Allele-specific transcription of the PAI-1 gene in human astrocytes

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Keywords: human astrocytes, haploChIP, plasminogen activator inhibitor-1, transcriptional activity.

SUMMARY

Background: The 4G allele of the PAI-1 -675(4G/5G) insertion/deletion promoter polymorphism has been associated with elevated plasma levels of PAI-1 and an increased risk of myocardial infarction. However, this allele has also been associated with a reduced risk of ischemic stroke (IS). In the brain, PAI-1 is mainly produced by astrocytes, and can reduce the neurotoxic effects exerted by tissue-type plasminogen activator during pathophysiologic conditions. *Objective:* The aim of the present study was to investigate whether the PAI-1 -675(4G/5G) polymorphism and the linked -844A/G polymorphism affect transcriptional activity of the PAI-1 gene in human astrocytes. *Methods:* Haplotype chromatin immunoprecipitation (haploChIP) was used in order to quantify allele-specific promoter activity in heterozygous cells. Protein-DNA interactions were investigated by electrophoretic mobility shift assay (EMSA). *Results:* A clear allele-specific difference in PAI-1 gene expression was observed in astrocytes, where the haplotype containing the 4G and the -844A alleles was associated with higher transcriptional activity compared to the 5G and -844G-containing haplotype. EMSA revealed an allele-specific binding of nuclear proteins to the 4G/5G site as well as to the -844A/G site. Supershift experiments identified specific binding of the transcription factors Elf-1 and Elk-1 to the -844G allele. The relative impact of the different sites on allele-specific PAI-1 promoter activity remains to be determined. *Conclusion:* We demonstrate that common polymorphisms within the PAI-1 promoter affect transcriptional activity of the PAI-1 gene in human astrocytes, thus providing a possible molecular genetic mechanism behind the association between PAI-1 promoter variants and IS.

INTRODUCTION

Genetic variation at the plasminogen activator inhibitor type 1 (PAI-1) locus has been implicated in cardiovascular disease. Several polymorphisms have been identified in the PAI-1 gene, among which the common PAI-1 -675(4G/5G) insertion/deletion promoter polymorphism (rs1799889) has been most extensively studied. The 4G allele has been associated with elevated plasma levels of PAI-1, and an increased risk for myocardial infarction (MI) (1) (2), although conflicting data exist (3) (4). The PAI-1 -844A/G polymorphism, which is in strong linkage disequilibrium (LD) with the -675(4G/5G) polymorphism (3) (5), has also been associated with plasma levels of PAI-1 and MI in some (6) (7), but not all (5) (8), studies.

Experimental studies conducted in order to functionally characterize the PAI-1 -675(4G/5G) polymorphism have suggested that this polymorphism is functional at the level of transcription. Transfection experiments using human umbilical vein endothelial cells (HUVECs) and HepG2 cells showed that cells transfected with a reporter gene construct harbouring the 5G allele variant exhibited a reduced transcriptional activity compared with cells transfected with the 4G allele (9). However, other studies have yielded conflicting results as to whether this polymorphism acts to modulate the level of PAI-1 gene transcription during basal conditions in these cells (10) (11). In addition, the PAI-1 -844A/G polymorphism has also been suggested as a potential functional candidate (5).

In contrast to the data showing an increased risk of MI in PAI-1 4G allele carriers, there seems to be a protective effect of the 4G allele in ischemic stroke (IS) (12) (13). The mechanisms behind this effect in IS remain unknown. In the brain, PAI-1 is mainly produced by astrocytes (14), and functions to inhibit tissue-type plasminogen activator (t-PA) (15). During physiologic conditions, t-PA is involved in the regulation of synaptic plasticity and remodeling (16). However, following brain injury or cerebral ischemia, excessive release of t-PA into the extracellular space can trigger neuronal degeneration (17) and enhance disruption of the blood-brain barrier (18). Astrocyte-derived PAI-1 may thus reduce the deleterious effects of excessive t-PA activity. Evidence supporting this notion comes from experimental models of cerebral ischemia, in which intraventricular infusion of PAI-1 results in a reduced infarct size (19), whereas mice deficient in PAI-1 display exacerbated brain damage (20). Moreover, astrocytic expression of PAI-1, induced by transforming growth factor-β (TGF-β), can protect neurons against t-PA-mediated excitotoxicity (21).

Against this background, it is of interest to examine whether the -675(4G/5G) and the -844A/G PAI-1 promoter polymorphisms affect PAI-1 gene expression in human astrocytes. In the present study, we used the haplotype chromatin immunoprecipitation (haploChIP) approach (22) in order to analyze allele-specific transcriptional activity of PAI-1. This method utilizes one of the key events involved in gene transcription, *i.e.* activation of RNA polymerase II (Pol II). As the amount of chromatin-bound active Pol II is related to the transcriptional activity of the corresponding gene (23), differences in active Pol II loading between the two alleles in a heterozygous sample provides an alternative measure of allele-specific gene expression. In contrast to conventional *in vitro* methods (*e.g.* transfection studies), the haploChIP approach allows for analysis of allele-specific gene expression in a normally functioning nucleus where the different alleles are subjected to the same genetic and environmental conditions.

MATERIAL AND METHODS

Cell culture

Native human astrocytes (ScienCell, San Diego, CA, USA) were cultured in astrocyte growth medium (ScienCell) supplemented with 3% fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere. Astrocyte cultures were >95% GFAP positive. Fresh umbilical cords were obtained from the maternity ward and HUVECs were prepared by collagenase digestion according to Jaffe *et al* (24), and cultured in endothelial cell growth factor containing medium-2 (Clonetics/BioWhittaker, Walkersville, MD, USA), supplemented with 2% FBS as described (25). The media was replaced every 2-3 days. All experiments were performed at passages 2-6. Human astrocyte cultures derived from two individuals were used in the haploChIP experiments.

DNA Sequencing

Total DNA was isolated using a tissue DNA isolation kit (PeqLab Biotechnology, Erlangen, Germany) according to manufacturer's protocol. DNA amplifications were carried out with 10 ng DNA in a mixture of 10 mM dNTP, 0.5 U *Taq* Gold polymerase (Roche, Basel, Schwizerland) and 10 μmol of each primer. A fragment of approximately 2 kb surrounding the PAI-1 transcription start site, and covering the -675(4G/5G) and the -844A/G (rs2227631) polymorphisms, was sequenced in both directions. The detailed sequences of the PCR primers are listed in Table 1. Amplifications were performed as follows: 95°C for 2 min followed by 30 cycles of 94°C for 30 s, 59°C for 30 s, 72°C for 30 s followed by 72°C for 5 min. The PCR products were purified by an AMpure PCR purification system (Agencourt, Beverly, MA, USA). Sequencing reactions were carried out in 10 μl volume containing 6 μl PCR product, 0.25 μl BDT (v 3.1), 1.875 μl Buffer ABI (5x) and 1.6 μmol of each primer. Conditions for the PCR reaction were as follows: 96°C for 1 min followed by 40 cycles of 96°C for 10 min, 50°C for 5 s and 60°C for 2 min. The products of the sequence reaction were purified with a CleanSEQ dye-terminator removal kit (Agencourt) before being analyzed on a 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Chromatin immunoprecipitation (ChIP)

Astrocytes (approximately $5x10^8$ cells per IP, *i.e.* $3x10^9$ cells in total per individual) were either unstimulated or stimulated with recombinant human TGF-β (1 ng/ml, Sigma-Aldrich, St Louis, MO, USA) for 3 h prior to the haploChIP experiments. The dosage and timing of TGF-β treatment was based on previous *in vitro* studies on human astrocytes (26). Cells were fixed with 1% formaldehyde for 10 min at 37°C and washed with ice-cold phosphate-buffered saline containing proteinase inhibitors. Chromatin was sonicated to produce DNA fragments between 400 and 1000 bp in length. The ChIP assay was performed using a ChIP-IT kit (Active Motif, Rixensart, Belgium), according to manufacturer's protocol. In brief, streptavidin-sepharose beads (GE Healthcare, Stockholm, Sweden) were incubated with antibodies directed against total Pol II (Active Motif, Carlsbad, CA, USA), active Pol II (phosphorylated Ser5 residue of the C-terminal domain (CTD) of Pol II, #MMS-134R, Covance Laboratories, Richmond, CA, USA) or normal mouse IgG antibodies (Upstate, Chicago, IL, USA). The Dynabeads-antibody complexes were then added to the

chromatin. After washing to remove unbound antibodies, the samples were treated with RNase A and proteinase K to reverse cross-links. The precipitated DNA fragments were then resuspended in TE buffer.

PCR amplification and Pyrosequencing

Nested-PCR was used to amplify a fragment surrounding the PAI-1 -675(4G/5G) polymorphism. For the first PCR, the reaction mixture contained 2 ng DNA, 2 mM $MgCl_2$, 10 mM dNTP, 0.5 U *Taq* Gold polymerase (Roche) and 2.5 pmol of each primer. Amplifications were performed as follows: 95°C for 10 min followed by 34 cycles of 94°C for 1 min, 63°C for 50 s, 72°C for 2 min followed by 72°C for 5 min. The following primer pairs were used: 5'-CATGGTAACCCCTGGTCCC-3' (sense) and 5'-CCACGTGATTGTCTAGGTTTTGTCTGTC-3' (antisense). For the second inner PCR, the reaction mixture contained a total of 0.5 μl PCR product, 2.5 mM $MgCl_2$, 10 mM dNTP, 0.5 U *Taq* Gold polymerase (Roche) and 2.5 pmol of each primer. The inner PCR used the following primer pair: biotinylated primer 5'-CCTCAGGGGCACAGAGAGAGTC-3' (sense) and 5'-CCCTCATCCCTGCCATGTGC-3' (antisense), with the annealing temperature of 66°C. After PCR amplification, the product was prepared for pyrosequencing according to the manufacturer's protocol (Pyrosequencing AB, Uppsala, Sweden). Pyrosequencing was performed using a Pyrosequencing Reagent kit (Pyro gold, Biotage, Uppsala, Sweden). Briefly, a total of 40 μl biotinylated PCR product was immobilized to streptavidin-coated sepharose beads, aspirated with Vacuum Prep Toll (Biotage), and washed with 70% ethanol, denaturing buffer and washing buffer. Subsequently, the beads were released into pyrosequencing reaction plates containing annealing buffer and sequencing primer (5'-TGATACACGGCTGACTCCCC-3'). Primer annealing was performed by heating the samples to 80°C for 2 min. The nucleotide dispensation order was TACACGATGACTG. Pyrosequencing data were quantified using the PSQ 96 MA version software (Pyrosequencing AB). Each sample was analyzed in duplicate on two separate occasions.

Preparation of nuclear extracts and labeling of oligonucleotides

Nuclear extracts from human astrocytes and HUVECs were prepared from 80-90% confluent cells as previously described (25) or using a nuclear/cytosol fractionation kit (BioVision Inc, Mountain View, CA, USA). Sequences of the oligonucleotides used as probes and competitors in the electrophoretic mobility shift assay (EMSA) are listed in Table 2. Labeling of HPLC-purified oligomers (100 ng), annealing, oligomer processing and preparation for EMSAs was performed as previously described (25).

EMSA

EMSA experiments were performed essentially as described (25). In brief, approximately 10 μg nuclear extracts in 4 μL Osborne buffer D was preincubated on ice for 15 min with 1 μL poly[d(I-C)] and 3 μL of SMK buffer. ^{32}P-labeled probe was then added and incubated for another 15 min on ice before being applied to a native 5% polyacrylamide gel. For competition and cross-competition titration experiments, nuclear extracts were incubated with unlabeled double-stranded PAI-1 -844G, -844A, -675(4G) or -675(5G) oligomers (5-, 50- or 500-fold molar excess) for 15 min on ice prior to addition of the probe. As nonspecific competitors, unlabeled scrambled oligonucleotides with the same base-composition as that of the corresponding probes

were used at 500-fold molar excess. Samples were loaded on a 5% native polyacrylamide gel. Electrophoresis was carried out at 200 V, 4°C. Gels were vacuum heat-dried and visualized using phosphoimaging technology (FLA-2000, Fuji, Stamford, CT, USA).

In silico transcription factor binding site analysis was performed using the MatInspector (Genomatix) and the PROMO (prediction of transcription factor binding sites) software tools. Supershift experiments were performed using antibodies directed against Ets-1 (#sc-350), Ets-2 (#sc-351), Elf-1 (#sc-631), Elk-1 (#sc-355), PU.1 (#sc-5949), IRF-2 (#sc-498), p53 (#sc-1315) and E2F-1 (#sc-193) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Supershifting was performed according to the same procedure as that described for standard EMSAs, except that 2 μl specific antibody (4 μg in total) was added to the nuclear extracts for 1 h after addition of the labeled probe.

RESULTS

Allele-specific transcriptional activity of the PAI-1 gene in human astrocytes

Relative allele-specific promoter activity of the PAI-1 gene was quantified in human astrocytes using the haploChIP approach. Five individuals were genotyped by DNA sequencing and two were found to be heterozygous for the PAI-1 -675(4G/5G) polymorphism, thus selected for the subsequent haploChIP experiments. Both individuals were also heterozygous for the PAI-1 -844A/G polymorphism, which is in strong linkage disequilibrium (LD) with the -675(4G/5G) polymorphism (3) (5). As any polymorphism located within 1 kb from the transcription start site can affect the haploChIP experiments, a 2 kb fragment surrounding the transcription start site was sequenced in the two individuals. No additional heterozygosity was identified.

Samples were immunoprecipitated with antibodies directed against Pol II, and the relative loading of Pol II onto the 4G and 5G alleles was quantified by pyroseqencing (Fig. 1). A clear allele-specific effect was observed in both individuals during unstimulated conditions, as demonstrated by a higher loading ratio of active Pol II to the haplotype containing the 4G allele as compared to the 5G containing haplotype (Table 3 and Fig. 2). A similar response was observed in cells pretreated with TGF-β (Table 3). In control samples, *i.e.* samples subjected to ChIP with total Pol II antibody, no marked difference in the Pol II loading onto the 4G or 5G allele was observed (mean 4G/5G ratio ± SEM, untreated cells 0.84 ± 0.07, and TGF-β treated cells 0.83 ± 0.05).

Binding of nuclear proteins from human astrocytes and HUVECs to the PAI-1 -675(4G/5G) site

EMSA was performed in order to assess specific nuclear protein binding to the PAI-1 -675(4G/5G) polymorphism. In addition to nuclear extracts from astrocytes, extracts from HUVEC were included for comparative purposes. Similar migration patterns were obtained when using both the 4G and 5G probes (Fig. 3A). However, a weak fast migrating complex (marked with an arrow in Fig. 3A) was observed in the presence of the 5G allele when incubated with nuclear extracts from both cell types. This complex was not as strong as that previously observed in extracts from HUVECs (9), and was only detected in some of the EMSAs. This complex was competed by addition of an excess of unlabeled specific 5G-competitor, but not with a specific 4G-competitor, suggesting that this is a 5G allele-specific complex (Fig. 3B).

The PAI-1 -844A/G polymorphism affects protein binding affinity

DNA-protein interactions were also studied using probes spanning the PAI-1 -844A/G site. Nuclear extracts from human astrocytes and HUVECs bound specifically to this DNA-region (Fig. 4A). Moreover, a prominent slow migrating G allele-specific band was observed when using extracts from both cell types. Cross-competition experiments using the -844G probe demonstrated that this DNA-protein interaction could not be competed by an excess of cold specific A allele-competitor, confirming that this complex indeed constitutes a G allele-specific protein (Fig. 4B and D). Competition and cross-competition experiments showed that the other proteins that associated with the -844 region bound with similar affinity to the two alleles (Fig. 4B-D), which was true for extracts derived from both cell types.

Elf-1 and Elk-1 bind to the PAI-1 -844G allele

In silico analysis of the -844G allele probe sequence predicted putative binding sites for factors belonging to the E-twenty six (Ets)- transcription factor family. The G allele-specific complex (Fig. 5A and B) disappeared following addition of Elk-1 or Elf-1 antibodies, indicating that these two transcription factors are present in the complex. Similar results were obtained for both the astrocyte and HUVEC-derived nuclear extracts (Fig. 5A and B, respectively).

IRF-2 and p53 bind to the PAI-1 -844 site

Supershift experiments were performed in order to identify the proteins present in the specific complex seen with both the -844G and A allele probes (marked with a thick arrow in Fig. 6). Antibodies directed against PU.1 and E2F-1 did not affect the mobility of the -844A/G-specific complex (Fig. 6). In contrast, addition of anti-IRF-2 and anti-p53-antibodies resulted in displacement of the -844A/G-specific band, indicating that these two proteins bind to this site (Fig. 6A-D). Similar results were obtained for both the astrocyte and HUVEC-derived nuclear extracts (Fig. 6A-B and C-D, respectively), although the weakening of the specific band and the IRF-2 and p53 supershifts were more readily seen for the HUVEC extracts. To overcome the high background present with the astrocytic extracts a cold nonspecific competitor was included in the control and supershift reactions (Fig. 6A and B).

DISCUSSION

The 4G allele of the PAI-1 -675(4G/5G) polymorphism has been associated with an increased risk of MI (1) (2), but with a reduced risk of IS (12) (13). These contradictory findings may be explained by the fact that while PAI-1 can have an antifibrinolytic effect in both conditions, in IS PAI-1 derived from astrocytes may also have neuroprotective effects. Therefore, the present study was undertaken to investigate whether there are allele-specific effects on PAI-1 gene expression in human astrocytes. Using heterozygous cells and the haploChIP approach, we found that the haplotype containing the 4G and the -844A alleles was associated with higher loading of active Pol II as compared to the 5G and -844G containing haplotype, thus indicating an increased transcriptional activity of the 4G and/or the -844A allele variants. A similar response was observed in cells treated with TGF-β, a potent activator of PAI-1 gene expression in human astrocytes (26). The present study provides the first evidence of an allele-specific expression of PAI-1 in human astrocytes.

In order to elucidate the molecular mechanisms behind this finding, EMSAs were performed. A specific binding of proteins to the PAI-1 4G/5G site was observed with nuclear extracts derived from human astrocytes. Two DNA-protein complexes could be identified, one common band that bound with similar affinity to both alleles, whereas the second very weak fast migrating bound only to the 5G allele. Similar DNA-protein migration patterns were observed with nuclear extracts from HUVECs, which is in accordance with previous findings (9). However, it is noteworthy that, in the present study, the 5G-specific complex was only observed in approximately half of the EMSAs, and even then it was very weak. Furthermore, although previous studies suggest that the 5G-specific complex contains a transcriptional repressor protein, attempts to identify this protein have so far been unsuccessful (9) (10).

As the human astrocytes were heterozygous for both the PAI-1 -675(4G/5G) and the -844A/G polymorphisms, the -844 variant may also have an influence on the haploChIP results. The -844A/G polymorphism was first identified by Grubic *et al* (5). Nuclear extracts from HUVECs appeared to bind to both alleles, suggesting a potential importance of this site (5). The results obtained in the present study confirm the binding of a common protein complex to the G and A alleles, and identifies IRF-2 and p53 as the major components of this complex. More importantly, we demonstrate for the first time an allele-specific protein binding to the -844G allele, both with nuclear extracts from human astrocytes and HUVECs, thus indicating a functional importance of the -844A/G polymorphism in these cells. The prominent G allele-specific band was observed in all EMSAs, and it was shown to contain the transcription factors Elf-1 and Elk-1, both which belong to the Ets-transcription factor family. As the -844G allele is in tight LD with the "low PAI-1 expressing -675(5G) allele" (7) (27), it is feasible that the binding of Elf-1 and Elk-1 to the -844G allele involves the interaction of another protein or proteins with inhibitory activities on gene transcription. Interestingly, Elk-1 appears to have two different repressor domains that can recruit other DNA binding inhibitors or co-repressors (28).

The present study has some limitations, but also some strengths, worth noting. Due to the limited access to astrocytes of human origin, and to the vast number of cells that is required for each haploChIP experiment, allele-specific expression was only investigated in cells derived from two individuals. These individuals were heterozygous for both the PAI-1 -675(4G/5G) and the -844A/G polymorphisms. Although not formally proven, we assume that they have the 4G/A and the 5G/G

haplotypes since the other haplotypes are very rare (7). Moreover, we were not able to evaluate the relative impact of the two individual polymorphisms on PAI-1 promoter activity. This has previously been investigated in a reporter-gene study using PAI-1 promoter constructs representing the four different -675(4G/5G) and -844A/G haplotypes in transfected endothelial and HepG2 cells (7). Although no difference in basal promoter activity was observed, this does not preclude a functional role of these variants *in vivo,* and/or in other cell types. The strength of the present study is that it shows an allele-specific effect on transcriptional activity of the PAI-1 gene within an intact chromatin structure where the different alleles are in their correct genomic context. Furthermore, analysis of allele-specific transcription in heterozygous cells provides an advantage as the relative expression of each allele is compared within the same sample.

In conclusion, the present study reveals the first evidence that common genetic variants within the PAI-1 promoter have a major impact on the transcriptional activity of the PAI-1 gene in human astrocytes, and suggests that the PAI-1 -675(4G/5G) and the -844A/G polymorphisms are of functional importance in these cells. As PAI-1 can have neuroprotective effects, it is feasible that an increased astrocytic expression of PAI-1, conferred by genetic PAI-1 promoter variants, may reduce brain damage following an ischemic event. Thus, our results provide a possible molecular genetic mechanism behind the association between PAI-1 promoter variants and IS.

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LEGENDS TO FIGURES

Figure 1. Pyrosequencing of the PAI-1 -675(4G/5G) polymorphism.
(A) Illustration of the complementary biotinylated DNA strand (●), and the primer used in the pyrosequencing reaction. (B) Pyrograms illustrating the pyrosequencing results for each genotype of the PAI-1 -675(4G/5G) polymorphism. The sequence of nucleotides added during the pyrosequencing reaction is depicted on the X-axis, where E denotes enzyme, S substrate, and T, A, C and G the different nucleotides. The signal peak corresponds to the number of incorporated nucleotides on the complementary DNA strand.

Figure 2. Higher loading ratio of active Pol II to the PAI-1 -675(4G) containing haplotype in heterozygous human astrocytes.
Representative pyrograms are shown for (A) untreated cells subjected to ChIP with nonspecific (IgG) antibodies, (B) before ChIP, and (C) after ChIP with antibodies directed against active Pol II (phosphorylated ser5 residues of the C-terminal domain (CTD) of Pol II). Representative programs of two independent pyrosequencing analyzes are shown. On each occasion samples from two different individuals were used.

Figure 3. Binding of unclear extracts from human astrocytes (H. Ast) and HUVECs to the PAI-1 -675(4G/5G) site.
(A) EMSA experiments were performed following incubation of ^{32}P-labeled 4G or 5G probes with nuclear extracts from H. Ast (lanes 1-2) or HUVECs (lanes 3-4). (B) Competition and cross-competition experiments were performed with a 5G probe and HUVEC extracts with 500-fold molar excess unlabeled (cold) nonspecific competitor, unlabeled 5G-competitor (self-competition) or unlabeled 4G-competitor (cross-competition) as indicated. NS = nonspecific.

Figure 4. The PAI-1 -844A/G polymorphism affects protein binding affinity.
(A) EMSA experiments were performed following incubation of ^{32}P-labeled G or A probes with nuclear extracts from human astrocytes (H. Ast, lanes 1-4) or HUVECs (lanes 5-8), in the absence of competitor (lanes 1, 3, 5, 7) or with 500-fold molar excess unlabeled (cold) nonspecific competitor (lanes 2, 4, 6, 8). (B) (C) (D) Competition and cross-competition experiments were performed with G or A probes and H. Ast or HUVEC extracts with increasing concentrations of unlabeled competitor corresponding to either the G or A allele variants (5-, 50-, or 500-fold molar excess) as indicated. NS = nonspecific.

Figure 5. Elf-1 and Elk-1 bind to the PAI-1 -844G allele.
(A) (B) Nuclear extracts from human astrocytes (H. Ast) or HUVECs were incubated with ^{32}P-labeled G or A probes with or without the addition of antibodies directed against Ets-1, Ets-2, Elk-1 or Elf-1 as indicated.

Figure 6. IRF-2 and p53 bind to the PAI-1 -844A/G site.
(A) (B) (C) (D) Nuclear extracts from human astrocytes (H. Ast) or HUVECs were incubated with ^{32}P-labeled G or A probes with or without the addition of antibodies directed against PU.1, E2F-1, IRF-2, p53, Elf-1, or Elk-1 as indicated. To overcome the high background present with the astrocytic extracts, an unlabeled (cold)

nonspecific competitor (500-fold molar excess) was included in the control and supershift reactions (Fig. 6A and B). NS = nonspecific.

Figure 1 A

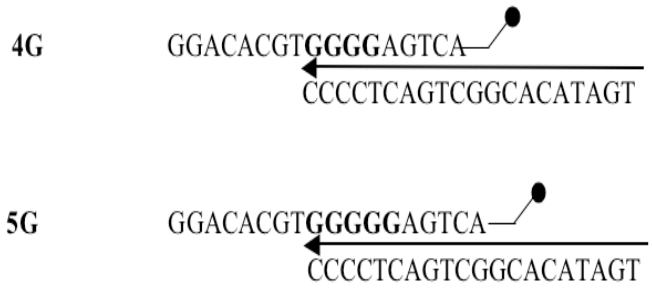

4G
GGACACGTGGGGAGTCA
CCCCTCAGTCGGCACATAGT
5G
GGACACGTGGGGGAGTCA
CCCCTCAGTCGGCACATAGT

Figure 1 B

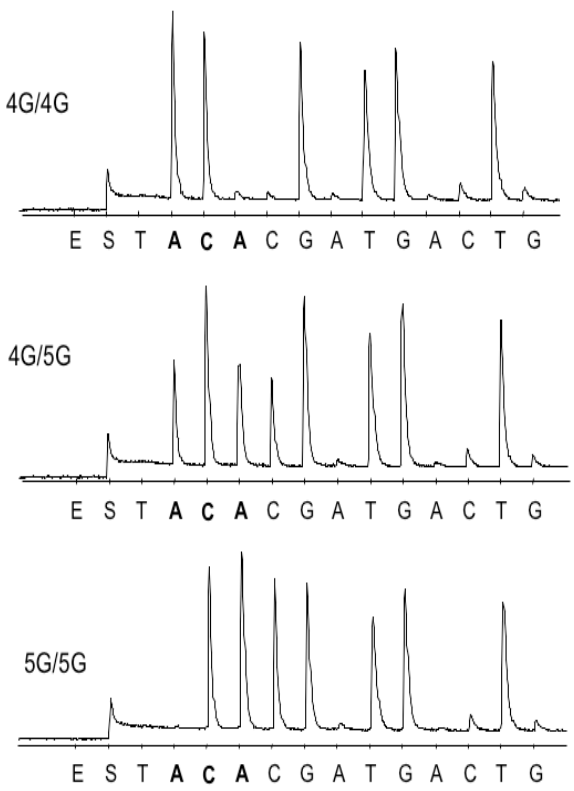

4G/4G
E S T A C A C G A T G A C T G
4G/5G
E S T A C A C G A T G A C T G
5G/5G
E S T A C A C G A T G A C T G

Figure 2

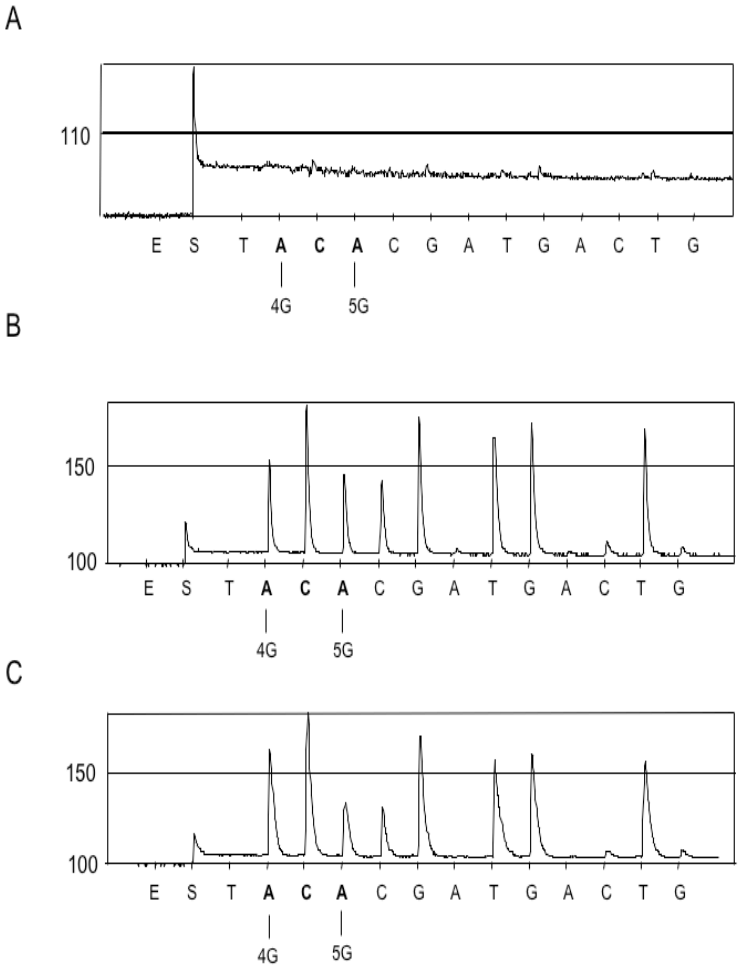

A
110
E S T A C A C G A T G A C T G
4G
5G
B
150
100
E S T A C A C G A T G A C T G
4G
5G
C
150
100
E S T A C A C G A T G A C T G
4G
5G

Figure 3

A

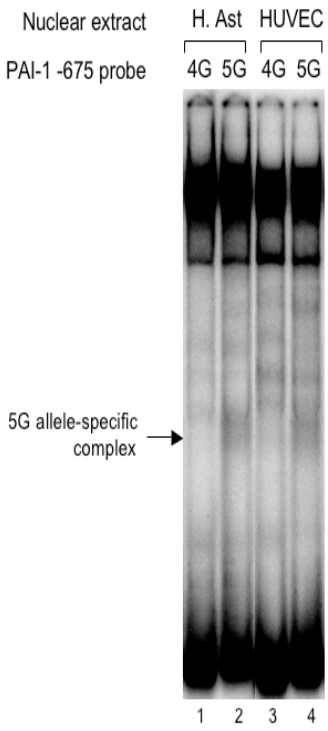

Nuclear extract
H. Ast
HUVEC
PAI-1 -675 probe
4G
5G
4G
5G
5G allele-specific complex
1
2
3
4

Figure 3

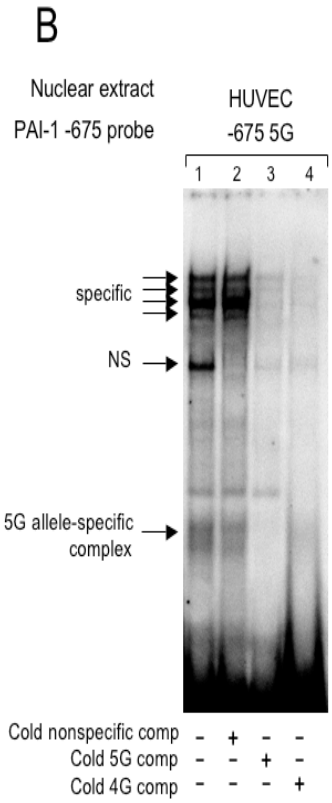

B
Nuclear extract
PAI-1 -675 probe
HUVEC
-675 5G
1 2 3 4
specific
NS
5G allele-specific complex
Cold nonspecific comp - + - -
Cold 5G comp - - + -
Cold 4G comp - - - +

Figure 4

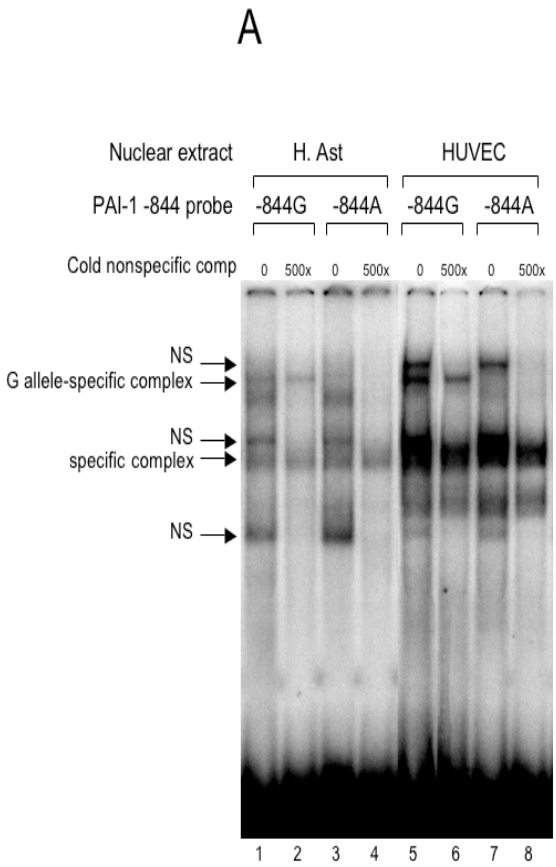

A
Nuclear extract
H. Ast
HUVEC
PAI-1 -844 probe
-844G
-844A
-844G
-844A
Cold nonspecific comp
0
500x
0
500x
0
500x
0
500x
NS
G allele-specific complex
NS
specific complex
NS
1
2
3
4
5
6
7
8

Figure 4

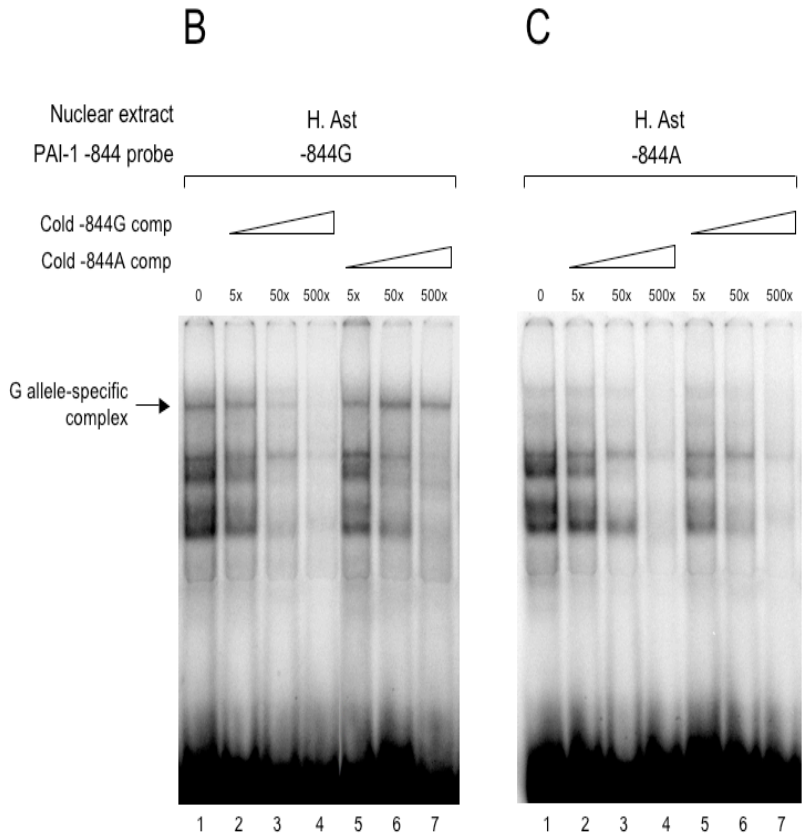

B
C
Nuclear extract
PAI-1 -844 probe
H. Ast
-844G
H. Ast
-844A
Cold -844G comp
Cold -844A comp
0 5x 50x 500x 5x 50x 500x
0 5x 50x 500x 5x 50x 500x
G allele-specific complex
1 2 3 4 5 6 7
1 2 3 4 5 6 7

Figure 4

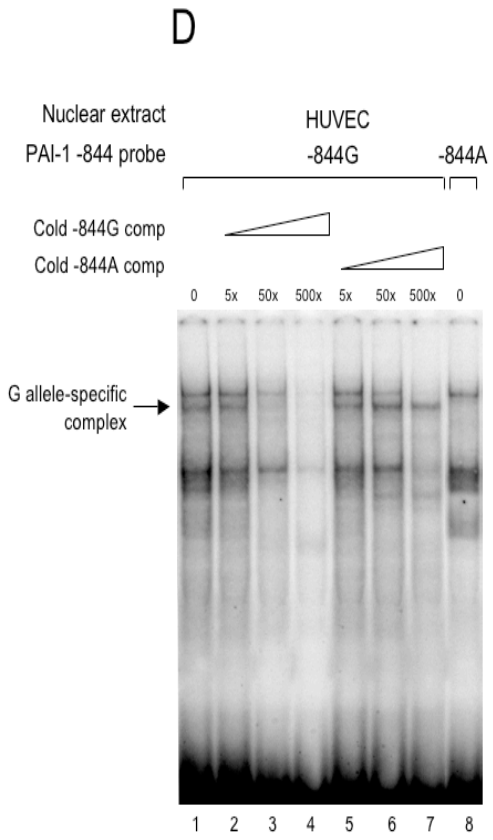

D
Nuclear extract
HUVEC
PAI-1 -844 probe
-844G
-844A
Cold -844G comp
Cold -844A comp
0 5x 50x 500x 5x 50x 500x 0
G allele-specific complex
1 2 3 4 5 6 7 8

Figure 5

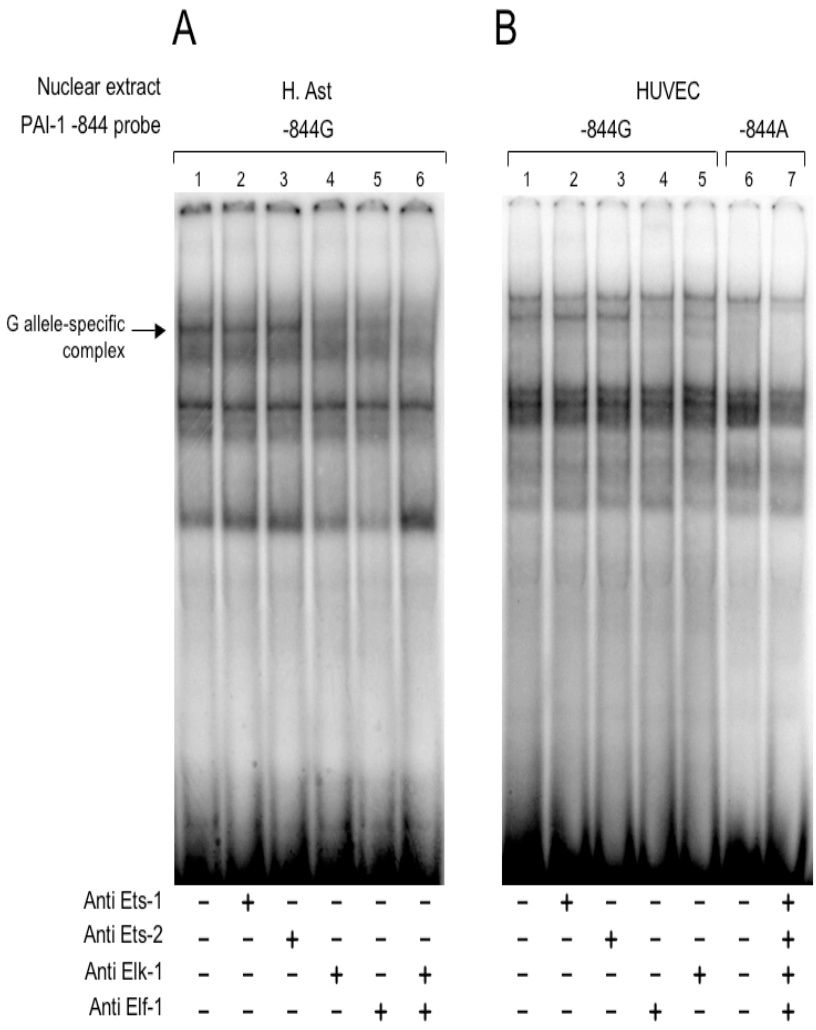

A
B
Nuclear extract
PAI-1 -844 probe
H. Ast
-844G
HUVEC
-844G
-844A
1 2 3 4 5 6
1 2 3 4 5 6 7
G allele-specific complex
Anti Ets-1 – + – – – – – + – – – – +
Anti Ets-2 – – + – – – – – + – – – +
Anti Elk-1 – – – + – + – – – – + – +
Anti Elf-1 – – – – + + – – – + – – +

Figure 6

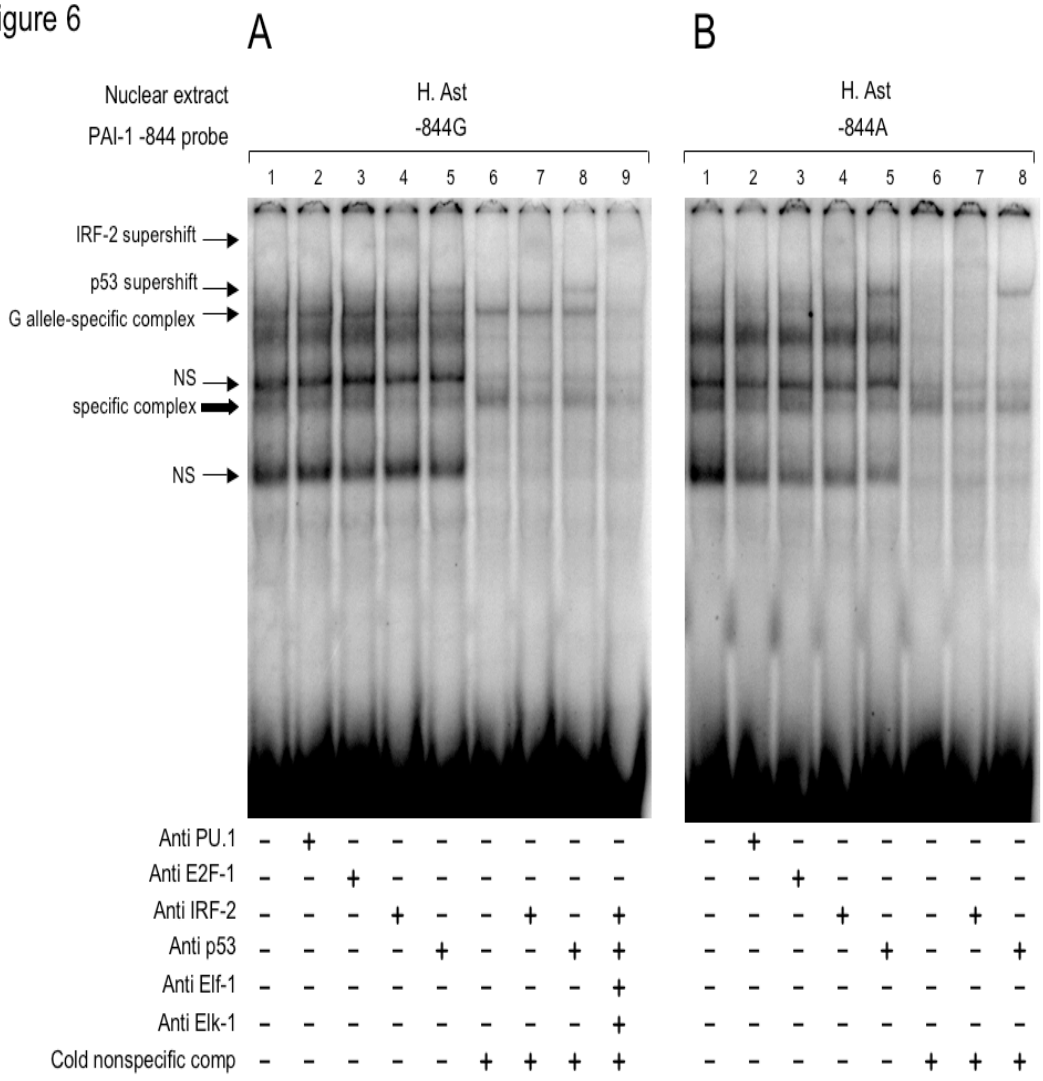
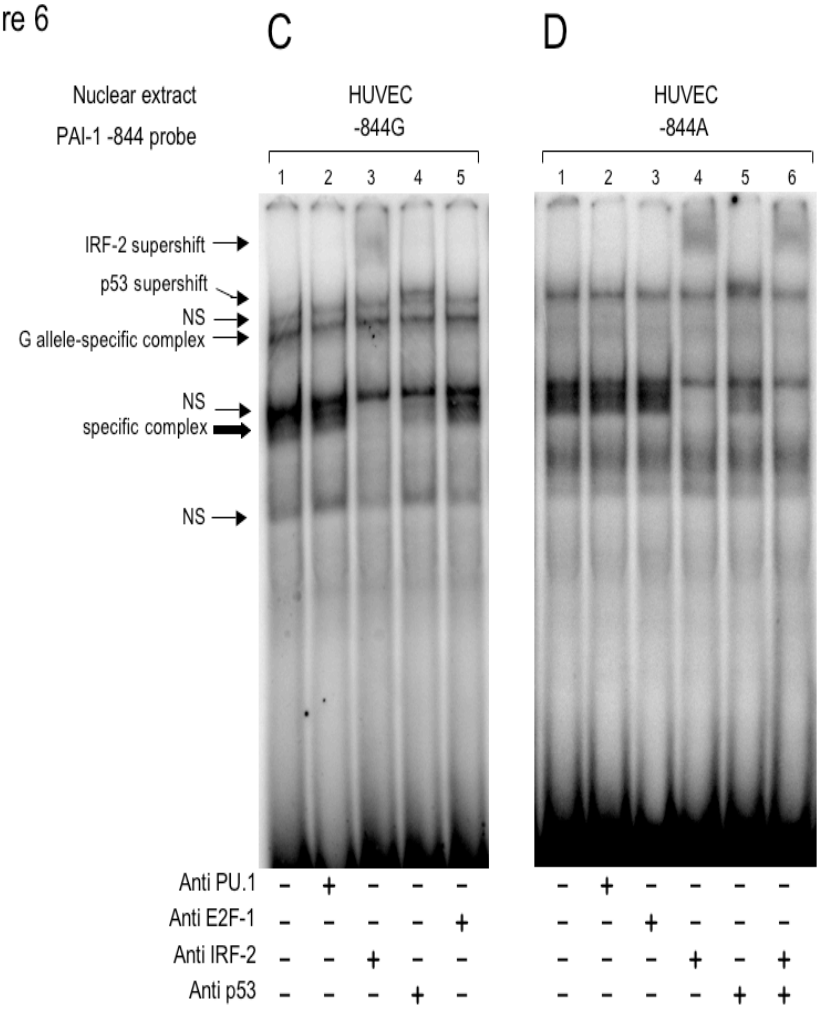

A
B
Nuclear extract
PAI-1 -844 probe
H. Ast
-844G
H. Ast
-844A
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8
IRF-2 supershift
p53 supershift
G allele-specific complex
NS
specific complex
NS
Anti PU.1 – + – – – – – – –
Anti E2F-1 – – + – – – – – –
Anti IRF-2 – – – + – – + – +
Anti p53 – – – – + – – + +
Anti Elf-1 – – – – – – – – +
Anti Elk-1 – – – – – – – – +
Cold nonspecific comp – – – – – + + + +
– + – – – – – –
– – + – – – – –
– – – + – – + –
– – – – + – – +
– – – – – – – –
– – – – – – – –
– – – – – + + +

Figure 6


C
D
Nuclear extract
PAI-1 -844 probe
HUVEC
-844G
HUVEC
-844A
1 2 3 4 5
1 2 3 4 5 6
IRF-2 supershift
p53 supershift
NS
G allele-specific complex
NS
specific complex
NS
Anti PU.1 – + – – – – + – – – –
Anti E2F-1 – – – – + – – + – – –
Anti IRF-2 – – + – – – – – + – +
Anti p53 – – – + – – – – – + +