Unique distance- and DNA-turn-dependent interactions in the human protein C gene promoter confer submaximal transcriptional activity

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Recent studies on the regulation of protein C gene transcription revealed the presence of three transcription-factor binding sites in the close proximity to the transcription start site. The proximal 40 bp upstream of the transcription-initiation site contain two, partly overlapping, binding sites for the liver-enriched hepatocyte nuclear factor (HNF)-3 and one binding site to which HNF-1 and HNF-6 bind in a mutually exclusive manner. In order to examine the functionality of the tight alignment of transcription-factor binding sites around the transcription-initiation site, we performed insertional mutagenesis experiments. Sequences were inserted at position −21, separating both HNF-3 binding sites from the HNF-1–HNF-6 binding site, and position −5, separating the HNF-3–HNF-1–HNF-6 complex from the transcription start site. All insertions were made in the context of the protein C gene −386/+107 promoter region and tested for activity by transient transfection experiments. Insertions at position −21 resulted in a combined distance- and DNA-turn-dependent increase in protein C gene expression. Insertions of variable length at position −5 decreased protein C gene expression in a DNA-turn-dependent manner. However, this turn-dependent decrease was accompanied by a distance-dependent increase in promoter activity. This is the first report in which changing the spacing between adjacent transcription-factor binding sites results in enhanced transcription, indicating the submaximal alignment of promoter elements in the wild-type protein C gene promoter region.

Key words: hepatocyte nuclear factor (HNF)-3, HNF-6, insertional mutagenesis, liver.

INTRODUCTION

Protein C is a vitamin K-dependent zymogen of a serine protease that plays an important role in the regulation of blood coagulation. After activation by the thrombin–thrombomodulin complex, activated protein C inhibits blood coagulation in the presence of its cofactor protein S [1], Ca^{2+} and phospholipids through the proteolytic inactivation of Factors Va and VIIIa [2,3]. Furthermore, activated protein C neutralizes plasminogen activator inhibitor-1 [4] and thereby activates fibrinolysis.

The physiological significance of the protein C anticoagulant pathway is clearly shown in newborns homozygous or compound heterozygous for protein C deficiency. These individuals suffer from massive disseminated intravascular coagulation or neonatal purpura fulminans [5–7]. Individuals affected by heterozygous protein C deficiency, although more mildly affected, have an increased risk of venous thrombo-embolism [8–11].

Eukaryotic gene transcription by RNA polymerase II involves both *cis*-acting DNA elements and *trans*-acting transcription factors that associate with these DNA elements [12–14]. Two functionally different groups of transcription factors are identified, namely basal transcription factors and specific transcription factors. The basal transcription factors are sufficient for a basal level of transcription and to define the transcription start site [15]. Specific transcription factors influence the efficiency of transcription initiation and thereby regulate transcription.

Analysis of the protein C gene promoter region to identify specific *cis*-acting DNA elements and the corresponding transcription factors involved in protein C gene expression has recently led to the identification of three such DNA elements. Located between nucleotide −26 to −37 and nucleotides −33 to −22 (relative to the transcription start site [16]) are two partly overlapping and inversely oriented HNF-3 binding sites. Both these binding sites were shown to be critically important for accurate protein C gene transcription [17]. The third transcription-factor binding site, located immediately downstream of the HNF-3 binding sites, binds either HNF-1 (nt −22 to −10) [18] or HNF-6 (nt −21 to −12) in a mutually exclusive manner [19]. It has been suggested that interactions between HNF-1 and HNF-3 are important in the transcriptional regulation of the protein C gene promoter [17].

The tight alignment of transcription-factor binding sites close to the transcription start site of the protein C gene made us wonder about the functional significance of the close proximity. To study the functional significance, we carried out insertional mutagenesis experiments of the protein C gene promoter region by introducing sequences of various lengths between the HNF-3 and HNF-1–HNF-6 recognition sites at position −21. Furthermore, we inserted sequences of various lengths at position −5 to examine the functional relationship between the transcription-start-site region and the upstream HNF-3–HNF-1–HNF-6 region.

Insertional mutagenesis experiments have been performed previously for a variety of genes with quite different results [20–25]. Changing the spacing between adjacent binding sites in these genes resulted in decreased promoter activities in a distance- [22], DNA-turn- [23,24] or combined distance- and DNA-turn-dependent manner [20,21]. Here we describe a novel phenomenon of insertional mutagenesis experiments that result in increased promoter activities.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides were synthesized on a Millipore 8909 Expedite nucleic acid-synthesis system. After purification of the single-

Abbreviations used: HNF, hepatocyte nuclear factor; CAT, chloramphenicol acetyltransferase; SV40, simian virus 40.

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Table 1 Constructs used in transient transfection assays and oligonucleotides used to create these constructs

Indicated in **bold** are the inserted nucleotides. Underlined are the nucleotides GAGCTC present in construct pCH1/H3+15, which create an *Eco*ICRI restriction site.

Construct	Oligonucleotide
pCwt	$^{-41}$GGCCAAGCAAATATTTGTGGTTATGGATTAACTCGAA^{-5}
pCH1/H3+3	$^{-41}$GGCCAAGCAAATATTTGTGGT**GGT**TATGGATTAACTCGAA^{-5}
pCH1/H3+5	$^{-41}$GGCCAAGCAAATATTTGTGGT**CTGGT**TATGGATTAACTCGAA^{-5}
pCH1/H3+8	$^{-41}$GGCCAAGCAAATATTTGTGGT**CGTCTGGT**TATGGATTAACTCGAA^{-5}
pCH1/H3+10	$^{-41}$GGCCAAGCAAATATTTGTGGT**TTACGTCTGGT**TATGGATTAACTCGAA^{-5}
pCH1/H3+15	$^{-41}$GGCCAAGCAAATATTTGTGGT**GAGCTCACGTCTGGT**TATGGATTAACTCGAA^{-5}
pCH1/ST+1	$^{-41}$GGCCAAGCAAATATTTGTGGTTATGGATTAACTCGAA**G**$^{-5}$
pCH1/ST+3	$^{-41}$GGCCAAGCAAATATTTGTGGTTATGGATTAACTCGAA**GAA**$^{-5}$
pCH1/ST+5	$^{-41}$GGCCAAGCAAATATTTGTGGTTATGGATTAACTCGAA**GCGAA**$^{-5}$
pCH1/ST+6	$^{-41}$GGCCAAGCAAATATTTGTGGTTATGGATTAACTCGAA**CCTCAG**$^{-5}$
pCH1/ST+8	$^{-41}$GGCCAAGCAAATATTTGTGGTTATGGATTAACTCGAA**CATGCGAA**$^{-5}$
pCH1/ST+10	$^{-41}$GGCCAAGCAAATATTTGTGGTTATGGATTAACTCGAA**CTTCAGCGAA**$^{-5}$

stranded oligonucleotides, they were treated with T_4 polynucleotide kinase for 2 h at 37 °C. The complementary oligonucleotides were mixed and annealed by heating to 90 °C and slowly decreasing the temperature to room temperature in 4–6 h.

Plasmid constructions

Two different fragments of the human protein C promoter region, spanning nucleotides −396 to −33 (fragment 1) and nucleotides −13 to +122 (fragment 2) were amplified from genomic DNA from a non-protein C-deficient individual. To perform the amplifications we used the following oligonucleotides: 5′-CAGCGTCCCCGGGCTTGTATGGTGGCACATAAATAC ATGT-3′ (primer 1; −396 to −357; all nucleotide numbering is relative to the transcription start site [16]) and 5′-TGCTTGGAGCTCAGCACTGAGGCCT-3′ (primer 2; −33 to −57) to create fragment 1 and 5′-CTCTTCTCTTCTCCCGGGGGCAGCCCTCCCTCCACACCCCTCATA-3′ (primer 3; +122 to +78) and 5′-TAACTCGAGCTCCAGGCTGTCATG-3′ (primer 4; −13 to +13) for fragment 2. The underlined nucleotides in the oligonucleotide sequences represent modified nucleotides which are not present in the wild-type protein C promoter region. These modified nucleotides introduce an *Sma*I site (-CCC/GGG-) in PCR fragment 1 at position −386 or an *Eco*1CRI site (-GAG/CTC-) at positions −41 and −5 in PCR fragments 1 and 2 respectively. Amplifications were performed in a 50 μl reaction mixture containing 10 mM Tris/HCl, pH 8.0, 1 mM $MgCl_2$, 50 mM KCl, 350 ng of primers, 100 ng of genomic DNA, 250 μM dNTPs, 60 μg/ml BSA and 0.3 unit of *Taq* polymerase. After an initial incubation at 91 °C for 4 min, 32 cycles were carried out at 91 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min.

Both PCR fragments 1 and 2 were digested with *Eco*1CRI, 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 bp fragment consisting of protein C promoter regions −396 to −42 and −5 to +107, separated by an *Eco*1CRI site, was digested by *Sma*I. Subsequently, the 457 bp fragment was cloned in the *Sma*I site of the CAT00 vector [26] (CAT is chloramphenicol acyltransferase). This reporter construct was named pCΔ−41/−5CAT493.

Cloning of the double-stranded oligonucleotides shown in Table 1 into the *Eco*1CRI site of the pCΔ−41/−5CAT493 construct resulted in the constructs used in the transient transfection experiments. Construct pCH1/H3+40 was constructed by insertion of 25 randomly chosen nucleotides (5′-GTCTTATGCGATCACAGACGTCAGC-3′) into the *Eco*1CR1 site of construct pCH1/H3+15. The integrity of all constructs was verified by sequencing.

Transient transfection

The differentiated human hepatoma cell line HepG2 (A.T.C.C. HB8065) and the simian-virus-40 (SV40)-transformed African-green-monkey kidney cell line Cos7 (A.T.C.C. 1651-CRL) were cultured in Minimal Essential Medium containing Earle's salts and non-essential amino acids supplemented with 15% (v/v) heat-inactivated foetal-calf serum. Cells were seeded at a density of approximately 1×10^5 cells/60-mm-diameter 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 [27]) and 1.5 μg of non-specific plasmid pUC13, was transfected into the cells by the calcium phosphate co-precipitation method [28]. For co-transfection experiments, 0.5 μg of HNF-3α expression vector, 1 μg of HNF-6 expression vector and/or 1 μg of HNF-1α expression vector was added. At 48 h after transfection, cells were harvested and β-galactosidase activity was measured [29]. The CAT activity of each construct was determined essentially as described by Seed and Sheen [30] and normalized to β-galactosidase activity. All transfections were repeated two to six times in duplicate, with at least two different plasmid preparations.

RESULTS

Four transcription factors with their corresponding binding sites have been claimed to be involved in protein C gene expression. At position −26 to −37 (bs-I) and −33 to −22 (bs-II) two partly overlapping and inversely orientated HNF-3 binding sites are located [17]. Immediately downstream at position −22 to −10 a HNF-1–HNF-6 binding site (bs-III) is located (Figure 1, pCwt) [18,19]. The tight alignment of these binding sites close to the transcription start site made us ask whether this close proximity is of functional importance.

Insertional mutagenesis at nt −21

In order to examine the importance of interactions between transcription factors binding to bs-I–bs-II and bs-III, we varied

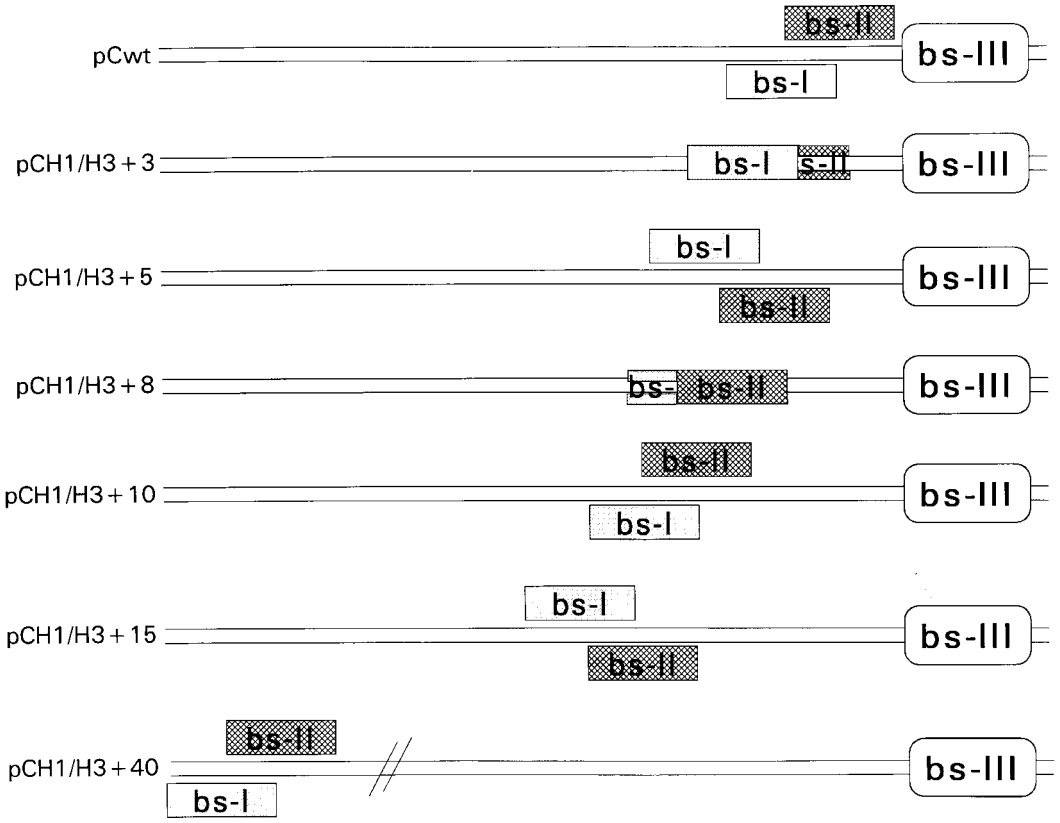

Figure 1 Schematic representation of the transcription-factor binding sites in the protein C gene promoter

Bs-I (nt −26 to −37) and bs-II (nt −33 to −22) correspond to the two partly overlapping and inversely orientated HNF-3 binding sites, whereas the HNF-1–HNF-6 binding site is indicated by bs-III (nt −22 to −10). The relative orientations of bs-I, bs-II and bs-III are based on the presumption that the DNA double helix has 10 bp per turn [32].

the spacing between these transcription-factor binding sites. Such changes have two effects on the relationship between adjacent binding sites: an effect on the distance along the helical axis, and a rotational effect on the alignment of the binding sites.

As shown in Figure 1 (pCH1/H3+3), the insertion of 3 bp at position −21 causes bs-I and bs-II to rotate a quarter turn along the DNA double helix. As a consequence, bs-I and bs-III become located on the same face of the DNA, whereas bs-II ends up on the opposite site. Rotating bs-I and bs-II a further quarter turn (or five quarter turns) brings the bs-I–bs-II complex back to the wild-type alignment except for the location of bs-I on top and bs-II on the bottom face of the DNA (Figure 1; pCH1/H3+5 and pCH1/H3+15). Increasing the spacing to a total of 8 bp aligns bs-II and bs-III on the same face of the DNA, whereas in this instance bs-I moves to the other site of the double helix (Figure 1, pCH1/H3+8). Since the DNA double helix has approx. 10 bp per turn [31], insertion of 10 bp or multiples thereof have no effect on the alignment of the binding sites (pCH1/H3+10 and pCH1/H3+40) and thus will only affect the distance between the binding sites.

The effect of insertions between bs-I–bs-II and bs-III on protein C promoter activity was determined using transient transfection experiments in HepG2 cells. As shown in Figure 2, the insertion of 3 bp resulted in an approx. 2.5-fold increase in promoter activity compared with wild-type activity. Increasing the spacing to 5 bp leads to an even higher promoter activity of about 460%. After this initial increase a gradual decrease in promoter activity, to approx. 250 and 145%, occurs for longer insertions of 8 and 10 bp respectively. The most likely explanation of these results is the presence of a turn-dependent increase in promoter activity that is maximal after the insertion of half a DNA turn between bsI–bsII and bsIII. Furthermore, it is likely that increasing the distance between the two binding sites releases steric hindrance between the transcription factors HNF-3 and HNF-1 and/or HNF-6, with, as a consequence, increased promoter activity. The initial increase in promoter activity changes into a gradual decrease due to larger insertions of 5, 8 and/or 10 nucleotides between bsI–bs-II and bs-III, reflecting the importance of a three-dimensional arrangement between HNF-3 and HNF-1 and/or HNF-6. The fact that even larger insertions of 15 and 40 nucleotides result in increased transcriptional activity indicate that the three-dimensional arrangement is diminished when the distance between the transcription factors becomes too large.

To distinguish between the two hypotheses mentioned above, we studied the consequence of larger insertions on protein C promoter activity. These experiments revealed that insertion of 15 bp results in activity levels of 700%, whereas increasing the spacing to 40 bp results in even higher transcriptional activities of approx. 2300%. Therefore we conclude that a combined distance- and turn-dependent increase in promoter activity is responsible for the observed data.

To characterize the functional relationship between the HNF-3 and HNF-1–HNF-6 binding site further, we performed co-transfection experiments in Cos cells, which do not express HNF-1, HNF-3 or HNF-6 endogenously. In these experiments,

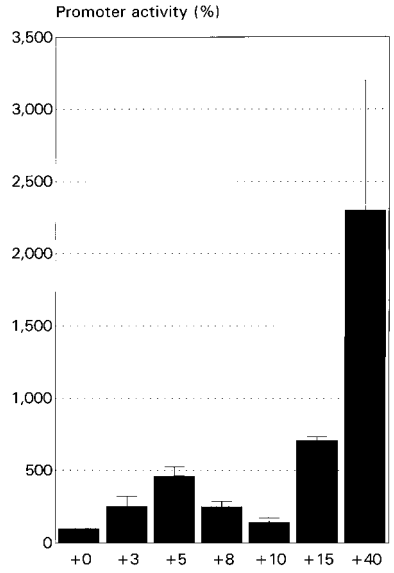

Figure 2 Effect of insertions at position −21 on protein C gene transcription in HepG2 cells

Plasmid constructs with the CAT gene under control of the wild-type protein C promoter (+0) or under the control of the different insertion mutants (+3, +5, +8, +10, +15 and +40) were tested for CAT activity. Shown are the means ± S.D. for six (+0, +3, +5, +8 and +10) or three (+15 and +40) individual experiments performed in duplicate.

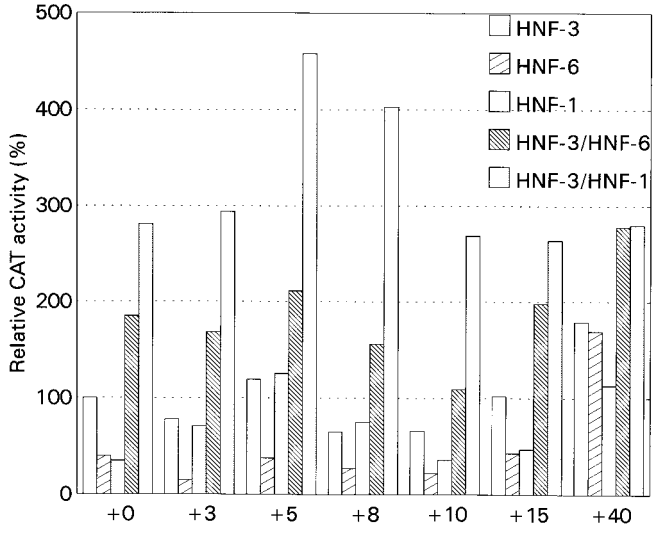

Figure 3 Effect of insertions at position −21 on protein C gene transcription in Cos cells

Plasmid constructs with the CAT gene under control of the wild-type protein C promoter (+0) or under the control of the different insertion mutants (+3, +5, +8, +10, +15 and +40) were tested for CAT activity in the presence of HNF-3, HNF-1, HNF-6, HNF-3 and HNF-1, and HNF-3 and HNF-6. All transfections were performed twice in duplicate.

we co-transfected the different insertion mutants with expression vectors for either HNF-1α, HNF-3α or HNF-6 alone or with a combination of HNF-3α and HNF-1α or HNF-6 expression vectors. As shown in Figure 3, similar results were obtained for co-transfection experiments with HNF-3 or HNF-6, except that the HNF-3-induced levels are higher. Introduction of 3, 8 or

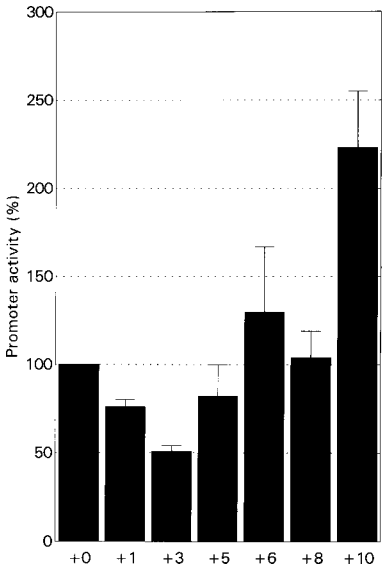

Figure 4 Effect of insertions at position −5 on protein C gene transcription in HepG2 cells

Plasmid constructs with the CAT gene under control of the wild-type protein C promoter (+0) or under the control of the different insertion mutants (+3, +5, +8 and +10) were tested for CAT activity. Shown are the means ± S.D. for six individual transfections performed in duplicate.

10 bp results in a decrease in promoter activity, whereas introduction of 5 or 15 bp has no effect on the promoter activity. After the insertion of 40 bp, protein C promoter activity is increased significantly. Co-transfection of the protein C promoter constructs with a combination of HNF-3 and HNF-6 results in the same turn- and distance-dependent effects as observed for one of the transcription factors alone. However, the combination of both factors results in a more-than-additive effect on promoter activity, indicating a co-operative effect between HNF-3 and HNF-6. Figure 3 also shows that co-transfection of HNF-1 results in an initial increase in promoter activity (pCH1/H3+3 and pCH1/H3+5), followed by a gradual decrease due to larger insertions (pCH1/H3+8 and pCH1/H3+10). Subsequently, the promoter activity increases again as the consequence of the introduction of 15 or 40 bp. Co-transfection of the protein C promoter constructs with HNF-3 and HNF-1 show comparable results as those obtained with co-transfection of HNF-1 alone. However, as observed for the combination of HNF-3 and HNF-6, the combination of HNF-3 and HNF-1 results in relatively high promoter activity levels, indicating the presence of co-operativity between HNF-3 and HNF-1.

Insertional mutagenesis at nt −5

In order to examine the importance of interactions between factors binding to the HNF-3- and HNF-1–HNF-6-responsive elements and factors of the basal transcription machinery that bind around the transcription start site, we inserted sequences of variable length at nt −5. Again, as for insertions at nt −21, these insertions influence both the distance along the helical axis and the relative alignment of adjacent binding sites.

As shown in Figure 4, introduction of 1 or 3 bp (pCH1/ST+1 and pCH1/ST+3) results in a slight decrease in promoter activity to approx. 80 and 51 % respectively. Due to the insertion of half a DNA turn (pCH1/ST+5 or pCH1/ST+6) the tran-

scriptional activity is restored (pCH1/ST + 5) or even elevated to 129 % (pCH1/ST + 6). As a consequence of inserting 8 bp (pCH1/ST + 8), the promoter activity is slightly reduced compared to the insertion of 6 bp. Finally, the insertion of a complete helical turn (pCH1/ST + 10) results in a maximal promoter activity of 225 %. These results clearly indicate a turn-dependent decrease in promoter activity. Furthermore, the observed distance-dependent increase in promoter activity suggests the presence of steric hindrance between the HNF-3–HNF-1–HNF-6 complex and factors of the basal transcription machinery.

DISCUSSION

The human protein C gene promoter region contains three partly overlapping transcription-factor binding sites (bs-I, bs-II and bs-III) which are located within the proximal 40 bp upstream of the transcription start site. The aim of the present study was to evaluate whether the tight alignment of binding sites close to the transcription start site has any functional significance.

The guanidine at nt −22 in the wild-type protein C sequence is part of both bs-II and bs-III. To prevent the disruption of either of the two binding sites due to the insertion at nt −21 we created all inserts with a duplicate of nt −23 to −21 at their 3′ end. In this instance both bs-II and bs-III are preserved completely. A general problem with insertional mutagenesis experiments is the risk of introducing or creating transcription-factor binding sites due to the inserted sequences. However, tedious inspection of the inserted sequences and the sequences created at their junction with the protein C gene sequence for similarity to a large variety of transcription-factor consensus sequences [32] minimized this risk. Another possible complication is that, due to the insertions, a transcription-factor binding site is abolished. Again, this risk was minimized by a close comparison of the region around the insertions with known transcription-factor consensus sequences.

The described HNF-6 binding site is quite similar to a number of HNF-3 binding sites in terms of nucleotide sequence [33]. As a consequence, some HNF-6 binding sites also bind HNF-3, whereas some HNF-3 binding sites also bind HNF-6. Therefore, it might be argued that bs-I–bs-II is a potential HNF-6 binding site and that bs-III is a potential HNF-3 binding site. However, transient transfection experiments show that abolishing bs-III makes the protein C promoter unresponsive to HNF-6 [19], whereas abolishing bs-I–bs-II makes the promoter unresponsive to HNF-3 [17]. This clearly suggest that bs-I–bs-II is not an HNF-6-responsive element and that bs-III is not an HNF-6-responsive element. Further evidence for the exclusive binding of HNF-3 to bs-I–bs-II and of HNF-6 to bs-III is obtained from electrophoretic-mobility-shift assays with liver nuclear extracts which show no binding of HNF-6 to bs-I–bs-II or binding of HNF-3 to bs-III (C. A. Spek, R. M. Bertina and P. H. Reitsma, unpublished work).

The results of the present study show that changing the spacing between the HNF-1–HNF-6 binding site and the two HNF-3 binding sites in the protein C promoter results in both a distance-dependent and a DNA-turn-dependent increase in promoter activity. The distance-dependent increase might be caused by release of steric hindrance between HNF-1 and/or HNF-6 and HNF-3 followed by a higher binding-site occupancy. Alternatively, it might be that moving the HNF-3 binding sites away from the HNF-1–HNF-6 binding site enables looping of the DNA between the two binding sites, resulting in more efficient complex formation between the two transcription factors with each other or with members of the basal transcription machinery. When considering that looping of the DNA requires more spacing than does the release of steric hindrance, the observation that introduction of 40 bp increases promoter activity approx. 100-fold more than introduction of 10 bp supports the latter explanation.

Introduction of half a DNA turn (or multiples thereof) between bs-I–bs-II and bs-III increases promoter activity significantly. This turn-dependent increase indicates that the transcription-factor binding sites are not optimally aligned in the wild-type protein C promoter. However, it also suggests an important role for protein–protein interactions in determining efficient protein C transcription. On the basis of co-transfection experiments in Cos cells, which show co-operativity between HNF-3 and both HNF-1 and HNF-6, we conclude that part of the protein–protein interactions occur between HNF-3 and HNF-1 and/or HNF-6. The fact that co-transfection with HNF-3 alone also results in distance- and turn-dependent variation in promoter activity suggests functionally important interactions between HNF-3 and downstream-located transcription factors. These downstream factors might be members of the basal transcription machinery or might be specific transcription factors interacting with the protein C promoter. To date, only a transcription-factor-Sp1 binding site has been localized downstream in the protein C promoter region (nt +46 to +53) [34], making this a possible candidate. Interactions between HNF-1 and/or HNF-6 with transcription factors located upstream of the HNF-3 binding sites also play an important role in wild-type protein C transcription. This follows from co-transfections with HNF-1 or HNF-6 alone, which show that insertions between bs-I–bs-II and bs-III result in turn- and/or distance-dependent variation in promoter activity.

The results presented here also show that changing the spacing between the HNF-3–HNF-1–HNF-6 complex and the transcription start site results in a distance-dependent increase and DNA-turn-dependent decrease in promoter activity. As mentioned above for the insertional mutagenesis experiments at nt −21, the distance-dependent increase might be explained in multiple ways. The increased spacing might release steric hindrance between the HNF-3–HNF-1–HNF-6 complex and members of the basal transcription complex. Alternatively, it might be that increasing the spacing leads to more efficient complex formation between the HNF-3–HNF-1–HNF-6 complex and basal transcription factors due to improved looping possibilities.

The observed DNA-turn-dependent decrease in promoter activity indicates that in the wild-type protein C promoter the HNF-3–HNF-1–HNF-6 complex is aligned optimally relative to the transcription start site. The introduction of half a DNA turn (5 or 6 bp) is less deleterious than the insertion of quarter DNA turns (3 and 8 bp). This again suggests the functional importance of protein–protein interactions between specific transcription factors (HNF-1, HNF-3 and HNF-6) and members of the basal transcription machinery. Correction for the distance-dependent increase in promoter activity shows that these suggested protein–protein interactions take place less efficiently when the relative orientation of the binding sites is changed.

We have shown that the transcriptional activity of the protein C gene promoter is enhanced dramatically when the transcription-factor binding sites are liberated from their tight clustering close to the transcription start site. This indicates that the alignment of binding sites is far from optimal for maximal promoter activity. Therefore the physiological importance of the tight alignment seems to be the prevention of excessive protein C expression. Previous insertional mutagenesis experiments did not reveal this unique feature of increased promoter activity levels [20–25]. However, unlike in the present study, the examined

binding sites were clearly separated. Insertions between two cell-specific footprint regions in the rat-growth-hormone promoter, which are separated 15 bp, resulted in combined distance- and DNA-turn-dependent decreases in promoter activity [21]. A combination of distance- and DNA turn-dependent reductions in promoter activity has also been reported for insertions between the enhancer and the 21 bp repeat region in the SV40 promoter [20]. Increasing the spacing between growth-hormone-factor-3 and thyroid-hormone- receptor binding sites, located at nt −239 to −219 and −190 to −166 respectively from downstream Pit-1 (nt −143 to −108 and −94 to −63) and Sp1 (−147 to −129) binding sites in the rat-growth-hormone promoter results only in a distance-dependent decrease in gene expression [22]. DNA-turn-dependent decreases in promoter activity have been reported for insertions or deletions between two proximal cell-specific elements in the rat prolactin promoter [23] and between the glucocorticoid response element and CACCC-box of the tryptophan oxygenase promoter [24]. Finally, insertions between binding sites for the liver-enriched HNF-4 and -3 in the apolipoprotein AI promoter did not influence the simultaneous binding of HNF-4 and HNF-3 nor did it influence their functional synergy [25].

This work was supported by a grant (no. 92.004) from the Trombosestichting Nederland.

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Received 11 January 1999/15 February 1999; accepted 9 March 1999