Regulation of human protein S gene (PROS1) transcription

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I can scarcely wait till tomorrow
When a new life begins for me,
As it does each day,
As it does each day.

~Stanley Kunitz~

Aan alle lieverds in mijn leven,
met Mams en Martijn voorop!

Contents

Abbreviations

AP	Activator Protein
APC	Activated Protein C
APR	Acute Phase Response
AR	Androgen Receptor
ATCC	American Type Culture Collection
ATF	Activating Transcription Factor
C4BP	Complement factor 4b binding protein
C/EBPβ	CCAAT/Enhancer-Binding Protein beta
ChIP	Chromatin immunoprecipitation
COS-1	African green monkey kidney cell line
CREB	cAMP-response element binding protein
CRP	C-reactive protein
DBP	D-binding protein
DIC	Disseminated Intravascular Coagulation
DPE	Downstream Promoter Element
ELISA	Enzyme-linked immunosorbent assay
EGF	Epidermal Growth Factor
EPCR	Endothelial Protein C Receptor
ER	Estrogen Receptor
ERE	Estrogen responsive element
EST	Expressed sequence tag
FBS	Foetal Bovine Serum
FOXA	Forkhead Box A
FV	Factor V
FVa	Activated factor V
FVi	Inactivated factor V
FVII	Factor VII
FVIII	Factor VIII
FIX	Factor IX
FX	Factor X
FXI	Factor XI
GAIT	Genetic Analysis of Idiopathic Thrombophilia
GAS6	Growth arrest-specific 6
GR	Glucocorticoid Receptor
GSP	Gene-Specific Primer
HeLa	Human cervix epithelial carcinoma cell line
HepG2	Human hepatocellular carcinoma cell line G2
HMEC-1	Human dermal microvascular endothelial cell line

HNF	Hepatocyte Nuclear Factor
HRP	Horseradish peroxidase
HuH7	Human hepatocellular carcinoma cell line 7
HUVEC	Human Umbilical Vein Endothelial Cells (primary cells)
IL	Interleukin
IL6-RE	Interleukin 6-Responsive Element
Inr	Initiator
ISTH	International Society of Thrombosis and Haemostasis
MCS	Multiple Cloning Site
Meg01	Human megakaryocytic cell line; chronic myeloid leukemia in megakaryocytic blast crisis established from bone marrow
LAP	Liver activating protein (alias for C/EBPβ)
LIP	Liver inactivating protein
NCBI	National Center for Biotechnology Information
NE	Nuclear extract
NF-IL6	Nuclear factor for IL6 (alias for C/EBPβ)
NFY	Nuclear Factor Y
OC	Oral contraceptives
PAI-1	Plasminogen Activator Inhibitor-1
PBGD	Phorphobilinogen deaminase
PCI	Protein C Inhibitor
PCR	Polymerase Chain Reaction
PMSF	phenyl methyl sulfonyl fluoride
PROS	Protein S gene
PS	Protein S
QPCR	Quantitative real-time PCR
RACE	Rapid Amplification of cDNA Ends
SAA	Serum amyloid A
SHBG	Sex Hormone Binding Globulin
Sp	Specificity protein
STAT	Signal Transducer and Activator of Transcription
T_a	Annealing temperature
TF	Tissue factor
TFPI	Tissue Factor Pathway Inhibitor
TNFα	Tumor Necrosis Factor alpha
t-PA	Tissue-type Plasminogen Activator
TSS	Transcription Start Site
UTR	untranslated region
VTE	Venous Thrombo-Embolism

Chapter 1

General introduction

Chapter 1

General Introduction

1.1 Haemostasis and Venous Thrombosis

Haemostasis refers to a physiologic process whereby bleeding is halted (reviewed in (1-3)). When a blood vessel is damaged, several processes occur to staunch the flow of blood. Firstly, vasoconstriction narrows the blood vessel, reducing vessel diameter and slowing bleeding. Then, during primary haemostasis blood platelets bind to collagen in the exposed sub-endothelium to form a haemostatic plug within seconds after an injury. This is followed by secondary haemostasis or coagulation, which involves the activation of a complex cascade of coagulation factors, ultimately resulting in the conversion of fibrinogen into polymerized fibrin, making a clot. Finally, the clot attracts and stimulates the growth of fibroblasts and smooth muscle cells within the vessel wall, and initiates the repair process, which ultimately results in the dissolution of the clot through fibrinolysis (tertiary haemostasis). Disorders of haemostasis can be roughly divided into platelet disorders, such as Glanzmann thrombasthenia and Bernard-Soulier syndrome, and disorders of coagulation, such as haemophilia or thrombosis.

Most coagulation factors circulate as the zymogen of a serine protease. The coagulation cascade is a series of reactions in which the zymogens and their glycoprotein cofactors are activated and then catalyze the next reaction in the cascade. Coagulation is initiated mainly in response to the interaction between factor VII (FVII) and exposed tissue factor (TF) from the vascular sub-endothelium. The TF-activated FVII (FVIIa) complex activates coagulation factors IX (FIX) and X (FX) (4), thereby activating the coagulation cascade (Figure 1). The final product of the cascade, thrombin, increases its own production by activating other components of the coagulation cascade, amongst which factors V (FV), VIII (FVIII), and XI (FXI) and so the cycle continues. The primary role of thrombin is the conversion of fibrinogen into fibrin fibres, the main component of the blood clot together with the platelets.

Activated clotting factors are the driving force of the coagulation cascade. To prevent excessive clotting, activated coagulation factors are inactivated by the circulating blood protease inhibitors; antithrombin (5) and heparin cofactor II (6), by tissue factor pathway inhibitor (TFPI) (7) and by Activated protein C (APC), the end product of the protein C anticoagulant pathway (8). Whereas blood protease inhibitors and TFPI bind to the activated

clotting factors and/or clotting factor complexes thereby rendering them inactive, APC inhibits the coagulation cascade through the proteolytic degradation of the two key coagulation factors activated FV (FVa) and activated FVIII (FVIIIa).

The coagulation cascade is tightly regulated since excessive production of fibrin would lead to the occlusion of blood vessels and thrombosis, whereas too little fibrin would cause (excessive) bleeding and impaired wound healing. When clots are formed in the venous system we refer to venous thrombo-embolism. These clots most often occlude veins in the extremities (e.g. legs; deep vein thrombosis), and may form emboli, which travel through the blood to the narrow pulmonary arteries, where they may cause life-threatening obstructions (pulmonary embolism). The annual incidence of venous thrombosis is 1-3 per 1000 individuals (9;10). A predisposition towards this disease may be genetic or acquired. Acquired risk factors include a.o. aging, immobilisation, trauma, pregnancy, and use of female hormones (11). Genetic risk factors include loss of function mutations in the genes coding for antithrombin, protein C, and protein S (PS) and gain of function mutations in the FV and prothrombin gene (12). The most common genetic risk factor is a mutation in the gene encoding FV, causing an amino acid substitution, R506Q, which renders this coagulation factor resistant to inactivation by APC (13). FV-R506Q is referred to as FV_{Leiden} and occurs in almost 50% of the patients with a family history of venous thrombosis (14).

1.2 The Protein C Anticoagulant Pathway

The protein C anticoagulant pathway is initiated by the activation of protein C to APC by a complex of thrombin and the transmembrane glycoprotein thrombomodulin. Thrombomodulin present in the membranes of vascular endothelial cells amplifies protein C activation more than 1000 fold (15), and when protein C is bound to the Endothelial Protein C Receptor (EPCR), this process is stimulated another 20-fold (16). Upon its generation, APC inactivates F Va and FVIIIa (Figure 1) thus inactivating the prothrombinase and tenase complexes (17;18). Both proteolytic reactions are greatly enhanced by the negatively charged phospholipids present on activated platelets and vascular endothelium, and by PS, the non-enzymatic cofactor to APC (19).

PS forms a complex with APC on the phospholipid surface and increases the affinity of APC for negatively charged phospholipids (17;20;21). Moreover, PS relocates the APC active site closer to the membrane surface, which contains the activated coagulation factors (22). In the inactivation of FVIIIa, PS and FV act as synergistic cofactors to APC (23). PS also has direct anticoagulant properties independent from APC. It was shown to directly inhibit the activity of the tenase and prothrombinase complexes, presumably by binding to factors VIIIa (24), Va and Xa (25-27). The physiological implications of these findings are the subject of active research (28-31).

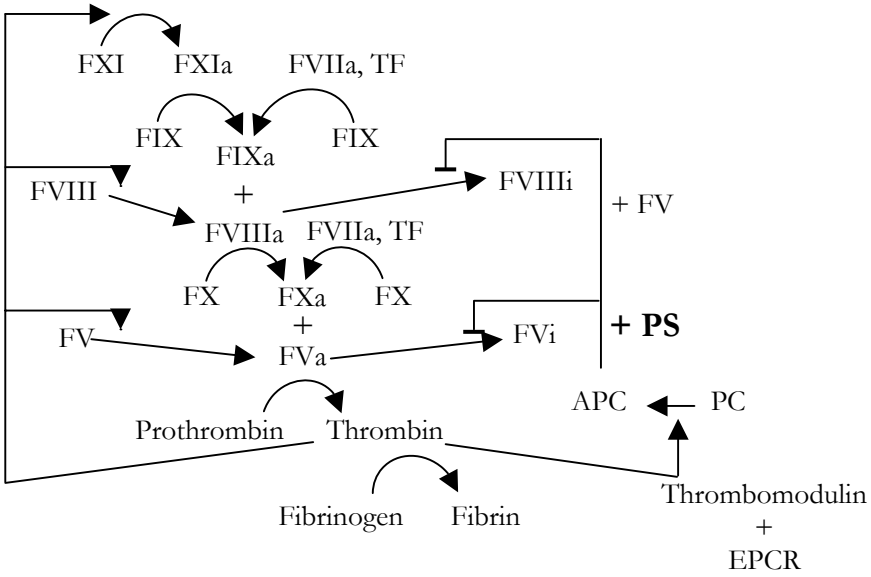

Figure 1 Schematic representation of the coagulation cascade and the protein C anticoagulant pathway. Blunted arrows represent inhibitory reactions.

Next to inhibiting the formation of thrombin, APC also has profibrinolytic properties. During fibrinolysis cross-linked fibrin, the main component of a blood clot, is solubilized by plasmin. Plasmin is generated from its precursor, plasminogen, by tissue-type plasminogen activator (t-PA). t-PA however, is strongly inhibited by plasminogen activator inhibitor-1 (PAI-1). APC is thought to stimulate fibrinolysis through binding and inhibition of PAI-1 (Figure 2) (32-34). The APC-PAI-1 interaction is greatly enhanced upon binding of the extracellular matrix protein vitronectin to PAI-1 (35).

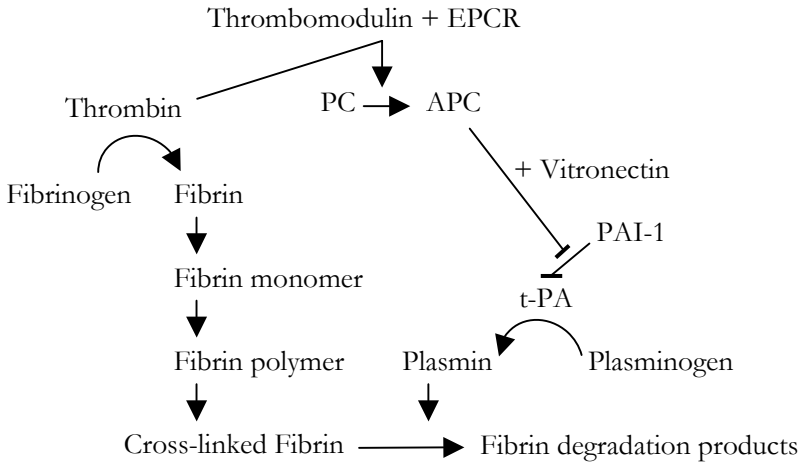

Figure 2 Schematic representation of the proposed role for APC in fibrinolysis. Blunted arrows represent inhibitory reactions.

In addition to their anticoagulant role, both APC and PS have been implicated in other major physiological processes. APC has been shown to have both anti-inflammatory and anti-apoptotic effects (36-38), and successful clinical trials have been conducted for development of its use in the treatment of sepsis (39;40). Similar functions have been allocated to PS (41). The emphasis of the research into a role beyond coagulation for this protein has focused mainly on its mitogenic properties (42;43) and its potential role in the regulation of cell survival (44-47).

Just as the coagulation cascade is under strict regulation by several inhibitory pathways, so are the main components of the protein C anticoagulant pathway. APC anticoagulant activity is inhibited after binding to protein C inhibitor (PCI) and α_1-antitrypsin, both members of the serpin family of blood protease inhibitors (48-50). Moreover, PCI prevents the formation of APC by inhibiting the thrombin-thrombomodulin complex (51). The PAI-1-vitronectin complex was also suggested to be important in limiting APC anticoagulant activity on the platelet and endothelial surface (35). The regulation of PS will be discussed in the following section.

1.3 Protein S

1.3.1 Protein

DiScipio and Davie in Seattle first isolated and named protein S(eattle) in 1977 (52). Not much later Walker described PS as the non-enzymatic cofactor to APC (17;19). PS (Figure 3) is a vitamin K-dependent glycoprotein with a molecular weight of 75 kilo Dalton that is present in plasma at a concentration of ~ 0.35 μM (53-55). The structure and function of its individual domains are well-documented and reviewed elsewhere (56-59) and will therefore not be covered here. Regretfully, a crystal structure of PS has not yet been successfully generated.

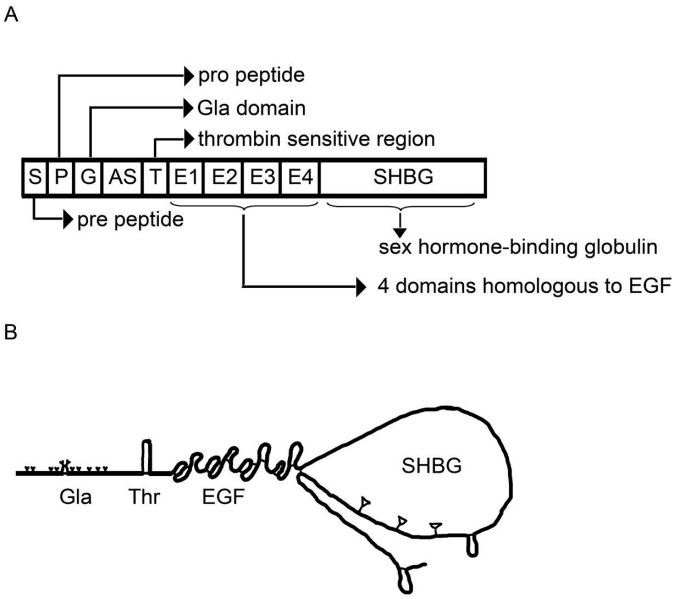

Figure 3 Schematic representation of human Protein S. (**A**) *Immature PS*. AS: aromatic stack, EGF: epidermal growth factor-like domain (**B**) *Mature post-translationally modified PS*. ϒ: carboxylated carboxyglutamic (Gla) residues, ⛛: glycosylation sites, illustration (**B**) taken from (60).

PS is produced and secreted mainly by hepatocytes (61) in the liver but also at low levels by various other cell types, such as megakaryocytes (62), endothelial cells (63;64), Leydig cells (65), osteoblasts (66) and cells of the nervous system (67). In addition, a small portion

(~2.5%) is stored in the α-granules of blood platelets (68). That extra-hepatically produced PS is an important source of plasma PS, is illustrated by the fact that in patients with liver disease PS levels are reduced but not to the same low levels as other vitamin K-dependent coagulation factors (69;70). The anticoagulant reactions in which PS participates (Figure 1), take place on the surface of endothelial cells and activated platelets, cell types that both produce and/or can release PS.

PS can bind to the surface of (endothelial) cells not only through its interaction with negatively charged phospholipids (71), but also by binding to a specific family of membrane receptor tyrosine kinases (Tyro/Axl) (67;72-74). This interaction was first shown for growth arrest-specific 6 (GAS6) (75), a protein structurally related to PS (76). The relevance of this finding is questionable though since in contrast to GAS6, which binds to and stimulates its receptor, PS seems to only bind, not activate, the receptor at physiological relevant concentrations (77).

Functional PS levels in plasma are mainly regulated in two different ways. Firstly, PS can be inactivated by proteolytic cleavage in the thrombin-sensitive region. *In vitro* PS is cleaved by thrombin after Arg 49 and Arg 70 in the thrombin-sensitive region (78). *In vivo* PS is protected against thrombin-mediated cleavage by the binding of calcium ions to the Gla-domain (79). Nevertheless, increased cleaved PS levels were found in patients with disseminated intravascular coagulation (DIC) (80). Long *et al.* demonstrated that *in vitro* PS is cleaved after Arg 60 by FXa (81). This cleavage site was later shown to be the actual cleavage site *in vivo* (82;83). It is therefore assumed that it is FXa that inactivates PS by proteolytic cleavage *in vivo*. Secondly, PS circulates in plasma in two forms; a free form (40%) and in complex with the complement inhibitor, C4b-binding protein (C4BP) (53-55). Multiple binding sites for C4BP are located in the SHBG domain of PS (84-87). The C4BP protein contains 6 or 7 identical α-chains and a single β-chain, although 17% of the C4BP molecules lack the β-chain (55). PS binds with high affinity to C4BP via the β-chain (Figure 4a) (88). Unbound PS circulating in plasma represents the molar excess of PS over C4BPβ (53;55).

Whereas only free PS functions as a cofactor to APC, the APC-independent anticoagulant properties of PS do not seem to be negatively influenced by complexation to C4BP (24;27;31).

Moreover, the newly proposed role for PS in the phagocytosis (44;45) and/or rescue (43;47) of early apoptotic cells is thought to be mediated at least in part by directing C4BP to the surface of apoptotic cells (Figure 4b).

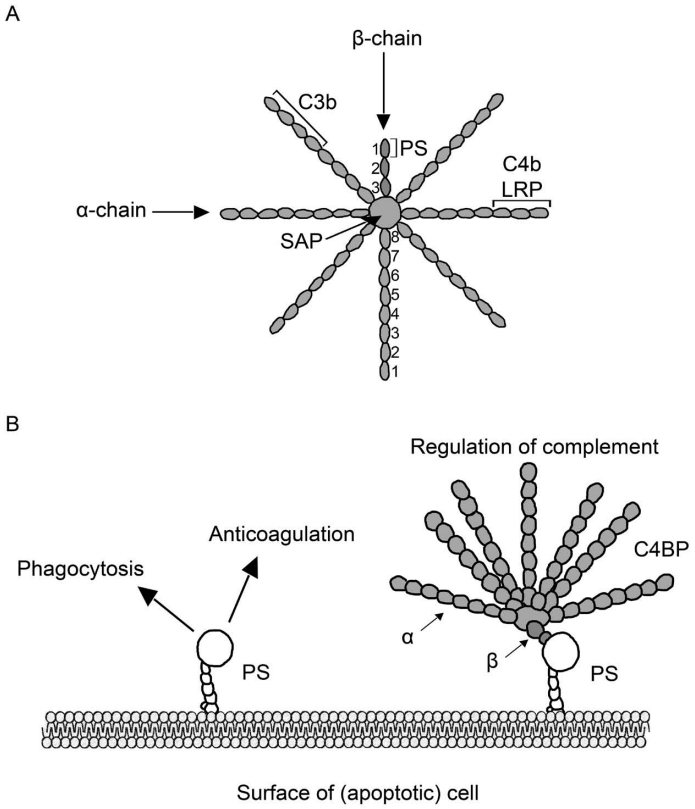

Figure 4 Interaction of PS with C4BP. (**A**) *Schematic representation of binding sites on C4BPβ^+.* PS binds to the β-chain of C4BP. The binding of other proteins at their specific binding sites does not affect PS binding to C4BP (89;90). C3b: activated complement factor 3, C4b: activated complement factor 4, LRP: low-density lipoprotein receptor-related protein, SAP: Serum Amyloid P component. Illustration adapted from (59). (**B**) *Properties of PS on the cell surface.* Illustration adapted from (91).

1.3.2 Gene

Two copies of the gene for PS, *PROS1* and *PROS2*, are located near the centromeric region of chromosome 3 (3p11.1-3q11.2) (92;93). *PROS* mRNA (94-96) is produced only from the *PROS1* gene, which spans a length of 80 kb and contains 15 exons and 14 introns (97-99). This is because *PROS2* is a pseudogene that lacks the promoter and the first exon. It

contains several frame-shift deletions leading to premature stopcodons that render this gene inactive. *PROS2* most likely originated from the functional gene, *PROS1*, through partial duplication (98). As with promoter regions from other genes coding for vitamin K-dependent coagulation proteins (100-106), a distinct TATA-box is absent from the *PROS1* promoter region. The finding of various transcription start sites reported for both published (94-96) and unpublished (NCBI-dbEST database entries (107)) *PROS1* cDNA sequences, suggests that transcription is initiated through multiple transcription start sites. In addition, the presence of an alternative promoter and first exon was postulated by Ploos van Amstel *et al* (98) upon their finding of two distinct start sites by primer extension analysis and the identification of two putative splice acceptor sites in the promoter region. This last hypothesis was, however, never borne out by experimental data.

In contrast to the coding region of the *PROS1* gene, which has been thoroughly investigated (reviewed in (108;109)), the promoter region has been poorly investigated. A promising abstract on the regulation of the *PROS1* promoter by various transcription factors, which was presented at a meeting of the International Society of Thrombosis and Haemostasis (ISTH) in 1995, was never followed by a paper in a peer reviewed journal (110). Since then, only a single report on the regulation of the promoter region has been published (111). In this last report, transcription directed from *PROS1* promoter-reporter gene constructs was stimulated in hepatoma HepG2 cells *in vitro* by binding of the ubiquitous transcription factor, Sp1. The liver-specific transcription factor, forkhead box A2 (FOXA2, HNF3β), also bound to the *PROS1* promoter, but *trans*-activation studies were not conducted with this transcription factor.

On a completely different level, Hooper and coworkers (112-114) measured PS levels after stimulation of cultured human hepatoma cell line HepG2, primary Human Umbilical Vein Endothelial Cells (HUVEC), and the human microvascular endothelial cell line, HMEC-1, with interleukin 6 (IL6), a mediator of the acute phase response during inflammation (115). IL6 stimulated PS production by all cell types and this could be suppressed by the addition of tumor necrosis factor α (TNFα). Although these authors did not directly investigate transcriptional regulation of *PROS1*, the results allude to the possible binding of the nuclear factors, which are induced by IL6 such as the signal transducer and activator of transcription 3 (STAT3) (116) and the CCAAT/enhancer-binding protein β (C/EBPβ) (117). In Figure 5 the

data from the various studies are combined and putative binding sites for STAT3 and C/EBPβ are included.

All in all, the components that determine (normal) variation in PS levels at the transcriptional level are still largely unknown.

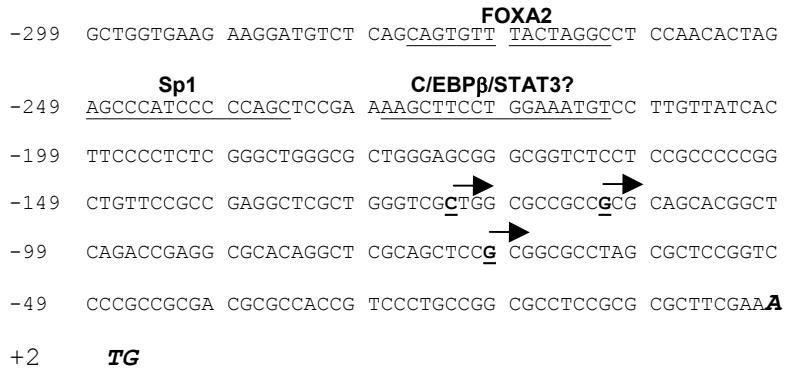

Figure 5 Partial *PROS1* 5' sequence. FOXA2 and Sp1 bind the *PROS1* promoter at the underlined regions (111). A putative binding site for C/EBPβ and STAT3 binding is depicted. The arrows indicate published transcription start sites derived from cDNA libraries (65;95;96). +1 is the first nucleotide of the translational startcodon, ATG.

1.4 Protein S Deficiencies

1.4.1 Hereditary PS deficiency

Partial PS deficiency was first reported to be associated with venous thrombotic disease in 1984 (118). In subsequent years it was established that in thrombophilic families heterozygous PS deficiency was associated with an increased risk of venous thrombosis. Homozygous or compound heterozygous PS deficiency is extremely rare and associated with severe purpura fulminans in the neonatal period. Hereditary PS deficiency is classified in three types (119). Type I deficiency corresponds to low levels of both free and complexed PS, type II PS deficiency is characterized by normal total and free PS levels but reduced PS activity, and type

III is defined by normal total PS levels but low free PS levels and activity. The disorder inherits as an autosomal dominant trait with incomplete penetrance (120) and is present in about 2% to 8% of families with hereditary thrombophilia (121;122). Many abnormalities in *PROS1* underlying hereditary PS deficiency have been described (reviewed in (108;109)). More recently Johansson *et al* reported a high incidence of large PS gene deletions in a group of Swedish PS deficient families (123). Not all familial PS deficiencies are explained by an abnormality in *PROS1* though (124). In this respect it must be noted that intronic sequences and 5', and 3' sequences are not routinely included in the investigation of familial PS deficiency and that possible functional mutations and polymorphisms in these regions are therefore not found. Of course, the remainder of unexplained inherited PS deficiencies may also be caused by variations in other genes. The Spanish Genetic Analysis of Idiopathic Thrombophilia (GAIT) project described the genetic linkage between free PS levels and the 1q32 genetic locus, which contains both genes for C4BPα and C4BPβ (125). This was not a surprising finding since PS binds to the C4BPβ-chain present in most C4BP molecules. This binding is of a 1:1 stoichiometric nature, which means that all C4BP-β^+ molecules are bound by one PS molecule. A drop or rise in C4BP-β^+ protein levels thus directly influences free PS levels either positively or negatively, respectively.

An example of a genetic abnormality/polymorphism is the relatively rare Ser 460 to Pro change in $PS_{Heerlen}$ (126) which, in some families, is associated with a type III PS deficiency (127;128). The anticoagulant properties of PS are not negatively affected by this amino acid change (29;31). A recent publication shows that instead, free $PS_{Heerlen}$ is cleared more rapidly than wild type PS from the circulation in mice (129).

1.4.2 Acquired PS deficiency

Whereas hereditary PS deficiency is relatively rare, many circumstances can lead to acquired deficiency, in which case it may be transient. Several non-genetic and environmental factors such as liver disease (130), DIC (80;130), and hormonal status (gender, contraceptive use, pregnancy) (131-133) influence PS levels. In liver disease PS levels as well as C4BP levels are decreased. Patients with DIC have decreased PS activity most likely due to increased cleavage of PS (80). Total PS levels remain similar as in controls and reports on the levels of

free PS in this hypercoagulable state are inconclusive (69;130). PS levels were shown to decrease with age (134), but when these data are corrected for gender the effect appears specific for females (135). It is evident from several studies that PS levels (total and free) are downregulated by female sex hormones, but the mechanism of this effect has not been clarified and would form an interesting area of research.

A more controversial issue is the regulation of PS levels during inflammation. C4BP is an established acute phase reactant with 2-3 fold elevated plasma levels during inflammation (136-139). Although PS levels are upregulated by the acute phase cytokine, IL6, *in vitro* (112), this finding was not confirmed by *in vivo* data. Several studies have demonstrated similar or only slightly increased total plasma PS levels (140-144). A controversy surrounds the levels of free PS during inflammation, with some studies showing reduced free PS levels in patient plasma (140-142), whilst others report stable free PS levels (143;144). A difference in the regulation of C4BP α- and β-chains in patient populations during the acute phase may explain the observed discrepancy (145).

1.5 Aim of this thesis

Overall the components that determine variations in PS levels at the transcriptional level are still largely unknown and the promoter region of *PROS1* has been poorly investigated. Knowledge of the *PROS1* promoter structure and the proteins regulating its transcriptional activity may help in acquiring a greater understanding of PS levels and PS function since;

a. Transcription factors that regulate *PROS1* transcription may be tissue-specific (e.g. liver-specific transcription factors), thereby explaining PS production in certain cell types,
b. Mutations or polymorphisms in the *PROS1* 5' sequence may lead to deficiencies if they are located in important binding sites for transcription factors or for the basal transcriptional machinery,
c. Deficiencies (qualitative or quantitative) in the regulatory factors of *PROS1* transcription may explain idiopathic hereditary PS deficiencies,
d. Transcription factors that upregulate *PROS1* transcription may themselves be triggered by a specific physiological process, thereby linking PS to this process.

The aim of this thesis was to identify the factors regulating PROS1 transcription, to determine whether PROS1 transcriptional regulation has a tissue/cell-specific component and to elucidate the underlying mechanisms.

In **Chapter 2** the transcriptional initiation of endogenous human *PROS1* is examined in liver and the relevant cell types that are exposed to the blood stream. Since PS is produced primarily by hepatocytes and to a lesser extent also by endothelial cells and megakaryocytes, we hypothesized that PS transcription might be regulated in a cell type-dependent manner. Cell-specific transcription may be regulated through a difference in the location of transcription start sites. Four major endogenous start sites were identified, the usage of which differed slightly between cell types. Furthermore, a minimal promoter with optimal transcriptional activity was identified in basal expression studies with *PROS1* promoter-reporter constructs in all cell types. It is this promoter construct that, in **Chapter 3**, was used in a pilot study in which the effect of a range of transcription factors on *PROS1* promoter activity was tested. Phylogenetic footprinting further provided a solid basis on which to select certain conserved regions within the human *PROS1* promoter for further research. **Chapter 4** expands the data presented in chapter 3 with a large scale investigation into the possible binding of nuclear proteins to the sequence contained in the *PROS1* promoter. Sp1 is identified as a transcription factor with multiple binding sites within the *PROS1* promoter. From further functional studies it became apparent that Sp1, and possibly also the related transcription factor Sp3, is almost solely responsible for the basal transcriptional activity of the *PROS1* promoter.

Previously published reports suggest that IL6 has a direct effect on *PROS1* transcription. In **Chapter 5** the IL6 responsive element within the *PROS1* promoter is identified. STAT3 binding to this element was essential for induction of *PROS1* transcription, which is illustrated by the absence of STAT3 binding and IL6 induction of *PROS1* transcriptional activity when this region is mutated. Major stimulatory effects on *PROS1* transcription were also observed upon cotransfection of another mediator of IL6 signalling, C/EBPβ. However, the role for C/EBPβ in *PROS1* promoter regulation was not fully elucidated during the course of this project.

Chapter 6 describes a classic case of serendipity. During the development of a real-time rtPCR analysis (QPCR) for the accurate measurement of *PROS1* transcipt levels, an additional unexpected PCR product was identified. The product was sequenced and resulted in the identification of a relatively abundant alternatively spliced *PROS1* mRNA. The alternative PS product from this alternative mRNA is compared to that of normal recombinant *PROS1* in COS1 cells *in vitro*.

In **Chapter 7** the results described in this thesis are discussed and related to insights from the literature.

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Chapter 2

Initiation of Protein S mRNA synthesis in human liver, various cell lines, and Protein S promoter-reporter gene Plasmids

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Summary

Protein S (PS), a cofactor of activated protein C (APC), is a vitamin K-dependent anticoagulant plasma protein, which is produced mainly in the liver but which is also produced extrahepatically. Therefore, the production of this protein may be regulated in a tissue-specific manner. Upon analysis we found that the 5'-flanking region of the human PS gene (*PROS1*) lacks characteristic "CCAAT" and "TATA" boxes. To determine the sites of transcriptional initiation *PROS1* transcripts from HepG2, HuH7, HeLa, HUVEC, and Meg01 cells and human liver were subjected to transcription start site (TSS) analysis by using 5'-rapid amplification of cDNA ends. In all cell types, as well as in human liver, transcription is initiated most frequently at one of three TSSs located 100 bp, 117 bp or 147 bp upstream from the translational startcodon. HUVEC cells contained an additional TSS at –200 bp. In all cell lines, reporter constructs containing a minimal promoter of 370 bp upstream of the translational startcodon demonstrated maximal promoter activity. Whereas three distinct TSSs were identified for the endogenous *PROS1* transcripts, no preferred TSSs could be determined for transiently transfected *PROS1* promoter luciferase constructs. This is consistent with our finding that deletion of the first 181 bp proximal to the translational start in these constructs did not result in complete deterioration of promoter activity. This suggests a relaxation of TSS regulation when using plasmid constructs in transient transfection studies.

Introduction

Reduced plasma Protein S (PS) levels were first reported to be involved in the development of venous thrombo-embolism (VTE) in 1984 (1-3). PS deficiency has been well established as a risk factor for VTE since then (4-7). PS is a vitamin K-dependent plasma protein that displays anticoagulant properties by acting as a non-enzymatic cofactor for activated Protein C (APC) in the proteolytic degradation of blood clotting factors Va and VIIIa (8-11). In human plasma, PS circulates in an active free form (40%) and a C4b-binding protein (C4BP)-bound inactive form (60%) (12-14). The major source of circulating plasma PS is the hepatocyte (15). However, PS is also known to be produced by a variety of other cell types such as megakaryocytes (16;17), endothelial cells (18;19), Leydig cells (20), osteoblasts (21) and cells of the nervous system (22). In mammals, PS mRNA was found in virtually all tissues and organs examined (23).

Two copies of the PS gene are located on chromosome 3. The active PS gene (*PROS1*) shares 96% homology with the inactive pseudogene (*PROS2*). The pseudogene, which lacks the promoter and the first exon also contains several frame-shift deletions leading to premature stopcodons that render this gene inactive (24-26). Mutational analysis of the exons of *PROS1* in patients with PS deficiency has shown various mutations within the PS gene to be responsible for low PS levels in plasma [reviewed by Gandrille *et al* (27;28)]. In contrast to the coding regions of the *PROS1* gene, the promoter region has been poorly investigated. As with other promoter regions from genes coding for vitamin K-dependent coagulation proteins, a distinct TATA-box is absent from the *PROS1* promoter region (29;30). The literature to date describes one primer extension assay (25) and multiple cDNAs derived from cDNA libraries (31-33). The databases at the National Center for Biotechnology Information (NCBI) provide multiple direct submissions of *PROS1* cDNAs (34). However, none of these cDNAs are necessarily full-length, whereas primer extension studies are notoriously difficult to interpret.

In the present study, we describe an analysis of the transcriptional control region of *PROS1*. To our knowledge for the first time we provide a transcription start site (TSS) distribution after analysis of multiple full-length *PROS1* cDNAs from various cell lines and human liver. Using a method that targets capped mRNA for reverse transcription we show

that there are a number of major start sites in the promoter of the endogenous *PROS1* gene. The cell types that were tested displayed small differences in TSS usage. In addition, we show that this preference for specific positions is lost in luciferase reporter constructs. The implications of these findings are discussed.

Experimental procedures

Plasmids --- The *PROS1* promoter reporter constructs used in this study, originated from a 7 kb *Eco*RI promoter fragment which was isolated from BAC clone #2513H18 from the CITBI-E1 genomic library (Research Genetics, Invitrogen, Carlsbad, CA) and ligated into the *Eco*RI site in the multiple cloning site region (MCS) of the pcDNA3 cloning vector (Invitrogen). This pcDNA3-*PROS1* construct contained 716 bp of sequence downstream from the translational startcodon. The complete sequence was determined through automated sequencing (ABI PRISM and Beckman CEQ2000 sequencers) and deposited in Genbank under accession number AY605182. Subsequently, pcDNA3- *PROS1* was modified to contain nucleotides -5948 to -1 from the translational startcodon as follows. First, the construct was digested with *Bam*HI and *Eco*RI (all restriction enzymes were obtained from New England Biolabs, Hertfordshire, UK) resulting in two fragments. Fragment -5948/-410 contained a pcDNA3-*Bam*HI and a *PROS1*-*Bam*HI terminus, and fragment -410/+716 contained a *PROS1*-*Bam*HI and a pcDNA3-*Eco*RI terminus. Fragment -410/+716 was ligated into pcDNA3, which had been linearized by digestion with *Bam*HI and *Eco*RI. The pcDNA3-*PROS1*-410/+716 was used to generate a product spanning from bp -410 to -1 by polymerase chain reaction (PCR) using the T7 primer in pcDNA3 and a mutant reverse gene-specific primer (GSP), PSstart, in which the initiation methionine was modified into an *Eco*RI site (Table 1). After digestion with *Bam*HI and *Eco*RI this PCR product was ligated into pcDNA3, which had been linearized by digestion with the same enzymes. *PROS1* fragment -5948/-410 was then religated into the *Bam*HI site connecting the 5' terminus of *PROS1*-410/-1 and pcDNA3. The resulting *PROS1* fragment (-5948/-1) was cloned directly 5' to the luciferase reporter gene in the pGL3basic vector (pGL3b, Promega, Madison, WI) after digestion with MCS restriction sites *Kpn*I and *Xho*I. This construct was named PS5948. PS5948 was linearized with *Kpn*I and *Nde*I (located at position –5798) and subsequently subjected to exonuclease III digestion (Erase a Base kit, Promega). The size of the resulting 5' deletion was determined by sequence analysis. The 5'-deletion constructs were used for transient transfection assays. After digestion of construct PS1062 with *Pst*I and *Eco*RI, exonuclease III

digestion was also used to obtain 3'-deletion fragments from this construct. Additional 3'-deletion constructs, PS-370/-181, PS-1062/-256 and PS-370/-256, were created using restriction enzymes. PS-370/-181 was created by combination of constructs PS-370 and PS-1062/-181 after digestion with *Hin*dIII. PS-1062/-256 and PS-370/-256 originated from the original PS370 and PS1062 constructs by religation after excision of a *Stu*I-*Eco*RI PS fragment (-256/-1). An overview of all luciferase reporter constructs is given in Figure 1.

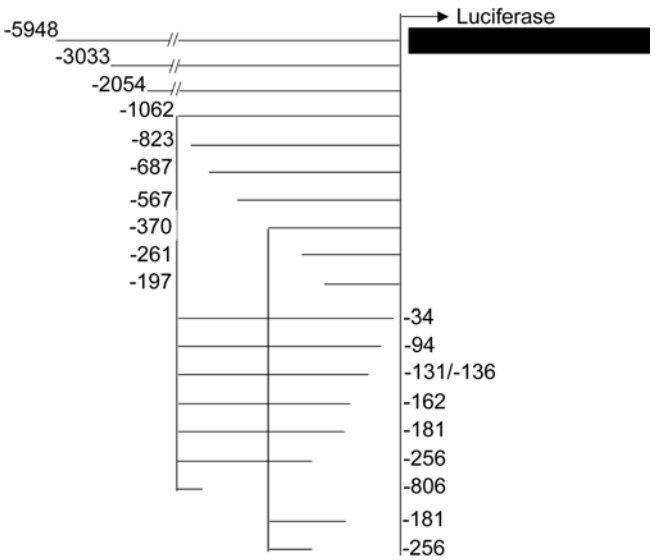


Figure 1 *PROS1* promoter-luciferase constructs in pGL3basic. The numbers indicate the length of the *PROS1* 5' flanking region proximal to the luciferase gene in pGL3basic. Constructs PS1062 and PS370 were also used in 3' deletion studies. The length of the 3' indentation is shown at the 3' end of the construct.

Human liver, cell lines and media --- A human liver sample was obtained from a deceased healthy donor and was a kind gift from E. de Wit at TNO Center for Prevention and Health, Leiden (TNO). The human hepatoblastoma cell line HepG2, and cervical adenocarcinoma cell line HeLa were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Megakaryocytic cell line Meg01 was a kind gift from G. van Willigen from the Utrecht Medical Centre. The hepatoma cell line HuH7 was a kind gift from M. Verschuur (TNO). Primary human umbilical vein endothelial cells, HUVEC cells, were a kind gift from J. Grimbergen (TNO). HepG2, HuH7, HeLa 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). HUVECs were grown in M199-medium (BioWhittaker, Walkersville, MD), 10% heat inactivated human serum (local bloodbank), 10% Newborn Calf Serum (home made), 10 IE/ml heparin (BioWhittaker), 150 U/ml endothelial cell growth factor, 100 µg/ml penicillin (BioWhittaker) and 100 µg/ml streptomycin (BioWhittaker). 24 hrs before transfection the medium was replaced with heparin-free medium to prevent interference with the transfection assay. For PS antigen level measurements HUVEC cells were grown in serum-free EBM medium (BioWhittaker). Meg01 cells were grown in RPMI 1640 medium, 20% FBS, 100 µg/ml penicillin, 100 µg/ml streptomycin (all purchased from Gibco, Invitrogen). For PS antigen level measurements Meg01 cells were suspended at $1*10^6$ cells/ml in RPMI 1640 medium with 10% FBS.

Reporter gene assays --- $1*10^6$ Meg01 suspension cells were used per transfection. All adherent cell lines (HepG2, HuH7, HeLa, HUVEC) were transfected at 60-80% confluency. HUVEC cells were transfected in passage 2-3, whereas the other cell types were used up to passage 25. Each transfection was performed in triplicate in 12-wells plates, all assays were conducted with two different DNA preparations of each construct. Transfections with HepG2, HeLa, HUVEC and HuH7 cell lines were carried out using 3 µl Tfx-20 lipids (Promega) per µg transfected DNA. Meg01 cells were transfected using 10 µg DAC-30 (Eurogentec, Seraing, Belgium) per 2 µg DNA. In each transfection an equimolar concentration of construct was used supplemented with pUC13-MCS vector to obtain a fixed amount of transfected DNA. In pUC13-MCS the MCS had been removed by digestion with *Pvu*II and religation. 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 in HepG2, HuH7 and HeLa cell lines, a 1:100 ratio in transfections with HUVEC and Meg01 cells. The cell extracts were harvested at either 24 hours (HepG2, HuH7) or 48 hours (Meg01, HUVEC, HeLa) after transfection. Luciferase activity was measured according to the Dual Luciferase Assay System Protocol (Promega). All cell lines were lysed in 250 µl Passive Lysis Buffer/well, after which 20 µl was used to measure luciferase activity in HepG2 and HuH7 cells, 100 µl was used for HeLa, HUVEC and Meg01 cells. Activity was measured using a Lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany).

PS measurements --- Total PS antigen levels in culture media were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (35), with the following

modifications. ELISA plates were coated with goat anti-human PS IgG (Kordia, Leiden, The Netherlands) overnight at 4°C. A second coating with 2.5% ovalbumin (Sigma-Aldrich, St. Louis, MO) at 37°C for 1 hour was performed to reduce background absorbance. Complexes were detected with horseradish peroxidase-conjugated rabbit anti-human IgG (Dako, Glostrup, Denmark). Absorbance at 450 nm was determined with an Organon Teknika plate reader (Turnhout, Belgium).

RNA-assays --- Total RNA was isolated from cell culture or frozen tissue using Trizol reagent (Invitrogen) according to the manufacturers recommendations. Samples were treated with RNAse-free DNAse I (Amersham, Roosendaal, The Netherlands) after which RNA was purified with the RNeasy mini kit (Qiagen, Hilden, Germany). For 5' RACE (Rapid Amplification of cDNA Ends) experiments polyA RNA was isolated from total RNA preparations with a PolyA isolation kit (Ambion, Austin, TX). *PROS1* RNA levels were determined by quantitative real-time PCR analysis (QPCR). First, 1μg total RNA from each cell line was reverse transcribed using Superscript II reverse transcriptase and random hexamers (Invitrogen). 1/20th of the obtained cDNA was subsequently used in a QPCR reaction with primers and probes specific for *PROS1*. The primers and probe sequence locations and lengths were determined by using the ABI Primer Express Program (Applied Biosystems, Foster City, CA), and custom synthesized at Eurogentec. *PROS1* QPCR reactions (Eurogentec) were performed in 0.5 ml thin-walled, optical-grade PCR tubes (Applied Biosystems) in a 50 μl final volume, by addition of the following components: 0.25 U AmpliTaq Gold DNA polymerase, 160 nM TaqMan probe, 300 nM of each primer, and 3 mM $MgCl_2$. A QPCR of the internal standard, the porphobilinogen deaminase gene (PBGD), was carried out in a similar fashion for each RNA sample with 4 mM $MgCl_2$. An Applied Biosystems Prism model 7700 sequence detection instrument monitored the reactions. Thermal cycling conditions consisted of 10 min at 95°C followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. Determinations of cycle threshold (C_T) were performed automatically by the instrument. The results are expressed as fold transcript relative to the internal standard PBGD ($=2^{\Delta Ct}$).

Determination of the TSS --- TSS analysis was performed on polyA RNA isolated from untransfected and transfected cell lines and human liver. The procedure was carried out using the Gene Racer™ kit (Invitrogen). The amount of polyA RNA used for the experiment varied from 300 to 2000 ng and was dependent on the amount of protein and mRNA detected in ELISA and QPCR analysis. After selection of full-length mRNA with the Gene Racer Kit,

first strand cDNA synthesis was performed with Superscript II reverse transcriptase and random hexamers (Invitrogen). For the untransfected cell lines and human liver the gene-specific amplification of full-length cDNA was performed with a 5'-primer provided in the Gene Racer kit and a 3' GSP located in the first exon of the *PROS1* mRNA (PSex1). For the cell lines transfected with a *PROS1* promoter reporter construct, the GL2 primer (Promega) was used as GSP. A *PROS1* primer in exon 2 was used for amplification of *PROS1* transcripts from a more downstream position (PSex2). The sequence and location of all non-commercially available primers are depicted in Table 1. The *PROS1* PCR cycle conditions were as follows; 5 cycles with an annealing temperature (T_a) of 72°C, 5 cycles with T_a 70°C, 25 cycles with T_a 68°C. After 20 cycles an equal volume of fresh PCR mix was added. For the construct PCR the annealing temperatures were, 68°C, 66°C, and 64°C respectively. The elongation time was set to 1.5 minutes to ensure complete elongation of all possible transcripts. After amplification of full-length *PROS1* transcripts all PCR products were ligated into sequencing vector pCR4.0. Approximately 40 clones of each cell type were analysed resulting in a TSS distribution estimate. The positional indications used throughout this article, are respective to the translational startcodon.

Target	Name[a]	Assay	Location	Primer/Probe sequence 5'-3'
PS	PS-F	QPCR	+32/+51 ex1	TGCTGGCGTGTCTCCTCCTA
	PS-R	QPCR	+109/+85 ex2	CAGTTCTTCGATGCATTCTCTTTCA
	PS-P	QPCR	+55ex1/+7ex2	CTCCCCGTCTCAGAGGCAAACTTTTTGTC
PBGD	PBGD-F	QPCR	+13/+28 ex1	GGCAATGCGGCTGCAA
	PBGD-R	QPCR	+25/+43 ex2	GGGTACCCACGCGAATCAC
	PBGD-P	QPCR	+30ex1/+23ex2	CTCATCTTTGGGCTGTTTTCTTCCGCC
PS	PSstart	GR	-1/-21	GAATTCGAAGCGCGCGGAGGCGCC
PS	PSex1	GR	+62/+33	ACGGGAAGCACTAGGAGGAGACACGCCAG
PS	PSex2	GR	+40/+21	CTTCCTAACCAGGACTTGTG

Table 1 Primer and probe sequences. F, forward primer; R, reverse primer; P, probe. All Taqman probes contain the reporter dye, 6-carboxy-4,7,2',7'-tetrachlorofluorescein, TET, and the quencher dye 6-carboxy-tetramethyl-rhodamine, TAMRA. GR= gene racer.

Results

PROS1 mRNA and PS protein are produced by HepG2, HuH7*, HeLa, HUVEC, Meg01 and human liver* --- PS circulates in normal plasma at a concentration of 0.33 μM (14). Hepatocytes are the largest contributor to the systemic concentration (15), but other cell types have also been shown to produce PS (16-18;20). ELISA analysis demonstrated that all cell lines used in this study produce PS (Figure 2). HepG2, and HuH7 cell lines were most productive whereas Meg01, HeLa, and HUVEC cells expressed smaller amounts of PS. Since mature megakaryocytes (platelets) are known to contain storage pools of coagulation factors in their α-granules, cell lysates were also analysed for PS antigen levels (36). Very low and comparable levels of PS antigen were found in lysates from all cell types including Meg01 ($\leq$10 fmol per 10^7 cells). *PROS1* transcript levels were determined by QPCR of total RNA from each cell line. All cell lines were found to contain *PROS1* transcripts, indicating *de novo* production of PS. The relative level of *PROS1* transcript in the various cell types correlated with the PS protein level found in each cell type, i.e. HepG2 and HuH7 cells produced high amounts of PS protein and contained high relative levels of *PROS1* transcript.

PROS1 mRNA contains multiple start sites --- For TSS determination, full-length *PROS1* transcripts were isolated from polyA RNA samples from all cell lines and from a human liver sample. Figure 3a shows the PCR products obtained after selection and amplification of full-length *PROS1* mRNA products. Specificity of the amplified products was confirmed with a nested *PROS1* PCR, in which an internal GSP was used on the PCR mix (Figure 3b). The size of the resulting *PROS1* fragments decreased by the expected number of base pairs (62 bp). Sequence analysis of the cloned cDNAs revealed the presence of multiple TSSs in *PROS1* mRNA in all cell types. The start site distributions (Figure 3c) generated from these data revealed that four distinct start sites, located at -200, -147, -117 and -100 bp upstream from the translational start, can be defined for *PROS1*. Other start sites were found, but most were encountered only once. HUVEC and HuH7 cells do not use the TSS at -100 bp, whereas all other mRNA samples do contain this starting point. Also, HUVEC cells use the initiation site at -200 bp more frequently than other cell types. Long transcripts commencing at -340 bp, -347 bp, and -360 bp were found for Meg01, HeLa and HuH7, respectively.

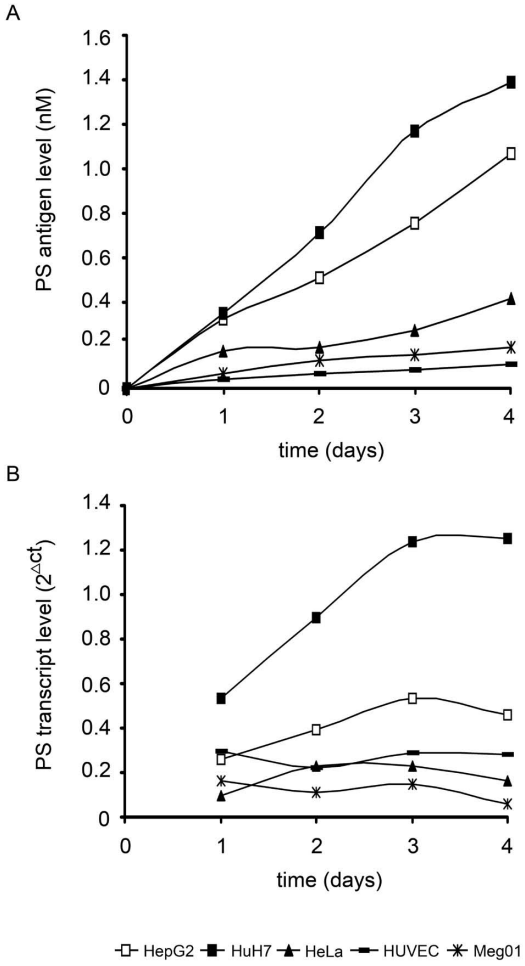
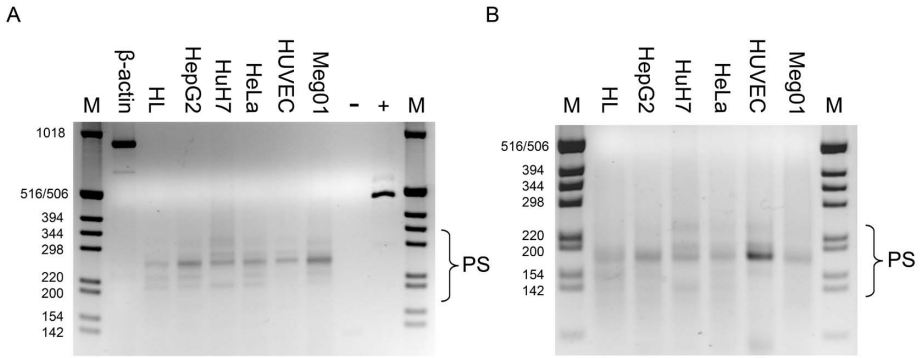


Figure 2 PS antigen and mRNA levels in various cell lines. (**A**) PS antigen levels were measured over time in media from cell culture. Cells were seeded in 12 well plates and incubated with 1 ml medium. Experiments were started at 80% confluency or, in the case of Meg01, at $1*10^6$ cells/ml. HepG2, HeLa, Meg01 and HuH7 medium included 10% FBS, HUVEC medium was serum-free. Fresh media in the appropriate dilutions were used as blanks to correct for background interference in the ELISA. Media and total RNA from triplicate samples were pooled. (**B**) PS mRNA levels are expressed as fold transcript relative to the internal standard PBGD ($2^{\Delta Ct}$).



C

Cell type	5' start site distribution (%)					Total	(n)	L
	-100	-117	-147	-200-210	Other			
HL	32	25	22	2	18	100	59	-267
HepG2	17	18	33	0	32	100	60	-177
HuH7	2	33	31	2	32	100	51	-360
Meg01	24	11	34	0	31	100	38	-340
HUVEC	2	12	45	12	29	100	42	-210
HeLa	24	12	24	2	37	100	41	-347

Figure 3 *PROS1* transcription is directed from multiple start sites. (**A**) Full-length *PROS1* cDNAs were amplified using the GeneRacer™ technique. M; DNA size standard, β-actin; positive 5' RACE control, -; negative PCR control, e.g. the reaction without cDNA, +; PS positive PCR control with primers spanning from exon 2 to exon 5. The PS primer is located in exon 1 at +62 to +32 (PSex1) (**B**) Nested PCR on the PCR mix which is depicted in Figure 3A. The PS primer is located in the 5' UTR at -1 to -21 (PSstart). M; DNA size standard. (**C**) Observed start site frequencies are given as percentage of the total. TSS numbering is relative to the translational startcodon. "other" indicates start sites different from the ones denoted, start sites in this category occurred no more than twice. (n) equals the number of PCR clones sequenced. L; longest transcript.

No evidence for an alternative promoter --- In 1986 Lundwall *et al* reported the presence of a *PROS1* transcript that starts in intron A (33). This could indicate the presence of an alternative promoter in intron A. The transcript has an intronic sequence of 236 bp and commences approximately 14.9 kb downstream from the first exon and 31.1 kb upstream from the second exon. This transcript is not prominently present in our HepG2 mRNA population. Figure 4 shows a 5' RACE PCR, which was conducted with a PS-specific primer in exon 2. The

products were not digested by *Mse*I, a restriction enzyme with a site in the alternative transcript that was found by Lundwall and co-workers (33). Moreover, all PCR products were digested by *Ava*II, for which restriction sites are present in exon 1.

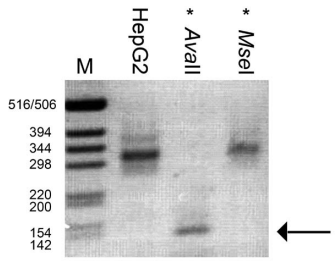


Figure 4 *PROS1* transcripts with a start in intron A are not present. Full-length *PROS1* transcripts were amplified from HepG2 mRNA using the GeneRacer™ technique. PSex2 was used as GSP for PS in the second exon. M; DNA size standard. The arrow indicates the specific digestion products after digestion with *Ava*II.

A minimal promoter of 370bp confers maximal transcriptional activity

5' deletion experiments --- Transient transfection studies were conducted in unstimulated cells with equimolar amounts of *PROS1* promoter constructs cloned upstream from the luciferase reporter gene (Figure 5a). Luciferase levels differed widely per transfected cell type and are dependent on transfection efficiency of the cell type and on the presence of the correct combination of nuclear transcription factors by which transcription is regulated. For comparison purposes, promoteractivity of the most active promoter construct in each cell type was adjusted to 100% after which the activity of the other constructs in the same cell type was normalised to this 100%. These studies pointed out that the first 370 bp of the *PROS1* 5' flanking region are sufficient for optimal promoter activity in all cell types. This activity was maintained up to a 5' region of 1062 bp. A marked difference in activity for longer constructs was observed among the various cell lines. In HuH7 and Meg01 cells promoter activity decreased only slightly with increasing construct size, whereas in HepG2 and HeLa cells promoter activity decreased more rapidly with increasing construct size.

3' deletion experiments --- Transient transfection experiments in HepG2 with *PROS1* 3' deletion reporter constructs confirmed the importance of the transcription start sites located at -100 bp and -117 bp. Deletion of the first 131 bp upstream from the startcodon decreased

promoter activity by approximately 50% (Figure 5b). The importance of the -147 bp TSS could not be confirmed in these experiments since promoter activity remained at a similar level after further deletion up to -181 bp. Transcriptional activity decreased significantly after deletion of the 5' region up to -256 bp from the startcodon, and deletion up to -806 bp completely reduced the level of activity to that of pGL3basic. Comparable results were obtained in all cell lines (results not shown). Since the -147 bp start site was one of the three most prominently found endogenous TSSs in all cell lines, we set out to validate the TSS usage in the transfected *PROS1*-luciferase constructs.

Construct start site determination --- The transcription start sites that were used in *PROS1* promoter constructs, PS370, PS1062, and PS5948 were determined after transfection in HepG2 cells. If the transfection system reflects the *in vivo* situation, the TSSs from the transfected constructs should be identical to those of the endogenous *PROS1* gene. Surprisingly, a large range of mostly unique TSSs was found for the three constructs tested (Table 2). Start sites -100 and -117, which were shown to be important sites in endogenous *PROS1* transcriptional initiation, were also used by the constructs. However, these sites were not used more frequently than the other TSSs.

Discussion

The experiments described in this study provide a more detailed understanding of the transcription start site usage during the initiation of *PROS1* transcription in various cell lines and human liver. Our results show that human liver has three prominent start sites, namely -100 bp, -117 bp and -147 bp upstream from the translational start. These TSSs were found in most cell types tested. Whereas hepatoma cell line HepG2 uses TSS -100 bp, this TSS was not found for the related cell line HuH7. Since human liver does contain this TSS we conclude that, for *PROS1* transcription, HepG2 cells are more representative of the *in situ* situation than HuH7 cells. HUVEC cells also do not use the aforementioned start site, moreover in this cell type transcription is regularly initiated from -200 bp. This TSS is rare in the other cell types suggesting that, in HUVEC, a different array of regulatory nuclear proteins may be present. Even though human liver, HepG2, HeLa, and Meg01 cell types all contain the same start sites, the contribution of the three main start sites to the total *PROS1* mRNA pool was different (Figure 3c).

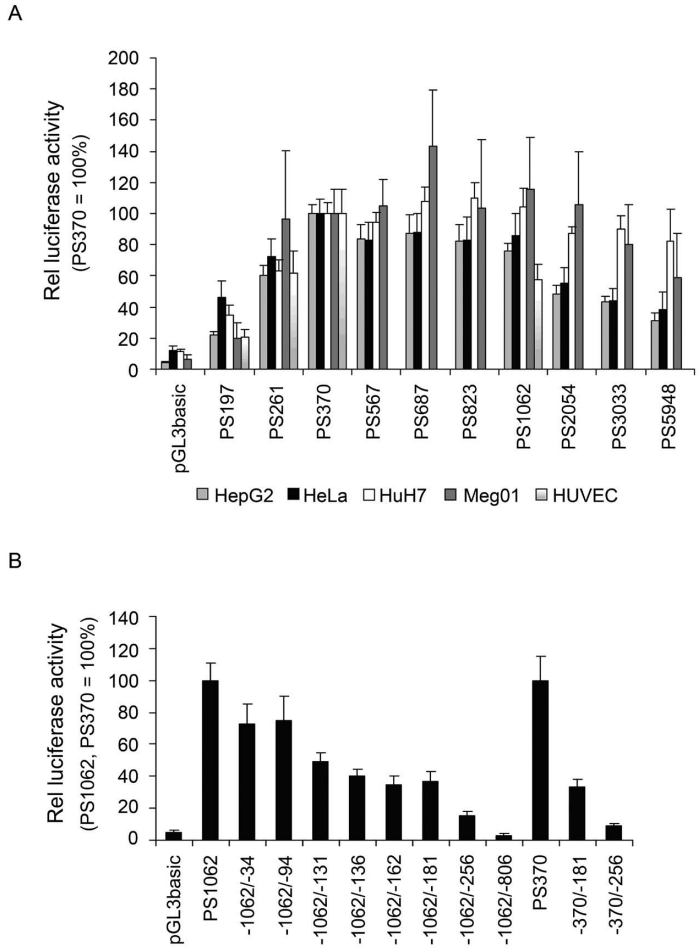


Figure 5 A minimal promoter of 370 bp is sufficient for maximal promoter activity. Cell lines were transiently transfected with the pGL3basic vector carrying *PROS1* promoter-luciferase gene constructs. Bars represent a mean of 2 triplicate assays with 2 different preparations of each plasmid (n = 12). (**A**) Luciferase activity is relative to the activity of construct PS370, which was designated as 100%. Note that the activity for PS370 was different for each cell line. Luciferase activities can therefore not be compared among cell lines, as they are also dependent on transfection efficiency in addition to promoter activity (**B**) HepG2 cells were transfected with 3' deletion constructs. Luciferase activity is relative to the activity of the 5' parent construct, i. e. PS1062 or PS370.

Construct	5' start position of cDNA				
	-1-100	-100-200	-200-300	-300-400	>-400
370	-36,-60,-73	-100,-115	-238,-242	-308,-320,-356	
1062	-59,-65,-87	-100,-119	-203,-242, -289	-308,-328,-332, -337,-395*	-412,-434, -464, -523
5948	-2	-100*,-119	-230,-238, -247, -290	-302,-318*, -332*	-424

Table 2 PS construct start site distribution. Full-length luciferase cDNAs were amplified from mRNA isolated from transfected HepG2 cells using the GeneRacer™ technique. Primer GL2 was used as GSP for luciferase mRNA. TSS numbering is relative to the luciferase translational startcodon. The start sites were all unique with exception of those with an asterisk (*), for these TSSs n=2.

Published and unpublished (NCBI database entries) *PROS1* cDNA sequences suggest the presence of multiple TSSs. To our knowledge for the first time, our results pinpoint exactly which TSSs are used for PS transcription in human liver and various cell lines. In accordance with some database entries we show that transcriptional initiation can take place from sites located more than 300 bp upstream from the translational start. However, these start sites are not used frequently. One can conclude that for GC-rich promoters, such as the *PROS1* promoter, the longest reported transcript does not necessarily represent the most abundantly used TSS.

The transcriptional regulation of the *PROS1* gene has, to date, been poorly investigated. According to a recent review this may be due to the presence of an additional exon 5' to what currently is considered exon 1 (37). Ploos van Amstel *et al* (25) identified two TSSs by primer extension analysis at -174 bp and -286 bp, respectively. Based on these findings and on the presence of a putative splice acceptor site, they postulated the presence of an alternative promoter and first exon. Also, Lundwall *et al* reported the presence of a *PROS1* transcript that starts in intron A (33). In our studies we found only uninterrupted *PROS1* sequences stretching from the 5' flanking region into exon 1. Also, restriction analysis of GeneRacer products generated with a GSP in exon 2 provided no support for the hypothesis of an alternative promoter.

At present, two DNA elements have been found to be involved in core promoter function in the transcriptional regulation from TATA-less promoters: the initiator (Inr), and the downstream promoter element (DPE)(38). The DPE is located precisely 28-30 nucleotides downstream from the transcription start site (for review see (39)). TSSs -200 bp, and -100 bp, form the center of an Inr consensus sequence ($YYA_{+1}NWYY$). However, the consensus sequence (RGWYV) for the DPE was not detected in the *PROS1* promoter at the designated positions. A schematic presentation of the TSSs and their interspecies conservation, is shown in Table 3. Phylogenetic footprinting revealed that the region surrounding the -100 bp TSS is poorly conserved, whereas the other regions have a higher degree of conservation.

Species	-200	-147	-117	-100
Human	.TATCACTTCC.	.CGGCTGTTCC.	.GGCGCCGCCG.	.GCTCAGACCG.
Chimpanzee	.TATCACTTCC.	.CGGCTGTTCC.	.GGCGCCGCCG.	.GCTCAGACCG.
Rhesus monkey	.TATCACTTCC.	.CGGCTGTTCC.	.GGCGCCGCCG.	.GCTCAGACCG.
Dog	.TATCACTTCC.	.CGGC(*)TGTTGG.	.GGCGCCGCCG.	.CCTCGGAGAG.
Pig	.TATCACTTCC.	.CGGCTCGGCA.	.GGCGCCGCTG.	.CTTCAGAGAG.
Mouse	.CACCGCTTCC.	.TGGCTGCTCC.	.GG.GCCGCCG.	.GCTGGGAGAG.
Rat	.CGCCGCTTC..	.TGACTGCTCC.	.GG.GCCGCAG.	.GTTGGGAGAG.
Cow	.TATCACTTCC.	.CGGCTCCGCT.	.GGCGCCCCCG.	.CCTTAC.TAG.

Table 3 Phylogenetic footprint for the most prominent *PROS1* TSSs. Gaps introduced to maximize alignment are indicated by dots. The TSS is underlined.

A region up to 370 bp upstream from the translational start is sufficient for optimal promoter expression in our model transfection system. We show that this activity is maintained up to a length of 1062 bp upstream. The subtle differences between the cell lines in these transfection studies do not allow a firm conclusion regarding tissue-specific regulation of the *PROS1* promoter under basal culture conditions. However, it can be concluded that the promoter region apparently does not contain additional upstream regulatory elements, e.g. enhancers and repressors that can be detected in our model system. Surprisingly, only a 60% decrease of promoter activity was shown after deletion of the three most prominent start sites in the 3' deletion experiments. This led us to investigate the TSS usage in three of the *PROS1* promoter constructs that were used in our transfection studies. The TSSs at -100 bp and -117

bp were observed for all constructs but the -147 bp and -200 bp start sites were not found amongst the cloned mRNAs derived from the transfected constructs. Moreover, even though similar TSSs were found for the three constructs that were tested, most identified transcripts were rare, indicating a relaxation of the transcriptional machinery (Table 2). A reduction to 10% of original promoter activity was obtained after deletion of the first 256 bp proximal to the translational start. The deleted region contained all of the endogenously important TSSs (-100, -117, -147 and -200). However, the absence of these sites is most likely not the sole cause of the abrogation of promoter activity. By deletion of this region also essential transcription factor binding sites may have been deleted. For instance, Tatewaki and co-workers (40) have recently shown transcription factor Sp1 to bind within the deleted region, at -247 to -238 bp. Since Sp1 is a major component in the transcriptional regulation from many genes with GC-rich promoters (41), deletion of this site may well be, at least in part, responsible for abrogation of promoter activity. A similar argument probably applies to the PS-1062/-806 construct, which has lost all promoter activity.

Our findings suggest that the mechanism of transcriptional initiation in this model system differs from the *in vivo* situation. A possible explanation for this phenomenon may be that transfected DNA is in plasmid form and not in a chromosomal form. It therefore may lack necessary structural elements that are present in normal genomic DNA. Based on our results, researchers should express great caution to use this model system for determining TSSs. We believe that even though transcriptional initiation is directed differently, i.e. more relaxed, this system remains a valuable tool for examining promoter regulation as the binding of essential transcription factors and their role in transcriptional activation is in general not affected as has been shown in numerous publications. However, it is clear from our results that transient transfection of luciferase reporter gene constructs may not be a suitable tool for dissecting subtle differences in promoter activity and TSS usage.

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Chapter 3

Regulators of *PROS1* transcription, a pilot study

De Wolf CJF, Bertina RM, and Vos HL

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

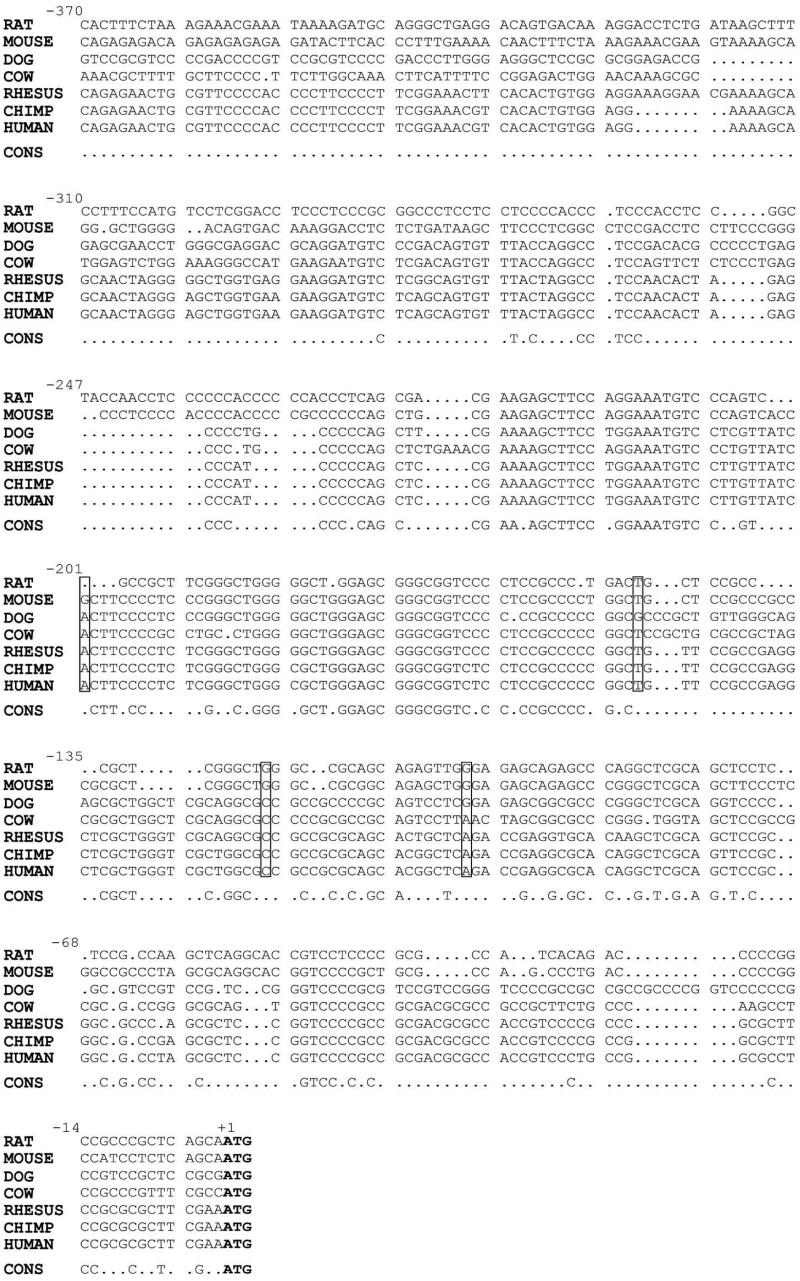

Figure 1 Phylogenetic alignment of the *PROS1* promoter sequence. Sequences from human, chimpanzee, rhesus monkey, cow, dog, mouse and rat were retrieved from the archives at the NCBI. Numbering is according to the human sequence with the first nucleotide of the translational startcodon being +1. Cons; 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.

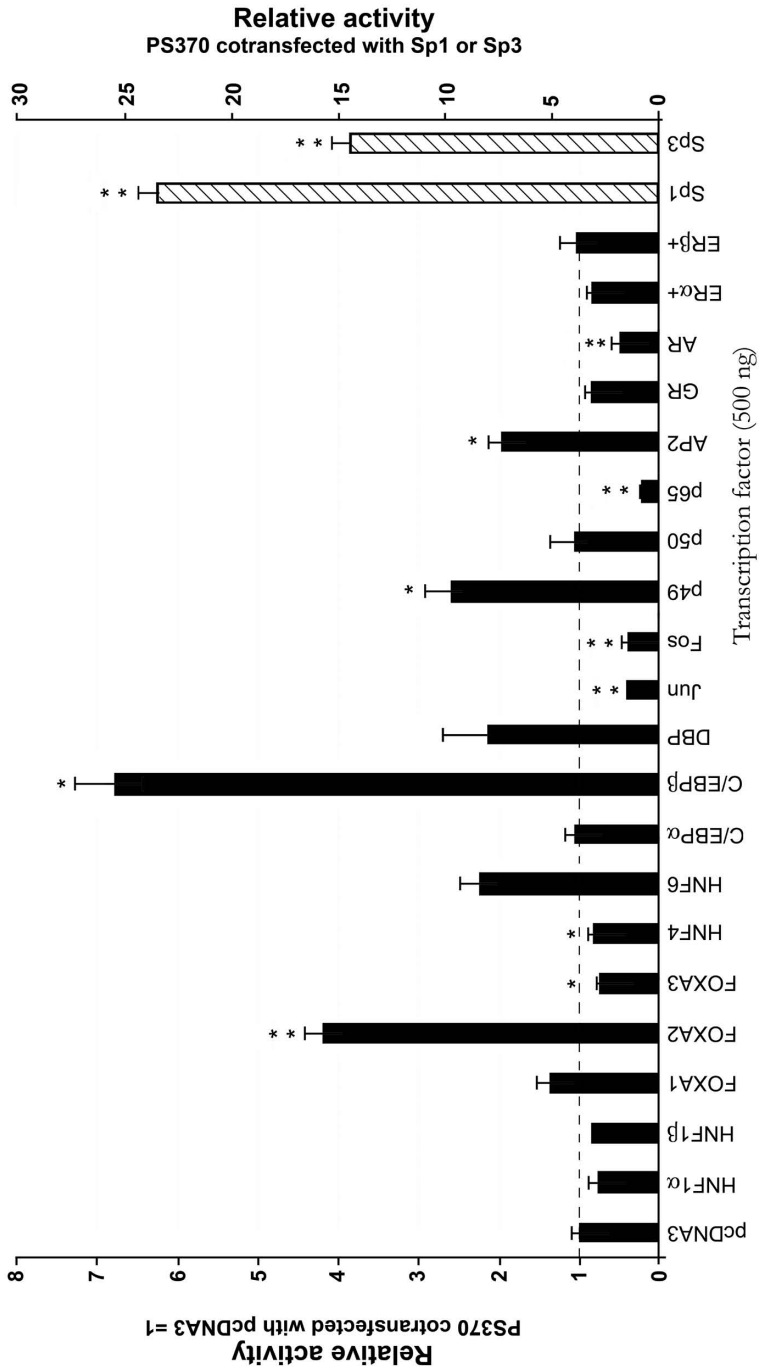

Figure 2 Cotransfection of HeLa cells with PS370 and transcription factors
HeLa cells were transfected as described in the experimental procedures section. Cells were harvested and luciferase activity was measured 48 hrs after transfection. Luciferase levels were not corrected for transfection efficiency 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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Chapter 4

The constitutive expression of anticoagulant Protein S is regulated through multiple binding sites for Sp1 and Sp3 transcription factors in the Protein S gene promoter

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Summary

Protein S (PS) is a vitamin K-dependent plasma protein that inhibits blood coagulation by serving as a non-enzymatic cofactor for activated protein C in the Protein C anticoagulant pathway. Low PS levels are a risk factor for the development of deep venous thrombosis. The regulation of PS protein levels through transcriptional regulation of the PS gene was investigated in this report. A minimal PS gene promoter of 370 base pairs upstream from the translational initiation codon was sufficient for maximal promoter activity in transient transfections regardless of the cell type. A pivotal role for Sp1 in the constitutive expression of the PS gene was demonstrated through EMSA experiments, transient expression of mutant PS promoter-reporter gene constructs and chromatin immunoprecipitations in HepG2 cells. A total of at least four Sp-binding sites were identified. The two sites most proximal to the translational startcodon were found to be indispensable for PS promoter activity, whereas mutation of the two most distal Sp-binding sites had a negligible influence on basal promoter activity. In addition, all other major promoter-binding proteins that were found by EMSA could be positively identified in supershift assays. We identified binding sites for the hepatocyte-specific forkhead transcription factor FOXA2, nuclear factor Y (NFY), and the cAMP-response element (CRE)-binding protein (CREB)/activating transcription factor (ATF) family of transcription factors. Their relevance was investigated using site-directed mutagenesis.

Introduction

The coagulation cascade is a complex system in which the consecutive activation of multiple coagulation factors leads to the production of thrombin and ultimately to the formation of fibrin polymers, the primary component of blood clots (for a recent review (1)). Protein S (PS) is a vitamin K-dependent plasma protein that functions as a nonenzymatic cofactor for Activated Protein C (APC) in the downregulation of the coagulation cascade via proteolytic inactivation of coagulant factors Va and VIIIa (2-5). PS has also been shown to display APC-independent anticoagulant activity in purified systems as well as in plasma (6-8). Recent studies indicate that PS may have a second function unrelated to coagulation in the clearance of apoptotic cells (9;10).

Over the past two decades low PS plasma levels have become a well-established risk factor for the development of deep venous thrombosis (11-13). However, not all mechanisms underlying low plasma PS levels have been fully characterized. Hereditary PS deficiency has been shown to be an autosomal dominant trait and many causative genetic mutations have been described in the PS gene (14;15). On the other hand, PS deficiency can also be acquired during life through conditions such as oral contraceptive use and liver disease (16). To better understand the different functions of PS and the possible causes of PS deficiency, more information on the regulation of the PS gene, mRNA and protein is needed.

The major source of circulating plasma PS is the hepatocyte (17), but PS is also produced constitutively at low levels by a variety of other cell types throughout the body (18-25). PS circulates in human plasma at a concentration of approximately 0.35 μM in a free form (40%) and a C4b-binding protein-bound form (60%) (26-28). The PS genetic locus, *PROS*, consists of an active PS gene (*PROS1*) and an inactive pseudogene (*PROS2*) which share 96% homology in their coding sequence. The promoter and the first exon are absent from the *PROS2* gene, however (29-31). Transcription from the *PROS1* promoter is directed from multiple start sites (32; Chapter 2), and recently the *PROS1* promoter was shown to contain a forkhead box A2 (FOXA2) binding site and an Sp1 binding site (33).

In this report, we further characterized the transcriptional regulation of the *PROS1* promoter. We identified binding sites for various transcription factors within the first 400 bp

proximal to the *PROS1* translational startcodon, among which multiple binding sites for the ubiquitous transcription factors Sp1 and Sp3, and single sites for nuclear factor Y (NFY), and the cAMP-response element-binding protein/activating transcription factor (CREB/ATF) family of transcription factors. Chromatin immunoprecipitations of chromatin from hepatocytic cell line HepG2 with an Sp1 antibody demonstrated the *in vivo* relevance of our findings. The results presented here show that Sp1 and Sp3 have a crucial role in the basal expression of the PS gene, whereas transcription factors FOXA2, NFY and CREB/ATF do not.

Experimental procedures

Plasmids --- The *PROS1* promoter-reporter constructs used in this study originated from a 7 kb *Eco*RI promoter fragment which was isolated from BAC clone #2513H18 from the CITBI-E1 genomic library (Research Genetics, Invitrogen, Carlsbad, CA). Their construction is described in Chapter 2 of this thesis.

Mutation of putative transcription factor binding sites --- Mutant constructs were generated by use of the QuikChange XL Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA). The sequence of the mutant oligonucleotides is depicted in Table 1. Successful incorporation of the mutations was confirmed by automated sequencing.

Oligo	Position*	Sequence‡
Sp1mta	-175/-144	GAGCGGGCGGTCTCATATGCCCCCGGCTGTTC
Sp1mtb	-262/-220	CCTCCAACACTAGAGCCCATATCATAGCTCCGAAAAGCTTCC
Sp1mtc	-306/-263	CTAGGGAGCTGGTGAATAGTCATGTCTCAGCAGTGTTTACTAGG
Sp1mtd	-366/-327	GAACTGCGTTCCCCACATCTTCATCTTTGGAAACGTCAC
NFYmt	-391/-353	CTGGAAGTTGTCTTGCCTTGTTTGAGAACTGCGTTCCCC
CREmt	-350/-321	CCCTTCCCCTTTGGAATGGTCACACTGTGG
FOXA2mt	-287/-253	GGATGTCTCAGCCGTGAGTACAAGGCCTCCAACAC

Table 1 Mutant oligonucleotides for site-directed mutagenesis. Nucleotide sequences of the primers used for site-directed mutagenesis of putative transcription factor binding sites within the *PROS1* promoter. * Numbering of the position is relative to the *PROS1* translational startcodon. ‡ Underlining of nucleotides denotes mutations.

Expression vectors --- Expression vectors containing human Sp1 and Sp3 (pCMV-Sp) transcription factors were a kind gift from J. Horowitz (Roswell Park Cancer Institute, USA) (34;35), and the expression vector containing murine FOXA2 (pcDNA3-FOXA2) was a kind gift from P. Holthuizen (University of Utrecht, NL). The pcDNA3-FOXA2 vector was created by inserting the cDNA for FOXA2, as reported by Lai and coworkers (36), into the Eco*RI* cloning site of vector pcDNA3.

Cell culture --- Leukocytes isolated from blood obtained from healthy donors (Sanquin Bloodbank, Leiden, NL) were a kind gift from E. Paffen (Leiden University Medical Center, Leiden, NL). The human hepatoblastoma cell line HepG2, and cervical adenocarcinoma cell line HeLa were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Megakaryocytic cell line Meg01 was a kind gift from G. van Willigen (Utrecht Medical Center, Utrecht, NL). The hepatoma cell line HuH7 was a kind gift from M. Verschuur (TNO Prevention and Health, Leiden, NL). Primary human umbilical vein endothelial (HUVEC) cells were a kind gift from J. Grimbergen (TNO Prevention and Health, Leiden, NL). HepG2, HeLa and HuH7 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 Invitrogen). Meg01 cells were grown in RPMI 1640 medium, 20% FBS, 100 µg/ml penicillin, 100 µg/ml streptomycin (all purchased from Invitrogen). HUVECs were grown in M199-medium (BioWhittaker, Walkersville, MD), 10% heat inactivated human serum (Sanquin Bloodbank), 10% Newborn Calf Serum (TNO Prevention and Health), 10 U/ml heparin (BioWhittaker), 150 U/ml endothelial cell growth factor, 100 µg/ml penicillin (BioWhittaker) and 100 µg/ml streptomycin (BioWhittaker). 24 hrs before transfection HUVEC medium was replaced with heparin-free medium to prevent interference with the transfection.

Reporter gene assays --- $1*10^6$ Meg01 suspension cells were used per transfection. All adherent cells (HepG2, HuH7, HeLa, HUVEC) were transfected at 60-80% confluency. HUVEC cells were transfected in passage 2-3, whereas the other cell types were used up to passage 25. Each transfection was performed in triplicate in 12-wells plates. All assays were conducted with two different DNA preparations of each construct. Transfections in HepG2, HeLa, HUVEC and HuH7 cells were carried out using 3 µl Tfx-20 lipids (Promega) per µg transfected DNA. Meg01 cells were transfected using 5 µg DAC-30 (Eurogentec, Seraing, BE) per µg DNA. In each transfection an equimolar concentration of construct was used, supplemented with pUC13-MCS vector to obtain a fixed amount of transfected DNA. In pUC13-MCS the MCS

had been removed by digestion with *Pvu*II and recircularisation. Control vector pRL-SV40 (Promega), expressing the Renilla luciferase, was co-transfected to correct for transfection efficiency in a 1:500 ratio to the total transfected amount (μg) of DNA in HepG2, HuH7 and HeLa cell lines, and a 1:100 ratio in transfections with HUVEC and Meg01 cells. 250 ng transcription factor expression vector was used for cotransfections, expression vector without the transcription factor cDNA was used as a negative control. Cell extracts were harvested at either 24 hours (HepG2, HuH7) or 48 hours (Meg01, HUVEC, HeLa) after transfection. Cells were lysed in 250 μl Passive Lysis Buffer (Promega) per well, after which 20-100 μl was used to measure luciferase activity. Luciferase activity was measured according to the Dual Luciferase Assay System Protocol (Promega) using a Lumat LB9507 luminometer (Berthold, Bad Wildbad, DE).

Preparation of nuclear extracts --- Nuclear extracts (NE) were prepared according to the method of Dignam *et al* (37). Nuclear extract buffer contained 20 mM Hepes (pH 7.9), 0.2 mM EDTA, 100 mM KCl, 0.5 mM DTT, 0.2 mM PMSF and the EDTA-free protease inhibitor cocktail (Roche, Mannheim, DE). NEs were aliquoted and frozen at -80^{o}C until further use. Protein concentration of the NE was measured with the BCA assay (Pierce Biotech, Rockford, IL).

Electrophoretic Mobility Shift Assays (EMSA) --- EMSAs were performed in a 13 μl binding reaction containing 10 μg NE and 195 ng denatured herring sperm DNA. EMSA buffers were purchased from Active Motif (Carlsbad, CA) and used according to the manufacturers recommendations. Double-stranded (ds) oligonucleotides were end-labeled using γ^{32}P-ATP and T4 polynucleotide kinase. The position numbers for the oligonucleotides in Figure 2 show their location respective to the *PROS1* translational startcodon. Reaction mixtures were incubated on ice for 20 min with or without an unlabeled competitor. Subsequently, the ^{32}P-labeled ds probe was added and the incubation was continued for another 20 min. In supershift experiments, NE was incubated on ice for 10 min with the ^{32}P-labeled ds probe after which an anti-Sp1 (sc59x), anti-Sp3 (sc664x), anti-NFYA (sc7711x), anti-CREB/ATF (sc270x) or anti-FOXA2 antibody (sc6554x) (Santa Cruz Biotechnology, Santa Cruz, CA) was added and the incubation was allowed to continue for another 10 min. Samples were loaded on a 3% or 5% non-denaturing polyacrylamide gel, which was electrophoresed for 2 hrs at 200V, after which gels were vacuum-dried and exposed to x-ray film.

Chromatin Immunoprecipitation Assay (ChIP) --- Chromatin immunoprecipitation assays were conducted with chromatin isolated from HepG2 cells with the Chip-IT kit (Active Motif,

Rixensart, Belgium) according to the manufacturers instructions. Briefly, HepG2 cells were grown to 80% confluency in 75 cm^2 flasks after which chromatin was fixed *in vivo* by addition of 1% formaldehyde in culture medium. Fixed chromatin was isolated and sheared for 5 x 20 seconds to an average fragment size of 500 bp using a Soniprep 150 homogenizer (MSE, Kent, UK) at 25% power. Approximately 20 µg sheared chromatin was incubated for 4 hours with 3 µg transcription factor-specific antibody at 4°C with gentle rotation, after which Protein G beads were added and the incubation was continued overnight. An antibody against TFIIB was used as a positive control, non-specific IgG was used as a negative control (both antibodies are included in the Chip-IT kit). The same Sp1 antibody was used for the ChIP experiments as for the supershift assays. The antibody-chromatin complexes on the Protein G beads were pelleted, washed extensively, eluted from the protein G beads, and treated with proteinase K and RNAse A. DNA was purified over a mini-column and resuspended in 100 µl H_2O. 3 µl was used as a template for PCR using primers surrounding the suspected transcription factor binding site. For the *PROS1*-specific PCR the following primers were used: -322/-299 (sense) `5′-GGAGGAAAAGCAGCAACTAGGGAG-3′`, -91/-106 (antisense) `5′-TCGGTCTGAGCCGTG-3′`. For the positive control primers located in the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) promoter were used. The background control consisted of a PCR with primers located in the chromosome condensation-related SMC-associated protein (*CNAP1*) gene.

	PS levels (nM)			
Cell Type	**24 hr**	**48 hr**	**72 hr**	**96 hr**
HepG2	0.315	0.498	0.752	1.064
HuH7	0.343	0.709	1.166	1.391
HeLa	0.171	0.189	0.268	0.405
HUVEC	0.042	0.072	0.089	0.112
Meg01	0.065	0.126	0.149	0.185

Table 2 PS protein levels over time in cell culture. PS levels were measured by ELISA analysis of cell culture medium at various time points. Cells were seeded in 12 wells plates and incubated with 2 ml medium. Experiments were started at 80% confluency of cell culture or, in the case of Meg01, at $1*10^6$ cells/ml. HepG2, HeLa, Meg01 and HuH7 medium included 10% FBS, HUVEC medium was serum-free. Fresh media were used as a blank to correct for background interference in the ELISA. Data are shown in graph format in Chapter 2.

Results

Protein S expression in cell lines --- Protein S levels in cell culture medium were determined by ELISA analysis for HepG2, HuH7, HeLa, Meg01, HUVEC cells (Table 2). Hepatocytic cell types HuH7 and HepG2 had the highest PS production. PS levels in HeLa and Meg01 cell culture were low when compared to levels in HepG2 and HuH7 culture medium. PS levels were lowest in medium from HUVEC cell culture.

PROS1 promoter activity in transient transfection of various cell types --- Transient transfection studies were conducted in unstimulated HepG2, HuH7, HeLa, HUVEC, and Meg01 cells with equimolar amounts of *PROS1* promoter constructs cloned upstream from the firefly luciferase reporter gene (Figure 1). These studies pointed out that the first 370 bp of the *PROS1* 5'-flanking region were necessary for maximal promoter activity in all cell lines. When compared with expression in other cell types, the shortest *PROS1* construct, PS197, had relatively high activity in HeLa and HuH7 cells. However, background pGL3basic activity was also higher in these cells. Optimal promoter activity was maintained up to a 5' region with a length of 1062 bp. Constructs longer than 1062 bp had reduced activity in transfections in HepG2 and HeLa cells, whereas in HuH7 and Meg01 cells promoter activity remained at a high level. This difference in expression indicates that tissue-specific expression of transcription factors that bind to sites in this region may play a role in the regulation of *PROS1* activity. Computational analysis of the *PROS1* promoter sequence with the MatInspector professional software (38) did not reveal the presence of distinct upstream inhibitory or stimulatory elements, however.

Multiple transcription factors bind to the PS promoter --- In a previous study we determined that *PROS1* transcription is driven from three possible start sites, namely -100, -117, and -147 (32). Here we show that maximal promoter activity is reached with a minimal *PROS1* promoter of 370 bp. On basis of these observations we investigated transcription factor binding to the *PROS1* region encompassing bp 100 to 370 upstream from the translational start. For this purpose, a series of overlapping ds oligonucleotides covering the aforementioned region was designed. The duplexes were all 24 bp long, each having a 12 bp overlap with its neighbouring oligonucleotides. The liver cell line HepG2 was chosen for these more detailed experiments, as it had a high PS expression level and has been more intensely investigated than the HuH7 cell line.

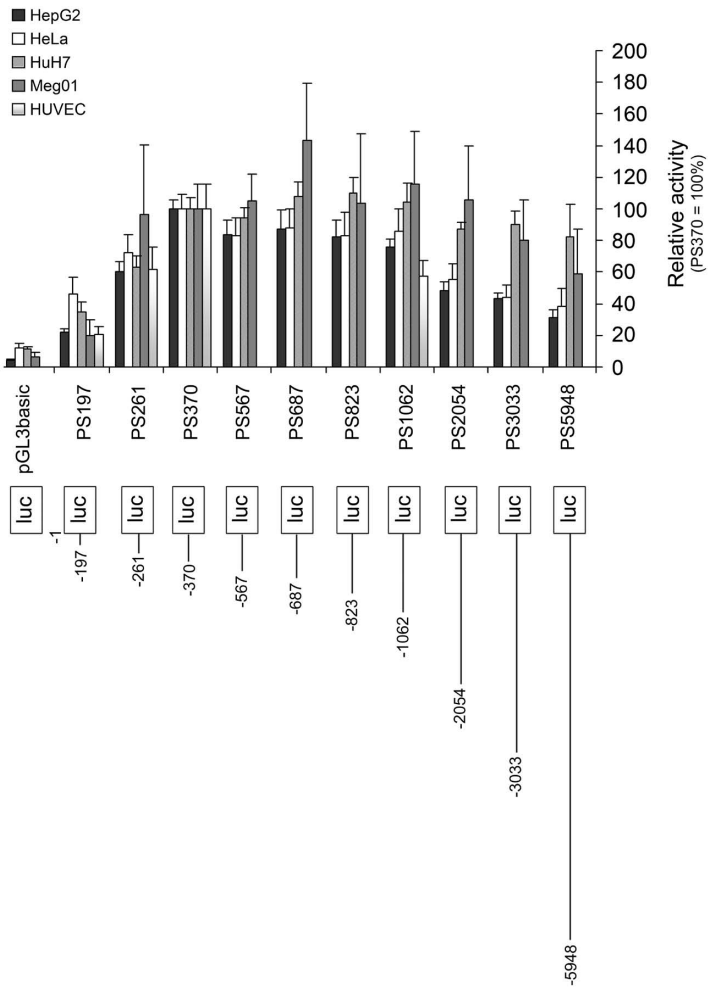


Figure 1 Transient expression of *PROS1* promoter-reporter gene constructs in cultured cells. Promoter activity of a series of 5' *PROS1* deletion constructs was measured as luciferase expression and is expressed as a percentage of the most active construct PS370. Note that the activity of PS370 was different for each cell line. Luciferase activities can therefore not be compared among cell lines, as they are also dependent on transfection efficiency in addition to promoter activity. The data are presented as the means (± SD) of at least two different experiments in triplicate with two independent plasmid preparations. Luc; luciferase. Numbering of the construct is relative to the *PROS1* translational startcodon. The data in this figure are also used in Chapter 2.

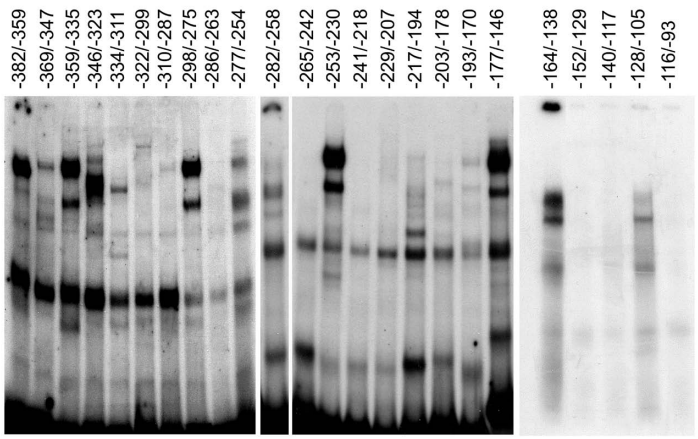


Figure 2 Primer walking: incubations of HepG2 Nuclear Extract and 24 consecutive ds oligonucleotide probes. 10 μg HepG2 Nuclear Extract was incubated with a single ds oligonucleotide probe for 20 minutes. Protein-DNA complexes were separated on 3-5% PAGE. The dried gel was exposed to film. Numbering of the nucleotide positions was as in Figure 1.

Incubation of HepG2 nuclear extracts with radio-labeled oligonucleotide duplexes located between positions -93 to -152 upstream from the *PROS1* translational start did not result in the formation of protein-DNA complexes (Figure 2). In contrast, almost all ds oligonucleotide probes from duplex -152/-129 up to -382/-359 were complexed with nuclear protein. In further experiments we focussed on the more pronounced complexes found with primer walking. Upon computational analysis of the first 400 bp of the *PROS1* promoter sequence with the MatInspector professional software (38), consensus binding sites for various transcription factors were found (Figure 3). A high degree (>60%) of interspecies conservation (human, chimpanzee, rhesus monkey, dog, bovine, pig, mouse, and rat comparison) was found from ~250 bp to ~100 bp upstream of the *PROS1* translational startcodon with the VISTA alignment tools (Chapter 3 this thesis) (39;40). Binding sites for Sp1 and STAT in this region were conserved by more than 95%.

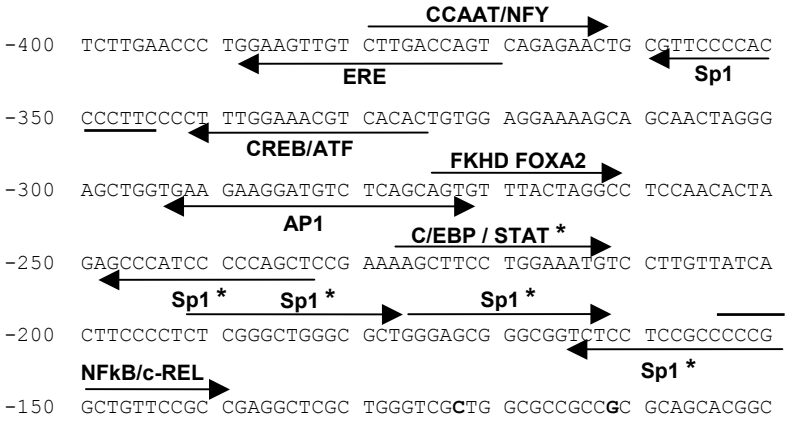


Figure 3 Putative transcription factor binding sites in the *PROS1* promoter.
Computational analysis of bp -400 to -100 of the *PROS1* promoter revealed the presence of several putative transcription factor binding sites within this region. *Interspecies conservation was above 95% for these sites.

The protein-DNA complexes observed in incubations containing HepG2 nuclear extract and radio-labeled oligonucleotide duplexes -359/-335, -298/-275, -253/-230, and -177/-146, all displayed a similar pattern of retardation on poly-acrylamide gel. Computational analysis pointed out that all oligonucleotides, with the exception of -298/-275, contained putative binding sites for the ubiquitous transcription factor Sp1. Sp3, another member of the Sp-family of transcription factors, has similar DNA-binding properties as Sp1 and thus often binds to the same sites as Sp1. Four Sp3 isoforms exist with different molecular weights that can all bind to the Sp1 consensus sequence. The resultant is the highly recognizable Sp1/Sp3 EMSA banding pattern (Figure 4) (41;42). The specificity of Sp-binding to the oligonucleotide duplexes was confirmed in competition experiments with unlabeled wild type oligonucleotide duplexes and the Sp-consensus oligonucleotide. Mutant oligonucleotides in which the putative Sp binding site had been modified did not compete for protein binding. Upon incubation with antibodies directed against either Sp1 or Sp3 a supershift of the specific bands, band a and bands b and c, respectively, was observed (Figure 4, panels A, B, and C). These experiments confirmed our hypothesis that all four oligonucleotide duplexes contained Sp-binding sites. Competition experiments demonstrated that not all Sp-binding sites in the *PROS1* promoter bind Sp1 with the same affinity (Figure 4, panels D and E). By addition of a 50-fold excess of

unlabeled competitor in the form of one of the three other duplexes to incubations with either labeled duplex -177/-146 or -298/-275, the following order of binding affinity was established: -177/-146 > -253/-230 > -298/-275 > -359/-335. The oligonucleotide for which the Sp-proteins displayed the highest affinity, -177/-146, was 8 bp longer than the other oligonucleotides. The computational analysis showed that more than one Sp-binding site may be located within this region. Moreover, the -177/-146 mutant oligonucleotide was still able to compete slightly for Sp1 binding (Figure 4, panel A). The higher affinity of Sp1 for -177/-146 may therefore be due to the presence of more than a single Sp-binding site.

All other oligonucleotide probe-protein complexes had a unique migratory behaviour. Computational data (Figure 3) and a previous publication (33) indicated that the protein complex attached to duplex -282/-258 could be the liver-specific transcription factor FOXA2. EMSA competition experiments with the wild type oligonucleotide and a mutant oligonucleotide in which the putative FOXA2 binding site had been altered, confirmed this. Moreover, incubation of the wild type oligonucleotide probe with a FOXA2-specific antibody resulted in the disappearance of the FOXA2-specific band (Figure 5 panel A).

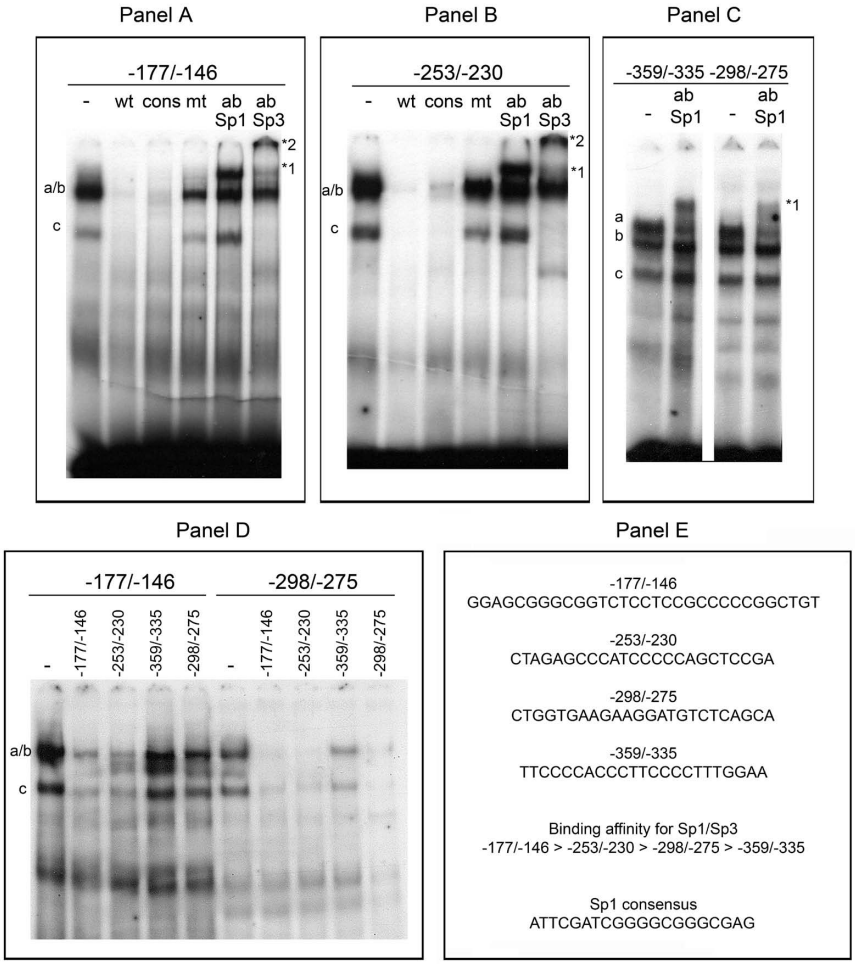


Figure 4 Sp1 and Sp3 bind to multiple sites in the *PROS1* promoter. 10 μg HepG2 Nuclear Extract was incubated with a single ds oligonucleotide probe for 20 minutes. Protein-DNA complexes were separated on 3% (panels C and D) or 5% (panels A and B) PAGE. The dried gel was exposed to film. In panels A and B competition experiments were carried out with a 50-fold excess of unlabeled wild type (wt) or Sp-consensus (cons) ds oligonucleotide. Sp1 (panels A, B, and C) and Sp3 (panels A and B)-specific antibodies were added to the reactions resulting in a supershift of the antibody-protein-oligonucleotide probe complex (bands are indicated *1 for Sp1, *2 for Sp3). Panel D depicts a competition experiment in which affinity of the Sp-proteins for the different oligonucleotides was investigated. Either oligonucleotide -177/-146 or -298/-275 was labeled and a 50-fold excess of the other (unlabeled) oligonucleotides was added. a: complex containing Sp1, b and c: complex containing Sp3 isoforms. In Panel E the sequences of the *PROS1* and Sp1 consensus ds oligonucleotides is depicted. The binding affinity of the four *PROS1* oligonucleotides is estimated.

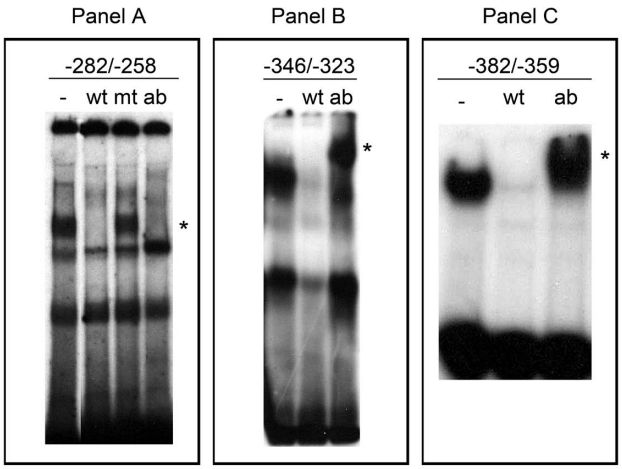


Figure 5 FOXA2, CRE/ATF and NFY bind to the *PROS1* promoter. 10 μg HepG2 Nuclear Extract was incubated with a single ds oligonucleotide probe for 20 minutes. Protein-DNA complexes were separated on 3% (panel A) or 5% (panels B and C) PAGE. The dried gel was exposed to film. Competition experiments were carried out with a 50-fold excess of unlabeled wild type (wt) or mutant (mt) ds oligonucleotide. FOXA2 (panel A), CRE/ATF (panel B) and NFYα (panel C)-specific antibodies were added to the reactions resulting in a supershift of the antibody-protein-oligonucleotide probe complex (bands are indicated with an asterisk).

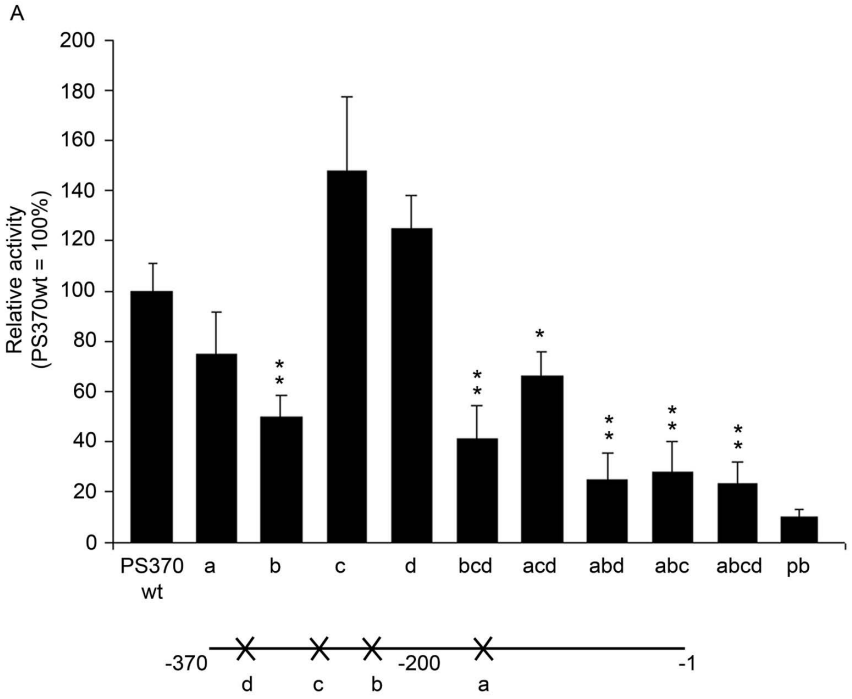
Computational analysis also revealed the presence of putative CREB/ATF and NFY sites around positions -346 to -323 and -382 to -359, respectively. Protein-DNA complexes were obtained in incubations with HepG2 nuclear extract and labeled primers at these positions. Evidence for binding of both families of transcription factors was subsequently established in supershift assays with transcription factor-specific antibodies (Figure 5, panels B and C).

Effects of transcription factor binding site mutations on PROS1 promoter activity – The contribution of each of the identified transcription factors in *PROS1* transcription was assessed by site-directed mutagenesis of their binding sites within the *PROS1* promoter-reporter gene constructs. First, it was confirmed that mutant oligonucleotide duplexes could not compete for transcription factor binding and that mutant probes could not bind nuclear protein in EMSA experiments (Figure 4 panel A, panel B and Figure 5 panel A and results not shown). Mutation of the Sp-binding sites had a pronounced effect on basal promoter activity. In the

experiments depicted in Figure 6A, construct PS370 was modified to contain either one, three or four mutated Sp-binding sites. Whereas alteration of site a (-177/-146) or b (-253/-230) had a strong negative effect on promoter activity, mutation of site c (-298/-275) or d (-359/-335) had a mildly positive effect. Mutation of site a or b alone resulted in a reduction of promoter activity to 75% and 50% of the wild type PS370 construct, respectively. No further beneficial or deleterious effects were seen when mutation of either site a or b was combined with alteration of both Sp-binding sites c and d (mutants acd and bcd). The quadruple mutant (abcd) and triple mutants, abc and abd, all displayed a residual promoter activity of approximately 20% (background pGL3b activity was 9%). From these experiments we conclude that Sp-binding sites a and b, but not c and d, are necessary for maximal *PROS1* promoter activity under basal conditions. It may even be cautiously concluded that sites c and d have an inhibitory influence on *PROS1* transcription. Finally, the influence of Sp-binding site b on basal activity is greater than that of site a.

Basal expression of *PROS1* construct PS370 or PS1062 in HepG2 was only slightly influenced by mutation of the binding sites for FOXA2, CREB/ATF or NFY (Figure 6B). The mutation in the FOXA2 binding site caused a slight decrease in *PROS1* promoter activity, whereas the NFY mutation caused a slight increase in promoter activity ($p<0.05$ for both mutations). Mutation of the CREB/ATF binding site caused no measurable alteration in promoter activity.

PROS1 *promoter expression is stimulated by Sp1, Sp3, and FOXA2* -- Cotransfection of HepG2 cells with PS370 and the Sp1-expression vector resulted in a 2.4 fold induction of promoter activity (Figure 7A). Sp3 had little or no effect on promoter activity. Cotransfections with triple mutants, in which only sites a, b, or c were still present, barely resulted in a decreased inducibility of PS370 by Sp1 (2.44, 2.07, and 2.15 fold for bcd, acd and abd respectively). Site a (mutant bcd) was sufficient for full Sp1 induction of PS370 and only a slight reduction of responsiveness to Sp1 was seen in triple mutants acd and abd. On the other hand, the triple mutant PS370abc and the quadruple mutant PS370abcd had low responsiveness comparable to that of the empty vector pGL3basic (1.37, 1.58, and 1.35 fold stimulation respectively). Apparently, Sp1-binding site d (-359/-335) is not able to mediate Sp1 induction of *PROS1* promoter activity, indeed this site may even exert an inhibitory effect (1.37 fold stimulation



Sp site	loc'n	original seq	alterations
a	-162/-153	CCTCCGCCCC	CATATGCCCC
b	-242/-233	CCCCCAGCTC	TACTAAGCTC
c	-292/-283	AAGAAGGATG	AATAGTCATG
d	-352/-343	ACCCCTTCCC	ACATCTTCAT

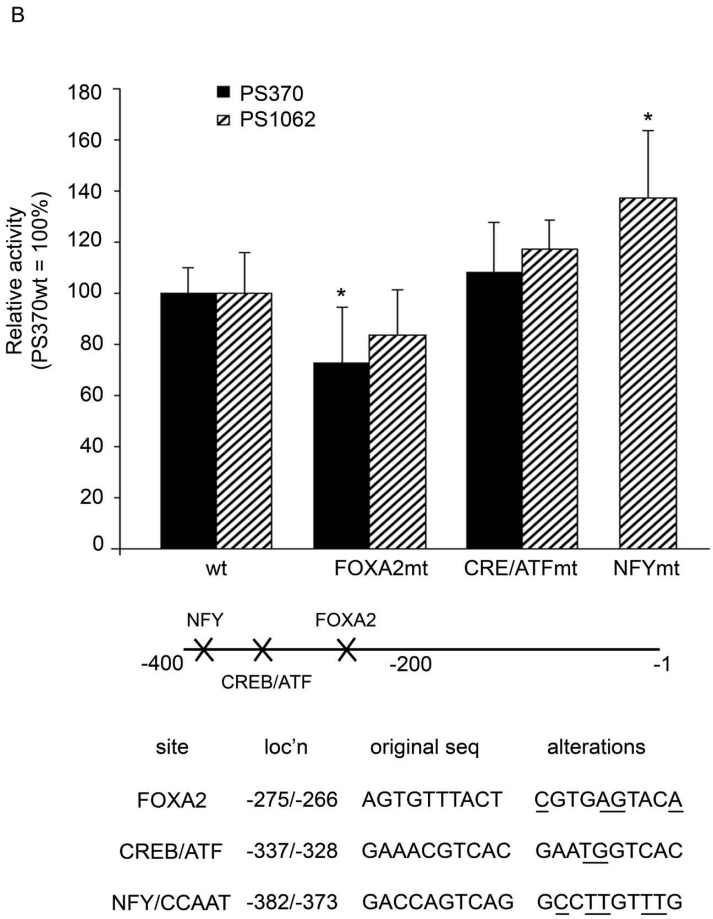


Figure 6 Effect of site-directed mutagenesis of transcription factor binding sites on *PROS1* promoter activity. HepG2 transient transfections with wild type and mutant *PROS1* promoter-luciferase contructs were carried out. The mutated site(s) are indicated below the bars. Luciferase expression of the wild type was set to 100% and promoter activity of the mutants is expressed as a percentage of the wild type construct. The data are presented as the means (± SD) of at least three different experiments in triplicate with two independent plasmid preparations. (**A**) Sp-binding sites in PS370 were mutated as shown. *p<0.01, **p<0.001 relative to the wild type construct. (**B**) Binding sites for FOXA2, CRE/ATF and NFY were mutated in the PS370 construct (FOXA2, CRE/ATF) and in the PS1062 construct (FOXA2, CRE/ATF and NFY) as shown. *p<0.05. pb; pGL3basic.

versus 1.58 for the quadruple mutant). Upon alteration of all four binding sites, not all Sp1 *trans*-activation potential was lost (1.58 vs. 1.35 background). This supports our earlier assumption that an additional Sp-binding site may be present nearby site a (-177/-146) that was not mutated. Similar results were obtained with cotransfections in HeLa cells, albeit that *PROS1* promoter induction was more pronounced in this cell type (results not shown).

The activity of *PROS1* promoter construct PS370 was stimulated slightly by cotransfection with the liver-specific transcription factor FOXA2 in HepG2 cells (p=0.06) (Figure 7b). Upon mutation of the FOXA2 binding site at position -282/-258 this trend was absent. The empty vector, pGL3basic, was also strongly responsive to cotransfection with the FOXA2 expression vector. Cotransfections in HeLa cells produced similar results for the *PROS1* constructs, but pGL3basic had an even higher background activity.

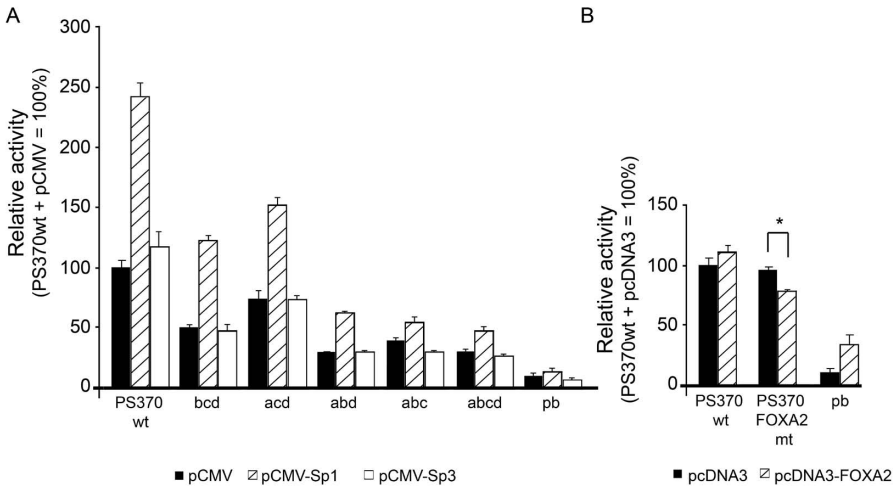


Figure 7 Cotransfections of HepG2 cells with PS370 variants and transcription factor expression vectors expressing Sp1, Sp3 or FOXA2. HepG2 transient transfections with wild type and mutant *PROS1* promoter-luciferase contructs were carried out. Luciferase expression of the wild type co-transfected with the empty transcription factor expression vector was set to 100% and all other data are expressed as a percentage thereof. The data are presented as the means (± SD) of a representative assay done in triplicate. (**A**) Cotransfections of PS370 variants with pCMV-Sp1 or pCMV-Sp3. (**B**) Cotransfections of PS370 variants with pcDNA3-FOXA2 *p<0.05. pb; pGL3basic.

Chromatin immunoprecipitation locates Sp1 to the PROS1 promoter in vivo -- A *PROS1*-specific PCR was performed on sheared HepG2 chromatin, which had been immunoprecipitated with an Sp1-specific antibody, with primers encompassing promoter region -322 to -91 upstream from the *PROS1* translational startcodon. As a positive control on the method, a PCR was performed on the *GAPDH* promoter after chromatin immunoprecipitation with the positive control, TFIIB antibody. The PCR on the *GAPDH* promoter also served as a control for the specificity of the Sp1 antibody, since the *GAPDH* promoter contains two Sp1 binding sites in the same region that was used for the TFIIB positive control PCR. The *PROS1*-specific PCR product that was obtained (Figure 8) provides evidence for direct *in vivo* association of Sp1 with the *PROS1* promoter in HepG2 chromatin.

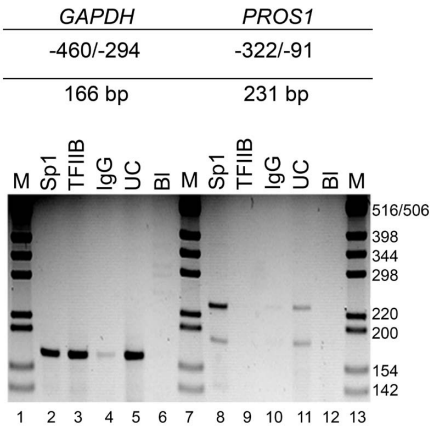


Figure 8 Sp1 binds to the *PROS1* promoter *in vivo.* ChIP assays were performed using sonicated chromatin isolated from HepG2 cells and an antibody specific for Sp1, TFIIB, or a non-specific IgG. The subsequently purified DNA samples were used as a template for PCR with primers specific for *GAPDH* (lanes 2-6) generating a 166 bp gene-specific product or *PROS1* (lanes 8-12), which generates a 231 bp fragment (bp -322 to -91). The following PCR results are shown: *lanes 2 and 8*, using DNA purified with the anti-Sp1 antibody; *lanes 3 and 9*, using DNA purified with the anti-TFIIB antibody; *lanes 4 and 10*, using DNA purified with the non-specific IgG; *lanes 5 and 11*, using untreated chromatin; *lanes 6 and 12*, H_2O; and. *Lanes 1, 7 and 13* represent a 1-kb plus marker.

Discussion

Heterozygosity for PS deficiency is found in 0.2% of the general population and in 1-2% of patients with deep vein thrombosis (43-45). Whereas hereditary PS deficiency is relatively rare, many other conditions lead to acquired PS deficiency. For instance, PS levels are strongly

influenced by hormonal state, which is illustrated by decreased PS levels during pregnancy and oral contraceptive use (46;47). Conditions known to negatively influence PS levels are liver disease, and disseminated intravascular coagulation (16;48;49). Overall, the regulation of PS levels (free and C4BP-bound) in normal and pathophysiological circumstances is still poorly understood.

This paper reports on the transcriptional regulation of *PROS1* by cell-specific and general transcription factors. A proximal promoter fragment of 370 bp with maximal activity was identified through transient expression of *PROS1* promoter-reporter constructs under basal conditions in all cell lines. Larger *PROS1* constructs had reduced activity in HepG2 and HeLa cells, whereas promoter activity was maintained at a high level in the same constructs in Meg01 and HuH7 cells. The finding of a minimal promoter with a high level of activity in all cell types suggested that ubiquitous transcription factors are involved in the regulation of *PROS1* transcription under basal cell culture conditions. On the other hand, the finding of differential activity of the longer constructs is an indication that also cell-specific transcription factors may be involved in *PROS1* transcription.

The hypothesis that *PROS1* transcription is driven for a large part by general transcription factors was confirmed by the identification of four binding sites for the ubiquitous transcription factor Sp1 (41) in the first 370 bp of the *PROS1* promoter. The affinity of Sp1 for the four sites was estimated by EMSA competition experiments and was categorized as follows; a (-177/-146) > b (-253/-230) > c (-298/-275) > d (-359/-335). Computational analysis showed that multiple Sp1/3-binding sites may be situated in the region surrounding site a, only one of which had been mutated during site-directed mutagenesis, however. Mutagenesis of site b had the most deleterious effect on basal *PROS1* promoter activity; PS370 activity in HepG2 cells was reduced by approximately 50% upon mutation of this site. Mutation of site a resulted in a reduction of PS370 activity to 75% of that of the wild type construct. Mutation of Sp-binding sites c or d had a slightly positive effect on PS370 promoter activity. Alteration of all Sp-binding sites resulted in 20% residual promoter activity.

We also tested the effectiveness of mutation of Sp-binding sites a and b in a smaller construct, namely PS261, and obtained similar results as for PS370 (40% (b) and 20% (ab)

residual activity (results not shown)). In this respect our study is in accordance with that of Tatewaki *et al* (33), who previously identified site b as an Sp1 binding site. Upon mutation of site b in a construct encompassing bp -282 to -161 of the *PROS1* 5' region they demonstrated a residual construct activity of approximately 15%. Site b is the only Sp-binding site present in the -282/-161 construct. This result is therefore best compared to the 20% residual activity of PS261ab and PS370abcd in our study. The finding that alteration of sites c and d in combination with either site a or b in triple mutants acd and bcd did not result in an additional effect on promoter activity over the single mutation of site a or b (Figure 5b) led us to conclude that sites c and d are redundant in *PROS1* transcriptional regulation by Sp1. The increased activity of the single c and d mutants relative to the wild type promoter construct suggests that these sites may nevertheless be important in *PROS1* promoter regulation, most likely through a different mechanism than *trans*-activation of the *PROS1* promoter by Sp1 binding. For instance, steric hindrance by Sp1 binding at site c (-298/-275) could impede simultaneous binding of FOXA2 at the overlapping site -282/-258 and vice versa. Sp1 has also been attributed a central role in transcriptional activation through histone acetylation and the prevention of gene silencing through DNA hypermethylation (50-53). A third possible explanation for the slight increase in promoter activity upon mutation of site c or d may be that these sites are bound with a higher affinity by Sp3 than by Sp1. Sp3 may act either as an inhibitor or activator of transcription depending on cell type, DNA-binding site context, and additional transcription factors that are present in the cell (41). In this case Sp3 may be repressing *PROS1* transcription through binding of Sp-binding sites c and d.

Cotransfection of Sp1 or Sp3 with PS370 triple mutant constructs confirmed that sites a and b are essential in *PROS1* transcriptional regulation by Sp1 (Figure 7). Mutant abd was responsive to Sp1 in the same manner as mutant acd. This was an unexpected finding since in basal activity experiments mutation of site c was found to have a stimulatory effect on *PROS1* promoter activity (Figure 6). However, this finding indicates that site c may indeed be bound with higher affinity by Sp3 under basal conditions, as suggested above. By addition of excess Sp1 in cotransfection experiments, most if not all Sp3 is most likely competed off of site c resulting in the observed induction of PS370abd promoter activity.

In vivo chromatin immunoprecipitation of Sp1-*PROS1* promoter complexes further confirmed the relevance of Sp1 in *PROS1* transcription. In an effort to further demonstrate *in*

vivo binding of transcription factors to the *PROS1* promoter region, *in vivo* genomic footprinting of *PROS1* promoter region -400 to -100 was attempted. Unfortunately, high levels of background signal in these experiments compromised the interpretation of the data. We believe that these high background levels were due to the fact that the endogenous *PROS1* promoter is not active in all cells, i.e. is not bound by transcription factors. This problem could probably be overcome if a major stimulator of *PROS1* transcription was added to the cell culture. To our knowledge such a compound has not yet been identified.

A previously identified binding site for the liver-specific transcription factor FOXA2 was confirmed at position -282/-258 (33). In contrast to these earlier findings, mutation of the FOXA2 site hardly resulted in reduced *PROS1* promoter activity (Figure 7c). The different construct sizes in both studies could cause the different effects of the mutations. As described above, Tatewaki *et al* used a construct, which ranged from -282 to -161, thereby lacking several of the previously identified transcription start sites (32), whereas the construct used in this report is larger, spanning nucleotides -370 to -1 in relation to the *PROS1* translational startcodon. Cotransfection of HepG2 cells with FOXA2 and either PS370 or the PS370-FOXA2 mutant resulted in a slight induction of the wild type PS370, but not of the FOXA2 mutant construct. In addition, the empty vector pGL3basic was strongly responsive to cotransfection with the FOXA2 expression vector. This complicated the interpretation of the data obtained with the *PROS1* construct. We conclude that FOXA2 may not be an important *trans*-activator of the *PROS1* promoter in our model system. We identified three putative FOXA2 binding sites within the first 100 bp upstream from the pGL3basic multiple cloning site (mcs) and a single binding site between the mcs and the luciferase translational startcodon. We therefore assumed that the cross-reactivity of pGL3basic was most likely due to FOXA2 binding to the vector.

A CREB/ATF binding site was discovered and confirmed by EMSA-analysis in the *PROS1* promoter at position -346/-323. Although no deleterious effect on basal PS370 activity was observed by mutation of the binding site, this does not rule out a role for CREB/ATF in the induction of *PROS1* transcription under stimulatory circumstances. The CREB/ATF family has at least 10 members, all of which bind to a DNA consensus sequence called the CRE (reviewed in (54;55)). The finding of a CRE element in the *PROS1* promoter, suggests that changes in intracellular cAMP levels may be involved in the regulation of PS

levels *in vivo*. Transcription of many cellular genes is regulated by changes in cAMP levels in response to extracellular stimuli, amongst which steroid hormones. This signalling pathway is associated with the protein kinase A-dependent phosphorylation of nuclear proteins that belong to the CREB/ATF family of transcription factors. It is tempting to speculate that cAMP and CREB/ATF are the messengers involved in the estrogenic/progesteronic effects on PS levels.

A binding site for the ubiquitous transcription factor NFY was found in the *PROS1* promoter at position -382/-373 through primer walking and antibody supershift EMSA experiments. NFY is primarily known as a transcriptional activator, although it has also been shown to inhibit transcription (56;57). NFY is unable to activate transcription by itself, but increases the activity of neighbouring enhancer motifs and participates in the correct positioning of other transcription factors at the start site. Mutation of the NFY binding site in *PROS1* promoter construct PS1062 had a positive effect on promoter activity in HepG2. Further research is needed to clarify the role of NFY and CREB/ATF in the transcriptional regulation of the *PROS1* promoter.

In conclusion, we show that constitutive expression of the minimal *PROS1* promoter PS370 is mainly directed by Sp1/Sp3. The two most proximal Sp-binding sites of the total of four binding sites that were identified in this study are pivotal in *trans*-activation of the *PROS1* promoter by Sp1, whereas such a role for the two most distal sites could not be established. Binding of either Sp1 or Sp3 to these last two sites may however be necessary for regulation of PS transcription at a different level, which could not be revealed by the methodology used in this report. The PS370 minimal promoter was further shown to contain many transcription factor binding sites, the most prominent of which were identified as sites binding transcription factors Sp1/Sp3, FOXA2, CRE/ATF and NFY.

Acknowledgements

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Chapter 5

IL6 induction of Protein S is regulated through Signal Transducer and Activator of Transcription 3 (STAT3)

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Submitted

Summary

The Protein C anticoagulant pathway is an essential mechanism for attenuating thrombin generation by the membrane-bound procoagulant complexes tenase and prothrombinase. In this pathway, Protein S (PS) serves as a cofactor for activated protein C (APC) in the inactivation of coagulation factors VIIIa and Va. The primary site of PS synthesis is the liver. PS circulates in plasma in a free form and in complex with complement component 4b-binding protein (C4BP), thus linking PS to the complement system. C4BP is a known acute phase reactant thereby also suggesting a relation between PS and the acute phase response. Interleukin 6 (IL6), a major contributor to the acute phase response of hepatocytes, has been shown to influence both PS and C4BP gene expression. We show here that IL6 upregulates PS mRNA and protein levels in HepG2 cell culture and that it exerts this effect by induction of PS promoter activity through a direct interaction of Signal Transducer and Activator of Transcription 3 (STAT3) with the PS promoter at a region spanning nucleotides 229 to 207 upstream from the translational start. A possible function for IL6-induced PS expression in cell survival is discussed.

Introduction

The Protein C anticoagulant pathway is indispensable in maintaining the hemostatic balance (1;2). In this pathway, Protein S (PS) functions as a non-enzymatic cofactor for Activated Protein C (APC) in the inactivation of coagulation factors VIIIa and Va (3-6). The clinical importance of PS became apparent by the association of venous thrombo-embolism (VTE) with (partial) PS deficiency (7-9). The major source of circulating plasma PS is the hepatocyte (10). In human plasma, PS circulates in an active free form (40%) and a C4b-binding protein (C4BP)-bound inactive form (60%) (11-13). The acute-phase response (APR) is characterized by a rapid increase of hepatic proteins, which serves to restore the physiological balance and limit the deleterious effects of tissue injury and infection. In the liver, synthesis of several plasma proteins, e.g. α1-antitrypsin, α2-macroglubulin, plasminogen, and fibrinogen, undergoes dramatic changes during the APR (reviewed by Ramadori *et al* (14)). Several of these proteins are also involved in coagulation or fibrinolysis. The changes during the APR are mainly mediated by the action of cytokines, such as IL6, IL1β, and TNFα.

In vitro studies in cultured human hepatoma cell line HepG2, primary Human Umbilical Vein Endothelial Cells (HUVECs), and in human microvascular endothelial cell line HMEC-1, have shown an increased PS production upon IL6 stimulation (15-17). C4BP is an established acute phase reactant with 2-3 fold elevated plasma levels during inflammation (18-21). The C4BP protein contains 6 or 7 identical α-chains and a single β-chain, although 17% of the C4BP molecules lack the β-chain (13). PS is bound to C4BP via the β-chain. Several studies have demonstrated similar or slightly increased total PS levels, but reduced free PS levels in plasma of patients during inflammation (22-24). The observed reduction in free PS levels has been explained as a direct consequence of an increase in the β-chain containing C4BP-form. Other studies, however, provide evidence for stable free PS levels during inflammation, showing only a rise in C4BP α-chain levels, but not in β-chain levels (21;25). The observed discrepancy between the various studies may be explained by a differential regulation of C4BP α- and β-chains in different subpopulations of patients during the APR (26).

The IL6 signalling pathway is well-defined (27-29). In short, IL6 is known to exert its stimulatory effect through IL6-responsive elements (IL6-RE) in the promoters of IL6-responsive genes. Two types of IL6-REs have been identified in genes encoding acute phase proteins. Type I is a binding site for CCAAT/enhancer binding protein (C/EBP) β (NF-IL6,

LAP), whereas the type II IL6-RE is bound by signal transducer and activator of transcription 3 (STAT3) (30). Whilst IL6 upregulates nuclear C/EBPβ levels through both elevated C/EBPβ gene transcription and C/EBPβ phosphorylation (29), increased nuclear STAT3 levels are mainly due to rapid STAT3 phosphorylation (28). C/EBPβ and STAT3 have been shown to affect IL6-induced transcription by binding to a.o. the promoters of the genes encoding C-reactive protein (CRP) (31;32), plasminogen (33), haptoglobin (34), and fibrinogen (35). In this report we investigate the mechanism by which IL6 upregulates PS protein levels.

Two copies of the PS gene are located on chromosome 3. The active PS gene (*PROS1*) shares 96% homology with the inactive pseudogene (*PROS2*) (36-38). Recently, constitutive expression of the *PROS1* promoter was shown to be regulated mainly by Sp1 *in vitro*, and to contain binding sites for various other transcription factors such as the hepatocyte-specific forkhead transcription factor FOXA2, which are possibly involved in tissue-specific or induced expression of the *PROS1* promoter (39). We confirmed earlier findings that PS is upregulated by IL6 in cultured HepG2 cells and complemented these with quantitative PS mRNA data thereby identifying the regulatory mechanism as pre-translational. Subsequently, we identified a region in the PS promoter spanning nucleotides 229 to 207 upstream from the translational start that binds STAT3, one of the nuclear messengers of IL6.

Experimental Procedures

Plasmids --- The generation of *PROS1* promoter reporter constructs is described in Chapter 2. *PROS1* promoter constructs were mutated at a putative STAT3 binding site by use of the QuikChange XL Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA), with forward primer 5'-CCAGCTCCGAAAAGCCGCCTGGTGCTGTCCTTGTTATCAC-3'and reverse primer 5'-GTGATAACAAGGACAGCACCAGGCGGCTTTTCGGAGCTGG-3'. Successful incorporation of the mutations was confirmed by automated sequencing (Beckman).

Cell culture --- The human hepatoblastoma cell line HepG2 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). For induction experiments, cells were washed twice with phosphate buffered saline, after

which fresh medium was added that lacked FBS but contained 0.1% human serum albumin and 5 or 100 ng /ml recombinant human IL6 (Strathmann)).

Preparation of nuclear extracts --- Nuclear extracts (NE) were prepared according to the method of Dignam *et al* (40). The final nuclear extract buffer contained 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 100 mM KCl, 0.5 mM DTT, 0.2 mM PMSF and a 1 x concentration of an EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany). Nuclear extracts were aliquoted and frozen at -80^{o}C until further use. Protein concentration of the NE was measured with the BCA assay (Pierce Biotech, Rockford, IL). Nuclear extracts were prepared from HepG2 cells cultured in the absence or presence (30 minutes or 14 hours) of 100ng/ml IL6.

Immunological detection of STAT3 and C/EBPβ --- Nuclear extracts (10 µg protein) were fractionated on a denaturing 7.5% poly-acrylamide gel and transferred onto a nitrocellulose membrane. After blocking for 1 hr in phosphate-buffered saline (PBS) containing 1% nonfat dry milk, 1% bovine serum albumin, and 0.05% Tween 20, the membrane was incubated for 1 hr at room temperature with the first antibody. C/EBPβ and p-STAT3 were detected with the polyclonal antibodies sc-150G (1:1000) and sc-7993 (1:1000), respectively (Santa Cruz Biotech, CA, USA). As secondary antibody, HRP-conjugated rabbit anti-goat IgG was used at a dilution of 1:1000. Enhanced chemoluminescence was used for detection by incubating the membrane for 1 min with freshly mixed 1.25 mM 3-aminophtalhydrazide, 0.2 mM p-coumaric acid, and 0.01% v/v H_2O_2 in 0.1M Tris pH 8.5.

Reporter gene assays --- Cells were transfected at 60-80% confluency. Each transfection was performed in triplicate in 12-wells plates. All assays were conducted with two different DNA preparations of each construct. Transfections were carried out using 3 µl Tfx-20 lipids (Promega) per µg transfected DNA. In each transfection, an equimolar concentration of construct was used, supplemented with pUC13-MCS vector to obtain a fixed amount of transfected DNA. In pUC13-MCS the MCS had been removed by digestion with *Pvu*II and religation. 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 at 24 hours (HepG2) after transfection. During induction experiments, medium was aspirated and replaced by fresh medium with or without IL6 eight hours after transfection and cell extracts were harvested 48 hrs after IL6 addition. Luciferase activity was measured according to the Dual Luciferase Assay System Protocol (Promega). Cells were lysed in 250 µl Passive Lysis Buffer/well, after which 20 µl was used to measure

luciferase activity. Activity was measured using a Lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany).

PS measurements --- Total PS antigen levels in IL6-stimulated and unstimulated culture media were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (41) with the following modifications. ELISA plates were coated with 10 μg/ml goat anti-human PS IgG (Kordia, Leiden, The Netherlands) overnight at 4°C. A second coating with 2.5% ovalbumin (Sigma-ALDRICH, St. Louis, MO) at 37°C for 1 hour was performed to reduce background absorbance. Immobilized Protein S was detected with horseradish peroxidase-conjugated rabbit anti-human IgG (Dako, Glostrup, Denmark). Absorbance at 450 nm was determined with an Organon Teknika plate reader (Turnhout, Belgium).

RNA-assays --- Total RNA was isolated from cell culture using Trizol reagent (Invitrogen) according to the manufacturers recommendations. Samples were treated with RNAse-free DNAse I (Amersham, Roosendaal, The Netherlands) after which RNA was purified with the RNeasy mini kit (Qiagen, Hilden, Germany). *PROS1* mRNA levels were determined by real-time quantitative PCR analysis (QPCR). First, 1 μg total RNA from each cell line was reverse transcribed using Superscript II reverse transcriptase and random hexamers (Invitrogen). 1/20th of the obtained cDNA was subsequently used in a QPCR reaction with primers and probes specific for *PROS1*. The forward primer was 5'-TGCTGGCGTGTCTCCTCCTA-3', the reverse primer was 5'-CAGTTCTTCGATGCATTCTCTTTCA-3', the probe was TET-5'-CTTCCC GTCTCAGAGGCAAACTTTTTGTC-3'-TAMRA. The primers and probe sequence locations and lengths were determined by using the ABI Primer Express Program (Applied Biosystems, Foster City, CA). *PROS1* QPCR reactions (Eurogentec) were performed in 0.5 ml thin-walled, optical-grade PCR tubes (Applied Biosystems) in a 50 μl final volume, by addition of the following components: 0.25 U AmpliTaq Gold DNA polymerase, 160 nM TaqMan probe, 300 nM of each primer, and 3 mM $MgCl_2$. A QPCR of the internal standard, the porphobilinogen deaminase gene (PBGD), was carried out in a similar fashion for each RNA sample with 4 mM $MgCl_2$. For this QPCR the following primers and probe were used; forward primer 5'-GGCAATGCGGCTGCAA-3', reverse primer 5'-GGGTACCCACGCGATCAC-3', and probe TET-5'-CTCATCTTTGGGCTGTTTTCTTCCGCC-3'-TAMRA. An Applied Biosystems Prism model 7700 instrument monitored the reactions. Thermal cycling conditions consisted of 10 min at 95°C followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. Determinations of cycle threshold (C_T) were performed automatically by the instrument. The results are expressed as fold transcript relative to the internal standard PBGD ($=2^{\Delta Ct}$).

Electrophoretic Mobility Shift Assays (EMSA) --- EMSAs were performed in a 13 µl binding reaction containing 10 µg NE and 195 ng denatured herring sperm DNA. EMSA buffers were purchased from Active Motif (Carlsbad, CA) and used according to the manufacturers recommendations. Double-stranded (ds) oligonucleotides were end-labeled using γ^{32}P-ATP and T4 polynucleotide kinase. The following ds oligonucleotides were used (only the sense strand is given): -229/-207wt 5'-AAAAGCTTCCTGGAAATGTCCTTG-3', -229/-207mt 5'-AAA AGCCGCCTGGTGCTGTCCTTG-3', STAT3cons 5'-GATCCTTCTGGGAATTCCTAGATC-3', C/EBPβ cons 5'-TGCAGATTGCGCAATCTGCA-3'. Reaction mixtures were incubated on ice for 20 min in the presence or absence of an unlabeled competitor. Subsequently the ^{32}P-labeled ds probe was added and the incubation was continued for another 20 min. In antibody supershift experiments, NE was incubated on ice 10 min with the ^{32}P-labeled ds probe after which an anti-STAT3 or anti-C/EBPβ antibody (sc-150x and sc-7993x, Santa Cruz Biotechnology) was added and the incubation was allowed to continue for another 10 min. Samples were loaded on a 5% non-denaturing polyacrylamide gel, which was electrophoresed for 2 hrs at 200V, after which gels were vacuum-dried and exposed to X-ray film.

Results

IL6 induces PROS1 mRNA and PS protein levels in HepG2 --- PS circulates in normal plasma at a concentration of about 0.33 µM (13). Hepatocytes are the largest contributors to the systemic levels of PS (10) therefore, hepatoma cell line HepG2 was used as a model system. PS mRNA and protein levels under basal culture conditions were compared with those after induction with 5 or 100 ng IL6 per ml culture medium (Figure 1). A dose-dependent increase in PS mRNA and protein levels in HepG2 by IL6 became detectable after 16 hrs. Total fibrinogen antigen levels were measured as a positive control. Fibrinogen is an established acute phase reactant (42-44), and its levels increased from 3.5 nM to 8.6 or 16.6 nM after induction with 5 or 100 ng/ml IL6 for 24 hours, respectively.

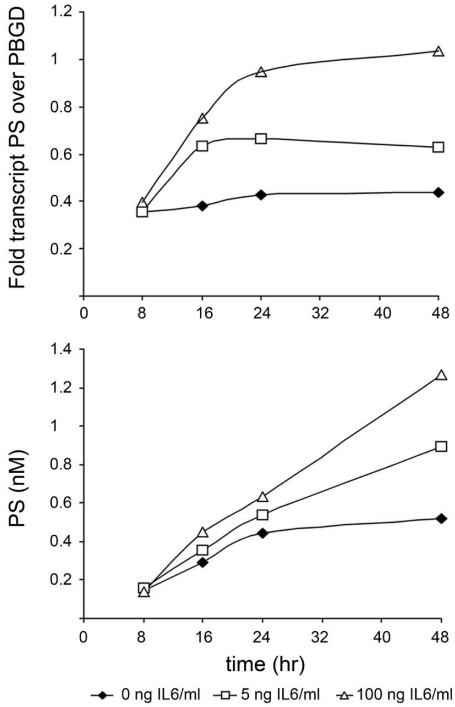

Figure 1 Induction of PS mRNA and protein expression by IL6. In the top panel, the expression of *PROS1*-derived mRNA in HepG2 cells is shown relative to that of the housekeeping gene PBGD, encoding porphobilinogen deaminase. The incubation time (x-axis) is plotted against the *PROS1:PBGD* transcript ratio (y-axis). The ratio of *PROS1* over *PBGD* transcripts was determined by real-time quantitative PCR (qPCR). In the lower panel, the production of PS over time is shown. The PS concentration in the culture medium was determined by ELISA. All values represent the average of at least 3 pooled independent experiments.

Phosphorylation of STAT3 is induced and maintained by IL6 in HepG2 cells --- The signal transduction pathway of IL6 signal has two main cellular targets: the transcription factors STAT3 and C/EBPβ that mediate most of the IL6-induced alterations in gene expression. Strongly increased nuclear STAT3 levels stimulate transcription of target genes and are due to rapid STAT3 phosphorylation by janus kinases (JAKS) that are stimulated by binding of IL6 to its cognate receptor (28). As a consequence, induction of gene transcription by binding of phosphorylated STAT3, which leads to dimerization, occurs rapidly after IL6 signalling. On

the other hand, increased C/EBPβ activity is achieved by increased transcription of the structural gene as well as by phosphorylation of the protein (29), hence increased *trans*-activation of gene promoters by C/EBPβ generally occurs later in time and lasts longer (45). Therefore we tested nuclear extracts from HepG2 cells, which had been stimulated with IL6, for the presence of both C/EBPβ and phosphorylated STAT3 (Figure 2). Whereas C/EBPβ levels remained similar, phosphorylated STAT3 levels were increased at both time points tested, 30 minutes and 14 hours. The presence of strong STAT3 phosphorylation after 14 hrs incubation with IL6 is in concordance with the sustained *PROS1* mRNA induction.

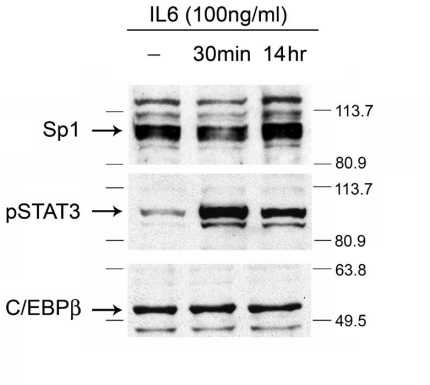

Figure 2 Effect of IL6 treatment on several transcription factors. The effect of IL6 treatment on the transcription factors Sp1 (as an unchanged loading control), STAT3 and C/EBPβ is shown using Western blot analysis. Antibodies specific for Sp1, C/EBPβ and the phosphorylated form of STAT3 were used.

An IL6 responsive element is present in the PROS1 promoter --- Subconfluent HepG2 cells were transfected with promoter-reporter gene plasmids containing a *PROS1* 5' region of varying length, after which the cells were either induced with IL6, or grown further under basal conditions (Figure 3a). A *PROS1* promoter region of 261 bp remained IL6-responsive. However, transfections with the shortest promoter-reporter gene construct, PS197, did not result in increased luciferase levels upon IL6 induction. These results indicate that an IL6-responsive element within the *PROS1* promoter is located between 261 and 197 bp upstream from the translational startcodon. The stimulatory effect of IL6 on PS261 was not augmented with increasing promoter size.

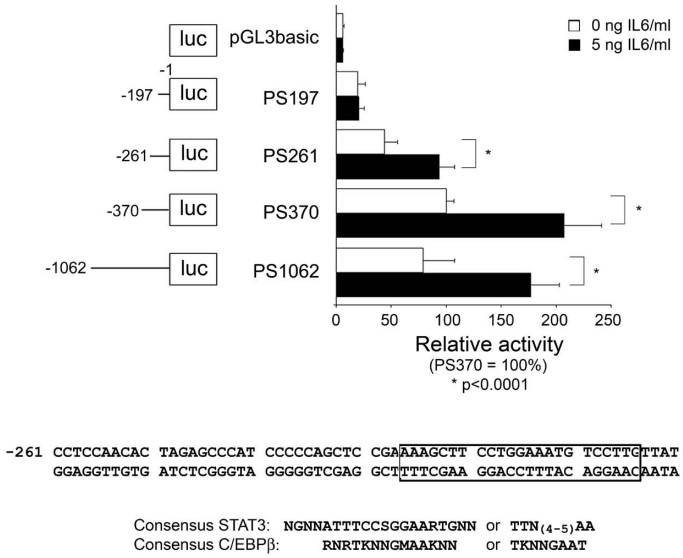

Figure 3 Localization of the IL6-responsive element in the *PROS1* promoter. (**A**) Histogram showing *PROS1* promoter activity, as assessed using luciferase constructs, in the absence or presence of a physiological concentration of IL6 (5 ng/ml). A schematic representation of each construct is shown on the left of the histogram. Statistical significance was determined with the Student's T-test. (**B**) Sequence of the *PROS1* promoter between -261 and -197 containing the IL6 responsive element. The ds oligonucleotide used in the EMSA assays is indicated.

The IL6 response is mediated by binding of STAT3 to the IL6-RE in the PROS1 promoter --- Computational analysis using various databases on the internet such as MatInspector professional (http://www.genomatix.com), Transfac (http://www.gene-regulation.com), and tfsitescan (http://www.ifti.org) showed that both a STAT3 and a C/EBPβ binding consensus are located in the -261 to -197 bp region in the *PROS1* promoter (Figure 3b). Increased binding of a protein complex to ds oligonucleotide probe -229/-207wt, was observed in extracts from IL6-treated HepG2 cells (Figure 4, left panel). The majority of this protein-DNA complex was shifted with a STAT3 antibody, leaving a weak signal at the original location. The mutated unlabeled ds oligonucleotide, -229/-207mt, was unable to compete with the PS wildtype probe for protein binding, whereas unlabeled ds oligonucleotides -229/-207wt and STAT3cons containing the wild type and STAT3 consensus sequence were efficient competitors (Figure 4). The C/EBPβ consensus oligo did not influence protein binding to the -229/-207 oligo probe. However, a slight shift from the original complex in nuclear extracts from untreated HepG2 cells was observed with a C/EBPβ antibody, suggesting that the

residual band present in the STAT3 supershift might contain comigrating C/EBPβ-probe complexes (Figure 4, right panel).

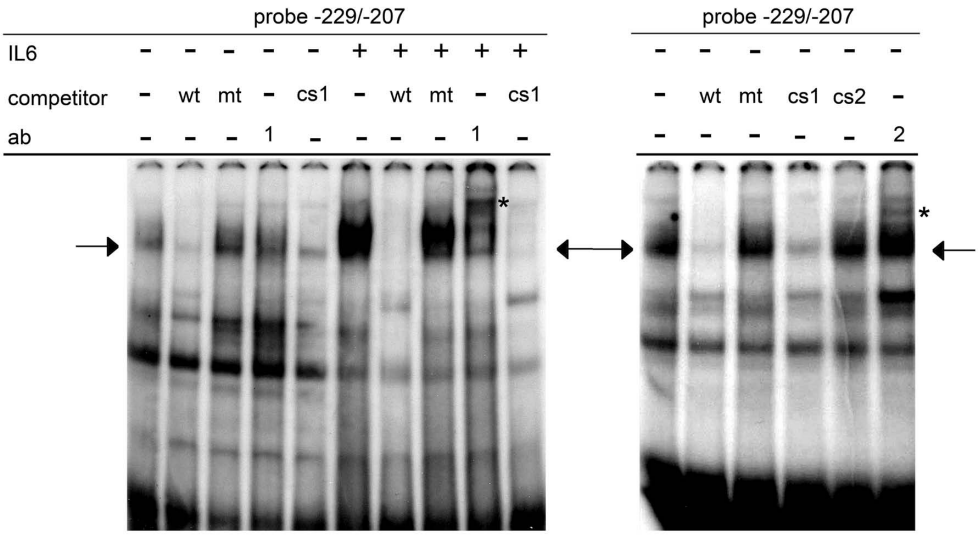

Figure 4 EMSA of the IL6-responsive element in the *PROS1* promoter. EMSA (Electrophoretic Mobility Shift Assay) using a ds oligonucleotide representing the IL6-responsive element and using nuclear extracts from HepG2 cells treated with or without 100 ng/ml IL6. The location of the IL6-specific band is indicated by an arrow. Non-labeled ds competitor oligonucleotides were used at 100 fold molar excess. wt = wild type, mt = mutant, cs1 is a STAT3 consensus oligonucleotide, cs2 is a C/EBPβ consensus oligonucleotide (see the experimental section for the sequences of the oligonucleotides). Antibody 1 was a p-STAT3-specific antibody and antibody 2 was a C/EBPβ-specific antibody.

The expression of mutated promoter-reporter gene constructs in HepG2 cell culture was analysed to investigate the contribution of STAT3 and possibly C/EBPβ to transcriptional upregulation of PS levels during IL6 induction. Although basal promoter activity remained unaltered upon mutation of the -229/-207 region, the mutations resulted in abolishment of the IL6 response in all promoter-reporter gene constructs (Figure 5). This underlines our preliminary conclusion from the data obtained with the wild type *PROS1*-reporter gene constructs that the site at -229/-207 is the only IL6-RE in the proximal *PROS1* promoter.

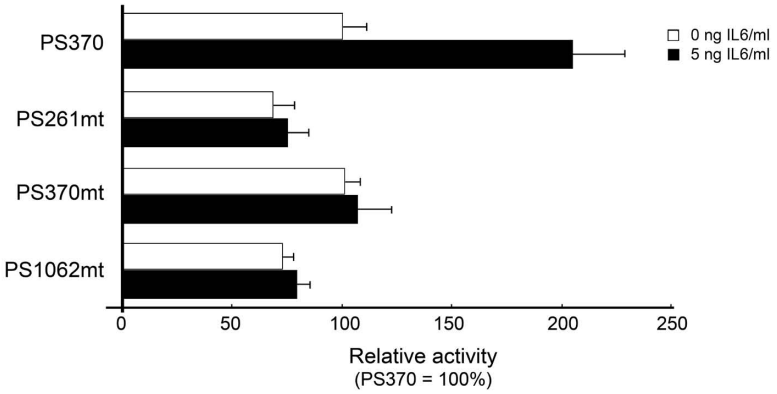

Figure 5 Activity of the *PROS1* promoter after mutagenesis of the IL6-responsive element. Histogram showing the luciferase activity of the wild type –370 construct and of three constructs with a mutated IL6-responsive element in the presence of a physiological concentration IL6 (5 ng/ml).

Discussion

Plasma proteins that are upregulated during the APR can be roughly divided into three major groups: proteins with an increased level of about 50% (e.g. ceruloplasmin and complement factor-3), those with an increase of two-three fold (haptoglobin, fibrinogen, C4BP), and a group of proteins which responds with a rapid increase of up to 5-fold to 1000-fold (CRP and serum amyloid A (SAA)) (18;46-49). We found a 2-fold increase in PS levels in medium after 48 hours incubation of HepG2 cell culture with 5 ng IL6 per ml medium (Figure 1), which is in agreement with a previous report (15). Both *PROS1* mRNA and PS protein levels were elevated up to 48 hours of IL6 stimulation. Protein S thus seems to belong to the second category of acute phase proteins.

That not only PS protein levels but also *PROS1* mRNA levels were upregulated by IL6 indicated that the mechanism by which IL6 upregulates PS levels was at the transcriptional level. Since IL6 induction is known to be mediated primarily by STAT3 and C/EBPβ, we tested the levels of these two transcription factors in IL6-induced nuclear extracts and found that total C/EBPβ levels remained unchanged throughout time, whereas phosphorylated STAT3 levels were increased, also after prolonged IL6 stimulation (Figure 2). This result is in

concordance with the sustained higher PS levels in IL6-treated versus untreated HepG2 cell culture.

In this study we identified an IL6-RE in the *PROS1* promoter at a region approximately 220 bp upstream from the *PROS1* translational startcodon. This region contains both the hallmarks of the STAT3 and the C/EBPβ binding sites (Figure 3). We identified STAT3 as the main transcription factor binding to the IL6-RE (Figure 4). C/EBPβ may also bind to this region, but its contribution appears to be relatively minor. Mutation of the STAT3 binding site in the *PROS1* promoter completely abolished the IL6 response in transfection experiments with promoter-reporter gene constructs (Figure 5). This indicates that there are no additional functional STAT3 or C/EBPβ binding sites in the region investigated through which the IL6 effect is mediated. The putative overlap between the STAT3 and the C/EBPβ binding sites around position –220 makes it difficult to make definitive statements about the role of the latter protein in the induction of *PROS1* transcription in HepG2 cells. Overlapping binding sites might constitute an efficient way to create an IL6-responsive element that is active both at early and late time points of IL6 stimulation.

Although *PROS1* is clearly IL6-responsive in HepG2 cell culture, patient studies indicate that total systemic PS levels are only slightly increased or remain at a similar level during inflammation. Moreover, in a subpopulation of patients free PS levels are reduced during inflammation due to increased C4BP β-chain levels (22). Also in patients with sepsis, free PS levels (measured as PS activity) were decreased (50). In both circumstances, inflammation or sepsis, IL6 is a major player and its induction of PS transcription does apparently not outweigh the above-mentioned *in vivo* effects. It could be argued that the effect on PS anti-coagulant activity would even be worse in the absence of the mild induction of *PROS1* transcription by IL6. Nevertheless, the possibility that the main function of *PROS1* transcriptional regulation by IL6 may lie in another aspect of the inflammatory response should also be considered.

IL6 has been implicated in a variety of cellular processes with a diverse array of regulatory roles. Dependent on the target cell, IL6 may induce various and sometimes contrasting responses. For instance, IL6 has a pro-inflammatory function and it can induce cytotoxic

effects, but it is also a cell survival factor. Several studies have demonstrated neuroprotective effects of IL6, which are mediated by the stimulation of growth factors and STAT3 (51-55). Recently, Liu *et al* reported a neuroprotective effect of intravenously injected PS on cortical neurons during ischemic injury in mice (56). The authors speculated that PS may be working through a similar mechanism as its structural homologue, growth arrest-specific gene 6 (GAS6), which induces cell survival through a signalling pathway mediated by the Tyro3/Axl membrane receptor family. Stimulation of local PS synthesis by IL6 may therefore explain part of the cell survival properties reported for IL6. In addition, increasing evidence suggests a function of PS in macrophage phagocytosis of apoptotic cells both directly (57;58) and indirectly by directing complement to the surface of apoptotic cells (59;60). Exactly what this new role for PS entails has not yet been fully clarified. Based on the cited literature, it seems plausible that the upregulation by IL6 of local and not systemic PS levels may contribute to processes such as the cell survival of cells in the inflamed region. Obviously, this hypothesis needs to be tested in future research.

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Chapter 6

Alternative splicing of Protein S pre-mRNA

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Submitted

Summary

For the first time we report the presence of a Protein S (PS) transcript resulting from alternative splicing of PS pre-mRNA. The splice variant contains an additional 96 base pairs, which maintain the reading frame and originate from an additional exon between exons 1 and 2. The transcript predicts a protein (PSalt) with an additional stretch of 32 amino acids between the pre- and propeptide containing four cysteine residues. The alternatively spliced PS transcript was found in human liver and various cell lines, such as hepatoma cell lines HepG2 and HuH7, megakaryocytic cell line Meg01, and human umbilical vein endothelial cells (HUVEC), and was shown to be relatively abundant. The ratio of wild type transcript versus the alternatively spliced variant was approximately 4 to 1 in human liver. Studies with Protein S expression vectors in COS-1 cells showed that the alternative transcript results in a PS protein, which is retained within these cells. Western blot analysis of cell lysates indicated that this protein S mostly forms high molecular weight aggregates within the cell that can be partly resolved by treatment with a reducing agent. However, in non-transfected HepG2 cells that contain the endogenous PSalt transcript, the high molecular weight species was not detected. The implications of these findings for the regulation of Protein S expression are discussed.

Introduction

Protein S (PS), a vitamin K-dependent plasma glycoprotein, functions as a co-factor for activated protein C (APC) in the proteolytic inactivation of coagulant factors VIIIa and Va by APC (1-4). PS also exhibits direct APC-independent anticoagulant activity by inhibition of the prothrombinase and tenase complexes (5-8). The primary source of circulating plasma PS is the hepatocyte (9), although PS is produced constitutively at low levels by a variety of cell types throughout the body (10-17). In human plasma, PS circulates in a free form (40%) and in complex with C4b-binding protein (C4BP) at a concentration of approximately 0.35 μM (18-20).

Partial PS deficiency was first reported to be associated with venous thrombotic disease in 1984 (21). The disorder inherits as an autosomal dominant trait with incomplete penetrance (22). PS deficiency is classified in three types (23). Type I deficiency corresponds to low levels of both free and total PS, type II PS deficiency is characterized by normal total and free PS levels, but reduced PS activity, and type III is defined by normal total PS levels, but low free PS levels and activity. Many abnormalities in the protein S gene (*PROS1*) have been described underlying hereditary PS deficiency (reviewed in (24;25)). PS deficiency can also be acquired, in which case it may be transient. Several non-genetic and environmental factors such as aging (26) and hormonal status (contraceptive use) influence PS levels (27).

Protein S is synthesized as a single-chain preproprotein of 676 amino acids (aa). Pre- and propeptide are consecutively removed from the aminoterminus of the preproprotein through proteolytic processing, before secretion of the 635 aa long mature protein from the cell. The signal peptide is first cleaved off by a signal peptidase during translocation of the nascent preproprotein to the endoplasmic reticulum. The propeptide is necessary for γ-carboxylation of the N-terminal vitamin K-dependent Gla-domain which is rich in carboxy-glutamic acid residues (28;29). In the trans-Golgi the propeptide is cleaved from the mature protein by a propeptidase or processing protease after completion of this process. The Gla-domain is followed by a thrombin-sensitive region, which is connected to four epidermal growth factor (EGF)-like domains. Whereas the C-terminal domain of other vitamin K-dependent plasma proteins consists of a serine protease domain, the C-terminal domain of PS has two laminin G-type repeats which share significant homology with sex hormone-binding globulin (30).

The PS genetic locus is located near the centromere on chromosome 3. It consists of an active PS gene (*PROS1*) and an inactive pseudogene (*PROS2*), which share 96% homology in their coding sequence. The promoter and the first exon are absent from *PROS2*, rendering it inactive (31-33).

We describe a protein S splice variant which was found in mRNA isolated from a human liver sample, hepatoma cell lines HepG2 and HuH7, megakaryocytic cell line Meg01, and human umbilical vein endothelial cells (HUVECs). The splice variant contains an additional 96 nucleotides, resulting in a PS protein with 32 aa extra located between the pre- and propeptide. Cloning of this alternative PS transcript in a eukaryotic expression vector and subsequent expression thereof in COS-1 cells resulted in a PS protein (PSalt), which was not secreted, but instead accumulated within the cell, mainly in high molecular weight aggregates.

Experimental procedures

Plasmids --- The PS wild type in expression plasmid pZEM229R (pZEM229R-PSwt) was described previously (34). The alternative PS splice variant was cloned in pZEM229R (pZEM229R-PSalt) as follows. First two overlapping PCR products were generated with one splice variant-specific primer and one wild type-specific primer. In the first PCR, primer PSwt(-14/+5), 5'-TCCGCGCCTTCGAAATGA G-3', which is located in the first exon was used in combination with alternative transcript-specific reverse primer PSalt(+171/+145) 5'-GCATAT AGAATAGAAAAAATTGCATCC-3'. The second PCR was performed with forward primer PSalt (+99/+120) 5'-TTTTATATACAACCGTGCATGC-3' and reverse primer PSwt (+1123/+1103) 5'-CCTCCAGTTGTGATTTTGGA-3'. The overlapping products were combined in a third PCR in which only the outermost primers were used, thereby generating a 1233 bp final product. This PCR product was digested with restriction enzymes BstBI and BlpI (New England Biolabs, Beverly, MA). The 858 bp PSalt BstBI-BlpI fragment was cloned into the pZEM229R-PSwt vector that had previously been linearized with BstBI and BlpI. The resulting pZEM229R-PSalt was sequenced and checked for errors through automated sequencing on the Beckman CEQ2000 system (Beckman Coulter, Fullerton, CA).

Human Liver Sample, Leukocytes and Monocytes --- A human liver sample was obtained from a deceased healthy donor and was a kind gift from E. de Wit at TNO Quality of Life, Leiden

(TNO). Leukocytes and monocytes were isolated from blood from healthy donors as previously described (35).

Cell culture --- The *Cercopithecus aethiops* (African green monkey) kidney cell line, COS-1, and human hepatoma cell line, HepG2, were purchased from the American Type Culture Collection (ATCC, Manassas, VA). COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM), HepG2 cells were grown in Minimal Essential Medium (MEM). Culture media for both cell lines were supplemented with 10% Fetal Bovine Serum (FBS), 100 μg/ml penicillin, and 100 μg/ml streptomycin (all cell culture reagents were purchased from Invitrogen, Carlsbad, CA).

PS measurements --- Total PS antigen levels in culture media were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (36) with the following modifications. ELISA plates were coated with 10 μg/ml goat anti-human PS IgG (Kordia, Leiden, The Netherlands) overnight at 4°C. A second coating with 2.5% ovalbumin (Sigma-ALDRICH, St. Louis, MO) at 37°C for 1 hour was performed to reduce background absorbance. Immobilized Protein S was detected with horseradish peroxidase-conjugated rabbit anti-human IgG (Dako, Glostrup, Denmark). Absorbance at 450 nm was determined with an Organon Teknika plate reader (Turnhout, Belgium).

RNA-assays --- Total RNA was isolated from cell culture and human liver using Trizol reagent (Invitrogen) according to the manufacturers recommendations. Samples were treated with RNAse-free DNAse I (Amersham, Roosendaal, The Netherlands) after which RNA was purified with the RNeasy mini kit (Qiagen, Hilden, Germany). PS transcript levels were determined by real-time quantitative PCR analysis (QPCR). First, 1 μg total RNA from each cell line was reverse transcribed using Superscript II reverse transcriptase and random hexamers (Invitrogen). 1/20th of the obtained cDNA was subsequently used in a QPCR reaction with probes specific for the wild type or alternative PS and a single primer set. The PS (+32/+51) forward primer was 5'-TGCTGGCGTGTCTCCTCCTA-3', the PS (+186/+162) reverse primer was 5'-CAGTTCTTCGATGCATTCTCTTTCA-3', the PSwt probe was TET-5'-CCT GTTGCTTGACAAAAGTTTGCCTCTGA-3'-TAMRA, the PSalt probe was TET-5'-AAGCCGACC AATACAGAAATGCATGCCA-3'-TAMRA. The optimal primer and probe sequences were determined using the ABI Primer Express Program (Applied Biosystems, Foster City, CA). *PROS1* QPCR reactions (Eurogentec, Seraing, Belgium) were performed in 0.5 ml thin-walled, optical-grade PCR tubes (Applied Biosystems) in a 50 μl final volume, by addition of the following components: 0.25 U AmpliTaq Gold DNA polymerase, 160 nM TaqMan probe,

300 nM of each primer, and 3 mM $MgCl_2$. A QPCR of the internal mRNA standard, the porphobilinogen deaminase gene (PBGD), was carried out in a similar fashion for each RNA sample using 4 mM $MgCl_2$. For this QPCR the following primers and probe were used; forward primer 5'-GGCAATGCGGCTGCAA-3', reverse primer 5'-GGGTACCCACGCGATCAC-3', and probe TET-5'-CTCATCTTTGGGCTGTTTTCTTCCGCC-3'-TAMRA. An Applied Biosystems Prism model 7700 sequence detection instrument monitored the reactions. Thermal cycling conditions consisted of 10 min at 95°C followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. Determinations of cycle threshold (C_T) were performed automatically by the instrument. The results are expressed as fold transcript relative to the internal standard PBGD ($=2^{\Delta Ct}$). PS transcript levels were also determined by densitometric measurement of PCR product using the same PCR conditions and primers as described above. The PCR was allowed to continue for 30, 33 or 36 cycles. The ratio of the alternative versus the wildtype PS transcripts was determined with the Syngene densitometric tools (Syngene, Cambridge, UK).

Transient transfection --- For PS production purposes, COS-1 cells were cultured in 75 cm^2 flasks. At 70% confluency cells were transfected with 7.2 pmol pZEM229R-PSwt or pZEM229R-PSalt and 100 µl transfection reagent Tfx-20 (Promega). Mock transfections contained 7.2 pmol pcDNA3 (Invitrogen) and 76 µl Tfx-20. Eight hours after transfection, cells were washed with phosphate buffered saline (PBS), after which cells were overlayed with 10 ml Opti-MEM Glutamax production medium (Invitrogen) containing 10 mg/l vitamin K (Konakion, Roche), 100 µg/ml penicillin, and 100 µg/ml streptomycin. Conditioned medium and cell lysates were harvested after a 10 hour incubation period. Three ml lysate buffer consisting of 2 mM $CaCl_2$, 50 mM Tris/HCl (pH 7.6), 0.5% Nonidet P40, 20% glycerol, 0.5 mM phenyl methyl sulfonyl fluoride (PMSF) and 4 x complete EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany), was used per 75 cm^2 flask. For immunofluorescence purposes, cells were grown to 70% confluency in 6 well plates, in which 18 mm glass dishes had been placed. Transfection conditions were identical as described above, but scaled down for transfections in 6 well plates. Eight hours after transfection cells were washed and medium was replaced with Opti-MEM. Cells were allowed to grow for another 5, 10, 15 or 20 hours. After the designated incubation time conditioned medium was aspirated and cells were washed twice with PBS.

In situ *immunofluorescent detection of PS* --- Cells were fixed *in situ* with 3% formaldehyde during a 30 min incubation period at 4°C. To allow penetration of antibodies into the cell, cells were permeabilized with 0.1% Triton in PBS. The glass dishes, which were overgrown

with cells, were then incubated at room temperature for 30 min with 50 µl 10 µg/ml primary antibody (goat-anti-human PS IgG (Kordia, Leiden, The Netherlands)). 50 µl 0.4 µg/ml FITC-conjugated secondary antibody (donkey-anti-goat IgG FITC (Santa Cruz)) was added after an additional three PBS washes, and reactions were incubated for 30 min in the dark at room temperature. The glass dish was put on a microscopic slide and coverslips were mounted over the sections with 2.5% 1.4-diazabicyclo-(2,2,2)-octane (DABCO).

Western Blot analysis --- Polyacrylamide gel electrophoresis was performed on 7.5% SDS/PAGE under reducing and non-reducing conditions according to Laemmli (37). Protein transfer to PVDF membranes (Millipore Corporation, Bedford, MA) was carried out overnight through a wet blot system (Bio-Rad, Hercules, CA). Goat-anti-human PS IgG (Kordia) was used to detect PS protein. Horseradish peroxidase (HRP)-conjugated rabbit-anti-human PS IgG (Dako, Glostrup, Denmark) in combination with a chemiluminescent peroxidase blotting substrate (SuperSignal West, Pierce, Rockford, IL) was used to visualize the immobilized antibodies.

Results

A novel transcript is produced from the PROS1 gene --- For the purpose of measuring PS protein and transcript levels in cell culture medium and RNA isolates, we used a sensitive PS ELISA and real-time quantitative PCR (QPCR), respectively. During optimisation of the real-time PCR assay on human liver cDNA with primers located in *PROS1* exon 1 and 2, the presence of a second PCR product was noticed; the expected 154 bp wild type PS (PSwt) product and an additional product of approximately 250 bp. Purification and sequence analysis of the unexpected product revealed that it was PS-specific and that the additional 96 base pairs originated from intron A (Figure 1A). The sequence was compared with the human expressed sequence tag (EST) database at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). From a total of 32 PS-specific ESTs covering this region, three entries contained (part of) the inserted sequence. The novel exon is located approximately 1.3 kb upstream from exon 2 and is flanked by consensus splice sites (Figure 1B).

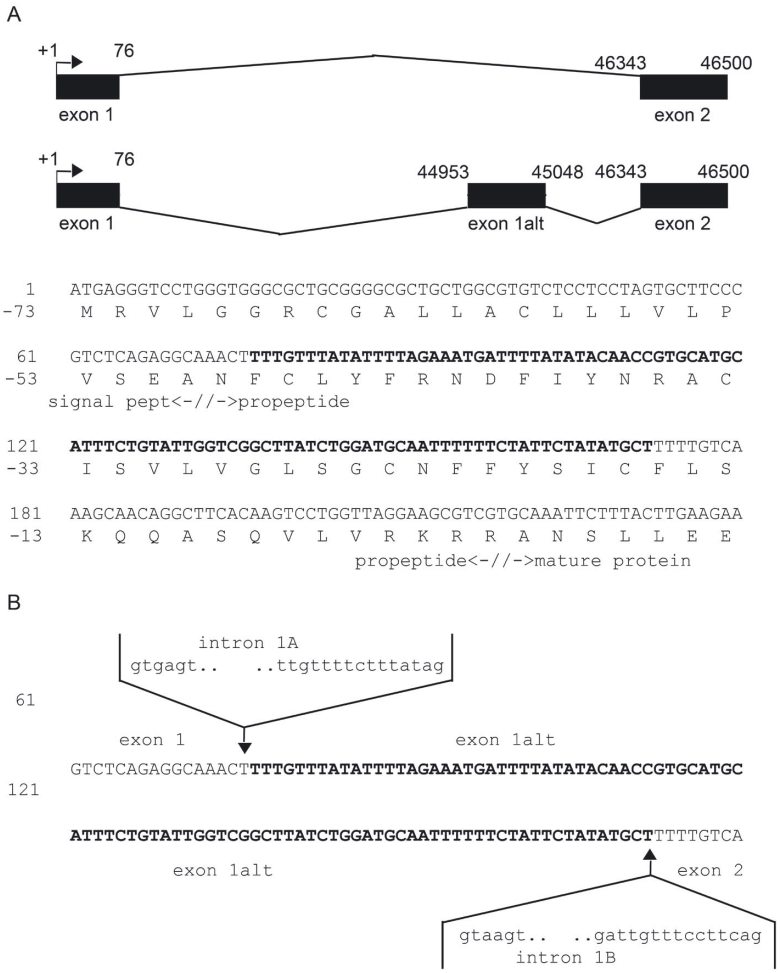

Figure 1 Location and sequence of the alternative exon in *PROS1.* (**A**) Schematic representation of the splice pattern of the standard and alternative transcript in the region of exons 1 and 2. The exons and introns are not shown to scale, the sequence coordinates are based on GenBank accession number AC117474. The sequence shows the relevant part of the alternative transcript. The nucleotide sequence of the alternative exon is shown in bold and is underlined. The putative junctions between signal peptide and propeptide and between propeptide and the mature protein have been indicated below the amino acid sequence. See the main text for a discussion on propeptide processing. Nucleotide numbering starts at the A of the startcodon, amino acid numbering starts at the first amino acid of the mature standard protein. (**B**) Partial cDNA sequence of the PSalt transcript showing the position of the introns and their splice consensus sequences.

PS splice variant PSalt is present in various cell lines and human liver --- cDNA from human liver and human hepatoma cell lines HuH7 and HepG2, megakaryocytic cell line Meg01, cervical carcinoma cell line HeLa, Human Umbilical Vein Endothelial Cells (HUVECs), and freshly isolated human leukocytes and monocytes was assayed for the presence of PSalt-encoding mRNAs (Figure 2). PS transcripts were found in all cell lines and human liver. Unexpectedly, low levels of the PSwt product were also found in reactions containing leukocytic and monocytic cDNA. However, this product did not appear until 33 or more PCR cycles had been performed. Therefore, we regard the potential of these cell types to produce PS mRNA as negligible. Quantitative analysis of transcript ratios through real-time QPCR and densitometric measurement of PCR products revealed that PSalt-specific transcripts represented approximately 20% of the total PS transcripts in the human liver sample (Table 1). Lowest PSalt transcript levels were found in cDNA isolated from HUVEC cells (0.3-5.7%). Overall, the alternative transcript represented a significant fraction of the total PS-specific mRNA pool in most cell types expressing PS.

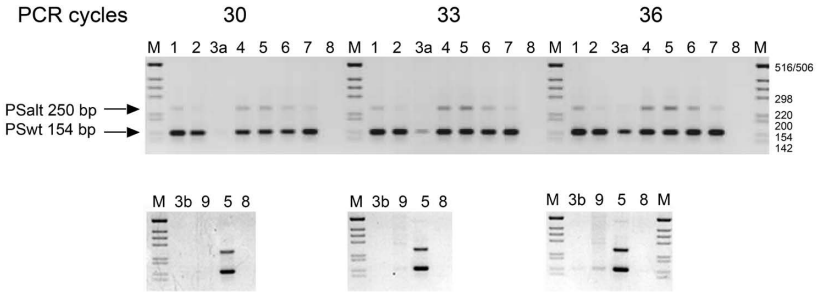

Figure 2 RT-PCR analysis of the standard and alternative transcripts in various cell types. First strand cDNA synthesis was primed using random hexamers and part of the product was amplified for a varying number of cycles using Protein S-specific primers located in exons 1 and 2. The products were separated on agarose gel. The lower panels represent a separate experiment. 1; HuH7. 2; HUVEC. 3a; PBMC leukocytes donor 1. 4; HeLa. 5; human liver. 6; Meg01. 7; HepG2. 8; blank. 3b; PBMC leukocytes donor 2. 9; 95% pure monocytes donor 3.

Recombinant PSalt protein expression --- The additional sequence in the PS splice variant was determined to be in frame with the rest of the PSwt sequence and encoded an additional 32 amino acids. This stretch of amino acids is located between the pre- and propeptide, just downstream of the predicted signal peptide cleavage site between the Ala and Asn-residues at

		% PSwt or PSalt transcript per cell type					
Technique	product	**HuH7**	**HUVEC**	**HeLa**	**Liver**	**Meg01**	**HepG2**
QPCR	PSwt	90.8	99.7	73.0	72.9	81.0	93.8
	PSalt	9.2	0.3	27.0	27.1	19.0	6.2
Densitometry	PSwt	91.3	94.3	86.2	84.8	89.8	93.9
	PSalt	8.7	5.7	13.8	15.2	10.2	6.1

Table 1 PS transcript composition per cell type. Transcript ratios were determined with two different methods; quantitative real-time PCR (QPCR) and densitometric analysis of PCR endproduct. In densitometric analysis a correction for product size was applied.

positions -50 and -49, respectively, and contains four cysteine residues (Figure 1A). This raised several questions regarding the intracellular processing of PSalt. A full-length PSalt-encoding cDNA was therefore cloned into a eukaryotic expression vector and protein expression of PSalt and Pswt was studied in COS-1 cells. COS-1 cells only produce very low levels of endogenous PS and readily produce high levels of protein from transfected expression vectors. PS levels were measured in conditioned media and lysates from COS-1 cells, which had been transfected with either pZEM229R-PSwt or pZEM229R-PSalt (Table 2). *In situ* immunofluorescent detection of PS confirmed the presence of PS protein in cells that had been transfected with either construct (Figure 3). After a 5 hour transfection with PS constructs, PSalt appeared to accumulate to a higher level within cells than PSwt. However, after a 10 hour transfection period the observed difference in fluorescence intensity between cells transfected with PSwt and PSalt had disappeared.

Incubation time (hr)	PS in medium (nM)			PS in lysate (nM)		
	-/-	PSwt	PSalt	-/-	PSwt	PSalt
10	0.028	1.037	0.030	0.076	0.222	0.423
20	0.045	1.612	0.055	0.089	0.264	0.573
30	0.048	1.865	0.066	0.103	0.232	0.460

Table 2 PS levels in COS-1 conditioned medium and cell lysate. Cells were grown in 75 cm^2 flasks. At 70% confluency cells were transfected with 7200 fmol pcDNA3 (-/-), pZEM229R-PSwt (PSwt), or pZEM229R-PSalt (PSalt), and then cultured in 10 ml Opti-MEM production medium for the designated incubation time.

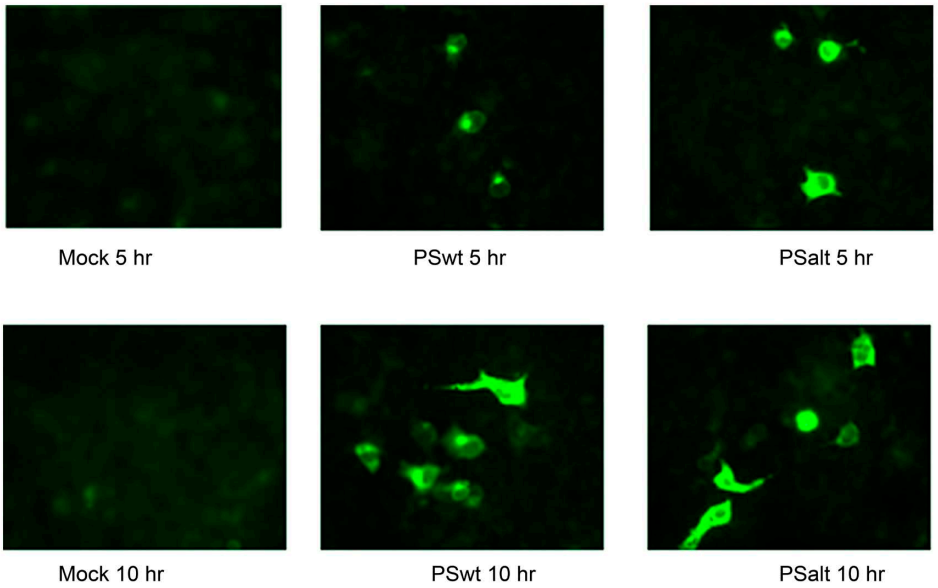

Figure 3 Immunofluorescent labeling of PS antigen in transfected COS-1 cells *in situ*. COS-1 cells were transfected with expression constructs expressing either the standard Protein S, PSwt, or the alternative Protein S, PSalt. Cells were grown on glass coverslips, fixed, permeabilized and stained with a PS-specific antibody 5 and 10 hrs after transfection.

PS-immunoreactivity was not present in medium from COS-1 cells that had been transfected with pZEM229R-PSalt, whereas it was present in medium from COS-1 cells transfected with pZEM229R-PSwt (Figure 4a). PS antigen was detected in lysates from COS-1 cells transfected with either the wild type or alternative construct. The PS protein in lysates from pZEM229R-PSalt transfections had a much higher propensity to form high molecular weight complexes. Some of the PSalt in these complexes could be reduced to the approximate size of the monomeric protein by addition of DTT, presumably by reduction of intermolecular disulfide bonds present in the cysteine-rich extra segment in the alternative protein (Figure 4b). The apparent molecular weight of the liberated PSalt appeared to be slightly greater than that of PS in lysates of cells transfected with PSwt, as is visible in the immunoblot analysis in Figure 4b. The larger molecular weight of PSalt, in combination with its propensity to form aggregates, might indicate that the cysteine-rich domain is still present at the aminoterminus of the protein, although another difference in post-translational

processing could also cause the observed migratory shift. Unfortunately we do not know whether the PS identified in the cell lysates represents fully processed PS or an intermediate form of PS, which still contains the propeptide. If the cysteine-rich domain is still present in the intracellular PSalt, the propeptide which is C-terminal from the PSalt-specific sequence, should also still be present in this protein. Interestingly, the high molecular weight complexes were not detected in non-transfected HepG2 cells, that also express the PSalt-specific mRNA (Figure 4a).

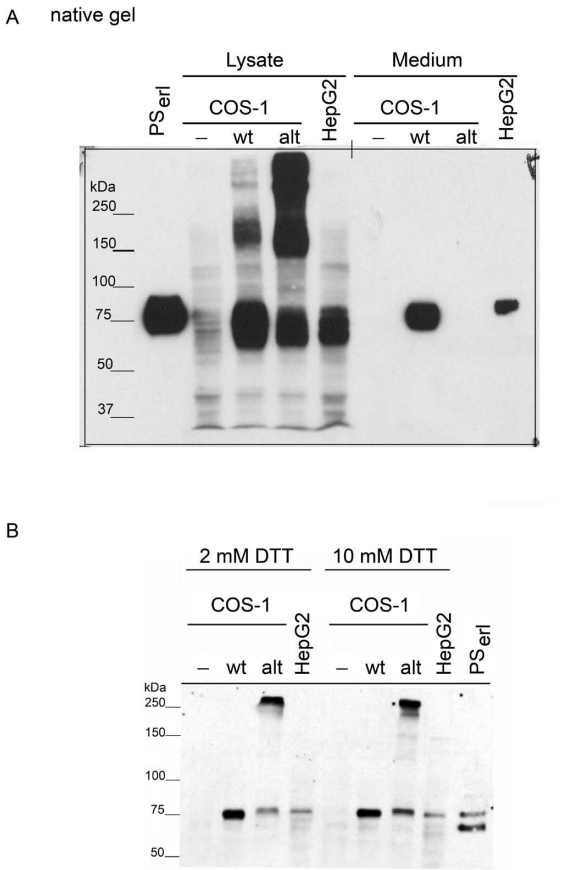

Figure 4 Western blot analysis of wild type and alternative Protein S. (**A**) shows a blot with denatured, but non-reduced proteins. Note the intense staining of the high molecular wight portion of the blot in the lane with PSalt and the absence of any staining in the lane with the growth medium of Psalt-transfected cells. PSerl represents purified Protein S from Enzyme Research Laboratories. HepG2 indicates lysate and medium from human HepG2 cells that show endogenous expression of Protein S and PSalt-encoding mRNAs. (**B**) shows a similar blot with reduced proteins. Note the somewhat higher molecular weight of the free PSalt compared to PSwt.

Discussion

We detected an alternatively spliced mRNA encoding a variant of the anti-coagulant Protein S in human liver and various cell lines. The Protein S splice variant, PSalt, contains an additional in-frame exon between the first two exons which encode the PS signal peptide (exon 1) and the propeptide and the Gla-domain (exon 2). Our results demonstrate that the additional 32 aa have a major impact on the maturation of the PSalt protein. The PSalt mRNA comprises a significant fraction of the total PS mRNA in most cell types that were tested and, most significantly, in human liver. Human liver mRNA had the highest level of PSalt transcript, whereas mRNA isolated from HUVEC cells had the lowest PSalt content. The difference between PSalt transcript levels in hepatocytic cell lines, HuH7 and HepG2, and those found in human liver may occur through various processes. Firstly, differences in growth conditions between cell culture and the *in vivo* liver may contribute to differences in levels of mRNA splice variants. Secondly, whereas mRNA from HuH7 and HepG2 culture contains only transcripts from a single origin, a human liver sample contains not only RNA from hepatocytes, but also from other cell types. Cell type-specific PS pre-mRNA splicing may be influenced by differences in composition of the spliceosome and splicing regulators, thereby resulting in different levels of mRNA splice variants (38;39).

Previously, we identified a transcription start site (TSS) in HUVECs, which is not employed for transcriptional initiation in other cell types (40). It is tempting to speculate that the TSS location in the *PROS1* promoter is somehow involved in the lower level of alternative splicing in these cells, as it has been shown that usage of different TSSs may lead to different splicing patterns downstream (41-44). However, alternative splicing of pre-mRNA is mostly due to the usage of multiple alternate promoters, leading to the incorporation of different first exons in the mature mRNAs. In the case of the *PROS1* transcripts in HUVECs, it is just the length of the 5' untranslated region (5'UTR) of the first exon that is different in the mRNAs.

Recombinant human PSalt was expressed by COS-1 cells, in which it was retained in large aggregates, whereas recombinant human PSwt was readily secreted into the medium. We believe that the accumulation of recombinant PSalt protein within the cell is a consequence of the altered sequence and folding of the region normally encoding the prepropeptide of

Protein S. In addition, some specific properties of the COS-1 cells may be responsible for the observed results, as similar high molecular weight complexes were not detected in HepG2 cells that apart from normal PS also should express PSalt from endogenous mRNAs. The presence of an extra sequence in this region of Protein S is not predicted to affect the cleavage site of the signal peptide. However, the cleavage site of the propeptide may be less accessible to the peptidase responsible for its cleavage, because of an altered three-dimensional structure of this region. This is expected to be mainly due to intra- or intermolecular disulfide bond formation by the four cysteine residues contained within the alternative peptide sequence. In addition, the γ-carboxylase complex may not be able to bind efficiently to the propeptide, because of steric hindrance by the cysteine-rich stretch located directly upstream. Fluorescent in situ antibody staining showed that at longer transfection times (10 hours) the observed difference in fluorescence intensity between cells that had been transfected with PSwt or PSalt had disappeared. Possibly, PSalt was actively degraded within the cell.

The high molecular weight complexes, which were found in immunoblot assays of lysates from COS-1 cells transfected with pZEM229R-PSalt, could be partly dissociated by addition of DTT. This may be explained if the cysteines within the alternative protein sequence form intermolecular disulfide bridges. We could not exclude the possibility that the observed complexes contained proteins other than PSalt, leaving open the possibility that PSalt interacts with other proteins within the cell. Cultured HepG2 cells contain both normally and alternatively spliced PS mRNA and secrete fully processed PS in medium. In contrast to what was observed in the lysates of COS-1 cells transfected with PSalt the lysates of HepG2 cells did not contain high molecular weight PS antigen. Whether this is related to differences between COS-1 cells and HepG2 cells or to the simultaneous presence of wt presence will need further investigation.

Alternative exons have been found in the same position in mRNAs from the Factor VII and Protein Z genes, which encode two other liver-expressed Vitamin K-dependent coagulation factors (45;46). The length and sequence of these exons is completely different, suggesting that it is mainly their location that matters. The significance of these findings has not yet been studied. GAS6 is a platelet protein with a domain structure highly similar to PS. No similar alternative transcripts have been reported for the GAS6 mRNA. However, the first

intron of *PROS1* is 46 kb long, whereas that of the GAS6 gene is only 91 bp in length, leaving no room for alternative splicing within the latter intron.

The presence of an alternative transcript resulting in a non-secreted form of PS suggests that alternative splicing might be used to regulate PS plasma levels *in vivo* and thus may contribute to the intraindividual variation in PS levels. However, it is as yet unknown whether this alternative splicing event is modulated *in vivo*. This awaits further investigations.

Acknowledgements

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Chapter 7

General Discussion and Conclusions

Chapter 7

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7.1 This Chapter

The association of familial protein S (PS) deficiency in the 1980s with an increased risk for venous thrombosis was followed by an extensive search for mutations in the *PROS1* gene. In contrast to the FV gene in which a single point mutation, FV Leiden, is responsible for over 90% of the APC-resistant phenotype, many different *PROS1* gene abnormalities were found to contribute to low PS levels. At the same time other studies investigated the causes and clinical significance of acquired PS deficiencies. Population-based studies revealed that PS levels are influenced by gender, oral contraceptive use, trauma, autoimmune disease, and liver disease, among others.

In functional studies, PS anticoagulant activity has been thoroughly investigated and the emerging functions of PS apart from those as an anticoagulant, e.g. in inflammation and apoptosis, present a wide area for ongoing and future research. Remarkably, not much interest has been shown in the transcriptional regulation of the PS gene.

This thesis describes our investigations into the transcriptional regulation of the protein S gene (*PROS1)* promoter. In this final chapter the results and conclusions from the preceding experimental chapters are integrated and discussed in a wider context. We will discuss the functions of the transcription factors that were found to bind to the *PROS1* promoter. Also, the use of *in vitro* studies, such as transfection studies, as a model system for the study of gene regulation will be discussed in light of our recent findings.

7.2 Basal expression of *PROS1*

About 50% of the promoter regions of a wide variety of genes is located in CpG islands, regions in which there is an abundance of CpG dinucleotides (1;2). The *PROS1* promoter region fits the characteristics of this category of promoters. Transcription from GC-rich promoters is generally initiated from multiple weak start sites that are distributed over a region of about 100 nucleotides (1;3) and it has been shown that binding of the ubiquitous transcription factor Sp1 together with the presence of an initiator element (Inr) is sufficient to activate transcription (4;5).

The main sites for initiation of *PROS1* transcription were identified in **Chapter 2**. This had been a long outstanding issue for various reasons. Different unique transcriptional start sites had been reported in literature (6-8) and many 5' cDNA ends have been deposited at the databases at the National Center for Biotechnology Information (NCBI). Whereas most reported *PROS1* cDNA start sites are located directly upstream from the first exon, a single cDNA was reported with a start in the first intron (9). Also, the results of PS primer extension experiments with total liver RNA led Ploos van Amstel *et al* to suggest that an additional exon from which transcription of *PROS1* could start, might be present upstream from what is now known as exon 1 (10). Recently, Kimura *et al* (2) showed that transcription from over 60% of all CpG-containing promoters may be directed from more than one promoter. In addition, they showed that these putative alternative promoters (PAPs), which are defined by a spacing of more than 500 bp between start sites, may be used in a tissue-specific manner.

We determined the sites for *PROS1* transcriptional initiation by RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE). With this technique we circumvented the problems of previous studies, since only full-length mRNAs were isolated and many mRNA products could be analysed (11). We did not find evidence for an alternative *PROS1* promoter in the cell types used for our studies, nor did we find a start in the first intron of *PROS1*. However, PS is also synthesized by Leydig cells in the testis (12), an organ that was shown to frequently contain tissue-specific PAPs (2). It would therefore be interesting to determine the *PROS1* transcription start sites in Leydig cells.

With the identification of three main transcription start sites (-147, -117, -100) and many additional unique sites in human liver, human umbilical vein endothelial cells (HUVEC), and cell lines HepG2, HuH7, Meg01 and HeLa we showed that initiation of *PROS1* transcription is directed from multiple start sites. Since then, Kimura *et al* have largely confirmed our results (2) by also identifying -147 as the major start site among many others. The different start site distribution per cell type, and the presence of an additional frequent start at -200 in HUVEC indicates that start sites may be used in a cell type-specific manner. The *PROS1* start sites at -200 and -100 form the center of an Inr, a *cis*-element which recruits the basal transcription machinery to the promoter (4;13;14). In **Chapter 3** we demonstrated transcriptional activation of the *PROS1* promoter by Sp1 and we extended this finding in **Chapter 4** where we identified four, possibly five, functional Sp1 binding sites in the *PROS1* promoter. Especially

the two most proximal Sp1 binding sites were shown to be crucial for *PROS1 trans*-activation *in vitro*. One of these sites may consist of two overlapping Sp1 sites.

Sp1 belongs to the superclass of transcription factors with a zinc-coordinating DNA-binding domain. Sp family members (Sp1-Sp5) contain three zinc fingers through which they bind and *trans*-activate DNA. Sp1 can form homotypic multimers (Sp-Sp), but it can also interact with various classes of other nuclear proteins, such as factors belonging to the general transcription machinery, AP1, NFkB, steroid receptors and many more (for reviews on Sp transcription factors (15-19)). It is possible that the multiple Sp1 and Inr pairs collectively generate the array of *PROS1* start sites that were observed in **Chapter 2**. Figure 1A shows the Sp1-binding sites relative to the main start sites. Start site -200 is located directly downstream to three of the four Sp1-sites whereas the other sites are downstream to all Sp1 sites.

Sp1 has been shown to mediate transcription initiation not only by acting in concert with the basal transcription factors but also by contributing to the maintenance of the hypomethylated state of CpG islands (20;21). The mechanism for this Sp1-mediated prevention of CpG methylation has thus far not been clarified. Methylation of CpG dinucleotides generally results in a loss of transcriptional activity by preventing the binding of transcription factors (22). Next to specific transcription start site usage, m^5-CpG-methylation of the *PROS1* promoter, may thus be a mechanism through which tissue-specific *PROS1* transcriptional activity is regulated. A hypothetical model is shown in Figure 1B. Preliminary experiments that were not included in this thesis suggested a difference in the degree of *PROS1* promoter methylation between cell types. Future research will point out if and how Sp1 is involved in determining the degree of methylation of the *PROS1* promoter.

In **Chapter 4**, the two most proximal Sp1-binding sites in the *PROS1* promoter, -177/-146 and -253/-230 were shown to be indispensible for promoter activity whereas mutation of sites -298/-275 and -359/-335, did not negatively influence *PROS1* promoter activity. The latter two sites therefore do not seem functional. On the other hand, these sites may be involved in preventing CpG-methylation, a level of regulation that cannot be examined with transfection experiments.

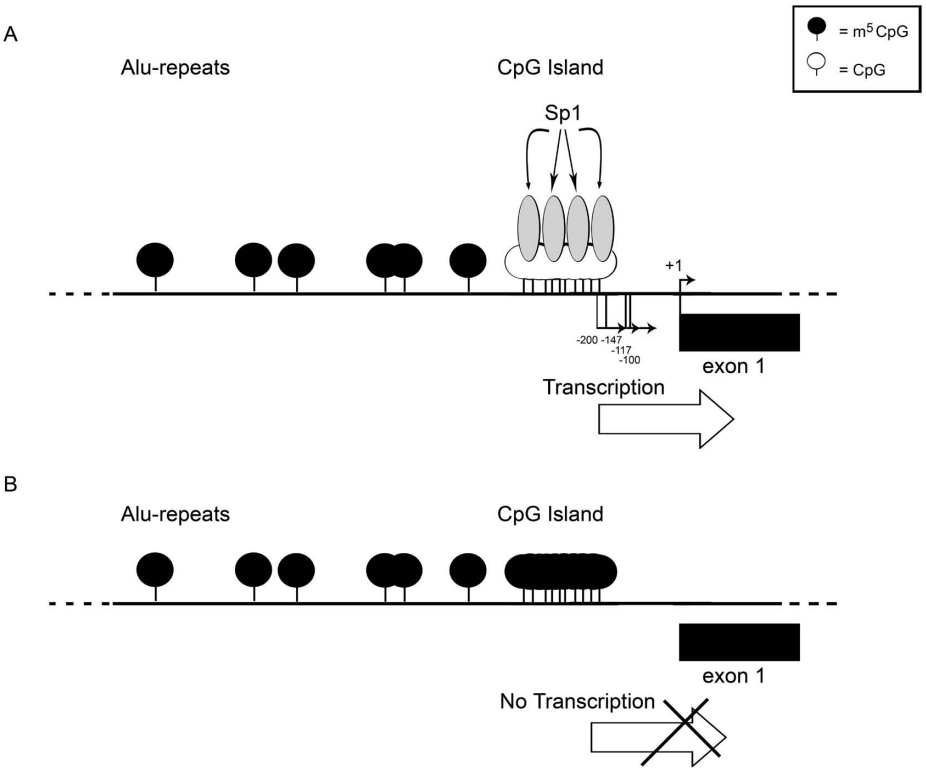

Figure 1 Schematic model for basal *PROS1* transcription. (**A**) Sp1 binding to the *PROS1* promoter relative to the transcription start sites, (**B**) Methylation of CpG dinucleotides results in loss of transcriptional activity. Note that this is a schematic representation of the *PROS1* promoter region, in fact 43 putative methylatable CpGs are located in the CpG island region. Many Alu-repeats are located in the region between 6 kb and 1 kb upstream of the *PROS1* translational startcodon. The Figure is adapted from a Figure by G. Brock (http://otir.nci.nih.gov/tech/slides-brock/slide-brock.html#title)

Another interesting issue concerning Sp1 is that, whereas Sp1 *trans*-activated the PS370 construct ~25 fold in HeLa cells, in HepG2 PS370 *trans*-activation was only ~2.5 fold (**Chapters 3 and 4**). This may be caused by a difference of endogenous Sp1 levels between these two cell types, which was not tested. On the other hand, these data indicate that Sp1 may be regulating *PROS1* activity in a cell-specific manner as well as at the constitutive level, either alone but more likely by interacting with (other) cell-specific transcription factors.

7.3 Induced expression of *PROS1*

7.3.1 Liver-specific expression of *PROS1*

In our pilot study in **Chapter 3** we found that liver-specific transcription FOXA2 (HNF3β) was a strong inducer of *PROS1* promoter activity. In **Chapter 4** we confirmed the presence of a previously identified FOXA2 binding site at position -282/-258 (23). In contrast to what was reported in the earlier study, mutation of this site resulted only in a slightly reduced basal activity of the PS370 promoter-reporter gene construct. An explanation for this discrepancy may be sought in a difference in *PROS1* promoter fragment length in the promoter-reporter gene construct in the two studies. Whereas we used a construct encompassing the first 370 bp proximal to the translational startcodon (-370/-1), Tatewaki and coworkers used a smaller *PROS1* promoter that ranged from -282 to -162. Cotransfection of HepG2 cells with FOXA2 and either PS370 or the PS370 FOXA2 mutant resulted in a slight induction of the wild type PS370, but not of the FOXA2 mutant construct. We concluded that FOXA2 may not be an important *trans*-activator of the *PROS1* promoter in our model system.

7.3.2 Inflammation

PS levels were upregulated in HepG2 cell culture by IL6 and Oncostatin M (OSM) (24). IL6- but not OSM-upregulated PS levels were downregulated by TNFα. The transcriptional mechanism of PS induction by IL6 was studied in **Chapter 5**. We identified an IL6-responsive element (IL6-RE) at -229/-207 in the *PROS1* promoter to which transcription factor STAT3 binds. When binding of STAT3 is prevented by mutation of the IL6-RE, all IL6 responsiveness was lost.

STAT3 may be the sole factor mediating the effects of IL6 on *PROS1*. On the other hand, the STAT3 binding site is imperative for the IL6 response but the response may be influenced by other transcription factors. In the fibrinogen β gene promoter for instance, a FOXA2 binding site just upstream from the IL6-RE was shown to augment IL6-induced transcription (25). A functional FOXA2 binding site is located in the *PROS1* promoter approximately 30 base pairs upstream from the IL6-RE at -275/-265 (**Chapter 4,** (23)). In preliminary experiments in HepG2 we found however that IL6-induced PS370 activity is only slightly

positively influenced by cotransfection of FOXA2. This argues against a prominent role for FOXA2 in IL6-mediated *PROS1* transcription.

In **Chapter 3,** another known mediator of the IL6 response, C/EBPβ, was shown to stimulate *PROS1* transcription in HeLa cells. Even though the IL6-RE in *PROS1* contains the consensus for a C/EBPβ binding site in addition to the STAT3 consensus, we could not establish a strong interaction of C/EBPβ with the IL6-responsive element, as detected by EMSA analysis (**Chapter 4**). Moreover, *PROS1* promoter-reporter gene plasmids with a mutated IL6-RE are still *trans*-activated by C/EBPβ in cotransfection studies in HeLa cells (Figure 2), which indicates that C/EBPβ mediates its effects on the *PROS1* promoter most likely through other C/EBPβ sites or through another, as yet unidentified, mechanism. In conclusion, we found no evidence for the involvement of C/EBPβ in the IL6-mediated stimulation of *PROS1* transcription in HepG2 cells.

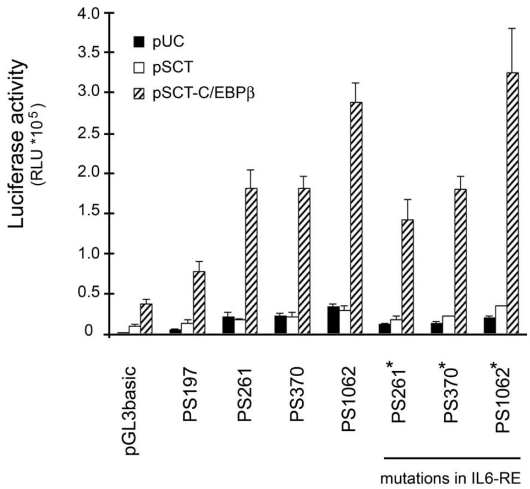

Figure 2 Induction of *PROS1* promoter activity by C/EBPβ in HeLa cells. 375 fmol *PROS1* construct and 25 ng expression vector was transfected into 60% confluent HeLa cell culture in 12 well plates. Total ng of transfected DNA was equalized by addition of pUC vector. Cells were grown for 48 hours after which cells were lysed and luciferase activity was measured. Each bar represents the average of three independent transfections. The exact mutations in the IL6-RE are described in Chapter 5.

The effects of IL6, OSM and TNFα on PS synthesis suggested that PS may function in the (anti-) inflammatory pathway. However, even though systemic total PS levels were increased

in patients during inflammation, free PS levels remained unchanged (26-28) or were even marginally reduced (29-32). It may be, however, that PS not so much mediates the effects of IL6 at the systemic level but instead is more important in mediating local IL6 effects through a local rise in PS levels. Support for such a hypothesis comes from the recent finding that intravenously injected PS has a neuroprotective effect on cortical neurons during ischemic injury in mice (33). The authors speculated that PS may be working through a similar mechanism as its structural homologue, growth arrest-specific gene 6 (GAS6), which induces cell survival through a signalling pathway mediated by the Tyro3/Axl membrane receptor family. In addition, increasing evidence suggests a function of PS in macrophage phagocytosis of apoptotic cells both directly (34;35) and indirectly by directing complement to the surface of apoptotic cells (36;37). Based on the cited literature, it seems plausible that the upregulation by IL6 of local and not systemic PS levels may contribute to survival of cells in the inflamed region. Obviously, this hypothesis needs to be tested in future research.

7.3.3 Unresolved Challenges

C/EBPβ

In the pilot study in **Chapter 3** C/EBPβ was found to be one of the major inducers of *PROS1* transcriptional activity. Moreover, C/EBPβ was first identified as the nuclear factor mediating IL6 effects (NF-IL6). We, therefore hypothesized that C/EBPβ would be the factor mediating the effects of IL6 on *PROS1* transcription. To the contrary, in **Chapter 5** we identified STAT3 and not C/EBPβ as the main IL6-signal transducer. This left us with the unresolved molecular mechanism of C/EBPβ-induced *PROS1* transcription.

We attempted to dissect the interaction of C/EBPβ with the *PROS1* promoter. In HeLa cells C/EBPβ induced *PROS1* promoter activity ~7 fold, whereas in HepG2 C/EBPβ only stimulated the *PROS1* promoter ~2 fold (**Chapter 3** and unpublished data). Surprisingly, in HepG2 *PROS1* promoter activity was stimulated more by the dominant negative variant of C/EBPβ, liver inactivating protein (LIP, unpublished results). This variant lacks the *trans*-activation domain (38;39) and has no effect in HeLa. C/EBPβ has a restricted expression pattern and is present in HepG2 but not in HeLa cells. Varying the C/EBPβ *versus* LIP ratio in these cell types resulted in opposite patterns of *PROS1* promoter induction; in HeLa C/EBPβ induced *PROS1* promoter activity and this induction was increasingly inhibited by increasing

amounts of LIP whereas in HepG2 LIP induced *PROS1* promoter activity and this induction was increasingly inhibited by increasing amounts of C/EBPβ. Due to the complexity of the cell-specific aspects and the failure to obtain a reliable functional assay for the detection of C/EBPβ, we were not able to resolve this intriguing issue in the course of this project.

Female hormones

Estrogens markedly affect the coagulation system, with increased levels of procoagulant factors VII, IX, X, XII and XIII, and reduced concentrations of the anticoagulant factors PS and antithrombin. The impact of female hormones on coagulation is underlined by the fact that women on oral contraceptives (OC) and hormone replacement therapy have an increased risk to develop venous thrombosis (reviewed in (40)). Gender and OC use are thus determinants of plasma PS levels, with women having lower levels than men and women using OC having lower levels than women who do not use OC (41;42). The mechanism through which PS levels are decreased has not been investigated but functional estrogen response elements (ERE) have been identified in the promoters of coagulation factors VII (43) and XII (44). The ERE in the factor VII promoter was shown to repress transcription, whereas the factor XII promoter was activated by estrogen.

To investigate the possible presence of a negatively acting ERE in the *PROS1* promoter we performed several pilot experiments. In the pilot study in **Chapter 3**, PS370 activity was inhibited by cotransfection in HeLa with the androgen receptor but not with the constitutively active estrogen receptor (ER). In a second experiment we measured endogenous PS levels in HepG2 culture medium after stimulation with 17β-estradiol and transfection with wild type and constitutively active ERs and found no significant effect (results not shown). In conclusion, we did not find evidence for an effect of ER and/or estrogen on *PROS1* transcription.

On the other hand, both a nuclear factor Y (NFY) binding site and a cAMP-response element (CRE)-binding protein (CREB)/activating transcription factor (ATF) binding site were discovered and confirmed by EMSA analysis in the *PROS1* promoter in **Chapter 4**. NFY is primarily known as a transcriptional activator, although it has also been shown to inhibit transcription (45;46). NFY was shown to prevent ER-mediated *trans*-activation of the

factor XII promoter in NIH-3T3 and HepG2 cells (47). CREB/ATF transcription factors bind promoter regions of many genes in response to changes in intracellular cAMP levels, which are regulated by extracellular stimuli, amongst which steroid hormones (48;49). It is tempting to speculate that NFY and CREB/ATF are somehow involved in the regulation of *PROS1* transcripton by estrogens. For now however, the mechanism of estrogen-mediated inhibition of *PROS1* transcription *in vivo* remains obscure.

7.4 *In vitro* studies as a model for *in vivo* gene transcription; how representative are they?

7.4.1 General issues

Plasmid DNA generated in *E. coli* culture lacks structural features of endogenous DNA such as a chromatin structure and CpG methylation. Transfection studies are thus useless for investigating the effect of these features on promoter activity and the lack of a chromatin structure and CpG methylation should be taken into account when analysing results. Instead, plasmid DNA is (super-) coiled and generally has prokaryotic dam (G_m**A**TC) and/or dcm (C_m**C**($^A/_T$)GG) methylation patterns which do not occur in eukaryotes. The effect of dam and dcm methylation on promoter activity, was studied by Allamane *et al* (50) who found that luciferase expression from a reporter gene plasmid is surprisingly greater when it is dam and dcm methylated than when it is unmethylated. This study showed that methylation affects promoter activity and therefore most likely transcription factor binding. Transcription factor binding was not studied however and, regretfully, no other studies have been published on the subject.

The luciferase assay system is wellsuited for establishing the effects of endogenous transcription factors on the promoter under investigation, although it should never be forgotten that it remains a model system, in which some aspects of promoter regulation, as discussed in the previous paragraph, cannot be investigated. However, cotransfection experiments with vectors expressing a transcription factor pose special challenges. These mainly have to do with the selection of the negative controls and the internal standard. Negative controls for both the transcription factor and for the promoter-reporter construct, e.g. pGL3basic, should be included. Generally, the empty expression vector or the

transcription factor cloned in the opposite direction is used as negative control for the transcription factor. In our hands some empty expression vectors, which mostly contain the strong cytomegalovirus (CMV) promoter, had a profound effect on promoter-reporter gene activity (see e.g. Figure 2 in this chapter). This is usually attributed to so-called squelching; the preferential binding of endogenous transcription factors to the CMV promoter at the cost of the weaker *PROS1* promoter. Although this usually leads to a lower promoter activity of the promoter under investigation, squelching may also stimulate the activity of a promoter, for instance when the cotransfected CMV promoter is bound by a negative factor. This effect can make it difficult to establish if an observed effect is caused by the transcription factor or by the CMV promoter that is regulating it. One should therefore never rely on just cotransfection experiments. They should be considered as pilot experiments that should be backed up by EMSA experiments and functional promoter assays using constructs in which the binding site of the implicated transcription factor has been mutated. We have followed this approach as often as possible.

Another problem of the cotransfection experiments is that the luciferase production from the "promoter-less" pGL3basic vector was sometimes strongly induced by the transcription factors (see Figure 2 in this chapter and Figure 7B in Chapter 4), presumably by the presence of spurious transcription factor binding sites in the bacterial sequences. This problem is not easily circumvented and we have been very cautious not to over-interpret the data whenever pGL3basic was induced by a co-transfected transcription factor, as was the case for the FOXA2 cotransfections.

A final problem has to do with the normalisation of the different experiments. Transfection efficiency may differ strongly from one experiment to another. Therefore normalisation is required to make the data from different experiments comparable. This is accomplished by cotransfection of an internal standard, a plasmid that encodes Renilla luciferase, another type of luciferase with different substrate requirements. The internal standard is an expression vector containing one of three promoters of variable strength (CMV>SV40>TK) upstream from the *Renilla* luciferase gene. Again, the addition of most transcription factor expression vectors influenced the production of *Renilla* luciferase, probably by the same mechanisms that have been previously delineated for the *PROS1* promoter. As a consequence, the internal standard could not be used to correct for

transfection efficiency and we had to assume that within a single experiment transfection efficiency was similar for all wells.

These problems have been circumvented by other scientists as follows: firstly, pGL3basic (or any other negative vector control) was mostly simply left out, secondly, wild type promoter activity in the absence of a co-transfected expression plasmid was not shown (only plus expression vector and empty expression vector), and lastly, the internal luciferase standard was omitted and/or transfection efficiency was estimated by β-galactosidase activity or cotransfection of a different internal standard. Whether these solutions are justified is open for discussion.

7.4.2 *PROS1*-specific issues

In **Chapter 2** we determined *PROS1* transcription start sites for transcription directed from the endogenous *PROS1* gene in HepG2 cells as well as start sites from *PROS1* promoter-reporter gene constructs that were transiently expressed in HepG2 cells. The starting points of transcripts derived from construct DNA were highly diverse and largely did not correspond to the starts found for the endogenous *PROS1* gene. Studies with TATA-box driven promoters have shown that transcription from promoter-reporter gene constructs is still directed from the correct start sites (51;52), however for TATA-less promoters this issue had not been previously addressed. The reason for this is unknown. Maybe such studies have not been conducted or researchers failed to report the (disappointing) results or results were not accepted by scientific journals. Whatever the reason, the de-regulated transcriptional initiation from TATA-less promoter-reporter constructs raises the question whether this model system is suitable for studying all the characteristics of this type of promoter.

The results presented in **Chapters 3 through 5** show that (co-)transfections are useful for the detection of large scale effects of transcription factors on promoter activity (e.g. Sp1). However, one has to keep in mind that an endogenous pool of transcription factors is always present and that the composition thereof differs per cell type. This is illustrated well by the fact that in **Chapter 3** Sp1 *trans*-activated the PS370 construct ~25 fold in HeLa cells whereas in HepG2 PS370 *trans*-activation was only ~2.5 fold. It would be interesting to compare the

results from EMSA experiments done with HepG2 nuclear extracts in **Chapter 4** with the same experiments done with nuclear extracts from HeLa cells.

7.5 Alternative splicing of *PROS1*

The alternative *PROS1* transcript, PSalt, that was found in human liver and various cell lines in **Chapter 6** contains an additional in-frame exon between the first two exons, which encode the PS signal peptide (exon 1) and the propeptide and the Gla-domain (exon 2). Our results demonstrated that the additional 32 amino acids have a major impact on the maturation of the PSalt protein. Recombinant human PSalt was expressed by COS-1 cells, in which it was retained in large aggregates, whereas recombinant human PSwt was readily secreted into the medium. We believe that the accumulation of recombinant PSalt protein within the cell is a consequence of the altered sequence and folding of the region normally encoding the prepropeptide of Protein S.

Even though HepG2 cells contained relatively large endogenous amounts of the alternative transcript, high molecular weight aggregates were not found in HepG2 cell lysates. The reason for this may be two-fold. First, the alternative mRNA may not be translated into protein. Second, the aggregates may be specific for COS-1 cells in which the recombinant alternative transcript was expressed. Future experiments will have to provide further insight into the relevance of our finding. Thus far, we do not know if the observed transcript is translated into protein in the cell lines in which it is expressed. If PSalt is indeed synthesized to the same extent as its mRNA, the accumulation of PSalt protein in the cell might restrict PS synthesis, thereby influencing systemic PS levels. Expression of the recombinant protein in a more relevant cell line, such as the hepatocytic cell line HepG2, would be a first step into understanding these processes. In this respect one could think of titration experiments in which ratios of transfected PSwt versus PSalt are varied. Also, in order to follow the protein maturation process in more detail, pulse chase experiments can be performed. Finally, the development of a PSalt-specific antibody would aid greatly in determining its in vivo relevance.

7.6 Implications of the findings presented in this thesis for the scientific community and for patients suffering from deep vein thrombosis.

Importance of our findings to the scientific community

According to Rezende *et al* (53) research into the cis-acting elements governing *PROS1* transcription has to date been hampered by a controversy regarding the presence of possible additional exons upstream to what is currently considered as exon 1. We clarified this issue and showed that no additional 5' exons are present in any of the *PROS1* transcripts that we isolated in our experiments. With the identification of the exact start sites for *PROS1* transcriptional initiation another long outstanding issue was resolved. These findings will certainly simplify future research into the *PROS1* promoter.

Subsequent identification of the ubiquitous transcription factor Sp1 as a major player in the basal transcriptional regulation of *PROS1*, through at least four and possibly five direct binding sites for Sp1 in the promoter, may explain why *PROS1* is transcribed at low levels by a large variety of cells throughout the body. Using cotransfection experiments, we found evidence that liver-specific transcription factors FOXA2, HNF6, C/EBPβ and DBP positively influence *PROS1* transcription. A partially different set of transcription factors was found to bind to the PS promoter in EMSA experiments. The discrepancies in the results may be the result of the limitations of the various test systems that are routinely employed, as was discussed in this chapter. No evidence was found for a direct interaction of the steroid hormone receptors; the estrogen, androgen or glucocorticoid receptors. Therefore, the mechanism by which female sex hormones downregulate PS plasma levels remains unresolved.

In experiments conducted in HepG2 cell culture, we showed that PS levels are positively influenced by IL6 through a direct interaction of the transcription factor STAT3 with the *PROS1* promoter. The changes in PS levels are most likely not of importance to plasma PS levels *in vivo,* since no major changes in PS levels have been reported during inflammation. However, locally upregulated PS levels at the site of inflammation may be of importance in light of the recently reported functions of PS in controlled cell death.

The full significance of our finding of an alternative PS transcript still needs to be determined. This transcript comprises 5 to 20% of the total transcripts, dependent on the cell type, suggesting an important *in vivo* role. Recombinant protein expression in COS1 cell culture pointed out that the alternative protein, which contains an additional 32 amino acid

stretch in between the pre- and propeptide of the immature PS protein, is retained within the cell and forms aggregates, possibly with other proteins. In the future, coexpression experiments with standard PS constructs in HepG2 cells need to be performed to determine if absolute and relative expression levels of PSalt affect its fate.

Importance of our findings to patients

The present research was mainly aimed at resolving several basic scientific issues regarding the regulation of transcription of the important anticoagulant Protein S. A thorough understanding of the transcriptional regulation of *PROS1* will contribute to a better understanding of how PS levels are regulated at the genetic level and may in the future provide important insights into as yet unexplained partial PS deficiencies. The present results can be used as a steppingstone in that direction. In addition, the role of the alternative exon in *PROS1* needs to be further explored, since similarly positioned exons occur in other coagulation genes.

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Summary

The goal of our research was to obtain more insight into the way in which Protein S (PS) expression is regulated. PS is a Vitamin K-dependent plasma protein with an important anticoagulant role. It is the cofactor of Activated Protein C (APC), which inactivates factors Va and VIIIa. PS is present in the circulation in two forms, as a free protein and bound to C4b-binding protein (C4BP). Only free PS is functional as an APC cofactor. A deficiency of free PS is associated with the development of deep venous thrombosis, which leads to obstruction of a blood vessel. This occurs most frequently in the deep veins of the leg. When (part of a) thrombus detaches, it travels via the heart to the lungs, leading to a pulmonary embolism, a complete or partial blockage of lung arteries.

A PS deficiency can be inherited or acquired. Inherited deficiency is relatively rare and occurs in 2-8% of the families with a hereditary propensity to thrombosis (thrombophilia). An acquired deficiency, which may be temporary in nature, occurs more frequently and may be caused by oral contraceptive use (female sex hormones), liver disease and diffuse intravascular coagulation (DIC). These effectors may directly or indirectly lower PS expression by altering transcription or by changing other factors, respectively. Knowledge of the transcriptional regulation of the gene encoding PS, *PROS1*, should enable us to understand the fluctuations in PS levels in various circumstances.

Careful study of the DNA sequence directly upstream of the translational start of *PROS1* showed that it does not contain a TATA box. The TATA box is a transcriptional element that directs initiation of transcription to a clearly defined start site at a fixed distance downstream from it. Genes without a TATA box often have several transcription start sites (TSSs) and may even contain multiple promoters. The use of these promoters and/or start sites is usually cell type-specific. Because PS is mainly produced by liver cells, but to a lesser extent also by other cell types, I investigated in **Chapter 2** the TSSs of *PROS1* transcripts in various cell types to determine their cell type-specificity. The mRNA was isolated from liver cells (human liver and the cell lines HepG2 and HuH7), megakaryocytes (cell line Meg01), and endothelial cells (human umbilical vein endothelial cells; HUVECs). These cell types share as a common property that they could in principle contribute to PS plasma levels, because they are in direct contact with blood and are known to be capable of PS expression. Epithelial cells (the HeLa cell line) were originally included as a negative control, but appeared to be capable of a low level of PS expression during the course of the studies. In addition, the activity of *PROS1*

promoter fragments cloned in luciferase reporter gene constructs was determined by transfection in the same cell lines. In these constructs, luciferase expression is entirely dependent on the strength of the *PROS1* promoter fragment. If the activity of the *PROS1* promoter would be mainly determined by cell-specific transcription factors, the relative strength of the various promoter constructions should be cell type-dependent. In contrast, if ubiquitous factors were responsible for the bulk of the promoter activity, then one would expect the activity of the various constructs to be similar in all cell lines.

All cell types express endogenous *PROS1* transcripts with TSSs at 100, 117 and 147 bp upstream from the startcodon. The various start sites are used at a different frequency among the cell lines, however. The HUVECs also showed an additional start site at 200 bp upstream of the translational start. These results show that the exact distribution of the start sites varies among the different cell types. This type of cell type specific regulation of transcription is probably caused by differences in the set of transcription factors in each cell type. We obtained no evidence for the existence of alternative *PROS1* promoters. The possible existence of additional transcription start sites in intron 1 was also excluded.

A *PROS1* promoter with maximal activity was identified using *in vitro* studies with *PROS1* promoter reporter-gene constructs. The maximally active promoter contained 370 bp upstream of the startcodon in all cell types. This region is present in construct PS370. Only small differences were found in the expression pattern of the various promoter constructs in the cell types used. Whereas the activity of the constructs in HepG2, HeLa and HUVEC cells decreased with an increasing length of the *PROS1* promoter fragment, the longer *PROS1* promoter constructs in the Meg01 and HuH7 cell lines retained a relatively high activity.

Contrary to our expectations, the maximally active promoter fragment still had an activity of 40% in HepG2 cells following deletion of all identified transcription start sites from the 3' end by exonuclease treatment. When we subsequently determined the TSSs in the parental PS370 construct, start sites were found to be apparently randomly spread over the *PROS1* promoter fragment. This is probably caused by a less stringent regulation of transcription initiation in our model system in comparison with the endogenous gene.

The results described in **Chapter 2** indicate that the basal activity of the *PROS1* promoter is regulated in approximately the same way in all cell types. The small differences in the endogenous TSSs and in the expression pattern of the various promoter constructs point at

some cell-specific regulation via start site choice, but argue against a major influence of these variables on the basal activity.

Chapter 3 describes a pilot study into the inducibility of the maximally active PS370-promoter described in **Chapter 2** by a number of carefully selected transcription factors. Co-transfection of the PS370 construct and expression vectors encoding a transcription factor in HeLa cells showed that *PROS1* promoter activity was increased 24- and 15-fold by the ubiquitous transcription factors Sp1 and Sp3, respectively. In addition, the liver-specific transcription factors C/EBPβ and FOXA2, and to a lesser extent HNF6 and DBP, had a stimulatory effect on the activity of PS370. A detailed study of the *PROS1* promoter sequence in PS370 and its conservation in various mammalian species resulted in the localisation of evolutionary conserved consensus binding sites for C/EBPβ, STAT, FOXA2 and transcription factors of the Sp-family.

The results of this pilot study were partially confirmed in **Chapter 4**, in which it was shown that FOXA2, Sp1 and Sp3 do indeed bind to the predicted locations in the *PROS1* promoter sequence. Four Sp1 binding sites were found at 177-146 bp, 253-230 bp, 298-275 bp and 359-335 bp upstream of the translational start site. The presence of the FOXA2 binding site at position 282-258 bp upstream of the startcodon had already been demonstrated by another group and could be confirmed. We also observed binding of the transcription factors NFY (at 382-359) and CREB/ATF (at 346-323) to the promoter sequence. Analysis of the functionality of the identified transcription factor binding sites via mutagenesis showed that the Sp1 binding sites at 177-146 and 253-230 were required for full basal activity of the PS370 construct in HepG2 cells. In constructs in which these sites had been mutated, the promoter activity of the PS370 construct was reduced to 70 and 50% of the wild-type construct, respectively. Alteration of just the sites at 298-275 and 359-335 had no negative effect, but a mutant in which all four Sp1-sites had been mutated had a remaining activity of just 20%.

Cotransfection of HepG2 with an Sp1 expression vector and the fourfold PS370-Sp1 mutant demonstrated that this mutant, despite its strongly decreased basal activity, could still be induced by Sp1, which indicates that not all functional Sp1 sites in the PS370 construct had been identified. Most likely, there is an additional non-mutated Sp1 binding site in the sequence 177-146. Finally, the Sp1-*PROS1* promoter interaction that was demonstrated *in vitro*

was confirmed by chromatin immunoprecipitation (ChIP) experiments using an antibody directed against Sp1.

Mutations in the binding sites of the transcription factors FOXA2, CREB/ATF and NFY only had a marginal negative effect on the basal expression of the luciferase reporter gene under the control of the *PROS1* promoter. Cotransfection of HepG2 cells with the PS370 FOXA2 mutant and a FOXA2 expression vector resulted in a decreased response of the mutant relative to the wild-type construct, but the interpretation of the data was complicated by the fact that the control vector without the *PROS1* promoter was strongly stimulated by FOXA2.

The results of **Chapter 4** confirm those of **Chapter 2**. The basal *PROS1* transcription is indeed regulated by an ubiquitous transcription factor, i.e. Sp1. The transcription factors that are presumably responsible for the high expression in the liver have not yet been identified, however. It is very well possible that part of this tissue-specific expression is caused by epigenetic modulation of the *PROS1* promoter via methylation and histone modification. Despite various attempts to study promoter methylation, we have not yet been able to obtain unequivocal results.

Another interaction between a transcription factor and the *PROS1* promoter described in **Chapter 3** was that between PS370 and C/EBPβ. C/EBPβ is known as the transcription factor that mediates the signalling of the inflammatory mediator interleukin 6 (IL6). STAT3 is a second nuclear factor that transduces the signal of IL6. In response to the binding of IL6 to its receptor on the cellular membrane, both STAT3 and C/EBPβ are phosphorylated. Subsequently, they enter the nucleus and stimulate the transcription of various genes by binding to their promoters. In the case of STAT3, phosphorylation is the only mode of activation, whereas the transcription of the C/EBPβ-gene itself is also upregulated by IL6. In **Chapter 5**, we investigated the effects of IL6 on PS levels in the medium of cultured HepG2 cells. We confirmed the results of other investigators, who previously showed that PS levels rise in cell culture upon stimulation by IL6. We subsequently demonstrated that this effect can also be detected at the level of the *PROS1* mRNA, which indicates that it is most likely caused by an effect on the promoter activity. The level of phosphorylated STAT3 (p-STAT3) in the nucleus of the HepG2 cells increased following incubation with IL6, while that of C/EBPβ

remained at the same level. This indicated that the observed effects were most likely mediated by STAT3.

The sequence element in the PS370 construct that could mediate the IL6-response contains a consensus binding site for both C/EBPβ and STAT3 at 229-207 bp upstream of the start site of translation. Mutation of this site in several *PROS1* promoter constructs resulted in the simultaneous disappearance of both binding sites and prevented stimulation of the promoter by IL6. Protein-DNA binding assays subsequently showed that STAT3 could indeed bind to the normal sequence, but not to that of the mutant, and that this interaction was much more obvious after stimulation of the cells with IL6. Binding of C/EBPβ to this sequence could not be detected, however. Moreover, the C/EBPβ consensus sequence was not capable to compete for protein binding to the *PROS1* promoter sequence, whereas the STAT3 consensus-sequence was capable of doing so. In addition, only a small part of the protein-DNA complex in the gel was supershifted by a C/EBPβ antibody, whereas incubation with an anti-STAT3 antibody resulted in a nearly complete supershift of the DNA-protein complex. This made clear that STAT3 plays an important role in the reaction of the *PROS1*-promoter toward IL6. A role of C/EBPβ could not entirely be discounted, however, on the basis of the results obtained in **Chapter 3**.

During inflammation the plasma levels of total Protein S are unaltered or slightly elevated, whereas free Protein S levels are slightly reduced to unchanged. An induction of plasma PS levels that is expected on the basis of our experiments, is probably counteracted by a simultaneous increase of the levels of the C4BP that is also stimulated by IL6. The induction of PS by IL6 is therefore probably not so much relevant to the levels of PS in plasma, but much more to local PS levels in tissues.

PS is synthesized as a preproprotein. The pre- and propeptide are removed during the subsequent maturation of the protein inside the cell. **Chapter 6** describes the discovery of an alternative *PROS1* transcript encoding 32 additional amino acids in between the pre- and propeptide of the immature protein. The novel transcript was detected during the optimalisation of a quantitative PCR aimed at the detection of the normal *PROS1* mRNA. The transcript was present in all cell types tested, also in human liver. The alternative transcript was converted to cDNA and cloned. The expression and size of the resulting alternative PS (PSalt) was compared to that of standard PS following transfection of both constructs in COS-1 cells. The alternative protein accumulated in the cell, whereas the

standard protein was secreted into the culture medium as expected. Western blot analysis indicated that the remaining PSalt was possibly larger than the standard protein, which suggested that the additional amino acids in between the pre- and propeptide disrupted the removal of these two peptides during the maturation of PSalt. PSalt did not just accumulate in the cell, it also formed aggregates that were easily detectable on Western blot.

The implications of these finding are unclear for the time being, since PSalt-aggregates were not observed in lysates of HepG2 cells that also produce significant levels of the alternative transcript. Possibly, the alternative *PROS1* mRNA is degraded before it is translated into protein. In that case, alternative splicing of *PROS1* transcripts may be used to regulate the levels of standard PS. Alternatively and perhaps more likely, PSalt might be more efficiently processed when it is expressed at relatively low levels in combination with standard PS in professional PS-expressing cells, such as HepG2 cells.

The regulation of *PROS1* transcription was the focus of the research presented in this thesis. Our results provide novel insights into the transcriptional regulation of this gene and thereby contribute to the basic knowledge required to understand the regulation and fluctuations in the PS levels of patients.

Nederlandse samenvatting

Het doel van het hier gepresenteerde onderzoek was om meer inzicht te verkrijgen in de wijze waarop de productie van Proteïne S (PS) geregeld wordt. PS is een eiwit in het bloedplasma dat een belangrijke rol speelt in de bloedstolling. Het is een helper (cofactor) van geactiveerd Proteïne C (APC). APC beperkt overmatige stolling door de stollingsfactoren Va en VIIIa onwerkzaam te maken. PS komt in bloed in twee vormen voor, als vrij eiwit en gebonden aan C4b-bindend eiwit (C4BP). Alleen de vrije vorm is functioneel als cofactor van APC. Een tekort aan vrij PS in het bloed draagt bij aan de ontwikkeling van diep veneuze trombose. Dit is een aandoening waarbij een bloedstolsel ontstaat in een ader (vene), waardoor de bloedbaan verstopt raakt. Dit komt het meest voor in de diepe venen van het been en staat bekend als een trombosebeen. Wanneer het stolsel of een stukje daarvan loslaat, kan het via het hart in de longen terechtkomen en een longembolie, een gehele of gedeeltelijke afsluiting van longslagaderen, veroorzaken.

Een tekort aan PS (een deficiëntie) kan aangeboren of verworven zijn. Een aangeboren deficiëntie is vrij zeldzaam en komt slechts voor in 2-8% van de families met een erfelijke tromboseneiging (trombofilie). Een verworven deficiëntie, die van voorbijgaande aard kan zijn, komt vaker voor en kan onder meer door pilgebruik (vrouwelijke geslachtshormonen), leverziekte en diffuse intravasale stolling (DIS) worden veroorzaakt. Het effect van deze factoren op PS spiegels kan direct (op het niveau van de PS productie) of indirect (door beïnvloeding van andere factoren) zijn. Het belangrijkste gereguleerde proces tijdens de eiwitproductie is doorgaans de transcriptie, waarbij een gen wordt overgeschreven in boodschapper RNA (mRNA), dat vervolgens in eiwit vertaald (getransleerd) wordt. De mRNA productie wordt geregeld door de promoter van een gen, die vlak voor de start van het gen gelegen is. De mate van transcriptie wordt bepaald door specifieke eiwitten, zogenaamde transcriptiefactoren, die aan de promoter binden en zo de transcriptie reguleren. Kennis van de transcriptionele regulatie van het gen dat codeert voor PS, *PROS1*, zal een bijdrage leveren aan ons begrip van de fluctuaties in PS spiegels in verschillende situaties.

Zorgvuldige bestudering van de DNA-volgorde in en rond de *PROS1*-promoter wees uit dat *PROS1* geen TATA-box heeft. De TATA-box is een DNA-element dat de transcriptie laat starten op een vast punt. Genen zonder TATA-box kennen vaak meerdere transcriptiestart-punten en hebben soms zelfs verschillende promoters. Het gebruik van deze promoters en/of startpunten is doorgaans celtype-specifiek. Omdat PS met name geproduceerd wordt door

levercellen, maar in mindere mate ook door andere cellen, onderzocht ik in **Hoofdstuk 2** de startplaatsen van *PROS1* transcripten in verschillende soorten cellen om zo te bepalen of de transcriptie startplaatsen van *PROS1* celtype-specifiek zijn. Het mRNA werd geïsoleerd uit levercellen (menselijke lever en de cellijnen HepG2 en HuH7), megakaryocyten (cellijn Meg01), en vaatwandcellen (humane vasculaire endotheelcellen uit navelstreng). Deze celsoorten hebben als gemeenschappelijke kenmerk dat zij in principe kunnen bijdragen aan plasma PS spiegels, omdat zij in direct contact staan met het bloed. Epitheliale cellen (cellijn HeLa) werden oorspronkelijk in de studie als een negatieve controle opgenomen, maar tijdens de studie bleek dat ook deze cellen in geringe mate PS produceren. Daarnaast werd de activiteit van *PROS1* genconstructen bepaald. In deze constructen regelt de *PROS1* promoter de transcriptie van een reporter-gen, dat codeert voor een gemakkelijk te meten eiwitproduct, in ons geval luciferase. De mate van luciferaseproductie is een maat voor de promoteractiviteit. Deze promoter-reportergenconstructen werden door middel van transfectie in de eerder genoemde cellijnen ingebracht. Indien de *PROS1* promoter gedreven wordt door celspecifieke transcriptiefactoren dan zou de relatieve sterkte van de promoterconstructen per celsoort moeten verschillen. Als *PROS1* transcriptie gereguleerd wordt door algemeen voorkomende factoren dan zou de relatieve promoteractiviteit van de verschillende constructen in alle cellijnen nagenoeg gelijk moeten zijn.

Alle celsoorten blijken eigen (endogene) *PROS1* transcripten te bevatten met een transcriptiestartplaats op 100, 117 en 147 basenparen (bp) voor de vaste startplaats van eiwitsynthese (translatie). De frequentie van gebruik van deze startplaatsen verschilde echter per celsoort. Ook bleken vaatwandcellen uit de navelstreng een extra startplaats van het *PROS1* transcript te vertonen op 200 bp voor de translationele start. Deze resultaten tonen aan dat de exacte verdeling van startpunten van *PROS1* transcripten in de bestudeerde celsoorten varieert. Deze vorm van celsoortspecifieke regulatie van transcriptie wordt mogelijkerwijs veroorzaakt door verschillen in de samenstelling van de groep eiwitten in de celkern die gezamenlijk de transcriptie reguleren. Voor alternatieve *PROS1* promoters werd geen bewijs gevonden en ook een eerder geopperde mogelijkheid dat *PROS1* transcriptie vanuit het eerste intron geïnitieerd zou kunnen worden, werd uitgesloten.

Een *PROS1* promoter met maximale activiteit werd geïdentificeerd door middel van *in vitro* studies met *PROS1* promoter-reportergenconstructen. De maximaal actieve promoter werd voor alle celsoorten vastgesteld op een lengte van 370 bp stroomopwaarts van de translationele start, zoals aanwezig in het *PROS1* promoterconstruct PS370. Er worden slechts

kleine verschillen gevonden in het expressiepatroon van de promoterconstructen in de geselecteerde celsoorten. Terwijl de activiteit van de constructen in HepG2, HeLa en HUVEC cellen met toenemende lengte van het *PROS1* promoterfragment sterk afnam, behielden de langere *PROS1* promoterconstructen in de cellijnen Meg01 en HuH7 een relatief hoge activiteit.

Tegen de verwachting in behield het maximaal actieve promoterfragment in HepG2 cellen 40% van zijn activiteit nadat alle gebruikelijke transcriptie startpunten vanaf de 3' kant waren verwijderd. Toen vervolgens de transcriptie startplaatsen bepaald werden op het PS370 construct bleek dat deze verschilden van de startpunten gevonden voor de transcriptie van het endogene *PROS1,* zoals dat op de chromosomen van de HepG2 cellen aanwezig was. Hieruit werd geconcludeerd dat transcriptie vanaf de getransfecteerde promoterconstructen vanuit veel meer punten werd geïnitieerd. Vermoedelijk wordt dit veroorzaakt door de minder nauwkeurige regulatie van transcriptie initiatie in het modelsysteem in vergelijking met het volledige gen in zijn normale chromosomale omgeving.

De bevindingen in **Hoofdstuk 2** wijzen uit dat de basale activiteit van de *PROS1* promoter in alle celsoorten min of meer op een zelfde wijze is gereguleerd. De kleine verschillen in transcriptie startplaatsen van het endogene *PROS1* en het expressiepatroon van de verschillende promoterconstructen in de verschillende cellijnen wijzen wel op een mogelijke celspecifieke regulatie van transcriptie via de startlocatie, maar spreken een grote invloed daarvan op de basale activiteit tegen.

Hoofdstuk 3 bevat een verkennende studie naar de induceerbaarheid van de in **Hoofdstuk 2** gevonden maximaal actieve promoter PS370 door verschillende zorgvuldig geselecteerde transcriptiefactoren. Cotransfectie van HeLa cellen met het PS370 construct en expressievectoren met daarin een gen coderend voor een transcriptiefactor, wees uit dat *PROS1* promoteractiviteit respectievelijk 24- en 15-maal gestimuleerd werd door de alom aanwezige transcriptiefactoren Sp1 en Sp3. Ook de leverspecifieke transcriptiefactoren C/EBPβ en FOXA2 en in geringere mate HNF6 en DBP hadden een positieve invloed op de PS370 activiteit. Nauwkeurige bestudering van de *PROS1* DNA-volgorde (sequentie) in PS370 en de conservering daarvan tussen verwante diersoorten, resulteerde in de lokalisatie van evolutionair goed bewaard gebleven consensus bindingsplaatsen voor onder andere C/EBPβ, STAT, FOXA2 en transcriptiefactoren van de Sp-familie.

De resultaten van de verkennende studie werden bevestigd in **Hoofdstuk 4**, waarin door middel van de bestudering van eiwit-DNA interacties aangetoond werd dat FOXA2, Sp1 en Sp3 inderdaad op de gevonden locaties binden aan de *PROS1* promotersequentie. Vier Sp1 bindingsplaatsen werden gevonden die op 177-146 bp, 253-230 bp, 298-275 bp en 359-335 bp stroomopwaarts van de translationele start werden gelokaliseerd. De aanwezigheid van een FOXA2 bindingsplaats werd al eerder door een andere groep aangetoond op 282-258 bp stroomopwaarts van de translationele start en kon worden bevestigd. Tevens werd een interactie van de transcriptiefactoren NFY (op 382-359) en CREB/ATF (op 346-323) met de promoter waargenomen. Analyse van de functionaliteit van de gevonden transcriptiefactorbindingsplaatsen middels mutagenese wees uit dat Sp1 bindingsplaatsen 177-146 en 253-230 van groot belang zijn voor de basale activiteit van het PS370 construct in HepG2 cellen. In constructen waarin deze bindingsplaatsen gemuteerd waren was de basale PS370 activiteit gereduceerd tot respectievelijk 70 en 50% van die van het onveranderde construct. Mutatie van uitsluitend de bindingsplaatsen op 298-275 en 359-335 had geen negatief effect, maar een mutant waarin alle vier de Sp1-posities werden gemuteerd had een restactiviteit van slechts 20%.

Cotransfectie van HepG2 cellen met een Sp1 expressievector en de viervoudige PS370-Sp1 mutant liet echter zien dat deze mutant ondanks de afgenomen basale activiteit nog door Sp1 geïnduceerd kan worden, hetgeen er op duidt dat niet alle functionele Sp1 bindingsplaatsen in het PS370 construct werden geïdentificeerd. Hoogstwaarschijnlijk is een extra niet-gemuteerde Sp1 bindingsplaats aanwezig in de sequentie 177-146. De *in vitro* aangetoonde interactie tussen Sp1 en de *PROS1* promoter werd tenslotte bevestigd door een chromatine immunoprecipitatie (ChIP) met een antilichaam specifiek voor Sp1.

Mutaties in de bindingsplaatsen voor de transcriptiefactoren FOXA2, CREB/ATF en NFY hadden niet of nauwelijks een negatief effect op de basale expressie van het luciferase reporter-gen onder de controle van de *PROS1* promoter. Cotransfectie van HepG2 cellen met de PS370 FOXA2 mutant en een FOXA2 expressievector resulteerde weliswaar in een afgenomen respons van de mutant ten opzichte van het normale construct, maar de interpretatie van de data werd bemoeilijkt door het feit dat de controle vector zonder *PROS1* promoter ook sterk op FOXA2 reageerde.

De resultaten in **Hoofdstuk 4** bevestigen die van **Hoofdstuk 2**. De basale *PROS1* transcriptie wordt inderdaad gereguleerd door een alomtegenwoordige transcriptiefactor, namelijk Sp1. De transcriptiefactoren verantwoordelijk voor de hoge leverspecifieke expressie

van PS werden echter vooralsnog niet geïdentificeerd. Het is goed mogelijk dat een deel van deze weefselspecifieke expressie wordt veroorzaakt door zogenaamde epigenetische modulering van de *PROS1* promoter middels promotermethylering en histonmodificatie. Ondanks pogingen om promotermethylering aan te tonen is het vooralsnog niet gelukt hieromtrent een eenduidig resultaat te verkrijgen.

Een andere interactie die naar voren kwam uit de studie in **Hoofdstuk 3** was die tussen PS370 en C/EBPβ. C/EBPβ staat bekend als de transcriptiefactor die de signalering van de ontstekingsmediator interleukine 6 (IL6) bewerkstelligt. STAT3 is de tweede nucleaire factor die IL6-effecten medieert. In reactie op binding van IL6 aan de zijn receptor op de celmembraan worden zowel STAT3 als C/EBPβ gefosforyleerd, waarna ze de kern ingaan en verschillende genen aanzetten tot (versterkte) transcriptie door aan hun promoters te binden. Voor STAT3 is deze fosforylering de enige manier van activatie, terwijl de transcriptie van het C/EBPβ-gen zelf ook door IL6 wordt opgereguleerd. In **Hoofdstuk 5** onderzochten wij het effect van IL6 op PS spiegels geproduceerd door gekweekte HepG2 cellen. Wij bevestigden de resultaten van andere onderzoekers, die eerder hadden gevonden dat PS spiegels in celkweek inderdaad toenemen tijdens stimulatie met IL6. Vervolgens toonden we aan dat dit effect ook zichtbaar is op het niveau van het *PROS1* mRNA en dus hoogstwaarschijnlijk wordt veroorzaakt door een direct effect op promoteractiviteit. In onze experimenten nam de concentratie van gefosforyleerd STAT3 (p-STAT3) toe in de kern van HepG2 cellen na incubatie met IL6, terwijl die van C/EBPβ gelijk bleef. Dit duidde erop dat de waargenomen effecten waarschijnlijk gemedieerd werden door STAT3.

De DNA-volgorde in het PS370 construct waardoor de IL6-respons gemedieerd zou kunnen worden, bevat een consensus bindingsplaats voor zowel C/EBPβ als voor STAT3 op 229-207 bp stroomopwaarts van de start van translatie. Nadat deze bindingsplaats in verscheidene *PROS1* promoterconstructen gemuteerd was, waardoor zowel de C/EBPβ als de STAT3 bindingplaatsen verdwenen waren, reageerde de promoter niet meer op de toevoeging van IL6. Eiwit-DNA bindingsassays toonden aan dat STAT3 inderdaad kan binden aan de normale sequentie, maar niet aan die van de mutant, en dat deze interactie versterkt wordt door stimulatie van de cellen met IL6. Voor C/EBPβ werd een dergelijke interactie echter niet waargenomen. De C/EBPβ consensussequentie was bovendien niet in staat binding van het eiwit aan de *PROS1* promoter sequentie te voorkomen, terwijl de STAT3 consensussequentie dat wel kon. Bovendien verschoof slechts een deel van het eiwit-DNA complex in de gel na

toevoeging van een C/EBPβ antilichaam, terwijl incubatie met een tegen STAT3 gericht antilichaam resulteerde in een nagenoeg complete verschuiving van het eiwit-DNA complex. Hierdoor is duidelijk geworden dat STAT3 een belangrijke rol speelt bij de reactie van de *PROS1*-promoter op IL6. Een rol van C/EBPβ kan echter niet volledig worden uitgesloten op basis van de resultaten in **Hoofdstuk 3**.

Tijdens ontsteking zijn de totale PS spiegels in het plasma van patiënten nagenoeg onveranderd tot licht verhoogd, terwijl vrije PS spiegels soms gereduceerd zijn of ook gelijk blijven. Een potentiële geringe inductie van plasma PS spiegels door IL6, zoals verwacht mag worden op basis van onze experimenten, wordt hoogstwaarschijnlijk tenietgedaan door de gelijktijdige stijging van de spiegel van C4BP, dat ook geïnduceerd wordt door IL6. Het belang van de inductie van PS door IL6 moet dan wellicht ook niet gezocht worden in een effect op de plasma PS spiegels, maar meer op lokaal (weefsel)niveau.

PS wordt aangemaakt als een prepro-eiwit, waarvan het pre- en propeptide verwijderd worden tijdens de verdere rijping van het eiwit in de cel. **Hoofdstuk 6** beschrijft de ontdekking van een alternatief *PROS1* transcript dat codeert voor 32 extra aminozuren tussen het pre- en propeptide van het onvolwassen eiwit. Dit transcript werd gevonden tijdens de optimalisatie van een kwantitatieve PCR voor het normale *PROS1* mRNA. Het nieuwe transcript was aanwezig in alle celsoorten die getest werden, waaronder een sample van een humane lever. Het alternatieve transcript werd gekloneerd en de expressie en grootte van het resulterende alternatieve PS (PSalt) werd vergeleken met die van het standaard PS na transfectie van beide constructen in COS-1 cellen. Het alternatieve eiwit hoopte zich op in de cel, terwijl het standaard PS product, zoals verwacht, werd uitgescheiden. Western blot analyse gaf aan dat het alternatieve eiwit dat in de cel aanwezig bleef mogelijk groter was dan het standaard product. Dit suggereerde dat de extra aminozuren tussen het pre- en propeptide de afscheiding van deze twee peptides in het rijpingsproces van PSalt verstoorde. Niet alleen bleef het alternatieve PS in de cel, het vormde ook aggregaten die zichtbaar waren op een Western blot.

De implicaties van deze bevindingen zijn vooralsnog onduidelijk, aangezien aggregaten met het alternatieve PS eiwit niet in lysaten van HepG2 cellen werden gevonden, die het alternatieve transcript ook produceren. Mogelijkerwijs wordt het alternatieve *PROS1* transcript afgebroken voordat het omgezet wordt tot eiwit. In dat geval bestaat de mogelijkheid dat vorming van het alternatieve transcript *in vivo* gebruikt wordt om de synthese van het

standaard PS te reguleren. Een andere en misschien meer waarschijnlijke mogelijkheid is dat PSalt efficiënter wordt verwerkt wanneer het in relatief lage hoeveelheden worden geproduceerd in de aanwezigheid van standaard PS en/of in cellen die gewend zijn PS te produceren, zoals HepG2 cellen.

De regulatie van *PROS1* transcriptie stond centraal in het onderzoek dat in dit proefschrift beschreven is. Dit proefschrift geeft nieuwe inzichten in die transcriptie en draagt daardoor bij aan de basiskennis die noodzakelijk is voor het begrijpen van de regulering en de schommelingen in de PS spiegels van patiënten.

Nawoord

Het promotieonderzoek beschreven in dit proefschrift werd uitgevoerd bij de sectie Trombose en Hemostase van de afdeling Hematologie aan het Leids Universitair Medisch Centrum onder supervisie van Dr. Hans Vos en Prof. dr. Rogier Bertina. De vier (and then some) jaren die ik hieraan besteed heb, zijn mooie en zware jaren geweest die ik nimmer vergeten zal mede dankzij mijn fijne collega's, vrienden, sportgenootjes en familie.

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Curriculum Vitae

Cocky de Wolf is op 16 september 1975 geboren te Leiden. Na het behalen van haar VWO diploma aan het Fioretticollege te Lisse, ging zij in 1993 Biologie studeren aan de Universiteit Leiden. Na het propedeuse jaar koos zij voor de differentiatie Organismale Biologie, waarna een specialisatie in de Medische Biologie volgde. Na een korte stage in de moleculaire endocrinologie bij Dr. J. Stalvey aan de Biological Sciences Department van Kent State University, Ohio (VS), volgde Cocky een bijvakstage aan het Instituut voor Moleculaire Plantkunde (IMP) te Leiden onder leiding van Dr. Leslie van der Fits en Dr. Johan Memelink. Tijdens haar hoofdvakstage (1 jaar) onderzocht zij het metabolisme van tamoxifen door cytochroom P450 enzymen in de lever aan de universiteit van Queensland (Australië) onder leiding van Dr. Elizabeth Gillam (Physiology and Pharmacology Department), met als Nederlandse begeleider John Meerman (Leiden/Amsterdam Center for Drug Research (LACDR). Voor dit onderzoek ontving zij een subsidie van het Koningin Wilhelmina Fonds. De studie Biologie werd afgerond met het behalen van het hoofdvaktentamen betreffende de Chemische Carcinogenese in 1999.

Gedurende de periode van 1 april 1999 tot 31 maart 2005 heeft Cocky haar promotieonderzoek onder leiding van Dr. Hans Vos en Prof. dr. Rogier Bertina verricht op de afdeling Hematologie van het Leids Universitair Medisch Centrum. Een groot deel van de resultaten van dit onderzoek staat beschreven in dit proefschrift.

Sinds 1 april 2005 is Cocky werkzaam als postdoctoraal onderzoeker in de groep van Prof. dr. Piet Borst op de afdeling Moleculaire Biologie van het Nederlands Kanker Instituut.

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