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Allele-specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction

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ABSTRACT **Increased plasminogen-activator inhibitor 1 (PAI-1) activity is a common finding in patients with coronary heart disease. Here we provide evidence for an independent, etiological role of PAI-1 in myocardial infarction. The 4G allele of a recently described common 4/5-guanine-tract (4G/5G) polymorphism in the PAI-1 promoter is associated with higher plasma PAI-1 activity. The prevalence of the 4G allele is significantly higher in patients with myocardial infarction before the age of 45 than in population-based controls (allele frequencies of 0.63 vs. 0.53). Both alleles bind a transcriptional activator, whereas the 5G allele also binds a repressor protein to an overlapping binding site. In the absence of bound repressor, the basal level of PAI-1 transcription is increased.**

Reduced fibrinolytic capacity, mainly due to increased plasminogen-activator inhibitor 1 (PAI-1) activity in plasma, is a common finding in patients with coronary heart disease (CHD) (1). Poor fibrinolytic activity has also been linked to increased risk of future cardiovascular events in men aged 40–54 years participating in a prospective epidemiological study (2). Increased PAI-1 is thought to predispose to coronary thrombosis due to its role as a rapid inhibitor of tissue-type plasminogen activator, the major proteolytic activator of plasminogen. Moreover, increased PAI-1 expression has been demonstrated in atherosclerotic plaques (3, 4) suggesting that PAI-1 may play a role in atherogenesis as well. However, the clinical evidence for a cause-and-effect relationship between PAI-1 activity and CHD is still limited and confined to a few longitudinal cohort studies of young post infarction patients (5, 6) and patients with CHD or peripheral atherosclerosis (7). Further, in mice transgenic for the PAI-1 gene, increased PAI-1 was shown to contribute to the development of venous but not arterial occlusions (8). Recently, the effects of gene inactivation of fibrinolytic components in mice have been studied. Mice with combined tissue-type and urokinase-type PA deficiency suffered from extensive spontaneous fibrin deposition with an associated shortened lifespan (9). Disruption of the PAI-1 gene induced a mild hyperfibrinolytic state and a greater resistance to venous thrombosis (10).

In the present study, we provide evidence for an independent, etiological role of PAI-1 in CHD on the basis of clinical and molecular biological studies of a common 4/5-guanine-tract (4G/5G) polymorphism in the PAI-1 promoter (11) (see Fig. 1*a*). The 4G allele of this polymorphism was found to be associated with higher plasma PAI-1 activity, and the prevalence of the 4G allele was significantly higher in young patients with myocardial infarction. Furthermore, the 4G allele-specific increase in plasma PAI-1 activity is related to a differential binding of transcription factors to the polymorphic site which increases the basal PAI-1 transcription.

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MATERIALS AND METHODS

Subjects. A total of 94 men with a first myocardial infarction before the age of 45 were included in the study. They belonged to a consecutive series of 131 male patients who were admitted to the 10 hospitals in the greater Stockholm area with a coronary care unit between April 1989 and April 1991 (12). The patients were subsequently referred to the Karolinska Hospital for metabolic, hemostatic, and cardiological investigations. Of the initial 131 patients, 3 died in the early postinfarction period. Patients on treatment with oral anticoagulants (n = 3) or who had heterozygous familial hypercholesterolemia (n = 3), severely impaired renal function (n = 5), or insulin-dependent diabetes mellitus (n = 4) were not included. Of the remaining 116 patients, 2 were excluded because of extensive ischemic stroke in connection with the myocardial infarction, 13 declined participation, and 7 were excluded for technical reasons (unavailable to the research team, deficient laboratory capacity, or referral later than 6 months after the infarction).

One hundred healthy men with the same age distribution were examined as controls. They were selected at random from a register containing all permanent residents in Stockholm County. Of those initially invited, 81% agreed to participate in the research program. All the men were interviewed to exclude individuals with a history of myocardial infarction, angina pectoris, or any other severe illness.

Blood Sampling, Biochemical Methods, and DNA Procedures. All patients were examined 4–6 months after the acute event, when it was expected that acute-phase reactions due to the myocardial infarction had declined. Blood sampling was as described (12). PAI-1 was assayed as activity in plasma (13) by means of a commercially available kit (TintElize PAI-1; Biopool, Umeå, Sweden). The major plasma lipoproteins were determined as described (14). Blood glucose was measured by a glucose oxidase method (Kodak Ektachem). Insulin and insulin propeptides were analyzed in the first consecutive 62 patients and 41 controls as described (15, 16).

For DNA procedures, nucleated cells from frozen whole blood were prepared according to Sambrook *et al.* (17), and DNA was extracted by a salting-out method (18). Genotyping for the 4G/5G polymorphism in the PAI-1 promoter region was conducted with an allele-specific oligonucleotide melting technique as described earlier (11), except that a slot blot (PR 648 Slot Blot; Hoefer) was used instead of a double blot.

Cell Cultures. Hep G2 cells were cultured in Ham's F12 medium supplemented with 10% newborn calf serum. Human umbilical vein endothelial cells (HUVECs) and smooth muscle cells were cultured as described (19, 20).

Abbreviations: CAT, chloramphenicol acetyltransferase; CHD, coronary heart disease; EMSA, electrophoretic mobility-shift assay; HUVEC, human umbilical vein endothelial cell; PAI-1, plasminogen-activator inhibitor 1.

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DNA Constructs. For electrophoretic mobility-shift assay (EMSA) and footprinting analysis, two sets of double-stranded oligonucleotides were designed. The oligonucleotides formed the 24/25-bp DNA fragment of the −687/−664 (4G) or the −688/−664 (5G) segment [GTCTGGACACGTGGGG-(G)AGTCAGCC] of the human PAI-1 promoter flanked by *Bam*HI and *Sal* I ends. The double-stranded oligonucleotides were ligated into *Bam*HI- and *Sal* I-digested plasmid pGEM-2 (Promega) to form the constructs pGEM-2-5G and pGEM-2-4G. Both constructs were tested for correct sequence by DNA sequencing. pGEM-2-4G/5G plasmids were end-labeled with [γ-^{32}P]ATP in either the *Bam*HI or the *Sal* I site by T4 polynucleotide kinase (21).

The 2×5G-HCAT and 2×4G-HCAT vectors were constructed as follows. Two sets of double stranded oligonucleotides constituting the same sequences as shown above but flanked by *Bam*HI and *Bgl* II ends were constructed. Two double-stranded oligonucleotides were ligated head to tail into *Bam*HI-digested HCAT vector (22). The correct sequence and orientation of the inserts were tested by DNA sequencing.

EMSA. Nuclear extracts were prepared according to Alksnis *et al.* (23). All buffers were freshly supplemented with leupeptin (0.7 μg/ml), aprotinin (16.7 μg/ml), phenylmethanesulfanyl fluoride (0.5 mM), and 2-mercaptoethanol (0.33 μl/ml). The protein concentration in the extracts was estimated by the method of Kalb and Bernlohr (24). Incubation for EMSA was conducted as described (11) and the reaction products were applied to a 7% (wt/vol) polyacrylamide gel (80:1 acrylamide/*N,N'*-methylenebisacrylamide weight ratio) and electrophoresed in 22.5 mM Tris/22.5 mM boric acid/0.5 mM EDTA buffer for 2 hr at 200 V. In the supershift assays the incubation followed the standard EMSA protocol with a subsequent incubation for 30 min at 25°C with 2–4 mg of polyclonal antibodies directed against either NF-κB p65 or c-Jun/AP-1 (Santa Cruz Biotechnology). Competitor DNAs used in the EMSA presented in Fig. 2 were as follows; 4G/5G sites denote purified *Bam*HI–*Sal* I fragments of pGEM-2-4G and pGEM-2-5G plasmids; nonspecific DNA denotes the *Sph* I–*Eco*RI fragment of pGEM-1; NF-κB, AP-1, and AP-2 sites denote 22, 21, and 26 bp, respectively, of double-stranded consensus oligonucleotides (Promega).

Methylation Interference and DNase I Footprinting. Methylation interference was conducted essentially as described (21). The methylated DNA fragments were cleaved with piperidine (25) and analyzed by electrophoresis in a denaturing 11% polyacrylamide gel (19:1 acrylamide/*N,N'*-methylenebisacrylamide weight ratio).

DNase I footprinting was conducted with the same DNA fragment, labeled at either end, as for EMSA. Partial DNase I digestion was as described (26, 27) except that the reaction was terminated with 0.02 M Na_2EDTA and the mixture was directly put on ice. The different protein/DNA complexes were separated by EMSA as described above. Retarded DNA fragments were electroblotted onto a Schleicher & Schuell DEAE membrane and analyzed in a denaturing 11% polyacrylamide gel (19:1).

Transfection Assay. Twenty-four hours before transfection, cells were plated in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum. Two to 4 hr before transfection, the dishes received fresh medium. Cells were incubated for 16 hr with calcium phosphate-precipitated DNAs (15 μg of plasmid per 90-mm dish) (17). After a 2-min 15% (vol/vol) glycerol shock, fresh medium was added. Cells were harvested for assay of transient expression 24–36 hr later. pSV-β-galactosidase (Promega) was cotransfected as an internal control.

Statistical Methods. A χ^2 test was used to compare the observed numbers of each PAI-1 genotype with those expected for a population in Hardy–Weinberg equilibrium. Allele frequency was estimated by gene counting and χ^2 analysis. Logarithmic or square-root (PAI-1) transformation was performed on all skewed biochemical variables to obtain a normal distribution before statistical computations and significance testing were undertaken. Differences in continuous variables between groups were tested either by Student's unpaired two-tailed *t* test or by analysis of variance with the Scheffé *F* test used as a posthoc test.

RESULTS AND DISCUSSION

Recently, the human PAI-1 promoter was searched for alterations that may affect the level of plasma PAI-1 activity. A single base-pair polymorphism, GACACGT($G_{4\ \mathrm{or}\ 5}$)AGT (4G/5G), was detected 675 bp upstream from the start of transcription of the PAI-1 gene (Fig. 1*a*) and found to be associated with plasma PAI-1 activity (11). To investigate whether the genotype-specific increase in PAI-1 activity is of etiological importance for coronary thrombosis and/or coronary atherosclerosis, we have genotyped a consecutive series of 93 men with myocardial infarction before the age of 45 for the 4G/5G polymorphism, along with 100 population-based age- and sex-matched control subjects (Fig. 1*b*). The frequencies of the two alleles in control populations were approximately 0.5/0.5 (Table 1 and ref. 11). There was a graded increase in plasma PAI-1 activity with the number of 4G alleles (Fig. 1*c*). Plasma PAI-1 activity was significantly higher (P = 0.037) in control subjects who were homozygous for the 4G allele than in individuals who were homozygous for the 5G allele. The frequency of the 4G allele was also significantly higher among young post infarction patients than in control subjects, and the genotype distribution differed significantly between the two

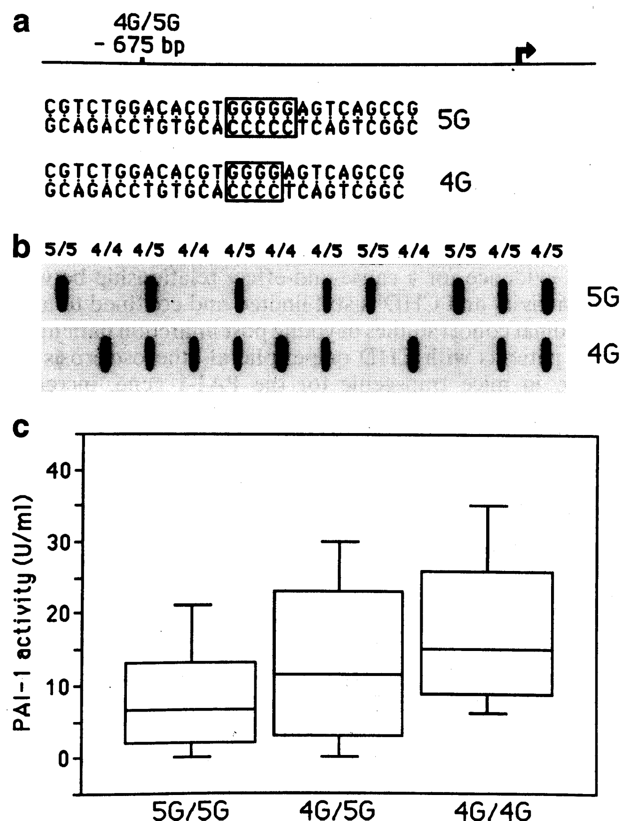


FIG. 1. The 4G allele of the PAI-1 promoter is associated with higher PAI-1 activity. (*a*) PAI-1 promoter sequences comprising the 4G/5G region. (*b*) Example of autoradiogram from genotyping of the 4G/5G polymorphic region by an allele-specific oligonucleotide melting technique. (*c*) Box plot displaying the 10th to the 90th percentiles of PAI-1 levels in the three 4G/5G genotypes of 100 control subjects. U, units.

Table 1. Distribution of 4G/5G genotypes in patients with myocardial infarction and controls

	Patients (n = 93)	Controls (n = 100)
No. with 5G/5G genotype	15	20
No. with 4G/5G genotype	38	54
No. with 4G/4G genotype	40	26
Odds ratio	2.15 (1.17–3.96)	
Frequency of 5G allele	0.37	0.47
Frequency of 4G allelle	0.63	0.53

Consecutive men with a first myocardial infarction before the age of 45 who had been admitted to the coronary care units in the greater Stockholm area, together with population-based age- and sex-matched control subjects, were recruited. The odds ratio is a measure of relative risk of myocardial infarction between subjects with the 4G/4G genotype and subjects with the 4G/5G or 5G/5G genotype. Allele frequency was determined by gene counting. The 95% confidence interval is shown in parentheses. A difference in allele frequency and genotype distribution between patients and controls was shown by χ^2 analysis with $\chi^2(1) = 4.3, P < 0.05$, and $\chi^2(2) = 6.2, P < 0.05$, respectively.

groups (Table 1). The odds ratio, a measure of relative risk of myocardial infarction between subjects with the 4G/4G genotype and subjects with the 4G/5G or 5G/5G genotype, was 2.15 with a 95% confidence interval of 1.17–3.96. No differences were found between genotypes in either patients or controls for parameters associated with plasma PAI-1 activity, such as body mass index, plasma lipoprotein concentrations, and basal and postload glucose, insulin, and insulin propeptide levels (data not shown). To our knowledge this is the first demonstration of an association between genotype and CHD for a hemostatic protein and supports the idea that PAI-1 is an etiological factor in myocardial infarction. In an earlier study (11), no significant difference in frequency of the 4G and 5G alleles was demonstrated between long-term survivors of myocardial infarction and matched controls, and the frequency of the 4G allele among patients was lower than in the present study. This discrepancy is not surprising, because of the selection bias inherent in the previous patient group, which was examined 5–7 years after the acute event. The 5-year mortality rate was 20% among those patients (6), and plasma PAI-1 activity has been found to predict recurrences in that particular cohort of young post infarction patients (5, 6). Accordingly, the discrepant allele frequency results between the present study, in which patients were investigated 3–6 months after the myocardial infarction, and that conducted by Dawson *et al.* (11) are likely to reflect the association between the 4G allele and myocardial infarction demonstrated in the present study. Nevertheless, the association between the 4G allele and myocardial infarction needs to be demonstrated in patients who have not been selected because of young age and in women before PAI-1 should be acknowledged as a major etiological factor underlying myocardial infarction in the general population.

The association of the 4G/5G polymorphism of the PAI-1 promoter with altered levels of plasma PAI-1 activity suggests a differential binding of protein(s) regulating the transcriptional activity of the gene. EMSA showed, in accordance with an earlier study (11), that this was indeed the case. Both alleles bound several factors present in a nuclear extract derived from Hep G2 cells, but the 5G allele bound an additional protein (Fig. 2*a*). As demonstrated in this study, only two proteins showed sequence-specific binding to the 4G and 5G sequences (Fig. 2*b*, lanes 1–5). Both alleles bound a common factor (factor A), while the 5G allele bound an additional factor (factor B). Further, these factors were present in liver cells (Hep G2) (Fig. 2*c*, lanes 2 and 6), endothelial cells (HUVECs) (lanes 3 and 7), and smooth muscle cells (lanes 4 and 8), cells that have been proposed as sources of the plasma PAI-1 (28–30). The abundance of the proteins varied. It is, however, questionable whether quantitative comparisons between different cell types are reliable, since the relative concentration/composition of specific/nonspecific proteins may vary between different types of cells when a crude extract preparation is used.

A methylation interference assay was used to map the binding sites of factors A and B on the DNA helix (Fig. 3*a*). Factor A bound upstream and adjacent to the polymorphic site. In contrast, factor B interacted with the sequence of five guanosine residues (−672 to −676) containing the polymorphic site, consistent with B's being the allele-specific factor. A methylation interference assay on the residual bands in the electromobility shift pattern (data not shown) showed no sequence-specific interactions, in accordance with the result in Fig. 2*b*. DNase I footprinting was also used in combination

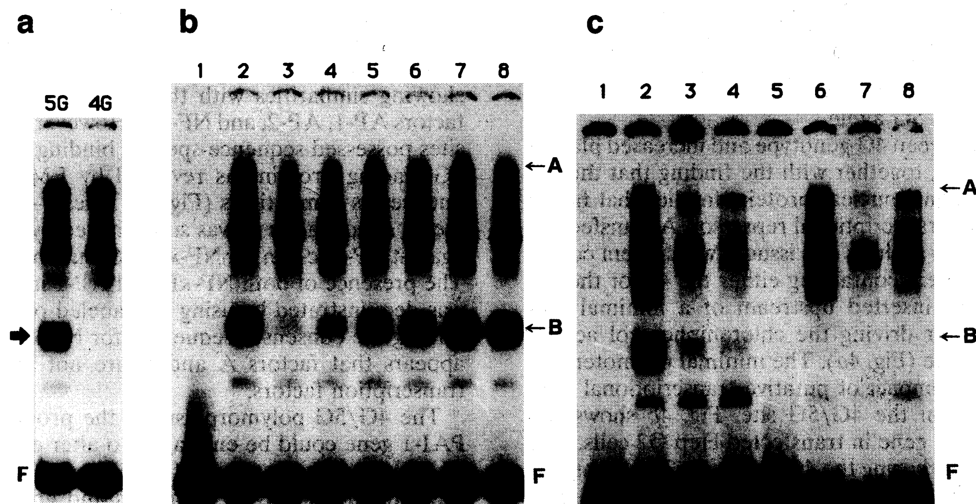


FIG. 2. The 5G allele binds an additional protein. (*a*) EMSA of Hep G2 nuclear extract proteins bound to a 30-bp DNA fragment containing either the 5G or the 4G site of the PAI-1 promoter. Arrow, 5G allele-specific factor; F, free DNA. (*b*) EMSA of HepG2 nuclear extract proteins bound to the 5G site in the presence of a 200-fold excess of various unlabeled DNA competitors. Lane 1, without extract; lane 2, with extract in the absence of competitor; lanes 3–8, with competitor: 5G site, 4G site, nonspecific DNA derived from pGEM-1 vector, NF-κB consensus site, AP-1 consensus site, and AP-2 consensus site, respectively. A and B denote the proteins showing sequence specificity; A refers to the common factor, and B refers to the 5G allele-specific factor. (*c*) EMSA using nuclear extract derived from various types of cells. Lanes 1–4: 5G site incubated with no extract (lane 1) or extract derived from Hep G2 cells (lane 2), HUVECs (lane 3), or smooth muscle cells (lane 4). Lanes 5–8: 4G site incubated with no extract (lane 5) or extract derived from Hep G2 cells (lane 6), HUVECs (lane 7), or smooth muscle cells (lane 8).

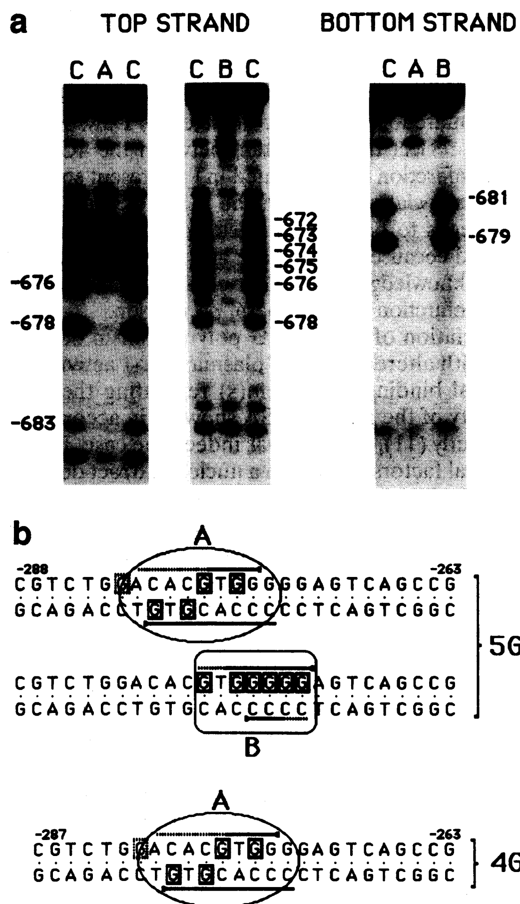
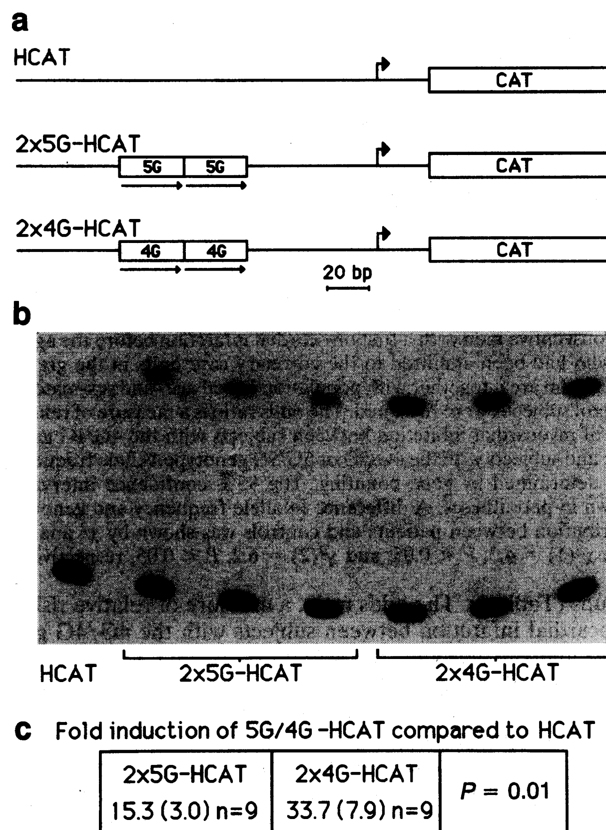


FIG. 3. Factors A and B bind to overlapping sites. (*a*) Methylation interference of factors A and B. C represents free methylated DNA. A and B represent methylated DNA bound to factors A and B. Numbers denote the position of interfering guanosine residues in relation to the start site of transcription. (*b*) Summary of footprinting data indicating the interfering guanine residues by boxed letters. Solid lines refer to the DNase I footprinting pattern; broken lines denote weak DNase I footprinting pattern.



2x5G-HCAT	2x4G-HCAT	P = 0.01
15.3 (3.0) n=9	33.7 (7.9) n=9	

FIG. 4. Factor A constitutes a transcriptional activator activity, whereas factor B interferes with this activity. (*a*) DNA constructs of the minimal and heterologous promoter constituting two copies of either the 4G or the 5G polymorphic site. (*b*) Example of autoradiogram from a CAT assay after transfection of HCAT, 2×5G-HCAT, or 2×4G-HCAT into Hep G2 cells. (*c*) Quantification of nine transfection experiments, showing the mean (and SE); n represents number of experiments. P = 0.01 denotes the result of a paired t test comparing the induction of 2×5G-HCAT with that of 2×4G-HCAT.

with an EMSA to separate the two complexes. The result from this assay (data not shown) was in agreement with the methylation interference pattern. Fig. 3*b* presents a summary of the footprinting data indicating the overlapping binding-regions of factors A and B in the 5G allele.

The association between 4G genotype and increased plasma PAI-1 activity (Fig. 1), together with the finding that the 5G allele binds an additional nuclear protein, implies that factor B could function as a transcriptional repressor. A transfection assay was conducted to address this issue. Two tandem copies of a 30-bp DNA segment containing either the 4G or the 5G allele sequence were inserted upstream of a minimal and heterologous promoter driving the chloramphenicol acetyltransferase (CAT) gene (Fig. 4*a*). The minimal promoter was used to delineate the impact of putative transcriptional activators or repressors on the 4G/5G site. Fig. 4*b* shows the expression of the CAT gene in transfected Hep G2 cells. The promoter construct harboring the 4G site had a 34-fold higher transcriptional activity than the minimal promoter alone (Fig. 4*c*). The 5G sequence also mediated an activator activity, although to a lower extent (15-fold compared with the minimal promoter). The ≈2-fold difference between the activities of the 4G and 5G constructs was statistically significant in a paired t test (P = 0.01). This suggests that factor A is a transcriptional activator whose activity is restricted by factor B, which thus acts as a transcriptional repressor. The binding of factors A and B to overlapping sites on the DNA helix suggests that factor B could decrease the binding of the common factor A by interference due to steric hindrance.

A database search (GENE WORKS 2.2.1; IntelliGenetics) with the binding sequences for the polymorphic region revealed that the binding region of factor B overlaps with regions showing similarities with the binding sites for transcription factors AP-1, AP-2, and NF-κB. However, none of these DNA sites possessed sequence-specific binding affinity for the 4G/5G-binding proteins as revealed by EMSA using these sequences as competitors (Fig. 2*b*, lanes 6–8). Further, neither factor A nor factor B was supershifted by antibodies directed against AP-1 (c-Jun) or NF-κB (p65 subunit) (data not shown). The presence of both NF-κB and AP-1 in the nuclear extract was demonstrated by using ^{32}P-labeled oligonucleotides containing the consensus sequences for NF-κB and AP-1. It thus appears that factors A and B are not identical with these transcription factors.

The 4G/5G polymorphism in the promoter region of the PAI-1 gene could be envisaged to alter either the basal level of PAI-1 or the response of PAI-1 to a specific physiological regulator. A variety of cytokines and growth factors induce PAI-1 production *in vitro* (31). In a previous study using a transfection assay in Hep G2 cells (11), it was suggested that the entire PAI-1 promoter responded to interleukin 1α (IL-1α) in an allele-specific manner. Incubation of the cells with IL-1α resulted in a 6-fold higher activity of the PAI-1 promoter containing the 4G allele compared with unstimulated controls, whereas the 5G construct did not respond to IL-1α. However, using a promoter fragment comprising the PAI-1 sequence

−804 to +17 of either genotype inserted into a pCAT-Basic vector (Promega), we were unable to reproduce these results. Both constructs, when transfected into either Hep G2 cells or HUVECs, responded to neither IL-1α nor IL-1β at 10–100 units/ml (data not shown). In accordance with earlier studies (32–34), both alleles responded to transforming growth factor β (TGFβ) at 5 ng/ml (4.3 ± 1.9-fold higher activity, mean ± SD, $n = 7$). This finding is also in contrast to the results of Dawson *et al.* (11), who found that neither the 4G nor the 5G allele responded to TGFβ. They demonstrated a TGFβ response in the 5G allele only when the cells were simultaneously stimulated by IL-1α, whereas the IL-1α-induced 4G allele expression was not further enhanced by TGFβ. In addition to the TGFβ response, our CAT constructs responded to phorbol 12-myristate 13-acetate at 42 ng/ml (2.8- and 5.3-fold higher activity, $n = 2$) (data not shown), which also is in accordance with an earlier study (35). EMSA was used to further study potential cytokine-induced activation of the transcription factors binding to the 4G/5G polymorphic site. However, nuclear extracts derived from cells stimulated by IL-1α or IL-1β did not show different binding patterns when compared with extracts derived from unstimulated control cells (data not shown). Thus, no evidence was obtained in this study for an allele-specific response of the 4G/5G polymorphism in either Hep G2 cells or HUVECs. Use of different Hep G2 strains may account for the discrepancy between our results and those of Dawson *et al.* (11). Since Hep G2 cells and HUVECs behaved identically in our experiments and in accordance with previous studies (32–35), we are inclined to conclude that the 4G/5G polymorphism is not related to an allele-specific response to IL-1α or IL-1β. Instead, the genotype-specific difference in plasma PAI-1 activity, together with the results from the transfection studies using the minimal promoter, suggests that the 4G/5G polymorphism of the PAI-1 promoter influences the basal level of transcription.

Since the discovery of PAI-1 in 1983 (13, 36), an abundance of cross-sectional studies of patients with myocardial infarction, angina pectoris, or angiographically ascertained significant coronary artery disease have linked increased PAI-1 to CHD (1). However, results from prospective studies of initially healthy individuals which include determinations of PAI-1 are lacking at this stage. The support for a cause-and-effect relationship between raised plasma PAI-1 activity and myocardial infarction is currently confined to a few longitudinal cohort studies of patients with manifest CHD (5, 6) or peripheral atherosclerosis (7). However, it could be argued that studies of patients with CHD are confounded by persisting acute-phase reactions, changes in lifestyle and body weight, and multiple disturbances of lipoprotein and carbohydrate metabolism that could result in spurious associations. Accordingly, a raised plasma PAI-1 level, although linked to CHD and presence of coronary artery disease, may not constitute an independent risk factor. In this context, the demonstration in the present study of an association between the 4G/5G polymorphism in the PAI-1 promoter and myocardial infarction and of an influence of this polymorphism on basal PAI-1 transcription provides strong evidence in favor of an independent, etiological role of PAI-1 in CHD.

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