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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Protein S (PS) is a vitamin K-dependent plasma protein that inhibits blood coagulation by serving as a nonenzymatic 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 levels through transcriptional regulation of the PS gene was investigated in this report. A minimal PS gene promoter 370 bp 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 electrophoretic mobility shift assay experiments, transient expression of mutant PS promoter-reporter gene constructs, and chromatin immunoprecipitations in HepG2 cells. At least four Sp-binding sites were identified. The two sites most proximal to the translational start codon 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 electrophoretic mobility shift assay could be positively identified in supershift assays. We identified binding sites for the hepatocyte-specific forkhead transcription factor FOXA2, nuclear factor Y, and the cAMP-response element-binding protein/activating transcription factor family of transcription factors. Their relevance was investigated using site-directed mutagenesis.

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 see Ref. 1). Protein S (PS) is a vitamin K-dependent plasma protein that functions as a nonenzymatic cofactor for activated protein C in the down-regulation of the coagulation cascade via proteolytic inactivation of coagulant factors Va and VIIIa (2–5). PS has also been shown to display activated protein C-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 throughout life by 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 is needed on the regulation of the PS gene, mRNA, and protein.

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 ~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) that 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), and recently the PROS1 promoter was shown to contain a forkhead box A2 (FOXA2)-binding site and an Sp1-binding site (33).

In this study, 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 transcriptional start codon, among which are multiple binding sites for the ubiquitous transcription factors Sp1 and Sp3, 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 EcoRI promoter fragment that was isolated from BAC clone 2513H18 from the CITBI-E1 genomic library (Research Genetics, Invitrogen).

The complete sequence was determined through automated sequencing (ABI PRISM and Beckman CEQ2000 sequencers) and deposited in GenBank™ under accession number AY605182. The PROS1 sequence was cloned into pcDNA3 (Invitrogen) and modified to contain nucleotides −5948 to −1 from the translational start codon.

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2 The abbreviations used are: PS, protein S; ChIP, chromatin immunoprecipitation; CREB/ATF, cAMP-response element-binding protein/activating transcription factor; EMSA, electrophoretic mobility shift assay; FOXA2, forkhead box A2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cell; MCS, multiple cloning site; NE, nuclear extract; NFY, nuclear factor Y; ELISA, enzyme-linked immunosorbent assay; CRE, cAMP-response element.
\textbf{Sp1 Regulation of Protein S Expression}

\textbf{TABLE 1}

\textit{Mutant oligonucleotides for site-directed mutagenesis}

Nucleotide sequences of the primers used for site-directed mutagenesis of putative transcription factor binding sites within the \textit{PROS1} promoter.

| Oligonucleotide | Position$^b$ | Sequence$^a$ |
|-----------------|-------------|-------------|
| Sp1mta          | −175/−144   | GAATGCGCGCGTTGCCCCGCTTTCC |
| Sp1mbb          | −262/−220   | CTCTCAGACCTCGAGTTCGCTGAGCC |
| Sp1mtc          | −306/−263   | CTTGAGAAGGTGGATAGGTCAGTCAAGTGGTTACTAGAG |
| Sp1mtd          | −366/−327   | CAACTGTGTCACACACCACCTCCTTGTAGG |
| NPYmt           | −391/−353   | TGAGAAGGTGGATAGGTCAGTCAAGTGGTTACTAGAG |
| CR4mt           | −350/−321   | GCGGACGCTGCGATTTCGAGAGAGG |
| FOXA2mt         | −287/−253   | GAGTTCCTCTACAGGTGTGACAGACCC |

$^a$ Numbering of the position is relative to the \textit{PROS1} translational start codon.

$^b$ Underlining of nucleotides denotes mutations.

\textit{PROS1} fragment −5948/−1 was cloned directly 5’ to the luciferase reporter gene in the pGL3basic vector (Promega, Madison, WI) after digestion with KpnI and XhoI that cut in the multiple cloning site. This construct was named PS5948-luc (contact authors for exact cloning details). PS5948-luc was linearized with KpnI and NdeI (at position −5798 in the promoter sequence) and was 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.

\textit{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.

\textit{Expression Vectors}—Expression vectors containing human Sp1 and Sp3 (pCMV-5p) transcription factors were a kind gift from J. Horowitz (Roswell Park Cancer Institute) (34, 35), and the expression vector containing murine FOXA2 (pcDNA3-FOXA2) was a kind gift from P. Holthuizen (University of Utrecht, Netherlands). The pcDNA3-FOXA2 vector was created by inserting the cDNA for FOXA2, as reported by Lai and co-workers (36), into the EcoRI cloning site of vector pcDNA3.

\textit{Cell Culture}—Leukocytes isolated from blood obtained from healthy donors (Sanquin Bloodbank, Leiden, Netherlands) were a kind gift from E. Paffen (Leiden University Medical Center, Leiden, Netherlands). The human hematoblastoma 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, Netherlands). The human umbilical vein endothelial (HUVEC) cells were a kind gift from J. Grimbergen (TNO Prevention and Health, Leiden, Netherlands). Primary human umbilical vein endothelial (HUVEC) cells were a kind gift from J. Grimbergen (TNO Prevention and Health, Leiden, Netherlands). HepG2, HeLa, and HuH7 cells were grown in minimal essential medium, 10% fetal bovine serum, 100 \( \mu \)g/ml streptomycin, 100 \( \mu \)g/ml penicillin, and 1× minimal essential medium nonessential amino acids (all purchased from Invitrogen). Meg01 cells were grown in RPMI 1640 medium, 20% fetal bovine serum, 100 \( \mu \)g/ml penicillin, 100 \( \mu \)g/ml streptomycin (all purchased from Invitrogen). HUVEC cells were grown in M199 medium (BioWhittaker, Walkersville, MD), 10% heat-inactivated human serum (Sanquin Bloodbank), 10% newborn calf serum (TNO Prevention and Health), 10 units/ml heparin (BioWhittaker), 150 units/ml endothelial cell growth factor, 100 \( \mu \)g/ml penicillin (BioWhittaker), and 100 \( \mu \)g/ml streptomycin (BioWhittaker). 24 h before transfection, HUVEC medium was replaced with heparin-free medium to prevent interference with the transfection.

\textit{PS Measurements}—Total PS antigen levels in culture media were determined by ELISA as described previously (37), 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) at 37 °C for 1 h 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).

\textit{Reporter Gene Assays}—1 × 10\(^6\) Meg01 suspension cells were used per transfection. All adherent cells (HepG2, HuH7, HeLa, and 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-well 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 \( \mu \)l of Tfx-20 lipids (Promega) per \( \mu \)g of transferred DNA. Meg01 cells were transfected using 5 \( \mu \)g of DAC-30 (Eurogentec, Seraing, BE) per \( \mu \)g of 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 PvuII and recircularization. Control vector pRL-SV40 (Promega), expressing the \textit{Renilla} luciferase, was co-transfected to correct for transfection efficiency in a 1:500 ratio to the total transfected amount (microgram) of DNA in HepG2, HuH7, and HeLa cell lines, and a 1:100 ratio in transfections with HUVEC and Meg01 cells. 250 ng of transcription factor expression vector was used for co-transfections, and expression vector without the transcription factor cDNA was used as a negative control. Cell extracts were harvested at either 24 (HepG2 and HuH7) or 48 h (Meg01, HUVEC, and HeLa) after transfection. Cells were lysed in 250 \( \mu \)l of Passive Lysis Buffer (Promega) per well, after which 20–100 \( \mu \)l 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, Germany).

\textit{Preparation of Nuclear Extracts}—Nuclear extracts (NE) were prepared according to the method of Dignam \textit{et al.} (38). Nuclear extract buffer contained 20 mm Hepes (pH 7.9), 0.2 mm EDTA, 100 mm KCl, 0.5 mm dithiothreitol, 0.2 mm phenylmethylsulfonyl fluoride, and the EDTA-free protease inhibitor mixture (Roche Applied Science). NEs were aliquoted and frozen at −80 °C until further use. Protein concentration of the NEs was measured with the BCA assay (Pierce).

\textit{Electrophoretic Mobility Shift Assays (EMSA)}—EMSAs were performed in a 13-\( \mu \)l binding reaction containing 10 \( \mu \)g of NE and 195 ng of denatured herring sperm DNA. EMSA buffers were purchased from Active Motif (Carlsbad, CA) and used according to the manufacturer’s recommendations. Double-stranded oligonucleotides were end-labeled using [\( \gamma \)-\textit{\textsuperscript{32}P}]ATP and T4 polynucleotide kinase. The position numbers for the oligonucleotides in Fig. 2 show their location respective to the \textit{PROS1} translational start codon. Reaction mixtures were incubated for 30 min with or without an unlabeled competitor. Subsequently, the \textit{\textsuperscript{32}P}-labeled double-stranded probe was added, and the incubation was continued for another 20 min. In supershift experiments, NE was incubated...
TABLE 2

PS protein levels over time in cell culture

| Cell type | PS levels (nM) | 24 h | 48 h | 72 h | 96 h |
|-----------|---------------|------|------|------|------|
| 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|      |

on ice for 10 min with the 32P-labeled double-stranded probe after which an anti-Sp1 (sc59x), anti-Sp3 (sc664x), anti-NFYA (sc711x), anti-CREB/ATF (sc270x), or anti-FOXa2 antibody (sc655x) (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% nondenaturing polyacrylamide gel, which was electrophoresed for 2 h at 200 V, 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 manufacturer’s instructions. Briefly, HepG2 cells were grown to 80% confluency in 75-cm² flasks after which chromatin was fixed in vivo by addition of 1% formaldehyde in culture medium. Fixed chromatin was isolated and sheared five times for 20 s to an average fragment size of 500 bp using a Soniprep 150 homogenizer (Sanyo/MSE, Kent, UK) at 25% power. Approximately 20 μg of sheared chromatin was incubated for 4 h with 3 μg of 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, and nonspecific IgG was used as a negative control. 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 protein K and RNase A. DNA was purified over a mini-column and resuspended in 100 μl of H₂O. 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’-GGA GGA AAA GCA GCA ACT AGG GAG-3’, −91/−106 (antisense) 5’-TGC GTC TGA GCC GTG-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.

RESULTS

Protein S Expression in Cell Lines—Protein S levels in cell culture medium were determined by ELISA analysis for HepG2, HuH7, HeLa, Meg01, and 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 with 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 PROS1 promoter constructs cloned upstream from the firefly luciferase reporter gene (Fig. 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-luc, 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 the 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 (39) 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, −114/−117, and −147/−150 (32). Here we show that maximal promoter activity is reached with a minimal PROS1 promoter of 370 bp. On basis of these observations we investi-
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gated transcription factor binding to the PROS1 region encompassing bp 100–370 upstream from the translational start. For this purpose, a series of overlapping double-stranded oligonucleotides covering the aforementioned region was designed. The duplexes were all 24 bp long, each having a 12-bp overlap with its neighboring probes. The liver cell line HepG2 was chosen for these more detailed experiments, because it had a high PS expression level and has been more intensely investigated than the HuH7 cell line. Incubation of HepG2 nuclear extracts with radiolabeled oligonucleotide duplexes located between positions −93 and −152 upstream from the PROS1 translational start did not result in the formation of protein-DNA complexes (Fig. 2). In contrast, almost all double-stranded oligonucleotide probes from duplex −152 to −129 up to −382 to −359 were complexed with nuclear protein. In further experiments we focused 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 (39), consensus binding sites for various transcription factors were found (Fig. 3). A high degree (>60%) of interspecies conservation (human, chimpanzee, rhesus monkey, dog, bovine, pig, mouse, and rat comparison) was found from −250 to −100 bp upstream of the PROS1 translational start codon with the VISTA alignment tools (40, 41). Binding sites for Sp1 and signal transducer and activator of transcription in this region were found to be conserved by more than 95%.

The protein-DNA complexes observed in incubations containing HepG2 nuclear extract and radiolabeled oligonucleotide duplexes.

![FIGURE 2. Primer walking, incubations of HepG2 nuclear extract and 24 consecutive double-stranded oligonucleotide probes. 10 μg of HepG2 nuclear extract was incubated with a single double-stranded oligonucleotide probe for 20 min. Protein-DNA complexes were separated on 3–5% PAGE. The dried gel was exposed to film. Numbering of the nucleotide positions is as in Fig. 1.](image)

![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.](image)
dence for binding of both families of transcription factors was subse-
quently established in supershift assays with transcription factor-spe-
cific antibodies (Fig. 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 construct. 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 (Fig. 4, panels A and B, and Fig. 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 Fig. 6A, construct PS370-luc was modified to contain either one, three, or four mutated Sp-binding sites. Although alteration of site a (−177/−146) or site b (−298/−275) 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-luc 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 ab and abd). The quadruplet mutant (abcd) and triple mutants, abc and abd, all displayed a residual promoter activity of ~20% (background pGL3basal 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 cau-
tiously 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-luc or PS1062-luc in HepG2 was only slightly influenced by mutation of the binding sites for FOXA2, CREB/ATF, or NFY (Fig. 6, panel B). 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—Co-transfection of HepG2 cells with PS370-luc and the Sp1 expression vector resulted in a 2.4-fold induction of promoter activity (Fig. 7, panel A). Sp3 had little or no effect on promoter activity. Co-transfections with triple mutants, in which only sites a, b, or c were still present, barely resulted in a decreased inducibility of PS370-luc 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-luc, 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 with 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, and indeed this site may even...
exert an inhibitory effect (1.37-fold stimulation versus 1.58 for the quadruple mutant). Upon alteration of all four binding sites, not all Sp1 trans-activation potential was lost (1.58 versus a background value of 1.35). This supports our earlier assumption that an additional Sp-binding site may be present close to site a (−177/−146) that was not mutated. Similar results were obtained with co-transfections 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-luc was stimulated slightly by co-transfection with the liver-specific transcription factor FOXA2 in HepG2 cells (p = 0.06) (Fig. 7, panel b). Upon mutation of the FOXA2-binding site at position −282/−258, this trend was absent. The empty vector, pGL3basic, was also strongly responsive to co-transfection with the FOXA2 expression vector. Co-transfections in HeLa cells produced similar results for the PROS1 constructs, but pGL3basic had even higher background activity.

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 start codon. 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, because 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 (Fig. 8) provides evidence for direct in vivo association of Sp1 with the PROS1 promoter in HepG2 chromatin.

**DISCUSSION**

In the general population, the prevalence of PS deficiency is estimated to be ~1 in 500 (44, 45). PS deficiency caused by genetic heterozygosity is present in 1–2% of the patients with deep vein thrombosis, however (46). Although 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 (47, 48). Pathological circumstances known to negatively influence PS levels are liver disease and disseminated intravascular coagulation (16, 49, 50). Overall, the regulation of PS levels (free and C4BP-bound) in normal and pathological circumstances is still poorly understood.

This study 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 (42) 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: site a (−177/−146) > b (−253/−230) > c (−298/−275) > d (−359/−335). Computational analysis showed that multiple Sp1-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-luc activity in HepG2 cells was reduced by ~50% upon mutation of this site. Mutation of site a resulted in a reduction of PS370-luc 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-luc, and obtained similar results as for PS370-luc (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 ~15%. Site b is the only Sp-binding site present in the −282/−161 construct. This result is therefore best compared with 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 c or b (Fig. 5, panel B) 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 (51–54). 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 (42). In this case Sp3 may be repressing PROS1 transcription through binding of Sp-binding sites c and d.

Co-transfection of Sp1 or Sp3 with PS370-luc triple mutant constructs confirmed that sites a and b are essential in PROS1 transcriptional regulation by Sp1 (Fig. 7). Mutant abd was responsive to Sp1 in the same manner as mutant acd. This was an unexpected finding because in basal activity experiments mutation of site c was found to have a stimulatory effect on PROS1 promoter activity (Fig. 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 co-transfection 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 (Fig. 7, panel b). The different construct sizes in both studies could cause the different effects of the mutations. As described above, Tatemaki et al. (33) used a construct that 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 start codon. Co-transfection of HepG2 cells with FOXA2 and PS370-luc or the PS370-luc FOXA2 mutant resulted in a slight induction of the wild type PS370-luc but not of the FOXA2 mutant construct. In addition, the empty vector pGL3basic was strongly responsive to co-transfection 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 start codon. 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 in the PROS1 promoter at position −346/−323. The CREB/ATF family has at least 10 members, all of which bind to a DNA consensus sequence called the CRE (reviewed in Refs. 55 and 56). Our finding suggests that 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, among which are steroid hormones. This signaling pathway is associated with the protein kinase A-dependent phosphorylation of nuclear proteins that belong to the CRE/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 (57, 58). NFY is unable to activate transcription by itself but increases the activity of neighboring enhancer motifs and participates in the correct positioning of other transcription factors at the start site. Mutation of the NFY-binding site in the 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 be necessary, however, 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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