The 92-kDa type IV collagenase (92-kDa gelatinase B also referred to as MMP-9), which plays a critical role in extracellular matrix degradation, is regulated by growth factors that mediate their effects through the ras proto-oncogene. The current study was undertaken to determine the transcriptional requirements for the induction of 92-kDa gelatinase B expression by an activated ras oncogene. Transfection of OVCAR-3 cells with an expression vector encoding an activated Ha-ras increased 92-kDa gelatinolytic activity and stimulated (over 10-fold) the activity of a CAT reporter driven by 670 nucleotides of 5′ flanking sequence of the 92-kDa gelatinase B gene. Transient assays using a CAT reporter driven by 5′ deleted segments of the 92-kDa gelatinase B promoter indicated that a region spanning –634 to –531 was required for optimal induction of the promoter. The individual deletion, or mutation, of a PEA3/ets (–540) motif, AP-1 sites (–533, –79), a NF-κB (–600) consensus sequence, and a GT box (–52) substantially reduced the activation of the promoter by ras. An expression vector encoding the PEA3 transcription factor caused a 3-fold stimulation of the wild type but not the PEA3/ets-deleted 92-kDa gelatinase B promoter. Coexpression of a dominant negative c-Jun antagonized the ras-dependent stimulation of the 92-kDa gelatinase B promoter-driven CAT reporter. The signaling pathway mediating the induction of 92-kDa gelatinase B promoter activity by ras was examined. The expression of a phosphatase (CL100) which inactivates multiple mitogen-activated protein kinase members abrogated the stimulation of 92-kDa gelatinase B promoter activity by ras. However, the expression of a kinase-deficient mitogen-activated protein kinase kinase 1 (MEK1) did not prevent the activation of the 92-kDa gelatinase B promoter by ras and a constitutively activated c-ras expression vector was insufficient for 92-kDa gelatinase B promoter activation. Thus, the stimulation of the 92-kDa gelatinase B promoter by ras requires multiple elements including closely spaced PEA3/ets and AP-1 sequences and is MEK1-independent.

The 92-kDa type IV matrix metalloproteinase (92-kDa gelatinase B also known as MMP-9) plays a major role in cell migration in both physiological and pathological processes (1–3) by facilitating the destruction of the type IV collagen-containing basement membrane which separates the epithelial and stromal compartments (4). The 92-kDa type IV collagenase is secreted as a proenzyme (5) and subsequently activated by multiple enzymes, including cathepsin G, trypsin, stromelysin 1 (6), and 72-kDa gelatinase A (7) by the removal of 73 amino acids from the amino terminus of the protease. The active enzyme, which is capable of digesting native type I, III, IV, and V collagens at nondenaturing temperatures (4, 6), consists of five domains: the aminoterminal and zinc-binding domains shared by all members of the metalloproteinase family, a collagen-binding fibronectin-like domain, a carboxyl-terminal hemepxin-like domain, and a unique 54-amino acid proline-rich domain homologous to the u2 chain of type V collagen (5).

The 92-kDa gelatinase B is encoded by a 7.7-kilobase pair gene, which spans 13 exons (8). Transcription of the 92-kDa gelatinase B gene, which yields a 2.5-kilobase mRNA (8, 9), is regulated by 670 bp2 of regulatory sequence, which includes binding sites for AP-1, NF-κB, AP-2, and Sp1 (9). Mutation, or deletion, of the NF-κB, AP-1, and Sp1 motifs located 600, 79, and 558 nucleotides upstream of the transcriptional start site, respectively, reduced or abolished the ability of tumornecrosis factor-α to stimulate the 92-kDa gelatinase B promoter in OST osteosarcoma and HepG2 hepatoma cells (9). On the other hand, activation of the 92-kDa gelatinase B promoter by v-src in HT 1080 fibrosarcoma cells was attributed to binding sites for AP-1 (–79) and a Sp1-binding GT box at –52 (10).

Several lines of evidence suggest an important role for ras in the regulation of 92-kDa gelatinase B expression. First, the amount of this collagenase is increased by growth factors including transforming growth factor-α and epidermal growth factor (11), which via their transmembrane receptors, signal through the Ras GTP-binding protein (12). Second, and equally import-

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1 The abbreviations used are: bp, base pair(s); MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; JNK, jun amino-terminal kinase; CAT, chloramphenicol acetyltransferase; MAPK, mitogen-activated protein kinase.
tant, the ras oncogene, itself, is a potent stimulus for 92-kDa gelatinase B secretion in rat embryo cells (13). However, while the role of ras in the regulation of this collagenase is clear, the events downstream of ras that lead to induction of 92-kDa gelatinase B synthesis have not been elucidated. Ras can stimulate multiple signaling pathways (14–17). One of these involves the sequential activation of the serine-threonine kinase c-raf, mitogen-activated protein kinase kinase (MEK1), and the extracellular signal-regulated kinases (ERKs) (18–21). The ERKs, in turn, increase the synthesis and/or activity of several transcription factors including c-fos (22) and cts (23, 24) family members. Alternatively, ras can modulate gene expression through other signaling pathways, which can be distinguished from each other on the basis of their utilization of the jun amino-terminal kinase (J NK) (25–27), a fos-related kinase (15), or phosphatidylidylinositolspecific kinase (17). Accordingly, the purpose of the current study was twofold: first, to identify the cis acting elements in the 92-kDa gelatinase B promoter that mediate the inductive effects of ras on the expression of this gene, and second, to examine the signaling events connecting ras with the nuclear regulators of 92-kDa gelatinase B expression.

MATERIALS AND METHODS

Vectors—A 6.6-kilobase pair BamHI fragment from the activated c-Ha-ras2 oncogene from T24 bladder carcinoma cells cloned into a pSV2 neo plasmid (28) was used for ras transfections. The TAM-67 vector, encoding a c-jun protein lacking the transactivation domain (amino acids 3–122 absent) of the molecule, has been described elsewhere (29). BXB is an in-frame deletion of amino acids 26–302 of c-raf rendering the serine-threonine kinase constitutively active (18). The K97M mutant expression vector encodes a catalytically inactive MEK1 in which a methionine is substituted for a lysine in the ATP-binding site (30). A full-length PEA3 cDNA (31) (kindly provided by Dr. J. H. Hall) was inserted between the HindIII and BamHI cloning sites of the cytosomal virus promoter-driven pCDNA3 expression vector (Invitrogen Corp, San Diego, CA) generating the construct PEA3 pCDNA3. The 3XAP1 pBLCat construct consists of three AP-1 tandem repeats upstream of a thymidine kinase minimal promoter-CAT reporter (pBLCat) (32). The 5′ deleted fragment and mutated (−600 NF-κB mt; −558-Sp1 mt; −79 AP-1 mt; −52 GT mt) 92-kDa gelatinase B promoter CAT reporter constructs have been described previously (9, 10).

Cell Lines—The OVCAR-3 cell line was derived from a malignant ascites of a patient with progressive adenocarcinoma of the ovary. Codons 12, 13, and 61 of c-Ha-ras are not mutated in the OVCAR-3 cells. All cells were maintained in McCoy’s medium 5A supplemented with 10% fetal bovine serum. For the collection of conditioned medium, cells were changed to serum-free medium (33) and 48 h later the conditioned medium was collected and the cells counted.

Chloramphenicol Acetyltransferase (CAT) Assays—OVCAR-3 cells were transfected by a calcium phosphate method (33) with CAT reporter constructs fused to the wild type, mutated, or 5′ deleted fragments of the human 92-kDa gelatinase B promoter (9, 10) without, or with, the c-Ha-ras expression vector. Transient transfections were performed in the presence of 4 μg of a β-galactosidase expression vector to correct for differences in transfection efficiencies. Briefly, DNA precipitate formed in the presence of 124 μM calcium chloride in a buffer (pH 7.1) containing 15 mM Hepes, 280 mM NaCl, 1.5 mM Na2HPO4 was added to 50% confluent OVCAR-3 cells. After 6 h, the cells were changed to fresh 10% fetal bovine serum-containing medium and cultured for an additional 48 h. The cells were harvested and lysed by repeated freeze-thaw cycles in a buffer containing 0.25 M Tris-Cl, pH 7.8. Transfection efficiencies were determined by assaying for β-galactosidase activity. CAT activity was measured by incubating cell lysates (normalization efficiency) at 37 °C for 8 h with 4 μM [3H]chloramphenicol and 1 mM acetyl coenzyme A. After 4 h, the acetyl coenzyme A was replenished. The mixture was extracted with ethyl acetate and acetylated products subjected to thin layer chromatography using chloroform:methanol (9:5) as a mobile phase. The amount of acetylated [3H]chloramphenicol was determined using a model 603 Betascope.

RESULTS

Activation of the 92-kDa Gelatinase B Promoter by ras—We first determined if an activated ras was inductive for 92-kDa gelatinase B synthesis. OVCAR-3 cells, which lack a mutated ras, were transiently transfected with various amounts of an expression vector encoding an activated c-Ha-ras (H-ras) or, as a control, the empty expression vector (pSV2 neo). After 24 h, the cells were changed to serum-free medium and cultured for an additional 48 h. After this time, the conditioned medium was collected and the cells enumerated. Conditioned medium clarified by centrifugation was normalized for any difference in cell number and assayed for gelatinase activity by zymography. The activated ras, but not the empty expression vector (pSV2 neo), caused a dose-dependent increase in a gelatinase activity the size (92 kDa) of which was indistinguishable from that of 92-kDa gelatinase B (Fig. 1A). In contrast, a 72-kDa gelatinase activity, which was identical in size (36) to that of the 72-kDa type IV collagenase (72-kDa gelatinase A), was unaltered by transfection with the mutated ras and served as an internal control for loading differences.

Using identical transfection conditions, OVCAR-3 cells were co-transfected with a CAT reporter driven by the wild type 92-kDa gelatinase B promoter (MMP-9 CAT) and the ras expression vector or the empty vector (pSV2 neo). The cells were harvested 48 h later and assayed for CAT activity. 92-kDa gelatinase B promoter activity was activated 13-fold with 4 μg of the ras expression vector over that achieved with the empty expression vector (pSV2 neo), while 1 μg of the oncogene caused a 6-fold induction of promoter activity (Fig. 1B). These data suggested that the increased synthesis of 92-kDa gelatinase B brought about by the activated ras is likely a reflection of a trans-activation of the 92-kDa gelatinase B promoter.

Activation of the 92-kDa Gelatinase B Promoter by ras Requires a Region Spanning Nucleotides −634 to −531 Relative to the Transcriptional Start Site—To determine the region of the promoter required for its stimulation by ras, OVCAR-3 cells were transiently transfected with a CAT reporter driven by 5′-deleted fragments (9) of the 92-kDa gelatinase B promoter and the ras expression construct (Fig. 2). The reporter driven by either 670 or 634 bp of 5′ flanking sequence was strongly stimulated (16- and 13-fold, respectively) by ras. In contrast, CAT reporter activity was dramatically reduced to a 3-fold stimulation with 531 bp of 5′ flanking sequence. A CAT re-

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Fig. 1. c-Ha-ras expression in OVCAR-3 cells increases 92-kDa gelatinolytic activity and 92-kDa gelatinase B promoter activity. Panel A, OVCAR-3 cells were transiently transfected at 50% confluency with varying amounts of a c-Ha-ras expression vector (H-ras) or the empty vector control (pSV2 neo). After 6 h, the cells were rinsed and the medium changed. The cells were replenished with serum-free medium 24 h later and cultured for an additional 48 h. Conditioned medium was collected, and cells were enumerated. Conditioned medium, normalized to cell number, was electrophoresed in a 7.5% SDS-polyacrylamide gel containing 0.1% gelatin. The gel was incubated at room temperature for 2 h in the presence of 2.5% Triton X-100 and subsequently at 37 °C overnight in a buffer containing 10 mM CaCl₂, 0.15 M NaCl, and 50 mM Tris (pH 7.5). The gel was then stained for protein with 0.25% Coomassie. Panel B, OVCAR-3 cells were transiently transfected, as for panel A with 6 μg of a CAT reporter driven by the wild-type (670 bp) 92-kDa gelatinase B promoter (MMP-9 CAT), 4 μg of a β-galactosidase-expressing vector in the presence or absence of varying amounts of a c-Ha-ras expression vector (H-ras) or the empty vector (pSV2 neo). Cells were cultured for an additional 48 h and lysed by freeze-thawing. Cell extracts (equal protein) were incubated with [14C]chloramphenicol for 8 h. The mixture was extracted with ethyl acetate and subjected to thin layer chromatography. The conversion of 14C chloramphenicol to acetylated derivatives was determined with a 603 Betascope. The data are typical of three separate experiments.

Fig. 2. Activation of the 92-kDa gelatinase B promoter by c-Ha-ras requires a region between –634 and –531 in the 5′ flanking sequence. OVCAR-3 cells were transiently transfected, as described in the legend to Fig. 1B using 4 μg of the c-Ha-ras expression vector or an equimolar amount of the empty expression vector (pSV2 neo), 6 μg of a CAT reporter driven by the indicated 5′-deleted fragment of the 92-kDa gelatinase B (MMP-9 CAT) promoter, and a β-galactosidase-expressing vector. The cells were harvested and extracts (equal protein) were incubated with [14C]chloramphenicol for 8 h. The mixture was extracted with ethyl acetate and subjected to thin layer chromatography. The conversion of 14C chloramphenicol to acetylated derivatives was determined with a 603 Betascope. The experiment was carried out three times.

porter driven by 90 or 73 bp of 5′ flanking sequence demonstrated little if any stimulation by ras over that achieved with the empty expression vector (pSV2 neo).

ras-dependent Stimulation of the 92-kDa Gelatinase B Promoter Requires Multiple Transcription Factor Binding Sites Including Closely Spaced PEA3/ets and AP-1 Motifs—Since deletion of the region from –634 to –531 was associated with a substantial reduction in the activation of the 92-kDa gelatinase B promoter by ras, we questioned whether transcription factor binding sites in this part of the promoter were required for its maximal activation by the oncogene. A computer search of this part of the sequence (9) indicated the presence of a previously unrecognized PEA3/ets binding site (–540) followed by an AP-1 motif at –533. Since these juxtaposed motifs have been shown to be responsible for basal and inducible expression of type I collagenase (37) as well as the urokinase-type plasminogen activator (38), we determined the role of these motifs in the regulation of 92-kDa gelatinase B promoter activity by ras. Removal of either of these sites either by mutation, or by deletion, substantially impaired the stimulation of the promoter by ras (Fig. 3). Thus, whereas ras caused a 11-fold stimulation of the wild type 92-kDa gelatinase B promoter (MMP-9 wt), deletion of the PEA3/ets site at –540 (–540 PEA3 del) reduced this by about two-thirds (Fig. 3), Similarly, the 92-kDa gelatinase B promoter mutated at the AP-1 site at –533 (–533 AP-1 mt) was induced only 2-fold by ras.

Additionally, a number of other transcription factor binding sites along the full length of the promoter were also required for its stimulation by ras. Thus, mutation of an Sp1 motif at –558 (–558 Sp1 mt), and an AP-1 binding site at –79 (–79 AP-1 mt) previously, shown to be required for the tumor necrosis factor-α or phorbol ester-dependent stimulation (9) of 92-kDa gelatinase B, all substantially reduced the ability of ras to stimulate the 92-kDa gelatinase B promoter (Fig. 3B). Also, the 92-kDa gelatinase B promoter mutated at an NF-κB site at –600 (–600 NF-κB mt) was stimulated far less (Fig. 3A) than the wild type promoter. Mutation of an Sp1-binding GT box (–52 GT mt) located 52 nucleotides upstream of the transcriptional start site and which is required for 92-kDa gelatinase B promoter stimulation by v-src (10), practically abolished the induction by ras (Fig. 3B). The observation that 90 bp of 5′flanking sequence was insufficient for ras stimulation (Fig. 2), combined with the finding that mutation of the AP-1 site at –79 or the GT box at –52 abrogated ras stimulation, suggests that these motifs are required for but, by themselves, insufficient to mediate the ras induction of 92-kDa gelatinase B. An NF-κB-like motif (39) located at –615 did not appear to be required for optimal 92-kDa gelatinase B promoter activity since mutation of this site (–615 NF-κB mt) did not diminish the stimulation by ras (Fig. 3A). We also found that deletion of a sequence spanning –175 to –146 (175–146 del), which was shown to mediate the induction of the osteopontin gene by ras...
Expression of PEA3 Stimulates 92-kDa Gelatinase B Promoter Activity—The observation that the deletion of the PEA3/ets site at −540 impaired the stimulation of the 92-kDa gelatinase B promoter by ras, suggested that this transcription factor binding site was mediating, at least in part, the induction by the oncogene. To further investigate this possibility, we determined if expression of the PEA3 transcription factor could itself stimulate the 92-kDa gelatinase B promoter. OVCAR-3 cells were transiently transfected with a PEA3-encoding expression vector (PEA3 pcDNA3) and a CAT reporter driven by either the wild-type 92-kDa gelatinase B promoter (MMP-9 wt) or the same vector lacking the PEA3 binding sites (pBL CAT). The amounts of the expression constructs were identical to that used in panel A. For both panels A and B, the cells were harvested and assayed for β-galactosidase activity. Cell extracts, corrected for differences in transfection efficiency, were incubated with [14C]chloramphenicol for 8 h. The mixture was extracted with ethyl acetate and subjected to thin layer chromatography. The conversion of [14C]chloramphenicol to acetylated derivatives was determined with a 603 Betascope. The experiment was carried out three and two times for panels A and B, respectively.

Expression of PEA3 Stimulates 92-kDa Gelatinase B Promoter Activity—The observation that the deletion of the PEA3/ets site at −540 impaired the stimulation of the 92-kDa gelatinase B promoter by ras, suggested that this transcription factor binding site was mediating, at least in part, the induction by the oncogene. To further investigate this possibility, we determined if expression of the PEA3 transcription factor could itself stimulate the 92-kDa gelatinase B promoter. OVCAR-3 cells were transiently transfected with a PEA3-encoding expression vector (PEA3 pcDNA3) and a CAT reporter driven by either the wild type 92-kDa gelatinase B promoter or the promoter that had the −540 to −50 site deleted. Expression of PEA3 increased the activity of a CAT reporter driven by either a PEA3-containing promoter (TORUCAT) (41) or the wild type 92-kDa gelatinase B (MMP-9 wt) promoter (Fig. 4A). The induction of the 92-kDa gelatinase B promoter was more modest than that achieved with ras (3-fold compared with 15-fold), suggesting that other transcription factors are also required (as indicated by mutational analysis; Fig. 3) and/or that the mouse transcription factor is less effective than the human homologue (31, 42). In contrast, deletion of the PEA3/ets site at −540 did not impair the stimulation of 92-kDa gelatinase B promoter activity by the oncogene (Fig. 3B).
Ras Activation of 92-kDa Gelatinase B Gene Expression

A

B

Fig. 5. Stimulation of the 92-kDa gelatinase B promoter by c-Ha-ras is attenuated by the expression of a transactivation domain-lacking c-jun mutant. Panel A, OVCAR-3 cells were transiently transfected, as described in the legend to Fig. 1B, using 6 μg of a CAT reporter driven by 670 bp of 5’ flanking sequence of the 92-kDa gelatinase B gene (MMP-9 CAT), a β-galactosidase-expressing vector, and, where indicated, expression vectors encoding c-Ha-ras (H-ras) and a transactivation domain-lacking c-jun protein (TAM-67) (or an equimolar amount of the empty vector-CMV vector). Panel B, OVCAR-3 cells were transiently transfected, as described in the legend to Fig. 1B using 3 μg of a reporter plasmid (pBL CAT) fused to three tandem AP-1 repeats (3X AP1 pBL CAT), a β-galactosidase-expressing vector, and, where indicated, 4 μg of the c-Ha-ras expression vector (H-ras), an equimolar amount of pSV2 neo. For panels A and B, the cells were harvested, equal amounts of extracted protein incubated with [14C]chloramphenicol for 8 (panel A) or 1.5 h (panel B). The mixture was extracted with ethyl acetate and subjected to thin layer chromatography. The conversion of [14C]chloramphenicol to acetylated derivatives was determined with a 603 Betascope. The data are typical of two different experiments.

Fig. 6. Abrogation of the c-Ha-ras-dependent stimulation of 92-kDa gelatinase B promoter activity by co-expression of a MAPK-inactivating phosphatase. OVCAR-3 cells were transiently transfected as described in the legend to Fig. 1B using 6 μg of a CAT reporter driven by the 92-kDa gelatinase B promoter (MMP-9 CAT), a β-galactosidase-expressing vector, and, where indicated, 4 μg of the c-Ha-ras expression vector (H-ras), an equimolar amount of pSV2 neo, with or without either an expression vector encoding CL100 (CL100 pSGS) or the empty vector (pSG5). Cell extracts (equal amounts of protein) were incubated with [14C]chloramphenicol for 8 h. The mixture was extracted with ethyl acetate and subjected to thin layer chromatography. The conversion of [14C]chloramphenicol to acetylated derivatives was determined with a 603 Betascope. The experiment was carried out twice. PEA3 del) in the 92-kDa gelatinase B promoter completely abolished the stimulation by the ets family member and reduced the ras-dependent activation of the 92-kDa gelatinase B promoter by over 80% (Fig. 4A). These data suggested that the stimulation of the 92-kDa gelatinase B promoter by ras is mediated, in part, through a previously unrecognized PEA3/ets site located 540 bp upstream of the transcriptional start site.

Expression of a Transactivation Domain-lacking c-jun Mutant &n; Ex...
Transfection of the OVCAR-3 cells with 1 μg of the CL100-encoding vector, but not the empty expression vector (pSG5), completely abrogated (Fig. 6) the stimulation of the 92-kDa gelatinase B promoter CAT reporter (MMP-9 CAT) by ras. Thus, it is likely that ras up-regulates 92-kDa gelatinase B promoter activity through one, or multiple, MAPK members.

Expression of a Dominant Negative MEK1 Does Not Impair the Stimulation of the 92-kDa Gelatinase B Promoter by ras—MEK1 is a specific activator of extracellular signal-regulated kinase 1 and 2 (14, 50), which are MAPK members. We determined the role of this ERK1/ERK2 activator in the ras-dependent stimulation of the 92-kDa gelatinase B promoter CAT reporter (MMP-9 CAT) by ras. OVCAR-3 cells were co-transfected with ras, the 92-kDa gelatinase B promoter-driven CAT reporter (MMP-9 CAT) with, or without, an expression vector encoding a dominant negative MEK1 (K97M) (30, 51). Expression of K97M did not decrease (Fig. 7A) the 16-fold induction of 92-kDa gelatinase B promoter activity by ras. However, this was not a consequence of a poor expression of the dominant negative vector since, in OVCAR-3 cells, the stimulation of a full-length urokinase promoter-driven CAT reporter by ras was practically abolished by co-expression of K97M (Fig. 7B).

To further address the role of MEK1 in the regulation of 92-kDa gelatinase B promoter activity by ras, we determined if the expression of a constitutively activated c-raf (BXB) (18) could induce the 92-kDa type IV collagenase promoter since c-raf lies directly upstream of MEK1 (19, 21). The BXB expression construct encodes an in-frame deletion of amino acids 26–302 in the regulatory region of c-raf (18). Expression of the constitutively activated serine-threonine kinase did not stimulate 92-kDa gelatinase B promoter activity (Fig. 8A) over that achieved with the empty expression vector (pMNC). However, the constitutively activated serine-threonine kinase caused over a 4-fold stimulation of a CAT reporter driven by the urokinase-type plasminogen activator promoter (Fig. 8B), indicating that the lack of effect on 92-kDa gelatinase B promoter activity was not a consequence of an ineffective expression of this construct in OVCAR-3 cells. Thus, the expression of a constitutively activated c-raf is an insufficient stimulus for 92-kDa gelatinase B expression in these cells.

**DISCUSSION**

92-kDa gelatinase B expression is regulated by ras (13) as well as by a number of growth factors, including transforming growth factor-α and epidermal growth factor, which mediate their effects through this GTP-binding protein (11, 12). However, the cis-acting elements within the 92-kDa gelatinase B promoter responsible for the induction by ras as well as the signaling pathway involved have not been elucidated. We report herein that the induction of 92-kDa gelatinase B promoter activity by an activated ras is mediated through mul-
least one real possibility in light of a report that the expression of at least one transcription factor, a termine if this was a direct effect of E1A-F or secondary to the expression of other transcription factors, a termine if this was a direct effect of E1A-F or secondary to the expression of other transcription factors, a termine if this was a direct effect of E1A-F or secondary to the expression of other transcription factors.

Our findings may explain the observation by Higashino et al. (53). Our findings may explain the observation by Higashino et al. (53). Our findings may explain the observation by Higashino et al. (53).

The involvement of the PEA3/ets binding site in mediating the induction of the 92-kDa gelatinase B promoter by ras is suggested from several observations. First, the ability of ras to stimulate the 92-kDa gelatinase B promoter was diminished by deletion of the PEA3/ets site at 540. Second, in OVCAR-3 cells an expression vector encoding PEA3 was sufficient to stimulate the ras, albeit to a lesser degree than that of ras, the wild type 92-kDa gelatinase B promoter, but not the promoter devoid of this consensus sequence. The contention that the PEA3/ets motif is involved in mediating the induction of the 92-kDa gelatinase B promoter by ras is given further support by reports from other investigators. Thus, ras increases the synthesis of several Ets proteins (52) and mutated Ets proteins inhibit ras activation of transcription at least in NIH3T3 fibroblasts (53). Our findings may explain the observation by Higashino et al. (54) that 92-kDa gelatinase B promoter activity is induced over 10-fold by the recently cloned human homologue of PEA3 (E1A-F) (42). However, in that study, the authors did not determine if this was a direct effect of E1A-F or secondary to the production and/or activation of other transcription factors, a real possibility in light of a report (55) that the expression of at least one ets family member (c-ets-1) stimulates the jun and fos promoters, thereby increasing AP-1 activity.

The PEA3/ets site at 540 was located just upstream of an AP-1 consensus sequence (533) and mutation of this site, like the PEA3/ets motif, practically abolished the stimulation of the 92-kDa gelatinase B promoter by ras. It may very well be that the cooperation of these two sites is necessary for the stimulation of 92-kDa gelatinase B promoter activity by ras. Indeed, the involvement of juxtaposed PEA3/ets/AP-1 elements in the regulation of several inducible genes including type I collagenase, urokinase, keratin 18, and tumor necrosis factor-α (37, 56-59) has been reported by this and other laboratories. Thus, we recently demonstrated that the stimulation of urokinase promoter activity by ras also required closely spaced intact PEA3/ets and AP-1 motifs as mutation of either of these sites abolished the induction by the GTP-binding protein (33). Similarly, the inducibility of the type I collagenase gene by either phorbol ester or fibronectin required the presence of an AP-1 sequence and a PEA3/ets-like motif (37, 60). However, while the closely spaced PEA3/ets and AP-1 motifs are required for the stimulation of the 92-kDa gelatinase B promoter by ras, it is unlikely that they are sufficient for optimal expression of this collagenase. Thus, the individual mutation of other motifs including an Sp1-binding site (558), a GT box (52), an AP-1 binding site (79), as well as a NF-κB motif (600)

**Fig. 8.** Effect of expressing a constitutively activated c-raf on 92-kDa gelatinase B promoter activity. OVCAR-3 cells were transiently transfected, as described in the legend to Fig. 1-B using 6 μg of a CAT reporter driven by the wild-type 92-kDa gelatinase B (MMP-9-CAT, panel A) or urokinase (uPA CAT; 62, panel B) promoters, a β-galactosidase-expressing vector, 4 μg of the c-Ha-ras expression vector (H-ras) (or an equimolar amount of the pSV2 neo vector), or the indicated amount of an expression vector encoding a constitutively active c-raf (BXB) or the empty vector (pMNC). The indicated amount (0.5, 1, 2 μg) of plasmid refers to molar equivalents with respect to the amount of CAT reporter. Cell extracts were assayed for β-galactosidase activity and equivalent amounts (after correcting for varying transfection efficiencies) incubated with [3H]chloramphenicol for 8 h. The mixture was extracted with ethyl acetate and subjected to thin layer chromatography. The conversion of [14C]chloramphenicol to acetylated derivatives was determined with a 603 Betascope. Mock, no DNA added. The results shown are representative of duplicate experiments.

**Fig. 9.** Diagrammatic representation of the transcription factor binding sites in the 92-kDa gelatinase B promoter. Transcription factor binding sites are represented as boxed areas. The position of the 5′ end of each motif is indicated relative to the transcription start site with the exception of the Sp1 site (9), where the number refers to the 3′ end of the motif.

multiple elements including two previously undescribed, closely spaced PEA3/ets and AP-1 motifs located 540 and 533 nucleotides upstream of the transcriptional start site, respectively. The involvement of the PEA3/ets binding site in mediating the induction of the 92-kDa gelatinase B promoter by ras is suggested from several observations. First, the ability of ras to stimulate the 92-kDa gelatinase B promoter was diminished by deletion of the PEA3/ets site at 540. Second, in OVCAR-3 cells an expression vector encoding PEA3 was sufficient to stimulate, albeit to a lesser degree than that of ras, the wild type 92-kDa gelatinase B promoter, but not the promoter devoid of this consensus sequence. The contention that the PEA3/ets motif is involved in mediating the induction of the 92-kDa gelatinase B promoter by ras is given further support by reports from other investigators. Thus, ras increases the synthesis of several Ets proteins (52) and mutated Ets proteins inhibit ras activation of transcription at least in NIH3T3 fibroblasts (53). Our findings may explain the observation by Higashino et al. (54) that 92-kDa gelatinase B promoter activity is induced over 10-fold by the recently cloned human homologue of PEA3 (E1A-F) (42). However, in that study, the authors did not determine if this was a direct effect of E1A-F or secondary to the production and/or activation of other transcription factors, a real possibility in light of a report (55) that the expression of at least one ets family member (c-ets-1) stimulates the jun and fos promoters, thereby increasing AP-1 activity.

The PEA3/ets site at 540 was located just upstream of an AP-1 consensus sequence (533) and mutation of this site, like the PEA3/ets motif, practically abolished the stimulation of the 92-kDa gelatinase B promoter by ras. It may very well be that the cooperation of these two sites is necessary for the stimulation of 92-kDa gelatinase B promoter activity by ras. Indeed, the involvement of juxtaposed PEA3/ets/AP-1 elements in the regulation of several inducible genes including type I collagenase, urokinase, keratin 18, and tumor necrosis factor-α (37, 56–59) has been reported by this and other laboratories. Thus, we recently demonstrated that the stimulation of urokinase promoter activity by ras also required closely spaced intact PEA3/ets and AP-1 motifs as mutation of either of these sites abolished the induction by the GTP-binding protein (33). Similarly, the inducibility of the type I collagenase gene by either phorbol ester or fibronectin required the presence of an AP-1 sequence and a PEA3/ets-like motif (37, 60). However, while the closely spaced PEA3/ets and AP-1 motifs are required for the stimulation of the 92-kDa gelatinase B promoter by ras, it is unlikely that they are sufficient for optimal expression of this collagenase. Thus, the individual mutation of other motifs including an Sp1-binding site (558), a GT box (52), an AP-1 binding site (79), as well as a NF-κB motif (600)
spread over the length of the promoter substantially impaired the ability of ras to induce 92-kDa gelatinase B promoter activity. These binding sites have been shown previously (9, 10) to be required for the stimulation of the 92-kDa gelatinase B promoter by v-src (−52 GT box, −79 AP-1) and by phorbol ester (−79 AP-1, −558 SP1, and −600 NF-κB). Since expression of PEAA3 stimulated the 92-kDa gelatinase B promoter, then, presumably, the basal level of transcription factors, which bind to the aforementioned sequences, is sufficient to allow activated expression of this metalloproteinase by this ras family member.

The signaling pathway connecting membrane-bound ras with the transcriptional control of 92-kDa gelatinase B expression in OVCAR-3 cells merits discussion. We initially hypothesized that this was accomplished via a pathway utilizing c-ras, MEK1, and MAPK family members (18, 20, 21). Indeed, the ability of CL100, which inactivates multiple MAPK members (47, 48), to abrogate the stimulation was in line with this contention. However, while these data suggested the involvement of a MAPK in mediating the stimulation of the 92-kDa gelatinase B promoter by ras, other experiments suggested that this was independent of MEK1 which activates the ERK1 and ERK2 group of MAPKs (19). Thus, interfering with the function of MEK1 failed to block the activation of the 92-kDa gelatinase B promoter by ras. Moreover, the expression of a constitutively activated c-ras, which lies upstream of MEK1 (20), while inducing the urokinase promoter in these cells, did not stimulate a 92-kDa gelatinase B promoter-driven CAT reporter.

In conclusion, we have shown that the induction of 92-kDa gelatinase B promoter activity by ras occurs through multiple transcriptional elements (see Fig. 9 for diagrammatic representation of these elements), including previously undescribed, closely spaced PEAA3/ets (−540) and AP-1 motifs (−533). Additionally, the ras-dependent expression of this metalloproteinase in OVCAR-3 cells is mediated through a MEK1-independent signaling pathway. Since the 92-kDa gelatinase B has been implicated in the invasiveness of different tumor types (1, 61), these findings may be relevant to the development of therapeutic agents for invasive cancer which modulate 92-kDa gelatinase B expression by interfering with its paracrine and/or autocrine activation through the ras pathway.

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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

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