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High Levels of MMP-1 Expression in the Absence of the 2G Single Nucleotide Polymorphism Is Mediated by p38 and ERK1/2 Mitogen-Activated Protein Kinases in VMM5 Melanoma Cells

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Abstract Matrix metalloproteinase-1 (MMP-1) is one of only a few enzymes with the ability to degrade the stromal collagens (types I and III) at neutral pH, and high expression of MMP-1 has been associated with aggressive and invasive cancers. We recently reported a single nucleotide insertion/deletion polymorphism (SNP) in the collagenase-1 (MMP-1) promoter (Rutter et al. [1998] Can. Res. 58:5321–5325), where the insertion of an extra guanine (G) at −1607 bp creates the sequence, 5′-GGAA-3 (2G allele), compared to the sequence 5′-GAA-3′ (1G allele). The presence of 2G constitutes a binding site for the ETS family of transcription factors, and increases MMP-1 transcription in fibroblasts and A2058 melanoma cells cultured in vitro. In addition, the presence of the 2G allele has been linked to several aggressive malignancies as well as to enhanced expression of MMP-1. In this study, we describe a melanoma cell line, VMM5, that is 1G homozygous, but that is invasive and expresses high levels of MMP-1 constitutively. The high level of MMP-1 expression in VMM5 cells is due to the utilization of both the p38 and ERK1/2 transduction pathways. In contrast, in the A2058 cell line, which also expresses MMP-1 constitutively and which is 2G homozygous, only the ERK pathway is activated. Thus, our data suggest that in the absence of 2G allele and in the presence of the appropriate transcription factors, tumor cells may use alternative signal/transduction pathways and cis-acting sequences to achieve high levels of MMP-1 expression, which contribute to the ability of tumor cells to invade, regardless of their genotype.
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Matrix metalloproteinases (MMPs) are a family of highly conserved enzymes that, collectively, degrade all components of the basement membrane and extracellular matrix [Parsons et al., 1997; Nagase and Woessner, 1999; Nelson et al., 2000]. Currently there are at least 26 known human MMPs and over expression of MMPs has been associated with several diseases, such as arthritis, periodontitis, atherosclerosis, and tumor invasion and metastasis [Vincenti et al., 1996; Basset et al., 1997; Chambers and Matrisian, 1997; Curran and Murray, 1999; Brinckerhoff et al., 2000; Koblinski et al., 2000; McCawley and Matrisian, 2000]. In particular, these MMPs include the collagenases, the only enzymes known to efficiently degrade the interstitial or fibrillar collagens I, II, II, V, and XI. In humans, type I collagen is the most abundant collagen found in skin and bone [Vincenti et al., 1996]. Of the collagenases, MMP-1 (collagenase-1) is

Abbreviations used: MMP, matrix metalloproteinase; uPA, urokinase plasminogen activator; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase.

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ubiquitously expressed in normal cells, including fibroblasts, keratinocytes, macrophages, smooth muscle cells, and endothelial cells [Vincenti et al., 1996]. These cells, however, produce low basal levels of this enzyme. Although the enzyme is readily induced by growth factors and cytokines [Borden et al., 1996; Vincenti et al., 1996; Borden and Heller, 1997], several tumor cell lines display high constitutive expression [Benbow et al., 1999a,b; McCawley and Matrisian, 2000], and enhanced expression of MMP-1 correlates with aggressive behavior in several types of cancer cells [Murray et al., 1996, 1998; Inoue et al., 1999; Ito et al., 1999; Benbow et al., 1999a,b; Brinckerhoff et al., 2000; McCawley and Matrisian, 2000]. While the expression of MMP-1 is often associated with fibroblasts surrounding the tumor cells [Heppner et al., 1996; Liotta and Kohn, 2001], histological studies also have shown expression of this enzyme within the tumor cells [Tsukifuji et al., 1999]. MMPs are secreted in latent form and are proteolytically cleaved by MMP-3 (stromelysin 1) and the serine proteinase uPA to generate enzymatically active forms. Thus, active MMPs are the product of a proteolytic cascade of enzymes that are produced by the tumor cells and/or by the adjacent stromal cells.

The concerted expression of the proteinases necessary for activating proMMP-1 plays a major role in facilitating the ability of tumor cells to invade the extracellular matrix. For example, the A2058 melanoma cells produce copious amounts of MMP-1 but are unable to invade a matrix of type I collagen because they can not activate proMMP-1 [Benbow et al., 1999b]. However, MMP-3 supplied by stromal cells can activate the latent collagenase, allowing the tumor cells to invade [Benbow et al., 1999b]. On the other hand, the breast cancer cell line, MDA-231, also produces high levels of MMP-1 constitutively, and these cells invade a collagen matrix without the need for stromal cells [Benbow et al., 1999a]. This indicates that these cells produce an assortment of proteinases that activates proMMP-1, even though the invasion is further enhanced by stromal cells [Benbow et al., 1999a]. The complement of proteinases produced by the tumor cells and by adjacent stromal cells can, therefore, influence the invasive behavior of the tumor cells.

Growth factors and cytokines induce MMP-1 expression, at least in part, by the activation of the mitogenic-activated protein (MAP) kinase pathways by activating AP-1 and ETS transcription factors [Westermarck et al., 1998, 2000; Westermarck and Kahari, 1999; Brauchle et al., 2000]. To date, three MAPK pathways have been described in detail: the stress activated protein kinases JNK (c-jun terminal kinases), the p38 activating kinases, and the extracellular signaling-related kinases (ERK1/2) [Lewis et al., 1998; Westermarck et al., 1998, 2000; Westermarck and Kahari, 1999; Brauchle et al., 2000]. The biological activators for the JNK and p38 pathways are cytokines and environmental stress, while ERK1/2 is activated by mitogenic growth factors via Ras [Frost et al., 1994]. In fibroblasts, both p38 and ERK1/2 are able to upregulate MMP-1 transcription independently, by either pathway [Brauchle et al., 2000], while in squamous cell carcinoma, expression of MMP-1 correlates with the activation of JNK and p38 [Westermarck et al., 2000]. These findings indicate that MMP-1 transcription is regulated by more than one signal/transduction pathway.

Recent studies by Rutter et al. [1998] describe a single nucleotide polymorphism (SNP) in the human MMP-1 promoter. This SNP is the absence or presence of a guanine (G) at −1607 bp, where the absence of the G creates a sequence, 5′-GAA-3′ (1G allele) and the presence of the extra G creates the sequence 5′-GGAA-3′ (2G allele). The frequency of these alleles in the population is approximately 25% 1G/1G; 25% 2G/2G; 50% 1G/2G [Rutter et al., 1998; Noll et al., 2001]. The sequence 5′-GGAA-3′ constitutes a consensus binding site for the Ets family of transcription factors. This DNA site at −1607 bp is adjacent to an AP-1 site at −1602 bp [Rutter et al., 1998]. Furthermore, transient transfection of promoter constructs containing either the 1G or 2G allele has shown that the 2G allele displays increased transcription when compared to the 1G allele. This increase was observed in fibroblasts and several tumor cells, including A2058 melanoma cells [Rutter et al., 1998]. It was, therefore, hypothesized that the increase in transcription of the 2G allele may be associated with a more aggressive phenotype in cancers, due to higher protein levels of MMP-1. Indeed, several studies have indicated an association between the 2G polymorphism and aggressive cancers [Kanamori et al., 1999; Nishioka et al., 2000; Gilhardi et al., 2001; Noll et al., 2001; Ye et al., 2001; Zhu et al., 2001].

In this article, we describe another melanoma cell line, VMM5, which is 1G homozygous, but which expresses high levels of MMP-1 constitutively. The mechanisms leading to high levels of MMP-1 transcription in these cells were compared to the A2058 melanoma cell line, which is 2G homozygous [Rutter et al., 1998], and were found to differ. While MMP-1 expression in the A2058 cells is mediated by the ERK1/2 pathway, the VMM5 cells utilize both the ERK1/2 and the p38 pathways. Since these pathways may target different cis-acting sequences in the MMP-1 promoter, it is possible that in the absence of the 2G allele, other elements in the promoter drive high levels of MMP-1 expression. Furthermore, using an in vitro invasion assay through type I collagen, we found that VMM5 cells, like the A2058 cells, were also invasive. However, unlike the A2058 melanoma cells, the VMM5 cells invaded without the aid of stromal cells, probably because these melanoma cells produce a panel of proteinases that can activate proMMP-1. Thus, our data suggest that melanoma cells are able to utilize alternative mechanisms to express MMP-1 and to invade a matrix of type I collagen.

MATERIALS AND METHODS

Reagents

The MEK1/2 inhibitor PD98059 and the p38 inhibitor SB203580 were purchased from Calbiochem (San Diego, CA).

Cell Culture

A2058 melanoma cells were purchased from ATCC, and VMM5 melanoma cells were a generous gift from Dr. Craig L. Slingluff, Jr., University of Virginia, Charlottesville, Virginia. The cells were grown in 150 mm diameter culture dishes in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) (37°C in 5% CO_2). After 3 to 4 days, when the cells were confluent, they were passaged 1:7 with 0.25% trypsin. For most experiments, cells were grown to the desired level of confluence, washed with Hanks balanced salt solution (HBSS; Gibco) to remove traces of serum and placed in serum-free DMEM supplemented with 0.2% lactalbumin hydrolysate (DMEM/LH) [Benbow et al., 1999b]. Human foreskin fibroblasts (HFFs) were obtained from the Birthing Pavilion at Mary Hitchcock Memorial Hospital (Lebanon, NH), and fibroblasts were isolated as previously described [Benbow et al., 1999b]. They were grown in 150-mm diameter culture dishes in DMEM, 10% FBS, penicillin/streptomycin, and used between passages 4–12 [Benbow et al., 1999b].

Western Blot Analysis

Nuclear extracts (20 μg) were separated by SDS–PAGE electrophoresis and analyzed by immunoblotting as described [Benbow et al., 1999a,b]. Primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For analysis of MAPK, cells were washed with HBSS, and then scraped in 2× denaturing SDS buffer. Extracts were heated for 10 min at 100°C and resolved on 10% SDS–PAGE and immunoblotted as described [Rutter et al., 1998]. Antibodies to p38, pp38, MEK1/2, ppMEK1/2, pp42/44, and p42/44 were purchased from England Biolabs (Beverly, MA).

Northern Blot Analysis

Confluent cultures of A2058 and VMM5 melanoma cells were placed in serum-free medium for 24 h in the absence or presence of varying concentrations of PD98059 or SB20350. Total RNA was harvested with a Trizol kit. RNA (10 μg/lane) was subjected to electrophoresis on 1% formaldehyde agarose gels and transferred to a GeneScreen Plus membrane. Membranes were hybridized with denatured [α-^{32}P] dCTP-cDNAs for MMP-1 and MT1-MMP, labeled by random oligo priming. Autoradiographs were quantitated by densitometric analysis [Rutter et al., 1998; Benbow et al., 1999a,b].

RT-PCR

Primers were designed according to published sequences [Saghizadeh et al., 2001]. RNA was treated with DNase using the Ambion DNA-free kit. RT reaction was carried out using AMV-RT (Boehringer Mannheim) and an oligo dT primer from Ambion. PCR was performed with Platinum Taq (GIBCOBRL) in the presence of 125 ng of the individual forward and reverse primers. For PCR, a three step program (denaturation: 94°C for 30 s; annealing: 50°C for 30 s; extension: 72°C for 1 min) was carried out for 35 cycles. PCR products were separated on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Real Time RT-PCR

Total RNA isolated from VMM5 and A2058 cells was treated with DNase using the Ambion DNA-free kit (Ambion, Austin, TX). Reagent concentrations and cycling parameters for RT-PCR reactions were as outlined in the standard protocol for SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). The RT reactions were performed on 500 ng of total RNA using oligo dT primers and Multiscribe reverse transcriptase (Applied Biosystems). The PCR to detect reverse transcribed MMP-1 mRNA was performed on the ABI PRISM7700 Sequence Detection System using Applied Biosystems' SYBR Green PCR master mix and primers designed with Applied Biosystems' primer express software (forward 5′-AGCTAGCTCAG-GATGACATTGATG-3′; reverse 5′-GCCGAT-GGGCTGGACAG-3′). In addition to detecting MMP-1 template in RNA samples, a clone of MMP-1 cDNA in pSP64 plasmid from American Type Culture Collection [Whitham et al., 1986] was used to generate a standard curve for quantitating MMP-1 message. Serial dilutions (1:10; from 2 ng to 0.2 pg) of the plasmid were used to generate a standard curve. The resulting curve was adjusted to indicate the amount of MMP-1 cDNA template present in each real time reaction, rather than the amount of total plasmid. This calculation was as follows; ratio of the length of MMP-1 insert to the length of complete construct (0.404) X pg of plasmid = pg of MMP-1 cDNA. MMP-1 quantities were normalized to GAPDH mRNA levels measured in 125 ng of total RNA with real time RT-PCR and Applied Biosystems' GAPDH pre-developed assay reagent.

Preparation of Oligonucleotides and Nuclear Extracts

Oligonucleotides (40 bp) spanning the region between −1619 to −1583 bp of the MMP-1 promoter and containing 1G or 2G were used as probes for electrophoretic mobility shift assays (EMSA) as described [Rutter et al., 1998]. Oligonucleotides for both the bottom and the top strand were synthesized on a BioSearch DNA synthesizer and purified by Nensorb chromatography (Dupont). One hundred picomoles of 5′-ends of single stranded oligomers were labeled and annealed to their unlabeled complementary oligomer. Annealed oligomers were separated on a 12% acrylamide gel. Oligomers were eluted from a gel slice, followed by ethanol precipitation. Nuclear extracts were prepared from confluent cells. Cells were scraped from the plate, pelleted, and the nuclear fraction isolated and stored in 10 μl volumes at −70°C as described by Rutter et al. [1998]. The protein content of the extract was determined by a modified Lowry assay (BioRad DcKit).

Electrophoretic Mobility Shift Assay

For EMSA, 5 μg (per reaction) of nuclear proteins were incubated with 30,000 cpm of γ-^{32}P ATP labeled double stranded probes for either the 1G or 2G DNA. The reactions were electrophoresed, and gels were dried and exposed to X-ray film overnight [Rutter et al., 1998].

Transient Transfections

Melanoma cells were plated at a density of 3×10^5 cells per well in 6-well tissue culture plates. The following day, 1 μg of DNA and 10 μl of GenePORTER^{TM} Transfection Reagent (Gene Therapy System, San Diego, CA) in 1 ml serum free media were added for 4 h. One milliliter of 20% DMEM was then added for overnight incubation. The next day, cells were placed in serum free medium, and treated with either PD98059 or SB203580. After 18 h, cells were washed three times with ice cold PBS and lysed in 25 mM glycylglycine, 4 mM EGTA, 15 mM $MgSO_4$, 1% Triton X-100, and 1 mM DTT. Luciferase activity was measured in a Dynatech ML2250 luminometer and reported as relative light units. Statistical significance was calculated using the Student's t test and the program is available at http://www. physics.csbsju.edu/stats/.

Evaluation of Tumor Cell Invasiveness by Invasion Chamber Assay and Confocal Laser Scanning Microscopy

The assay utilizes a modified Boyden chamber [Benbow et al., 1999a,b, 2000] with the two compartments separated by a nitrocellulose filter (Schleicher & Schuell AE100, 25 mm diameter, 12 μm pore size). Briefly, filters were autoclaved and coated with collagen type I (1 mg/ml; Sigma) diluted in sterile DMEM in the presence of 1% antibiotics and successively applied to the membrane (3×150 μl, 1×550 μl, allowed to gel at 37°C for 30 min, air dried for 1 h at room temperature). The efficiency of coating was monitored by confocal laser scanning microscopy at the Image Facility at Dartmouth

Medical School. Single cell suspensions, of tumor cells were washed three times in HBSS, resuspended in serum free media, and counted. Cell viability was monitored by Trypan Blue exclusion. The lower chamber was filled with serum free media, and 10^5 cells in 1 ml were added to the upper chamber. The chamber was cultured at 37°C and 5% CO_2 for 48 h, and the degree of cellular invasion was assessed by confocal laser scanning microscopy as previously described [Benbow et al., 1999a,b, 2000].

RESULTS

Expression of Matrix Metalloproteinases (MMPs) and uPA in VMM5 and A2058 Melanoma Cells Lines

As a first step in comparing these two cell lines, we developed a profile of MMP expression (Fig. 1). We found that both the 1G VMM5 and the 2G A2058 cells expressed similar levels of the membrane bound MMP, MT1-MMP. Northern analysis revealed that MMP-1 RNA levels were similar in the two cell types (Fig. 1A). However, real time RT-PCR demonstrated that VMM5 cells actually contained slightly more MMP-1 mRNA than A2058 cells ($P = 0.035$) (Fig. 1B), supporting the Northern blot analyses. The type IV collagen degrading enzymes, MMP-2 and MMP-9, could not be detected in VMM5 cells, while high to moderate levels were observed in A2058 cells. We also found that VMM5 cells expressed low levels of MMP-3 and high levels of uPA, enzymes that can activate latent MMPs [Parsons et al., 1997; Nagase and Woessner, 1999; Nelson et al., 2000], while A2058 cells did not express detectable levels of mRNA for either of these proteinases (Fig. 1C). Expression of MMP-13, often restricted to cartilage, bone, and squamous cell carcinoma [Mengshol et al., 2000], was not detected in either cell line (data not shown). Thus, MMP-1 is the primary interstitial collagenase produced by these cells, and it may be largely responsible for mediating the invasive ability of these tumor cells.

DNA Binding and Differential Expression of Transcription Factors in VMM5 and A2058 Melanoma Cells

We have previously demonstrated that nuclear proteins from the A2058 melanoma cells preferentially bind to an oligonucleotide containing 2G versus 1G [Rutter et al., 1998]. To compare the ability of transcription factors present in the VMM5 and A2058 cells to bind to DNA containing 1G or 2G, we used EMSA. As shown in Figure 2A, a similar binding pattern of nuclear factors to the 1G and 2G probes was observed for the two cell lines, suggesting that each cell type contains transcription factors capable of binding to both the 1G and 2G DNAs. Further, binding to the 2G probe was slightly more pronounced than binding to the 1G probe with extracts from both cells, and includes a novel retarded complex (*). However, despite the similar binding pattern, Western analysis of nuclear extracts indicates differential expression of transcription factors. For example, c-Jun, a member of the AP-1 family of transcription factors, was not detected in A2058 cells while c-Jun was present in VMM5 cells (Fig. 2B). Differential expression was also observed for a member of the Ets family of transcription factors. Ets1/2 was expressed by both cell types, with higher levels observed in the VMM5 cells, while Erg was only observed in A2058 cells (Fig. 2B). Furthermore, by RNA analysis, we found that both cell lines produce similar amounts of Fra1, while Fra2 was not detected in either cell line (Fig. 2C, data not shown). The Fra1 mRNA consists of double bands due to differential splicing [Schreiber et al., 1997; Tower et al., 2002]. In contrast, only A2058 cells produce large amounts of JunD. Thus, these two cell lines display a different profile of transcription factors capable of binding to AP-1 and ETS sites, which may contribute to the differential regulation of MMP-1 seen in these cells.

Transient Transfection of 1G and 2G Promoter Constructs Into VMM5 and A2058 Melanoma Cells

To investigate the mechanism(s) controlling MMP-1 transcription in these two cell lines, we transiently transfected them with the −4372 bp and the −2002 bp constructs, containing either 1G or 2G. We chose these two constructs because they represent the longest and the shortest promoter fragments that contain the SNP. We found that the 1G constructs displayed a similar pattern of expression in each cell line (Fig. 3), with the 2002 bp construct significantly less responsive ($P < 0.01$) than the 4372 bp construct. This finding suggests that upstream sequences contribute to the transcriptional activation of the MMP-1 promoter with 1G. In

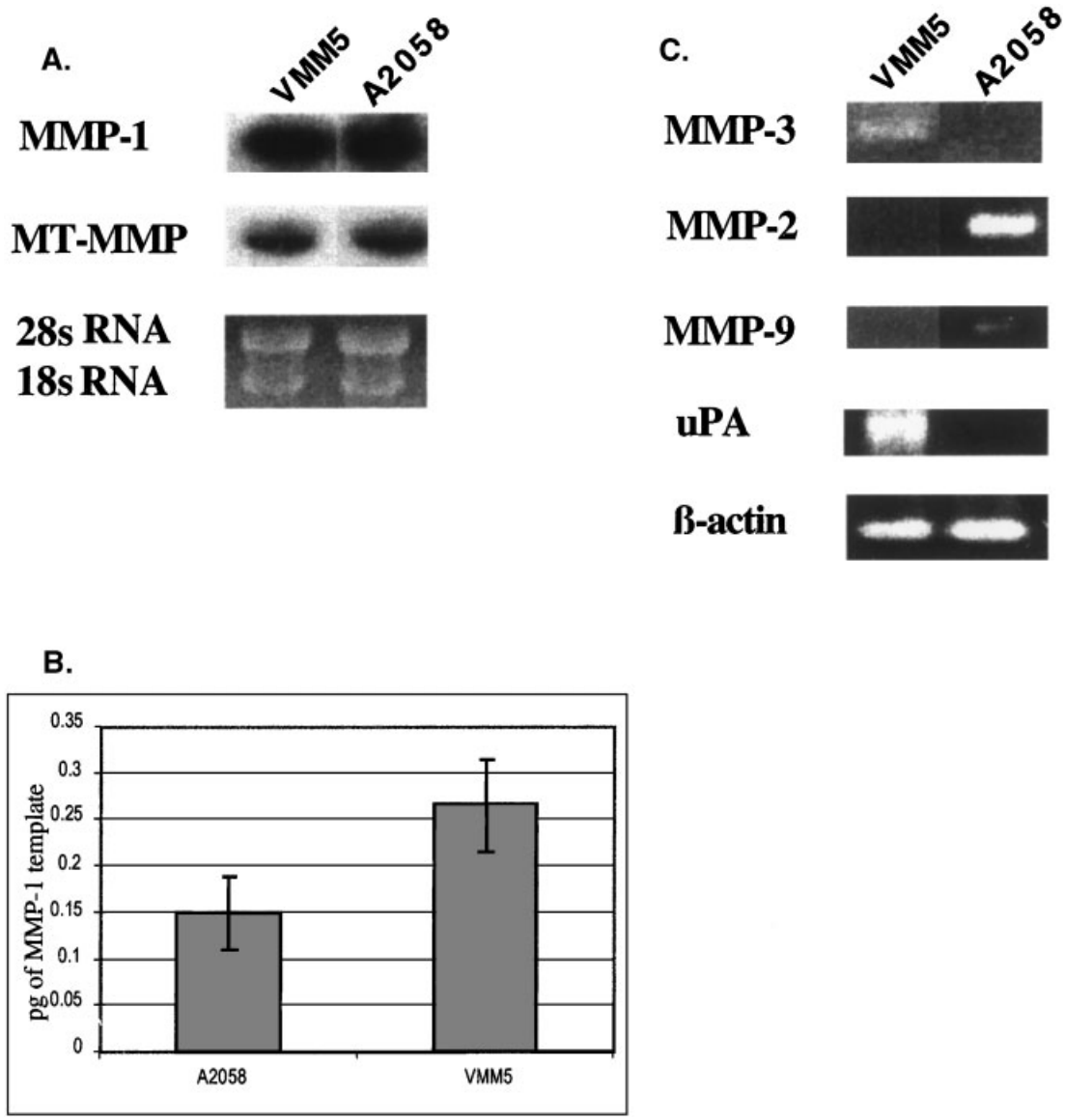

Fig. 1. Constitutive expression of matrix metalloproteinases and uPA by A2058 and VMM5 melanoma cells. (**A**) Northern analysis and (**B**) RT-PCR. Confluent cells were incubated in serum free-medium, and RNA was harvested after 18 h. Ribosomal RNA and β-actin are shown as loading controls. (**C**) Real time RT-PCR. MMP-1 mRNA template levels are calculated from a cDNA standard curve ($n = 3$; $P = 0.035$).

addition, all constructs containing the 2G DNA were more transcriptionally active than the 1G constructs in both cells lines. However, in the A2058 cells, the activity of the 2002 bp 2G construct was significantly decreased ($P < 0.01$) compared to the 4372 bp construct, suggesting that upstream sequences may further enhance expression of the 2G allele in these cells. In contrast, both constructs containing 2G were similarly expressed in the VMM5 cells. These data indicate that both cell lines contain the factors necessary to sustain transcription of the 2G allele, and they substantiate the EMSAs, which show similar binding patterns of nuclear extracts from each cell type to the 1G and 2G oligonucleotides (Fig. 2A).

Differential Activation Pathways Regulate MMP-1 Expression in the 1G VMM5 and 2G A2058 Cells

To determine whether high expression of MMP-1 in VMM5 and A2058 cells depends on the same or different signal transduction

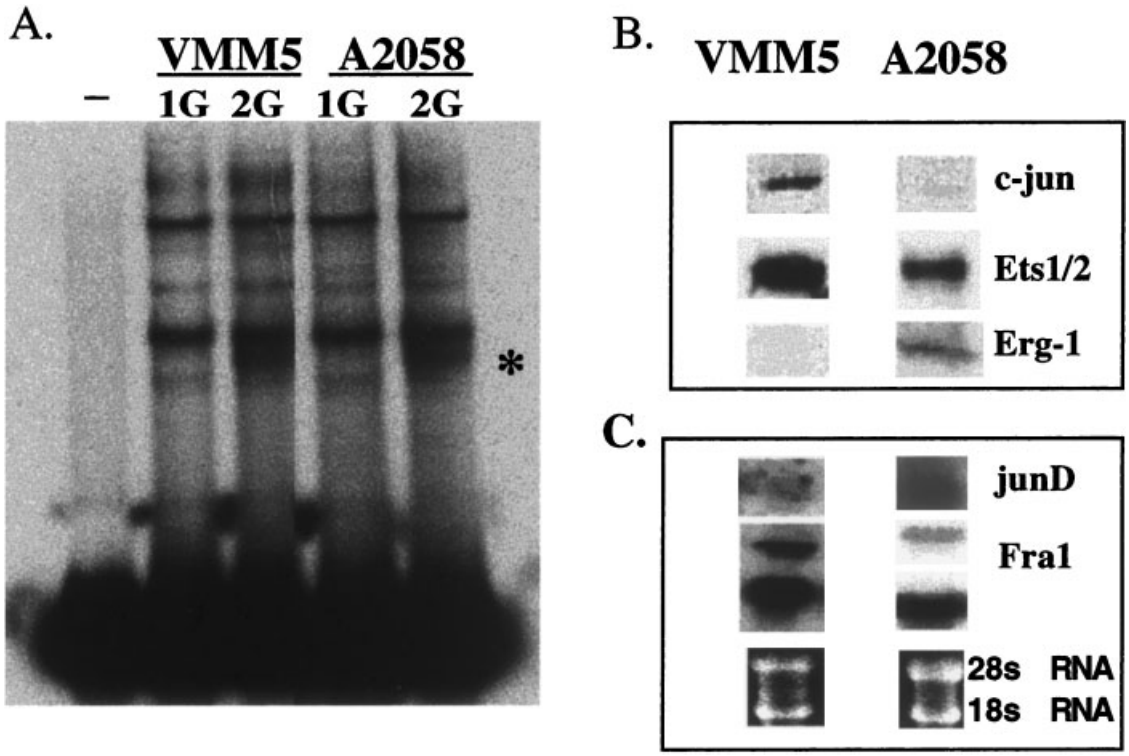

Fig. 2. Differential binding and expression of transcription factors in VMM5 and A2058 cells. (**A**) Electrophoretic mobility shift assay using 1G and 2G radiolabeled oligonucleotides. Nuclear extracts (5 μg) from VMM5 and A2058 cells were incubated with the corresponding radiolabeled probes. Lane (–) indicates free probe alone. The asterisk (*) indicates differential binding of nuclear proteins to the 2G probe. (**B**) Western blots for nuclear proteins. Assays were performed using 20 μg of nuclear extracts and blots were probed for the proteins indicated. (**C**) Northern blot analysis. RNA from VMM5 and A2058 cells was analyzed for expression of transcription factors.

pathways, we used SB203580, an inhibitor of the p38 MAPK pathway, that often targets AP-1 sites, and PD98059, an inhibitor of the ERK1/2 activation pathway that targets ETS sites [Frost et al., 1994; Lewis et al., 1998; Garrington and Johnson, 1999]. For these experiments, cells were treated with increasing concentrations of the inhibitors for 18 h and levels of MMP-1 mRNA were measured by Northern analysis. We found that at low concentrations (5 and 10 μM) the ERK1/2 inhibitor effectively repressed MMP-1 mRNA in both cell

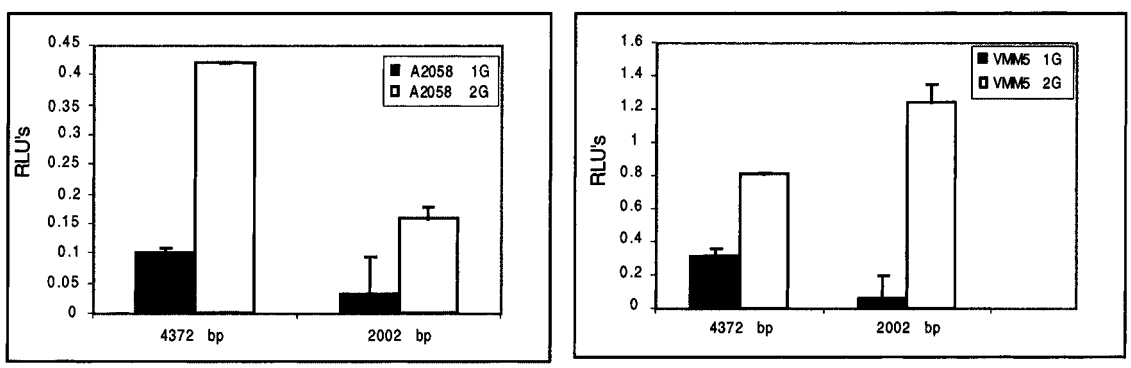

Fig. 3. Transient transfection of 1G and 2G containing promoter constructs into VMM5 and A2058 melanoma cells. Cells were transiently transfected with 4372 or 2002 bp promoter constructs linked to a luciferase reporter. Luciferase activity was determined using a luminometer and expressed as relative light units (RLU's) as described in Materials and Methods. The data are representative of three independent experiments that gave similar results.

lines (Fig. 4A), with densitometric analysis revealing about a 50% inhibition at a concentration of 5 μM in both cell lines. In contrast, the p38 inhibitor SB203580, at a concentration of 5 μM, which is specific for p38 [Westermarck et al., 2001] partially repressed MMP-1 mRNA in VMM5 cells to about 50%, but was not effective in A2058 cells at any of the concentrations tested. These results suggest that different signal/transduction mechanism(s) are responsible for activating MMP-1 transcription in the VMM5 and A2058 cells.

To confirm the inhibition of MAPK in VMM5 and A2058 cells, we examined the activation of the ERK1/2 and p38 by Western blot analysis, using phospho-specific antibodies. In the VMM5 cells, PD98059 (5 μM) decreased the phosphorylation of ppMEK1/2 and pp38, while total MEK1/2 and p38 were not affected (Fig. 4B). The decreased phosphorylation of pp38 in response to PD98059 in the VMM5 cells is in agreement with a recent report by Westermarck et al. [2001], which showed that p38 activation inhibits MEK1/2 activity and MMP-1 gene

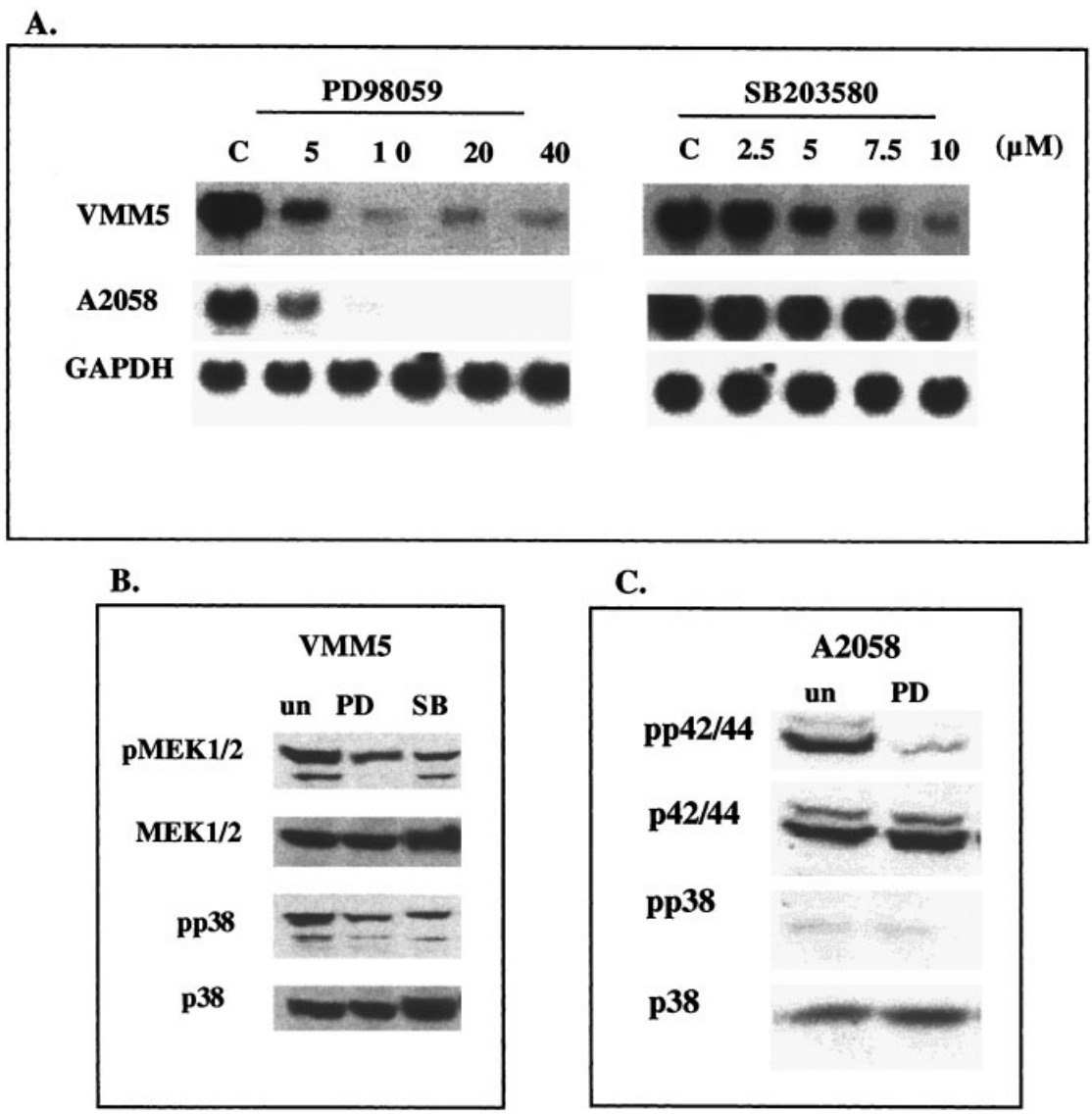

Fig. 4. Effects of inhibition of the ERK1/2 pathway by PD98059, and of the p38 MAPK by SB203580 on MMP-1 mRNA expression and transcription. (**A**) Northern blot analysis. Confluent cells were placed in serum free media in the absence or presence of increasing concentration of the inhibitors. After 24 h, RNA was isolated and probed for MMP-1. GAPDH was used as loading control. (**B**,**C**) Western blot analysis of nuclear extracts from untreated cells or from cells treated with PD98059 (5 μM) for 24 h. (B) Extracts from VMM5 cells were probed for ppMEK1/2, total MEK1/2 and pp38 and total p38. (C) Extracts from A2058 cells were probed for and pp42/44, p42/44, pp38, and p38.

expression in human skin fibroblasts. In the A2058 cells, PD98059 decreased the phosphorylation of ERK1/2 (pp42/44), but there was no effect on p38, since this pathway is not activated in these cells (Fig. 4C).

Effect of PD98059 and SB203580 on Transiently Transfected 1G and 2G Promoter Constructs

Previously, we examined the effect of PD98059 on 1G and 2G promoter constructs transiently transfected into A2058 melanoma cells [Tower et al., 2002]. We showed that when the constructs containing 1G were tested, the −4372 bp construct displayed a decrease in promoter activity in the presence of PD98059, but that the −2002 bp DNA was not inhibited, suggesting that upstream sequences mediate the inhibitory effects with 1G promoter. In contrast, transfection of the 2G promoter constructs in the presence of the inhibitor resulted in decreased promoter activity of both constructs [Tower et al., 2002].

To examine the role of the ERK1/2 and p38 pathways on MMP-1 transcription in the VMM5 cells, we measured the transcriptional activity of the 1G and 2G constructs in the presence of PD98059 and SB203580. PD98059 modestly inhibited the expression of both the 4372 bp and 2002 bp constructs containing 1G (Fig. 5A). Although significant compared to untreated values ($P = 0.01$ or less), the repression was about 30% for 4372 bp and only about 20% for the 2002 bp construct. These findings substantiate our hypothesis that in promoter DNA containing 1G, PD98059 may inhibit transcriptional activity, at least in part, through upstream cis-acting sequences. In the context of the 2G DNA, transcription was more strongly inhibited, with a 50% inhibition of the 4372 bp construct and a 70% inhibition of the 2002 bp construct, suggesting that ERK1/2 may target the 2G SNP. Finally, we measured the effect of the p38 inhibitor, SB203580, on promoter activity in the VMM5 cells (Fig. 5B). The inhibitor modestly reduced transcription of both the 1G and 2G promoter constructs, a result in keeping with the effect on MMP-1 mRNA (Fig. 4A).

Comparison of the VMM5 (1G) and the A2058 (2G) Melanoma Cells With Respect to Invasion Into a Type I Collagen Matrix

The potential biological significance of MMP-1 expression by the two cell lines was studied using a quantitative in vitro invasion assay. In previous studies [Benbow et al., 1999a,b], we determined that if tumor cells express the appropriate complement of serine proteinases and MMPs, they can mediate their own invasion through a matrix of type I collagen. Therefore, we used confocal laser scanning microscopy [Benbow et al., 1999a,b, 2000] to compare the ability of VMM and A2058 cells to invade type I collagen, and the invasion profile of the two cell types is shown in Figure 6. When cultured in serum free medium (LH), 90% of the A2058 cells were detected on top of the collagen matrix, and invaded only when cultured in the presence of fibroblast-conditioned medium. This finding confirms our previous observation that MMP-3 produced by the fibroblasts is necessary for the full activation of the proMMP-1 produced by the tumor cells, since A2058 cells do not make this enzyme (Fig. 1) [Benbow et al., 1999b]. In contrast, the VMM5 cells produce both uPA and MMP-3, along with MMP-1, and more than 50% of these cells invade the collagen matrix to a depth of 2 to 6 μm in the absence of fibroblast conditioned medium, although conditioned medium further enhances invasion. Thus, the invasive behavior of VMM5 cells may be due to the expression of MMP-3 and of uPA, (Fig. 1C), which are able to fully activate proMMP-1 [Nagase and Woessner, 1999].

DISCUSSION

Malignant melanoma accounts for 1–3% of all new cancers in the USA, with approximately 48,000 new cases and 8,000 deaths in the year 2000, numbers that have steadily increased over the past 50 years [Rigel et al., 1996; Hoffmann et al., 1998; Greenlee et al., 2000]. The greatest prognostic factor in this disease is the depth of invasion of the initial lesion [Breslow, 1970]. Although degradation of type IV collagen in basement membrane by MMP-2 and MMP-9 is an important component of this invasion, so is the degradation of the stromal collagens (types I and III) present within the dermis. Since this degradation is accomplished primarily by the interstitial collagenases and since collagenase-1 (MMP-1) is the most ubiquitously expressed of these collagenases, it is not surprising that this enzyme may play a major role in tumor invasion and metastasis. Indeed, increased expression of MMP-1 has been associated with the progression of several cancers, including malignant melanoma [Benbow et al.,

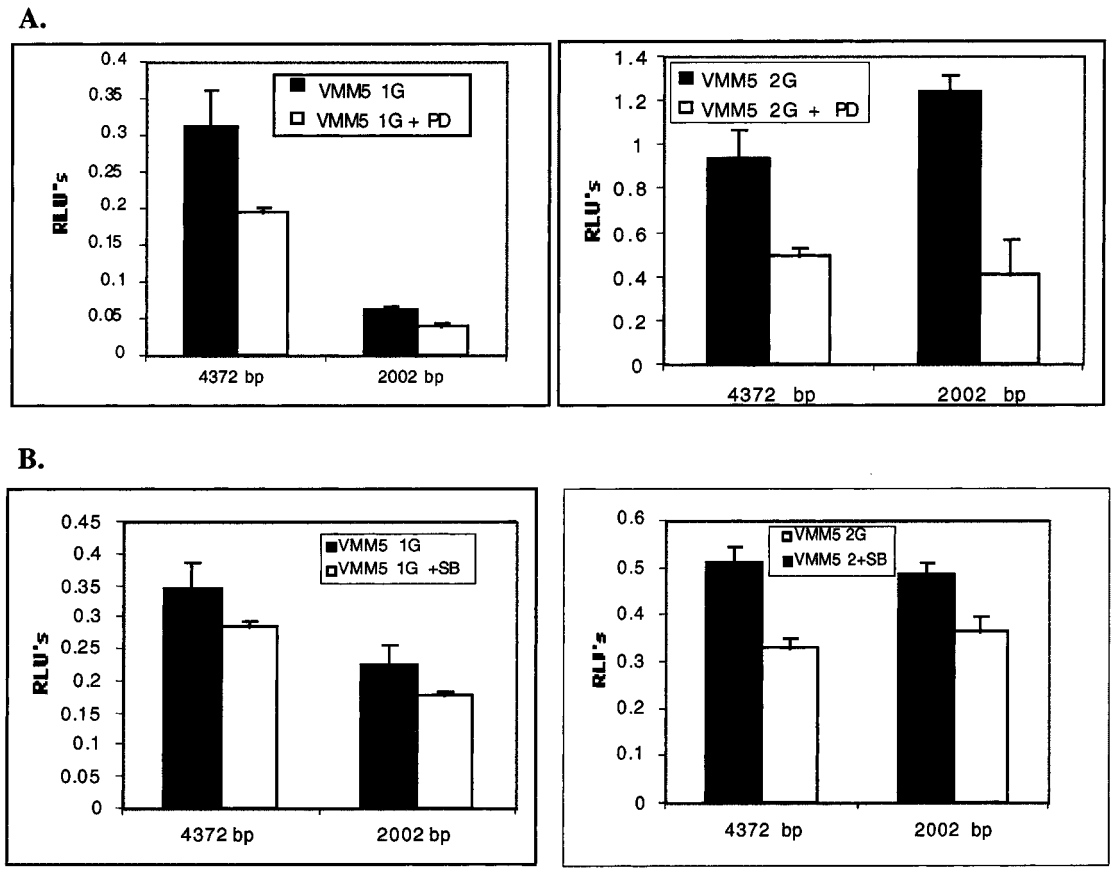

Fig. 5. Transient transfection of the 1G and 2G promoter constructs into VMM5 cells in the presence of the ERK1/2 inhibitor, PD98059 or the p38 inhibitor SB203580. (**A**) VMM5 cells treated with PD98059 (5 μM). (**B**) VMM5 cells treated with SB203580 (7.5 μM). Luciferase activity is expressed as relative light units (RLU's). The data are representative of three independent experiments that gave similar results.

1999b; Brinckerhoff et al., 2000; Ye et al., 2001; Noll et al., 2001].

Recently, we showed that the level of MMP-1 expression can be influenced by a genetic variation in the MMP-1 promoter [Rutter et al., 1998]. This variation is a SNP (2G allele) at −1607 bp that is present in the general population [Rutter et al., 1998; Noll et al., 2001]. The presence of the 2G allele creates a binding site for Ets family of transcription factors, which is associated with enhanced transcriptional activity of MMP-1 in A2058 melanoma cells and in fibroblasts [Rutter et al., 1998]. These findings led to the hypothesis that the presence of the 2G allele may favor tumor invasion and metastasis, and several studies have linked this allele to aggressive cancers. Three reports have indicated that the 2G polymorphism could be a prognostic marker with respect to susceptibility to cancer, as demonstrated for ovarian, endometrial, and lung cancer [Kanamori et al., 1999; Nishioka et al., 2000; Zhu et al., 2001]. In another study, with cutaneous malignant melanoma, the 2G allele was associated with an increased tendency to invade and with tumor progression [Ye et al., 2001]. This finding has been substantiated by our investigation of loss of heterozygosity (LOH) at the MMP-1 locus, chromosome 11q 22–23 [Noll et al., 2001]. This locus also harbors a putative tumor suppressor gene, and LOH at 11q 22–23 is a common event in melanoma [Herbst et al., 1992]. We hypothesized that although loss of either the 1G or 2G allele from 1G/2G heterozygotes would be random, if the 2G allele were retained, tumor metastasis would be favored. In fact, 83% of metastatic melanomas displaying LOH at this locus retained the 2G allele, further supporting the concept that this allele may facilitate tumor invasion [Noll et al., 2001].

However, in the present study we demonstrate that high levels of MMP-1 expression in

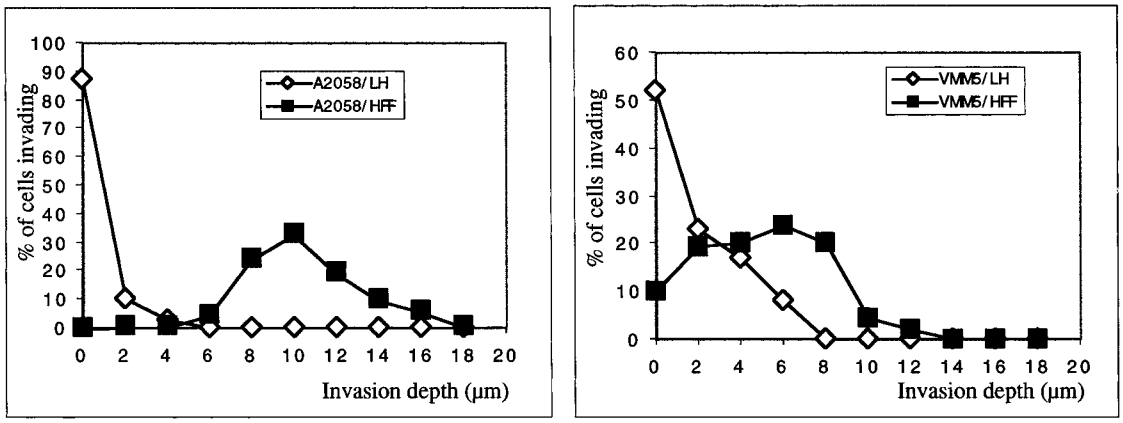

Fig. 6. Quantitative invasion profiles of A2058 and VMM5 cells. Melanoma cells were seeded on type I collagen. Five milliliters of serum-free media (LH) or fibroblast-conditioned media (HFF) was added to the lower invasion chamber. The invasion assays were terminated after 48 h, and invasion was quantified by CLSM. A representative of quadruplicate assays is shown.

melanoma cells can be seen in cells homozygous for the 1G allele, and that the high level of expression in these cells is mediated by MAPK pathways that do not target the 2G allele. Indeed, transient transfections of 1G and 2G containing promoter constructs into melanoma cells showed that the greatest inhibition of the ERK1/2 pathway occurs in the context of the 2G promoter constructs. This suggests that in the absence of the 2G allele, cells may compensate by using an alternative pathway, such as p38, as seen in VMM5 cells. Indeed, we have shown that in the absence of this allele the VMM5 cells utilize differential pathways with respect to MMP-1 expression when compared to the A2058 2G melanoma cells. Perhaps the activation of different signal/transduction pathways in these two different melanoma cell lines results from the constant changes that occur in tumor cells as they respond to genetic, physiological, and pharmacological pressures [Heimbrook and Oliff, 1998; van Dam and Castellazzi, 2001]. For example, in progressing breast cancer, there is a transition to a more mesenchymal phenotype due to the loss of keratin and e-cadherin, which is accompanied by an increase in vimentin and expression of MMP-1 [Gilles et al., 1997]. Consequently, perhaps as a result of cumulative genetic changes, the A2058 and VMM5 melanoma cells display high constitutive levels of MMPs in the absence of stimuli.

In addition to using different signal/transduction pathways, the A2058 and VMM5 cells may also use diverse transcription factors to drive MMP-1 gene expression (Fig. 2). These cells express different members of the AP-1 and Ets families of transcription factors, and these different proteins may bind preferentially to certain AP-1 or ETS sites, with disparate regulation of transcription [Chamberlain et al., 1993; White and Brinckerhoff, 1995]. However, any differences in regulation are not reflected by the binding patterns seen with the gel shift experiments, where nuclear extracts from both cells showed similar binding patterns and were equally capable of binding more strongly to the 2G DNA than to the 1G. Since gel mobility shift assays measure the ability of proteins to bind to DNA, rather than transcriptional activity, the only conclusion we can make from this experiment is that both cells contain a complement of transcription factors that are able to bind to 2G DNA. More meaningful data are derived from the transient transfections and the studies with the signal/transduction inhibitors, which implicate different pathways and cis-acting elements in the MMP-1 promoter in order to mediate transcription in the two cell lines.

Even in the absence of genotypic analysis for the 2G SNP, MMP-1 expression has been inversely correlated with survival in several cancers [Murray et al., 1996, 1998; Inoue et al., 1999; Ito et al., 1999], indicating that elevated MMP-1 expression may be a poor prognostic marker [Brinckerhoff et al., 2000]. Indeed, a few melanomas with the 1G allele have displayed metastatic ability in vivo [Noll et al., 2001]. In support of this concept, we found that levels of MMP-1 mRNA in the VMM5 cells (1G) are as great as in the A2058 cells (2G). Further, the

VMM5 cells readily invaded a matrix or type I collagen, probably because they express the proteinases needed to activate the proMMP-1 that they produce. Nonetheless, tumor growth and metastasis involve interactions between tumor cells and fibroblasts of the surrounding stromal tissue [Heppner et al., 1996; Benbow et al., 1999b; Liotta and Kohn, 2001]. It has been suggested that the stromal cells, rather than the tumor cells, are the primary source of MMPs [Heppner et al., 1996, Liotta and Kohn, 2001], and these cells are, therefore, important facilitators of invasion. Thus, despite the ability of tumor cells with the 1G genotype to produce abundant amounts of MMP-1 and to display invasive behavior, perhaps the documented association between the 2G allele and enhanced cancer progression is due to the adjacent stromal cells, where this allele contributes to heightened expression of MMP-1 and to tumor invasion and metastasis in vivo.

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