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Murine *Asb-17* expression during mouse testis development and spermatogenesis

Kye-Seong Kim, Myung-Sun Kim, Soo-Kyung Kim and Kwang-Hyun Baek
Cell and Gene Therapy Research Institute, Graduate School of Life Science and Biotechnology, Pochon CHA University, CHA General Hospital, Seoul, Korea

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Summary

In this study we isolated a murine *mAsb-17* from mouse testis by RT-PCR using primers designed based on the sequences from the GenBank database. The sequence analysis showed that *mAsb-17* encodes a 295 amino acid polypeptide with a molecular weight of approximately 34 kDa containing two ankyrin repeats and one SOCS box. The amino acid sequence of mASB-17 showed 87.5%, 98.3% and 92.9% identity with that of human, rat and dog, respectively. Interestingly, northern blot analysis showed that *mAsb-17* was expressed only in the testis. The expression analysis by RT-PCR for *mAsb-17* in mouse indicates that *mAsb-17* is expressed from the fourth week after birth to adult, with the highest expression in round spermatids. Both northern blot and RT-PCR analyses suggest that mASB-17 may play essential roles in testis development and spermatogenesis.

Keywords: Ankyrin repeat, Asb-17, SOCS box, Spermatogenesis

Introduction

The suppressor of cytokine signalling (SOCS) box motif, extending approximately 40 amino acids, has been identified in a number of proteins. It has been shown that these proteins inhibit signalling pathways initiated by cytokines, hormones and growth factors through direct interactions with JAK kinases or activated cytokine receptors (Endo *et al*., 1997; Starr *et al*., 1997; Kile *et al*., 2001). Previous reports suggest that the SOCS box plays a role in ubiquitin-mediated proteasomal degradation via interaction with the elongin B and elongin C complex (Kamura *et al*., 1998; Zhang *et al*., 1999). In addition to SOCS family members, ASB proteins (ankyrin repeat), WSB proteins (WD-40 repeat), SSB proteins (SPRA domain) and RAR-like proteins (GTPase domain) also contain the SOCS box motif, which is known to be involved in protein–protein interaction (Kile *et al*., 2001).

The ankyrin repeat, extending approximately 33 amino acids, is found in proteins with a wide variety of functions including receptors, transcription factors, tumour suppressors and cell cycle regulators (Wharton *et al*., 1985; Breeden & Nasmyth, 1987; LaMarco *et al*., 1991; Serrano *et al*., 1993). Even though ankyrin repeats have been found in a number of proteins, their cellular functions are poorly understood. Only the function of *Asb-1* has been studied in genetically modified mice. In the absence of *Asb-1*, mice showed a reduction in spermatogenesis with less complete filling of seminiferous tubules (Kile *et al*., 2001).

Design-wise, spermatozoa are a highly organized, sleek vehicle of paternal genetic materials and are a marvel of differentiation. Formation of the male gamete occurs in sequential mitotic division of spermatogonial stem cells, two meiotic divisions by spermatocytes, and unique morphological remodelling of the spermatid during spermiogenesis – a process that continues throughout adult life in most mammals, including humans. This process occurs in seminiferous tubules containing an epithelium populated by a mixture of germ cells and Sertoli cells, surrounded by a thin wall of peritubular cells. Many germ-cell-specific gene expressions have been observed during this process. Such expression is developmentally regulated and stage-specific. Understanding the genetic regulation of male gametogenesis will provide insight into the cellular process of differentiation.

All correspondence to: Kwang-Hyun Baek, PhD, College of Medicine, Pochon CHA University, CHA General Hospital, 605 Yeoksam 1-dong, Kangnam-Gu, Seoul 135-081, Korea. Tel: +82 2 3468 3197. Fax: +82 2 3468 3264. e-mail: baek@cha.ac.kr

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Based on the fact that *Asb-17* is expressed in the testis of human and *Macaca fascicularis*, we predicted that the orthologue exists in mice. We report here the cloning and characterization of a member of the ankyrin repeat-containing SOCS box protein family, *mAsb-17*, and its tissue- and stage-specific expression in mouse germ cells.

Materials and methods

Animals

Male ICR mice (5 days, 2, 4, 6 and 10 weeks old) were purchased from Daehan Biorink (Chungbuk, Korea).

Isolation of spermatogenic cell populations

Mixed populations of spermatogenic cells were obtained from mouse testes using the collagenase dissociation method (Bellve *et al*., 1977). Purified populations of spermatogenic cells were isolated following collagenase treatment of testes and trypsin digestion of isolated seminiferous tubules using unit gravity sedimentation velocity in a bovine serum albumin (BSA) gradient (Romrell *et al*., 1976; Bellve *et al*., 1977). Tubes containing each cell population (pachytene spermatocyte (PS), >90% pure; round spermatid (RS), >90% pure; and condensing spermatid (CS)–residual body mixture) were selected by phase-contrast microscopy.

RT-PCR for expression analysis

Total cellular RNA was isolated from mouse testes and mouse embryonic fibroblasts using TRIzol (Gibco BRL, Rockville, MD). Whole mouse testes were obtained from 5 day-old, 2-, 4-, 6- and 10-week-old mice, respectively. PS, RS and CS were separated from the whole testis of 10-week-old mice by STA-PUT. The cDNA synthesis from obtained total RNA was performed in accordance with the protocol of the SuperScript Preamplification system (Gibco BRL, Rockville, MD). After incubation of 3 μg total RNA with 0.5 μg Oligo $(dT)_{12–18}$ primer at 70 °C for 10 min, the reaction was carried out in 5 × first strand buffer (containing 250 mM Tris-HCl, 375 mM KCl and 15 mM $MgCl_2$) from the kit, 10 mM DTT (dithiothreitol) and 0.5 mM dNTP in a final volume of 20 μl mixture. Mixed contents of the tube were incubated at 42 °C for 2 min. Then SuperScript II (Gibco BRL, Rockville, MD) was added and incubated for 50 min at 42 °C. After the reaction, the enzyme was denatured at 70 °C for 15 min.

In order to obtain the complete coding region of *mAsb-17*, we performed PCR using a pair of primers designed after a BLAST search (GenBank accession no. AF425641). The nucleotide sequences of these primers are as follows: upstream primer (5′-GCTCTT-CCTACCCTTAAGAAGCAATG-3′) and downstream primer (5′-AGGGACAGTGTAGAGATGCATGC-3′). PCR was performed at 94 °C for 1 min, 54 °C for 2 min and 72 °C for 1 min for a total of 25 cycles. The expression analysis for *mAsb-17* also was carried out using RT-PCR. We performed PCR using a pair of primers (upstream primer: 5′-TGGTTGACCACGAG-TTGATTGAC-3′; downstream primer: 5′-AGGGA-CAGTGTAGAGATGCATGC-3′). PCR analyses for *mAsb-17* and *G3PDH* were performed at 94 °C for 1 min, 54 °C for 45 s and 72 °C for 30 s for a total of 22 cycles.

Northern blot analysis

Expression of the *mAsb-17* mRNA was analysed in mouse tissues by northern blot analysis. Mouse multiple tissue blot was purchased from Seegene (Seegene, Seoul, Korea). For the stage-specific mouse blot, pachytene spermatocyte, round spermatid and condensing spermatid RNA samples (20 μg each) were electrophoresed and blotted onto Hybond-N+ membranes (Amersham Biosciences, Buckinghamshire, UK). B-lymphocyte RNA sample and whole testis of 2-week-old mice were used as negative controls. The immobilized nucleic acids were hybridized with a radiolabelled DNA probe corresponding to nucleotides 670–996 bp of the *Asb-17* clone or nucleotides 476–996 bp of G3PDH cDNA as a positive control. The probe used for northern blot analysis was labelled by random primers with [α-^{32}P]dCTP (Amersham Biosciences, Buckinghamshire, UK). Hybridization was performed in a bag containing ExpressHyb hybridization solution (Clontech, Palo Alto, CA) at 68 °C overnight. Hybridized membrane was washed at room temperature in 2× SSC and 0.1 % SDS, and then in 0.1× SSC and 0.1% SDS. The membrane was exposed to Kodak X-ray film with an intensifying screen for 5 h at −70 °C.

Results

In order to screen a putative mouse homologue of *Asb-17* that contains ankyrin repeat domains, databases from GenBank were accessed and searched using the BLAST algorithm (Altschul *et al*., 1997) at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/). The *mAsb-17* cDNA was isolated by RT-PCR from murine testis and subcloned into an expression vector, which was subjected to further analysis. We performed RT-PCR using a pair of primers designed with reference to sequences from the cDNA library clone (GenBank accession nos. NM_080868 for human and AF425641 for mouse). Both strands of DNA sequences were

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A

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1   M N N S S K L C R K T S F P R S N I F C N L V D K I V K R P    Mouse ASB-17
1   M S K S T K L C G K T S C P R S N I F C N L L D K I V K R P    Human ASB-17
1   M N N S S K L C R K T S F P R G N I F C N L I D K I V K R P    Rat ASB-17
1   M S K S S K L C R K T S C P R S N I F C N L I D K I V K R P    Dog ASB-17

31  S L Q F L G Q W G Y H C Y E P R I Y R T L A K I L R Y V D L     Mouse ASB-17
31  S L Q F L G Q W G Y H C Y E P R I Y R S L A K I L R Y V D L     Human ASB-17
31  S L Q F L G Q W G Y H C Y E P R I Y R T L A K I L R Y V D L     Rat ASB-17
31  S L Q F L G Q W G Y H C Y E P R I Y R T L A K I L R Y V D L     Dog ASB-17

61  D G F D I L L T D Y I A F V E K S G H R F E L N F N L E F T     Mouse ASB-17
61  D G F D A L L T D Y I A F V E K S G Y R F E V S F N L D F T     Human ASB-17
61  D G F D I L L T D Y I A F V E K S G H R F E L N F N L E F T     Rat ASB-17
61  D G F D I L L S D Y I A F V E K S G Y R F E L N F N L E F T     Dog ASB-17

91  E I C V N T I L Y W V F A R K G N P D F V E L L L K K T K D     Mouse ASB-17
91  E I C V N T I L Y W V F A R K G N P D F V E L L L K K T K D     Human ASB-17
91  E I C V N T I L Y W V F A R K G N P D F V E L L L K K T K D     Rat ASB-17
91  E I C V N T I L Y W V F A R K G N P D F V E L L L K K T K D     Dog ASB-17

121 Y V Q D R S C S L A L I W R T F T P V Y C P S P L S G I T P     Mouse ASB-17
121 Y V Q D R S C N L A L I W R T F T P V Y C P S P L S G I T P     Human ASB-17
121 Y V Q D R S C S L A L I W R T F T P V Y C P S P L S G I T P     Rat ASB-17
121 Y V Q D R S C N L A L I W R T F T P V Y C P S P L S G I T P     Dog ASB-17

151 L L Y V A Q T R Q S N I L K I L L Q Y G I L E R E K N P I N     Mouse ASB-17
151 L F Y V A Q T R Q S N I F K I L L Q Y G I L E R E K N P I N     Human ASB-17
151 L L Y V A Q T R Q S N I L K I L L Q Y G I L E R E K N P I N     Rat ASB-17
151 L L Y V A Q T R Q S N I L K I L L Q Y G I L E R E K N P I N     Dog ASB-17

181 I V L T I L L Y P S R V R I M V D H E L I D I Q E D A K T C     Mouse ASB-17
181 I V L T I V L Y P S R V R V M V D R E L A D I H E D A K T C     Human ASB-17
181 I V L T I L L Y P S R V R I M V D H E L I D I Q E D A K T C     Rat ASB-17
181 I V I T I L L Y P S R V R I M V D H E L V D I Q E D A K T C     Dog ASB-17

211 L M L C S R V L S T I S V R E I E T Q L S L G R R P I I Q N     Mouse ASB-17
211 L V L C S R V L S V I S V K E I K T Q L S L G R H P I I S N     Human ASB-17
211 L T L C S R V L S V I S V R E I E T Q L N L G R R P I I Q N     Rat ASB-17
211 L V L C S R V L S A I S I R E I E T Q L S L G R R P I I S N     Dog ASB-17

241 W L D Y I P P T R Y K D P C E L V H L C R I T I R T Q L L A     Mouse ASB-17
241 W F D Y I P S T R Y K D P C E L L H L C R L T I R N Q L L T     Human ASB-17
241 W L D Y I P P T R Y K D P C E L V H L C R I T I R T Q L L A     Rat ASB-17
241 W L D Y I P S T R Y K D P C E L S H L C R I T I R A Q L L T     Dog ASB-17

271 N N M L P N G I F S L L I P T R L Q N F L N L E S .            Mouse ASB-17
271 N N M L P D G I F S L L I P A R L Q N Y L N L E I .            Human ASB-17
271 N N M L P N G I F S L L I P T R L Q N F L N L E S .            Rat ASB-17
271 N N M L P N G I F S L Q I P X R L Q K Y L N L E S .            Dog ASB-17
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B

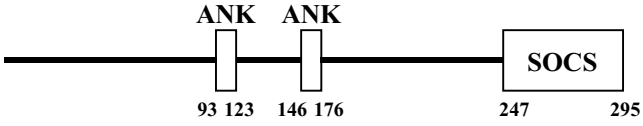


Figure 1 (A) Alignment of amino acid sequences for mouse ASB-17 (AF425641), human ASB-17 (NM_080868), rat ASB-17 (AJ518866) and dog ASB-17 (AJ518867) using MegAlign software (Clustal method) from DNASTAR (LASERGENE). Different amino acids are blocked in all four sequences. (B) Domain composition of mouse ASB-17, indicating two ankyrin repeats (ANK: amino acids 93–123 and 146–176) and one SOCS box (amino acids 247–295).

analysed, confirming the fidelity of the sequence information. The putative mouse *Asb-17* is 885 bp in length, with open reading frame encoding a protein of 295 amino acids (Fig. 1A). Fig. 1A shows the amino acid sequences of ASB-17 family members derived from GenBank aligned using the Clustal method with the

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Table 1 The nucleotide and amino acid sequence identities of mAsb-17 with other members of Asb-17 family

Mouse (mAsb-17)	Nucleotide sequence identity (%)	Amino acid sequence identity (%)
Human	83.1	87.5
Rat	94.3	98.3
Dog	85.0	92.9

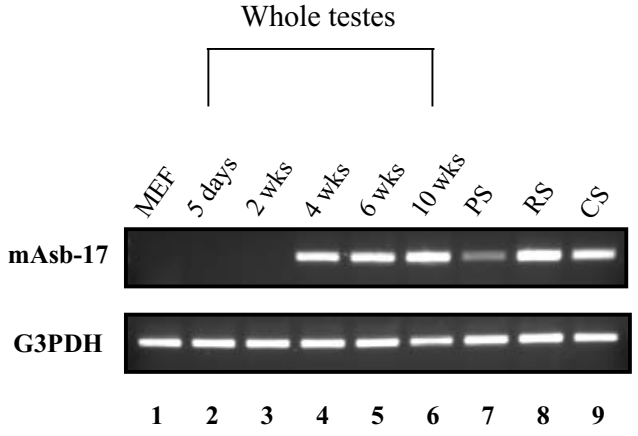


Figure 2 Expression analysis during mouse testis development and spermatogenesis by RT-PCR. Lanes 2–6 are testicular cDNA samples from 5-day-old, 2-, 4-, 6- and 10-week-old mice. Also, cDNA samples from pachytene spermatocytes (PS; lane 7), round spermatids (RS; lane 8) and condensing spermatids (CS; lane 9) were used. Mouse embryonic fibroblasts (MEF; lane 1) were used as a control. To determine RNA integrity, a G3PDH positive control was included.

DNASTAR program from LASERGENE. The amino acid sequence for mouse ASB-17 revealed 87.5%, 98.3% and 92.9% identity with that of human, rat and dog, respectively (Table 1). Computer analysis (http://smart.embl-heidelberg.de) revealed that the deduced amino acid sequence of the mouse ASB-17 contains two ankyrin repeats and one SOCS box (Fig. 1B). As expected, the amino acid sequence analysis by the PSORT online method (http://psort.nibb.ac.jp/) predicted that mASB-17 protein is localized mainly in the cytoplasm (K value = 9/23, 47.8% cytoplasmic, 26.1% nuclear).

The expression of *mAsb-17* was confirmed by both RT-PCR and northern blot analyses (Figs. 2, 3). A PCR product for *mAsb-17* during testis development and spermatogenesis was not shown until the fourth week of mouse development (Fig. 2, lanes 4–9). Interestingly, *mAsb-17* expression was relatively less in pachytene spermatocytes and then gradually increased in round spermatids, suggesting that mASB-17 may play a role in spermatogenesis. In addition, the expression of *mAsb-17* was also analysed for a variety of mouse tissues by northern blot analysis. Strikingly, northern blot analysis revealed that *mAsb-17* was expressed in testis (Fig. 3A). In addition, the high expression of 1.35 Kb *mAsb-17* transcript was detected in round spermatids and condensing spermatids, with moderate expression in pachytene spermatocytes (Fig. 3B).

Discussion

Cellular growth and differentiation are regulated by a number of cytokines, hormones and growth factors. These exert biological functions through interacting with receptors on the cell surface, triggering cytoplasmic signal transduction pathways that transmit specific signals to the nucleus. The JAK signalling pathway is one of these signal transduction pathways that is well characterized (Kisseleva *et al*., 2002). However, this signal transduction has to be negatively regulated for homeostasis. The SOCS box-containing proteins inhibit the JAK signalling pathway, thereby regulating the appropriate level of response to external stimuli (Nicholson *et al*., 2000). The SOCS box has been identified in four other protein families: ankyrin repeat containing ASBs, WD-40 repeat containing WSB, SPRY domain containing SSB, and GTPase domain containing RAR-like proteins (Hilton *et al*., 1998; Kile *et al*., 2001).

The ankyrin repeat domain has been found in more than 400 proteins in eukaryotes, bacteria and viruses (Bork, 1993). However, fewer than 20 ASB family members containing the ankyrin repeat motif have been identified (Kile *et al*., 2000; Liu *et al*., 2003). Other than ASB-1 and ASB-2, the cellular functions of ASB family members are poorly understood. In the absence of ASB-1, mice revealed a diminution of spermatogenesis with less complete filling of seminiferous tubules (Kile *et al*., 2001). Exogenous expression of ASB-2 in myeloid leukemia cells led to growth inhibition and chromatin condensation

Figure 3 (A) Expression of *mAsb-17* mRNA in mouse organs. Total RNA was prepared from mouse brain (lane 1), heart (lane 2), lung (lane 3), liver (lane 4), spleen (lane 5), kidney (lane 6), stomach (lane 7), small intestine (lane 8), skeletal muscle (lane 9), thymus (lane 10), testis (lane 11), non-pregnant uterus (lane 12), placenta (17.5 d.p.c.) (lane 13) and embryonic stem cells (ES; lane 14). Strikingly, expression was shown only in testis. (B) Northern blot hybridization of *mAsb-17* mRNA from pachytene spermatocytes (PS; lane 3), round spermatids (RS; lane 4) and condensing spermatids (CS; lane 5) during spermatogenesis. mRNA from 2-week-old testis (lane 2) and pro-B cells (Ba/F3; lane 1) were used as controls. To determine RNA integrity, a G3PDH positive control was included for both analyses.

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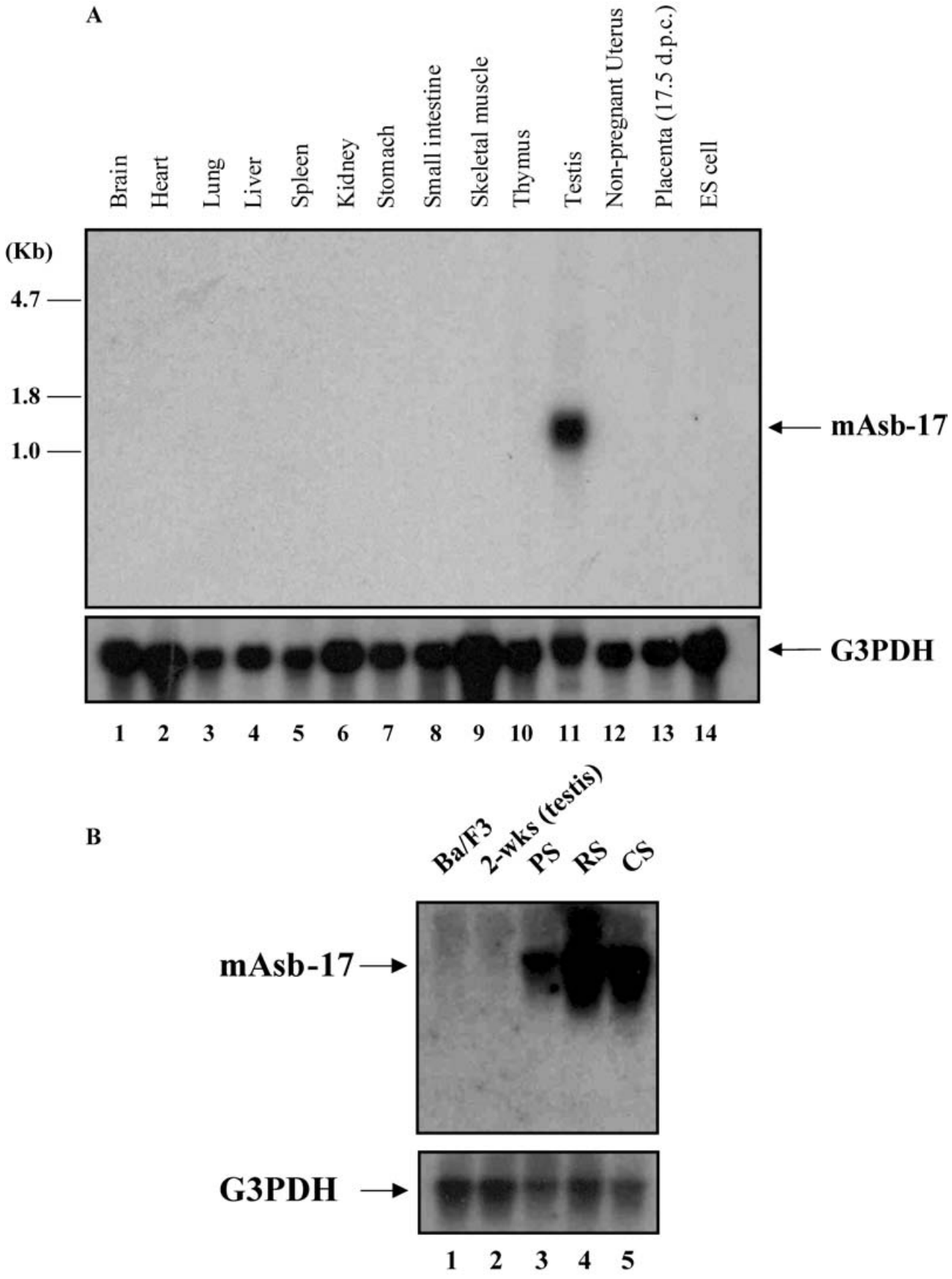


Figure 3 For legend see facing page.

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(Guibal *et al*., 2002). Taken together, it is expected that ASB family members may have pleiotropic functions through protein–protein interactions.

It is of interest to find that *mAsb-17* is expressed exclusively in the mouse testis (Fig. 3A). Notably, the extent of its expression differs during the various stages of spermatogenesis. The production of mature spermatozoa from original spermatogonial stem cells in mouse takes about 5 weeks from birth, and they reach sexual maturity at between 6 and 8 weeks of age. Therefore, RT-PCR results in our experiments are well correlated with mouse germ cell developmental stages and spermatogenesis, suggesting that *mASB-17* may play a role in this cellular process.

The high conservation of amino acid sequences among ASB-17 family members (Table 1) and their tissue- and stage-specific expressions suggest that they may play essential roles in testis development and spermatogenesis in mammals. In order to better understand the developmental role of ASB-17 in mouse testis, the functional roles of SOCS box motif and ankyrin repeat domains during testis development and spermatogenesis should be elucidated.

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