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Promoter paper

Isolation, sequencing, and functional analysis of the TATA-less murine ATPase II promoter and structural analysis of the ATPase II gene☆

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Abstract

The P-type Mg^{2+}-ATPase, termed ATPase II (Atp8a1), is a putative aminophospholipid transporting enzyme, which helps to maintain phospholipid asymmetry in cell membranes. In this project we have elucidated the organization of the mouse *ATPase II* gene and identified its promoter. Located within chromosome 5, this *gene* spans about 224 kb and consists of 38 exons, three of which are alternatively spliced (exons 7, 8 and 16), giving rise to two transcript variants. Translation of these transcripts results in two ATPase II isoforms (1 and 2) composed of 1164 and 1149 amino acids, respectively. Using RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) we identified multiple transcription start sites (TSS) in messages obtained from heart, lung, liver, and spleen. The mouse *ATPase II* promoter is TATA-less and lacks a consensus initiator sequence. Luciferase reporter analysis of full and core promoters revealed strong activity and little cell type specificity, possibly because more flanking, regulatory sequences are required to cause such tissue specificity. In the neuronal HN2, N18, SN48 cells and the NIH3T3 fibroblast cells, but not in the B16F10 melanoma cells, the core promoter (−318/+193 with respect to the most common TSS) displayed significantly higher activity than the full promoter (−1026/+193). Serial 5′ deletion of the core promoter revealed significant cell type-specific activity of the fragments, suggesting differential expression and use of transcription factors in the five cell lines tested. Additionally distribution of the TSS was organ specific. Such observations suggest tissue-specific differences in transcription initiation complex assembly and regulation of *ATPase II* gene expression. Information presented here form the groundwork for further studies on the expression of this gene in apoptotic cells.

Keywords: P-type ATPase; Promoter; Mouse ATPase II; Apoptosis; Aminophospholipid translocase; Phospholipid-translocating ATPase; Phospholipid-transporting ATPase

1. Introduction

The distribution of phospholipids in the animal cell plasma membrane is not random. Instead, the aminophospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE) are commonly present in the inner leaflet of the phospholipid bilayer whereas zwitterionic phospholipids, phosphatidylcholine (PC) and sphingomyelin are present in the outer leaflet [1–3]. Polar head groups present in plasma membrane lipids cannot cross a hydrophobic membrane interior freely and for this reason a system of sophisticated proteins evolved in eukaryotic cells to facilitate such transbilayer movements of phospholipids (reviewed in [4]).

At least three distinct protein-based activities are involved in the regulation of membrane lipid asymmetry and in generation

Abbreviations: APLT, aminophospholipid translocase; ATF, Activating transcription factor; gDNA, genomic DNA; HGP, Human Genome Project; htgs, unfinished High Throughput Genomic Sequences: phases 0, 1 and 2; NCBI, National Center for Biotechnology Information; RefSeq, NCBI Reference Sequence (see: http://ncbi.nih.gov/RefSeq/); RLM-RACE, RNA ligase-mediated rapid amplification of cDNA ends; TF, transcription factor; TSS, transcription start site; USF, upstream stimulating factor; WDG, Wilson disease gene

☆ The sequence data presented in this article have been entered into the EMBL/GenBank database under accession numbers DQ503476-DQ503480.

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and maintenance of the aminophospholipid gradient. These are energy dependent inwardly directed ('flippases') and outwardly directed transporters ('floppases') as well as energy independent bi-directional transporters ('scramblases') (reviewed in [3]). Flippases are highly specific for PS and are responsible for sequestering it from the cell surface. Floppases are, with a few exceptions, nonspecific toward phospholipids head groups and have been associated with the ABC (ATP binding cassette) class of membrane transporters. Scramblases are also nonselective toward head groups and play a major role in randomizing the distribution of the newly synthesized phospholipids [2,5,6]. The interplay between these specific and nonspecific transporters results in generation and maintenance of asymmetry and gradient phospholipids distribution, which is responsible for multiple morphological and functional characteristics of living cells as well as homeostasis that is essential for their survival (reviewed in [1]).

As mentioned earlier, phosphatidylserine (PS) is normally sequestered to the inner leaflet of the plasma membrane due to the action of a flippase [1,7–9], which is chemically named as aminophospholipid translocase (APTL), phospholipid-translocating ATPase or aminophospholipid flippase [1–3]. During programmed cell death, the loss of the aminophospholipid asymmetry, which is typical for healthy cells, leads to the appearance of PS on the cell surface. This externalization of PS serves as a signal for phagocytosis of apoptotic lymphocytes, neutrophils, and neuronal cells by macrophages and microglia [6–8,10]. Our earlier studies to analyze the mechanism of this PS externalization established that the enzymatic activity of APLT is inhibited during apoptosis, which could be the prime reason for the observed PS externalization [10].

Several lines of experimental evidence have been provided linking the PS-translocating enzymatic activity of APLT to the ATPase activity of the mammalian enzyme ATPase II [aminophospholipid transporter, class I, type 8A, member 1 (Atp8a1)], first cloned from bovine chromaffin granules [11]. Both APLT activity and the ATPase II molecule require PS and Mg^{++} to carry out ATP hydrolysis. Furthermore, both the activities are inhibited by vanadate (VO_4^{2-}) and N-ethylmaleimide. Both activities are also inhibited in the presence of increased intracellular Ca^{2+} concentration (>0.2 μM) and selective depletion of intracellular Ca^{2+}, which causes an inhibition of the Ca^{2+}-ATPase, leaves APLT and ATPase II completely active [3,12]. Of mammalian ATPase II cDNAs, in addition to the bovine ATPase II cDNA, its murine and human homologs have been cloned [13,14]. A yeast strain with a mutant gene (*Drs2*), which is homologous to the mammalian *ATPase II* gene, showed inhibited translocation of PS across the plasma membrane [15]. *Drs2* is one of sixteen yeast genes arranged in five classes and bearing characteristics of P-type ATPases. The bovine and murine ATPase II proteins are most homologous to the yeast gene product Drs2 [16].

Our earlier studies demonstrated that overexpression of mouse ATPase II cDNA [14] in the mouse hippocampus-derived hybrid neuroblastoma (HN2) causes an increase in APLT activity measured by PS translocation [17,18]. In the same cell line, overexpression of ATPase II rendered cells insensitive to stress-induced inhibition of APLT activity, which is otherwise inhibited in the cells expressing only endogenous ATPase II. ATPase II is a member of a type-4 subfamily of the P-type ATPases. This evolutionarily conserved superfamily of P-type of ATPase is widely expressed in eukaryotes and prokaryotes and consists of 5 subfamilies. The type-4 subfamily is expressed only in the higher eukaryotes while the members of the type-3 subfamily are almost exclusively found in plants and fungi. The core of a typical P-type ATPase contains a P-type signature sequence DKTGT[L,I,V,M][T,I,S] and also harbors 10 putative transmembrane domains. Its molecular weight ranges from 70 to 150 kDa. With an exception of the type-4 subfamily, which includes proteins believed to translocate phospholipids, members of four other subfamilies are involved in translocating cations across the plasma membrane. The aspartic acid residue (D) in the P-type signature sequence accepts γ-phosphate from ATP during a phosphorylation cycle and this phosphotransfer is an essential driving force behind all P-type ATPases mediated transport [19,20].

Tissue specific expression of ATPases have been well characterized [14,21]. Surprisingly, among the multiple members of P-type ATPases known so far [11,16,19,21], the promoters for only a few P-type ATPase genes have been identified. Among others, these genes are, respectively, human *ATPase II* and *ATPase7B* [22,23]. *ATPase7B* and the closely related homolog *ATPase7A* yield homologous, copper transporting, P-type ATPases. A defect in *ATPase7A* results in Menke's disease whereas malfunction of the *ATPase7B* gene product leads to Wilson disease [23–27]. Menke's disease is associated with overall copper deficiency due to impaired export of copper from intestinal cells. Wilson's disease, in contrast, is caused by copper accumulation predominantly in the liver, brain, and kidneys. Previous studies by our research group characterized the promoter and the 5′UTR in human P-type aminophospholipid transporting *ATPase II* [22]. In the present study we report isolation and characterization of mouse *ATPase II* promoter as well as its 5′UTRs from various organs and compare and contrast its function with those of the human homolog. Intriguingly, notwithstanding significant homology between them, some differences exist in the organization of mouse and human genes and their promoter elements, which underscore the importance of the current study.

2. Materials and methods

2.1. Reagents

The GeneRacer™ Kit for full-length, RNA ligase-mediated rapid amplification of 5′ ends (RLM-RACE), the TOPO-TA™ Cloning Kit and Thermozyme™ thermostable DNA polymerase, the THERMOSCRIPT™ RT-System, the Platinum® Taq High Fidelity thermostable DNA polymerase, agarose and a dNTPs mixture were purchased from Invitrogen Corporation (Carlsbad, CA). Proteinase K (fungal) was purchased from Invitrogen. Prior to use, proteinase K was dissolved in 10 mM Tris–Cl (pH 7.5)–20 mM $CaCl_2$–50% glycerol and stored in single use aliquots at −20 °C. The QIAprep Miniprep plasmid DNA isolation kit, QIAquick PCR purification kit, QIAquick gel extraction kit, the Effectene™ Transfection Reagent, RNeasy Mini kit including QIAShredder™ columns and on-column DNase digestion kit were purchased from QIAGEN (Valencia, CA). The pGL3-Basic (no promoter) and pGL3-Promoter (SV40 promoter) firefly luciferase reporter plasmids, pRL-TK vector and the Dual