

Binding of upstream stimulatory factor 1 to the E-Box regulates the 4G/5G polymorphism-dependent plasminogen activator inhibitor 1 expression in mast cells

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Background: Plasminogen activator inhibitor (PAI)-1 is a key regulator of the fibrinolytic system. PAI-1 levels are markedly elevated in the asthmatic airways. The 4G/5G polymorphism of the PAI-1 gene is associated with allergic asthma.

Objective: To characterize the mechanisms of the 4G/5G-dependent PAI-1 expression in mast cells (MCs), a major source of PAI-1 and key effector cells in asthma.

Methods: Transcription of PAI-1 was assessed by transiently transfecting human MC line (HMC-1) cells with the luciferase-tagged PAI-1 promoters containing the 4G or 5G allele (4G-PAI-1 or 5G-PAI-1 promoter). Upstream stimulatory factor (USF)-1 and the E-box interactions were studied by electrophoretic mobility shift assays and supershift assays.

Expression of USF-1 was determined by Western blot analysis. Results: The 4G-PAI-1 promoter has higher promoter activity than the 5G-PAI-1 promoter in stimulated HMC-1 cells, and the E-box adjacent to the 4G/5G site (E-4G/5G) regulates the genotype-specific PAI-1 transcription. USF-1 binds to the E-4G with greater affinity than to the E-5G. USF-1 level is increased in HMC-1 cells after stimulation, and elevated USF-1 enhances PAI-1 transcription. Overexpression of wild-type USF-1 or dominant-negative USF remedies the 4G/5G-dependent PAI-1 transcription.

Conclusion: Binding of USF-1 to the E-4G/5G regulates the 4G/5G polymorphism-dependent PAI-1 expression in MCs. (J Allergy Clin Immunol 2007;119:1101-1110.)

Key words: Plasminogen activator inhibitor 1, 4G/5G polymorphism, transcription, human mast cells, upstream stimulatory factor 1, E-box

Plasminogen activator inhibitor (PAI)-1 is a key regulator of the fibrinolytic system.¹ We previously demonstrated that the PAI-1 levels are markedly elevated in the airways of human and murine allergic asthma.^{2,3} Others also demonstrated that PAI-1 expression is increased in nasal tissues of human and murine allergic rhinitis.^{4,5} Depletion of PAI-1 reduces the infiltration of

Abbreviations used

bHLH:	Basic helix-loop-helix
DN:	Dominant-negative
E-4G/5G:	E-box adjacent to the 4G/5G allele
EMSA:	Electrophoretic mobility shift assay
hMC:	Human mast cell
MC:	Mast cell
PAI:	Plasminogen activator inhibitor
TFE3:	Transcription factor E3
USF:	Upstream stimulatory factor
WT:	Wild-type
Zic2:	Zinc finger transcription factor member 2

inflammatory cells and collagen deposition in these models. These findings strongly suggest that PAI-1 promotes the development of allergic diseases by inducing allergic inflammation and tissue remodeling.

Mast cells (MCs) are key effector cells in asthma and other allergic diseases. We discovered that the PAI-1 gene is induced at the highest level among the inducible human MC (hMC) genes analyzed with DNA microarray.² Nakajima et al⁶ also demonstrated PAI-1 is one of the most highly upregulated genes in IgE-stimulated hMCs. We previously showed that MCs secrete abundant amounts of PAI-1 in the airways of the ovalbumin-challenged murine model of allergic asthma.³ PAI-1 production is 2-fold lower in the airways of MC-deficient W/W^v mice after ovalbumin challenge compared with that in wild-type (WT) mice (S.H. Cho and C.K. Oh, unpublished data, December 2003). These findings suggest that MCs are a major source of PAI-1 in asthma.

Molecular regulations of PAI-1 expression have been studied in various cell types. A single guanosine nucleotide insertion/deletion polymorphism (4G/5G) at -675 bp of the PAI-1 gene has been extensively studied. The plasma level of PAI-1 is higher in patients with the 4G/4G genotype than in those with the 5G/5G genotype, whereas the 4G/5G has intermediate values.⁷⁻¹³ The 4G allele frequency is higher than the 5G (0.63-0.71 vs 0.29-0.37) in Asian people,¹⁴ whereas in healthy white people, they are similar.^{7,10-13} However, the frequency of the 4G is significantly higher than the 5G in patients with asthma. We reported that in Nottingham families of United Kingdom, the 4G frequency of the children with asthma is 0.67, but the 5G frequency is as low as 0.33.¹⁵ In Czech population, the 4G and 5G frequency is similar, but in patients with asthma or other allergic diseases, the 4G frequency is significantly higher than the 5G (0.6 vs 0.4).¹⁶ In house dust mite-sensitive patients with allergic asthma, the 4G frequency is 0.7, whereas in healthy controls, it is 0.55.¹² In patients with the 4G/4G, the serum total IgE and PAI-1 levels during allergen

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challenge are significantly higher than in patients with the 5G/5G.^{12,17} These findings suggest that genetically elevated PAI-1 levels are linked to a propensity to develop asthma and other allergic diseases. However, the molecular mechanisms involved in the genotype-specific PAI-1 expression in asthma or other allergic diseases are unknown. Angiotensin II,¹⁸ very-low-density lipoproteins,¹⁹ IL-1,^{8,14} and circadian clock proteins BMAL/CLOCK⁹ are known to induce higher levels of PAI-1 expression with the 4G allele compared with the 5G allele in human hepatocellular carcinoma (HepG2) cells, endothelial cells, or monkey kidney fibroblast-like cell (COS)-7 cells. In the current study, we report the first evidence that the 4G allele renders higher PAI-1 promoter activity in stimulated hMCs by binding to upstream stimulatory factor (USF)-1 with greater affinity than the 5G allele.

METHODS

Reagents and materials

DMRIE-C transfection reagent and 6% DNA Retardation Gels were purchased from Invitrogen (Carlsbad, Calif). Dual-Luciferase Assay Kit and luciferase report vectors pGL3-Basic and pRL-CMV were from Promega (Madison, Wis). Ionomycin and poly (dI-dC) were from Sigma (St Louis, Mo). Antibodies against human USF-1 (C-20), USF-2 (C-100), transcription factor E3 (TFE3; H-300), Wilms tumor suppressor (C-19), Zinc finger transcription factor member 2 (Zic2; N-13), and histone H1 (FL-219), and Western Blotting Luminol Reagent were from Santa Cruz Biotechnology (Santa Cruz, Calif).

HMC culture and stimulation

Human mast cell 1 (HMC-1) cells (a kind gift from J. H. Butterfield, Mayo Clinic, Rochester, Minn) were maintained as described² and were stimulated with 2 μ M/L ionomycin for the indicated times.

Plasmids constructs and site-directed mutagenesis

Plasminogen activator inhibitor 1 promoter luciferase construct containing 5' flanking -800 bp to +71 bp of human PAI-1 promoter inserted in pGL3-Basic vector (pGL3-Basic-800), which contains the 4G allele, was a kind gift from Dr Anh D. Le, University of Southern California (Los Angeles, Calif). To generate pGL3-Basic-800 with the 5G allele, site-directed mutagenesis was performed by using QuikChange Site-Directed Mutagenesis Kit (Stratagene Inc, San Diego, Calif). To engineer 5'-deletion PAI-1 promoter luciferase reporter constructs containing 5' flanking -698 bp and -41 bp, PCR products with 4G-pGL3-Basic-800 or 5G-pGL3-Basic-800 as template were inserted into pGL3-Basic at *Sac* I and *Bgl* II sites. Site-directed mutagenesis was also performed to introduce dinucleotide substitution (CACGTG→CAATTG) into the E-box at -682 bp to -677 bp of PAI-1 promoter in 4G-pGL3-Basic-800 and 5G-pGL3-Basic-800. The primers used for these constructions are summarized in this article's Table E1 in the Online Repository at www.jacionline.org. Each construct was sequenced for verification and orientation.

Transient transfection of HMC-1 cells and luciferase assay

Human mast cell 1 cells (2×10^6 cells per well) were transfected in 6-well tissue culture plates by using DMRIE-C according to the manufacturer's protocol. Briefly, cells were cotransfected with 4 μ g pGL3-Basic containing different PAI-1 promoter fragments and 20 ng *Renilla* luciferase reporter vector pRL-CMV as internal control. Transfected cells were stimulated with 2 μ M/L ionomycin or an equal volume of ethanol 24 hours after transfection, and the luciferase activities were measured by using the Dual-Luciferase Reporter Assay System (Promega, Madison, Wis) 12 hours after stimulation. Final firefly luciferase activities were normalized to corresponding *Renilla* luciferase activities, and the relative transcriptional activity was expressed as the ratio of the normalized luciferase activities between the constructs assayed and

pGL3-Basic-41 (minimal PAI-1 promoter construct). To study the effects of USF-1 on PAI-1 transcription, HMC-1 cells were cotransfected with 4G-pGL3-Basic-800 or 5G-pGL3-Basic-800 PAI-1 promoter constructs and human USF-1 expression vector pSG5-hUSF-1 or dominant-negative (DN) USF construct p566-A-USF, the kind gifts of Dr Nanyue Chen (MD Anderson Cancer Center, Houston, Texas). Experiments were performed at least 3 times independently.

Nuclear extracts purification and radiolabeled probe preparation

Briefly, 2×10^7 HMC-1 cells were stimulated with 2 μ M/L ionomycin or equal ethanol for 2 hours. Nuclear extracts were prepared using Qproteome Nuclear Protein Kit (QIAGEN, Valencia, Calif). Native PAI-1 promoter fragments -687/-664 (4G) or the -688/-664 (5G) segment [GTCTG GACA CGTGGGG(G)AGTCAGCC] and 4T-substituted 5G segment GTCTGG ACACGTGTTTATGTCAGCC (1G) were synthesized and labeled with [α -³²P]CTP by using a Rediprime II Labelling System (Amersham Biosciences, Piscataway, NJ).

Electrophoretic mobility shift assays

Nuclear extracts 10 μ g were incubated with ³²P-labeled 24-bp or 25-bp PAI-1 promoter probes in 20 μ L binding buffer (10 mmol/L TRIS-HCl, pH 7.5, 50 mmol/L NaCl, 1 mmol/L dithiothreitol, 1 mmol/L EDTA) containing 1 μ g poly (dI-dC) for 20 minutes at room temperature, and the resulting complexes were then separated on 6% DNA Retardation Gels. For competition assay and supershift assay, 50-fold molar excess of unlabeled oligonucleotides or 2.0 μ g antibodies against USF-1, USF-2, TFE3, Wilms tumor suppressor 1, and Zic2 were incubated with nuclear proteins for 20 minutes before adding radiolabeled probes. Intensities of protein-DNA complexes were quantified with densitometric analysis.

Western blot analysis

Nuclear proteins (10 μ g) from HMC-1 cells stimulated with 2 μ M/L ionomycin for 0, 2, 4, or 8 hours were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were incubated for 1 hour at room temperature with the primary rabbit antibodies against human USF-1 (1:1000), followed by incubation with the secondary antibody of goat antirabbit IgG. The Western Blotting Luminol Reagent was used for detection. Histone H1 was used as the internal control to show similar amounts of samples were loaded at each condition. Signal intensities of USF-1 proteins were quantified with densitometric analysis.

Statistical analysis

Results were expressed as means $\pm$ SEs. Differences between 2 groups were identified with a paired Student *t* test. Significance was defined as *P* < .05.

RESULTS

4G-PAI-1 promoter has higher promoter activity than 5G-PAI-1 promoter in stimulated HMC-1 cells

On the basis of the kinetics assay that showed PAI-1 promoter activity reaches its peak 12 hours after ionomycin stimulation (see this article's Fig E1 in the Online Repository at www.jacionline.org), transfected HMC-1 cells were stimulated for 12 hours for promoter assays in the current study.

To determine the 4G/5G polymorphism-dependent regulation of PAI-1 expression, HMC-1 cells were transiently transfected with 4G-pGL3-Basic-800 or 5G-pGL3-Basic-800. Promoter activity was 32% higher in 4G-pGL3-Basic-800 than 5G-pGL3-Basic-800 in ionomycin-stimulated HMC-1 cells (*P* < .001), whereas promoter activities of these constructs were

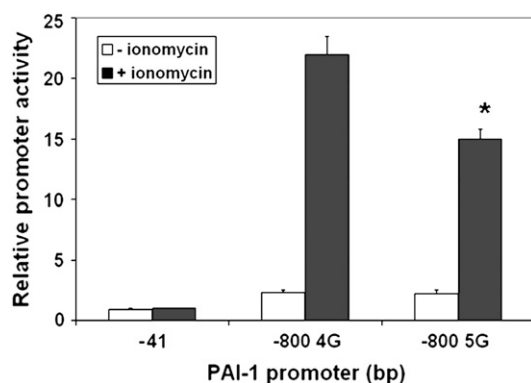


FIG 1. Effects of the 4G/5G polymorphism on transcriptional activity of PAI-1 promoters in HMC-1 cells. HMC-1 cells were transfected with PAI-1 promoter luciferase constructs and then were stimulated with ionomycin or same volume ethanol for 12 hours. The luciferase activities were measured. Results represent means $\pm$ SEs of 5 separate replicates. * $P < .001$ vs 4G constructs.

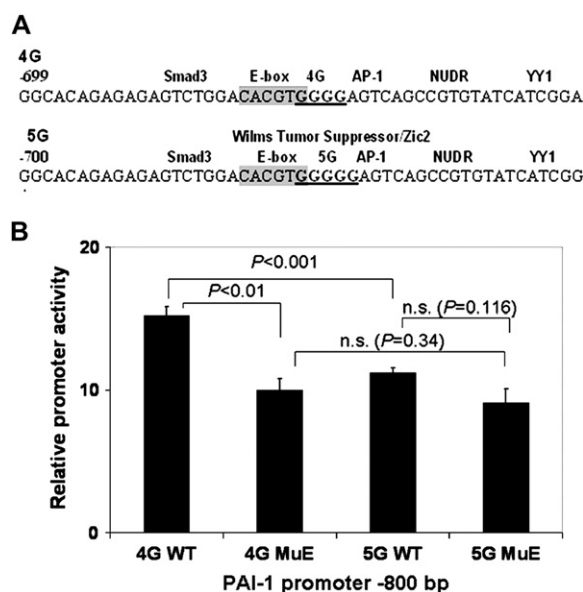


FIG 2. Roles of the E-4G/5G in the 4G/5G polymorphism-dependent PAI-1 transcription in HMC-1 cells. **A**, DNA sequence and putative binding sites adjacent to 4G/5G site of human PAI-1 promoter. **B**, HMC-1 cells were transfected with 4G-pGL3-Basic-800 or 5G-pGL3-Basic-800 with wild-type (WT) or mutated E-4G/5G (MuE). Values were means $\pm$ SEs of 4 individual experiments. AP-1, Activator protein-1; NUDR, nuclear DEAF-1 related transcriptional regulator; NS, not significant.

comparable in unstimulated HMC-1 cells (Fig 1). Further deletion of sequences from -800 bp to -699 bp had no effect on promoter activity of these constructs, suggesting that the sequences from -800 bp to -698 bp may not significantly contribute to PAI-1 transcription (data not shown).

E-box adjacent to the 4G/5G site enhances PAI-1 promoter activity and is critical for regulation of 4G/5G polymorphism-dependent PAI-1 transcription

Bioinformatics analysis using MatInspector 7.4.2 and Matrix MatBase database (Munich, Germany; <http://www.genomatix.de>)

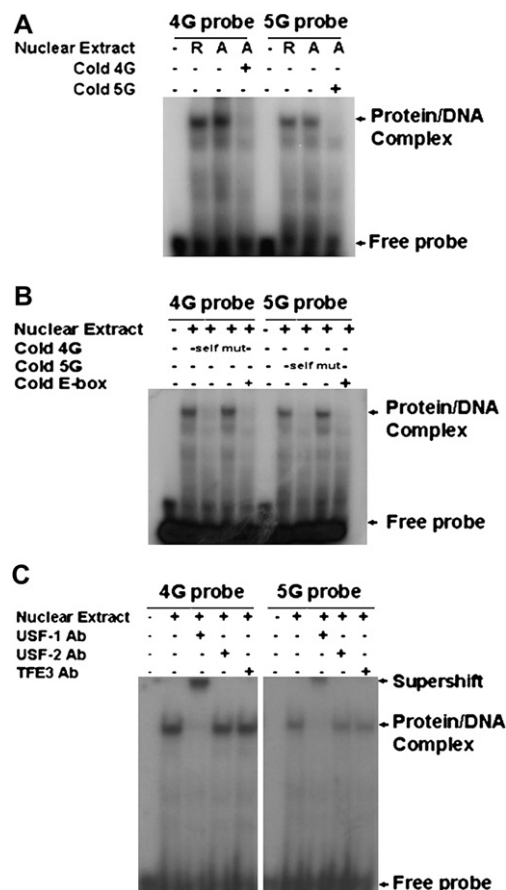


FIG 3. Protein-DNA interactions of 4G-PAI-1 or 5G-PAI-1 promoter. **A**, EMSA with nuclear extracts from resting (R) and activated HMC-1 cells (A) and PAI-1 promoter fragment $-687/-644$ (4G) or $-688/644$ (5G). **B**, EMSA without or with the unlabeled self competitor (self), competitor containing mutated E-box (mut), and consensus E-box. **C**, Supershift assay with antibodies against USF-1, USF-2, or TFE3.

showed that there is an E-box motif (5' CACGTG3') at -682 bp to -677 bp overlapping with the 4G/5G site by a guanosine nucleotide (Fig 2, A). Mutagenesis of the E-box adjacent to the 4G/5G allele (E-4G/5G) in 4G-pGL3-Basic-800 and 5G-pGL3-Basic-800 decreased promoter activities by 34% and 18%, respectively, compared with their corresponding WT constructs ($P < .01$; $P = .116$). The promoter activities between 4G-pGL3-Basic-800 and 5G-pGL3-Basic-800 were comparable after mutagenesis ($P = .34$; Fig 2, B).

USF-1 binds to the E-4G/5G

To characterize the protein-DNA interactions involved in 4G/5G polymorphism-dependent PAI-1 expression, electrophoretic mobility shift assay (EMSA) was performed. A protein-DNA complex was observed with PAI-1 promoter fragments containing the 4G or 5G allele and nuclear proteins from both resting and activated HMC-1 cells. Specificity of this complex was confirmed by adding 50-fold molar excess of unlabeled 4G or 5G probes, which eliminated the complex (Fig 3, A). Also, 50-fold molar excess of unlabeled E-box consensus sequence, but not the mutant E-4G/5G sequence, eliminated the complex (Fig 3, B). These

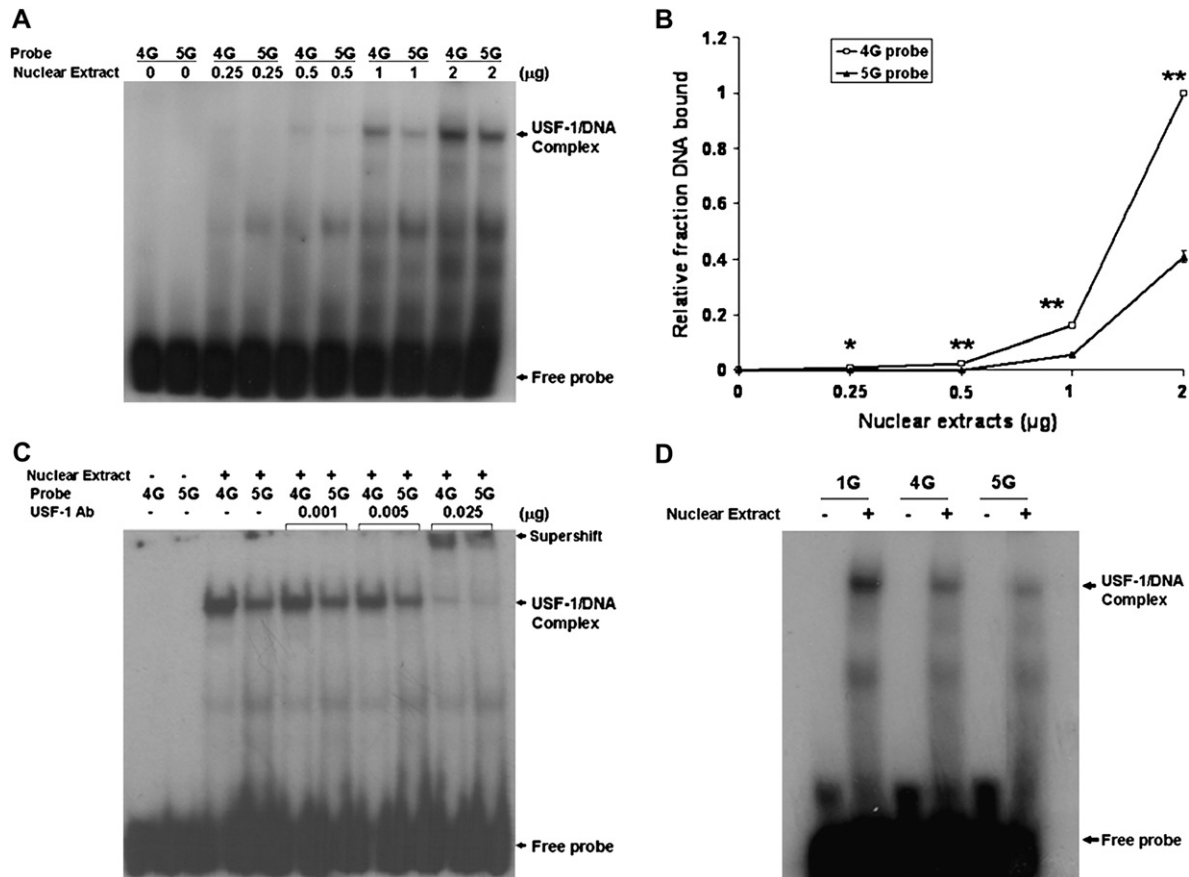


FIG 4. Binding of USF-1 to the E-4G/5G. **A**, EMSA with 0 to 2 µg nuclear extracts. **B**, Densitometric analysis. Results represented means $\pm$ SEs of 3 independent experiments. * $P < .05$, ** $P < .01$ vs 5G. **C**, Supershift assay with anti-USF-1 antibodies. **D**, EMSA with 4G, 5G, and 1G probes.

results indicate that the E-4G/5G is crucial in the formation of this complex.

To identify the transcription factor binding to the E-4G/5G, supershift assays were performed with antibodies against putative E-box-binding transcription factors. Preincubation of nuclear extracts with antibodies against USF-1 produced a specific supershift band with the 4G probe and the 5G probe, but not with antibodies against USF-2 and TFE3 (Fig 3, C). Bioinformatics analysis suggests that Zic2 and Wilms tumor suppressor 1 may bind to the 5G allele but not to the 4G allele of the PAI-1 promoter (Fig 2, A). However, supershift assays with antibodies against Zic2 and Wilms tumor suppressor 1 did not show any supershift band (data not shown).

USF-1 binds to the E-4G with higher affinity than to the E-5G

The band intensity of protein-DNA complex was stronger with the 4G probe than with the 5G probe (Fig 3). This suggests that USF-1 binds to the E-4G with higher affinity than to the E-5G. To characterize further the binding affinity of USF-1 to the E-4G and the E-5G, 0 to 2 µg nuclear extracts were used for EMSA. As shown in Fig 4, A and B, USF-1 binds to the E-4G and the E-5G in a dose-dependent manner. The protein-DNA complex was formed with the 4G probe at as low as 0.25 µg nuclear extracts, but not with the 5G probe ($P < .05$), and at all other

amounts of nuclear extracts used, binding of USF-1 to the 4G probe is significantly greater than to the 5G probe ($P < .01$). Supershift assay using a series of amounts of anti-USF-1 antibodies showed that 0.025 µg anti-USF-1 antibody completely supershifted the USF-1–5G complex, but not the USF-1–4G complex (Fig 4, C). These results indicated that USF-1 binds to the E-4G with higher affinity than to the E-5G.

To determine whether the additional guanosine nucleotides lead to the decreased binding of USF-1 to the 5G–PAI-1 promoter, EMSA was performed with mutant 25-bp 5G–PAI-1 promoter in which the last 4 guanosine nucleotides were substituted with thymine nucleotides (1G) and WT 4G and 5G probes (Fig 4, D). The DNA-binding activity of USF-1 was strongest with the 1G probe and weakest with the 5G probe, suggesting that guanosine nucleotides downstream of the E-4G/5G are critical to determine the binding affinity of USF-1 to the E-4G/5G.

Expression of nuclear USF-1 is increased in stimulated HMC-1 cells

To determine whether USF-1 is induced in nuclei of hMCs after stimulation, HMC-1 cells were treated with ionomycin for 0, 2, 4, and 8 hours. Nuclear USF-1 protein was constitutively expressed at high level and was increased after stimulation in a time-dependent manner (Fig 5).

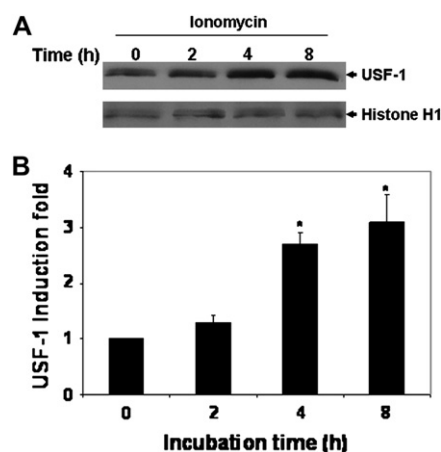


FIG 5. Expression of nuclear USF-1 in HMC-1 cells. **A**, Cells were stimulated with ionomycin for 0 to 8 hours. Nuclear extracts were analyzed by immunoblotting with histone H1 as the internal control. **B**, Densitometric analysis. Results are reported as folds to 0 hours and represent means $\pm$ SEs of 6 independent experiments. * $P < .05$ vs 0 hours.

USF-1 regulates the 4G/5G polymorphism-dependent PAI-1 expression in HMC-1 cells

Promoter activities of 4G-pGL3-Basic-800 were higher than 5G-pGL3-Basic-800 ($P < .01$; Fig 6). After overexpression of USF-1, promoter activities of 4G-pGL3-Basic-800 and 5G-pGL3-Basic-800 were increased by 34% and 63%, respectively ($P < .05$), and after overexpression of DN USF, activities of 4G-pGL3-Basic-800 and 5G-pGL3-Basic-800 were decreased by 57% and 39%, respectively ($P < .05$). Promoter activities between 4G-pGL3-Basic-800 and 5G-pGL3-Basic-800 were not significantly different after overexpression of WT USF-1 or DN USF.

DISCUSSION

The plasma level of PAI-1 is higher in patients with the 4G allele than in patients with the 5G allele, and the 4G/5G polymorphism is linked to a propensity to develop asthma. However, the underlying molecular mechanisms are unknown. In the current study, we report the first evidence that the 4G allele has higher PAI-1 promoter activity than the 5G allele in stimulated HMC-1 cells. We also report that the E-4G/5G regulates the 4G/5G polymorphism-dependent PAI-1 transcription in HMC-1 cells on the basis of our finding that mutation of the E-4G/5G eliminated the difference of transcriptional activities between the 4G-PAI-1 and 5G-PAI-1 promoters. Finally, we report for the first time that the molecular mechanism of the 4G allele-dependent higher PAI-1 expression level in hMCs is a result of increased binding of USF-1 to the E-4G than to the E-5G.

The E-4G/5G has been found to be important in the regulation of PAI-1 expression. In human keratinocytes, the E-4G/5G is known to render growth-arresting-dependent repression of PAI-1 expression by binding of USF-1 and growth stimulation-dependent activation of PAI-1 expression by binding of USF-2.²⁰ It is also found to involve hypoxia-induced PAI-1 expression through USF-2 in HepG2 cells.²¹ Moreover, the circadian expression of PAI-1 may be regulated through binding of circadian clock proteins BMAL2/CLOCK to the E-4G/5G.⁹ In the current study, we identified a novel function of the E-4G/5G in hMCs as the key *cis*-acting element responsible for the genotype-specific PAI-1 expression through binding of USF-1 but not USF-2.

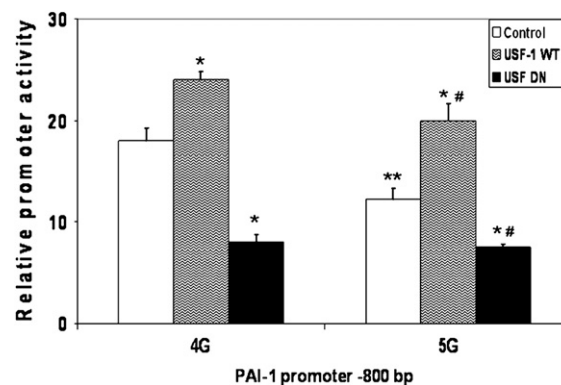


FIG 6. Effects of USF-1 on 4G/5G-dependent PAI-1 transcription. HMC-1 cells were cotransfected with 4G-pGL3-Basic-800 or 5G-pGL3-Basic-800 and USF-1 overexpression vector pSG5-hUSF-1 (USF-1 WT) or USF DN construct p566-A-USF. Results are means $\pm$ SEs of 5 separate replicates. ** $P < .01$ vs 4G control; * $P < .05$ vs corresponding controls; # $P > .05$ vs 4G constructs under the same conditions.

The cellular USFs are ubiquitously expressed and consist of 43-kd (USF-1) and 44-kd (USF-2) polypeptides.²² USFs belong to the basic helix-loop-helix (bHLH) family of transcription factors and function as a transcriptional enhancer or suppressor through binding to the E-box as homodimers/heterodimers of USF-1 and USF-2. In addition to USFs, other transcription factors such as c-Myc, c-Max, and TFE3 are known to bind to the E-box.²³ In the current study, we demonstrated that USF-1 binds to the E-4G/5G of the PAI-1 promoter in HMC-1 cells as homodimers. We also found that the E-4G/5G is constitutively bound by USF-1 in HMC-1 cells, and ionomycin-mediated stimulation does not affect the binding under the current assay conditions. This is in agreement with the finding that binding of USF-1 to the E-boxes in the PAI-1 promoter in human keratinocytes is not affected by TGF- β 1-mediated or serum-mediated stimulation.^{20,24} We previously reported that PAI-1 mRNA and protein expression levels are below the level of detection in resting HMC-1 cells, whereas both mRNA and protein expression of PAI-1 is sharply induced after stimulation with ionomycin.² In the current study, we showed that overexpression of WT USF-1 and DN USF increases and decreases ionomycin-mediated PAI-1 transcription in HMC-1 cells, respectively. These findings indicate that ionomycin-induced PAI-1 expression is dependent on the function of USF-1. PAI-1 transcription is induced in HMC-1 cells stimulated with ionomycin, although USF-1 binds to the E-4G/5G in both resting and stimulated HMC-1 cells. These findings suggest that ionomycin-mediated stimulation triggers the function of USF-1 as a transcriptional activator without affecting its DNA binding activity through a novel mechanism that remains to be identified. Furthermore, the DNA-binding affinity of USF-1 is higher with the E-4G than with the E-5G. This is consistent with our finding that the 4G allele renders higher PAI-1 promoter activity than the 5G allele. In adipocytes, the E-4G/5G is occupied by both USF-1 and USF-2, and the binding of USFs to the E-4G and E-5G is similar, which is consistent with the observation that PAI-1 transcription is not influenced by the 4G/5G allele in adipocytes.²⁵ These findings suggest that 4G/5G-mediated PAI-1 expression regulation is cell type-specific. Taken together, our results suggest that the greater binding of USF-1 to the E-4G leads to the higher level of transcription of

the PAI-1 promoter with the 4G allele, which ultimately causes a higher plasma PAI-1 level.

Eriksson et al⁷ and Dawson et al⁸ reported that a protein factor binds to the 5G–PAI-1 promoter but not to the 4G–PAI-1 promoter in HepG2 cells, which may work as a repressor to decrease PAI-1 expression. However, a report using the same cell type (HepG2) did not show a difference in the binding of nuclear proteins to the 4G and 5G probes.²⁶ In adipocytes, such a repressor protein was not observed with the 5G–PAI-1 promoter.²⁵ We also did not observe this additional protein preferentially binding to the 5G–PAI-1 promoter in hMCs. Bioinformatics analysis shows that Wilms tumor suppressor 1 and Zic2 may preferentially bind to the 5G–PAI-1 promoter. However, our supershift assay suggests that neither Wilms tumor suppressor 1 nor Zic2 binds to the 5G–PAI-1 promoter in HMC-1 cells. These results indicate that this additional protein may not be present in hMCs and therefore may not be the mechanism of the 4G/5G polymorphism-dependent PAI-1 expression in hMCs.

Our EMSA results with the 1G–PAI-1 promoter suggest that the 3' flanking sequences of the E-4G/5G affect USF-1 DNA-binding activity. This is consistent with the report that the 3' flanking sequences of the E-box core motif contribute to the binding affinity of USFs and provide discrimination between CACGTG-binding proteins.²⁷ Similar results were also reported with other bHLH family proteins, including yeast protein PHO4 and the *Drosophila* bHLH protein Enhancer-of-split, whose binding to the E-box is associated with both the E-box core sequence and the flanking sequences.^{28,29} Moreover, the 3' downstream flanking sequences are found to be more important to the function of the E-box than the 5' upstream sequences.^{30,31} Our results also suggest that the difference of the 3' downstream sequences of E-4G/5G causes the differential binding affinity of USF-1 to the E-4G and E-5G. The detailed structural elements that determine the binding affinity of USF-1 to E-4G or E-5G remain to be identified.

We demonstrated that nuclear USF-1 protein is expressed at a high level in resting HMC-1 cells by Western blot, which is consistent with our EMSA results that showed there are bodies of USF-1 binding to the PAI-1 promoter even under the resting status. The level of nuclear USF-1 is increased in activated HMC-1 cells. We also found that overexpression of WT USF-1 leads to increased PAI-1 promoter activity in HMC-1 cells, and after overexpression of DN USF, PAI-1 transcription is decreased. Finally, we demonstrated that overexpression of DN USF or WT USF-1 remedies the significant difference between the promoter activities of the 4G–PAI-1 and 5G–PAI-1 promoters. These *in vivo* results further indicate that USF-1 is the positive regulator of PAI-1 expression, and more importantly, it is the key transcription factor that regulates the 4G/5G allele-dependent PAI-1 transcription in hMCs.

In summary, we present the first evidence that the 4G allele renders higher PAI-1 transcription in hMCs, which may explain the clinical association of the 4G/5G polymorphism with plasma PAI-1 level and a propensity to develop asthma and other allergic disorders. Furthermore, for the first time, we demonstrate that 3' downstream sequences-dependent differential binding affinity of USF-1 to the E-4G and the E-5G is the mechanism responsible for the regulation of the 4G/5G-dependent PAI-1 transcription in hMCs. Binding of USF-1 to the E-4G/5G is the major mechanism of the regulation of PAI-1 transcription in hMCs, and the increased binding of USF-1 to the 4G allele leads to the higher

transcriptional activity of PAI-1 promoter with the 4G allele compared with the 5G allele. Further analysis of the 4G/5G polymorphism-dependent differential expression of the PAI-1 gene may provide better insight into the increased prevalence of asthma and other allergic diseases in specific populations and lead to the development of a novel therapeutic target for treatment and prevention of asthma and other allergic diseases.

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Clinical implications: The results of this study may help identify individuals who are at high risk for diseases associated with elevated PAI-1 levels, such as asthma, and may lead to the development of a novel therapeutic target to control PAI-1 expression.

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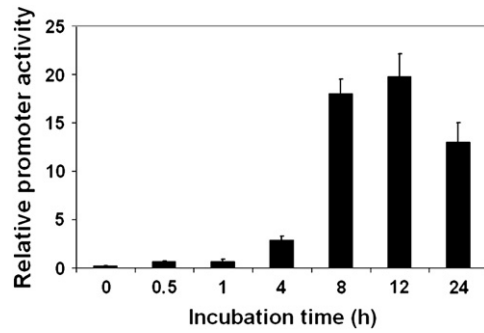


FIG E1. Time-dependent PAI-1 transcription in ionomycin-stimulated HMC-1 cells. HMC-1 cells were transfected with pGL3-Basic-800 and then stimulated with 2 μ mol/L ionomycin. Cells were harvested at indicated times. The luciferase activities were measured. Results represent means $\pm$ SEs of 3 independent experiments.

TABLE E1. Primers used for construction of human PAI-1 promoter luciferase reporter plasmids and site-directed mutagenesis

Region amplified	Primer sequences (5' → 3')*
−41 bp	CGCGAGCTCTGCCACATCTGGTATAAAA
−698 bp	CGGGAGCTCCACAGAGAGAGTCTGGACACG
+71 bp	CGCAGATCTCAGCTGCTGGAGGGGGGCGT
5G-pGL3-Basic-800	GAGTCTGGACACGT GGGGG AGTCAGCCGTGTATC
4G-pGL3-Basic-800EMu CG → AT	TGTCTGGACAATT GGGG AGTCAGCC
5G-pGL3-Basic-800EMu CG → AT	TGTCTGGACAATT GGGG AGTCAGCC

*The *underlined* sequences represent the restriction enzyme sites used for subcloning (*Sac* I and *Bgl* II) or mutant site. 4G and 5G sequences appear in *boldface*.