Two Common, Functional Polymorphisms in the Promoter Region of the β-Fibrinogen Gene Contribute to Regulation of Plasma Fibrinogen Concentration

Ferdinand M. van 't Hooft, Sara J. F. von Bahr, Angela Silveira, Anastasia Iliadou, Per Eriksson, Anders Hamsten

Abstract—Plasma fibrinogen is a major risk factor for coronary heart disease, stroke, and peripheral artery disease. There is evidence that genetic variation in the β-fibrinogen gene contributes to the rate of synthesis of fibrinogen, but the molecular mechanism underlying the genetic heritability of the plasma fibrinogen concentration is largely unknown. We evaluated the physiological roles of 5 common nucleotide substitutions in the promoter region of the β-fibrinogen gene at positions −148, −249, −455, −854, and −993 from the transcriptional start site. Electrophoretic mobility shift assays revealed distinct differences in the binding characteristics of nuclear proteins between wild-type and mutant fragments of both the −455G/A and −854G/A polymorphisms, whereas no clear differences were observed for the −148C/T, −249C/T, and −993C/T sites. Transfection studies in HepG2 cells showed increased basal rates of transcription for both the G-to-A substitution at position −455 (+50%, P<0.05) and the G-to-A substitution at −854 (+51%, P<0.05). Additional transfection studies using proximal promoter constructs confirmed that both the −455A and −854A alleles independently enhance the basal rate of transcription of the β-fibrinogen gene. The rare alleles of the nonrelated −455G/A and −854G/A polymorphisms were also associated with significantly increased plasma fibrinogen levels in healthy middle-aged men. Overall, the 2 polymorphisms together explained ≈11% of the variation in plasma fibrinogen concentration. It is concluded that the −455G/A and −854G/A polymorphisms of the β-fibrinogen gene are physiologically relevant mutations with a significant impact on the plasma fibrinogen concentration. (Arterioscler Thromb Vasc Biol. 1999;19:3063-3070.)

Key Words: DNA ■ fibrinogen ■ β-fibrinogen gene ■ −455G/A polymorphism ■ −845G/A polymorphism

The notion that fibrinogen is related to cardiovascular disease was originally voiced in the late 1950s, when increased plasma fibrinogen levels were found in patients with coronary heart disease.1,2 Since then, substantial epidemiological evidence has accumulated suggesting that the plasma fibrinogen concentration is a major risk factor for coronary heart disease,3–7 (for review, see Ernst and Resch8), stroke,3,9 and peripheral artery disease.10 However, it remains to be established whether plasma fibrinogen elevation is a cause or consequence of atherosclerotic disease.8

An elevated plasma fibrinogen concentration in an otherwise healthy individual could be due to the presence of predisposing environmental and/or genetic factors. There is general agreement that smoking is an important environmental factor associated with increased plasma fibrinogen levels.3,5,11,12 In addition, there is evidence that gender, age, body mass index, and plasma lipoprotein levels may affect the plasma fibrinogen concentration.11 However, there are conflicting reports regarding the role of specific genetic components in determining plasma fibrinogen levels. Some investigators14–16 have reported a substantial contribution of genetic effects on plasma fibrinogen levels, whereas others17,18 have reported only minimal genetic influences on plasma fibrinogen levels.

Human fibrinogen is composed of 3 pairs of nonidentical polypeptide chains, denoted α, β, and γ, assembled in a bilaterally symmetrical arrangement connected by disulfide bonds. The 3 chains of fibrinogen are encoded by 3 independent genes grouped in a cluster of ≈50 kb on chromosome 4 at 4q23 to 32.19 It has been demonstrated that synthesis of the β-fibrinogen protein in hepatocytes is the rate-limiting step in the overall synthesis of the mature fibrinogen protein.20 Several recent studies have therefore evaluated potential associations between polymorphisms in the β-fibrinogen gene and plasma fibrinogen levels. At least 10 common polymorphisms have been described.10,21–23 Several of these are associated with increased plasma fibrinogen concentrations, suggesting that genetic variation in the β-fibrinogen gene can contribute to the regulation of plasma fibrinogen levels. However, it was also observed that some of these polymorphisms are in linkage disequilibrium,23,24 which makes it difficult to determine the physiological significance of the different mutations.
In the study described here, we screened the proximal promoter of the human β-fibrinogen gene in search of common genetic variants with distinct effects on the transcriptional activity of the gene. We demonstrate that 2 nonrelated polymorphisms in the promoter of the β-fibrinogen gene (the G-to-A substitution at position −455 and the G-to-A substitution at −854) have a substantial impact on the basal rate of transcription of the gene encoding the β-fibrinogen chain in vitro. The rare alleles of both polymorphisms are associated with a significantly increased plasma fibrinogen concentration in middle-aged men. Overall, the 2 polymorphisms explained ~11% of the variation in plasma fibrinogen level. It is concluded that the −455G/A and −854G/A polymorphisms are physiologically relevant mutations with a significant impact on the plasma fibrinogen concentration.

Methods

Subjects
A total of 210 men aged 35 to 50 years (mean±SD, 44.9±5.3 years) were studied. They were recruited randomly from a register of all permanent residents of Stockholm County, Sweden. Of those initially invited, 81% agreed to participate in the research program. All subjects were interviewed to exclude individuals with a history of cardiovascular disease. Additional exclusion criteria were the presence of severely impaired renal function, arteritis, collagenosis, or diabetes mellitus; a history of alcohol abuse or other forms of addiction; and non-Swedish origin.

Blood Sampling, Biochemical Methods, and DNA Procedures
Blood sampling, preparation of plasma, and quantification of major fasting plasma lipoproteins were performed as described by Moor et al.25 Plasma fibrinogen was measured as described by Clauss26 using fasting plasma lipoproteins were performed as described by Moor et al.25 Plasma fibrinogen was measured as described by Clauss26 using.

Gene Sequencing
For nucleotide sequencing of the promoter of the β-fibrinogen gene, 19 were used for analysis of overlapping sections of 200 to 300 bp in both directions. Oligonucleotides were designed to represent the 30-bp sequences around the polymorphic region. Pairs of oligonucleotides were ligated head to tail into a pCAT basic vector as described by the supplier (Promega Corporation). Promoter fragments were obtained by PCR amplification of DNA samples from subjects homozygous for the various polymorphisms using the forward primer 5′-AACTGAGACCTCTGATAAACC-TGCCATC and reverse primer 5′-GCTCTAGAAGAGCTCCAAGAACCACATCC. The correct sequence and orientation of the inserts were tested by DNA sequencing.

Electrophoretic Mobility Shift Assay
Nuclear extracts were prepared according to the method of Alksnis et al.29 All buffers were freshly supplemented with leupeptin (0.7 μg/mL), aprotinin (16.6 μg/mL), PMSF (0.2 mmol/L), and 2-mercaptoethanol (0.33 μL/mL). The protein concentration in extracts was estimated by the method of Kalb and Bernlohr.30 Oligonucleotides were designed to represent the 30-bp sequences around the polymorphic region. Pairs of oligonucleotides were annealed at equimolar ratios. The double-stranded oligonucleotides were end-labeled using the T4 polynucleotide kinase method.27 Incubation for the electrophoretic mobility shift assay (EMSA) was conducted as described by Dawson et al.,32 and the reaction products were applied to 7% (wt/vol) polyacrylamide gel (80:1 acrylamide/N,N′-methylene-bisacrylamide weight ratio), after which electrophoresis was performed in a solution of 22.5 mmol/L Tris, 22.5 mmol/L boric acid, and 0.5 mmol/L EDTA buffer for 2.5 hours at 200 V. Nonradioactive competitor DNAs, either identical, of the opposite allelic variant, or of nonspecific origin, were added in 100-fold excess of the labeled DNA.

The −455G/A and −854G/A polymorphisms were analyzed using 2 sets of oligonucleotides and 4 annealing reactions in more than 10 EMSAs.

Transient Transfection Assay
Human hepatoblastoma (HepG2) cells were cultured in 90-mm dishes in DMEM supplemented with 10% FCS. Confluent cells were transfected using the calcium-phosphate DNA coprecipitation method essentially as described by Sambrook et al.24 The pSV–β-galactosidase gene (Promega) was cotransfected as an internal control. In all experiments, 5 μg of CAT construct and 5 μg of β-galactosidase plasmid were added to the medium. CAT activity was analyzed as described by Sambrook et al.24 and quantified using a PhosphorImager (bioimaging analyzer BAS-2500, Fuji Photo Film Co). β-Galactosidase activity was determined according to the supplier’s instructions (Promega). CAT levels were expressed in arbitrary units after standardization for β-galactosidase activity. All constructs were tested in triplicate in 4 independent transfection experiments using 2 plasmid preparations.

Statistical Methods
Distribution of continuous variables in groups was expressed as mean±SD or mean±SEM. Logarithmic transformation was performed on all skewed variables to obtain a normal distribution before statistical computations and significance testing were performed. Allele frequencies were estimated by gene counting. A χ2 test was used to compare the observed numbers of each β-fibrinogen genotype with those expected for a population in Hardy-Weinberg equilibrium. The normalized linkage disequilibrium coefficient (D′) for the −455G/A and −854G/A polymorphisms was calculated according to the method of Ott.33 One-way ANOVAs (with age or age and smoking as covariates) and 2-way ANOVAs performed by the general linear model procedure were performed to test whether genetic variation within the β-fibrinogen promoter was associated...
polymorphic sites.

When comparisons of fibrinogen concentrations were observed in several subjects, differences were noted. Five common nucleotide substitutions in DNA samples from 24 subjects with a broad range of plasma fibrinogen concentrations were evaluated using nuclear extracts derived either from HepG2 cells bound to a 30-bp DNA fragment containing the wild-type or the mutant site for the promoter of the β-fibrinogen gene might affect the transcriptional activity between promoter constructs. EMSA studies showed that the 1109-bp section of the proximal promoter of the β-fibrinogen gene was sequenced in both directions using DNA samples from 24 subjects with a broad range of plasma fibrinogen concentrations. Five common nucleotide substitutions were observed in several subjects when comparisons were made with the published nucleotide sequence: a C-to-T substitution at position −148, a C-to-T substitution at position −249, a G-to-A substitution at position −455, a G-to-A substitution at position −854, and a C-to-T substitution at position −993.

**Results**

**Detection of Common Nucleotide Substitutions in the β-Fibrinogen Promoter**

A 1109-bp section of the proximal promoter of the β-fibrinogen gene was sequenced in both directions using DNA samples from 24 subjects with a broad range of plasma fibrinogen concentrations. Five common nucleotide substitutions were observed in several subjects when comparisons were made with the published nucleotide sequence: a C-to-T substitution at position −148, a C-to-T substitution at position −249, a G-to-A substitution at position −455, a G-to-A substitution at position −854, and a C-to-T substitution at position −993.

**Allele-Specific Binding of 1 or More Nuclear Proteins to the −455G/A and −854G/A Polymorphic Sites**

We explored the possibility that the nucleotide substitutions in the promoter of the β-fibrinogen gene might affect the interaction with nuclear proteins by using an EMSA. The binding characteristics of 30-bp DNA fragments containing either the wild-type or the mutant site for the 5 polymorphisms were evaluated using nuclear extracts derived from HepG2 cells. No differences were observed between the binding characteristics of the wild-type and the mutant DNA fragments of the −148C/T, −249C/T, −993C/T polymorphisms (data not shown). In contrast, distinct differences between the binding patterns of the wild-type and mutant DNA fragments were observed for the −455G/A and −854G/A polymorphisms.

As indicated by the arrows in Figure 1, 1 major and 1 minor protein-DNA complex were found to be associated with the −455G allele. The major protein-DNA complex with differences in plasma fibrinogen concentration. The Scheffé multiple comparisons test was used as a post hoc test. The percentage of genotype-based variation in plasma fibrinogen concentration was calculated according to the method of Sing and Davignon.

**Figure 1.** Difference in the binding of nuclear proteins to the −455G and −455A alleles. EMSA of nuclear extract derived from HepG2 cells bound to a 30-bp DNA fragment containing either the −455G (lanes 1 to 4) or −455A site (lanes 5 to 8) of the β-fibrinogen promoter. Lanes 1 and 5, without extract; lanes 2 and 6, 0.05 mg/mL HepG2 extract; lanes 3 and 7, 0.10 mg/mL HepG2 extract; and lanes 4 and 8, 0.20 mg/mL HepG2 extract. Arrows 1 and 2 indicate the protein-DNA complexes associated with the −455G/A site.

**Figure 2.** Binding of nuclear protein to the −455G allele is specific. EMSA of nuclear extract derived from HepG2 cells bound to the −455G allele (lane 6) or the −455G allele in the presence of unlabeled DNA as competitor (lanes 1 to 5). Lane 1, without extract; lanes 2 and 6, 0.20 mg/mL HepG2 extract in the absence of competitor; and lanes 3 to 5, 0.20 mg/mL HepG2 extract in the presence of a 100-fold excess of unlabeled DNA as competitor. Competitors used were −455G site (lane 3), −455A site (lane 4), and nonrelated 30-bp fragment (lane 5). Arrows 1 and 2 indicate the protein-DNA complexes associated with the −455G/A site.

(Figure 1, arrow 2) was not detected when the −455A allele was analyzed. In contrast, substantial quantities of protein-DNA complex 1 (Figure 1, arrow 1) were observed in association with the −455A allele. There was a distinct difference in size between protein-DNA complexes 1 and 2, as can be seen more clearly in Figure 2 (compare lanes 2 to 5 and 6). Competition studies showed that a 100-fold excess of unlabeled −455G fragment substantially reduced the interaction of the labeled −455G fragment with the nuclear proteins (Figure 2, lane 3). In contrast, no clear reductions were observed for the major protein-DNA complex when a 100-fold excess of unlabeled −455A fragment (Figure 2, lane 4) or unrelated DNA fragment (Figure 2, lane 5) was added as competitor for the binding of labeled −455G fragment to the nuclear proteins.

Three protein-DNA complexes were found to be associated with the −854G-allele (Figure 3A and 3B, complexes 1 to 3). However, only 1 of the protein-DNA complexes was observed in relation to the −854A allele (Figure 3A and 3B, complex 2). A 100-fold excess of unlabeled −854G fragment effectively reduced the interaction of the labeled −854G fragment with the nuclear proteins (Figure 4, lane 3). In contrast, a 100-fold excess of unlabeled −854A resulted in a reduction in protein-DNA complex 2 only, whereas no changes were observed in protein-DNA complexes 1 and 3 (Figure 4, lane 4).

Similar results were observed with 2 sets of DNA fragments, generated from oligonucleotides purchased from different commercial suppliers and annealed in 4 reactions. Moreover, comparable results were obtained when the binding characteristics of the 3 nuclear extracts were analyzed. Together, the results of the EMSA studies indicate that the nucleotides at positions −455 and −854 of the promoter of the β-fibrinogen gene are involved in the binding of several different nuclear proteins. The EMSA studies also provided strong evidence of distinct differences between the wild-type
Polymorphisms Increase Transcription of the \(-854G/A\) and \(-455G/A\) sites of the \(\beta\)-fibrinogen promoter. Lanes 1 and 6, without extract; lanes 2 and 7, 0.05 mg/mL HepG2 extract; lanes 3 and 8, 0.10 mg/mL HepG2 extract; lanes 4 and 9, 0.15 mg/mL HepG2 extract; and lanes 5 and 10, 0.20 mg/mL HepG2 extract. The vertical bar indicates the section of the gel enlarged in B. B, Enlarged sections of the gel shown in A: lane 5 for \(-854G\) and lane 9 for \(-854A\). Arrows 1 to 3 indicate the protein-DNA complexes associated with the \(-854G/A\) site.

Figure 3. Difference in the binding of nuclear proteins to the \(-854G\) and \(-854A\) alleles. A, EMSA of nuclear extract derived from HepG2 cells bound to a 30-bp DNA fragment containing either the \(-854G\) (lanes 1 to 5) or \(-854A\) site (lanes 6 to 10) of the \(\beta\)-fibrinogen promoter. Lanes 1 and 6, without extract; lanes 2 and 7, 0.05 mg/mL HepG2 extract; lanes 3 and 8, 0.10 mg/mL HepG2 extract; lanes 4 and 9, 0.15 mg/mL HepG2 extract; and lanes 5 and 10, 0.20 mg/mL HepG2 extract. The statistical significance of differences was determined by using Student’s paired \(t\) test.

and mutant DNA fragments of both the \(-854G/A\) and \(-854G/A\) polymorphisms regarding the specific binding of 1 or more nuclear proteins.

Rare Alleles of the \(-854G/A\) and \(-854G/A\) Polymorphisms Increase Transcription of the \(\beta\)-Fibrinogen Gene

Transfection studies in HepG2 cells were conducted to explore whether the \(-854G/A\) and \(-854G/A\) polymorphisms influence the rate of transcription of the \(\beta\)-fibrinogen gene. CAT activities were compared between promoter constructs harboring a 30-bp fragment of the \(\beta\)-fibrinogen promoter containing either the wild-type or mutant sites of the \(-854G/A\) and \(-854G/A\) polymorphisms upstream of a minimal and heterologous promoter coupled to a CAT reporter gene. As shown in Figure 5, significantly higher CAT activities were observed for the \(-455A\) construct than for the \(-455G\) construct (150±11%, \(P<0.005\)). Moreover, as shown in Figure 6, significantly higher CAT activities were also observed for the \(-854A\) construct than for the \(-854G\) construct (151±26%, \(P<0.05\)).

The rates of transcription of 1104-bp fragments of the proximal promoter spanning from \(-1069\) to +35 of the \(\beta\)-fibrinogen gene containing either the \(-249T\) site, the \(-854A\) site, or the haplotype combination of the \(-148T/-455A/-993T\) sites were compared with a fragment containing the wild-type promoter. As shown in Figure 7, comparable CAT activities were observed for the wild-type promoter and the promoter containing the \(-249T\) site (94±17%). In contrast, both the plasmid containing the \(-854A\) site (134±21%, \(P<0.05\)) and the plasmid containing the \(-148T/-455A/-993T\) haplotype (163±30%, \(P<0.05\)) had significantly higher CAT activities than the wild-type
plasmid. Together, the results from the transfection studies indicate that both the G-to-A substitution at the 249T site, the haplotype combination of the −148T/−455A/−993T sites (indicated as −455A), or the −854A site were compared with a fragment containing the wild-type (WT) promoter. Constructs were tested in triplicate in 4 independent experiments in transfection studies using HepG2 cells. CAT activities of the constructs were expressed relative to the activity of the wild-type construct. Bars indicate mean values with SDs. The statistical significance of differences was determined by Student’s paired t test.

Allele Frequencies and Degree of Linkage Disequilibrium
Genotyping for the −148C/T, −249C/T, −455G/A, and −854G/A polymorphisms was performed in 210 healthy, population-based men aged 35 to 50 years. All polymorphisms were found to be in Hardy-Weinberg equilibrium. The frequencies of the rare −148T, −249T, −455A, and −854A alleles were 21.4%, 20.2%, 21.4%, and 18.6%, respectively. Complete allelic association was observed between the −148C/T and −455G/A polymorphisms. In contrast, the −249C/T polymorphism was in complete negative linkage disequilibrium with the −148C/T and −455G/A polymorphisms. The normalized linkage disequilibrium coefficient (D') was 0.27 (P<0.001) for the 2 functional −455G/A and −854G/A polymorphisms. In fact, the 2 polymorphisms were in complete negative linkage disequilibrium in this population; eg, the rare −854A allele was always present together with the common −455G allele and vice versa.

Associations Between the −455G/A and −854G/A Polymorphisms and Plasma Fibrinogen Concentration
The relationships between the −455G/A and −854G/A polymorphisms and plasma fibrinogen concentration were determined in the 210 healthy, population-based middle-aged men. As shown in Table 1, the rare −455A and −854A alleles were associated with significantly higher plasma fibrinogen levels than the common −455G and −854G alleles. Furthermore, subjects who were homozygous for the −455A or −854A allele had higher plasma fibrinogen concentrations than subjects who were heterozygous for these alleles. Smoking had a significant effect on plasma fibrinogen level. Overall, smokers (n=75) and nonsmokers (n=135) had plasma fibrinogen concentrations of 2.89±0.45 and 2.72±0.43 g/L (mean±SD, P<0.001), respectively. No specific interactions between smoking and either the −455G/A or −854G/A polymorphism were observed (data not shown).

The influences of the −455G/A and −854G/A polymorphisms on the plasma fibrinogen concentration were about equally strong, accounting for 5.4% and 4.4% of the variation in plasma fibrinogen, respectively (Table 1), when the effects of age and smoking habits were considered in the ANCOVA. To further determine the impact of genetic variation on fibrinogen levels in plasma, genotypes for the 2 polymorphisms were defined and ranked in order of mean variation in plasma fibrinogen, respectively (Table 1), when the effects of age and smoking habits were considered in the ANCOVA. To further determine the impact of genetic variation on fibrinogen levels in plasma, genotypes for the 2 polymorphisms were defined and ranked in order of mean fibrinogen values (Table 2). The highest plasma fibrinogen concentrations were encountered in subjects who were homozygous for the −455G and −854A alleles. ANCOVA controlling for the influence of age and smoking habits showed that the mean differences in fibrinogen level between

**Table 1. Associations Between −455G/A and −854G/A Polymorphisms in Promoter Region of β-Fibrinogen Gene Locus and Plasma Fibrinogen Concentrations in Healthy Middle-Aged Men**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Polymorphism</th>
<th>Phenotype Variation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>−455</td>
<td>−455G/G</td>
<td>−455G/A</td>
</tr>
<tr>
<td>n</td>
<td>133</td>
<td>64</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>2.70±0.43</td>
<td>2.88±0.46*</td>
</tr>
<tr>
<td>−854</td>
<td>−854G/G</td>
<td>−854G/A</td>
</tr>
<tr>
<td>n</td>
<td>138</td>
<td>66</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>2.73±0.46</td>
<td>2.85±0.40</td>
</tr>
</tbody>
</table>

Values are mean±SD. One-way ANCOVA (with age and smoking habits as covariates) was performed to test whether genetic variation within the β-fibrinogen promoter was associated with differences in plasma fibrinogen concentration. The percentage of genotype-based variation in fibrinogen levels was calculated according to the method of Sing and Davignon.34 The Scheffe multiple comparisons test was used as a post hoc test to assess differences between genotype groups.

*P<0.05 compared with homozygotes for the common allele.
TABLE 2. Mean Plasma Fibrinogen Concentrations in Groups of Healthy Middle-Aged Men With Different Combinations of \(\beta\)-Fibrinogen \(-455G/A\) and \(-854G/A\) Genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Fibrinogen, g/L</th>
<th>Covariance Analysis</th>
<th>Phenotype Variation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>((-455G/A))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>((-854G/A))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG/AA</td>
<td>2.9</td>
<td>3.20 (\pm) 0.16*</td>
<td>8.07</td>
</tr>
<tr>
<td>AA/GG</td>
<td>6.2</td>
<td>3.07 (\pm) 0.11*</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>GA/GA</td>
<td>8.1</td>
<td>3.02 (\pm) 0.10*</td>
<td></td>
</tr>
<tr>
<td>GG/GG</td>
<td>22.4</td>
<td>2.62 (\pm) 0.06</td>
<td></td>
</tr>
<tr>
<td>GG/GA</td>
<td>3.3</td>
<td>2.78 (\pm) 0.06</td>
<td></td>
</tr>
<tr>
<td>AA/GG</td>
<td>37.1</td>
<td>2.62 (\pm) 0.05</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean \(\pm\) SEM. One-way ANCOVA (with age and smoking habits as covariates) was performed to test whether genetic variation within the \(\beta\)-fibrinogen promoter was associated with differences in plasma fibrinogen concentration. The percentage of genotype-based variation in fibrinogen levels was calculated according to the method of Sing and Davignon. The Scheffe multiple comparisons test was used as a post hoc test to assess differences between genotype groups.

\*\(P<0.05\) compared with the \(-455GG/-854GG\) genotype.

combined genotypes were highly significant (Table 2). The genetic variation associated with the joint \(-455G/A\) and \(-854G/A\) polymorphism accounted for 11.3% of the variation in plasma fibrinogen concentration.

**Discussion**

The primary aim of this study was to define functional polymorphisms in the human \(\beta\)-fibrinogen gene to elucidate the molecular mechanism underlying the genetic heritability of the plasma fibrinogen concentration. Because the synthesis of the \(\beta\) chain is the rate-limiting step in the production of the mature fibrinogen molecule, we concentrated our analysis on the proximal promoter of the \(\beta\)-fibrinogen gene. Five common polymorphisms were detected: \(-148C/T\), \(-249C/T\), \(-455G/A\), \(-854G/A\), and \(-993C/T\). The \(-148C/T\), \(-249C/T\), \(-455G/A\), and \(-854G/A\) polymorphisms have been described previously, whereas the \(-993C/T\) polymorphism, to the best of our knowledge, has not been reported previously. Strong linkage disequilibrium between the \(-148C/T\) and \(-455G/A\) polymorphisms has been demonstrated by several groups. The \(-854G/A\) polymorphism, on the other hand, which was recently described by Behague et al., was found to be in complete negative linkage disequilibrium with the \(-148C/T/-455G/A\) polymorphisms; eg, the rare \(-854A\) allele was always present with the common \(-455G\) allele, and vice versa, in the population examined in this study.

EMSA studies were conducted as an initial step in the evaluation of the physiological relevance of the 5 promoter polymorphisms. No differences in the binding patterns of the wild-type and mutant probes for the \(-148C/T\), \(-249C/T\), and \(-993C/T\) polymorphisms were observed, suggesting that these polymorphisms do not affect the rate of transcription of the \(\beta\)-fibrinogen gene. In contrast, significant changes were found in the complex binding patterns of the wild-type and mutant probes for both the \(-455G/A\) and \(-854G/A\) polymorphisms. These allele-specific differences were consistently observed using different probes and different nuclear extracts from HepG2 cells. Moreover, competition studies provided evidence that the changes in binding patterns are related to the specific interaction of several protein-DNA complexes. Together, the results from several protein-DNA complexes. Together, the results from several protein-DNA complexes. Together, the results from several protein-DNA complexes. Together, the results from several protein-DNA complexes. Together, the results from several protein-DNA complexes. Together, the results from several protein-DNA complexes. Together, the results from several protein-DNA complexes.

The physiological roles of \(-455G/A\) and \(-854G/A\) polymorphisms were subsequently analyzed in transfection assays. These studies demonstrated that the basal rates of transcription in HepG2 cells for minimal promoter constructs containing either of the rare \(-455A\) or \(-854A\) sites were higher than the transcriptional activity of constructs containing the wild-type \(-455G\) and \(-854G\) sites, respectively. Essentially similar results were obtained with CAT constructs containing a 1104-bp section of the proximal promoter of the \(\beta\)-fibrinogen gene. These results indicate that the rare alleles of the \(-455G/A\) and \(-854G/A\) polymorphisms are both associated with an increase in the basal rate of transcription of the \(\beta\)-fibrinogen gene. The nature of the nuclear proteins involved in these protein-DNA complexes and the detailed molecular mechanisms remain to be defined. The present data suggest that the nuclear protein contained in complex 1 that binds preferentially to the \(-455A\) allele is either a stronger transcriptional activator than the nuclear protein contained in complex 2 or that complex 2 acts as a transcriptional repressor. For the \(-854 site, on the other hand, it appears that the nuclear proteins contained in complexes 1 and 3 are transcriptional repressors, interfering with the binding of the nuclear protein contained in complex 2, which acts as a transcriptional activator.

There is evidence that the plasma fibrinogen level is influenced by factors like sex, age, chronic disorders (eg, coronary heart disease, hypertension, and diabetes mellitus), and possibly racial background. Potential associations between the \(-455G/A\) and \(-854G/A\) polymorphisms and plasma fibrinogen concentrations were therefore evaluated in a well-defined group of apparently healthy, middle-aged men of Swedish origin. In this population, the \(-455G/A\) and \(-854G/A\) polymorphisms were independently associated with plasma fibrinogen concentration and explained 11.3% of the variation in plasma fibrinogen levels.

A significant relationship between the \(-455G/A\) polymorphism and plasma fibrinogen levels has been documented in several reports. It appears from these studies that the overall impact of the \(-455G/A\) polymorphism on the plasma fibrinogen concentration is rather small. For instance, investigators from the Copenhagen City Heart Study reported that the \(-455G/A\) polymorphism explained only 1% of the variation in plasma fibrinogen level. The relationship between the \(-854G/A\) polymorphism and plasma fibrinogen level, on the other hand, has thus far been evaluated in only 1 study. It was reported that the effect of the \(-854G/A\) polymorphism was insignificant in univariate analysis but became significant after adjustment for the \(-455G/A\) polymorphism. Previous reports thus suggest that the impact of the \(-455G/A\) and \(-854G/A\) polymorphisms on the plasma fibrinogen concentration is limited. However, in our study, we found a significant contribution of both the \(-455G/A\) and
−854G/A polymorphisms to the plasma fibrinogen concentration. Moreover, the 2 polymorphisms together explained ≈11% of the plasma fibrinogen concentration. This indicates that the impact of the −455G/A and −854G/A polymorphisms on the plasma fibrinogen concentration may be larger than previously reported. It is likely that discrepancies between studies are in part due to differences in selection criteria of the populations analyzed. However, it is noteworthy that in the only study in which the 2 polymorphisms were compared directly,23 it was found that the −455G/A and −854G/A polymorphisms were incompletely dissociated and not in complete allelic dissociation as was observed in this study. This points to subtle genetic differences between Swedish subjects and other European populations, although inaccuracies in genotyping may have also contributed to this phenomenon.

It seems reasonable to assume that the observations described above regarding the −455G/A and −854G/A polymorphisms are interrelated and part of a sequence of events starting at the level of the β-fibrinogen gene and ultimately leading to increased plasma fibrinogen concentration. We thus propose that the 2 mutations affect the binding of hepatic nuclear factors to the promoter of the β-fibrinogen gene, resulting in an increase in β-fibrinogen transcription and enhanced secretion of mature fibrinogen by the liver, ultimately leading to increased plasma fibrinogen levels. However, it must be stressed that we cannot formally exclude the possibility that other mutations, linked to either the −455G/A or −854G/A polymorphism, may influence the metabolism of fibrinogen.

Acknowledgments

We thank Karin Husman, Tobias Söderman, and Mårten Toverud for their help with DNA isolation and genotyping. This study was supported by the Swedish Medical Research Council (grant 8691), the Swedish Heart-Lung Foundation, the Marianne and Marcus Wallenberg Foundation, the European Commission (grant BMH4CT96-0272), the Petrus and Augusta Hedlund Foundation, the King Gustaf V 80th Birthday Foundation, and the Foundation for Old Servants. Dr Eriksson holds a postdoctoral research fellowship from the Swedish Medical Research Council.

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Ferdinand M. van 't Hooft, Sara J. F. von Bahr, Angola Silveira, Anastasia Iliadou, Per Eriksson and Anders Hamsten

doi: 10.1161/01.ATV.19.12.3063
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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