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Biochimica et Biophysica Acta 1731 (2005) 209 – 214
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Promoter paper

Involvement of upstream stimulatory factor in regulation of the mouse *Prnd* gene coding for Doppel protein

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Received 5 May 2005; received in revised form 4 October 2005; accepted 10 October 2005
Available online 26 October 2005

Abstract

Promoter of the *Prnd* gene coding for the Prion-like protein Doppel contains two critical cis-regulatory elements, NF-Y consensus motif and canonical E-box. Here, we studied a role of the upstream stimulatory factor (USF) in the E-box-mediated activation of *Prnd* transcription. Co-expression of USF-1 with the luciferase reporter gene driven by the −185/+27 *Prnd* promoter fragment resulted in several fold increase of the luciferase activity. Conversely, mutations within the E-box led to a significantly reduced *Prnd* promoter activation. USF-1 binding was supported by the gel shift assay, supershift with USF-1 antibody and UV cross-linking. The activation capacity of the related USF-2, c-Myc and HIF-2α proteins was lower compared to USF-1 suggesting that USF-1 is the major E-box-binding transcription factor regulating the *Prnd* promoter.

Keywords: Doppel; Promoter; Gel shift; Supershift, USF; E-box

Doppel (Dpl) was identified as a protein with the structural homology to Prion protein (PrP^c) [1,2]. Whereas PrP^c expression is mainly observed in the central nervous system and its conversion into pathological PrP^{Sc} form leads to development of the prion disease [3], Dpl does not share these characteristics [4]. Under physiological conditions, high Dpl level is consistently observed only in the male reproductive tract. However, Dpl can be transiently detected in the brain endothelial cells of mice around one week old [5], suggesting that physiological expression of Dpl is not only tissue-specific but also developmentally-regulated. Although the exact biological function of Dpl is still unclear, its deregulation has serious consequences: absence of Dpl due to homozygous disruption of the *Prnd* gene causes the male sterility [6] and ectopic expression of Dpl in the CNS of PrP knockout mice leads to death of the Purkinje cells accompanied by ataxia in elderly animals [7,8]. Moreover, Dpl expression has been recently reported in the human astrocytomas and its increased level was found in the malignant brain tumours [9]. Albeit all these data suggest a high complexity in the Dpl regulation, there is a little information on how this complexity is being accomplished at the molecular level. Involvement of Brn-3a and Brn-3b transcription factors in the control of *Prnd* gene expression has been proposed on the basis of experiments with the Brn-3a knock-out mice and with the neuroblastoma cell lines overexpressing Brn-3a or Brn-3b, but the genomic position of their binding site(s) has not been determined [10]. On the other hand, functional analysis of the *Prnd* promoter revealed importance of NF-Y transcription factor for the basal Dpl expression in the cells of testicular origin [11]. The deletion analysis localized the core *Prnd* promoter region to a −185/+27 position with respect to the transcription start site and targeted mutagenesis disclosed two critical regulatory elements [Nagyova et al, 2004]. The binding of NF-Y transcription factor to the −121/−96 region containing a CCAAT box was confirmed by the gel shift experiments, but regulatory proteins which could operate via the −182/−177 region containing a typical E-box with a canonical CACGTG sequence have remained unknown. This study was aimed at the identification of transcription factor(s) binding to this motif.

Based on our previous experimental experience and taking into account the fact that Dpl expression is mostly detected in the testis, we decided to use two mouse cell lines. The GC1-spg cell line of the testicular origin that corresponds to a stage between the type B spermatogonia and the primary spermatocytes was obtained from the American Type Culture Collection

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doi:10.1016/j.bbaexp.2005.10.002

(ATCC cat. No. CRL-2053). The 15P-1 cell line with the characteristics of Sertoli cells was kindly provided by Prof. F. Cuzin, (University of Nice, France). Both cell lines were maintained in DMEM with 10% FCS (BioWhittaker, Verviers, Belgium), 2 mM glutamine and 40 μg/ml gentamicine (Lek Slovenia) at 37 °C in 5% CO_2.

Computer search for the regulatory elements using the MatInspector Professional program (threshold 0.75 http://www.genomatix.de) revealed the highest predictive significance for the binding of an upstream stimulatory factors (USF1/USF2) to the E-box sequence at −182/−177 position upstream of the transcription start site. To prove the computer prediction, we co-transfected the GC1-spg cells by the −185/+27 *Prnd*-Luc construct, which was prepared by placing the core *Prnd* promoter in front of the luciferase reporter gene in pTAL-Luc vector as described earlier [11], with either pCMV-USF-1 or pCMV-USF-2 eukaryotic expression plasmids (a generous gift from Prof. Michele Sawadogo, University of Texas, TX). Transfection was performed using the GenePorterII reagent (Gene Therapy Systems) and the reporter expression was evaluated with the Dual-Luciferase Reporter Assay System (Promega). As shown in Fig. 1A, activity of the −185/+27 *Prnd*-Luc promoter construct increased almost three fold upon co-expression with the USF-1 transcription factor, whereas the positive effect of USF-2 was significant, but much lower than that of USF-1. Similar data were obtained using the 15P-1 cells line (not shown).

USF transcription factors are basic helix–loop–helix proteins that generally act as transcriptional enhancers of diverse genes with tissue-restricted and/or developmentally-related expression pattern [12]. Their activity is accomplished via the mutual homo- and heterodimerization events. In most cell lines and tissues, heterodimers between USF-1 and USF-2 are the dominant species, followed by USF-1 homodimers, while USF-2 homodimers are less abundant [13]. The clear preference of *Prnd* promoter for activation by USF-1 may indicate that it predominantly binds the USF-1 homodimers.

To confirm a direct involvement of the CACGTG E-box sequence in the USF-1 mediated activation, we also examined the activity of −185/+27 *Prnd-mut*-Luc reporter construct containing the CA**TTA**G sequence with a mutation that disturbs the canonical E-box motif [Nagyová et al., 2004]. In accord with our expectation, the E-box mutation resulted in a significant down-regulation of the reporter gene expression to 57–58% of the wild-type promoter irrespective of whether USF-1 transcription factor was co-transfected or not (Fig. 1A). This fact suggests that the E-box mutation abolished the binding of relevant transcription factors inherently present in the cells and that it also completely prevented the activation by the ectopically supplied USF-1. It seems likely that at least a part of the intrinsic *Prnd* E-box binding molecules corresponds to USF-1. The remaining *Prnd* promoter activity is then conferred by the NF-Y transcription factor binding to its intact consensus motif located downstream of the E-box sequence, as demonstrated previously [11].

The above data indicated that USF-1 actively participates in the transcriptional activation of the *Prnd* promoter via the E-box sequence. This assumption was further strengthen by a co-transfection of the −185/+27 *Prnd*-Luc promoter construct with increasing amounts of the USF-1 expression

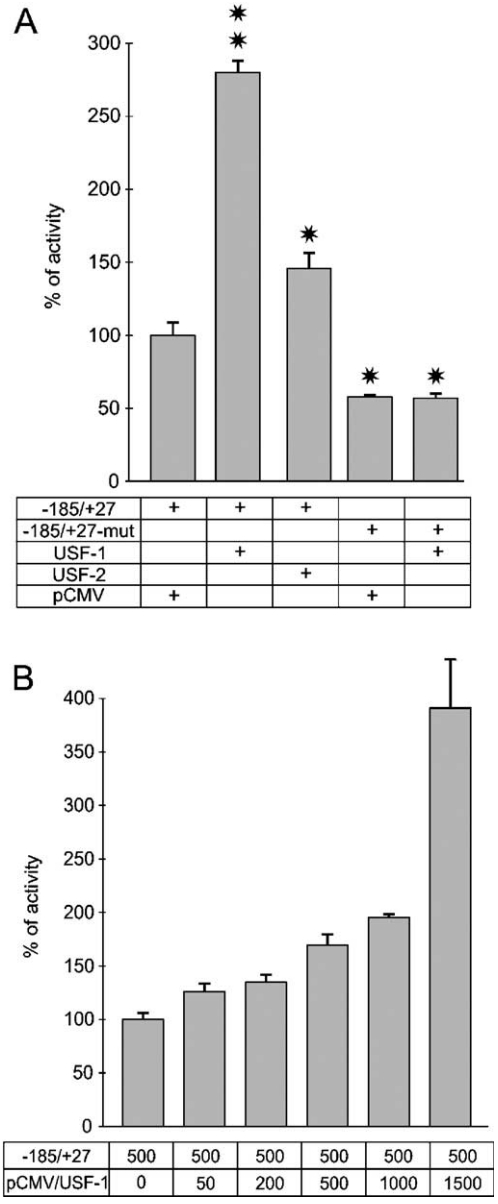

Fig. 1. Transcriptional activities of the −185/+27 Prnd-Luc promoter construct in GC1-spg cells. (A) The cells grown in a subconfluent monolayer were co-transfected with the equivalent quantities of the wild type promoter construct (0.75 μg) and either USF-1 or USF-2 (1.5 μg) expression plasmids. The promoter construct containing an E-box mutation (0.75 μg) was used as a control of the DNA-binding specificity. Promoter activities are expressed in % relative to the inherent activity of the wild type promoter construct in the absence of USF, which was set to 100%. Mean values with standard deviation were calculated from at least two independent experiments performed in triplicates. Asterisks indicate the level of significance related to the wild type promoter construct co-transfected with the empty pCMV plasmid: *for $P < 0.01$ and **for $P < 0.001$ calculated by Student's t test. (B) The cells were co-transfected with the constant amounts of the promoter construct in combination with increasing amounts of the USF-1 expression vector and decreasing amounts of an empty pCMV plasmid to balance the total DNA input as indicated below the chart. Resulting promoter activities are illustrated as in part A.