regulation of MMP-9 gene expression not only at a basal level but also in response to v-Src. Substitution of GT for the GC box, which is located upstream of AP-1 site and is essential for induction by TPA, abolished TPA inducibility (data not shown). These results suggest distinct roles for GT/A and GC boxes in transcriptional regulation of the MMP-9 promoter. Consistent with this, Bruggeman et al. (56) demonstrated that nuclear extracts isolated from tissues that variably express type IV collagen showed distinctly different gel shift patterns with respect to GA box binding characteristics. It is important to note that promoters of MMP-9 and the major structural components of epidermal growth factor, e.g., type IV collagen, laminin B1, laminin B2, and fibronectin, share GT/A box, or homologous elements. Alterations in matrix protein metabolism are critical to the process of tumor metastasis, suggesting a specific role of the GT box homologous to RCE in the regulation of genes associated with tumor invasion.

**Acknowledgments**—We thank Masahiro Fujiy and Peter K. Sanders for helpful discussions and advice.

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