Type I Protein C Deficiency Caused by Disruption of a Hepatocyte Nuclear Factor (HNF)-6/HNF-1 Binding Site in the Human Protein C Gene Promoter*

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Protein C is a vitamin K-dependent zymogen of a serine protease that inhibits blood coagulation by proteolytic inactivation of factors Va and VIIIa. Individuals affected by protein C deficiency are at risk for venous thrombosis. One such affected individual was shown earlier to carry a $-14 \, T \rightarrow C$ mutation in the promoter region of the protein C gene. It is shown here that the region around this mutation corresponds to a binding site for the transcription factor hepatocyte nuclear factor (HNF)-6 and that this site completely overlaps an HNF-1 binding site. HNF-6 and HNF-1 bound in a mutually exclusive manner. The $-14 \, T \rightarrow C$ mutation reduced HNF-6 binding. In transient transfection experiments, HNF-6 transactivated the wild-type protein C promoter and introduction of the mutation abolished transactivation by HNF-6. Similar experiments showed that wild-type protein C promoter activity was reduced by co-transfection of an HNF-1 expression vector. This inhibiting effect of HNF-1 was reversed to a stimulatory effect when promoter sequences either upstream or downstream of the HNF-6/HNF-1 site were deleted. It is concluded that HNF-6 is a major determinant of protein C gene activity. Moreover, this is the first report describing the putative involvement of HNF-6 and of an HNF-6 binding site in human pathology.

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brinolysis through the neutralization of plasminogen activator inhibitor-1 (3). The physiological significance of the anticoagulant activity of protein C is shown in individuals homozygous or compound heterozygous for protein C deficiency. These individuals suffer from massive disseminated intravascular coagulation or neonatal purpura fulminans (4–6). Individuals affected by heterozygous protein C deficiency, although more mildly affected, are at risk of thrombophlebitis, deep vein thrombosis, or pulmonary embolism (7–9).

Transcription of eukaryotic genes by RNA polymerase II involves DNA elements located within the promoter region and transcription factors that associate with these DNA elements (10). Certain transcription factors, such as the TATA box-binding protein TBP, specify the transcription initiation site (11), whereas others regulate the efficiency of transcription initiation. This latter group of transcription factors comprises both ubiquitous and tissue-specific factors. Protein C promoter activity is liver-specific and controlled by liver-specific and by ubiquitous transcription factors. Binding sites have been identified in this promoter for the liver-enriched factors hepatocyte nuclear factor (HNF)-1 and HNF-3 and for the ubiquitous factor NF-1, and the activity of these factors was shown to be synergistic (12–16).

Genetic analysis of individuals suffering from hereditary protein C deficiency suggested that HNF-1 (13–15) and HNF-3 (12, 14, 15) are involved in protein C gene transcription, by binding to nucleotides $-10$ to $-22$ and $-26$ to $-37$ and $-33$ to $-22$, respectively. HNF-1 binding to and HNF-1 transactivation of the protein C promoter was abolished in a promoter construct where the naturally occurring $-14 \, T \rightarrow C$ mutation (13) had been introduced. This mutation is associated with type I protein C deficiency (13).

It is shown here that this HNF-1 binding site also binds HNF-6, a recently cloned liver-enriched transcription factor (17). HNF-6 contains a bipartite DNA binding region consisting of a novel type of homeodomain and of a single cut domain (17). In the liver, HNF-6 controls the activity of genes that code for plasma carrier proteins and for enzymes regulating glucose metabolism (17). This work shows that HNF-6 stimulates the protein C gene promoter and that a mutation in this promoter, which is associated with protein C deficiency in patients, leads to a loss of HNF-6 binding and activity. Finally, promoter sequences located both upstream and downstream of the HNF-6/HNF-1 binding site are important for transactivation by both HNF-6 and HNF-1.

1 The abbreviations used are: HNF, hepatocyte nuclear factor; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay.
Experimental Procedures

Polymerase Chain Reaction (PCR) Amplification—Figments of the human protein C promoter region were PCR-amplified, using pCwtCAT (12) as template, with different combinations of the following oligonucleotides: 5′-CAGGTTCCCGGGCTTTATGTTGGCGACATAATC-3′ (5′ pr1, −396 to −357, all nucleotide numbering is relative to the transcription start site) (18); 5′-TGGACACTTCTGTGCGGTGCAGCCTCCCTGTCGTTAGAT-3′ (5′ pr3, −179 to −153); 5′-GGCAAGCCGGCGCTTCCGGGAGAACAG-3′ (5′ pr4, −139 to −112); 5′-TAGAGCGAGGATCGCCGGGCC-3′ (5′ pr5, −100 to −76); 5′-CAGGTCGCCGGCGCTTGGTTGGGACCATATAATCTG-3′ (5′ pr6, −396 to −357); 5′-TAACTCTCTCCACCTGCTGCTCC-3′ (5′ pr7, −13 to +13); 5′-CTCTTTTCTTCTCTCCACCTCCACCTTCCCTGATGACAGATGACAGA-3′ (5′ pr8, +122 to +222). The nucleotides underlined in the sequences are not present in the human protein C promoter region and introduce Smal or EcoCRI sites in the PCR fragments. Amplifications were performed in a 50-μl reaction mixture containing 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 0.5 mM EDTA, 2.5 μM bovine serum albumin, and 1 mg/ml RNase gene. PK-H1, 5′-TGGTTGATGATGGACTAACTCGAACT-3′, was used. The −14 mutation in pCAT493, two fragments of the human protein C promoter region, spanning nucleotides −396 to −33 (fragment 1) and nucleotides −13 to +122 (fragment 2), were amplified with primers 5′ pr6/3′ pr4 and 5′ pr7/3′ pr5, respectively. Both PCR fragments 1 and 2 were digested by EcoCRI and equal amounts were ligated for 16 h at room temperature. The ligation mixture was PCR-amplified with primers 1 and 3 and the 472-base pair fragment, consisting of protein C promoter regions −396 to −42 and −5 to +107 separated by an EcoCRI site, was digested by Smal. Subsequently, the 453-base pair fragment was cloned into the Smal site of the CAT vehicle vector. This reporter construct was named pCA-41-5CAT493. The double-stranded oligonucleotide 5′-GGCCAGAAGAATTTGTTGGTTAGTGATGTAAGAGA-3′ (−41 to −5) was cloned into the EcoCRI site of pCwt00. The integrity of all constructs was verified by sequencing.

Transient Transfection—The human hepatoma cell line HepG2 (ATCC no. HB8065) and the SV40-transformed African green monkey kidney cell line COS 7 (ATCC no. 1651-CRL) were cultured in minimal essential medium containing Earle’s salts and nonessential amino acids supplemented with 15% heat-inactivated fetal calf serum. Cells were seeded at a density of approximately 1 × 10^6 cells/60-mm tissue culture dish. After 24 h, a DNA mixture containing 6 μg of protein C-CAT reporter construct, 2 μg of β-galactosidase expression vector (pCH110) (20), and 1.5 μg of nonplasmid plasmid pUC13, was transfected into the cells by the calcium phosphate co-precipitation method (21). For co-transfection experiments, 1 μg of HNF-1a expression vector was added, unless stated otherwise. Forty-eight hours after transfection, cells were harvested, and β-galactosidase activity was measured (22). The CAT activity of each construct was determined as described previously (23) and normalized to β-galactosidase activity. All transfections were repeated two to six times in duplicate, with at least two different plasmid preparations, and data from representative experiments are shown.

In Vitro Transcription/Translation—HNF-6 was in vitro transcribed and translated using the Tnt SP6 coupled wheat germ system (Promega Corporation BNL, The Netherlands) according to the manufacturer’s protocol, in a final volume of 50 μl (17). The crude wheat germ extract containing translated protein was used directly in electrophoretic mobility shift assays (EMSA).

Preparation of Cell Extracts—Rat liver nuclear extracts were prepared as described (24). HNF-1α or HNF-6-containing COS 7 cell extracts were prepared as follows. The cells (6 × 10^6) were transfected for 6 h in Dulbecco’s modified Eagle’s medium without fetal calf serum by lipofection using DOTAP and 10 μg of pRSV-HNF-1α or pcDNA-HNF-6. Forty-eight hours after transfection, the COS 7 cells were washed with phosphate-buffered saline and harvested in 1 ml of 40 mM Tris-Cl (pH 7.5), 1 mM EDTA, 150 mM NaCl. The cells were pelleted and resuspended in 200 μl of 50 mM Tris-Cl (pH 7.9), 500 mM NaCl, 0.5 mM EDTA, 2.5 μg/ml leupeptin, 1 mM dithiothreitol, 0.1% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 20% (v/v) glycerol. After three freeze-thaw cycles, the lysates were centrifuged and the supernatants were collected. Human hepatoma HepG2 cell extracts were prepared following the same procedure as for COS 7 cell extracts.

Oligonucleotides Used in EMSAs—Human protein C gene: pCwt 5′-TTTGTGGTTAGATTACTGCACCT-3′ (−28 to −3); pCwt 5′-TTTGTGGTTATGGACTAACTCGAACT-3′ (−96 to −73); rat HNF-3β gene: HNF-3β, 5′-AGCTTACCCGCTATCCGATTCATATTGGATTTTACCTCC-3′ (−150 to −118); rat o2-urinary globulin gene: 5′-TTTGTGGTATGGACTAACTCGAACT-3′ (−28 to −3); rat pCwt 5′-TTTGTGGTATGGACTAACTCGAACT-3′ (−96 to −73); 5′-AAATATTATTTAGAATAATC-3′ (−202 to −179). The −14 T → C mutation is underlined in the pCwt oligonucleotide.

EMSA—EMSA’s were performed with 3 μl of liver nuclear extract, 3 μl of in vitro transcribed/translated HNF-6, and 5 μl of COS 7 or HepG2 cell extract, in a 20-μl reaction mixture containing 20 mM HEPS (pH 7.6), 2 mM MgCl2, 0.5 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol, and 1 μg of 32P-end-labeled oligonucleotide. After an incubation of 20 min on ice, free DNA and DNA-protein complexes were separated by electrophoresis on a 6 or 8% polyacrylamide gel with 0.5× TBE buffer at 4°C. Competing oligonucleotides (50-fold molar excess) or preimmune or immune serum were added to the incubation mixture 20 min prior to addition of the 32P-labeled probe. After electrophoresis, the gel was dried and subjected to autoradiography at −80°C for 16 h.

Results

As shown in Fig. 1, the sequence of the HNF-1 site identified previously (13) in the human protein C gene promoter is compatible with the consensus sequence reported for HNF-6 binding (17, 25). To determine whether HNF-6 actually binds to this region of the protein C promoter, EMSAs were performed with recombinant HNF-6. As shown in Fig. 2A (lane 2), HNF-6 transcribed/translated in a wheat germ extract formed a complex with the pCwt oligonucleotide. This complex was absent when unprogrammed wheat germ extracts were used as a source of proteins (Fig. 2A, lane 1). To confirm that this complex was due to HNF-6 binding, the mixture was repeated with a labeled probe (UG-HNF-6) known to bind HNF-6 (25). As shown in Fig. 2A (lanes 5–7), the complex formed between HNF-6 and the UG-HNF-6 oligonucleotide was competed not only with the corresponding unlabeled oligonucleotide, but also with the unlabeled pCwt oligonucleotide.

Next, the pCwt oligonucleotide was tested in EMSAs with liver nuclear extracts as a source of proteins. Several complexes were detected (Fig. 2B, lane 1). One complex (labeled HNF-1)
was prevented by an excess of the unlabeled HNF-1-binding oligonucleotide PK-H1 (26) (Fig. 2B, lane 2). Also, this complex co-migrated with that formed between the probe and recombinant HNF-1 produced in COS 7 cells transfected with an HNF-1 expression vector (Fig. 2B, lanes 1, 6, and 8). Consistent with previous results (13), it was concluded that this complex, seen with liver extracts, results from HNF-1 binding to the probe. Another complex (labeled HNF-6) was prevented by the addition of a competing amount of oligonucleotide HNF-3β, previously shown to bind HNF-6 (25) (Fig. 2B, lane 3). This complex was inhibited by addition of anti-HNF-6 serum in the binding reaction, but not by addition of preimmune serum (Fig. 2B, lanes 4 and 5). In addition, the complex formed between the probe and recombinant HNF-6 obtained by transfection of COS 7 cells, co-migrated with the complex seen with liver nuclear extracts (Fig. 2B, lanes 1, 6, and 7). The conclusion from these observations was that the complex seen with liver nuclear extracts results from binding of HNF-6 to the pCwt probe. Two minor complexes (Fig. 2B, open arrowheads) were also observed. These complexes are specific as an excess of unlabeled probe (data not shown) prevented them. The identity of the proteins involved was not investigated in the present work. Finally, incubation of the pCwt probe with HepG2 cell extracts produced two complexes that co-migrate with those obtained with recombinant HNF-1 and HNF-6, suggesting that the latter proteins are also expressed in this human hepatoma cell line (Fig. 2B, lane 9).

The question as to whether HNF-1 and HNF-6 binding to the probe is mutually exclusive was then investigated. EMSAs were performed with binding reactions containing a fixed amount of HNF-6-containing COS 7 cell extracts and increasing amounts of HNF-1-containing extracts. As shown in Fig. 3 (lanes 1–6), increasing amounts of HNF-1 inhibited formation of the HNF-6-probe complex, without production of a ternary complex. The reverse experiment was also performed. Addition of increasing amounts of HNF-6-containing COS 7 cell extracts to a mixture containing a fixed amount of HNF-1-containing extracts led to inhibition of HNF-1-probe complex formation (Fig. 3, lanes 7–12). These experiments showed that binding of HNF-6 and HNF-1 to the −22 to −10 region of the protein C gene promoter is mutually exclusive.

To determine the influence of the −14 T → C promoter mutation on the binding affinity of HNF-6 for the protein C promoter, EMSAs were performed with an oligonucleotide containing an identical mutation (pCmt)). This mutation clearly reduced HNF-6 binding (Fig. 2A, lane 4). Moreover, HNF-6 binding to the UG-HNF-6 oligonucleotide could be totally prevented by the corresponding unlabeled oligonucleotide and by the unlabeled pCwt oligonucleotide, but not by the pCmt oligonucleotide (Fig. 2A, lanes 5–8).

The next question was whether HNF-6 was able to transactivate the protein C promoter. HepG2 and COS 7 cells were transfected with the protein C reporter construct pCAT493 and an expression vector for HNF-6. As shown in Fig. 4A,
transfection of HepG2 cells with HNF-6 increased transcription from the wild-type protein C promoter approximately 3-fold. When a construct containing the −14 T → C mutation was used, basal activity was severely reduced and transactivation by HNF-6 was abolished. Similarly, in COS 7 cells the wild-type, but not the mutant promoter, was also stimulated by HNF-6 (Fig. 4B). As expected, HNF-1 also transactivated the reporter construct in COS 7 cells. However, overexpression of HNF-1 in HepG2 cells resulted in a significant decrease in protein C promoter activity. Dose-response experiments (Fig. 5) showed that this inhibitory effect started with as little as 0.5 μg of HNF-1 expression vector. These observations contrasted with those of Berg et al. (13), who showed that the protein C gene promoter is transactivated 1.5-fold by HNF-1 in HepG2 cells. This discrepancy could have been due to the absence of the nontranslated first exon (nucleotides 1 to 152) or the first part of intron 1 in the protein C reporter construct used by Berg et al. (13), which ranges from nucleotides −618 to +7. The experiments were therefore repeated in HepG2 cells with constructs partially devoid of exon/intron 1 (pCCAT469* and pCCAT434*). As shown in Fig. 6, both deletion constructs displayed an approximately 2-fold lower protein C promoter activity compared with the wild-type promoter. The effect of HNF-1 on these 3’ deletion constructs was then determined. In contrast to the exon/intron 1-containing pCCAT493 construct, both pCCAT469* and pCCAT434* were transactivated about 3-fold by the addition of HNF-1 (Fig. 6).

The observation that HNF-1 activity was controlled by neighboring 3’-cis sequences led to a search for other modulatory sequences. A number of 5’-deletion constructs were tested for both basal activity and transactivation by HNF-1. As shown in Fig. 6, deletion of the −386 to −185 region (pCCAT291) slightly reduced basal transcriptional activity, while deletion of the −184 to −161 region (pCCAT267) had no effect on basal activity. Cotransfection experiments with HNF-1 resulted for both pCCAT291 and pCCAT267 in a clear reduction in promoter activity. Further deletion of nucleotides −160 to −131 (pCCAT237) slightly reduced basal activity. However, cotransfection with the HNF-1 expression vector up-regulated the activity of pCCAT237. Deletion of nucleotides −130 to −84 (pCCAT190) had no effect on basal activity, whereas the dele-
different protein C promoter fragments were tested for CAT activity in transiently transfected HepG2 cells with 1 μg of HNF-1 expression vector (white bar), 2 μg of HNF-6 expression vector (hatched bar), or without expression vector (black bar). At present, there is no good explanation for the difference in HNF-1 effects observed with different promoter constructs. One possibility is that in transfected cells HNF-6 displaces HNF-1 from the protein C promoter and acts as a stronger transactivator. In this case, displacement may depend on the length of the promoter in the construct. This would be in line with the observations by Tsay et al. (16) who showed that binding of transcription factors to the protein C promoter involves synergistic interactions. Alternatively, factors that bind to the longer promoter constructs, which tend to possess stronger basal activity, may specifically be squelched by overexpressed HNF-1. Whatever the explanation, the question remains as to whether HNF-1, HNF-6 or both are important for protein C promoter activity in vivo. Experiments aimed at identifying the occupancy of the protein C promoter in intact tissues, based on in vivo footprinting, might resolve this intriguing issue.

When introduced in a promoter context that cannot be activated by HNF-1, the −14 T → C mutation still induced a severe drop in basal activity. This mutation also led to the loss of HNF-1 effects observed with different promoter constructs. The activity of HNF-6 was clearly less context-dependent than that of HNF-1. This study therefore questions the role of HNF-1 in protein C promoter activity and uncovers a function for HNF-6. In addition, this is the first report describing the putative involvement of HNF-6 and of an HNF-6 binding site in human pathology.

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