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Cloning of the 5′ regulatory regions and functional characterization of the core promoters of ovine PLAU (u-PA) and SERPIN1 (PAI-1)

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ABSTRACT

The activation of plasminogen plays a crucial role in various extracellular proteolytic events (fibrinolysis, cell migration, ovulation and involution of the mammary gland). In the present study we describe the isolation of the 5′ proximal and distal promoter regions of ovine PLAU (urokinase plasminogen activator, u-PA) and SERPIN1 (plasminogen activator inhibitor 1, PAI-1) genes for the first time in ruminants. Analysis of the 5.645 kb 5′-flanking region of u-PA revealed a putative TATA-less promoter. In contrast the isolated 2.787 kb 5′-flanking region of PAI-1 included a TATA-box. It should be noted that both genes lack the initiator motif around the transcription start site. The two genes share a number of transcription factor binding sites, namely Nuclear Factor-kappa B, Stimulating Protein 1 and Activating protein 1, suggesting co-expression of the two genes. Moreover, additional, not shared, transcription factor binding sites were identified in u-PA and PAI-1. More important of these are the cis-regulatory elements for plasminogen activator inhibitor 2 located in the distal promoter region of u-PA, suggesting an involvement of the other specific inhibitor in the regulation of ovine u-PA gene expression, and the three stress response elements sites present in the proximal and distal promoter of PAI-1. Different genomic fragments of the two 5′ flanking regions were directionally cloned into a suitable reporter vector upstream of a promoter-less luciferase gene. Transient transfection into bovine mammary epithelial (BME-UV) cells demonstrated that the regions of −384/+27 and −382/+22 for the u-PA and PAI-1genes, respectively, potentially function as core promoter regions.

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1. Introduction

The cascade of reactions leading to plasminogen activation is controlled by a complex network of molecular interactions between plasminogen activators (urokinase-type, u-PA and tissue-type, t-PA) and at least two types of plasminogen activator inhibitors (PAI-1 and PAI-2). Plasminogen activation plays a crucial role in the control of fibrinolysis, cell migration, wound healing, matrix degradation and cell signaling (Alfano et al., 2005; Toriseva and Kahari, 2009; Watanabe et al., 2006). The serine protease u-PA catalyzes the conversion of inactive zymogen plasminogen to the enzymatically active plasmin. The conversion of plasminogen to plasmin plays a crucial role in maintaining vascular patency while also contributing to a variety of extracellular proteolytic events and the activation of other growth factors. Plasmin, also, participates in fibrinolysis by degrading fibrin and in tissue remodeling by degrading extracellular matrix (ECM) and activating other matrix degrading proteases (Fay et al., 2007; Mondino and Blasi, 2004; Politis, 1996).

The mammary gland is a prime example of tissue that undergoes extensive tissue remodeling throughout its growth and its development. At puberty, mammary development accelerates with ductal elongation and branching, followed by lobulo-alveolar development and maturation during pregnancy. The final result of this process is the development of the secretory epithelium during lactation. After weaning or following cessation of milking in ruminants, major well-controlled degenerative events occur as the mammary gland is remodeled in preparation for the next lactation. It is well known that the

Abbreviations: Ang II, Angiotensin II; AP1, Activator Protein 1; AP4, Activator Protein 4; BME, Bovine Mammary Epithelium; C/EBPb, CCAAT/Enhancer Binding Protein beta; CRE-BP, cAMP Response Element Binding Protein; DEX, Dexamethasone; ECM, Extracellular Matrix; EGF, Epidermal Growth Factor; ETS, E-twenty six; FBS, Fetal Bovine Serum; GATA-1, GATA Binding Factor 1; GATA-2, GATA Binding Factor 2; GATA-3, GATA Binding Factor 3; gDNA, genomic DNA; IGF-1, Insulin-like Growth Factor 1; Inr, transcriptional initiator; MyoD, Myogenic Differentiation 1; NF-κB, Nuclear Factor-kappa B; NF-1, Nuclear Factor 1; PAI-1, Plasminogen Activator Inhibitor 1; PAI-2, Plasminogen Activator Inhibitor 2; PRL, Prolactin; Sp1, Stimulating protein 1; STRE, Stress Response Element; t-PA, tissue-type Plasminogen Activator; TGF-β, Transforming Growth Factor-β; TSS, Transcription Start Site; u-PA, urokinase Plasminogen Activator; u-PAR, urokinase Plasminogen Activator Receptor.

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characteristics of mammary gland involution are very different in ruminants compared to other species. The main difference lies in the fact that the involution process is less extensive in ruminants (Politis, 1996). Because u-PA related genes are implicated in the regulating aspects of the involution process (Rabot et al., 2007; Theodorou et al., 2009) it is reasonable then to examine whether the less extensive involution in ruminants could be related to difference in the organization of genes implicated the plasminogen activating cascade.

The main physiological inhibitor of u-PA is PAI-1. Besides, inhibiting plasminogen activation, PAI-1 has a multi-faceted role with relation to cell adhesion/migration and thus is involved in wound healing (Lijnen, 2005). PAI-1 is a secreted protein belonging to the serpin (serine protease inhibitor) family. Serpins exist in two states. The first is the native state, also known as S-state. The second state, also known as R-state, is accompanied by a dramatic conformational change of the molecule due to the interaction of the serpin with a target protease or the cleavage of the region that interacts with the protease (reactive center loop) (Bruch et al., 1988).

During the last few years, u-PA and PAI-1 have been extensively analyzed at the molecular level, especially in human and rodents. Completely characterized cDNA and genomic sequences, including the promoter region, have been previously reported for human, mouse, rat, pig and chicken. In more detail, the 5′ regulatory region of PLAU (henceforth referred to as u-PA gene) has been characterized in human (Riccio et al., 1985), mouse (Degen et al., 1987), pig (Irigoyen et al., 1997) and chicken (Leslie et al., 1990), while the 5′ regulatory region of SERPIN1 (henceforth referred to as PAI-1 gene) has been characterized in human (Bosma et al., 1988), mouse (Prendergast et al., 1990) and rat (Bruzdzinski et al., 1990). Recently, the ovine cDNAs for both u-PA and PAI-1 have been characterized by our group (Theodorou et al., 2009; Theodorou et al., 2010) but information concerning the sequence and functional characterization of the promoter regions is lacking.

According to our current knowledge, no relevant information regarding the transcriptional regulation of u-PA and PAI-1 genes has been reported in ruminants so far. In view of the key biological role of the plasmin-plasminogen system that is mediated by u-PA and PAI-1 and the wide variability of u-PA and PAI-1 expression levels observed between different types of tissues (Theodorou et al., 2009; Theodorou et al., 2010), the characterization of the promoter regions of the ovine family members is an important task, in the process to elucidate the cellular mechanisms regulating ovine u-PA and PAI-1 genes transcription activity. Molecular dissection of the ovine u-PA and PAI-1 promoter regions would definitely provide the concrete basis for an investigation of the gene regulation mechanism of these important enzymes. In the present study, we describe the isolation of the 5′ regulatory region and the functional characterization of the proximal promoter of ovine u-PA and PAI-1 genes.

2. Materials and methods

2.1. Isolation of the ovine u-PA and PAI-1 (distal/proximal) 5′ flanking region via a genome walking approach

The Universal GenomeWalker kit (Clontech, USA) was used to generate new ovine u-PA and u-PAR-specific genomic PCR fragments. Genomic DNA (gDNA) was isolated using the Illustra blood genomicPrep Mini Spin Kit (GE Healthcare, England) and separately digested with the restriction enzymes *EcoRV*, *DraI*, *PvuII* and *StuI* to produce four ovine genomic DNA pools, termed DL-1, DL-2, DL-3 and DL-4, respectively. According to the manufacturer's instructions, each pool of digested genomic fragments was ligated to the GenomeWalker adaptor provided by the kit. The ligated DNA pools were used as templates for two rounds of Touchdown PCR (Don et al., 1991) (primary and nested) with gene-specific primers and adaptor primers (Table 1 and Fig. 1). The amplification reactions were performed in a 50 μl total volume, with the 50x Advantage Genomic Polymerase mix (Clontech, USA), on a 2720 thermal cycler (Applied Biosystems). The first round of PCR was carried out with an initial denaturation step at 94 °C for 5 min, followed by: (a) 7 cycles at 94 °C for 25 s, 72 °C for 3 min and (b) 32 cycles at 94 °C for 25 s, 67 °C for 3 min and a final polymerization step at 67 °C for 7 min. The second round of PCR was carried out with an initial denaturation step at 94 °C for 5 min, followed by: (a) 5 cycles at 94 °C for 25 s, 72 °C for 3 min and (b) 20 cycles at 94 °C for 25 s, 67 °C for 3 min and a final polymerization step at 67 °C for 7 min. For the first genome walking step, gene-specific primers were designed according to the submitted cDNA sequences of the ovine u-PA and PAI-1 (FJ803199 and GQ855215, respectively). Subsequently, the second and third genome walking steps were performed using ovine u-PA and PAI-1 gene-specific primers that were appropriately synthesized according to the nucleotide sequence obtained of the previous genome walking step. Three and two sequential rounds of genome walking were performed to ultimately isolate the 5′ flanking region presumably containing ovine u-PA and PAI-1 cognate promoter, respectively.

2.2. Molecular cloning and DNA sequencing analysis of the ovine u-PA and PAI-1 PCR genomic fragments

The PCR fragments obtained through the genome walking approach were agarose gel purified using the Qiaquick Gel Extraction Kit (Qiagen, USA) and directly sequenced for confirmation through aligning against known, homologous sequences. It was then TA-ligated to vector pGEM-T Easy (Promega, USA), transformed into *Escherichia coli* JM109 competent cells and successful transformants were selected through blue-white screening. Plasmid derived DNA was isolated using the Qiaprep Miniprep Kit (Qiagen, USA) and three independent

Table 1
Nomenclature, nucleotide sequence and corresponding length of the oligonucleotide primers utilized for the molecular cloning and isolation of the ovine uPA and PAI-1 5′ flanking regions, through a genome walking approach.

	Name	Oligonucleotide sequence (5′➔3′)	Length	Technique course
u-PA	GW_u-PA_outer	CAGGACCTGGACTGTGGGGACTTGTAG	27 nt	1st walking
	GW_u-PA_inner	ATCTCCGCCGGGGTCTAGGGCTCCAGTCTC	30 nt	1st walking
	GW_u-PA_outer (2nd)	TGTGTTCATCTTTTGGTGCAGTCATCTTG	29 nt	2nd walking
	GW_u-PA_inner (2nd)	AGGAATCCATGAACAAGTGAGAACAAAGC	29 nt	2nd walking
	GW_u-PA_outer (3 rd)	CCTTGTGTGTGTCAGGCAGAGTTCTAG	27 nt	3rd walking
	GW_u-PA_inner (3rd)	CAGGATTCCAGTCAAGTGGCAATGCTAC	28 nt	3rd walking
PAI-1	GW_PAI-1_outer	GTTTGTTCCTGCTGCCACTGCGGGTGTTG	29 nt	1st walking
	GW_PAI-1_inner	GTGTGGCTCTCTCTGGACGGTGCTCTTG	28 nt	1st walking
	GW_PAI-1_outer (2nd)	GATTCCCTCCCGTCTTTCTCTCTTTTCTGC	30 nt	2nd walking
	GW_PAI-1_inner (2nd)	AGGTGTGCTGGGTGGGGTGGTCGTCTTAC	29 nt	2nd walking
	AP1 (outer)	GTAATACGACTCACTATAGGGC	22 nt	1st, 2nd and 3rd walking
	AP2 (inner)	ACTATAGGGCACGCGTGGT	19 nt	1st, 2nd and 3rd walking

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