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Advances in Brief

A Single Nucleotide Polymorphism in the Matrix Metalloproteinase-1 Promoter Creates an Ets Binding Site and Augments Transcription[1]

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Abstract

Matrix metalloproteinases (MMPs) facilitate cellular invasion by degrading the extracellular matrix, and their regulation is partially dependent on transcription. Binding sites for members of the Ets family of transcription factors are present within *MMP* promoters and are potent positive regulators. We report a single nucleotide polymorphism at −1607 bp in the *MMP-1* promoter, where an additional guanine (G) creates an Ets binding site, 5′-GGA-3′. This polymorphism displays significantly higher transcription in normal fibroblasts and in melanoma cells than the 1 G polymorphism, and it binds substantially more nuclear extract and recombinant ETS-1. Analysis of control DNAs from the Center d'Etude du Polymorphisme Humain pedigrees reveals that this polymorphism is not a mutation, with a frequency of the 2 G polymorphism at 30%. In contrast, in eight tumor cell lines, this frequency increased to 62.5% ($P < 0.0001$). Thus, this *MMP-1* polymorphism contributes to increased transcription, and cells expressing the 2 G polymorphism may provide a mechanism for more aggressive matrix degradation, thereby facilitating cancer progression.

Introduction

MMPs[3] are a family of at least 15 enzymes that degrade the ECM (1). They have essential roles in modeling and remodeling the ECM in normal physiology and disease pathology. Several of these enzymes have the unique ability to degrade the interstitial collagens (types I, II, and III), the body's most abundant proteins. MMP-1 is the most ubiquitously expressed interstitial collagenase, thereby assigning it a prominent role in collagen degradation. Overexpression of MMP-1 is associated with several pathological conditions, including the irreversible degradation of cartilage, tendon, and bone in arthritis (2) and the degradation of collagens I and III in tumor invasion and metastasis (3, 4). Indeed, patients with tumors that express MMP-1 have an overall poorer prognosis than patients with tumors that do not (4, 5). This overexpression of MMP-1 may be due to the juxtaposition of transcription factor binding sites within the promoter of this gene and to the cooperativity among the factors that bind these sites (6–9).

Most normal cells express modest, but detectable, levels of MMP-1 constitutively, and this expression increases substantially in the presence of cytokines or growth factors (2, 10–12). In contrast, the A2058 melanoma cells constitutively express high levels of *MMP-1* (13), making them an attractive model for studies on the transcriptional regulation of this gene and for comparative studies with normal cells. Previously, we isolated and sequenced over 4 kb of the *MMP-1* promoter DNA from a leukocyte genomic library (10). DNA sequence analysis revealed that this clone contained only 1 G at position −1607 bp, resulting in the sequence 5′-AAGAT-3′ (10). This sequence differs from that reported by others (11, 14), who described 2 Gs at that location, with the sequence 5′AAGGAT-3′. The presence of 2 Gs at this site creates the sequence 5′-GGA-3′, which is a consensus sequence (15) for a functional PEA3/EBS. Thus, this site constitutes an SNP in the human *MMP-1* promoter, and we investigated the effect of this SNP on the transcriptional activity, protein/DNA binding activity, and frequency of this SNP in normal fibroblasts and in melanoma tumor cells.

Materials and Methods

Cells and Plasmids. Primary HFFs were prepared, as described previously (10), and used during passages 4–8. HFFs and A2058 cells were cultured in DMEM (Life Technologies, Inc.) with 10% fetal bovine serum (Sigma Chemical Co.), penicillin (100 units/ml), and streptomycin (100 μg/ml).

A 4.3-kb *MMP-1* promoter DNA fragment containing only 1 G at −1607 bp has been described (10). Primers were made to amplify the endogenous promoter from the A2058 cells (−4008 bp to −3988 bp sense primer: 5′-GTGGAAGCTTACACCTATAATCCCAACACTC-3′ and −511 bp to −543 bp antisense primer: 5′-CTGCCTGGTACCCTATTGCGATAGCAC-CATGGC-3′). Two A2058 PCR amplified clones were subcloned into the pBL5CAT (Promega) vector and sequenced (10) to ensure the absence of PCR artifacts. We next constructed reporter clones in which the only difference between the two pGL3-MMP-1 vectors was the SNP at position −1607 bp. First, the *MMP-1* promoter insert from the pXP2 vector (10) was subcloned into the pGL3 Basic vector (Promega). Unique sequences flanking the G variation were restricted by AatII (5′) and *Eco*RV (3′), thereby isolating a 450/451-bp fragment from the leukocyte clone in pGL3 Basic (1 G) and A2058 melanoma DNA in pBL5CAT (2 Gs), respectively, and excluding any other sequence variations found in the A2058 promoter. Finally, the 451-bp insert containing the 2 Gs was "swapped" and ligated into the pGL3-MMP-1 construct containing AatII/*Eco*RV ends, thereby generating the two SNP constructs.

Northern Analysis. Confluent cultures in 150-mm diameter tissue culture dishes were washed and placed in 10 ml of serum-free DMEM plus 0.2% lactalbumin hydrolysate. Immediately (time 0) and at 24 h, total RNA was isolated using the TRIzol reagent (Life Technologies, Inc.), and 20 μg were subjected to Northern analysis and hybridized with cDNA-specific probes for MMP-1 or glyceraldehyde-3-phosphate dehydrogenase. Probes were random prime labeled with α (32)P-dCTP (12.5 μCi/reaction of 3,000 Ci/mmol) and hybridized for 20 h at 56°C.

Transfection and Luciferase Assay. Transient transfections were performed in triplicate as described (10) with the LipofectAMINE PLUS reagent (Life Technologies, Inc.) using 2 μg of the chimeric *MMP-1* promoter/reporter plasmids, 5 μl of the PLUS reagent, and 5 μl of LipofectAMINE. Luciferase

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[3] The abbreviations used are: MMP, matrix metalloproteinase; ECM, extracellular matrix; EBS, Ets binding site; SNP, single nucleotide polymorphism; HFF, human foreskin fibroblast; AP-1, activator protein-1; EMSA; electromobility shift assay; CEPH, Centre d'Etude du Polymorphisme Humain; G, guanine; RLU, relative light unit; oligo, oligonucleotide.

activity is reported as RLUs. Hirt's analyses were performed and normalized to RLUs to control for any variations in transfection efficiency (16, 17). Statistics were performed using the InStat Program (GraphPad Software) using the Welch's alternate *t* test, a modification of the unpaired *t* test.

Nuclear Extract Preparation and EMSAs. Extracts of nuclear proteins were prepared, and EMSAs were performed as described previously (18) with 1×10^5 cpm of γ^{32}P-ATP end-labeled oligo incubated with nuclear extract (5 μg) and/or recombinant ETS-1 protein (2 μM; a generous gift from Dr. Barbara Graves, University of Utah School of Medicine, Huntsman Cancer Institute, Salt Lake City, Utah) and c-JUN protein (1 μg; Promega), as indicated in the figure legends. The samples were subjected to 5% PAGE at 150 V, dried, and autoradiographed. Oligos used for EMSAs were: 1G sense, 5′-AAATAATTAGAAAGATATGACTTATCTCAAATCAA-3′; 2 G sense, 5′-AAATAATTAGAAAGGATATGACTTATCTCAAATCAA; −88/−73, sense, 5′-TTCATTGTTAATCAAGAGGATGTTATAAAGCATGAGTCACACCCTCAGCTT-3′. The −88/−73 oligo spans the region −110 to −61 bp and includes the locations within the oligo that correspond to the proximal PEA3/AP-1 sites at −88/−73, respectively.

Radiolabeled PCR Assay. Primers that flank the SNP in *MMP-1* were used for PCR amplification (sense primer, 5′-GTTATGCCACTTAGATGAGG-3′; antisense primer 5′-TTCCTCCCCTTATGGATTCC-3′). A typical reaction consisted of ~20 ng of DNA template; 0.2 mM dGTP, dATP, and dTTP, 2.5 μM dCTP; 10 × buffer and *Taq* DNA polymerase (Sigma); and α (32)P-dCTP (Dupont/NEN). Reactions were PCR amplified (MJ Research PTC100™ Thermocycler) in 25 cycles (4 min at 94°C; 45 s at 94°C, 45 s at 58°C, and 45 s at 72°C; followed by a brief extension (10 min) at 72°C). Following amplification, the reactions (2.5 μl) were mixed with 10 × loading buffer and denatured for 2 min at 80°C. Samples were loaded onto an 8% denaturing PAGE and electrophoresed for 3 h. Gels were dried and autoradiographed for ~15 min. Control samples generated from the plasmid clones were loaded on each gel for accurate scoring of the alleles. Statistics were calculated using the Instat program (GraphPad Software) and were based on the percentage of the total number tested.

Results

Levels of MMP-1 Expression Differ in Normal Fibroblasts and A2058 Melanoma Cells. We measured constitutive mRNA expression of the endogenous *MMP-1* gene in normal foreskin fibroblasts (HFFs) and in the A2058 melanoma cells over a 24-h period. In keeping with previous studies, we found that HFFs express low levels of MMP-1 mRNA, as detected by the 2.1-kb transcript (10, 12), and that the A2058 cells express higher levels (Ref. 13; Fig. 1). To determine whether the A2058 cells contained a difference in the endogenous *MMP-1* promoter, we used PCR to amplify the promoter from these cells, and compared this sequence with the previously isolated leukocyte clone (10) and with promoters from other sources (11, 14). Several substitutions were observed, but were considered

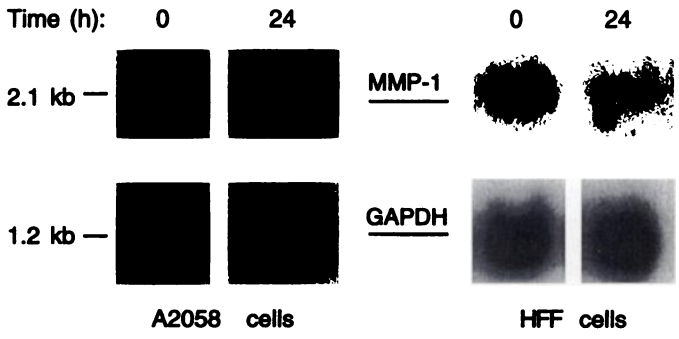


Fig. 1. *MMP-1* gene expression in A2058 melanoma cells and HFFs. Total RNA was isolated immediately (time zero) and 24 h after cells were transferred to DMEM/lactalbumin hydrolysate, and 20 μg were loaded onto a formaldehyde gel for Northern analysis. The blots were probed for MMP-1 expression using an α (32)P-dCTP-labeled fragment of the human MMP-1 cDNA and autoradiographed for 24 h, detecting a 2.1-kb band. Glyceraldehyde-3-phosphate dehydrogenase (1.2 kb) is shown to correct for loading differences.

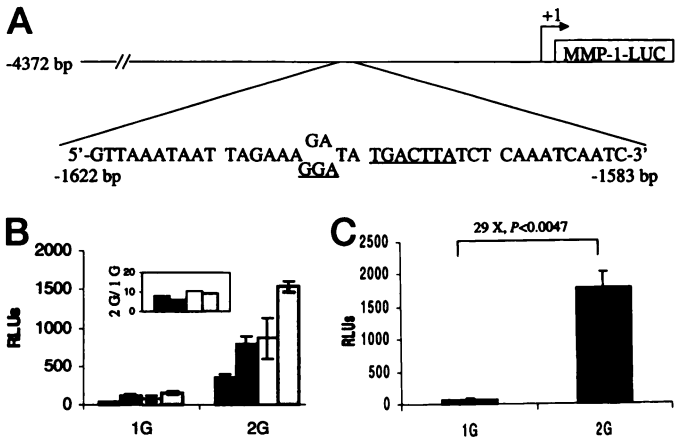


Fig. 2. Transcriptional effects of the *MMP-1* SNP at −1607 bp in HFF or A2058 cells. *A*, a segment of the *MMP-1* promoter containing the SNP is shown. The EBS-SNP and the AP-1 sites are underlined. *B* and *C*, basal transcription of the human *MMP-1* promoter with either 1 or 2 Gs located at position −1607 bp in HFF cells (*B*). The inset represents the fold induction of the 2 G construct over the 1 G construct for each transfection ■, HFF-1; ■, HFF-2, □, HFF-3; □, HFF-4. *C*, A2058 melanoma cells. The fold induction of the 2 G construct over the 1 G construct, along with the *P* is shown in the figure. These results are representative of at least five separate experiments and at least four different primary HFF sources. The *P*s for each sample were significant. (HFF-1, $P < 0.0065$; HFF-2, $P < 0.0093$; HFF-3, $P < 0.0384$; and HFF-4, $P < 0.0007$). The nucleotide sequence for the *MMP-1* promoter clone has been deposited in the GenBank database under GenBank Accession Number AF023338 (10).

insignificant because they did not create or delete any known binding sites for transcription factors within the promoter. However, one major difference was detected: the A2058 promoter DNA contained an additional G at position −1607 bp, which was flanked by a guanidine (5′) and an adenine (3′), thus creating an EBS (15). The presence/absence of this G at −1607 bp has been reported previously (10, 11, 14), suggesting that this variation may constitute a true SNP.

MMP-1 Transcription Is Influenced by the SNP That Creates an EBS. To specifically test the role of the 1 G/2 G variation in regulating transcription, we generated two luciferase reporter constructs driven by a large (4.3 kb) fragment of the *MMP-1* promoter with the only difference being 1 G or 2 Gs at −1607 bp (Fig. 2*A*). Note that this SNP is adjacent to an AP-1 site at −1602 bp (10, 14), which may also influence transcription (Refs. 6 and 7; see below). We transiently transfected these clones into HFFs and measured the effect on basal transcription. With at least four separate donors of HFFs, we consistently observed a significant increase (ranging from 2–10-fold) in transcription with the 2 G promoter construct compared with the 1 G promoter construct (Fig. 2*B* and data not shown). Hirt's analysis (17) of transfected DNA demonstrates that these differences were not attributable to transfection efficiency (data not shown). When these two constructs were transfected into the A2058 melanoma cells, we observed a 29-fold increase in transcription of the 2 G construct over the 1 G construct (Fig. 2*C*). Other experiments showed similar increases ranging from 16–37-fold (data not shown). We then tested these constructs in other tumor cell lines (MDA231 breast cancer cells, and two primary melanoma lines) to assess their transcriptional response. In these cells, the 2 G promoter construct augmented transcription at least 4-fold over the 1 G construct (data not shown). Thus, the increase in transcription is dependent on the presence of 2 Gs at −1607 bp, and it is seen in both normal and malignant cells.

Recombinant Ets-1 Binds to the 2 G-SNP. We analyzed the ability of an oligo probe containing either 1 G or 2 Gs at −1607 bp to bind to nuclear extracts from the A2058 cells (Fig. 3*A*). A striking difference in binding intensity is seen, with the 2 G oligo binding more nuclear proteins compared with the 1 G oligo (compare *Lanes 2* and *7*). Nonetheless, both oligos bind a similar profile of proteins, as

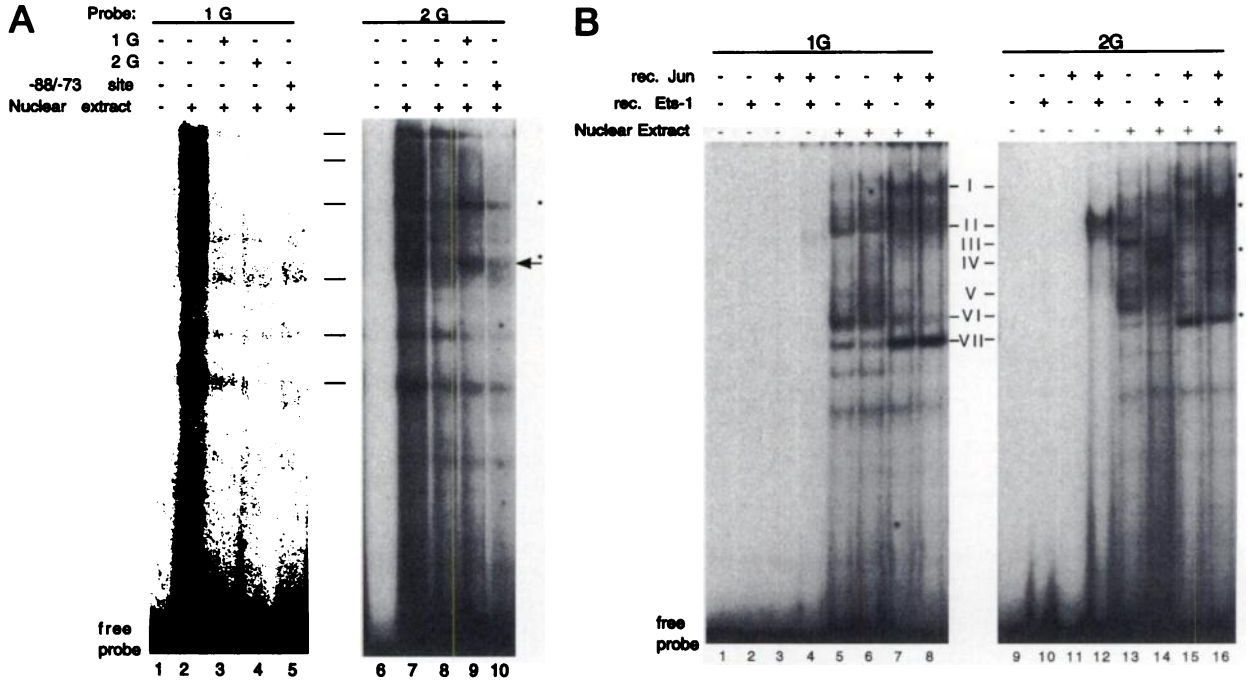


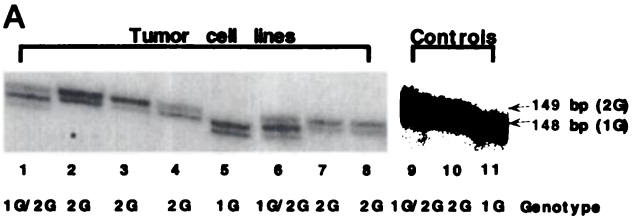
Fig. 3. Nuclear extracts from A2058 melanoma cells show different DNA-binding activity with probes containing 1 G or 2 Gs at −1607 bp and ETS-1 binding is dependent on 2 Gs. Nuclear extracts were prepared from confluent cultures of A2058 cells. Nuclear extract (five μg) was incubated with 100,000 cpm of radiolabeled oligos corresponding to the −1619 bp and −1585 bp regions of the *MMP-1* promoter. Complexes were separated on a 5% nondenaturing PAGE, dried, and autoradiographed. *A*, Competition analysis. Extracts were incubated with radiolabeled oligo in the presence of 200 × molar excess of unlabeled "self" oligo (*Lanes 3* or *8*), unlabeled "non-self" competitor (*Lanes 4* or *9*), or the proximal EBS/AP-1 site (*Lanes 5* and *10*) at −88/−73, respectively. The center lines represent bands in common between the two oligos. Bands specific for the 2 G oligo (*arrow*) and bands that are enhanced by the presence of the EBS (*) are shown on the right. *B*, Ets and Jun proteins are necessary for maximal DNA-protein binding. Complexes were assigned Roman numbers I-VII for reference, and *dashes* indicate whether the complexes are the same with both oligos or are oligo-specific. *, complexes that are enhanced by the addition of recombinant proteins.

indicated by the shared bands (*center lines*), suggesting that the 1 G oligo may be a weak binding site and that the presence of the 2 Gs augments this binding. Cross competition experiments were used to determine the specificity of binding to each labeled oligo (*Lanes 3–5* and *8–10*). Binding to the 1 G oligo (*left panel*) was readily competed, suggesting that these DNA/protein interactions are weak (compare *Lanes 3–5* with *Lane 2*). Competition studies with the 2 G oligo (*right panel*) revealed that the "self" oligo (*Lane 8*) only mildly competed the binding of the shared bands (*middle lines*), although it did compete two bands (denoted by asterisks), demonstrating that these proteins bind preferentially (upper asterisk) and/or specifically (*arrow*) to the 2 G oligo. Furthermore, the 1 G oligo did not compete well (*Lane 9*), but was effective in competing the shared bands (*center lines*). Finally, the proximal EBS/AP-1 competitor shows a pattern similar to that seen with the 1 G oligo (compare *Lanes 9* and *10*). Taken together, these observations suggest that the 2 G "self" oligo competes for the proteins able to bind specifically to the 2 G sequence, and that the other bands may represent proteins or protein complexes that are not 2 G-dependent (*e.g.*, the AP-1 proteins). Importantly, these data also suggest that the presence of the EBS in this region of *MMP-1* creates an environment where DNA/protein interactions can strongly occur.

Because the SNP at −1607 bp is located adjacent to an AP-1 site at −1602 bp (Refs. 10 and 14; Fig. 2*A*), we investigated binding to the 1 G and 2 G oligos by recombinant ETS-1 and c-JUN (Fig. 3*B*), proteins that are likely candidates for binding to these sites (6, 7, 19). The recombinant proteins, by themselves, were not able to bind to either oligo (*Lanes 2* and *3*, *Lanes 10* and *11*). The combination of both proteins, however, bound to the 2 G oligo (*Lane 12*, complex II), but only faintly to the 1 G oligo (*Lane 4*), suggesting that additional proteins were required for optimal DNA binding. Indeed, incubation with nuclear extract from A2058 cells resulted in distinctive binding patterns for each probe (compare *Lanes 5* and *13*, and Fig. 3*A*). Complexes I, II, VI, and VII were present in both panels, whereas complexes III, IV, and V were specific for the 2 G oligo. When ETS-1 and c-JUN were added together with nuclear extract, binding to the 2 G oligo was more pronounced compared with the 1 G oligo (complex II, *Lanes 8* and *16*).

In the presence of recombinant c-JUN, complex I became more apparent with both oligos, suggesting that complex I may represent AP-1 proteins binding to the DNA (compare *Lane 7* with *5* and *Lane 15* with *12*). Complex VII also became more intense when c-JUN was added (*Lanes 7*, *8*, *15*, and *16*) suggesting that it, too, contains AP-1 related proteins. When both recombinant proteins were added, the binding pattern in the 1 G panel did not differ from the reactions with c-JUN alone (compare *Lanes 8* and *7*), supporting the importance of 2 Gs in creating the EBS. When nuclear extracts and recombinant ETS-1 were added to the 2 G oligo, a new complex was formed (complex IV). Furthermore, when both proteins and nuclear extract were added to the 2 G probe, several complexes (I, III, IV, V, and VI) were diminished and seemed to combine into a much stronger complex II (*Lane 16*), again demonstrating the influence of the 2 Gs in creating the EBS. We used antibodies to several members of the Ets family of transcription factors (Ets-1/2, Erg-1/2, Elk-1, and ERM) in "super shift" reactions to identify the proteins binding to this site. However, binding of these antibodies was not detected due to limitations of the antibodies, or because the complexes did not allow for the antibody to have access to the epitope, and/or because these proteins did not bind (data not shown). These data suggest that the oligo containing 2 Gs represents a *bona fide* EBS that binds an Ets family member(s) in a complex with AP-1 protein members. However, it is important to emphasize that although the data show that recombinant ETS-1 binds, it is likely that this is not the only Ets family member that can bind to this site (19).

A



B

n (per sample)	Sample Group	Genotype (% of total number tested)		
		1G/1G	2G/2G	1G/2G
100	CEPH Controls	31	30	39
8	Tumor cell lines	12.5	62.5 *	25

*$P < 0.0001$

Fig. 4. SNP frequency detection by radiolabeled PCR. *A*, primers that flank the SNP (see "Materials and Methods") and a mixture of unlabeled dNTPs, with radiolabeled α (32)P-dCTP, were used in the PCR reaction. Controls consisted of the plasmids used for transfections containing either 1 G (148-bp product), 2 Gs (149-bp product), or a 1:1 mixture of both to represent a heterozygote (*Lanes 9* and *10*). The lowest band in all lanes represents a "shadow" band, an artifact of the radiolabeled PCR reaction, which does not influence the interpretation of the results. Tumor cell lines are shown (*Lanes 1–8*) with their respective genotype shown below. *, the A2058 cell line. The *MMP-1* SNP genotype for each cell line is given below each lane. *Lane 1*, MCF-7 breast cancer cells; *Lane 2*, A2058 melanoma cells; *Lane 3*, BC8701 breast cancer cells; *Lane 4*, MDA231 breast cancer cells; *Lane 5*, VMM5 melanoma cells; *Lane 6*, VMM12 melanoma cells; *Lane 7*, VMM39 melanoma cells; and *Lane 8*, IM3 melanoma cells. *B*, a summary of the data are shown along with the frequency in the CEPH controls. *, *P* based on the percentage of the total number of samples tested.

The MMP-1 SNP Is Not a Mutation. Finally, we confirmed that this 1 G/2 G difference in the leukocyte clone sequence and the A2058 melanoma sequence is a SNP and not a mutation. We developed a radiolabeled PCR assay using primers that flank the variation to amplify a product of either 148 bp (1 G), 149 bp (2 G), or both (heterozygous) in genomic DNA (Fig. 4*A*). With this assay, we analyzed 100 control DNAs derived from the CEPH pedigrees (http://www.cephb.fr/cephdb/) to determine the frequency of this variation within a population (Fig. 4*B*). Only the parents in the pedigrees were used to avoid biasing the results through inheritance. In addition to the CEPH control DNAs, we also assessed the frequency of this SNP in several tumor cell lines, including the A2058 melanoma cells (denoted by the asterisk; Fig. 4*A*). We found that the occurrence of 2 G homozygotes in the CEPH controls is approximately 30%, but in the tumor cells lines, it is 62.5% ($P < 0.0001$).

Discussion

On the basis of our data, we hypothesize that this 1 G/2 G SNP may influence the transcriptional responsiveness of the human *MMP-1* promoter in cancer, where excessive production of MMP-1 is a major contributor to the stromal degradation involved in tumor invasion (3–5). As with other genes, expression of *MMP-1* is mediated by multiprotein complexes that bind to DNA in a sequence-specific manner, and these complexes often cooperate to achieve maximal activation (6–8, 20, 21). DNA elements containing a single EBS are often not sufficient for Ets induction, and require a nearby AP-1 site to which Fos and Jun proteins bind (6, 7, 22). Furthermore, the transcriptional environment within the cells may influence the level of gene expression, as evidenced by differences in the level of transcription of the *2 G* allele in HFFs *versus* melanoma cells. These differences may be due to various extracellular stimuli, such as growth factors and cytokines, as well as to cell-type-specific nuclear factors within the cell. Thus, the combination of *cis*-acting sequences in the *MMP-1* promoter and specific *trans*-acting factors can dramatically increase transcription, and this increase may provide one molecular mechanism for enhanced ECM degradation.

The abundance of SNPs in the genome make these genomic variations powerful tools for identifying disease genes, particularly in loss of heterozygosity studies in tumors. A large effort is presently underway for identifying these SNPs, but most research is focused on the coding regions of genes (23). Instead, we have isolated a SNP located in the promoter of the interstitial collagen degrading enzyme, MMP-1. Because this variation is not located in a coding region, it does not alter the structure of the enzyme. However, the location of this SNP in the promoter region of *MMP-1* has profound effects on the production/regulation of the enzyme. Perhaps the increase in the 2 G frequency in the tumor cell lines indicates an increase in invasive behavior due to high levels of MMP-1 expression. MMP-1 is implicated in tumor cell invasion and metastasis due to its ability to cleave the interstitial collagens types I and III at neutral pH (3, 4). Therefore, a structural variation with the potential to influence the level of expression is important to understanding how this enzyme modulates ECM metabolism and tumor cell invasion and metastasis.

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