Regulation of human protein S gene (PROS1) transcription

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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°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.

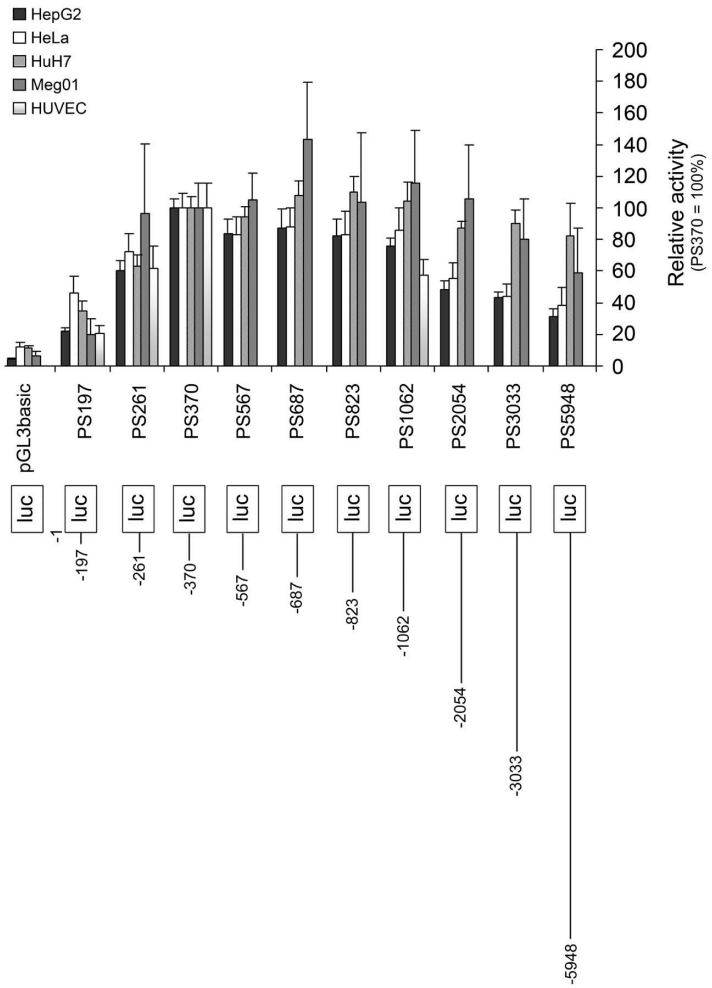


Figure 1 Transient expression of *PROS1* promoter-reporter gene constructs in cultured cells. Promoter activity of a series of 5' *PROS1* deletion constructs was measured as luciferase expression and is expressed as a percentage of the most active construct PS370. Note that the activity of PS370 was 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.

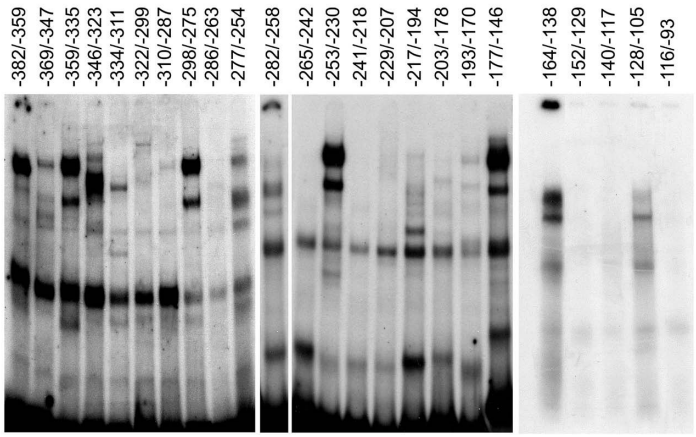


Figure 2 Primer walking: incubations of HepG2 Nuclear Extract and 24 consecutive ds oligonucleotide probes. 10 μg HepG2 Nuclear Extract was incubated with a single ds oligonucleotide probe for 20 minutes. Protein-DNA complexes were separated on 3-5% PAGE. The dried gel was exposed to film. Numbering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

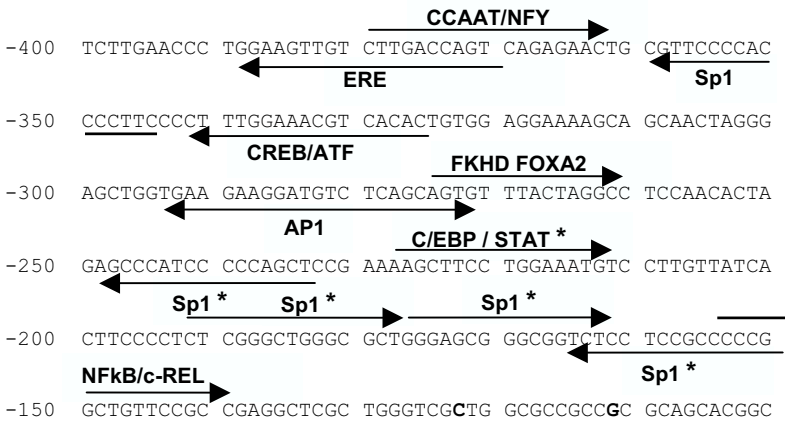


Figure 3 Putative transcription factor binding sites in the *PROS1* promoter.
Computational analysis of bp -400 to -100 of the *PROS1* promoter revealed the presence of several putative transcription factor binding sites within this region. *Interspecies conservation was above 95% for these sites.

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).

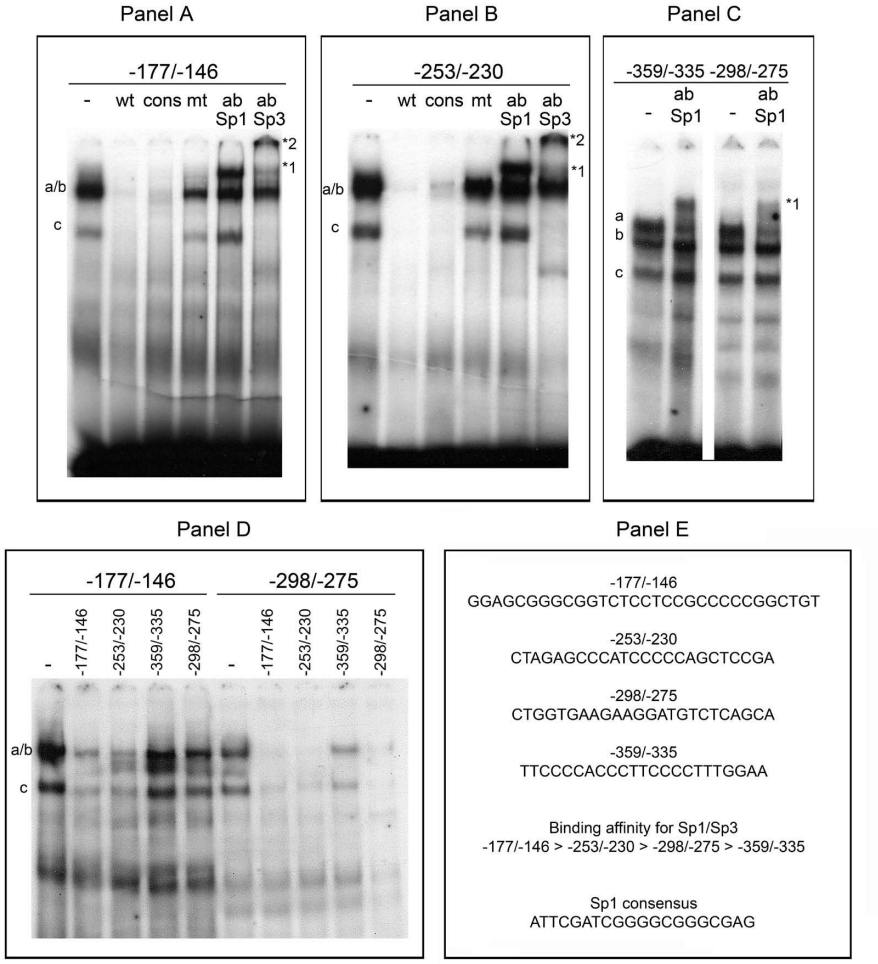


Figure 4 Sp1 and Sp3 bind to multiple sites in the *PROS1* promoter. 10 μg HepG2 Nuclear Extract was incubated with a single ds oligonucleotide probe for 20 minutes. Protein-DNA complexes were separated on 3% (panels C and D) or 5% (panels A and B) PAGE. The dried gel was exposed to film. In panels A 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.

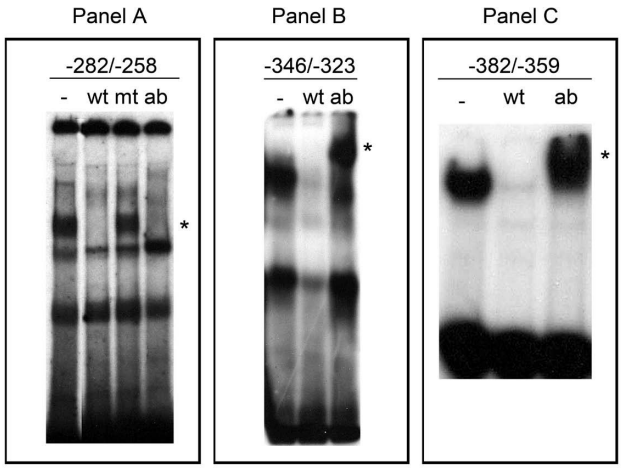


Figure 5 FOXA2, CRE/ATF and NFY bind to the *PROS1* promoter. 10 μg HepG2 Nuclear Extract was incubated with a single ds oligonucleotide probe for 20 minutes. Protein-DNA complexes were separated on 3% (panel A) or 5% (panels B and C) PAGE. The dried gel was exposed to film. Competition experiments were 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).

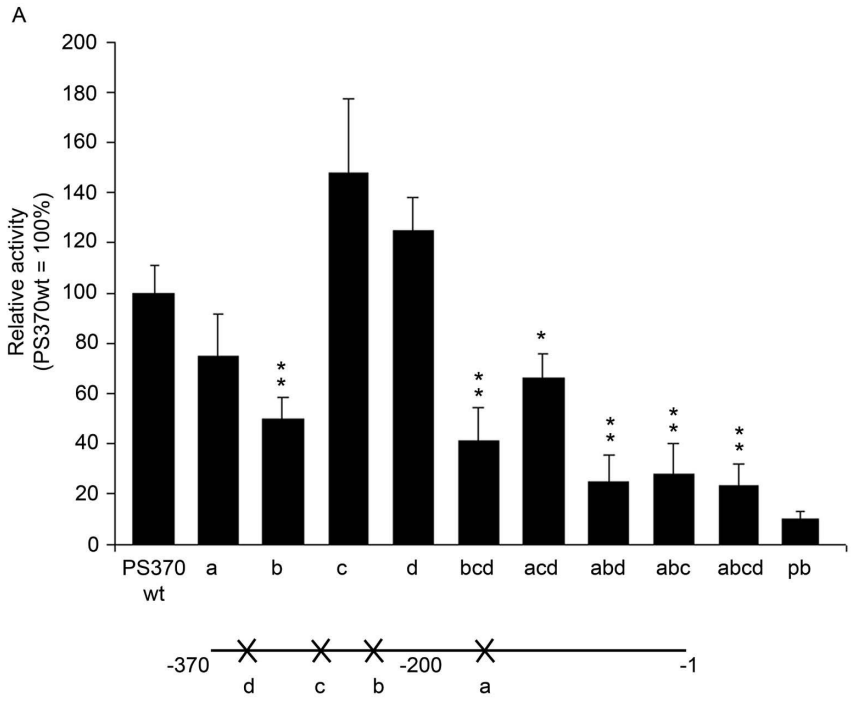
Computational analysis also revealed the presence of putative CREB/ATF and NFY sites around positions -346 to -323 and -382 to -359, respectively. Protein-DNA complexes were obtained in incubations with HepG2 nuclear extract and labeled primers at these positions. Evidence for binding of both families 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

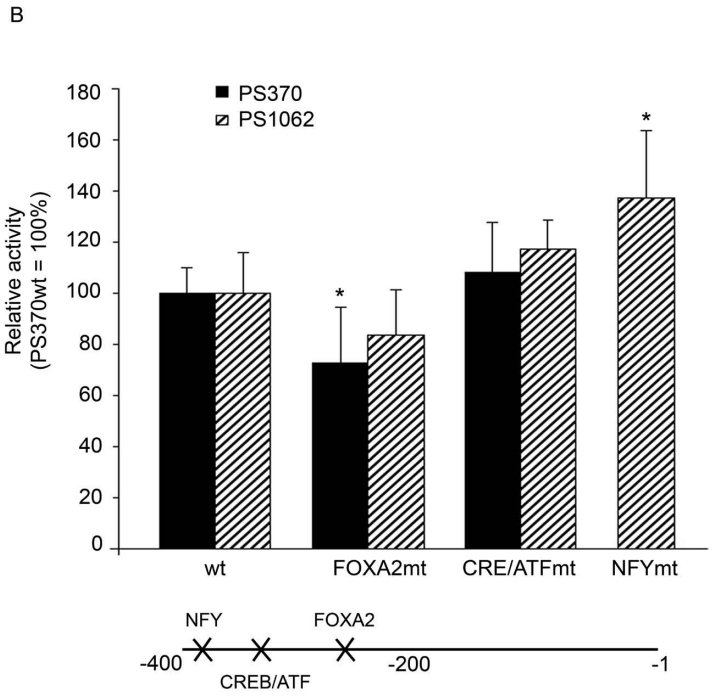
experiments depicted in Figure 6A, construct PS370 was modified to contain either one, three or four mutated Sp-binding sites. Whereas alteration of site a (-177/-146) or b (-253/-230) had a strong negative effect on promoter activity, mutation of site c (-298/-275) or d (-359/-335) had a mildly positive 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



site	loc'n	original seq	alterations
FOXA2	-275/-266	AGTGTTTACT	CGTGAGTACA
CREB/ATF	-337/-328	GAAACGTCAC	GAATGGTCAC
NFY/CCAAT	-382/-373	GACCAGTCAG	GCCTTGTTTG

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.

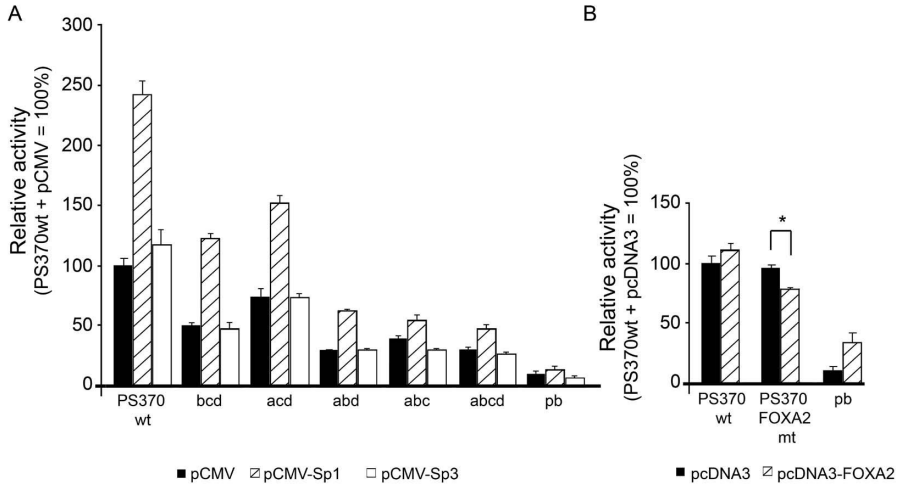


Figure 7 Cotransfections of HepG2 cells with PS370 variants and transcription factor expression vectors expressing Sp1, Sp3 or FOXA2. HepG2 transient transfections with wild type and mutant *PROS1* promoter-luciferase contructs were carried out. Luciferase expression of the wild type co-transfected with 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.

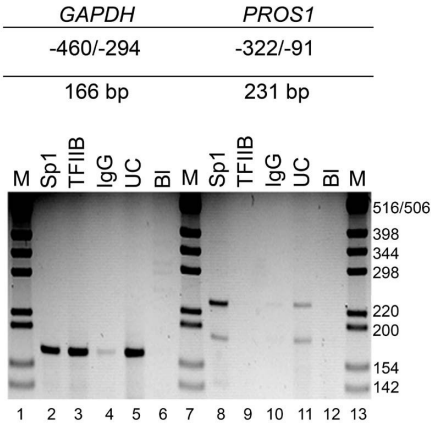


Figure 8 Sp1 binds to the *PROS1* promoter *in vivo.* ChIP assays were performed using sonicated chromatin isolated from HepG2 cells and an antibody specific for Sp1, TFIIB, or a non-specific IgG. The subsequently purified DNA samples were used as a template for PCR with primers specific for *GAPDH* (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.

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