

A Hepatocyte Nuclear Factor-3 Site in the Fibrinogen β Promoter Is Important for Interleukin 6-induced Expression, and Its Activity Is Influenced by the Adjacent $-148C/T$ Polymorphism*

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An elevated plasma fibrinogen level is an independent risk factor for cardiovascular disease. Therefore, an understanding of the regulation of fibrinogen expression is important. Inflammation and genetic variation of the fibrinogen β gene regulate plasma fibrinogen levels, and there are indications that inflammation and genetic variation interact. The aim of our study was to gain more understanding of the regulation of the inflammatory response of the fibrinogen β gene and to determine the effects of genetic variation. Luciferase reporter gene assays in hepatoma cells, mutation analysis, and electrophoretic mobility shift assays were used to investigate the transcriptional regulation of the fibrinogen β promoter. We identified a hepatocyte nuclear factor-3 (HNF-3) site located just upstream of previously identified interleukin-6 (IL6)-responsive sequences. This HNF-3 site is essential for a full response of the promoter to IL6, which is a new function for HNF-3. The activity of the CCAAT box/enhancer-binding protein site (located 18 nucleotides downstream of the HNF-3 site and important to the IL6 response) depends on the integrity of the HNF-3 site and vice versa, explaining the necessity of HNF-3 in the IL6 response of the fibrinogen β promoter. Furthermore, small interfering RNA to HNF-3 reduces the fibrinogen β mRNA levels. The rare T allele of the $-148C/T$ polymorphism, which is present between the binding sites of HNF-3 and CCAAT box/enhancer-binding protein, interferes with this mechanism, and this polymorphism is in our assay system the only genetic determinant of IL6-induced promoter activity among six polymorphisms in the fibrinogen β promoter.

Inflammation is an important process in the development of cardiovascular disease, and increased plasma levels of inflammatory factors such as fibrinogen are consistently associated with an increased risk of cardiovascular disease (1–4). Inflammatory factors may play a dual role. On the one hand their plasma levels reflect the severity of inflammatory processes in the vessel wall, and on the other hand they can contribute

directly to the development of the disease. Fibrinogen is present in the atherosclerotic plaque where it can contribute to the progression of atherosclerosis, for example by increasing the chemotaxis of smooth muscle cells and affecting the stability and structure of the plaque (5–8). Because of the relationship of fibrinogen with atherosclerosis, much attention has been paid to the regulation of fibrinogen levels under both basal and inflammatory conditions.

The mature fibrinogen molecule is composed of three pairs of polypeptide chains: two α chains, two β chains, and two γ chains; and *in vitro* functional studies showed that synthesis of the β chain is rate-limiting (9). Fibrinogen is expressed by the liver. Fibrinogen levels can strongly increase in response to intense acute phase stimuli such as trauma, surgery, or strenuous exercise, and fibrinogen levels are chronically elevated in the presence of mild (inflammatory) stimuli such as smoking or severe atherosclerosis (10–12). Interleukin-6 (IL6)¹ is the main mediator of acute phase-induced fibrinogen synthesis, and sequences responsive to IL6 are present in the promoter regions of the genes coding for the three fibrinogen chains. In the promoter region of the fibrinogen β gene, several DNA sequences that are required for full IL6-induced expression have been identified as follows: an hepatocyte nuclear factor-1 (HNF-1) site at ~ 85 nucleotides upstream of the transcription start site; a CCAAT box/enhancer-binding protein (C/EBP)-binding site; and an IL6-responsive element (IL6 RE) (13–15). The C/EBP-binding site and the IL6 RE are located adjacent to each other at ~ 125 nucleotides upstream from the transcription start site. C/EBP β (also named LAP (liver-enriched activating protein) or NF-IL6 (nuclear factor for interleukin-6 expression)) has been identified as a transcription factor important in mediating the IL6 response of many liver acute phase genes, and binding of C/EBP β to the fibrinogen β promoter has been demonstrated (14, 16). The IL6 RE is located 4 bp upstream of the C/EBP β site, and it has been shown repeatedly that sequence changes in this element result in loss of response of the gene to IL6 (13–15). However, no transcription factor binding to this IL6 RE has been identified yet, leaving the regulation of the hepatic IL6-induced fibrinogen β expression partly unexplained.

The relationship between genetic variation of the fibrinogen β chain and plasma fibrinogen levels has been studied on many occasions. The A allele of the fibrinogen β $-455G/A$ variation

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¹ The abbreviations used are: IL, interleukin; HNF, hepatocyte nuclear factor; C/EBP, CCAAT box/enhancer-binding protein; IL6 RE, IL6-responsive element; DMEM, Dulbecco's modified Eagle's medium; HSA, human serum albumin; EMSA, electrophoretic mobility shift assay; siRNA, small interfering RNA; Fib, fibrinogen.

TABLE I
Fibrinogen β promoter variants present in pGL3-basic constructs

Fibrinogen β promoter variants are present as 1797- or 414-bp promoter fragments in pGL3-basic. In the upper part of the table the natural haplotypes and their frequency in the Caucasian population are shown (41).

	Fibrinogen β promoter variations						Population frequency	Fragment size (bp)
	-1420 G/A	-993 C/T	-854 G/A	-455 G/A	-249 C/T	-148 C/T		
Natural haplotypes								
FGB Haplo a	G	C	G	G	C	C	≈ 0.45	1797
FGB Haplo b	G	C	A	G	C	C	≈ 0.15	1797
FGB Haplo c	G	C	G	G	T	C	≈ 0.17	1797
FGB Haplo d	A	T	G	A	C	T	≈ 0.17	1797
Artificial constructs								
FGB Haplo b'-148T	G	C	A	G	C	T		1797
FGB Haplo d'-148C	A	T	G	A	C	C		1797
FGB Haplo b HNF3mut	G	C	A	G	C	C		1797
FGB Wild-type (15)	-	-	-	-	C	C		414
FGB C/EBPmut (15)	-	-	-	-	C	C		414

has been associated consistently with elevated habitual fibrinogen levels, and an interaction between the -455G/A polymorphism and acute phase stimuli has been reported as well (17–23). It has been demonstrated that subjects with at least one A allele respond to trauma, surgery, or strenuous exercise with a stronger rise in fibrinogen levels than -455GG homozygotes, and in most of these reports a critical role is attributed to the inflammatory mediator IL6 (24–27). Besides the -455G/A polymorphism, there are several other polymorphisms in the promoter of the fibrinogen β gene. The -1420G/A, -993C/T, and -148C/T variations are in complete linkage disequilibrium with -455G/A in Caucasians, and any of these variations therefore could influence fibrinogen levels (28, 29). Knowing the functional variation(s) is important from an epidemiological point of view, as these polymorphisms are not always co-inherited in other ethnic groups (30). Furthermore, identification of the functional variations and understanding of the mechanism are essential for insight into the causes of the inter-individual variation in the inflammatory response.

The aim of our study was to gain more understanding of the acute phase response of fibrinogen and to determine the influence of genetic variation on these mechanisms. We identified a novel HNF-3 site located just upstream of the IL6 RE and C/EBP site. We demonstrate requirement of this HNF-3 site for full IL6 response of the fibrinogen β promoter, which can be explained by an interaction between the HNF-3 site and the IL6-responsive C/EBP site. In addition, we show interference of the adjacent -148C/T polymorphism with this mechanism, explaining its effect on IL6-induced fibrinogen β promoter activity. Finally, we establish that, among six common polymorphisms present in the fibrinogen β promoter, it is only the -148C/T variation that is functional in our study.

EXPERIMENTAL PROCEDURES

Materials

Cell Lines—The human hepatoma cell lines HepG2 (American Type Culture Collection) and HuH7 (31) were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin (BioWhittaker Europe), and 10% fetal bovine serum (Invitrogen).

pGL3-Fibrinogen β Promoter-Reporter Gene Constructs—Approximately 1800 bp of the promoter region of the fibrinogen β gene (-1788 to +8, taking position 1499 in GenBank™ accession number X05018 as -1) was amplified by PCR on genomic DNA. In this PCR, 10 pmol of each primer (forward primer, 5'-TCT TAC GCG TGA AGA ATG CCA ATC AGA GTA-3'; reverse primer, 5'-TCA TCT CGA GTA GAC TTA ACT GAG AGA TCT TCA-3') and 50 ng of genomic DNA was used at an annealing temperature of 72 °C. PCR products were digested with MluI and XhoI (the restriction sites introduced are underlined in the primer sequences) and cloned into pGL3-basic (Promega). Finally, four pGL3-basic constructs, each containing 1800 bp of the wild-type fibrinogen β promoter with a different common promoter haplotype, were obtained: pGL3-FGB Haplo a, pGL3-FGB Haplo b, pGL3-FGB Haplo c, and pGL3-FGB Haplo d (Table I). In addition, artificial promoter haplotypes were constructed to discriminate between the effects of the individual polymorphisms. By exchanging the BsaBI-MluI restriction fragment between pGL3-FGB Haplo b and pGL3-FGB Haplo d, two additional constructs (pGL3-FGB Haplo b'-148T and pGL3-FGB Haplo d'-148C) were derived (Table I). For creation of the HNF-3-binding site mutants, pGL3-FGB Haplo b was used as parental construct. The putative HNF-3 site at -159/-151 in the fibrinogen β promoter was mutated by site-directed mutagenesis (QuikChange® site-directed mutagenesis kit, Stratagene), exactly as described by the manufacturer, using the mutagenic primers with sequences 5'-GCA ACA TCT TCC CAG CAA AGC TGA AGT ACT TGT CAT ACA AC-3' and 5'-GT TGT ATG ACA AGT ACT TCA GCT TTG CTG GGA AGA TGT TGC-3'. This resulted in a pGL3-basic-derived construct including 1800 bp of the human fibrinogen β promoter, with the core of the putative HNF3 site at -159/-151 changed from TATTTACTT to GAAGACTT (pGL3-FGB Haplo b

HNF3mut). The identity of the clones was verified by sequencing.

In addition to the constructs described above, constructs designed to study the C/EBP site at $-133/-125$ were also used. These constructs were already available at our institute and are described elsewhere (15). These pGL3-basic-derived constructs include 400 bp of the wild-type fibrinogen β promoter (pGL3-FGB wild-type) or a 400-bp fibrinogen β promoter fragment with the core C/EBP site at $-133/-125$ changed from GTTGCTTAA to GTTTAGTAA (pGL3-FGB C/EBPmut). The polymorphisms in this construct (at -249 and -148) are represented by their common alleles. The DNA sequences of the regions spanning from -166 to -118 in the mutant and wild-type constructs are shown in Table II.

Vectors Expressing Transcription Factors—Vectors expressing HNF-3 α , HNF-3 β , HNF-3 γ , and C/EBP β (pCDNA3.1-HNF3 α , pCDNA3.1-HNF3 β , pCDNA3.1-HNF3 γ , and pSCT-C/EBP β , all from rat origin) and their empty counterparts (pCDNA3.1- and pSCT-) were the kind gifts from Dr. P. Holthuis (Department of Physiological Chemistry, University of Utrecht, The Netherlands).

Antibodies—Polyclonal antibodies directed against HNF-3 β (M-20 sc-6554X) and HNF-3 γ (N-19 sc-5361X) were obtained from Santa Cruz Biotechnology, and antibodies directed against HNF-3 α (2000007) were obtained from Active Motif.

Luciferase Reporter Gene Assays

Transfection Conditions—HepG2 and HuH7 cells were plated in 24-well plates in DMEM with 10% fetal bovine serum at a density of 1.0×10^5 cells/well. After allowing the cells to attach overnight, the medium was replaced by DMEM with 0.1% human serum albumin (HSA, Cealb®). After 2 h, cells were transfected using FuGENE 6 (Roche Applied Science), according to the manufacturer's protocol. 200 ng of pGL3 construct and 4 ng of pRL-tk (*Renilla* luciferase expression construct, Promega) to correct for differences in transfection efficiency were used per well. If applicable, vectors expressing HNF-3 α , HNF-3 β , HNF-3 γ , C/EBP β , or a molar equivalent of the empty expression vector as control were added, and the total amount of DNA was kept at 400 ng per well with carrier DNA (herring sperm, Invitrogen). The effect of the empty expression vectors on fibrinogen β promoter activity and on the *Renilla* luciferase expression was determined for all conditions, and no effects of the empty control vectors were detected. For each construct at least two independent DNA preparations were used, and all DNA preparations were transfected at least twice in triplicate. 24 h after transfection, the medium was replaced by DMEM with 0.1% HSA, containing IL6 concentrations ranging from 0 to 15 ng/ml (recombinant human IL6, h).

Luciferase Assay—After culturing the cells for 24 h in the presence of IL6, cells were washed with 500 μ l of phosphate-buffered saline and lysed with Passive Lysis Buffer (Promega). The firefly luciferase reporter and *Renilla* luciferase internal control activities were measured with a luminometer (Berthold), using the Dual-Luciferase® Reporter Assay System (Promega).

Electrophoretic Mobility Shift Assay (EMSA)

Preparation of Nuclear Extracts—HepG2 cells were cultured under serum-free conditions (DMEM with 0.1% HSA) for 24 h prior to preparation of the nuclear extracts. For the preparation of nuclear extracts of IL6-stimulated cells, cells were incubated with 5 ng/ml IL6 for 15 min. Cells were washed and lysed, and nuclear extracts were prepared according to the method of Slomiany *et al.* (32). Buffers were supplemented with a protease inhibitor mixture (Complete™ Mini, Roche Applied Science) and with phosphatase inhibitors (250 μ M sodium orthovanadate and 25 mM β -glycerophosphate). The protein concentration in the extracts was estimated using the BCA micro kit (Pierce), and the samples were stored at -80°C for future use.

Oligonucleotides—Double-stranded 26-bp oligonucleotides including the wild-type HNF-3 site at $-159/-151$, (Fib β HNF3wt (wild type)) or the mutated HNF-3 site (Fib β HNF3 mut) were designed. In addition, a double-stranded 21-bp HNF-3 consensus oligonucleotide was designed according to the consensus sequence provided by Locker (33) (Table II). Pairs of oligonucleotides were annealed at equimolar amounts and radioactively labeled at the 5' ends with [γ - ^{32}P]ATP (Amersham Biosciences) using T4 kinase (Invitrogen). The double-stranded and labeled oligonucleotides were purified by using Micro-Spin G-25 columns (Amersham Biosciences) and stored at -20°C for future use.

Binding Reaction and Electrophoresis—To demonstrate the presence of HNF3 α , β , and γ , Western blot analysis was performed. Nuclear extracts from HepG2 cells grown in the absence or presence of IL6 (10

ng/ml), and rat liver nuclear extracts (Active Motif) were separated on 8% SDS-polyacrylamide gels. Gels were blotted on Protran BA83 cellulose-nitrate filters (Schleicher & Schuell), and the filters were incubated first with HNF3 α rabbit IgG (Active Motif), HNF3 β goat IgG, or HNF3 γ goat IgG (Santa Cruz Biotechnology) and subsequently with peroxidase-labeled swine anti-rabbit or anti-goat antibodies (DAKO Cytomation). Bands were visualized by using chemiluminescence (Western Lightning™ Chemiluminescence Reagent Plus, PerkinElmer Life Sciences).

For each binding reaction, 3 μ g of nuclear extract was preincubated for 30 min on ice with 5 μ g of poly(dI-dC) (Amersham Biosciences) in a 12- μ l reaction mixture containing 10 mM Hepes (pH 7.9), 100 mM KCl, 25 mM MgCl₂, 1 mM dithiothreitol, 0.05 mM EDTA, 0.1% (v/v) Nonidet P-40, 10% (v/v) glycerol, 0.03 mg/ml bovine serum albumin, 250 μ M sodium orthovanadate, and 25 mM β -glycerophosphate. Subsequently, the labeled oligonucleotides were added to the prebinding reactions, and this mixture was incubated for another 30 min on ice. If applicable, 3 μ g of antibody directed against HNF-3 was added, and the reaction was incubated for an additional 30 min on ice. Competition assays were performed by adding a 100-fold molar excess of unlabeled oligonucleotides to the radioactive oligonucleotides, prior to their addition to the binding reaction. The reaction products were loaded onto 5% nondenaturing polyacrylamide gels and run in 0.25 \times TBE (1 \times TBE: 0.1 M Tris, 0.09 M boric acid, 0.001 M EDTA). Gels were blotted onto Whatman paper, and the bands were visualized by autoradiography.

RNA Interference

The inhibition of endogenous HNF3 β expression was performed with 0.32 μ mol/liter siRNA targeted to exon 3 of HNF3 β (Silencer™ Pre-Designed siRNA, Ambion), which was transfected into HepG2 cells using siPORT™ Lipid Transfection Agent (Ambion) following the manufacturer's protocol. Four hours after transfection, 1 ml of medium was added, and cells were incubated in the absence or presence of 10 ng/ml IL6. After 18 h, culture medium was harvested, and mRNA was isolated from the cells.

The effect of siRNA on mRNA expression of HNF3 β and fibrinogen β was established by quantitative reverse transcription-PCR (Taqman) with predesigned primers from Applied Biosystems. Porphobilinogen deaminase mRNA concentration was used for normalization. The effects of lowered levels of the transcription factor HNF3 β on endogenous fibrinogen protein production by HepG2 cells was investigated by measuring the fibrinogen concentration in the culture medium with an in-house fibrinogen enzyme-linked immunosorbent assay using polyclonal antibodies to fibrinogen (Dako).

Statistical Methods

Firefly luciferase activity was normalized for transfection efficiency by using *Renilla* luciferase activity as the internal standard. These normalized luciferase activity levels were expressed as a percentage of the normalized luciferase activity of the pGL3-FGB Haplo b (Figs. 2-4a), pGL-3 FGB wild-type (Figs. 4b and 5), or pGL3-FGB Haplo d (Fig. 7) constructs at base line, depending on the combination of constructs used in the experiments. Normalized relative luciferase expression levels were compared by analysis of variance testing followed by LSD post hoc testing, or with Student's *t* test. SPSS 11.0 for Windows was used.

RESULTS

Identification of an HNF-3-binding Element Adjacent to the -148C/T Polymorphism—A nearly perfect HNF-3 consensus site was identified in the fibrinogen β promoter after a search for putative transcription factor-binding sites with MatInspector Professional (34). This putative HNF-3 site is located at $-159/-151$ relative to the transcription start site, just upstream of the C/EBP-binding site, the IL6 RE, and the -148C/T polymorphism (Table II), in a DNA sequence highly conserved during mammalian evolution.

To verify whether this putative HNF-3 element is capable of binding HNF-3 isoforms, EMSA experiments were performed (Fig. 1). When oligonucleotides representing the wild-type HNF-3 element (Fib β HNF3wt), the mutant HNF-3 element (Fib β HNF3mut), or an HNF-3 consensus site were incubated with basal HepG2 nuclear extracts, binding of a nuclear complex to the HNF3 consensus oligonucleotide (Fig. 1, lane 1) and to the fibrinogen β wild-type oligonucleotide (Fig. 1, lane 3) was

TABLE II
Sequences of the wild-type and mutant fibrinogen β promoter variants

The sequences of the wild-type fibrinogen β promoter, the mutant fibrinogen β promoters, the EMSA oligonucleotides, and the HNF-3 and C/EBP consensus sequences are shown. Note that different HNF-3 consensus sequences exist, and the one present in the fibrinogen β promoter is different from the one represented by the HNF-3 consensus oligonucleotide (33, 46).

Wild-type fibrinogen β promoter sequence	
	HNF-3 -148C/T IL6 RE C/EBP 5' -GTATGACAAGTAAATAAGCTTTGCTGGGAAGATGTTGCTTAAATGATAA-3' 3' -CATACTGTTTCATTTATTCGAAACGACCCCTTCTACAACGAATTTACTATT-5'
Consensus sequence (14;33)	3' -YTYRKTAT-5' 5' -CTTGCNAA-3'
Mutations created in fibrinogen β pGL3-basic constructs	
pGL3-FGB HNF3mut	5' -GTATGACAAGTactTcAGCTTTGCTGGGAAGATGTTGCTTAAATGATAA-3' 3' -CATACTGTTcATgaAgTCGAAACGACCCCTTCTACAACGAATTTACTATT-5'
pGL3-FGB CEBPmut	5' -GTATGACAAGTACTTcAGCTTTGCTGGGAAGATGTTtagTAAATGATAA-3' 3' -CATACTGTTcATGAAGTCGAAACGACCCCTTCAcAAatcATTACTATT-5'
Sequences of oligonucleotides used in EMSAs	
Fib β wild-type oligo	5' -TATGACAAGTAAATAAGCTTTGCTGG-3' 3' -ATACTGTTTCATTTATTCGAAACGACC-5'
Fib β HNF3mut oligo	5' -TATGACAAGTactTcAGCTTTGCTGG-3' 3' -ATACTGTTcATgaAgTCGAAACGACC-5'
HNF3 consensus oligo	5' -GCCCATTTGTTTTTAAAGCC-3' 3' -CGGGTAACAAACAAAATTCGG-5'
Consensus sequence (46)	5' -VAWTRTTKRYTY-3'

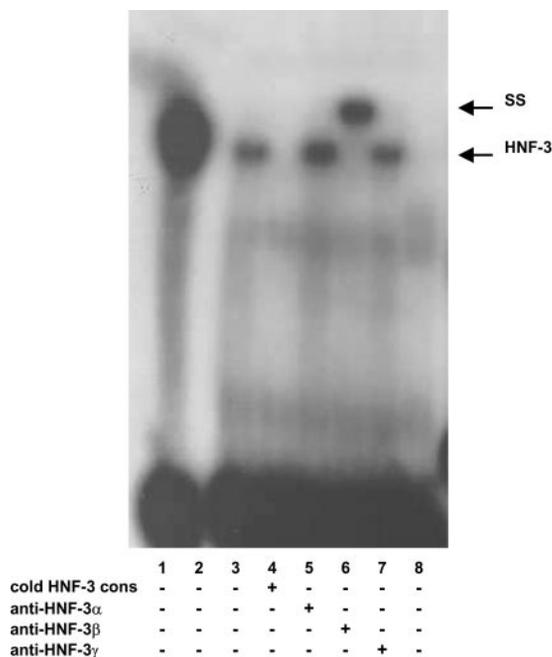


FIG. 1. Binding of HNF-3 β to the HNF-3 element in the fibrinogen β promoter. Labeled oligonucleotides representing the HNF-3 site in the wild-type fibrinogen β promoter (lanes 3–7), the mutated HNF-3 site (lane 8), or a HNF-3 consensus oligonucleotide (lane 1) were incubated with nuclear extracts derived from HepG2 cells cultured under basal conditions. 100-Fold molar excess of cold HNF-3 consensus oligonucleotide was added in lane 4, and antibodies against HNF-3 α (lane 5), HNF-3 β (lane 6), HNF-3 γ (lane 7) were added. Lane 2 was left empty. The locations of HNF-3 and the supershifted (SS) complexes are indicated by arrows.

detected. However, no binding of nuclear proteins to the fibrinogen β oligonucleotide with the mutated HNF-3 site (Fib β HNF3mut) was observed (Fig. 1, lane 8). The complex on the

fibrinogen β wild-type oligonucleotide disappeared when a 100-fold excess of cold HNF3 consensus oligonucleotide was added, showing that the nuclear proteins binding the fib β wild-type oligonucleotide also recognize this HNF3 consensus sequence (Fig. 1, lane 4). The mobility of the complex was retarded when an antibody directed against HNF-3 β was added (Fig. 1, lane 6) but not when antibodies directed against HNF-3 α (lane 5) or HNF-3 γ (lane 7) were added. Together, these results show that in HepG2 nuclear extracts, HNF-3 β binds the HNF-3 element at -159/-151 in the fibrinogen β promoter. HNF-3 β and - γ were clearly present in nuclear extracts of HepG2 cells that were cultured in the presence or absence of IL6, but more HNF-3 β than HNF-3 γ was present. HNF-3 α could not be seen.

The HNF-3 Site in the Fibrinogen β Promoter Is Functional, and Its Activity Is Influenced by the -148C/T Polymorphism—To investigate whether the putative HNF-3 site at position -159/-151 is functional, several reporter gene constructs, including 1800-bp fragments of the fibrinogen β promoter (pGL3-FGB Haplo b, pGL3-FGB Haplo b'-148T, and pGL3-FGB Haplo b HNF3mut), were transfected into HepG2 cells, together with vectors expressing HNF-3 isoforms. These experiments revealed that disruption of the putative HNF-3 site resulted in a major decrease of the response of the fibrinogen β promoter to overexpression of HNF-3 α , HNF-3 β , or HNF-3 γ (Fig. 2), showing that the HNF-3 site at -159/-151 in the fibrinogen β promoter is functional.

Change from C to T at position -148C/T also had a clear effect on the response of the fibrinogen β promoter to overexpression of HNF-3 isoforms, although the effect of the polymorphism was less severe than the effect of mutation of the HNF-3 site, and it was most apparent when intermediate concentrations of HNF-3 expression vectors were cotransfected (Fig. 2). No significant effects on basal promoter activity of either the mutation of the HNF-3 site or change from C to T at position

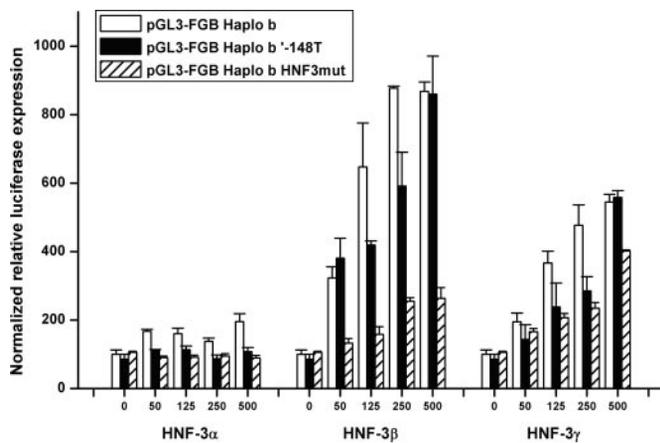


FIG. 2. Transactivation of fibrinogen β promoter variants by HNF-3 isoforms. Fibrinogen β promoter-reporter gene constructs were transfected into HepG2 cells in the presence of increasing amounts of vectors expressing HNF-3 α , HNF-3 β , or HNF-3 γ . Differences in activity between the fibrinogen b promoter haplotype b and the HNF-3 mutant (Haplo b HNF3mut) were significant at all HNF-3 α concentrations, at all HNF-3 β concentrations, and at concentrations above 50 pg of HNF-3 γ expression vector ($p < 0.005$ in all these cases). The differences in response to HNF-3 between the -148C/T alleles (Haplo b and Haplo b'-148T) were statistically significant at all HNF-3 α concentrations ($p < 0.01$ in all cases), when 125 or 250 pg of HNF-3 β expression vector was cotransfected ($p = 0.037$ and $p = 0.007$, respectively) and when 125 and 250 pg of vector expressing HNF-3 γ was cotransfected ($p = 0.046$ and $p = 0.011$, respectively). Normalized luciferase activities are expressed relative to base-line activity of the haplotype b construct. Means (\pm S.D.) of triplicate transfections are shown.

-148 could be detected in these experiments. These results in HepG2 cells were confirmed in HuH7 cells, although the response of the fibrinogen β promoter to overexpression of HNF-3 isoforms was weaker in HuH7 cells than in HepG2 cells (data not shown).

Integrity of the HNF-3 β Site Is Important for IL6-induced Fibrinogen β Promoter Activity—The HNF-3 site is located next to the IL6-responsive sequences described previously, and therefore the function of the HNF-3 site in IL6-induced fibrinogen β promoter activity was studied using several pGL3-basic-derived constructs containing an 1800-bp fragment of the fibrinogen β promoter. Addition of 2.5 ng/ml IL6 to HepG2 cells transfected with these fibrinogen β promoter-reporter gene constructs resulted in an 11-fold increase of promoter activity of the natural haplotype b, but the activity of the promoter variant with the mutated HNF-3 site increased only 5-fold (Fig. 3). Also at lower IL6 concentrations, a similar effect of mutation of the HNF-3 site on the IL6 response of the fibrinogen β promoter was detected. This shows that an intact HNF-3 site is required for a full response of the fibrinogen β promoter to IL6.

The change from C to T at position -148 strongly reduced the IL6 response of the fibrinogen β promoter as well. However, the effect of the polymorphism was milder than the effect of disruption of the HNF-3 site at -159/-151 (Fig. 3). Also in this experiment, no effect of mutation of the HNF-3 site or the -148C/T polymorphism on basal fibrinogen β promoter activity was observed. These results were confirmed in HuH7 cells, but the response of the promoter variants to IL6 was again weaker in HuH7 cells than in HepG2 cells (data not shown).

The effect of a 15-min IL6-treatment on binding of HNF-3 to its binding site was investigated using EMSA experiments. In these assays, basal HepG2 nuclear extracts were compared with IL6-treated HepG2 nuclear extracts, in experiments similar to the one shown in Fig. 1. The results obtained with the IL6-treated nuclear extracts were identical to the results obtained with the basal nuclear extracts (data not shown). There-

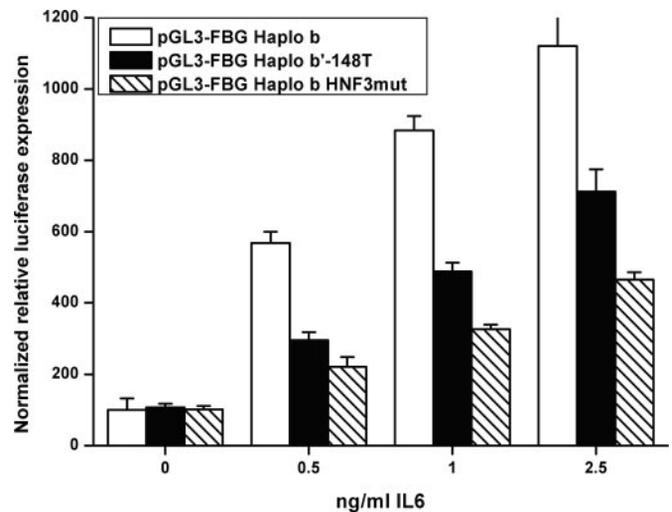


FIG. 3. Response of fibrinogen β promoter variants to IL6. Fibrinogen β promoter-reporter gene constructs were transfected into HepG2 cells, and cells were subsequently treated with IL6. At all IL6 concentrations, the IL6-induced activity of the fibrinogen b promoter haplotype b was significantly higher than the induced activity of the HNF-3 mutant promoter (Haplo b HNF3mut) ($p < 0.0003$ at all IL6 concentrations) and was higher than the induced activity of the haplotype b'-148T promoter variant (Haplo b'-148T) ($p < 0.001$ at all IL6 concentrations). The IL6-induced promoter activity of the Haplo d-148T in this experiment is also shown. Normalized luciferase activities are expressed relative to base-line activity of the fibrinogen β promoter haplotype b construct, and means (\pm S.D.) of triplicate transfections are shown.

fore, there was no qualitative effect of IL6 treatment on binding of HNF-3 β to the HNF-3 element.

Interaction between the HNF-3 and C/EBP Sites and the Effect of the -148C/T Polymorphism—To investigate whether the HNF-3 site and the C/EBP β site interact, cotransfection experiments were performed with the same 1800-bp fibrinogen β promoter constructs and a C/EBP β expression plasmid. Mutation of the HNF-3 site severely reduced the response of the fibrinogen β promoter to C/EBP β . The activity of the fibrinogen β haplotype b increased up to 7-fold in the presence of 250-pg vectors expressing C/EBP β , whereas the activity of the promoter variant with the mutated HNF-3 site increased only up to 3-fold (Fig. 4a). A similar effect of the mutation was also observed at lower C/EBP β concentrations, showing that the response of the fibrinogen β promoter to C/EBP β is largely dependent on the integrity of the HNF-3 site at -159/-151.

In addition, the -148C/T polymorphism also affected the response of the fibrinogen β promoter to overexpression of C/EBP β . The activity of fibrinogen β haplotype b increased up to 7-fold in the presence of overexpressed C/EBP β , whereas the haplotype b'-148T increased only up to 4-fold (Fig. 4a). Similar differences were also present at lower C/EBP β concentrations, showing that the change from C to T at position -148 results in a decreased response of the fibrinogen β promoter to C/EBP β . In this experiment, a minimal reduction in basal promoter activity was observed after mutation of the HNF-3 site or a change from C to T at position -148.

Subsequently, we examined whether an intact C/EBP site at position -133/-125 is necessary for the response of the gene to HNF-3 β . In these experiments, the pGL3-basic constructs were used, including 400 bp of the proximal fibrinogen β promoter (15). In the presence of 1000 pg of HNF-3 β expression vector, the activity of the wild-type fibrinogen β promoter increased 2-fold, whereas the activity of the fibrinogen β promoter with the mutated C/EBP site was barely induced by HNF-3 β , and similar results were obtained at lower HNF-3 β concentrations

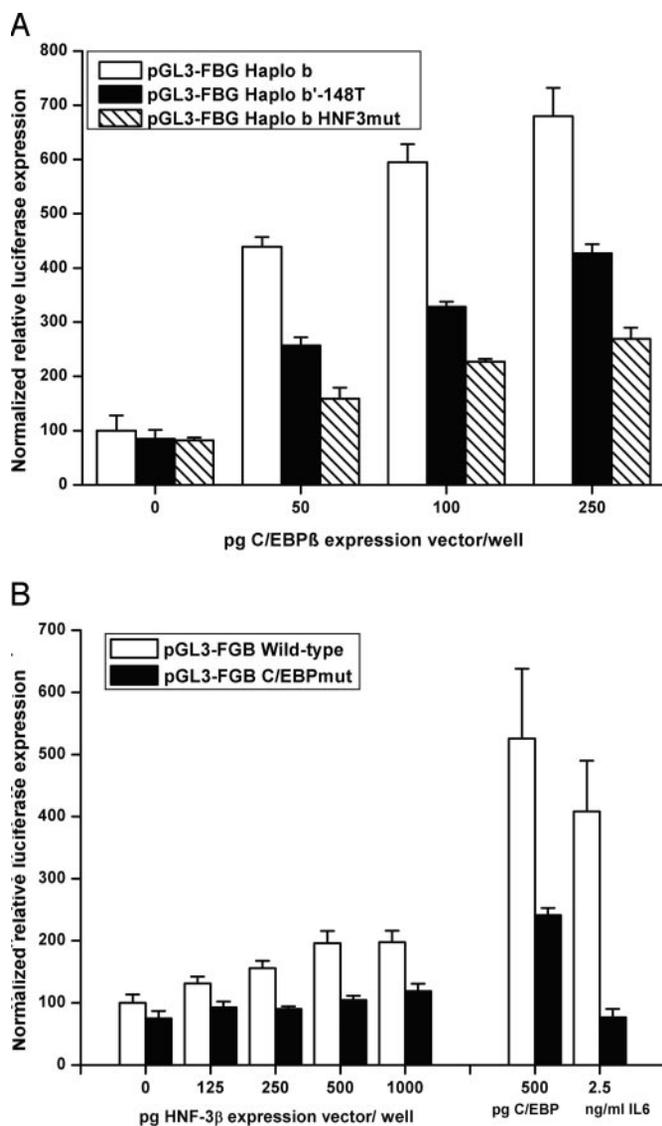


FIG. 4. Interdependency of the HNF-3 site and the C/EBP site in the fibrinogen β promoter. Fibrinogen β promoter-reporter gene constructs were transfected into HepG2 cells, together with the indicated amount of vectors expressing C/EBP β or HNF-3 β . If applicable, the transfected cells were subsequently treated with IL6. *A*, normalized luciferase activities expressed relative to base-line activity of the pGL3-FBG Haplo b construct are shown. At all C/EBP β expression vector concentrations, the C/EBP β -induced activity of the haplotype b promoter was significantly higher than the induced activity of the HNF-3 mutant (Haplo b HNF3mut) ($p < 0.0003$ at all C/EBP β concentrations). The C/EBP β -induced promoter activity of haplotype b was also significantly higher than the C/EBP β -induced promoter activity of haplotype b'-148T ($p < 0.0005$ at all C/EBP β concentrations). *B*, the (induced) promoter activities of the pGL3-basic constructs including the 400-bp promoter fragments (FGB wild-type and FGB C/EBPmut) are shown. Normalized luciferase activities are expressed relative to base-line activity of the FGB wild-type construct. Differences in activity between the wild-type promoter and the C/EBP mutant were significant at all concentrations of HNF-3 β expression vector ($p < 0.005$) (left panel). In addition, the FGB wild-type promoter responded significantly stronger than the C/EBP mutant to 500 pg of cotransfected C/EBP β expression vector ($p = 0.002$), and to 2.5 ng/ml IL6 ($p = 0.01$) (right panel).

(Fig. 4*b*, left panel). This shows that the response of the fibrinogen β promoter to HNF-3 depends on a functional C/EBP site at -133/-125.

Finally, additional transfection assays were performed to confirm the previously reported requirement of the C/EBP site for the IL6 response of the fibrinogen β promoter (13, 14). Disruption of the C/EBP site reduced the response of the promoter to overexpression of C/EBP β from 5.5- to 2.5-fold, and

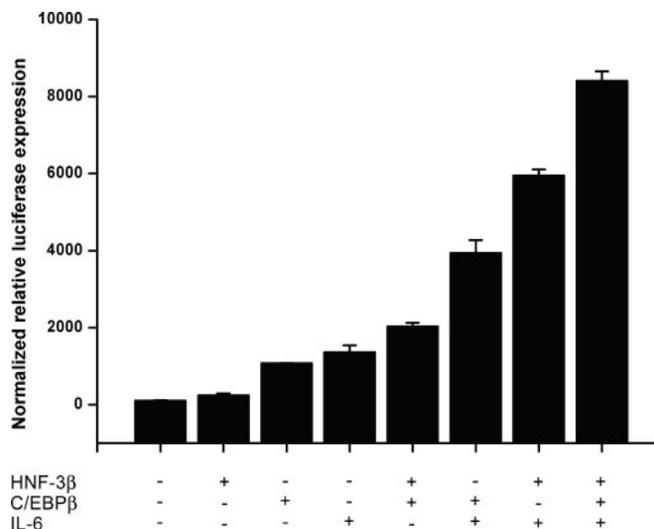


FIG. 5. Enhancement of fibrinogen β promoter activity by HNF-3 β , IL6, and C/EBP β . Reporter gene constructs, including 400 bp of the wild-type fibrinogen β promoter (FGB wild-type), were transfected into HepG2 cells in the presence or absence of 500 pg of HNF-3 β expression vector, and/or 250 pg of C/EBP β expression vector, and/or 2 ng/ml IL6. Normalized luciferase activities are expressed relative to base-line activity and means (\pm S.D.) of triplicate transfections are shown.

eliminated the response to 2.5 ng/ml IL6 (Fig. 4*b*, right panel). These results underline and confirm the important role of the C/EBP site for the IL6 response of the fibrinogen β gene promoter.

Synergistic Effects of HNF-3 β , IL6, and C/EBP β on Fibrinogen β Promoter Activity—To investigate the combined effect of HNF-3 β , IL6, and C/EBP β on fibrinogen β promoter activity, the pGL3-basic constructs were used, including 400 bp of the wild-type fibrinogen β promoter (15). The activity of the fibrinogen promoter increased 2.5-fold in the presence of 500-pg vectors expressing HNF-3 β and increased 13-fold in response to 2 ng/ml IL6 (Fig. 5). However, when HNF-3 β and IL6 were added simultaneously, the activity of the fibrinogen β promoter increased 40-fold, showing that HNF-3 β and IL6 synergistically enhance fibrinogen β promoter activity. For C/EBP β (250 pg of expression vector) and IL6, and for HNF-3 β and C/EBP β , similar synergistic effects were observed. When HNF-3 β , C/EBP β , and IL6 were all present, fibrinogen β promoter activity increased 85-fold compared with the basal expression level. Enhanced stimulation of IL6-induced fibrinogen β promoter activity by HNF-3 β and C/EBP β suggests that both HNF-3 β and C/EBP β are involved in the regulation of the IL6 response of the fibrinogen β gene. Similar results were observed for ratios 1:1, 1:2, 1:3, 2:1, and 3:1 of HNF-3 β :C/EBP β (results not shown).

RNA Interference—Twenty four hours after addition of siRNA to HNF-3 β , the amount of HNF-3 β mRNA was clearly decreased in quantitative reverse transcription-PCR experiments, both in the absence ($63 \pm 19\%$ decrease) and in the presence of 10 ng/ml IL6 ($76 \pm 9\%$ decrease) (Fig. 6). As a result, the amount of fibrinogen β mRNA was decreased, also in both the absence ($83 \pm 9\%$ decrease) and in the presence of 10 ng/ml IL6 ($72 \pm 19\%$ decrease). No significant effects were seen on the protein concentration of fibrinogen in the culture medium at the 24-h time point. This experiment shows that the siRNA-mediated HNF-3 β knock-down results in severely blunted IL6 response of the endogenous fibrinogen β gene in these cells.

The -148C/T Polymorphism Is the Functional Variant in the Fibrinogen β Promoter—In the previous experiments, we ob-

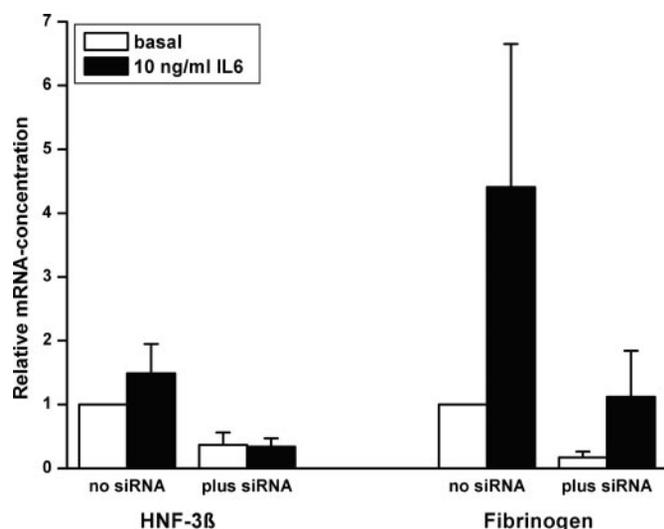


FIG. 6. **Results of siRNA experiments.** Effect of transfection of HNF-3 β siRNA on the concentration of HNF-3 β and fibrinogen β mRNA, under basal conditions, and after addition of 10 ng/ml IL6 is indicated. Mean (\pm S.D.) relative mRNA concentrations (with basal situation without siRNA defined as 1) of sextuplet experiments are shown.

served effects of the $-148C/T$ polymorphism on the response of the fibrinogen β promoter to IL6, overexpression of HNF-3, and overexpression of C/EBP β . Next, we investigated whether the $-148C/T$ polymorphism is the only functional variant or that other polymorphisms in the fibrinogen β promoter are also relevant. Transfection experiments were performed with 1800-bp fragments of the four common fibrinogen β promoter haplotypes cloned into pGL3-basic (Table I). IL6-treatment of HepG2 cells transfected with these constructs resulted in a strong induction (up to 25-fold) of the fibrinogen β promoter variants (Fig. 7a). However, the IL6-induced expression of promoter haplotype d (including the $-148T$ allele) was \sim 50% lower than the IL6-induced expression of haplotypes a, b, and c. Besides the $-148T$ allele, also the $-1420A$, $-993T$, and $-455A$ alleles are specific to haplotype d, which implies that any of these single nucleotide polymorphisms could be responsible for the lower IL6-induced promoter activity of fibrinogen β promoter haplotype d. To investigate the isolated effect of the $-148C/T$ variation, artificial haplotypes that differed from their natural counterparts only at position -148 were used in the next set of transfection experiments. The IL6-induced expression of haplotypes b and d'-148C (both have C at -148) was significantly higher than the IL6-induced expression of haplotypes d and b'-148T (both have T at -148) (Fig. 7b). There were no significant differences between the activity of constructs carrying the same -148 allele were but different at all other polymorphic sites. This confirms that the $-148C/T$ polymorphism is the main determinant of the lowered IL6 response of haplotype d.

In these experiments, the basal promoter activity of fibrinogen β promoter haplotypes carrying the $-148T$ allele were slightly lower than the basal promoter activity of the haplotypes carrying the $-148C$ allele. In all experiments, the absolute and relative differences in promoter activity between constructs with the $-148C$ or the $-148T$ allele were much larger in response to IL6 than under basal conditions.

The \sim 50% lower IL6-induced promoter activity of haplotypes d and b'-148T was confirmed in HuH7 cells, even though the IL6 response of the fibrinogen β promoter variants was not as strong in HuH7 cells as in HepG2 cells (data not shown).

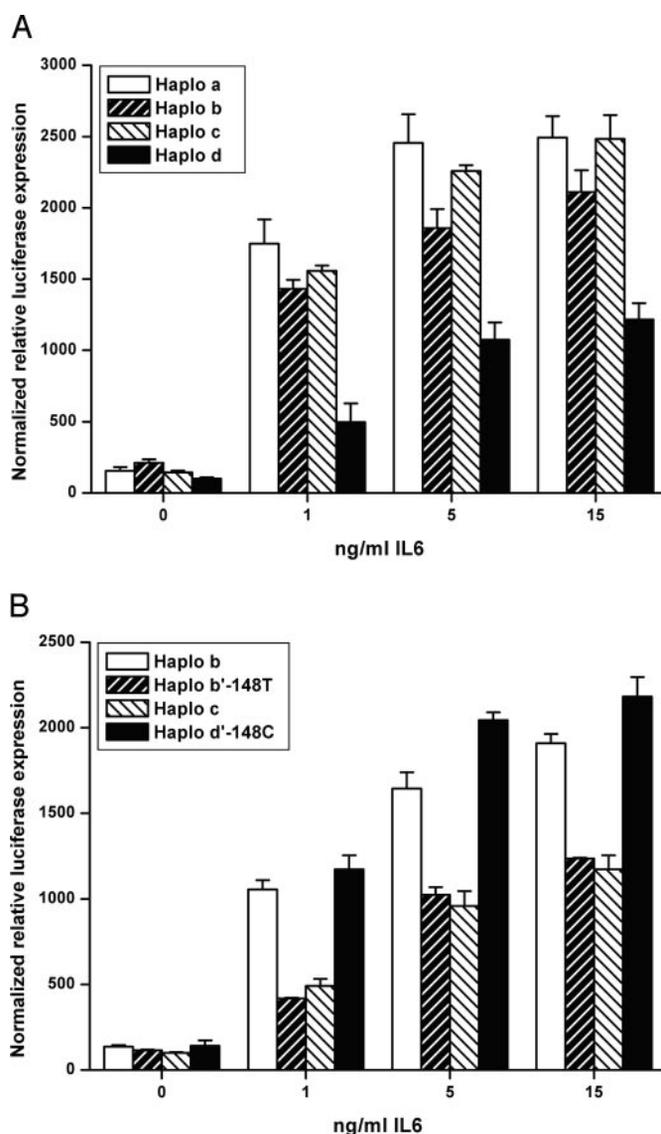


FIG. 7. **Identification of the functional polymorphism in the fibrinogen β promoter.** Fibrinogen β promoter-reporter gene constructs were transfected into HepG2 cells, including the several haplotypes. Normalized luciferase activities are expressed relative to baseline activity of the haplotype d construct and means (\pm S.D.) of triplicate transfections are shown. A, cells transfected with the natural haplotypes were treated with IL6, and the IL6-induced promoter activity of haplotype d was significantly lower than the IL6-induced promoter activity of the other natural haplotypes ($p < 0.001$ at all IL6 concentrations). B, cells transfected with the artificial fibrinogen β promoter haplotypes were treated with IL6, and the IL6-induced promoter activity of the haplotypes d and b'-148T was significantly lower than the IL6-induced promoter activity of the haplotypes b and d'-148C ($p < 0.001$ at all IL6 concentrations).

DISCUSSION

The present work includes studies on the regulation of the IL6 response of the fibrinogen β promoter and the effects of genetic variation on this mechanism. We describe a new functional HNF-3 element located at position $-159/-151$ in the fibrinogen β promoter, and we explain the importance of this site for the IL6 response of the promoter by indicating its interaction with the adjacent C/EBP site. Our experiments suggest that the common $-148C/T$ polymorphism, which is located between the novel HNF-3 site and previously identified IL6 RE and C/EBP elements, influences the response to IL6 by

interfering with the cooperation between the HNF-3 and C/EBP sites.

Regulation of the IL6 Response of the Fibrinogen β Promoter—Functional C/EBP β -binding motifs have been characterized in the promoters of numerous acute phase genes, such as those encoding haptoglobin, C-reactive protein, serum amyloid A, fibrinogen α , and fibrinogen β . The activity of C/EBP β is mainly regulated by its phosphorylation status, and IL6 is able to activate C/EBP β by phosphorylation. Phosphorylation of C/EBP β leads to increased transactivating potency, thus conferring early acute phase signals to the promoters of C/EBP-regulated acute phase genes (16). The HNF-3 transcription factors (HNF-3 α , - β , and - γ) are regulators of genes important in inflammation and development, and in contrast to C/EBP, HNF-3 is regulated at the transcriptional level. Cytokine-responsive C/EBP β and C/EBP δ proteins induce the HNF-3 β promoter upon activation by cytokines and can pass delayed IL6- and IL1-mediated stimulation onto HNF-3 β -responsive promoters (35). It has been shown *in vitro* that HNF-3, but not C/EBP, can bind to its binding site in compacted chromatin and open the local nucleosomal structure. The ability of HNF-3 to open chromatin is mediated by a high affinity DNA-binding site and a C-terminal domain of the protein that binds histones (36). This property of HNF3 may explain the strongly lowered basal fibrinogen β mRNA levels observed in the HNF-3 β siRNA experiment, in which HNF-3 β action on chromosomal DNA may be important.

The mutual enhancement of fibrinogen β promoter activity by HNF-3 and C/EBP that we have observed in our experiments suggests that HNF-3 and C/EBP synergize, and such cooperation between HNF-3 and C/EBP, in similar cotransfection experiments, has been observed before (37). The different mechanisms of gene activation by HNF-3 and C/EBP, briefly described above, might provide a hypothetical mechanism for this cooperation. HNF-3 could make the IL6-responsive DNA elements in the fibrinogen β promoter accessible to IL6-activated transcription factors such as C/EBP β , which subsequently increase promoter activity.

HepG2 cells express much less HNF-3 γ than HNF-3 β . This may explain the difference between the luciferase expression experiments where the effect of added HNF3 was studied and the EMSA experiments that depend on the available amount of HNF3 in the cells.

Between the HNF-3 site at position -159/-151 and the C/EBP site at position -143/-137, an IL6 RE is located (Table II). Mutations in this IL6 RE result in loss of the IL6 response of the fibrinogen β promoter, but no transcription factor binding to the IL6 RE has been identified (13-15). This IL6 RE may bind a transcription factor of its own, or it may be required for a correct positioning of the HNF-3 and C/EBP sites relative to each other or both. The cooperation between the HNF-3 and C/EBP sites that we observe in our reporter gene experiments might also involve the IL6 RE. Therefore, for a complete understanding of the IL6 response of the fibrinogen β gene, the exact role of the IL6 RE also needs to be clarified.

In this study we have focused on the HNF-3 site located at position -159/-151, but there can be other functional HNF-3 sites present in the fibrinogen β promoter. Indeed, our initial *in silico* analysis (with MatInspector Professional) revealed that there are several other possible HNF-3-binding motifs present in the fibrinogen β promoter. These putative additional HNF-3 elements might be responsible for the residual response to overexpressed HNF-3 of the fibrinogen β promoter with the disrupted HNF-3 site (Fig. 2). In addition, these putative elements may also account for the stronger response to HNF-3 by the 1800-bp fibrinogen β promoter fragments compared with the 400-bp fibrinogen β promoter fragments (compare Fig. 2 with Fig. 4b).

Finally, the presence of additional HNF-3 sites may also provide an explanation for the severely down-regulated basal fibrinogen β mRNA levels in the experiment with anti-HNF-3 β siRNA that were not seen in the luciferase expression experiments with mutations in the HNF-3 β -binding site at -159/-151. The siRNA experiment will affect all HNF-3 β sites, whereas the mutations will only affect a single site. However, as indicated previously, the lowered basal fibrinogen β levels may also be caused by a larger dependence of the endogenous gene on the activity of HNF-3 β as compared with the reporter gene construct. These options do not exclude one another.

The involvement of HNF-3 in cytokine-regulated gene response has not been described previously, but this novel function of HNF-3 may well be shared by other genes. Inspection of promoter regions of several other acute phase genes revealed that the C-reactive protein promoter and the fibrinogen α promoter also have putative HNF-3 sites located within a few base pairs from cytokine-responsive C/EBP β elements (38, 39). This indicates that involvement of HNF-3 in the IL6 response may in fact be a more general regulatory mechanism of the hepatic acute phase response. The physiological role of HNF-3 in fibrinogen regulation was supported by the results of our siRNA experiments, which showed that a decrease of HNF-3 β mRNA resulted in greatly reduced levels of fibrinogen β mRNA.

Genetic Variation of the Fibrinogen β Promoter; the -148C/T Polymorphism—We observed that the -148C/T variation in the fibrinogen β promoter influences fibrinogen β promoter activity, especially in response to IL6. The identification of -148C/T as the functional variation is supported by other groups (40, 41), but on some other occasions a functional role has also been indicated for the -455G/A, -854G/A, and the -1420G/A variations (21, 41, 42). Clearly, in our assay system, we observed functional effects for the -148C/T variation only, and the effects of this polymorphism on the response of the promoter to IL6 or overexpression of HNF-3 isoforms or C/EBP β were strong and reproducible.

The identification of -148C/T as a functional variation is important from an epidemiological point of view, as the degree of linkage disequilibrium between the fibrinogen β promoter polymorphisms may differ between ethnic groups. In Caucasians, the linkage disequilibrium between -1420G/A, -993C/T, -455G/A, and -148C/T is nearly complete, and thus each of the four variations can be used as a marker for the functional -148C/T polymorphism. In other ethnic groups, however, the situation can be different. Cook *et al.* (30) showed that the -455G/A and the -148C/T variations were not in complete linkage disequilibrium in an Afro-American population, and in this population the -148C/T polymorphism showed a stronger association with plasma fibrinogen levels than the -455G/A polymorphism. These data support our conclusion that -148C/T is a functional polymorphism, and therefore, we recommend that the fibrinogen β -148C/T variation is determined in epidemiological surveys.

In our transfection assays, haplotype d, which is characterized by the -148T and -455A alleles, showed weaker IL6-induced and basal expression. Most surprisingly, in epidemiological studies, increased basal and increased acute phase-induced fibrinogen levels have generally been associated with the -455A allele (and the -148T allele), which is opposite to our findings. In this respect, there are some issues that are worth discussing. First, we performed our studies with both HepG2 and HuH7 cells. Although there are large differences between these cells with respect to the magnitude of their IL6 response, fibrinogen β promoter fragments with haplotype d (-148T) had lower transcriptional activity in both HepG2 and HuH7 cells. A lower transcriptional activity of haplotype d in

reporter gene experiments has also been observed in HepG2 and Hep3B cells by another research group (41, 43). Similar results in three different hepatoma cell lines indicate that the discrepancy between the epidemiological reports and our experimental data are not caused by a peculiarity of any of the cell lines used. Second, we used IL6 to mimic the acute phase response in our experiments. In case of an acute phase event *in vivo*, however, several other inflammatory mediators are also expressed, and IL6 interacts with several of these factors. Concerning the acute phase response of fibrinogen, interactions between IL6 and IL1 β , IL4, IL10, and IL13 have been described, underlining the complexity of the inflammatory response of fibrinogen *in vivo* (44, 45). Therefore, our model system might have been too simplified relative to the real life situation. Third, we need to be aware of the fact that we only studied the effects of six polymorphisms located in the promoter region of the fibrinogen β gene, which only is a part of the complete haplotype (28, 29). Therefore, other variations located outside of the region present in our constructs may also contribute to the effects observed *in vivo*.

Basal Fibrinogen β Promoter Activity—In our experiments we observed on some occasions that the $-148C/T$ polymorphism slightly influenced basal fibrinogen β promoter activity, but the effect of mutation of the HNF-3 site on basal promoter activity was even smaller, if detectable at all. This may seem surprising, because many of our data indicate that the $-148C/T$ polymorphism should be regarded as a mild mutation of the HNF-3 site, because in our experiments the IL6-, HNF-3-, and C/EBP β -induced activity of the $-148T$ promoter is between that of the $-148C$ promoter and the HNF3mut promoter. However, due to the location of the $-148C/T$ polymorphism and the HNF-3 site with respect to the other regulatory elements, it is possible that the $-148C/T$ polymorphism affects the activity of downstream regulatory elements (e.g. the C/EBP element and the IL6 RE) more strongly than does the mutation of the HNF-3 site. As mutation of the C/EBP element reduces basal activity of the fibrinogen β promoter (Fig. 4b, left panel) (14, 15), it is plausible that the $-148C/T$ polymorphism has a stronger effect on promoter activity in the absence of IL6 than mutation of the HNF-3 site. However, we should make the proviso that the effects of the polymorphism and mutation of the HNF-3 site on basal promoter activity were very small, and in some of the experiments not even detectable. In all experiments, the absolute and relative effects of the $-148C/T$ polymorphism and mutation of the HNF-3 site were much stronger on IL6-induced fibrinogen β promoter activity than on basal promoter activity, underlining the importance of our observations for IL6-induced fibrinogen β expression.

In conclusion, with this study we have contributed to the understanding of the inflammatory response of fibrinogen, a cardiovascular risk factor. We have demonstrated the role of HNF-3 in the IL6 response of the fibrinogen β gene, and we show the effects of the common $-148C/T$ polymorphism on this mechanism.

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