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Functional mapping of the bovine Doppel gene promoter region

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Abstract

The *PRND* gene encodes Doppel (Dpl), a protein that is strongly expressed in testis and at much lower levels in other tissues. Despite the recent discovery of Dpl involvement in spermiogenesis and in apoptotic death of cerebellar neurons, respectively in *wild type* and transgenic mice, the physiological role of this prion-like protein remains unknown. To better understand which factors may contribute to the modulation of *PRND* activity, a study of the bovine promoter region was performed. First, the transcription start site of *PRND* mRNA was identified using an innovative fluorescently labelled oligonucleotide extension (FLOE) method. The initiation site mapped 129 nt upstream of the protein coding sequence and represents a refinement of a previous assignment based on RACE. Second, deletion mutants of the 4530 nt encompassing 2704 nt 5′ of the bovine *PRND*, exon 1, intron 1, and the first 6 nt of exon 2, have been investigated with CAT-reporter assays in order to identify critical elements for the activation of the gene. The results showed that the region −323/+32 (+1 is the transcription start site mapped by FLOE) represents the promoter region and contains positive *cis*-acting elements (CCAAT and E box) confirming previous reports with the mouse gene. Additional regulatory elements, including binding sites for repressor molecules, have been identified upstream of that region and in the first portion of intron 1, suggesting a complex tissue-specific regulation of Doppel gene expression.

Keywords: *PRND*; CAT assay; FLOE; Primer extension

1. Introduction

Doppel was first discovered in knock-out $Prnp^{0/0}$ mice, which express unusual chimeric transcripts generated by intergenic splicing with *Prnp* (Moore et al., 1999). The structure of *Prnd* is known in *M. musculus*, *H. sapiens*, *B. taurus* and *O. aries* (GenBank Acc. Nos. U29187, AF106918, AY017310, AY017311): the murine gene has three exons, and all the other species two exons including the entire ORF in the second exon (Comincini et al., 2001). Prion (PrP) and Doppel (Dpl) proteins share common biochemical and structural features, with approximately 25% amino acid identity and the same backbone topology (Mo et al., 2001). Despite their molecular similarities, the expression patterns of the proteins are quite different. PrP is ubiquitous but predominantly expressed in the central nervous system; whereas Dpl is primarily located in the testis although it has also been detected at low levels in the heart and transiently in brain endothelial cells of newborn mice (Li et al., 2000). One peculiar feature of Dpl is the toxicity that follows ectopic expression in the brain, causing a specific ataxic phenotype (Moore et al., 1999; Rossi et al., 2001). However, unlike PrP, Dpl is incapable of transition to a neuropathogenic protease-resistant form and there is no evidence of its implication in prion diseases (Mo et al.,

Abbreviations: nt, nucleotide(s); βGal, β-galactosidase; CAT, Cm acetyltransferase; Dpl, Doppel protein; DMEM, Dulbecco's modified eagle medium; ELISA, enzyme-linked immunosorbent assay; ORF, open reading frame; FBS, fetal bovine serum; FLOE, fluorescently labelled oligonucleotide extension; kb, kilobase(s) or 1000 bp; kDa, kilodalton(s); MCS, multiple cloning site(s); *Prnd*, murine Doppel gene; *PRND*, Doppel gene; *Prnp*, murine Prion gene; *PRNP*, Prion gene; PrP, Prion protein; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate; SV40, simian virus 40; UTR, untranslated region(s).

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2001). Instead, the study of *Prnd* knock-out murine lines has uncovered an essential role for Dpl in spermiogenesis (Behrens et al., 2002), which has also been confirmed in humans (Peoc'h et al., 2002).

Numerous studies have been performed at the gene level and the protein has been structurally analysed in various species, however investigations on the regulation of *PRND* expression are lacking. Two recent works addressed the problem in the murine model, showing that the expression of *Prnd* is regulated by the Brn-3a and Brn-3b transcription factors (Calissano et al., 2004) and that two *cis*-acting elements (a CCAAT box and an E box) are critical for the expression of the murine gene (Nagyovà et al., 2004).

Following this line of study we characterized the regulatory signals at the bovine *PRND* locus. We identified the exact transcription start site performing a primer extension on testicular mRNA and refined the position mapped previously by RACE (Tranulis et al., 2001). In addition, we developed a set of deletion mutants covering a 4530-nt genomic region and comprising 2.5 kb 5′ of the gene, exon 1 and the entire intron 1 and tested the mutants in transfected cells with a CAT-reporter assay. The results allowed us to define the promoter region of the bovine *PRND* gene and to highlight regulatory signals acting at this locus.

2. Materials and methods

2.1. Primer extension by fluorescently labelled oligonucleotide extension (FLOE)

FLOE is an innovative technique that makes use of fluorescently labelled oligonucleotides in the primer extension reaction, thus producing data in a faster and easier way compared to radioactive labelling (Fekete et al., 2003). In brief, total RNA extracted from bovine testis with TRIzol® (Invitrogen) was used for the extension reaction: in a volume of 25 μl, 8 μg RNA was reverse-transcribed with 5 pmol 5′ FAM TGTGTCGGGATCTTAGGGTCT DPLB1L primer (MWG), designed on the bovine *PRND* mRNA sequence, using SuperScript™ II Reverse Transcriptase (Invitrogen). The reaction mixture was lyophilized and sent to a sequencing service (CRIBI, http://bmr.cribi.unipd.it), they returned the GeneScan® electropherogram runs (Applied Biosystems). ROX-500 Size Standards (Applied Biosystems) were used in the capillary electrophoresis as described (Fekete et al., 2003), and also in a parallel standard dideoxy sequencing reaction performed with 5 pmol of the FAM labelled primer used for reverse transcription; this served to estimate the exact transcription start site. Dideoxy sequencing was done with Sequenase® Version 2.0 (United States Biochemical) on a PCR fragment amplified on a plasmid construct as described below (see Section 2.2). A second sequencing reaction was performed on the same template with an ABI PRISM® Big Dye™ Terminator v1.1 kit (Applied Biosystems) and the unlabelled DPLB1L primer to assist in the correct interpretation of the primer extension results. The analysis of the electropherograms was performed with Chromagna V1.0.3 (kindly provided by D. Miller).

2.2. PCR amplification and construction of CAT-reporter plasmids

The primers employed for the PCR amplifications were designed with the available bovine genomic sequence and are shown in Table 1. The amplifications that yielded the *PRND* CAT-reporter constructs were done on genomic DNA or on a BAC clone containing the *PRND* region (Hills et al., 2003) (30 cycles at 94 °C 1 min, 58 °C 1 min, 72 °C 3.5 min); additional constructs were derived from parent clones by PCR and/or by subcloning. In particular, the PCR product used in the standard dideoxy sequencing reaction was obtained priming 1 ng pCAT®3-Basic/Δ6 plasmid with 10 pmol DPLB1L (32 cycles at 94 °C 20 s, 64 °C 40 s, 72 °C 40 s). PCR products were purified with the GeneElute™ PCR Clean-up (SIGMA); pCAT®3 vectors (Promega) were

Table 1
Oligonucleotides used in the design of deletion mutants

Primer	Sequence (5′ to 3′)	Primer pair—corresponding deletion mutant
1U	CAACCG**CTCGAG**CACAGTCTTTGTGTCGTGGAGC [−2675/−2653]	1U/3L−Δ1
5U	CAACCG**CTCGAG**GGGTGGACAGAACACAGAGTTC [−2175/−2153]	5U/3L−Δ2
6U	CAACCG**CTCGAG**CTCTTCTGGCATCATCTGCTGG [−1652/−1630]	6U/3L−Δ3
7U	CAACCG**CTCGAG**CTGGCAAGTGCTCTCATTTGTC [−1186/−1164]	7U/3L−Δ4
8U	CAACCG**CTCGAG**GCCCTAACCAGACTCCTTGAAC [−569/−547]	8U/3L−Δ5
2353U	CG**ACGCGT**CGAGAGTGATGTAACCCCACACTC [−323/−301]	2353U/3L−Δ6
9U	CAACCG**CTCGAG**ATAGCTGGGCAGGTCCAGGAGT [+30/+52]	9U/3L−Δ7
3208U	CAACCG**CTCGAG**GAGAAAGCTTGTCTGAGCTCACTG [+533/+557]	3280U/3L−Δ8
2353U	CG**ACGCGT**CGGAGTGATGTAACCCCACACTC [−323/−301]	2353U/10L−Δ355
10L	CAACCG**CTCGAG**TATGTAGGAGCTGCAGGGGGAG [+11/+32]	
3L	CAACCG**CTCGAG**CGGATTCTGCAACAGAAAAGGCTGG [+1855/+1831]	

Primers are based on the AY017310 sequence (underlined); nucleotide position refers to the transcription start site mapped in this paper; the extra sequence at the 5′ end contains *Xho*I or *Mlu*I restriction sites (bold); the right column shows the primer pair used to assemble a deletion mutant and its code.