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Regulation of human protein S gene (PROS1) transcription

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Chapter 7

General Discussion and Conclusions

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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.

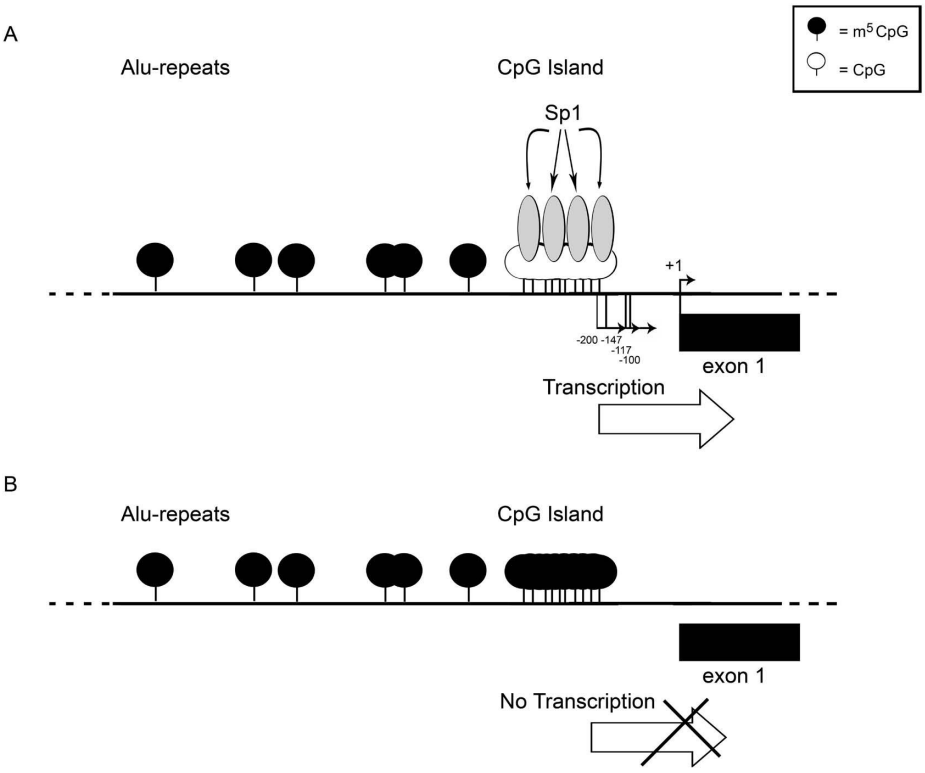

Figure 1 Schematic model for basal *PROS1* transcription. (**A**) Sp1 binding to the *PROS1* promoter relative to the transcription start sites, (**B**) Methylation of CpG dinucleotides results in loss of transcriptional activity. Note that this is a schematic representation of the *PROS1* promoter region, 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.

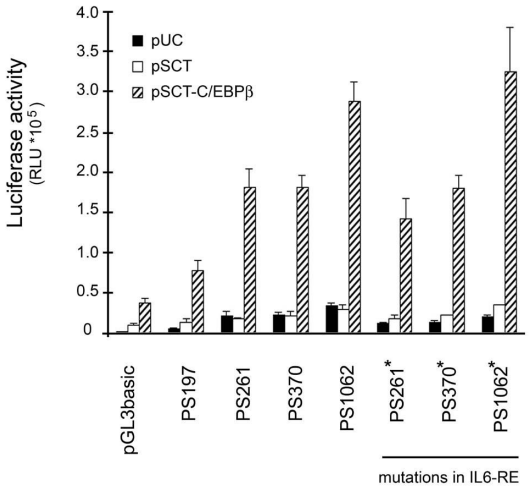

Figure 2 Induction of *PROS1* promoter activity by C/EBPβ in HeLa cells. 375 fmol *PROS1* construct and 25 ng expression vector was transfected into 60% confluent HeLa cell culture in 12 well plates. Total ng of transfected DNA was equalized by addition of pUC vector. Cells were grown for 48 hours after 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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