J Hum Genet (2003) 48:194–198
DOI 10.1007/s10038-003-0009-3

ORIGINAL ARTICLE

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Predominant maternal expression of the mouse *Atp10c* in hippocampus and olfactory bulb

Received: 5 November 2002 / Accepted: 9 February 2003 / Published online: 12 March 2003
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Abstract The human chromosome 15q11-q13 region is one of the most intriguing imprinted domains, and the abnormalities inherited are associated with neurological disorders including Prader-Willi syndrome (PWS), Angelman syndrome (AS) and autism. Recently we have identified a novel maternally expressed gene, *ATP10C*, that encodes a putative aminophospholipid translocase within this critical region, 200 kb distal to *UBE3A* in an imprinted domain on human chromosome 15. *ATP10C*, with *UBE3A*, displayed tissue-specific imprinting with predominant expression of the maternal allele in the brain. In this study, we demonstrated that the mouse homologue, *Atp10c/pfatp,* showed tissue-specific maternal expression in the hippocampus and olfactory bulb, which overlapped the region of imprinted *Ube3a* expression. These data suggest that the imprinted transcript of *Atp10c* in the specific region of CNS may be associated with neurological disorders including AS and autism.

Keywords Angelman syndrome · *Atp10c/pfatp* · Tissue-specific imprinting · Maternal expression · P-type ATPase

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Introduction

Angelman syndrome (AS [MIM 105830]) is a neurodevelopmental disorder, characterized by mental retardation, epilepsy, seizures, frequent smiling and laughter, absence of speech, and abnormal gait, with an occurrence of approximately 1:15,000 live births (Williams et al. 1995). This syndrome is one of the best examples of human disease involved in genomic imprinting, caused by the absence of a normal maternal contribution to the human chromosome 15q11-q13 region. The E6-AP ubiquitin protein ligase gene, *UBE3A*, has been strongly implicated as the AS gene because of genetic mutation and tissue-specific imprinting with preferential maternal expression in human brain, fibroblasts, and lymphoblasts, and in specific regions in the mouse brain (Matsuura et al. 1997; Rougeulle et al. 1997; Albrecht et al. 1997; Herzing et al. 2002). However, the phenotypes of AS patients and a model mouse with a 15q11-q13 deletion are more severe than that with an *UBE3A* mutation (Jiang et al. 1998).

Recently, human *ATP10C* encoding a putative aminophospholipid translocase was identified from the adjacency to *UBE3A*, and also exhibited maternal expression in human brain and lymphocytes and lack of expression in AS patients, suggesting that it may contribute to AS phenotypes (Meguro et al. 2001; Herzing et al. 2001). Moreover, abnormal phenotypes, such as language delays and autism spectrum disorders, were observed with the maternal 15q11-q13 duplication, while the paternal duplication has no obvious phenotypes (Cook et al. 1997). It was also suggested that chromosome 15q11-q13 contains maternally expressed autism associated gene(s). Since *ATP10C* is presumed to function as an aminophospholipid-transporting ATPase, it may play a role in cell signaling in the central nervous system (CNS) (Halleck et al. 1999). Moreover, many human genetic disorders caused by loss of function of members of the P-type ATPase family display neurological symptoms

(DiDonato and Sarkar 1997). Thus, it is possible that *ATP10C* may contribute to chromosome 15 associated neurological disorders including AS and autism.

A recent report demonstrated that *Atp10c/pfatp*, which is a mouse homologue of *ATP10C*, was associated with obesity, and maternal inheritance of the deletions resulted in increased body fat when compared with the inheritance of the deletion from the father, suggesting it was involved in genomic imprinting (Dhar et al. 2000). Although *Atp10c* was not imprinted in testis and adipose tissues, it is possible that it could be imprinted tissue specifically as with *Ube3a*. To determine whether mouse *Atp10c* is imprinted in the CNS, we examined the allele-specific expression and methylation status of *Atp10c* in brain tissues. Although the parent-of-origin-specific methylated region was not observed, mouse *Atp10c* was imprinted in a tissue-specific manner, with predominant expression of the maternal allele in hippocampus and olfactory bulb, overlapping with regions where *Ube3a* is imprinted.

Materials and methods

Strains and matings

Mouse strains used were C57BL/6J and JF1. (B6 x JF1)F1 mice were generated by mating a female B6 with a male JF1 mouse. (JF1 x B6)F1 mice were generated by mating a female JF1 with a male B6 mouse. These reciprocal crosses to generate F1 mice were used for allele-specific expression analysis. Each of the F1 mice was 30 weeks old.

Expression analysis

Total RNA was isolated from each tissue using the AGPC method. Total RNA (6 μg) was treated with DNase I (Wako Nippon Gene, Tokyo, Japan) and the reaction was subsequently used to synthesize first-strand cDNA with random primers (Roche Diagnosis Co., Indianapolis, IN), with or without reverse transcriptase (Invitrogen Co., Carlsbad, CA). RT-PCR was performed on the cDNA with Ampli Taq Gold (Roche Diagnosis Co., Indianapolis, IN) using a step-down protocol. The reaction parameters were as follows: an initial denaturation at 95°C for 10 min, three cycles of 95°C for 30 s, 64°C for 30 s, 72°C for 30 s, three cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 30 s, three cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 27 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s. Primer sequences were as follows: Atp10cF2, 5'-GCAATGTGCATTGTTTCTTCA-3' and Atp10cR2:5'-TCAGACCCATGAGGTGAACTT-3'. PCR products were analyzed on a 2% agarose gel followed by SYBR green I (Bio-Whittaker Molecular Applications, Rockland, ME) staining.

Identification of polymorphism

Genomic DNA was prepared from both B6 and JF1 mice. DNA samples were amplified by PCR using primers corresponding to *Atp10c* 3' UTR (Atp10cF1:5'-AGGAAGCCAGAGGTACCAAA-3' and Atp10cR1:5'-GGACCCCCACTCTTCTTACC-3'). PCR products were purified and directly sequenced.

PCR-RFLP

Total RNA from hippocampus, olfactory bulb, cerebellum, cerebral cortex, and brain stem of adult (B6 x JF1) F1 and (JF1 x B6) F1 mice was used for PCR-RFLP analysis. RT-PCR was performed using the primers Atp10cF2 and Atp10cR2. PCR products were digested with *Msp*I (Nippon Gene, Tokyo, Japan). The digested products were separated in a 5% polyacrylamide gel and stained with SYBR green I followed by quantification using a phosphoimager.

Intron-spanning RT-PCR

Intron-spanning RT-PCR analysis was performed using the primers spanning an *Atp10c* intron 20 (Atp10cF3:5'-TGTCTCATCG-CACCTATTGC-3' and Atp10cR2). Genomic DNA was prepared from liver from (B6 x JF1)F1 and cDNA were prepared from CNS tissues from (B6 x JF1)F1. The PCR reaction parameters were as follows: an initial denaturation at 95°C for 10 min, three cycles of 95°C for 30 s, 66°C for 30 s, 72°C for 30 s, three cycles of 95°C for 30 s, 64°C for 30 s, 72°C for 30 s, three cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 30 s, and 26 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s. PCR products were analyzed on a 1.2% agarose gel followed by SYBR green I staining.

Methylation Southern blot analysis

Southern blot analysis was performed to verify the methylation status of the CpG island of *Atp10c* intron 1, predicted by EMBROSS/EMBL-EBI (http://www.ebi.ac.uk/emboss/cpgplot/). Genomic DNA was isolated from B6 (♂/10 weeks) various brain tissues and testis. The 10-μg genomic DNA was digested with or without 50 U methylation-sensitive enzyme *Not*I after 20 U *Apa*I digestion, separated on 0.8% agarose gel and transferred onto Hybond N^+ membrane (Amersham Pharmacia, Bucks., England). Radiolabeled PCR probes were generated using the following primers, Atp10cF4:5'-AAGCTGGAGGGTAGGGTGTT-3' and Atp10cR3:5'-TCAAAAACACTGCAGCAAGG-3'. Hybridizations were performed in ×5 SSPE, 0.5% SDS and 200 μg/ml salmon sperm DNA at 55°C and a final wash in ×0.1 SSC and 0.1% SDS at 55°C. Autoradiography was analyzed with a BAS-2500 phosphoimager (Fuji Film).

Results

The database searches permitted us to identify the sequence of the mouse ATPase class V type 10A gene, which was the mouse ortholog of human *ATP10C*. We first examined the tissue-specific expression of mouse *Atp10c*. It was expressed at high level in the CNS including cerebral cortex, cerebellum, hippocampus, olfactory bulb and brain stem as well as lung, kidney and testis, and at a low level in thymus, heart, liver, pancreas and muscle (Fig. 1). A faint signal was detected in spleen. These results of tissue-specific expression suggested that *Atp10c* had significant functions in the CNS. To determine the imprinting status of *Atp10c*, we searched for sequence polymorphisms between C57BL/6J and JF1 mice. By direct sequence analysis, we found a single nucleotide polymorphism within the 3' UTR region of the *Atp10c* gene, which abolished a *Msp*I restriction site in JF1 that is present in B6 (Fig. 2A). Using this polymorphism, we verified the parental origin of the transcripts in reciprocal crosses between B6 and JF1 mice. RNAs from hippocampus, olfactory bulb, cerebellum, brain stem and cerebral cortex were subjected to RT-PCR and the PCR products were digested with

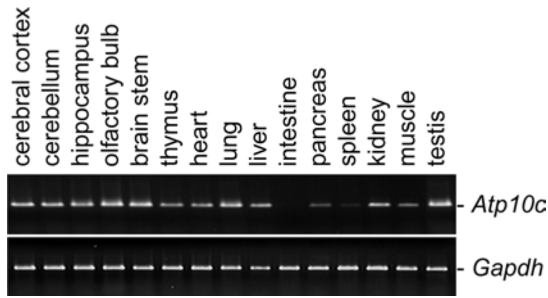

Fig. 1 Expression analysis of the mouse *Atp10c* gene in multiple tissues. RT-PCR with the specific primers for *Atp10c* showed a high level of expression in brain tissues, suggesting that it has significant functions in CNS. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as a control

*Msp*I. Allelic expression bias with preferential maternal expression was shown in hippocampus and olfactory bulb with a small amount of paternal expression, but not in other brain tissues (Fig. 2B). Parent-of-origin-specific expression was not observed in any other tissues examined (data not shown). These results suggested that mouse *Atp10c* was imprinted in a region-specific distribution in the same manner as *Ube3a*.

An antisense transcript extending to the *Atp10c* locus has not yet been identified, but it is tempting to speculate that it may also be associated with the imprinted expression of *Atp10c*. To study the mechanism of the imprinted expression of *Atp10c*, we performed RT-PCR analysis using primers spanning an *Atp10c* intron 20 (Fig. 2C). Only the spliced RNA was expressed in any tissues, suggesting the antisense transcript was not extending to this locus. Then, we analyzed methylation status in the CpG island of *Atp10c* intron 1. The *Not*I site (Fig. 3) and the *Sma*I site (data not shown) in this CpG island were unmethylated in any tissues, whether *Atp10c* was imprinted or not. While it is possible for other CpG dinucleotides in *Atp10c* to display allele-specific

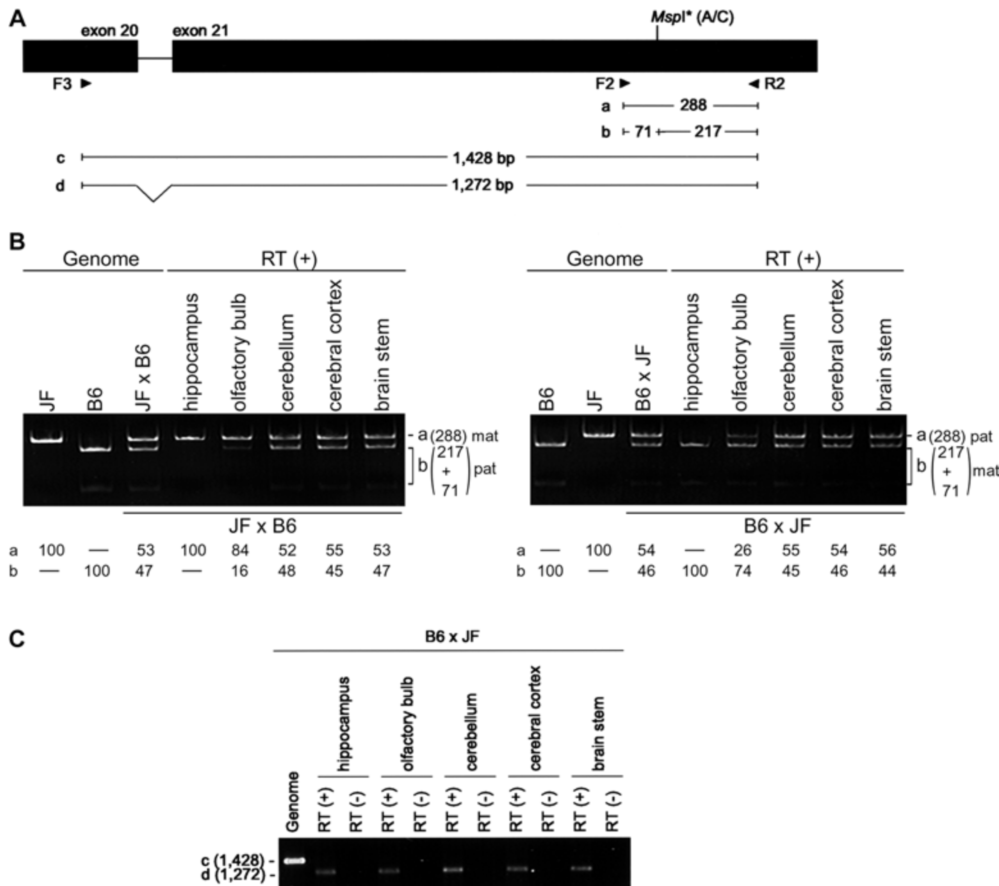

Fig. 2A–C Imprinted expression of *Atp10c* in the F1 mice of reciprocal crosses (JF1 x B6) and (B6 x JF1). **A** Schematic of the transcribed sequence polymorphisms in the *ATP10c* 3' UTR and the primers used for amplification of *ATP10c*. An *asterisk* denotes the polymorphic site. *Arrowheads* represent oligonucleotide primers. **B** Imprinted expression of *Atp10c* in brain tissues. The imprinted expression was assessed by PCR-RFLP analysis. PCR products were digested with *Msp*I, producing the undigested 288-bp fragment (*a*) for the B6 allele and the digested 217-bp and 71-bp fragments (*b*) for the JF1 allele. Densitometric values are given below each lane. Maternal allele-specific transcription is seen for *Atp10c* in hippocampus and olfactory bulb, in contrast to biallelic expression in the other samples. **C** Intron-spanning RT-PCR analysis of *Atp10c*. Genomic DNA and cDNAs were amplified using the primers spanning the intron 20 (F3 and R2). Only the spliced transcripts were amplified, indicating that the antisense transcript does not lie in the 3' UTR region of *Atp10c* (*RT+* reverse transcriptase-positive lane, *RT−*reverse transcriptase-negative)

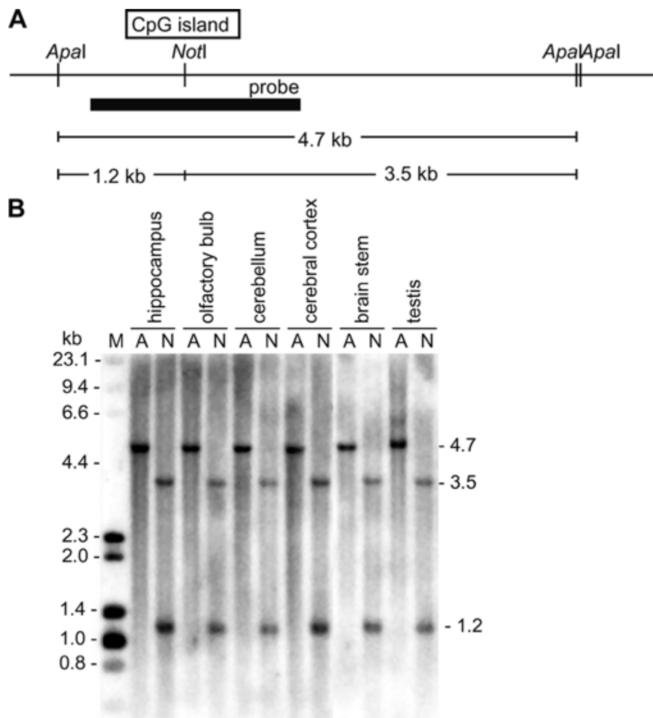

Fig. 3A, B DNA methylation analysis of the CpG island of *Atp10c* intron 1. **A** Restriction map of the genomic fragment in the CpG island of *Atp10c*. The probe generated by PCR amplification is indicated by the *black box*. **B** Southern blot analysis of the *Atp10c* CpG island. Genomic DNA was digested with *Apa*I (*A*) or *Apa*I plus *Not*I (*N*). The 4.7-kb *Apa*I fragment was completely digested by the methylation-sensitive endonuclease *Not*I in all tissues, indicating absence of methylation on both alleles (*M* marker)

differential methylation, our result may be related to no DMR identified in *Ube3a*.

Discussion

In the present study, expression analysis of *Atp10c* also showed higher levels in the CNS including cerebral cortex, cerebellum, hippocampus, olfactory bulb and brain stem. A previous study showed the localization of *Atp10c* in mouse CNS to subiculum, cerebellar granule cells, hippocampus, olfactory bulb mitral cells, hypothalamus by in situ hybridization (Halleck et al. 1999), and expression overlapped with regions where *Ube3a* was imprinted (Albrecht et al. 1997). These findings suggest that *Atp10c* had significant functions in the CNS.

UBE3A is the only gene where mutations have been found in AS patients, strongly supporting its causative role in AS. However, there is evidence to suggest that another gene may play a role either directly in AS or indirectly by regulating *UBE3A*. We previously reported that *ATP10C* was preferentially expressed from the maternal allele in human lymphoblasts and brain tissues (Meguro et al. 2001). A recent study demonstrated the preferential maternal expression of *UBE3A* in human fibroblasts, lymphoblasts and neural precursor cells by FISH, while RT-PCR analysis could not detect allelic expression bias (Herzing et al. 2002; Nakao et al. 1994). Here we demonstrated that mouse *Atp10c* was imprinted in a tissue-specific manner, with a predominant expression from the maternal allele in hippocampus and olfactory bulb, where mouse *Ube3a* also shows imprinted expression. This overlap suggests that the imprinted expression of these two genes is coordinately regulated in the CNS.

Recently, a paternally expressed *Ube3a* antisense transcript was demonstrated, which is under control of an imprinting center (IC) (Rougeulle et al. 1998; Chamberlain and Brannan 2001; Runte et al. 2001). Although the role of this antisense transcript is unknown, it may regulate the imprinted expression of *Ube3a*. However, the antisense transcript extending to the *Atp10c* locus has not been identified. The DMR also has not been identified in *Atp10c*, as well as *Ube3a*. Although the mechanism of the imprinted expression of *Atp10c* is unknown, it is possible that *Atp10c* may play an important role in CNS development, and the absence of maternally expressed *Atp10c* may be causative of the phenotypes of AS and autism.

Acknowledgements We thank Dr. Haruaki Ninomiya, Division of Neurobiology, Department of Biomedical Sciences, School of Life Sciences, Faculty of Medicine, Tottori University, and Dr. Kaoru Inokuchi, Mitsubishi Kasei Institute of Life Sciences, for discussion and technical advice. This study was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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