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Methylation of the prominin 1 TATA-less main promoters and tissue specificity of their transcript content

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ABSTRACT

Prominin 1 (PROM1, CD133) is a unique transmembrane glycoprotein encoded by the *PROM1* gene. It is a cell surface marker of various stem cells including hematopoietic, prostatic epithelial, pancreatic, leukemic, liver cancer, and colorectal cancer stem cells. Here, we studied tissue specificity of *PROM1* transcription isoforms and the methylation level of its two main promoters (P1 and P2) in different human cell lines. Only transcripts lacking the 4th exon (the CD133.s1 form) were expressed in cell lines studied. Moreover, these transcripts, if sufficiently abundant, were initiated simultaneously and independently from both promoters P1 and P2. In cell lines with low levels of the total *PROM1* transcript, the transcription was likely initiated from other promoters. Promoter P1 was hypermethylated in all cell lines under study, and therefore, methylation can hardly play an important role in its regulation. In contrast, the methylation of promoter P2 was tissue specific, and hypomethylation of this promoter is probably necessary but not sufficient for efficient transcription of the *PROM1* gene. Therefore, we report an unusual instance of different mechanisms of transcription activity regulation for two closely located promoters of the same gene.
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1. Introduction

Prominin 1 (PROM1, CD133, AC133) [1] has been first detected in murine neuroepithelial stem cells [2]. A homologous human protein was described by [3].

PROM1 is a cell surface marker [4,5] expressed in progenitor and stem cells [6,7] including hematopoietic, prostatic epithelial, pancreatic, leukemic, liver cancer and colorectal cancer stem cells (for recent review see [8]). Transcripts of the *PROM1* gene were found in many human tissues and cell lines, but not in mature peripheral blood leucocytes [9], human embryonic stem (ES) cells, trachea, small intestine, NT2 cells, diffuse-type gastric cancer, and colorectal cancer [8]. The presence of the PROM1 protein is more characteristic of non-differentiated cells of embryonic epithelium [2,10], precursors of endothelial cells [11], prostate epithelial stem cells [12], and myogenic cells [13]. It is also present in some tumors, e.g. in retinoblastoma [3,14] and teratocarcinoma [3], as well as in leukemia [4].

The *PROM1* gene transcription regulation is rather complicated and poorly understood. A possible involvement of five alternative TATA-less promoters was suggested to explain the pattern of transcripts differing in the lengths and sequences of 5′ untranslated regions (UTRs). Two of them (P1 and P2, see Fig. 1A) were shown to be active in *in vitro* tests with a reporter gene. A common transcription initiation site was assigned to exon 1A (Fig. 1) by RACE technique [9], and an mRNA transcribed from exon 1A was found to be the major transcript [8]. Other major transcription start points were also reported, one of them belonging to exon 1B (Fig. 1). As reported by Shmelkov et al. [9], the choice between the transcription start sites depended on tissue. In particular, liver, kidney, pancreas, placentas lung spleen, and colon expressed both exon 1A and exon 1B containing transcripts, whereas brain and ovary contained only exon 1B transcripts, and prostate, fetal liver and small intestine — only 1A transcripts. Additional start sites were reported by these authors to exist in testis. Only 3 of the 5 hypothetical promoters (P1, P2, and testis specific promoter P4) were experimentally proved to drive transcription. Exons 1A and 1B were shown [9] to be spliced to exon 2 (Fig. 1B, C).

Transcripts of the *PROM1* gene containing first translated exons 2 (contains translation start site), 3, 4 and 5 were found to be represented by two products of alternative splicing (Fig. 1B–D) [15]. A shorter transcript lacks 27 nucleotides of the 4th exon which corresponds to the deletion of 9 amino acid residues from the N-terminal extracellular domain of the protein [15]. It can be mentioned that each of these isoforms is represented by a group of transcripts variantly spliced in the distal region of the gene [16]. The murine prominin gene, ~60% identical with the human counterpart, is also represented by transcripts alternatively spliced in the same gene region [17]. However, the *PROM1* genes of rodents lack loci homologous to P1 or P2 [8], suggesting that they are regulated differently.

The regulation of the *PROM1* gene transcription can be further complicated by epigenetic factors, as suggested by experiments with artificial *in vitro* methylation of both promoters P1 and P2 leading to suppression of a reporter gene expression [9].

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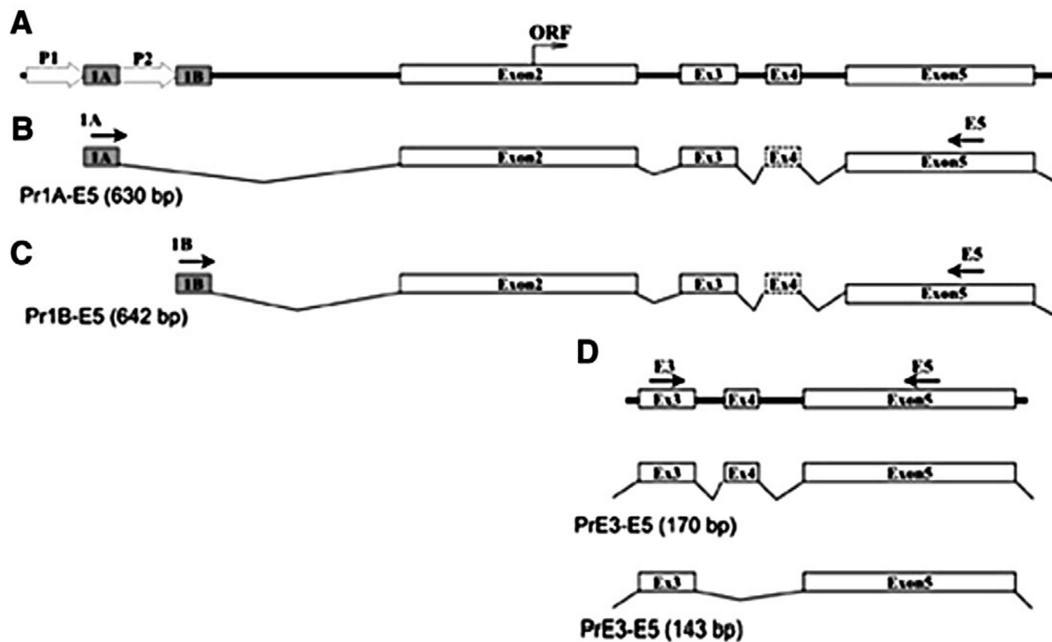

Fig. 1. Schemes of the *PROM1* gene 5′ terminus (A) and corresponding transcript variants (B, C). The 4th exon (Ex4) boxed by a dotted line can be spliced out in some transcripts. 1A/E5 and 1B/E5, the primer pairs used to selectively amplify transcripts from P1 and P2, respectively. (D) part of the PROM1 gene containing exons 3–5, and two possible PCR amplification products (below), containing and lacking the 4th exon; E3/E5, the primer pair used to amplify this part of the *PROM1* gene. 5′-UTR exons and other exons are designated by gray and empty rectangles, respectively; promoters P1 and P2 are depicted as empty arrows; introns are shown as thick straight lines; ORF, translation initiation site; PCR primers are depicted as horizontal arrows. The size of the expected amplified fragments is indicated in parentheses.

To get a deeper insight into the regulatory features of the *PROM1* gene expression, we studied tissue specificity of the *PROM1* isoform transcription using a panel of 12 human cell lines of different origins. We have shown that (*i*) in all cell lines studied, *PROM1* transcripts were represented solely by the shortened form lacking exon 4, and (*ii*) the cell lines with a relatively high *PROM1* gene transcription level contained transcripts of this gene initiated from both promoters, P1 and P2.

For several cell lines with various levels of the *PROM1* transcript, we determined the methylation status of the gene promoters P1 and P2 *in vivo*. Methylation of the “minimal” P1 promoter was not tissue specific thus probably being of no importance to the transcripts level in the cells. On the contrary, methylation of promoter P2 is tissue specific. Hypermethylation of the promoter unambiguously correlated with a low level, whereas its hypomethylation typically but not always correlated with high level of transcripts. Therefore, hypomethylation of the promoter is probably necessary, but insufficient, for high level transcription of the *PROM1* gene.

2. Materials and methods

2.1. Cell cultures

Human cell lines HEK293 (CRL-1573), A549 (ATCC CCL-185), A431 (ATCC CRL-1555), HepG2 (ATCC HB-8065), Hela (CCL-2), Jurkat (TIB-152), NT2/D1 (CRL-1973), NCI-H23 (ATCC CRL-5800), NCI-H460 (ATCC HTB-177) and Tera-1 (HTB-105) were cultured in the media recommended by ATCC. The HaCaT cell line was kindly provided by Christine Leib-Mösch and grown as described previously [18]. The NGP-127 cell line was kindly provided by Paul S. Meltzer and grown as described previously [19]. All cell lines were cultured with addition of 10% fetal calf serum (FCS) at 37 °C and 5% CO_2.

2.2. RNA isolation and RT-PCR

Total RNA was isolated from cell lines using an RNeasy Mini RNA purification kit (Qiagen). All RNA samples were further treated with DNase I to remove residual DNA. cDNA synthesis was performed according to the manufacturer's protocol using random hexamer primers (Perkin Elmer), with (+RT) or without (−RT) addition of PowerScript II reverse transcriptase (Clontech). The efficiency of cDNA synthesis was equal in all preparations, as verified by RT-PCR with *β-actin* and *GAPDH* gene specific primers (see Table 1).

A cDNA equivalent of 20 ng total RNA was used as template in each PCR, and the amplification was carried out using specific primers (Table 2; Fig. 1B–D). The standard reaction was performed as follows: 95 °C for 30 s, T_m °C for 30 s, 72 °C for 60 s (where T_m is the annealing temperature in Table 1), in a 50 μl volume containing 10 pmol of each primer and 1 U Taq DNA polymerase, recombinant (GibcoBRL). After 18 cycles of RT-PCR, 6 μl of the reaction mixture was taken every three cycles, and the amplification was continued up to the 39th cycle. The amplification products were analyzed by gel electrophoresis in 1.5–2.0% agarose gels. Gel images obtained by charge-coupled device (CCD) camera systems were quantitated using the Gel-Pro analysis software (Media Cybernetics). Expression levels were estimated from

Table 1
Primers used in the experiments

Primer	Primer sequence, 5′–3′	Annealing temperature
E3	ATATGAATCCAAAATTGATTATGAC	55
E5	CACATTTGTTACAGCAACGACAC	55
1A	GCCATGCTCTCAGCTCTCC	56
1B	TGACTAGGGCGGGAGCA	56
BisF1	GTTATTAGTATTGGTATTGTTGTATATTAAGG	54
BisR1	CTCCTCACTATACACCCCCAATAC	54
BisF	GAGGGTAGTTGTATTTTAAGTAAGG	51
BisR	AAATAAAAACCCAACTACTCACC	51
P2F	TGGGAGGCGGGCTCTCCGA	66
P2R	GCTCCTGCTCCCGCCCTAG	66
WDHD1-For	GTAACCTGGTCTCCCTGTGG	57
WDHD1-Rev	GATGTCTTTCCACTGGGGTC	57
GAPDH-For	TTAGCACCCCTGGCCAAGG	55
GAPDH-Rev	CTTACTCCTTGGAGGCCATG	55
β-actin For	GAAGATCAAGATCATTGCTCCTCC	55
β-actin Rev	CTGGTCTCAAGTCAGTGTACAGG	56

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