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Promoter Paper

Promoter analysis of the bovine gene for seminalplasmin

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Abstract

In this study we mapped the transcriptional initiation site of the gene for seminalplasmin (SAP) by primer extension analysis, situated 125 nucleotides upstream of the translational initiation site of the SAP-specific mRNA. We showed that the TATA-box in position −30 of the SAP gene is part of a functional promoter. A 280 bp region of the 5′-flanking region exerted a strong positive effect on promoter activity. In this region we identified consensus sequences for the transcriptional control elements AP1, AP2, PEA3 and GATA.

Keywords: Seminalplasmin gene; Transcriptional initiation site; Primer extension analysis; Consensus sequence; Promoter activity; (Bovine)

The bovine accessory sex gland seminal vesicle produces the majority of seminal plasma proteins. A number of these proteins have been characterized at the mRNA level by cDNA cloning: SVSP109 [1], seminalplasmin (SAP) [2], ribonuclease BS1 [3], monocyte chemoattractant protein-1 (MCP-1) [4], acidic seminal fluid protein (aSFP) [5] and bovine C-type natriuretic peptide (CNP) [6]. Expression of the genes must be controlled by different mechanisms since the proteins SVSP109, SAP, aSFP und ribonuclease BS1 are apparently expressed exclusively in sex tissues of male sexually mature animals [7,2,5,3], whereas MCP-1 and CNP are also expressed beyond the sex tissues [4,8].

SAP is a member of the neuropeptide Y gene family which also contains neuropeptide Y, peptide YY and pancreatic polypeptide [9]. Although all genes of this family have a highly conserved structural organization, their gene products are functionally distinct [10]. SAP belongs to the group of acquired spermatozoal surface antigens [11]. The defined topical localization has been demonstrated using immunohistochemical techniques [11]. SAP provides a number of biological properties including inhibition of the uptake of Ca^{2+}-ions by epididymal spermatozoa [12], calmodulin antagonist function [13], inhibition of lymphocyte proliferation [14] and antimicrobial activity [15]. Expression of this species-specific protein is apparently under androgen control [2]. The compact gene with the approximate size of 2.1 kb is organized into four exons and three introns (Fig. 1). The sequence of the gene has been described elsewhere [16]. To evaluate the role of transcriptional mechanisms in controlling the expression of SAP we characterized the promoter region of the SAP gene.

Poly(A)$^+$ RNA of bovine seminal vesicles was isolated using the *PolyATtract mRNA Isolation System* from Promega (Heidelberg, Germany). 10 nmol of a ^{32}P-labelled [17] antisense exon 1 oligonucleotide (PE; 5′-GACAGCTCTAGAGGCAG-3′; position 87–69) was annealed to 5 μg poly(A)$^+$ RNA for 30 min at 55°C. Primer

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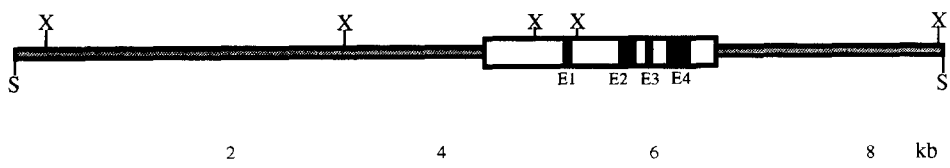

Fig. 1. Restriction map of the SAP specific clone pSAP23 [16]. The sequenced part of the genomic clone was marked by a solid box, exons were indicated by black bars. S = *Sma*I, X = *Xba*I.

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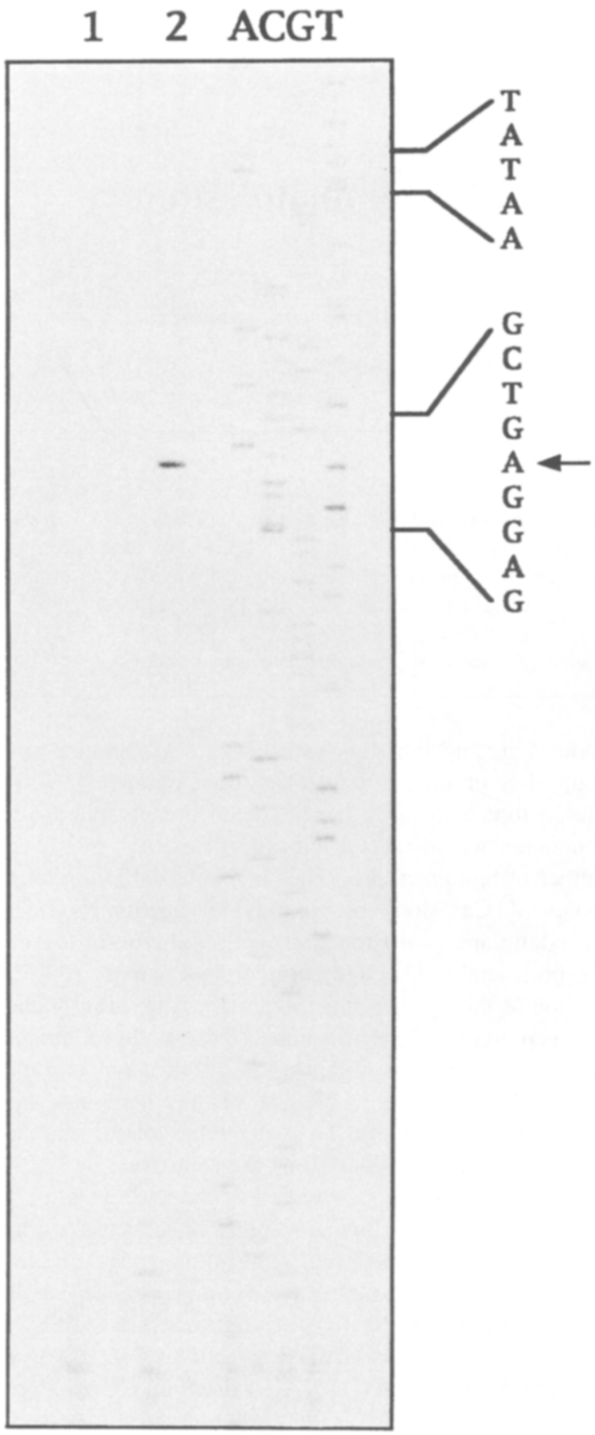

Fig. 2. Definition of the transcription initiation site of the bovine seminalplasmin gene by primer extension analysis. Lane 2: primer extended products from 5 μg seminal vesicles poly(A)$^+$ RNA primed with ^{32}P-end-labelled antisense exon 1 oligonucleotide (PE: 5′-GACAGCTCTAGAGGCAG-3′; position 87–69). Lane 1: no mRNA. A dideoxy sequencing reaction [21] of the SAP gene 5′ flanking region using the same primer is also shown. The putative transcription initiation site is marked by an arrow. The TATA-box is indicated. DNA sequence analysis was performed using the computer program of the University of Wisconsin Genetics Computer Group [22].

extension reaction with Reverse Transcriptase Superscript from Gibco BRL (Eggenstein, Germany) was performed according to the protocol of Ausubel et al. [18]. The primer extension product was precipitated and co-electrophoresed on a 6% polyacrylamide-urea gel with a sequence reaction in which the same oligonucleotide was used as a primer and pSAP23 [16] was used as template. The nucleotide 'A', 125 nucleotides upstream of the translational start of SAP mRNA, was identified as the starting-point (Fig. 2).

In order to define the functional role of the DNA region upstream of the structural part of the SAP gene we amplified fragments of this region by PCR and cloned them upstream of the chloramphenicol acetyltransferase (CAT) reporter gene into the plasmid pCATenhancer (Promega). Taq polymerase, substrates and reaction buffer for PCR were from Pharmacia (Freiburg, Germany). The protocol for amplification was: 5 min at 94°C, followed by 30 cycles of 45 s at 94°C, 45 s at 55°C, 3 min at 72°C and finally 45 s at 94°C, 45 s at 55°C, 15 min at 72°C. The SAP specific clone pSAP23 [16] was used as a template. The oligonucleotide primers for amplification of the various fragments are listed as follows:

ON1: 5′-tgact**ctgcag**TGAATGTGTATGTGTCAGGC-3′ (nt −700 to −681)

ON2: 5′-tcagt**ctgcag**TCTAGAGGCAGAGTGGTCA-3′ (antisense nt 81 to 61)

ON3: 5′-tcagt**ctgcag**TCTAGACCGAATGGTTAAC-3′ (nt −280 to −262)

ON4: 5′-tcagt**ctgcag**GAGTTGAAGGGAGGGGGCTC-3′ (antisense nt −51 to −71).

All primers bear *Pst*I-sites at their 5′-end which were used for cloning into the pCATenhancer. The primer combinations for PCR were ON1 + ON2, ON3 + ON2 and ON3 + ON4, yielding pCATeSAP14, pCATeSAP4 and pCATeSAP32. All constructs were transiently transfected into HeLa cells (ATCC No. CCL2) by calcium phosphate transfection [19]. Plasmids pCATenhancer and pCATcontrol (Promega) served as negative and positive controls. A plasmid containing the β-galactosidase gene (pSVβgal, Promega) was cotransfected with the pCAT constructs. Cell extracts were prepared and assayed for chloramphenicol acetyl transferase and β-galactosidase activity following the instructions of the manufacturer (Promega). The results from the β-galactosidase assay were used to normalize the CAT activity. Fig. 4 shows the results of the CAT assay. pCATeSAP14 (base pairs −700 to 81) possessed a promoter activity similar to that of pCATcontrol. Deletion of 416 bp from the 5′-end of the insert (construct pCATeSAP4 (base pairs −280 to 81)) improved promoter activity significantly. Further deletion of the TATA-box region (construct pCATeSAP32 (base pairs −280 to −51)) resulted in the complete loss of promoter activity. These results provide strong evidence that the TATA box at position −30 upstream of the transcriptional initiation site of the SAP gene is part of the functional promoter of this gene. Based upon the observation that pCATeSAP14 (base

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-869  tctttgaccctgagcccagtggagattgtaacgggctagggatggtggcatgttgttgggcgttcctggcatcactcagtcctgtaagac

-779  atcagtgcccaagtgacccctggatggaaatgggggcacattgccttgcaaggggtctgtataccaggcctgctggacctgaatgtgtat

          Sp1                                        AP2
-689  gtgtcaggctgggtgtacaggtggctgactcagctgcttctggggaggggactgaccttggcctcctcccaccccctctcccaactcttga

-599  acagagggagacatagtggagtgtccagaggggtccctcgtccaagacttcacagacagcaggtcaagagccaaggttatctctcccatc

-509  ctgcctccagtggagagagattttccccccacaggtcttgtcactcctcagttctcctccaacatattatgaagcaggggctgaaaagac

-419  tggcttttggaagatgcagctacaggtcccacagactggaaaaaatattctggcttcctgcctgcatctcctctcccccaccatggacag

-329  gctagaaaaagacagaatgtcaaccctggtcctaggggtcgtcccagaaatctagaccgaatggttaacttcctcaggaggctacacctgc

          AP2                                                                  AP1
-239  tgggggagggctgtagggtgaagatggggttctgactagacatcctgtccagaggtaccctgagtacaagtgtgggggctccctcctcgg

                    GATA                                                                PEA3     AP2
-149  ctccttggacgtggagatatcatgtactgtctgagtgtaatcacagcgatctgctgtgggggggaggggaagaggaagagccccctccc

            AP2                      TATA                              +1
-59   ttcaactccccctcccctttcagaccaggatataagccccacacggaatgcactgagctgAGGAGGCCACAGCCCCACCTGTAGAAGTCCA   exon1

 32   GCCCTTCGGACTCCTCTGGCCTTCCATCTCCTGACCACTCTGCCTCTAGAGCTGTCGCTgtgagtggcctgggacccagcaatggtctgg
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Fig. 3. Nucleotide sequence of the 5′ end of the bovine SAP gene. The transcription initiation site is indicated by (+1). The locations of potential binding sites for transcriptions factors [20] are indicated.

pairs −700 to 81) directed a lower CAT activity than pCATeSAP4 (base pairs −280 to +81), we hypothesize that the region −700 to −280 contains binding sites for factors that inhibit promoter activity. A binding site for an inhibitory transcription factor could not be identified by sequence comparison to consensus sequences of known transcription factors [20]. Fig. 3 shows a number of transcriptional control elements such as AP1, AP2, PEA3 and GATA which were localized in the region up to −280.

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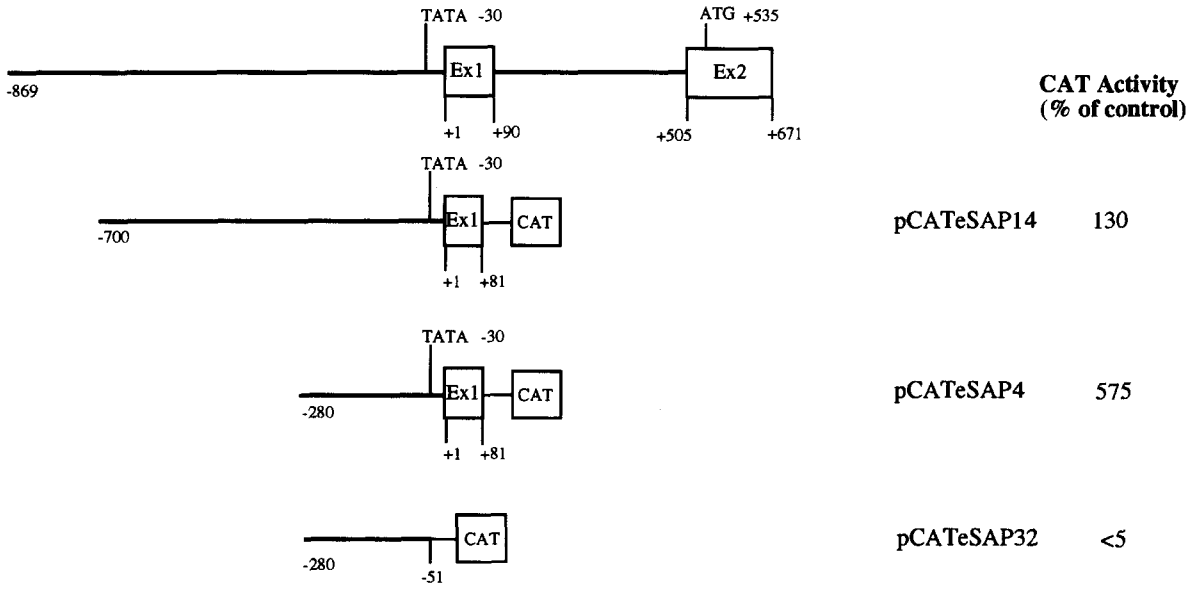

Fig. 4. Promoter analysis of SAP gene. pCAT derivates were obtained by amplification of SAP specific plasmid pSAP23 [16] by PCR, using primer pairs with restriction sites, restriction of the products and cloning into pCATenhancer (Promega). HeLa cells were grown in Dulbecco's modified Eagle's medium containing 4.5 mg/ml glucose and 10% fetal calf serum at 37°C in 5% CO_2-air [18]. The DNA mixture employed for transfection comprised 5 μg pCAT construct and 6 μg pSVβgal (Promega) purified by Qiagen 100 columns (Diagen, Hilden, Germany). All constructs were verified by double strand sequencing of both strands using the dideoxy chain termination method of Sanger et al. [21]. The transfection efficiency was estimated from 21 transfection experiments with pSVβgal. The mean normalized β-gal activity was 1.25, with an S.D. of 0.15 and a CV of 12.75. The 95%-confidence interval of the mean was $1.14 \leq 1.25 \leq 1.36$.

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