Regulation of human protein S gene (PROS1) transcription

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Chapter 3

Regulators of *PROS1* transcription, a pilot study

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Summary

The function of anticoagulant protein S (PS) as a cofactor for activated protein C in the inactivation of coagulation factors Va and VIIIa has been well described. A deficiency in PS may either be hereditary or acquired and is associated with an increased risk for venous thrombosis. It is for this reason that much research has been conducted into the genetic structure of the gene encoding PS; *PROS1*. Many mutations in the exons encoding for the PS protein have been found to contribute to lowered plasma PS levels. On the other hand, the *PROS1* promoter through which transcription is regulated has been poorly investigated. We set out to tackle this outstanding issue by analysing the *PROS1* promoter sequence through phylogenetic footprinting. The promoter sequences of six different species were compared with the human *PROS1* sequence and the compiled data were analysed for conserved regions. Especially the region between base pairs -300 and -100 upstream from the translational startcodon showed a high degree of similarity amongst species. In this region sites were found for DNA-binding proteins such as the Sp-family of transcription factors and C/EBPβ.

Transfection into HeLa cells of a *PROS1* promoter-reporter gene construct with a promoter length of 370 bp and candidate transcription factors confirmed that both Sp1 and C/EBPβ are potent activators of the *PROS1* promoter. *PROS1* promoter activity was also influenced, either positively or negatively, by FOXA2, AP2 and AP1 and the androgen receptor. The results of this study provide indications as to which effectors may play a role in *PROS1* transcriptional regulation *in vivo* and therefore provide a starting point for future research into the transcriptional regulation of PS levels.

Introduction

Anticoagulant Protein S (PS) is a vitamin K-dependent plasma glycoprotein which functions as a non-enzymatic cofactor to activated protein C (APC) in the protein C anticoagulant pathway (1-3). PS is produced mainly by the hepatocytes in the liver (4), but also at low levels by many other cell types in the body (5-11). In patients with liver disease PS plasma levels are not reduced to the same extent as those of the structurally related vitamin-K dependent coagulation factors (12). In these circumstances significant amounts of PS are thought to be produced and released by megakaryocytes (6;7;13) and the vascular endothelium (5;8).

Protein S mRNA is transcribed from the *PROS1* gene on chromosome 3 (14-17). Early studies into the transcriptional initiation of *PROS1* indicated that transcription is generated from multiple start sites within the TATA box-less promoter of this gene (17-20). We recently confirmed and extended these assumptions by demonstrating that *PROS1* transcription is generally directed from one of four major transcription start sites located 100 bp, 117 bp, 147 bp, and 200 bp upstream from the translational startcodon, respectively (21). Hall and coworkers presented an abstract at the meeting of the International Society of Thrombosis and Haemostasis (ISTH) in 1995 reporting the regulation of *PROS1* transcription by multiple transcription factors (22). An induction of *PROS1* promoter activity *in vitro* was demonstrated in cotransfections of *PROS1* promoter-reporter gene constructs with expression vectors containing cDNAs for CCAAT/Enhancer-Binding Protein (C/EBP) α, D-Binding Protein (DBP), Forkhead Box A (FOXA), Hepatocyte Nuclear Factor (HNF) 4, androgen receptor (AR), estrogen receptor (ER) and glucocorticoid receptor (GR). The only other study that investigated the regulation of the *PROS1* promoter, showed binding to and induction of the *PROS1* promoter by Sp1 and binding of FOXA (23).

In this pilot study, we tested selected candidate transcription factors for the ability to influence *PROS1* promoter activity *in vitro*. Cotransfections of epithelial carcinoma cell line HeLa with a *PROS1* promoter-reporter gene construct and expression vectors containing the cDNA for C/EBPβ, FOXA2 (HNF3β), HNF6 (Onecut1), DBP, p49, Activator Protein (AP) 2, Specificity protein (Sp) 1 and Sp3 resulted in increased promoter activity. In contrast,

promoter activity was decreased in the presence of FOXA3 (HNF3γ), HNF4, jun, fos, p65 and the AR.

Experimental procedures

Phylogenetic footprinting and promoter predictions for the PROS1 promoter region --- The 5' upstream sequences for *PROS1* from human, chimpanzee, rhesus monkey, dog, mouse, rat, pig and cow were either generated by automated sequencing (human) or assembled through alignment of known sequences with the nucleotide and trace databases at the National Center for Biothechnology Information (NCBI) (www.ncbi.nlm.nih.gov). The sequences were aligned and analyzed with the VISTA software (genome.lbl.gov/vista), where necessary the alignments were adjusted manually. Additional transcription factor binding site analyses were done with the IFTI mirage software (www.ifti.org) and MatInspector professional (www.genomatix.de). Promoter predictions were done with the proscan software at the bimas.dcrt.nih.gov web site.

Plasmids --- The *PROS1* promoter-reporter gene (luciferase) construct, PS370, was described previously (Chapter 2 of this thesis). The expression vectors containing cDNAs for transcription factors of murine origin; pSCT-C/EBPβ (24), pMSV-C/EBPα (25), pECE-HNF6 (26), pCMV-DBP (27), pcDNA3-FOXA (1, 2, 3) (HNF3α, β, γ) (28;29), were described previously. pcDNA3-HNF4 was generated from pLEN4S-HNF4 (30) and pcDNA3-HNF1α was generated from pBJ5-HNF1α (31). The expression vectors containing cDNAs for transcription factors of human origin; pSV-ARO (32), pCMV-AP2 (33), pCMV4-Sp1 (34) and pCMV4-Sp3 (35), pRSV-cjun and pRSV-cfos (36), pCMV-p49 (37), pCMV-p50 and pCMV-p65 (38), were previously described. pTracer-GR was generated from pRSV-GR (39) and pcDNA3-HNF1β was generated by insertion of the original HNF1β cDNA (40) into pcDNA3. The cDNAs for constitutively active human ERα and ERβ in expression vector pShuttle were generated at the Human and Clinical Genetics department of the Leiden University Medical Centre and were kindly provided by Dr Willems van Dijk.

Cell culture --- The human cervix epithelial carcinoma cell line HeLa was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in Minimal essential medium (MEM), 10% Fetal Bovine Serum (FBS), 100 μg/ml penicillin, 100 μg/ml streptomycin, and 1x MEM non-essential amino acids (all purchased from Gibco, Invitrogen).

Reporter gene assays --- Cells were transfected at 60-80% confluency. Each transfection was performed in triplicate in 12-wells plates. Transfections were carried out using 3 µl Tfx-20 lipids (Promega) per µg transfected DNA. In each transfection 500 ng PS370 and 500 ng transcription factor expression vector was used. Control vector pRL-SV40 (Promega), expressing the Renilla luciferase, was co-transfected for correction of transfection efficiency in a 1:500 ratio to total transfected DNA. The cell extracts were harvested 48 hours after transfection. Luciferase levels were measured with the dual-luciferase assay kit (Promega, Leiden, the Netherlands) in the LB9507 luminometer (Berthold, Bundoora, Australia) according to the manufacturers recommendations.

Results

Conserved sequences in the PROS1 promoter region --- The 370 bp 5' upstream sequences for *PROS1* from human, chimpanzee, rhesus monkey, dog, mouse, rat, pig and cow were aligned with the mVISTA software and analysed for putative transcription factor binding sites (Figure 1). A high degree of conservation amongst all species was found in a region approximately encompassing bp -230 to -100. Upstream from this point the *PROS1* promoter sequence from mouse and rat deviated from the other species, which kept a high homology up to -300 bp. Sequences from rhesus monkey, chimpanzee and human were highly homologous throughout the examined region.

In the first region (-230/-100) two stretches of sequence with a very high homology were found, namely -240/-205 and -185/-155. GC-boxes are located at -170 and -155 bp and are putative binding sites for the Sp-family of transcription factors, the consensus sequence being KGGGCGGRRY (5'-3') (41). At positions -230 through -210 sites for STAT (42), consensus TT(n)$_{4\text{-}5}$AA, and C/EBPβ (24), consensus RNRTKNNGMAAK, were identified. Since these sites have remained in the *PROS1* promoter throughout evolution it may be assumed that they areimportant in the regulation of the transcriptional activity of the *PROS1* promoter. Therefore, these sites are obvious candidates for future studies.

Further computational analyses were done for the *PROS1* promoter region also taking into account the location of transcription start sites. The proscan software calculated a putative promoter with a start site at -148 but did not identify the other start sites that were

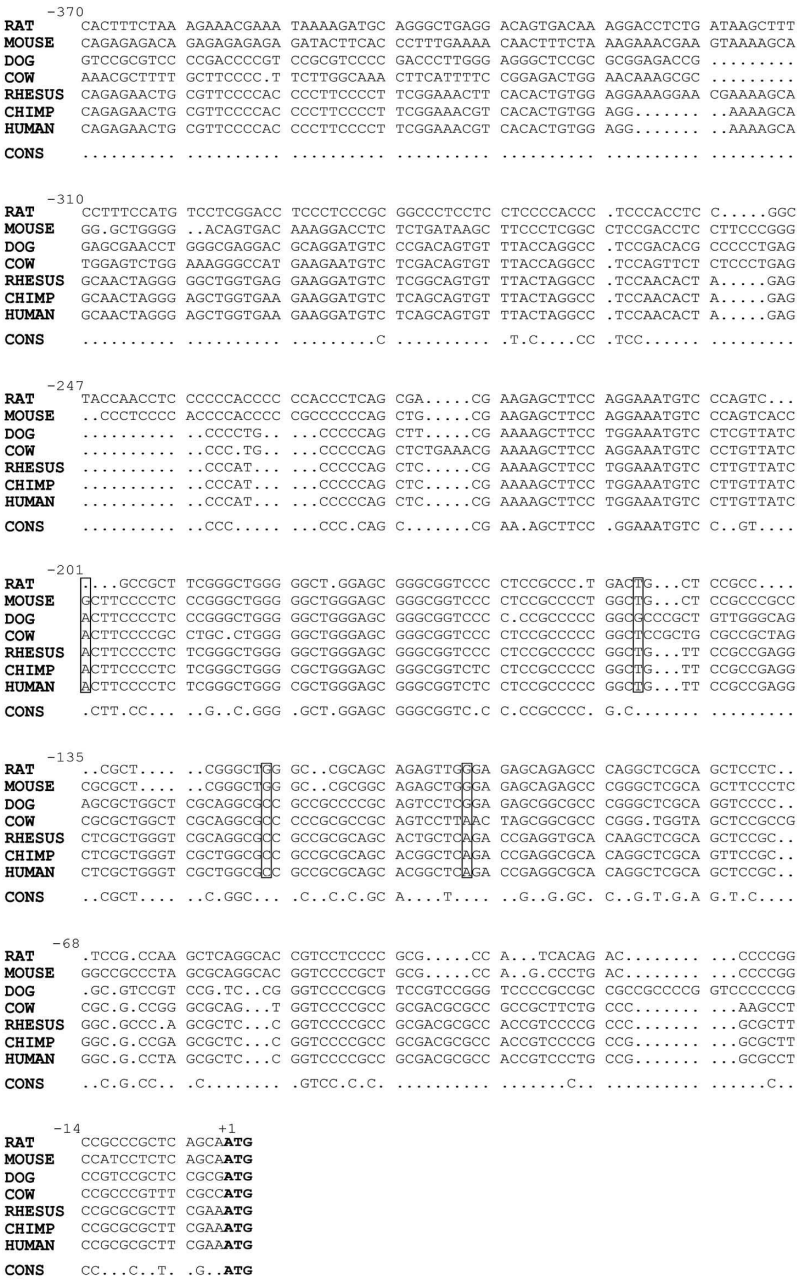

Figure 1 Phylogenetic alignment of the *PROS1* promoter sequence. Sequences from human, chimpanzee, rhesus monkey, cow, dog, mouse and rat were retrieved from the archives at the NCBI. Numbering is according to the human sequence with the first nucleotide of the translational startcodon being +1. Cons; 100% conserved sequence. The enboxed nucleotides are the transcription start sites found in reference 21.

experimentally found by us previously (21). Table 1 gives the consensus, binding site and conservation level of selected transcription factor binding sites within the -370/-1 *PROS1* sequence using various programs.

TF	Location	Consensus	Conservation	Software
AP2	-360	YCSCCMNSSS	h,ch,r	3, 4
Sp1	-360/-345	KGGGCGGRRY	h,ch,r	2, 4
CREB/ATF	-340/-325	TGACGTMA	h,ch,r	2, 3, 4
FOXA2	-275/-265	TRTTTRYTT	h,ch,r,co,d	2, 4
STAT, C/EBPβ	-225/-215	TTNNNN(N)AA RNRTKNNGMAAK	All	1, 2, 4
GR	-215	TGTNCT	All	1
Sp1	-190	KGGGCGGRRY	All	1
AP2	-190/-180	CCCMNSSS	All	1, 3, 4
Sp1	-170	KGGGCGGRRY	All	1, 2, 3, 4
Sp1	-160/-150	KGGGCGGRRY	All	1, 2, 3, 4

Table 1 Putative transcription factor binding sites in the *PROS1* promoter. Software: 1; rVISTA, 2; Matinspector professional matrix library, 3; proscan, 4; ifti mirage. Abbreviations: TF: Transcription factor, h; human, ch; chimpanzee, r; rhesus monkey, co; cow, d; dog, GR: glucocorticoid receptor. For consensus binding sites, see the Transfac public database at www.gene-regulation.com.

Many more possible binding sites are routinely found by computational analysis, however most can be ruled out because of tissue-specific expression of the transcription factor or an incomplete consensus. On the other hand, when an incomplete binding site is preserved throughout evolution it may nevertheless be functional. For instance, incomplete sites were found for AP1 (fos), C/EBPα, NFκB and the neurally expressed silencer NSRF. These sites are not included in the table. On the other hand, the GR-site, which is also incomplete, ís mentioned because it is in a highly conserved region.

Rationale for the selection of transcription factors --- Transcription factors were selected on basis of the presence of a putative binding site in the -370/-1 *PROS1* sequence (Sp1, Sp3, FOXA2, C/EBPβ, AP2, GR), on their relation to the prior category (FOXA1, FOXA3, C/EBPα, ER, AR) or their liver-specific expression (HNF1, HNF4, HNF6, FOXA1, FOXA2, FOXA3, C/EBPβ, DBP). An additional argument for including the steroid receptors ER and AR is

given by the fact that plasma PS levels are negatively influenced by female steroid hormones (43-45). The mechanism by which this downregulation of PS levels occurs is unknown.

The major pro-inflammatory cytokines interleukin 1 (IL1) and Tumor Necrosis Factor α (TNFα) activate transcription factors AP1 (jun and fos), and NFκB (p49, p50, p65) (46;47). Incomplete binding sites for these DNA-binding proteins are located in the PS370 sequence. Even though the consensuses are incomplete we included the expression vectors for these transcription factors in our study mainly for two reasons. Firstly, PS levels in hepatoma (HepG2) and endothelial (MVEC, HUVEC) cell culture are upregulated by interleukin 6 (IL6) and downregulated by TNFα thus linking PS to the inflammatory response (48;49). Secondly, recent publications indicate that free plasma PS may act on inflammation by facilitating the uptake of early apoptotic cells by macrophages (50) and that PS in complex with complement 4b binding protein (C4BP) directs complement to the surface of apoptotic cells (51;52).

PROS1 promoter activity is modulated by various liver-specific and ubiquitous transcription factors --- HeLa epithelial carcinoma cells were co-transfected with *PROS1* promoter-reporter construct, PS370, and a transcription factor expression vector (Figure 2). Transcription factors Sp1 and Sp3 upregulated *PROS1* promoter activity 23- and 15-fold, respectively. Lower, but also significant, stimulatory effects were shown for C/EBPβ (7-fold), FOXA2 (4-fold), HNF6 (2-fold), p49 (2-fold) and AP2 (2-fold). Negative effectors were the components of AP1 (jun and fos), the p65 subunit of the NFκB complex and the AR. FOXA3 and HNF4 slightly downregulated *PROS1* promoter activity, the measured reduction in promoter activity was significant but the effect was marginal.

Identical transfection assays were also carried out with hepatocytes (HepG2 cells), which are the main source of plasma PS *in vivo,* resulting in a similar albeit less-pronounced pattern of promoter induction (results not shown). Because endogenous liver-specific transcription factors are already present in the nucleus of hepatocytes, possibly in sufficient levels for maximal *PROS1* promoter regulation, the effect of this category of transcription factors on promoter activity is quenched and routinely underestimated.

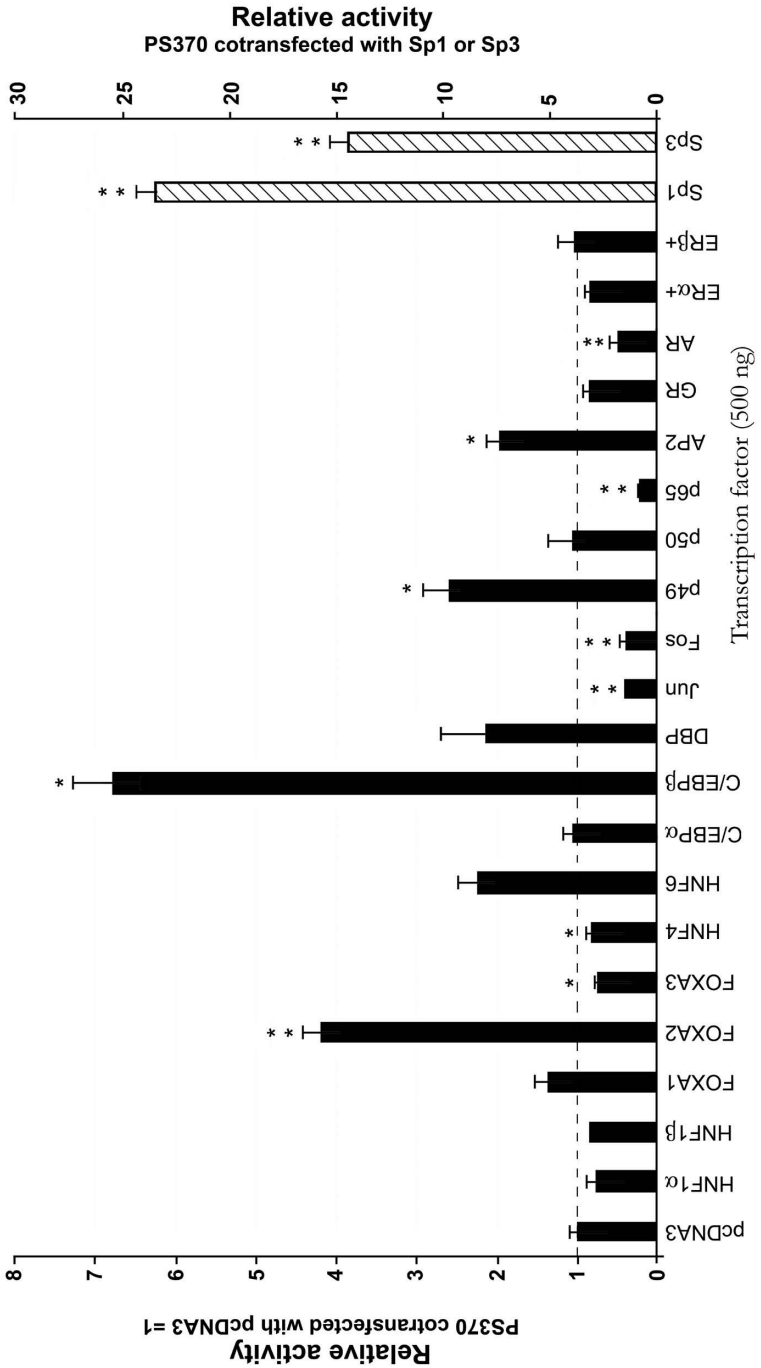

Figure 2 Cotransfection of HeLa cells with PS370 and transcription factors
HeLa cells were transfected as described in the experimental procedures section. Cells were harvested and luciferase activity was measured 48 hrs after transfection. Luciferase levels were not corrected for transfection efficiency since the cotransfected transcription factors also affected the Renilla luciferase undercontrol of the SV40 promoter. All levels are relative to transfections with PS370 and the respective empty expression vectors; only PS370 + pcDNA3 is depicted. *;$p<0.05$, **; $p<0.001$

Discussion

This pilot study is a good example of the added value of phylogenetic footprinting in locating and identifying potential transcriptional effectors in promoter studies. In our species comparison for the PS370 *PROS1* promoter two major sites, at -230/-200 and -190/-155, proved to be highly conserved throughout evolution (Figure 1). The factors C/EBPβ and Sp1/Sp3 that have consensus binding sites within these regions were subsequently found to be the major transcriptional activators of the PS370 promoter-reporter gene construct (Figure 2). Most likely Sp1 *trans*-activates the *PROS1* promoter by binding to multiple sites within the promoter, whereas only a single binding site for C/EBPβ is contained in the PS370 sequence (Table 1). Sp1 is a ubiquitously present transcription factor (41) and may therefore be involved in the constitutive expression of *PROS1*. On the other hand, C/EBPβ is restricted to certain tissues, amongst which the liver (24;53), and is probably involved in tissue-specific regulation of PS levels. The same holds for the liver-specific transcription factor FOXA2 (54) of which the binding site is also highly conserved and which also upregulated PS370 promoter activity. HNF6 mildly induced PS370 activity. We did not find the consensus for HNF6 (5'-AARTCA ATAW-3') in the PS370 promoter region.

The AP1 proteins jun and fos as well as the *trans*-activation subunit p65 of NFkB negatively influenced PS370 activity (Fig 2). On the other hand, C/EBPβ, a known mediater of IL6 signalling, enhanced PS370 activity. The stimulatory and inhibitory effects on PS levels *in vitro* described for IL6 and TNFα, respectively, may thus be mediated through these proteins (48;49). Interestingly, APC was shown to inhibit the activation and translocation of the NFκB complex and AP1 to the nucleus thereby inhibiting TNFα production and release by monocytes (55). Although there is no evidence to suggest a regulation of PS levels by APC, it is tempting to speculate that APC could positively influence *PROS1* transcription through a similar mechanism.

What has been clearly shown is that PS plasma levels are downregulated by female hormones. The mechanism by which this occurs has not been studied, thus cotransfections of PS370 with the steroid receptors were of special interest. We did not find a good consensus for any nuclear steroid receptors in the *PROS1* -370/-1 sequence (the GR-site in Table 1 is rather weak/poor), but nevertheless included expression vectors for the constitutively active

ERα and ERβ, the GR, and the AR in our pilot study. In contrast to the results obtained by Hall and coworkers (22) we found that the AR downregulated PS370 activity, whereas the GR and ER had no influence. Although the ER was long considered to be the sole mediator of estrogen action, several findings show that estrogen can directly activate AR function (56-58). Our results for the AR are thus in line with the inhibitory influence of female steroid hormones on PS plasma levels, however we had expected to see an effect with the ER as well.

As a cell differentiation factor, the transcription factor AP2 is imperative for the normal development of the neural crest in embryogenesis (59). In adulthood AP2 mRNA is present in several tissues with highest levels occurring in female reproductive organs, eye, skin and liver (http://harvester.embl.de/harvester). The *PROS1* promoter has a conserved AP2 binding site and, in cotransfections, AP2 enhanced *PROS1* promoter activity, albeit marginally (2 fold). This is of interest since PS is present in brain (60-62) and has been shown to protect against neuronal cell death upon ischemic injury (63).

In conclusion, transcription factors affecting *PROS1* transcription were found amongst ubiquitously expressed factors, as well as amongst tissue-specific transcription factors. The results of this pilot study give a sneak preview into the regulation of PS protein levels at the transcriptional level and provide a starting point for in depth research into the regulation of *PROS1* promoter activity.

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