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A newly identified AMSH-family protein is specifically expressed in haploid stages of testicular germ cells

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Abstract

*A*ssociated *M*olecule with *SH*3 domain of STAM (AMSH) plays a critical role in the cytokine-mediated intracellular signal transduction downstream of the Jak2/Jak3-STAM complex. We newly identified a family molecule of AMSH, AMSH-FP (*AMSH-F*amily *P*rotein) in the mouse brain. AMSH-FP encodes the intracellular protein and has a highly conserved *J*AB1 *S*ubdomain *H*omologous (JSH) region, suggesting that AMSH-FP may act as adaptor of gene transcription and/or regulation system. AMSH-FP has two splicing forms, one is expressed in various tissues, whereas the other one is restricted to expression in testis. We named the abundant type AMSH-FPα and the testis type AMSH-FPβ. AMSH-FPβ is a variant lacking N-terminal 166 amino acid residues of AMSH-FPα. Analysis of the 5′-untranslated regions in AMSH-FPα and AMSH-FPβ mRNAs and exon–intron structure of AMSH-FP gene suggests that testis-specific transcripts are generated due to alternative promoter usage and/or alternative splicing. Importantly, AMSH-FPβ mRNA was not detected in juvenile and infertile mouse testis but was restrictively expressed in the haploid stage of testicular germ cells in the normal mature testis. We suggested that AMSH-FPβ had a functional role in the spermiogenesis.

Keywords: AMSH; JSH region; Signal transduction; Spermatogenesis

Spermatogenesis, a highly specialized process of germ cell differentiation in testis, leads to production of functional sperm (reviewed in [1]). The process is separated into distinct cellular phases, including stem cell mitosis, spermatocyte meiosis, and a post-meiotic cytodifferentiation stage (spermiogenesis). Spermatogonial stem cells mitotically proliferate and subsequent meiotic divisions give rise to secondary spermatocytes and round spermatids. The resulting haploid cells, termed round spermatids, undergo an elaborate cytodifferentiation before being released as viable sperm into the lumen of the seminiferous tubules. These unique cellular events depend on the precise and ordered expression of genes specific for spermiogenesis [2].

Lack of conventional tissue and cell culture systems that precisely recapitulate coordinated processes of spermatogenesis has made it difficult to elucidate molecular mechanisms governing spermatogenesis. In recent years, a number of genes, the expression of which is restricted to meiosis and/or spermiogenesis, have been identified [3], and targeted disruption of genes, including A-myb, p53, Bax, MLH1, Hsp70.2, and Limk2, revealed abnormal spermatogenesis and/or infertility with impaired meiosis [4–9]. Likewise, impaired sperm formation was noted in mice deficient in HR6B, CAMK4, and CREM [10–12]. We have now identified and characterized novel testis-specific genes, the expression of which is limited to spermatids.

We report here the cloning and characterization of a novel molecule termed AMSH-FP, *AMSH-F*amily

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*P*rotein. AMSH-FP has high homology with *A*ssociated *M*olecule with *SH*3 domain of STAM (AMSH) [13]. AMSH was originally cloned as an adaptor molecule associated with STAM, another adaptor protein that is tyrosine-phosphorylated and directly interacts with Jak2 and Jak3 in interleukin (IL)-2 and granulocyte-macrophage colony stimulating factor (GM-CSF) signaling systems [13]. We identified two distinct forms of AMSH-FP; AMSH-FPα distributed in somatic tissues and AMSH-FPβ specifically expressed in testis. Interestingly, AMSH-FPβ is predominantly expressed in the haploid stage of testicular germ cells. Together with its structural similarity with AMSH, the potential involvement of AMSH-FPβ in intracellular signal transduction that regulates the spermiogenesis can be considered.

Materials and methods

Molecular cloning. Yeast two-hybrid screening was carried out using Matchmaker Two-Hybrid System 2 (Clontech, Palo Alto, California) with the bait plasmid containing cDNA for rat LIM kinase-2 (LIMK2) [9], and the mouse embryonic fibroblast-derived cDNA library. Two of positive clones contained a 1.3 kb of cDNA fragment encoding a novel protein lacking the N-terminus. To obtain the full length cDNA, mouse brain- [14] or mouse testis- [15] derived cDNA libraries were screened, using a 1.3 kb cDNA fragment as a probe and plaque hybridization. The nucleotide sequences of the positive clones were determined using the DNA-sequencer model 377A and Dye Terminator cycle sequencing kits (PE Applied Biosystems, Foster City, California). The subsequent homology analysis was done using DNASIS software (Hitachi Software Engineering, Tokyo, Japan).

Northern blot hybridization. Poly(A)$^+$ RNA was prepared from various mouse tissues by standard protocol, electrophoresed, transferred onto Hybond-N$^+$ nylon membranes (Amersham–Pharmacia Biotech, Piscataway, New Jersey), and then hybridized with the ^{32}P-labeled cDNA fragment of AMSH-FP (corresponding to 670–1978 bp in AMSH-FPα; refer to Accession No. AB066211 in DDBJ/EMBL/GenBank DNA databases). Filters were washed under high stringency condition and subjected to autoradiography.

Immunoblotting. cDNA fragments containing the entire coding region of AMSH-FPα and AMSH-FPβ were, respectively, subcloned into the pUC/SRα-myc expression vector, such that the C-termini of AMSH-FPs are fused with a Myc epitope tag. COS-7 cells grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum were transfected with 10 μg SRα-AMSH-FPα-myc or SRα-AMSH-FPβ-myc by electroporation. Forty-eight hours after transfection, COS-7 cells were lysed, immunoprecipitated, and immunoblotted under reducing conditions, using an anti-myc antibody, as described elsewhere [16].

In situ hybridization. Freshly dissected testes were fixed in Bouin's solution, embedded in paraffin, and sectioned at 10 μm thickness. AMSH-FP cDNA fragments (997 bp: corresponding to 982–1978 bp in AMSH-FPα and 331–1327 bp in AMSH-FPβ), which were subcloned into pBluescript SK(−), served as templates for in vitro transcription. In situ hybridization was carried out as described previously [17]. Briefly, hybridization was done using 1×10^6 cpm/slide of probe at 55 °C for 16 h. The slides were then washed in high stringency buffer, dehydrated in a graded series of ethanol, and air-dried. Sections were coated with emulsion and exposed for one day.

Results

Molecular cloning of an AMSH-family Protein

During the cloning of molecules that interact with LIMK2, using a yeast two-hybrid system, we initially obtained 10 clones capable of growing in selective media and displaying a strong β-galactosidase signal that were identified when we screened 3.8×10^6 independent colonies. Among them, two clones contained an identical 1.3 kb cDNA insert encompassing the coding region at the C-terminus and the 3′-untranslated region. Our preliminary Northern blot analysis showed that this cDNA-derived mRNA was expressed in the brain, lung, and liver and a smaller size of transcripts was detected in testis (data not shown). Accordingly, we first screened a mouse brain cDNA library to obtain full length cDNA. After screening of 5×10^5 pfu mouse brain cDNA library, we obtained three positive clones, two of which contained an entire coding region of 1311 bp (437 amino acid residues) (Fig. 1).

In these clones, there were two potential ATG translation initiation codons upstream of a single, long open reading frame. Since both ATG codons were in good agreement with the Kozak criteria [18], we tentatively numbered the first ATG as a start codon (+1). The deduced amino acid sequence of this gene revealed the absence of long stretches of hydrophobic residues which could serve as a signal sequence for protein secretion and a membrane-spanning domain, suggesting that the cDNA encoded an intracellular protein. Using homology search analysis, this protein was found to be novel but structurally related to AMSH [13]. As there is 56% homology in amino acid sequence between this gene and AMSH, this protein was termed "*AMSH-F*amily *P*rotein (AMSH-FP)" (Fig. 1). In AMSH-FP, a putative nuclear localization signal (NLS) and a unique domain structure *J*AB1 *S*ubdomain *H*omologous (JSH) region which are characteristic of AMSH were conserved. The JSH region of AMSH-FP (corresponding to residues 335–381) shared 89% amino acid identity to that of AMSH (corresponding to residues 323–369). AMSH has two potential PXXP motifs known as possible binding sites for the SH3 domain, while AMSH-FP does not.

To isolate an AMSH-FP isoform or AMSH-FP specifically expressed in testis, we screened a testis cDNA library using the cDNA fragment described above. After screening of 2×10^6 pfu mouse testis cDNA library, we obtained five positive clones containing 1.3 kb cDNA insert mostly identical to AMSH-FP derived from the brain cDNA library. However, testis-derived AMSH-FP clones had unique 181 bp of untranslated sequence at their 5′-terminus, which was not comparable to ordinary AMSH-FP cDNA, and did not have 832 bp sequence at the 5′-terminus region of brain-derived AMSH-FP (Figs. 2A and B). We hereafter term the AMSH-FP

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AMSH-FP  MEQPFTVNSLKKLAAMPDHTDVSLSPEERVRALSKLGCNISINEDITPRRYFRSGVEMER  60
AMSH     ----------------MSDHGDVSLPPEDRVRALSQLGSAVEVNEDIPPRRYFRSGVEIIR  45

AMSH-FP  MASVYLEEGNLENAFVLYNKFITLFVEKLPSHRDYQQCAVPEKQDIMKKLKEIAFPRTDE  120
AMSH     MASIYSEEGNIEHAFILYNKYITLFIEKLPKHRDYKSAVIPEKKDTVKKLKEIAFPKAEE  105

AMSH-FP  LKTDLLRKYNIEYQEYLQSKNKYKAEILKKLEHQRLIEAERQRIAQMRQQQLESEQFLFF  180
AMSH     LKAELLKRYTKEYTEYNEEKKKEAEELARNMAIQQELEKEKQRVAQQKQQQLEQEQFHAF  165

AMSH-FP  EDQLKKQELARGQIR-----GQDSPVLSE----QTDGSALSCFSTHQSNSLRNAFADHPH  231
AMSH     EEMIRNQELEKERLKIVQEFGKVDPGLGGPLVPDLEKPSLDVFPTLTVSSI------QPS  219

AMSH-FP  KSDGSNFANYSPPVNRALKPAATLSAVQNL-VVEGLRCVVLSRDLCHKFLLLADSNTVRG  290
AMSH     DCHTTVRPAKPPVVDRSLKPGA-LSNSESIPTIDGLRHVVVPGRLCPQFLQLASANTARG  278

AMSH-FP  IETCGILCGKLTHNEFTITHVVVPKQSAGPDYCDVENVEELFNVQDQHGLLTLGWIHTHP  350
AMSH     VETCGILCGKLMRNEFTITHVLIPKQSAGSDYCNTENEEELFLIQDQQGLITLGWIHTHP  338

AMSH-FP  TQTAFLSSVDLHTHCSYQLMLPEAIAIVCSPKHKDTGIFRLTNAGMLEVSTCKKKGFHPH  410
AMSH     TQTAFLSSVDLHTHCSYQMMLPESVAIVCSPKFQETGFFKLTDHGLEEISSCRQKGFHPH  398

AMSH-FP  TKDPKLFSICSHVLVKDIKTTVLDLR*                                    437
AMSH     SKDPPLFCSCSHVTVVDRAVTITDLR*                                    415
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Fig. 1. Sequence alignment of mouse AMSH-FP(α) with human AMSH. The cDNA sequence has been submitted to the DDBJ/GenBank DNA databases under Accession No. AB066211. The residues are numbered on the right and gaps (-) are inserted to achieve maximum similarity. Identical residues are in bold letters. The putative translation initiation position of AMSH-FPβ is indicated by an arrow. NLS and JSH regions are indicated with dashed and solid boxes, respectively. In AMSH, two PXXP motif sites are doubly underlined.

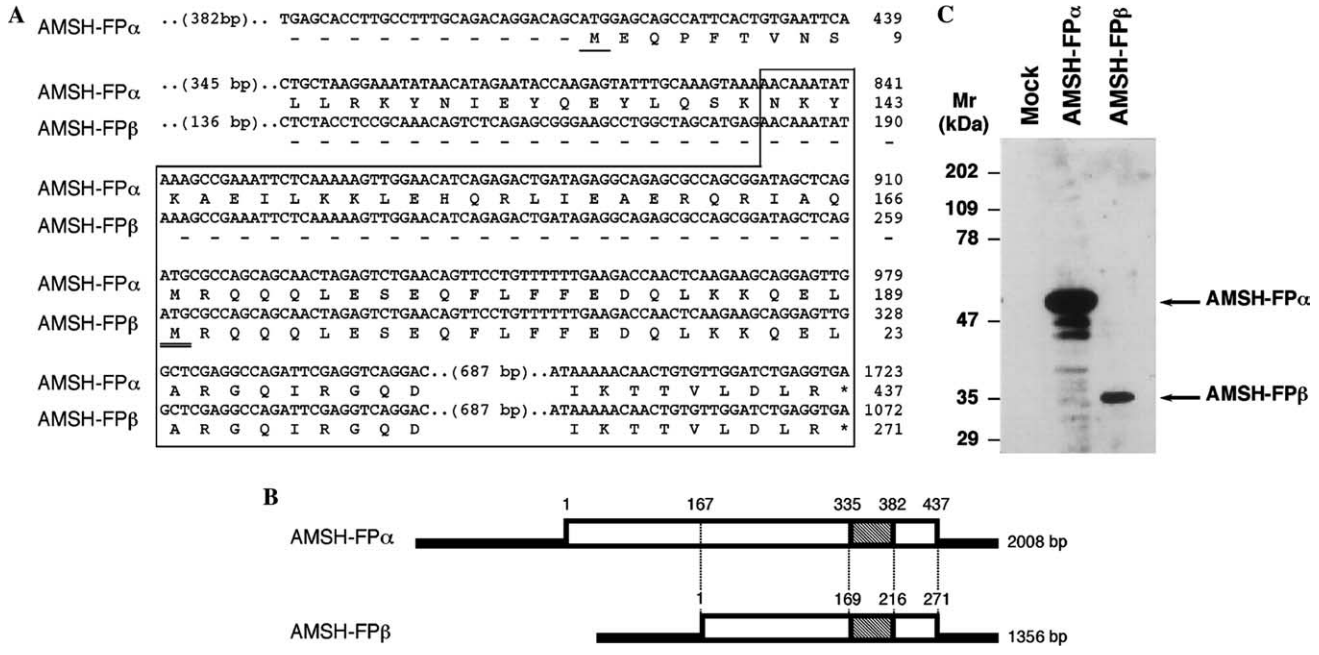

Fig. 2. Identification of two AMSH-FP isoforms. (A) The cDNA and amino acid sequences of the 5′-end of AMSH-FPβ in comparison with the corresponding region of AMSH-FPα. The identical cDNA region is boxed. The initiation codon in AMSH-FPα is underlined and in AMSH-FPβ is double-underlined. (B) Schematic representation of mouse AMSH-FPα and AMSH-FPβ mRNAs. Numbers of amino acid residues of each product are shown. JSH regions are shaded. Thin lines indicate the 5′- and 3′-untranslated regions. (C) Immunoblot analysis of AMSH-FPα and AMSH-FPβ expressed in COS-7 cells. Cell lysates of COS-7 cells transfected with empty plasmid (Mock) and expression plasmid for AMSH-FPα and AMSH-FPβ were, respectively, immunoprecipitated with an anti-myc antibody, electrophoresed, and immunostained with an anti-myc antibody.

subtypes isolated from brain and testis AMSH-FPα and AMSH-FPβ, respectively. In AMSH-FPβ, there was only one potential ATG codon upstream of a long open reading frame (271 residues), while it did not meet the Kozak criteria. Therefore, we examined the M_r of recombinant AMSH-FPα and AMSH-FPβ proteins expressed in COS7 cells. Expression plasmids for myc-tagged AMSH-FPα and AMSH-FPβ were, respectively, transfected into COS7 cells and these cell lysates were subjected to SDS–PAGE and subsequent Western blots using an anti-myc antibody. Approximately 50 and 35 kDa proteins, respectively, derived from cells transfected with AMSH-FPα and AMSH-FPβ expression plasmids were clearly detected (Fig. 2C).

Because these M_r values of AMSH-FPα and AMSH-FPβ were comparable to those predicted from their respective sequences, it is highly likely that the ATG codon numbered as +1 is an initiation codon. When we checked the binding potential of AMSH-FPα and AMSH-FPβ with LIMK2, no specific associations were seen (data not shown).

Genomic organization and tissue expression of AMSH-FP gene

When we searched BLAST genomic sequence database MGSCV3 with the mouse AMSH-FP cDNA, genomic sequences containing open reading frames for AMSH-FP were found. The AMSH-FP gene is located on chromosome 19C1 (Accession No. NT_039689.1) and is composed of 11 exons interrupted by 10 introns (Fig. 3). The sequence at exon–intron boundaries completely fits the donor/acceptor splicing rule (Table 1) [19]. The transcription unit contains 11 exons plus an alternative exon, named exon 1β (Fig. 3). Exons 1α to 5 collectively encode first 832 bp of AMSH-FPα cDNA containing the translation start site and 166 amino acids at the N-terminus. Exon 1β includes entire 5′-terminal sequences specific for AMSH-FPβ cDNA and can be spliced to exon 6, which encodes the 5′-end of cDNA region common to AMSH-FPα and AMSH-FPβ. Accordingly, it is assumed that AMSH-FPβ mRNA is transcribed from exon 1β due to alternative promoter usage (Fig. 4). Conventional TATA or CAAT box is not found in 5′-upstream sequences of exons 1α and 1β, however, several binding motifs for the transcription factors, CdxA, p300, AP-1, Oct-1, c-Myc, and MZF1, are found in the upstream region of exon 1α. Likewise, putative binding sites for Oct-1, CdxA, HSF, Tst-1, GATA, CREB, v-Myb, and C/EBPα are found in the upstream region of exon 1β.

Expression of AMSH-FP mRNA in mouse tissues was examined by Northern blot analysis, using a cDNA fragment of the common C-terminal region of AMSH-FPα and AMSH-FPβ as a probe. The probe detected transcripts of 2.0 kb in various adult organs; strong expression was seen in thymus, and to a lesser extent in brain, lung, spleen, stomach, intestine, colon, and ovary (Fig. 5). AMSH-FP transcripts were hardly detectable in heart, liver, kidney, and skeletal muscle. In contrast, a 1.3 kb transcript was strongly and restrictedly detected in testis. This 1.3 kb transcript could not be detected when cDNA sequence specific to AMSH-FPα was used as a probe (data not shown).

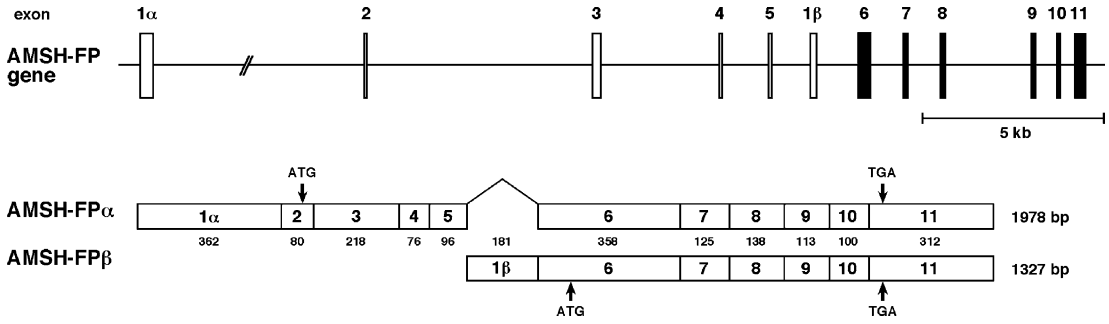

Fig. 3. Genomic organization of mouse AMSH-FP gene. The linear map of exon–intron structure is schematically shown. Exons are represented as numbered boxes. Closed boxes indicate those common to AMSH-FPα and AMSH-FPβ. Two alternative splicing patterns, which give rise to AMSH-FPα and AMSH-FPβ transcripts, respectively, are shown in the bottom. For numbers of nucleotides in each exon, see Table 1. "ATG" and "TGA" indicate the positions of initiation and termination codons, respectively.

Table 1
Exon–intron junctions of the mouse AMSH-FP gene[a]

Exon	Exon size (bp)	Position in cDNA	Splice donor site	Intron size (kb)	Splice acceptor site
1	362	1–362	CCGAG gtgag	27.64	aacag ATGAA
2	80	363–442	CACTG gtaag	6.28	tgcag AAAAA
3	218	443–660	ATAAC gtaag	3.53	tctag GTTAT
4	76	661–736	TGAAG gtaca	1.26	gaaag AAACT
5	96	737–832	GTAAA gtaag	2.44	cttag AACAA
6	358	838–1190	TCAGA gtaag	0.89	tttag ATTTG
7	125	1191–1315	AACTG gtatg	0.94	tctag ACACA
8	138	1316–1453	TCCAT gtatg	2.36	tgcag ACACA
9	113	1455–1566	AAAGA gtaag	0.62	tttag CACCG
10	100	1567–1666	TCAGT gtgag	0.42	tctag ATATG
11	312	1667–1978			
		Consensus	C/AAG **gt**[a]/gagt		C/tc**ag** G

[a] For exon and intron sequences, capital and small letters are used, respectively.

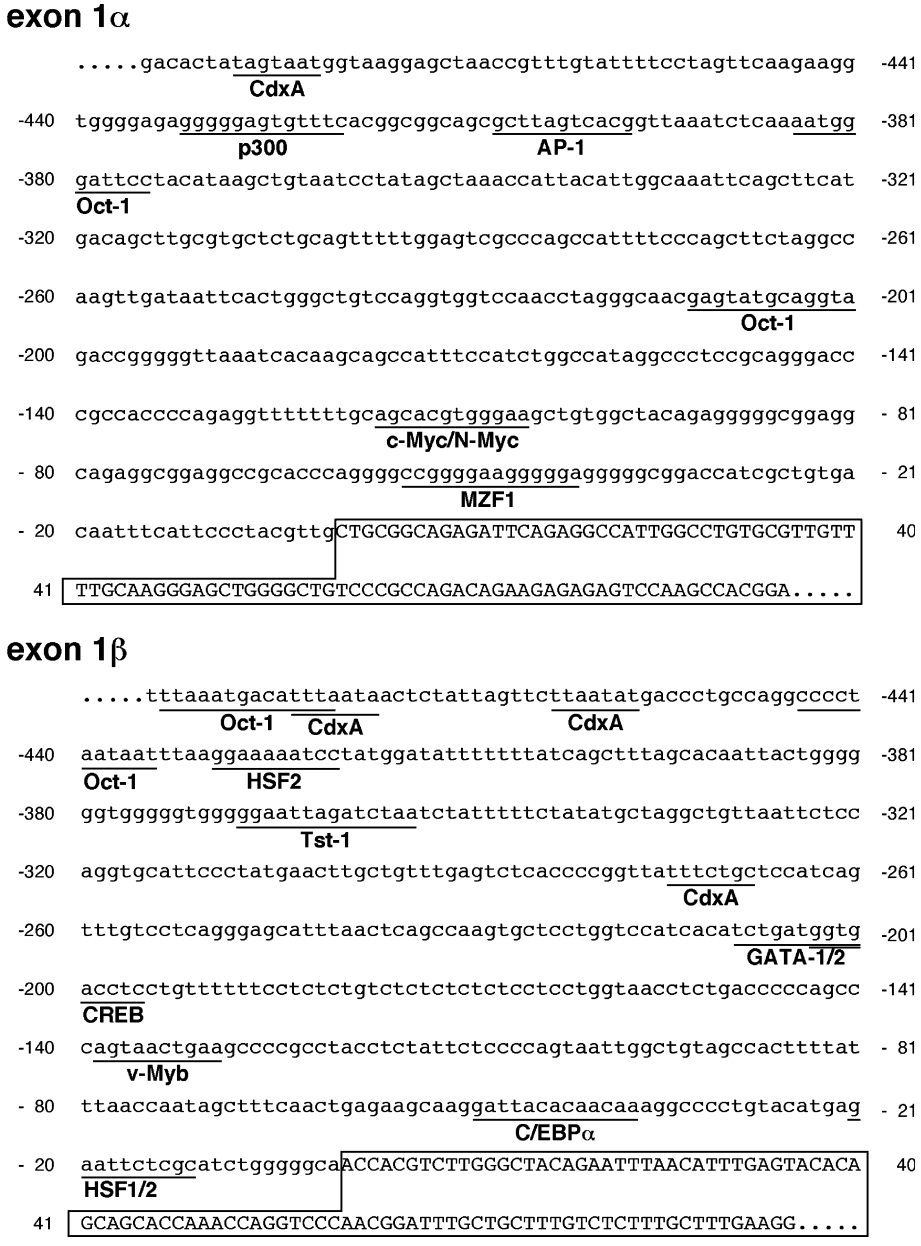

Fig. 4. Nucleotide sequences of the 5′-upstream regions of mouse AMSH-FP gene. These sequences were taken from MGSCV3 database. Nucleotides encoding the cDNA region are in capitals. The 5′-ends of AMSH-FPα and AMSH-FPβ cDNA cloned by us are tentatively numbered as 1 and nucleotide numbers in exons are shown in left. Consensus binding motifs for CdxA, p300, AP-1, Oct-1, c-Myc/N-Myc, MZF1, HSF, Tst-1, GATA, CREB, v-Myb, and C/EBPα were identified using the Transcription Factor Database (TRANSFAC).

Haploid expression of testis-specific AMSH-FP isoform

Because AMSH-FPβ was specifically expressed in testis in adult tissues, we analyzed testicular cell types in which AMSH-FPβ was expressed. To determine if AMSH-FPβ is transcribed in testicular germ cells or in somatic cells, we first analyzed the expression of AMSH-FP mRNA in three distinct types of testes, which were devoid of differentiated germ cells. In W/Wv mice, abnormalities in c-kit expression led to different types of abnormalities during spermatogenesis (e.g., decreases in primordial germ cell migration and/or spermatogonia proliferation) [20]. Juvenile spermatogonial depletion (jsd) and the cryptorchid testis (Crp) result in spermatogonial arrest after the first wave of spermatogenesis [21,22]. As described above, 1.3 kb transcripts of AMSH-FPβ were abundant in normal adult testis (Fig. 6A). However, AMSH-FPβ (and also AMSH-FPα) mRNA was not detectable in agametic testis derived from W/Wv, jsd/jsd, and Crp mice, thus indicating that AMSH-FPβ was transcribed in differentiated germ cells in testis. We then examined AMSH-FPβ mRNA expression in

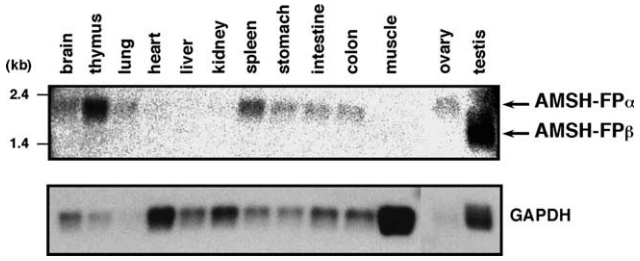

Fig. 5. Expression of AMSH-FP mRNA in adult mouse tissues. Poly(A)$^+$ RNA (2 μg per each lane) from various tissues was subjected to Northern blot analysis, using the ^{32}P-labeled C-terminal fragment of mouse AMSH-FP (upper panel). The same blot was rehybridized with ^{32}P-labeled glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as a control (lower panel). Positions of molecular weight markers are on the left.

various stages of mouse testes, using Northern blots, since the first wave of spermatogenesis was well characterized by accumulation of germ cells at progressively more advanced stages (Fig. 6B). An AMSH-FP-specific signal was undetectable in testes of mice within 16 days after birth. In case of a long exposure, AMSH-FPβ transcript was first detectable as a faint band in testis at 20 days (data not shown) and this corresponded to the first appearance of haploid germ cells (spermatids). As the expression of AMSH-FPβ mRNA was drastically increased during 20–60 days, the predominant expression of AMSH-FPβ in spermatids seems likely.

Finally, localization and identification of AMSH-FP-expressing cells in testis was done using in situ hybridization. Consistent with data from Northern blot analysis, AMSH-FP transcripts were predominantly detected in layers of differentiated germ cells in testis. In certain populations of seminiferous tubules (about 40%) at 20 days, specific signals were only found in the most advanced stages of germ cells, that is the round spermatids (Figs. 6D and E). Similarly, in adult testis, AMSH-FP transcripts appeared in ring-like layers of germ cells located in the central part of tubules. AMSH-FP (possibly AMSH-FPβ) transcripts were detected in round spermatids of tubules at stages I–IV (Figs. 6F–I). In these tubules, no significant signals were found in other stages of spermatogenic cells, such as pachytene spermatocytes and elongated spermatids.

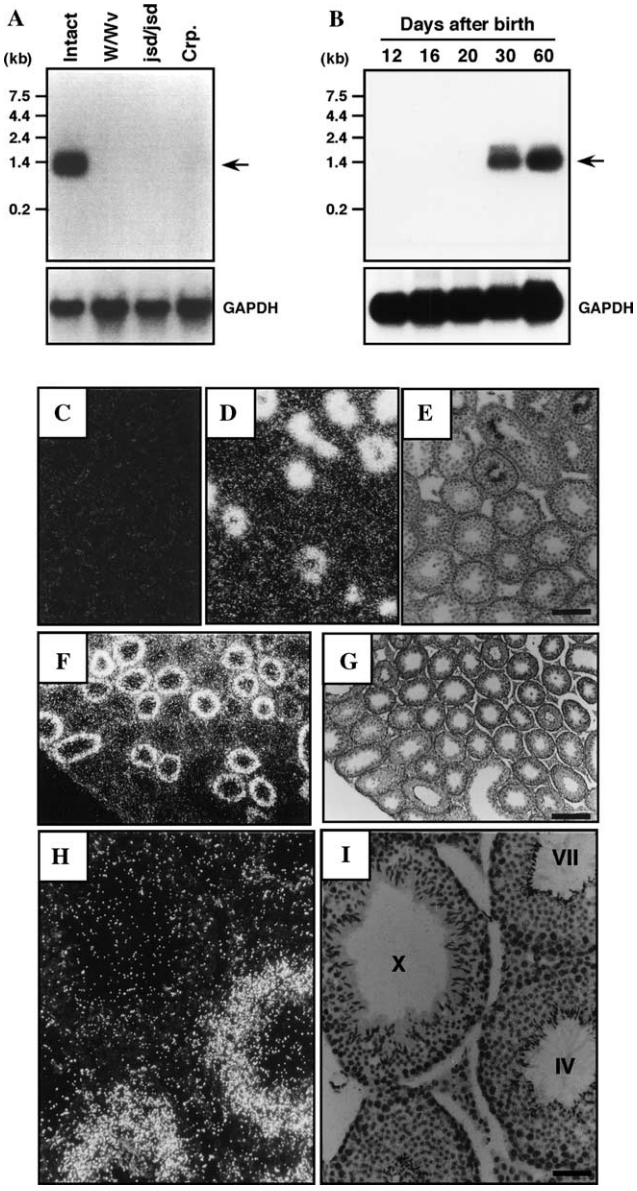

Fig. 6. Expression and in situ localization of AMSH-FPβ mRNA in mouse testes. (A,B) Poly(A)$^+$ RNA (2 μg per each lane) prepared from agametic testes (A) or various stages of mouse testes (B) was subjected to Northern blot analysis, using the ^{32}P-labeled C-terminal fragment of mouse AMSH-FP (upper panel). The same blot was rehybridized with ^{32}P-labeled GAPDH, as a control (lower panel). Arrows indicate the AMSH-FPβ transcripts and positions of molecular size markers are to the left. (C–I) In situ localization of AMSH-FP mRNA in the testis. The sections of testis in 20 dayed mice (C–E) and those in adults (F–I) were hybridized with AMSH-FP riboprobes (C, sense; D–I, antisense). E, G, and I are the corresponding bright-field images to D, F, and H, respectively. The stage of the seminiferous tubules in different stages is indicated in Greek in I. Bars, 100 μm in E, 250 μm in G, and 50 μm in I, respectively.

Discussion

In the present study, we identified a gene encoding an AMSH-related protein termed AMSH-FP and found that two AMSH-FP variants were differently expressed in tissues. During preparation of this manuscript, the cloning of a putative human homolog of AMSH-FP was reported; human AMSH-FP (AMSH-LP in the report) is ubiquitously distributed in various tissues and has a functional similarity with AMSH without binding to the SH3 domains of STAM1 and Grb2 [23]. However, it is noteworthy that cloning and expression of the short form of AMSH-FP variant (here termed AMSH-FPβ) was not noted in human, including in testis. On the other hand, in mouse the

long form of AMSH-FP (termed AMSH-FPα) was widely expressed in brain, thymus, spleen, and digestive organs, while the short form AMSH-FPβ transcripts were restrictedly expressed in testis, and more definitely in round spermatids.

Various genes are commonly expressed in testis and other tissues, whereas the size of transcripts in testis is often distinct from that found in somatic cells [17,24]. In these cases, testis-specific transcripts are generated due to alternative promoter usage and/or alternative splicing. In addition, since only the first 181 bp of the 5′-untranslated region in AMSH-FPβ is unique and the following sequence is completely the same as that in AMSH-FPα, the presence of AMSH-FPβ transcript in testis can be attributed to alternative promoter usage and/or alternative splicing. Analysis of potential binding sites for transcription factors in the upstream of the 5′-termini of the AMSH-FPα and AMSH-FPβ genes indicated that some transcription factors seem to be commonly used but others seem to be distinctly used between AMSH-FPα and AMSH-FPβ. Therefore, distinct usage of these transcription factors may confer distinct tissue specificity between AMSH-FPα and AMSH-FPβ.

It is considered that such testis-specific isoforms have functions different from those for original, somatic-type gene products. For example, although cAMP-responsible transcriptional factor CREB promotes cAMP-induced gene expression, testis-type CREB can act as transcription repressor [25]. In addition, we recently found that the original type LIMK2 acts as a cofilin-kinase in the cytoplasmic region, regulating cytoskeletal actin dynamics, while testis-type LIMK2 (tLIMK) is predominantly distributed in the nucleus of spermatogenic cells and may control cofilin and/or actin turnover in the nucleus [17,26].

Northern blots and in situ hybridization analyses clearly showed exclusively restricted AMSH-FPβ transcription in round spermatids; AMSH-FPβ signals were rarely evident in primary/secondary spermatocytes and elongating spermatids. Previous reports demonstrated that several round spermatid-specific products, including HR6B, CAMK4, and CREM [10–12], play an essential role in spermiogenesis, including chromatin condensation, and acrosomal biogenesis. Hence, such restricted localization of AMSH-FPβ in round spermatids strongly suggests the functional significance of the AMSH-FPβ product in sperm formation.

Since the amino acid sequence similarly between AMSH-FP and AMSH is considerably high through the entire region, the functional contribution of AMSH-FP to certain signal transduction system is implicated. AMSH was first identified as an adaptor molecule participating in cytokine (IL-2 and GM-CSF)-mediated signaling pathways [13]. AMSH has two SH3 binding motifs PXXP and interacts with STAM, a Jak3-associating adaptor, via these sites. However, PXXP motif was not found in the AMSH-FP protein, suggesting that AMSH-FP could not act as an SH3-mediated adaptor molecule. Another structural feature of AMSH is the presence of the JSH region which is highly conserved in both AMSH-FPα and AMSH-FPβ. An AMSH mutant lacking the JSH region dominant-negatively suppressed c-*myc* induction, thereby indicating involvement of the JSH region in transcriptional regulation [13]. Moreover, the JSH region is also seen in JAB1 (Jun activation domain-binding protein-1) and Pad1/Poh1 [27,28], co-activator proteins for the transcription of AP-1 target genes. Although further biochemical characterization of AMSH-FP has to be done, our view is that AMSH-FP may be involved in regulating some types of gene transcription through interactions with other molecules via JSH region. Together with findings that AMSH-FPα is relatively strongly expressed in thymus and involved in IL-2-mediated signal transduction [23], involvement of AMSH-FPα in signal transduction in immune functions is suggested.

Itoh et al. [29] reported the contribution of AMSH to bone morphogenetic protein (BMP)/transforming growth factor (TGF)-β signaling. AMSH directly interacted with Smad6/7, potent antagonists of the BMP/TGF-β-mediated signal transduction, and antagonized their inhibitory actions. Some lines of evidence suggest the functional role of BMP/TGF-β signaling in spermatogenesis. BMP8a/b are specifically produced in round spermatids and loss of BMP-8a/b critically impaired spermatogenesis in mice [30]. TGF-β1, -β2, -β3, and their type I and II receptors are expressed in differentiated germ cells and/or supporting somatic cells (Sertoli cells) [31]. Since AMSH is not expressed in post-meiotic germ cells (our unpublished data), we suspect that AMSH-FP may regulate BMP/TGF-β signaling during spermiogenesis, instead of AMSH.

Although AMSH-FP was cloned as a LIMK2-binding molecule in a yeast two-hybrid system, we could not obtain data showing a physical interaction between them. Like AMSH-FP, LIMK2 has a testis-specific alternative splicing variant, called tLIMK2 [17]. We recently found that gene-disruption of LIMK2 led to abnormal spermatogenesis in mice [9]. Since the localization of AMSH-FPβ partly overlapped with that of tLIMK2, the possibility of functional interaction of AMSH-FP with LIMK2 could not be excluded. Studies to define biological roles of AMSH-FP, particularly in signal transduction and spermatogenesis, are in progress.

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