

Identification of a Glucocorticoid Response Element in the Human γ Chain Fibrinogen Promoter

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Summary

The effect of the synthetic glucocorticoid hormone dexamethasone on human γ chain fibrinogen gene expression was examined. The whole promoter region of 3.8 kb of this gene and progressive 5'-deletions were inserted into a promoterless expression vector, upstream of the luciferase gene and transiently transfected into the human hepatoma HepG2 cells, in the presence or in the absence of dexamethasone stimulation. Deletion analysis allowed to identify a region located between -1359 and -954 bp upstream from the transcription start site, involved in hormone inducibility. On the basis of a computer-assisted analysis, a putative GRE was found in this region at bases -1116 to -1102. Specific point mutations eliminating this putative GRE led to complete loss of glucocorticoid inducibility, thus indicating its functional role. Binding of the rat glucocorticoid receptor to this site was demonstrated by mobility-shift assays.

Introduction

Fibrinogen is a plasma glycoprotein mainly synthesized by liver parenchymal cells. It is secreted as a dimeric molecule, composed of pairs of three different polypeptides, A α , B β and γ (for review, see 1). Fibrinogen chains are encoded by separate genes, which in humans are clustered on chromosome 4q28 (2), organised in a γ , α and β sequence with the β gene in opposite transcriptional orientation (3). Each gene is separately transcribed and translated and it has been demonstrated in different experimental systems, ranging from humans to *Xenopus*, that expression of the three fibrinogen chains is a co-ordinate process, regulated at the transcriptional level (4, 5).

Fibrinogen plays a crucial role in blood clotting process both by polymerisation to a fibrin network (6) and by mediating platelet aggregation (7). High plasma fibrinogen levels are also considered as an independent risk factor for cardiovascular disease (for review, see 8) and fibrinogen is considered a model protein in epidemiological studies (9).

Fibrinogen is also involved in the acute phase response to injury and stress (for review, see 10) and it has been suggested that circulating fibrinogen may be involved in regulation of the inflammatory response (11).

The expression of a subset of hepatic proteins is regulated during the acute phase of inflammation. The three fibrinogen genes belong to class II acute-phase proteins regulated by glucocorticoids and interleukin-6 (IL-6) (12, 13). Most of the studies concern the induction of fibrinogen gene expression by IL-6. IL-6 responsive elements have been identified in α , β and γ fibrinogen genes, both in humans (14-18) and in rat (19-21). Moreover, the molecular mechanism underlying this response is being elucidated (20, 22).

Less information is available on the molecular analysis of glucocorticoid induction of mammalian fibrinogen genes. The only data refer to the response to glucocorticoids of the genes coding for α and β fibrinogen chains in humans (14, 17) and for the three genes in rat (19). However, the specific glucocorticoid response elements (GREs) have not been precisely located and therefore no data are available on the regulation at the molecular level of human fibrinogen γ chain expression by glucocorticoids.

In this paper, we describe, for the first time, the identification of a GRE in the human γ chain fibrinogen promoter. Transient expression of constructs containing sequentially deleted 5'-flanking sequences of the γ chain gene fused to the luciferase reporter gene showed that the promoter was inducible by the synthetic glucocorticoid dexamethasone and allowed to identify the shortest DNA fragment (-1359 to -954 bp, from the transcription initiation site), mediating hormone-induced expression. This region contains a putative GRE at bases -1116 to -1102. The functional relevance of this element was confirmed by site-directed mutagenesis and binding of the recombinant rat glucocorticoid receptor (GR) to this site was demonstrated by electrophoretic mobility-shift assays.

Materials and Methods

Genomic DNA cloning. A human genomic library, inserted in the bacteriophage vector EMBL3 SP6/T7 (Clontech) was screened, using as probes a previously described 637 bp long cDNA encoding for human γ chain fibrinogen (23) and a set of oligonucleotides. The cDNA probe encodes from amino-acid 49 to 269 of the mature fibrinogen γ chain and recognises part of the exon III, exons IV to VII and part of exon VIII of this gene. Oligonucleotide sequences were derived from the known sequence of the human γ chain fibrinogen gene (24).

Library screening, DNA isolation, restriction mapping, Southern blotting and filter hybridization were performed according to standard techniques (25). Restriction endonucleases were from New England Biolabs (Beverly, Massachusetts); all the other enzymes were from Boehringer (Mannheim, Germany).

Oligonucleotides were purchased from PE-Applied Biosystems (Warrington, Cheshire, United Kingdom); poly (dI-dC) was from Sigma-Aldrich GmbH (Steinheim, Germany).

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Plasmid constructions. Plasmids containing sequential 5'-deletions of the upstream region of the γ fibrinogen gene were constructed according to the following strategy: the *XhoI-HpaII* fragment of λ HF2 of about 3.8 Kb, containing the 5'-flanking region of γ fibrinogen gene up to 13 bp upstream from the start codon, was first subcloned, after filling in of cohesive ends, into the *SmaI* site of Bluescript SK(-) vector (Stratagene, San Diego, California). The recombinant plasmid, designated as BSSK3.8, was used for sequencing and as starting material for subsequent insertional cloning of deleted promoter regions into the promoterless expression plasmid vector pGL2-basic, containing the luciferase gene of *Photinus pyralis* (Promega, Madison, Wisconsin). pGL2-basic will be hereafter referred to as pGL2. To allow subsequent directional cloning in pGL2, orientation of insertion in BSSK3.8 was analyzed by sequencing and by restriction enzyme mapping.

For subcloning the whole 5'-flanking region, BSSK3.8 was partially digested with *EcoRI*, whose site is located both in the promoter region and in the polylinker of BSSK3.8. To generate 5'-deleted fragments, the BSSK3.8 plasmid was singly digested with the restriction enzymes *EcoRI*, *AccI*, *HindIII*, or *AvaII*. After filling in of the cohesive ends by Klenow enzyme, each linearized plasmid was digested with *BamHI*. Each plasmid was then destroyed by digestions with *XbaI* and *KpnI*. The deleted fragments were then directionally inserted in pGL2 digested with *SmaI* and *BglIII*. The boundaries of insertion of each construct were verified by sequencing.

Plasmids were transformed into XL1-blue competent cells (Stratagene, San Diego, California) and DNA was isolated by Qiagen-tips 500 (Qiagen GmbH, Hilden, Germany).

DNA sequencing. DNA sequencing was carried out on both strands with a 370A automated DNA sequencer by the Dye dideoxy-terminator cycle sequencing kit with ampli-Taq FS (Applied Biosystems Division, Perkin Elmer), according to the manufacturer's instructions.

Computer DNA analyses. Computer-assisted analyses of DNA sequences were accomplished by using the software PCGene (Intelligenetics).

Cell cultures. The human hepatoma cell line HepG2 was cultured in Dulbecco-modified Eagle's (DMEM) and Ham's F12 media (1:1, vol/vol), supplemented with 10% foetal calf serum. HeLa cells were cultured in DMEM containing 10% calf serum. Antibiotics (100 i.u./ml penicillin and 100 μ g/ml streptomycin) were added to the growth medium. Cells were grown in a humidified atmosphere of 5% CO₂ and 95% air, at 37°C and cultured according to standard procedures.

Transfections. Transfections were performed by the calcium phosphate technique essentially as described by Wigler et al. (26). HepG2 cells (2.5 \times 10⁶ per 10 cm-diameter dish) or HeLa cells (2 \times 10⁶ per 10 cm-diameter dish) were plated 24 h prior to transfection. Transfection was carried out in growth medium containing 10% charcoal-stripped foetal calf serum (Hyclone, Logan, Utah) and CaPO₄-DNA precipitate, containing 15 μ g of the test plasmid and 5 μ g of the carrier plasmid (pUC18), was applied to the cells. 16 h later, this medium was removed, cells were washed with phosphate-buffered saline (PBS) and the medium replaced with growth medium with or without dexamethasone (Sigma)(10⁻⁶ M). Cells were harvested for luciferase assay after 24 h.

Luciferase assays. Cells were washed twice with PBS, scraped with a rubber policeman in 1 ml of TEN (40 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl), pelleted and resuspended in 100 μ l of 0.25 M Tris pH 7.8. After three cycles of freezing and thawing, samples were centrifuged at 4°C for 10 min at 5000 g. Protein content of the supernatant of each sample was determined spectrophotometrically using the Bio-rad protein assay (München, Germany). Amounts of supernatants, containing 100 μ g of proteins, were aliquoted and after addition of 350 μ l of a solution containing 25 mM glycylglycine pH 7.8, 2 mM ATP pH 7.8 and 0.01 M MgSO₄, tubes were placed in a Berthold luminometer. The reaction was initiated by the injection of 100 μ l of a solution containing 20 mM glycylglycine pH 7.8 and 0.2 mM luciferine (Sigma) and the peak light emission, expressed as relative light units (RLU), was recorded after 30 sec.

Site-directed mutagenesis. Mutations in the GRE were produced, in the -1359 construct, by the Quickchange site-directed mutagenesis kit (Stratagene), according to the manufacturer's instructions, using the oligonucleotide GREm (see Fig. 3, top).

Electrophoretic mobility-shift assays. Mobility-shift assays were performed using the purified rat glucocorticoid receptor DNA-binding domain, kindly provided by dr. K. Yamamoto (S. Francisco, CA). This recombinant protein consists of aminoacids from 440 to 525 of the rat GR (27). Double-stranded DNA fragments used in mobility-shift and competition assays were generated by self-annealing of complementary oligonucleotides. Equimolar amounts of each strand were heated at 95°C and then slowly cooled down to room temperature. Binding reactions were carried out as described by Roberts et al. (28); double stranded oligonucleotide probes were end labelled with T4 polynucleotide kinase. For nucleotide sequence of probes and competitors, see Fig. 3, top.

In competition assays, an excess (10 to 1000 fold) of unlabelled double-stranded competitors was preincubated in the binding reactions for 10 min. After the addition of the radioactive probe, the reactions were incubated for additional 30 min at room temperature.

Reaction mixtures were run on 5% polyacrylamide non denaturing gels in 0.5 \times TBE (25) at 4°C at a constant voltage of 225 V. Dried gels were exposed to Kodak (Rochester, New York) XAR5 film for one hour at -80°C with intensifying screens.

Results

Isolation of the 5'-flanking Region of Human Fibrinogen Gene

Out of approximately 10⁶ plaques of a human genomic library screened with a human γ -fibrinogen cDNA probe (23), one positive clone, designated as λ HF2 was isolated. The insert of this recombinant phage spans about 14.2 Kb. A series of oligonucleotides, mapping in different regions of the gene, were synthesised on the basis of the known sequence of fibrinogen gene (24), and used to characterise this genomic fragment. All oligonucleotides gave a positive hybridization signal with λ HF2 insert excised from the phage vector with *XhoI* (data not shown), confirming that λ HF2 contains the whole human γ fibrinogen gene. The absence of rearrangements was checked by restriction enzyme analysis and hybridization with the cDNA probe.

The 5'-flanking region of this clone spanned 3799 bp. In order to isolate this region as a whole fragment, λ HF2 was double digested with *HpaII* and *XhoI*, whose sites are located 16 bp upstream from the ATG start codon of the γ fibrinogen gene and in the polylinker of the phage vector, respectively. The excised insert was subcloned into the Bluescript SK(-) vector for subsequent sequencing and insertional cloning of promoter deletions into the vector pGL2.

The promoter region was completely sequenced on both stands; two nucleotide differences with the previously published sequence (18) were identified: an A instead of a G and a G instead of an A at positions -3790 and -3725, respectively.

Identification of an Upstream Region Responsible for Dexamethasone Stimulation

The response of the human hepatoma HepG2 cells to glucocorticoid stimulation, as far as the γ fibrinogen gene transcription is concerned, was verified by Northern blot analysis. After 24 h of dexamethasone stimulation (10⁻⁶M), an increase in the level of γ fibrinogen mRNA was observed (data not shown).

In order to test whether the analyzed promoter region of γ fibrinogen gene contained the regulatory *cis*-acting element/s for achieving a dexamethasone response, the whole promoter of 3799 bp was fused to the luciferase reporter gene into the vector pGL2. This construct was transiently transfected into HepG2 and HeLa cells (negative control)

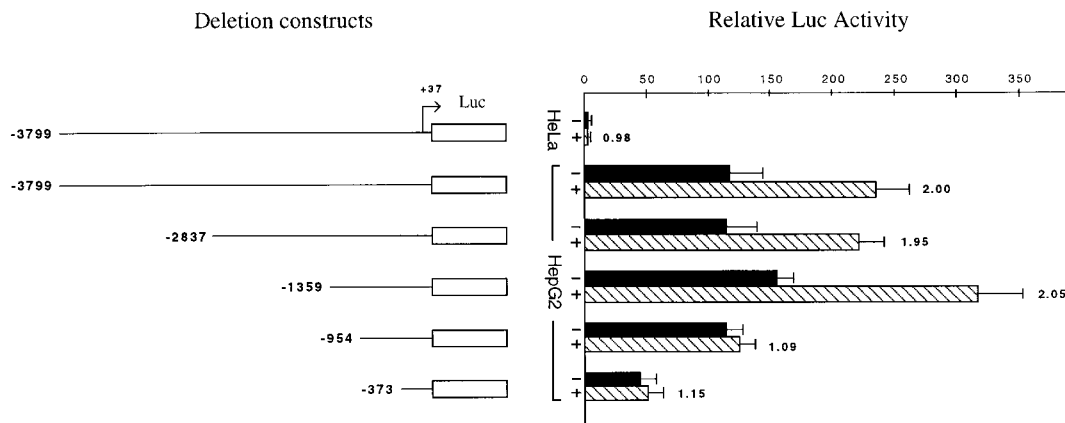


Fig. 1 Identification of the region responsible for dexamethasone stimulation in the promoter of human γ fibrinogen gene. A series of DNA fragments containing progressive 5'-deletions of the human γ fibrinogen promoter were cloned into the plasmid expression vector pGL2 upstream of the luciferase gene. All constructs contained the γ fibrinogen gene transcription initiation site (indicated by the bent arrow) and sequences up to position +37. Each construct was transiently transfected into HepG2 cells and the construct -3799 was transfected also in HeLa cells. The transfected cells were treated with (+) or without (-) dexamethasone (10^{-6} M) for 24 h, before measuring luciferase activity, as described in Materials and Methods. Luciferase expression levels are expressed as percentages of the uninduced pGL2 vector. The fold-increase in luciferase activity by dexamethasone stimulation for each construct is indicated beside each bar. Values are the mean of five independent experiments for each construct, each performed in duplicate. Error bars represent the standard errors

and luciferase activity was assayed after 24 h in culture with or without dexamethasone. As shown in Fig. 1, when this construct was transfected in HepG2 cells, a 2-fold increase in the expression of the reporter gene following hormonal induction was observed, while no hormonal stimulation could be detected in HeLa cells.

To determine the location of the *cis*-acting GRE/s within this 3.8 kb long promoter, a set of constructs with progressive 5'-deletions were generated. The basal and induced activities of constructs containing 3799, 2837, 1359, 954 or 373 bp of the 5'-flanking sequence fused to the luciferase gene were tested after transfections into HepG2 cells. The results, reported in Fig. 1, showed that the region from -1 to -954 bp did not contain sequences responding to dexamethasone. The region from position -954 to -1359 supported a hormonal stimulation of about 2-fold. Hormonal induction was no further increased by sequences from -1359 to -3799 bp. These data therefore allowed to localise the putative GRE/s in the γ fibrinogen promoter region comprised between position -1359 to -954.

Search for the Presence of Sequences Homologous to the Consensus GRE

In order to identify sequences homologous to the reported consensus GREs, GTACANNNTGTTCT (29) or AGAACANNNTGTTCT (30), we performed a computer-assisted search on the 3799 bp long 5'-flanking region of fibrinogen gene with the program Matscan (PcGene). The consensus GRE consists of 12 conserved nucleotides arranged as two hexameric half-sites, at least partially palindromic, separated by 3 non critical nucleotides. Since the two 15 bp long consensus differ in the first and third nucleotide, both consensus sequences were taken into account in the matrix construction. 4 putative GREs were identified by this procedure at positions -3489/-3475, -2809/-2795, -2634/-2620 and -1116/-1102, respectively. Only one of them (-1116 to -1102, TGTTCACTTTGTTAT) lies within the region shown to be responsible for dexamethasone inducibility on the basis of the deletion analysis (Fig. 1) and is identical to the consensus sequences in 9 out of 12 positions.

Mutational Analysis of the GRE Element

To confirm functionality of this putative GRE at position -1116/-1102, both half-sites of the GRE (Fig. 2, top) were mutated in the construct containing the first 1359 bp of the upstream sequence,

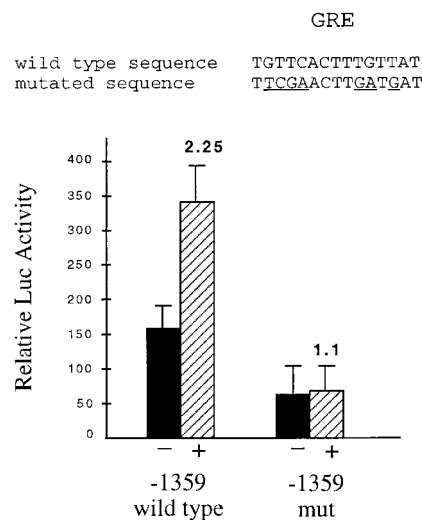


Fig. 2 Effect of mutations in the putative GRE of human γ fibrinogen promoter on dexamethasone-stimulated expression. Top: Comparison of nucleotide sequences of the wild type and mutated GREs. The putative GRE at positions -1116 to -1102 in the construct -1359 w. t. was mutated by site-directed mutagenesis by replacing 4 out of 6 nucleotides in one half-site and 3 of out 6 in the other half-site (mutated nucleotides are underlined), to produce the mutated construct (-1359 mut). Bottom: Wild type and mutated constructs were transiently transfected into HepG2 cells, as described in Materials and Methods, with (+) or without (-) dexamethasone (10^{-6} M). Luciferase expression levels are expressed as percentages of the uninduced pGL2 vector. The fold-increase in luciferase activity by dexamethasone stimulation is indicated above each bar. Values are the mean of two independent experiments, each performed in duplicate. Error bars represent the standard errors

GRE 5' AATCTGGTTTTGATGTGTTCACTTTGTTATAAATTATTGATTATTTAC 3'

GREm 5' AATCTGGTTTTGATGTTCGAACTTGGATGATAAATTATTGATTATTTAC 3'

GREm2 5' AATCTGGTTTTGAGAAATTCAACTTGGATGATAA 3'

palGRE 5' CTACGCAGAACATGATGTTCTAGTCTT 3'

Unrelated Sequence (US) 5' AAAAAATGGCTAATAGATCTCTCTTTGGCCCTC 3'

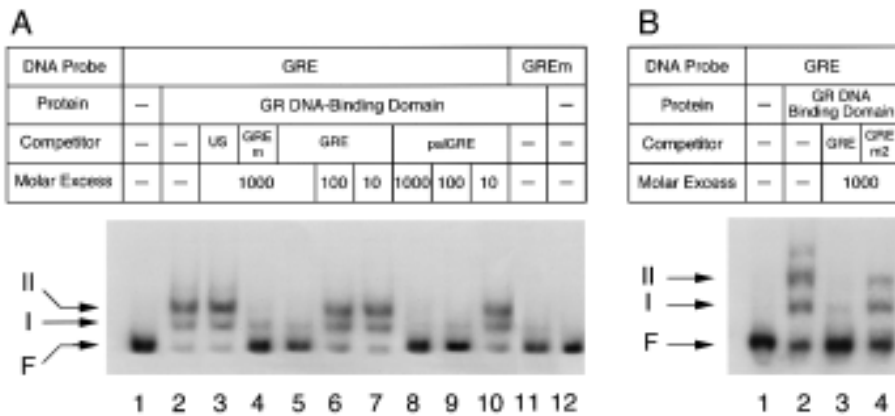


Fig. 3 Binding of the DNA-binding domain of rat glucocorticoid receptor (GR) to the GRE of human γ fibrinogen subunit gene, evaluated by gel mobility-shift assays. Top: Nucleotide sequences of DNA probes and of competitors used in the assays. For each DNA sequence, only the forward strand is reported. The hexameric half-sites of each glucocorticoid response element are boxed. Mutated nucleotides are underlined. In the GREm oligonucleotide, mutagenesis introduced a new GRE half-site (TGTTTCG) shifted two bases upstream from the wild type one. Pal GRE is an oligonucleotide containing a perfectly palindromic GRE. A) An end-labelled double-stranded synthetic oligonucleotide, including the wild type γ fibrinogen GRE and surrounding nucleotides from -1131 to -1085 (GRE probe) or a mutated oligonucleotide containing the same mutations as in the construct -1359 mut used in transfection experiments (GREm probe), were incubated with the purified recombinant DNA-binding domain (aminoacids 440-525) of the rat GR (38). Complexes were resolved on a non denaturing polyacrylamide gel. Arrows indicate the free DNA probe (F) and the DNA occupied by one (I) or two (II) protein molecules respectively. B) Electrophoretic mobility-shift assay performed as in A, using a different competitor, GREm2, in which also the artificially introduced GRE half-site present in GREm, was abolished

generating the mutated construct -1359 mut. Wild type and mutated constructs were transiently transfected into HepG2 cells. Upon dexamethasone stimulation, the wild type construct elicited the expected glucocorticoid response of approximately 2-fold (Fig. 2, bottom), whereas hormone stimulation was completely abolished in the construct with the mutated GRE. Basal expression driven by the -1359 construct containing the wild type GRE was higher than that driven by the -1359 mut construct.

Binding of the Recombinant Rat Glucocorticoid Receptor DNA-binding Domain to the Glucocorticoid Response Element

Binding of glucocorticoid receptor to the GRE identified in the human γ fibrinogen promoter was assessed by mobility-shift assays using a recombinant truncated rat GR, containing the DNA-binding domain (27). An end-labelled double-stranded synthetic oligonucleotide designed as GRE and including the γ fibrinogen GRE and surrounding nucleotides from -1131 to -1085 (Fig. 3, top), was incubated with the purified receptor fragment and run on a non denaturing polyacrylamide gel. Figure 3a shows bands representing the free DNA probe (F) and DNA occupied by one (I) or two (II) protein molecules, according to La Baer and Yamamoto (31). The fainter band observable above complex II (Fig. 3b, lane 2) should correspond to the complex with three proteins (31).

The results confirmed binding of the protein to the wild type GRE (Fig. 3a, lane 2), the stronger intensity of complex II band revealing that dimer binding was predominant. In competition assays, only a 1000 \times molar excess of the same unlabelled oligonucleotide was able to compete off the binding (Fig. 3a, lane 5), while a perfectly palindromic

GRE resulted to compete binding already at a molar ratio of 1:100 (Fig. 3a, lanes 8-10). A completely unrelated sequence was unable to affect the binding of GRE to the recombinant GR (Fig 3a, lane 3).

Unexpectedly, the double-stranded oligonucleotide GREm (Fig. 3, top), whose sequence is identical to that present in the construct -1359 mut used in transfection experiments, appeared to compete the DNA protein complex (Fig. 3a, lane 4), and was also able to bind the receptor, even if showing a fainter complex II as compared to wild type double-stranded GRE oligonucleotide (lane 11). The affinity of GREm for the receptor could be explained by considering that the mutagenesis step inadvertently introduced a new GRE half-site (TGTTTCG), shifted two bases upstream from the identified GRE (Fig. 3, top). This hexamer matches the consensus in 5 positions out of 6, including the two critical nucleotides (32) at positions 2 and 5.

In order to verify this hypothesis, competition assays were repeated using a new mutated double stranded oligonucleotide (GREm2), in which the artificially introduced GRE half-site in GREm was completely mutated (Fig. 3, top). This new competitor was unable to affect the binding of GRE to the recombinant GR (Fig 3b, lane 4).

Discussion

In this paper, we report, for the first time, the identification and characterization of a glucocorticoid response element (GRE) in the human γ chain fibrinogen promoter. Up to now, no GREs were characterized in any mammalian fibrinogen gene.

This study was performed on a γ fibrinogen promoter region of 3799 bp. In nucleotide sequence determination of this region, two

discrepancies with the sequence reported by Mizuguchi et al. (18) were identified. Transition at position –3790 abolishes restriction sites for *ApaI*, *AsuI*, *HaeIII* and *NlaIV*, while transition at position –3725 does not create nor abolish any restriction site. These nucleotide differences could reflect a polymorphism (the genomic fragments analyzed by our group and by Mizuguchi et al. (18) were isolated from different libraries) or a sequencing artefact. Since we confirmed the absence of *HaeIII* restriction site at position –3790 by restriction enzyme analysis, we tend to favour the first hypothesis, taking also into account that allelic variants in the coding region of this gene have been reported (23).

The approximate location of the GRE element was identified by transfections of constructs containing progressively deleted 5'-flanking sequences of the human γ chain fibrinogen gene fused to the luciferase reporter gene into the human hepatoma HepG2 cells.

Since glucocorticoid receptor levels in some clonal lines of HepG2 can be too low for efficient stimulation of GRE-containing episomal plasmid vectors (19), we first demonstrated by Northern blot analysis that our HepG2 cells were a suitable cell test system for studying glucocorticoid stimulation of the human γ fibrinogen gene. Moreover, when a plasmid containing the whole human γ fibrinogen 5'-region of 3.8 kb was transfected into these cells, an increase in the expression of the reporter gene was observed after hormonal induction, while no hormonal induction could be detected after transfection of the same construct in non hepatic cells (HeLa cells). In addition, these experiments indicated the presence of regulatory *cis*-acting elements, i.e. of GREs, in the γ fibrinogen promoter we were analyzing.

The 2-fold increase in the expression of the reporter gene we observed following hormonal induction is identical to that reported by Huber et al. (14) for human β fibrinogen constructs and comparable to that observed in preliminary studies for human α fibrinogen gene (17) and for rat fibrinogen genes (19).

Number, spacing and orientation of GREs greatly vary between different glucocorticoid-regulated genes (for review, see 33). This holds true also for fibrinogen genes. In *Xenopus laevis*, a single GRE at bases –148 to –162 of the β fibrinogen gene is present (28), while glucocorticoid regulation of the γ fibrinogen gene requires at least three nearby GREs and an accessory factor binding site, all within the first 187 bp of the promoter (34). In the human γ fibrinogen gene, we have shown that a GRE at position –1116 to –1102 confers dexamethasone inducibility. In human α and β fibrinogen genes, hormone responsiveness has been located in the first 217 bp of the 5'-flanking sequence (17) and in a fairly large region between –2900 and –1503 (14), respectively. Rat α and γ subunit genes have been shown to contain glucocorticoid-responsive sequences within 600 and 800 bp of the promoter, respectively, while in rat β gene, hormone inducibility was conferred by a fragment from position –349 to –193 (19). However, the GRE elements of these mammalian fibrinogen genes were no further analyzed in detail and their exact location was not established.

Like other characterised GREs, which do not match the consensus sequences at all positions (28, 34–39), the human γ fibrinogen GRE shows three mismatches with the consensus GRE reported by Beato (29) and four mismatches with that reported by Faisst and Meyer (30). Three out of four bases which has been demonstrated to be most critical for glucocorticoid receptor binding, at least in the distal GRE of rat TAT gene (32) are conserved also in the human γ fibrinogen GRE (positions 2, 5, 11), while position 14 is an A instead of a C. In this respect it should be noted that, also in other characterised GREs, these four critical nucleotides are not strictly conserved (35, 37).

In addition to deletion analysis, the functional significance of the human γ fibrinogen GRE at position –1116/–1102 was confirmed by site-directed mutagenesis and electrophoretic mobility-shift assays.

Mutations within both half-sites of the GRE element completely abolished glucocorticoid responsiveness of a γ fibrinogen-luciferase plasmid construct containing 1359 bp of the upstream sequence. Even if mutagenesis inadvertently introduced a new GRE half-site (TGTTTCG) shifted two bases upstream from the wild type GRE half-site (Fig. 3, top), the presence of a single GRE half-site was unable to sustain hormone induction, in agreement with the observations reported by Raju et al. (36) and Argenton et al. (40). No evidence for an overlapping binding site for an accessory transcription factor reported by Woodward et al. (34) in *Xenopus* γ fibrinogen gene was detected by a computer assisted search.

In mutational analysis experiments, a variation in the level of basal expression of constructs containing the mutagenised GRE was observed by our and other groups (34, 36, 41). This finding doesn't seem to be restricted to the GRE elements, since for instance mutations in the IL-6 responsive elements of fibrinogen genes led to a reduction in the basal level of the mutagenized constructs in transfected cells (16, 17, 21). According to Butz and Hoppe-Seyley (41), this variation can be explained by supposing that mutations in these critical DNA sequences would inactivate other possible regulatory *cis*-acting elements involved in mediating induction of gene expression, as also suggested by the decrease in basal expression of the construct containing the deletion of this region (–1359 to –954, see Fig. 1). In this respect, one of us (S.D.) noticed that one of the consensus sequences for the liver-enriched transcription factor HNF-3 (42) overlaps the human γ fibrinogen GRE site. At position –1116/–1106, a sequence matching the HNF-3 consensus in 9 nucleotides out of 11 is present. It is interesting to note that it has been recently reported that HNF-3 acts as a tissue-specific accessory factor in supporting glucocorticoid-induced transcription of other genes such as those coding for phosphopyruvate carboxykinase, insulin-like growth factor-binding protein 1 (43, 44) and tyrosine aminotransferase (45). The possible role of HNF-3 proteins in the regulation of glucocorticoid-induced expression of the human γ fibrinogen subunit remains to be clarified. This would increase our understanding of how induction of γ fibrinogen gene is regulated by glucocorticoids.

Mobility-shift assays showed that a protein-DNA complex is formed by the γ fibrinogen GRE with the DNA-binding domain of the rat GR, indicating that this GRE physically interacts with the GR. The observation that binding affinity of the γ fibrinogen GRE was approximately 10 fold lower than that of a perfectly palindromic GRE is concordant with the data of Roberts et al. (28) for *Xenopus* β fibrinogen GRE. The mutated double-stranded GRE oligonucleotide (GREm) was also able to bind the GR and to compete off the binding of the wild type GRE to the GR. This unexpected finding could be due to the presence of a new GRE half-site introduced during mutagenesis (Fig. 3, top). In agreement with this conclusion is the observation that in the binding of the GR to GREm, the monomeric complex is predominant. Involvement of this artificially introduced GRE half-site in the competition with the binding to GR was demonstrated by a further mutagenesis step. Removal of this half-site by means of nucleotide substitutions, leading to GREm2 oligonucleotide, completely abolished the ability to compete off the binding of GR to GRE.

The identification of a specific GRE in the promoter of the human γ fibrinogen gene offers the first "building block" for the elucidation of the molecular mechanism underlying the increase in plasmatic fibrinogen levels in the acute phase response to injury and stress.

Acknowledgments

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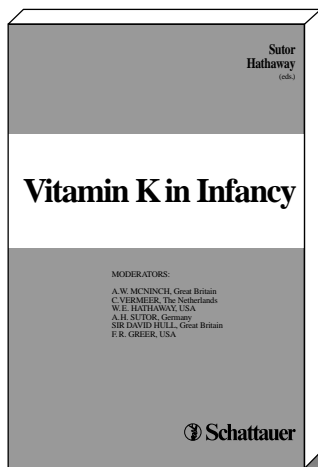
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