

Structure and Characterization of AAT-1 Isoforms

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A novel protein, AAT-1, was identified as a AMY-1-binding protein and three splicing variants of AAT-1, AAT-1 α , - β and - γ were identified. The function of AAT-1 is thought to be related to spermatogenesis. In this study, we further identified other splicing isoforms of AAT-1, AAT-1L, AAT-1M and AAT-1S, consisting of 767, 603 and 252 amino acids, respectively. These isoforms were found to use a promoter different from that used by AAT-1 α , - β and - γ in the *aat-1* gene, which contains 20 exons. Only 60 amino acids in the C-terminal portion of AAT-1 derived from exons 15–17 are common among AAT-1L, AAT-1M, AAT-1S and AAT-1 α . While AAT-1 α is specifically expressed in the testis, AAT-1L, AAT-1M, AAT-1S were found to be differentially expressed in human tissues. All of the isoforms of AAT-1 were found to bind to and colocalized with AMY-1 in human cells. While AAT-1L and AAT-1M were found to be localized diffusely in the cytoplasm, AAT-1S, like AAT-1 α , was found to be localized in the mitochondria-like structure, suggesting different roles of AAT-1 isoforms in cells.

Key words AAT-1; AMY-1; promoter usage; spermatogenesis

AMY-1 has been identified by us as a c-Myc-binding protein and was found to stimulate c-Myc transcription activity. AMY-1 was also found to be associated with A-kinase anchor protein 84/149 (S-AKAP84/AKAP149) in the mitochondria in somatic cells and sperm, suggesting that it plays a role in spermatogenesis. To elucidate the functions of AMY-1, a cDNA encoding a novel protein named AAT-1 was obtained. Three isoforms of AAT-1, AAT-1 α , - β and - γ were found to be derived from an alternative splicing of the transcripts of the *aat-1* gene, which was mapped at human chromosome 3q13–3q21. AAT-1 is specifically expressed in the testis during the course of spermatogenesis and is also present in the spermatid and mature sperm, as is AMY-1.^{1–3)} AAT-1 α binds to and is colocalized in mitochondria with AMY-1 in human HeLa and mouse GC-1 cells.⁴⁾ Furthermore, AAT-1 α binds to the N-terminal half of S-AKAP84/AKAP149 in a complex with AMY-1 and a regulatory subunit (RII) of cAMP-dependent kinase (PKA), in which AAT-1 α is associated with RII *via* S-AKAP84/AKAP149, in the rat testis and HeLa cells. AAT-1 α was found to stimulate a phosphorylation activity of PKA and AAT-1 itself was phosphorylated by PKA *in vivo* and *in vitro*.⁴⁾ These results suggest that both AAT-1 and AMY-1 play roles in spermatogenesis. In this study, to further characterize structure and functions of AAT-1, other splicing isoforms of AAT-1 were identified. Binding activities of AAT-1 isoforms to AMY-1 and their cellular localizations were analyzed.

MATERIALS AND METHODS

Cell Culture Human cell lines of HeLa, 293 and 293T, mouse cell lines of GC-1, TM3, TM4 and NIH3T3 and hamster CHO cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum.

In Vivo Binding Assay Ten micrograms of pcDNA3-AAT-1 α isoforms-FLAG together with 10 μ g of pEF-AMY-1-HA was transfected into human 293T cells 60% confluent

in a 10-cm dish by the calcium phosphate precipitation technique.⁵⁾ Forty-eight hours after transfection, the whole cell extract was prepared by the procedure described previously.¹⁾ Approximately 2 mg of the 293T cell proteins was first immunoprecipitated with a mouse anti-FLAG antibody (M2, Sigma) or with non-specific mouse IgG in a buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 7.5), 0.05% BSA and 0.1% NP-40. After washing with the same buffer, the precipitates were separated in a 15% polyacrylamide gel containing SDS, blotted onto a nitrocellulose filter, and reacted with a rabbit anti-HA antibody (Y11, Santa Cruz) or with the mouse anti-FLAG antibody.

Indirect Immunofluorescence Human HeLa cells transfected with pcDNA3-AAT-1 α isoforms-FLAG together with pEF-AMY-1-HA were fixed with a solution containing 4% paraformaldehyde and reacted with a rabbit anti-HA antibody (Y11, Santa Cruz) or with a mouse anti-FLAG antibody (M2, Sigma). The cells were then reacted with an rhodamine-conjugated anti-rabbit IgG or with an FITC-conjugated anti-mouse IgG and observed under a fluorescent microscope.

RT-PCR Analysis Total testis RNAs were prepared from human tissues (OriGene) or cultured cells by the acid guanidine thiocyanate–phenol–chloroform method, and cDNA was synthesized using the oligo dT primer and BcaBEST polymerase (Takara Co., Ltd). The first strand of cDNA products was amplified with specific primers for the first 5 min at 95 °C and then for 30 cycles of 1 min at 95 °C, 2 min at 58 °C and 2 min at 72 °C. The nucleotide sequences of the sense and antisense primers were

AAT-1L (antisense): 5'-CAACTTCCCTTCTATGTTCT-TTCC-3',

AAT-1M-mouse (antisense): 5'-GCAGGTGCGCAAC-TCCTGTATCAA-3',

AAT-1M (sense): 5'-CCAGAATATGTAGTATGTCAG-GAC-3',

AAT-1S-mouse (antisense): 5'-CAGGCGCTGTTTTTC-CACCTGGCG-3',

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AAT-1 M-human (antisense): 5'-CTCCTGGATCAACT-CCAGTCTGCTT-3',

AAT-1 S-human (antisense): 5'-ACCACTCTCTTCA-GCCTCTCGTAT-3',

AAT-1 large (sense): 5'-GCGAATTCATGAGCCACGC-AGTA-3',

G3PDH-up (sense): 5'-GAAATCCCATCACCATCTTCC-AGG-3',

G3PDH-low (antisense): 5'-CAGTAGAGGCAGGGATG-ATGTT-3'³⁾, β -actin sense: 5'-TACCACGGGCATTGTGA-TGG-3'; β -actin antisense: 5'-GATCTTGATCTTCATGGT-GC-3'.

The amplified products were separated on a 2% agarose gel and stained with ethidium bromide.

RESULTS AND DISCUSSION

We have identified a transcriptional start site of the *AAT-1* α gene, which is mapped at human chromosome 3q12—q13,3, by primer extension analysis, and other two splicing isoforms, AAT-1 β and AAT-1 γ , were also identified (Fig. 1).⁴⁾ Promoter activity was also observed using a luciferase construct linked with the region upstream of the transcriptional start site of the *AAT-1* α gene (data not shown). After a search for human and monkey EST clones that contain sequences homologous to that of AAT-1 α , we identified one human and three monkey clones, whose accession numbers are AK05558 (human), AB071132 (monkey), AB069991 (monkey) and AB070102 (monkey). These clones contain 3334, 2554, 2092 and 1704 nucleotides and encode 767, 767, 603 and 252 amino acids, respectively. Sequences of these clones, AAT-1 α , AAT-1 β and AAT-1 γ , were compared to those of the human genome, and organization of the structure of the

AAT-1 gene was clarified (Fig. 1). Although all the monkey genome sequences were not determined, three monkey cDNAs were found to be almost perfectly matched with human sequences, suggesting that human mRNAs corresponding to these monkey cDNAs are present. Although the sizes of mRNAs of the clones of AK05558 and AB071132 were different due to different usage of polyA adenylation signals, these clones were found to encode identical proteins. Proteins encoded by clones of AB071132, AB069991 and AB070102 were therefore named AAT-1L, AAT-1M and AAT-1S, respectively. The *AAT-1* gene comprises 20 exons and 19 introns, in which the GT-AG rule at the boundary between exons and introns were protected, and two promoters seem to be present. The first promoter may be located upstream of exon 1, which regulates expression of AAT-1L, AAT-1M and AAT-1S, and the second one is located upstream of exon 16, which regulates expression of AAT-1 α , AAT-1 β and AAT-1 γ (Fig. 1). Amino acid sequences from the latter part of exon 15 and whole exons 16 and 17 are common among AAT-1L, AAT-1M, AAT-1S and AAT-1 α As described previously,⁴⁾ long-sized mRNAs corresponding to AAT-1L, AAT-1M or AAT-1S were detected by Northern blot analysis using labeled AAT-1 α cDNA as a probe and a pre-made filter into which mRNA from various human tissues had been blotted (data not shown).

The expressions of AAT-1L, AAT-1M and AAT-1S mRNAs were examined by RT-PCR using various RNAs extracted from human tissues or cultured cells (Fig. 2). The results showed that human mRNA corresponding to monkey AAT-1L, AAT-1M and AAT-1S existed, and that AAT-1L was expressed ubiquitously in all of the tissues tested. AAT-1M was, on the other hand, found to be strongly expressed in the liver, and AAT-1S was found to be expressed strongly in the

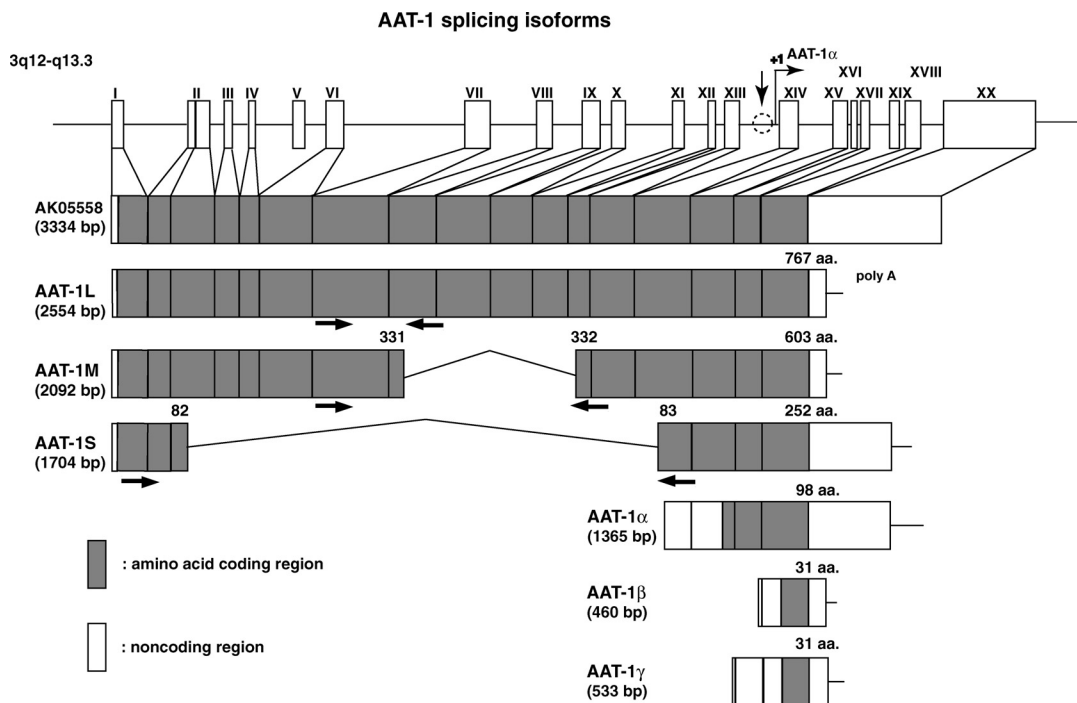


Fig. 1. Organization of the Human *AAT-1* Gene

Physical map of the human *AAT-1* gene is shown. Exons are represented as squares, in which the regions coding for proteins are represented as black boxes, and the numbers above the boxes representing genomic DNA indicate the exon numbers for the AAT-1 gene transcripts. Positions on which PCR primers were set to amplify the regions of AAT-1L, AAT-1M and AAT-1S were indicated by arrows.

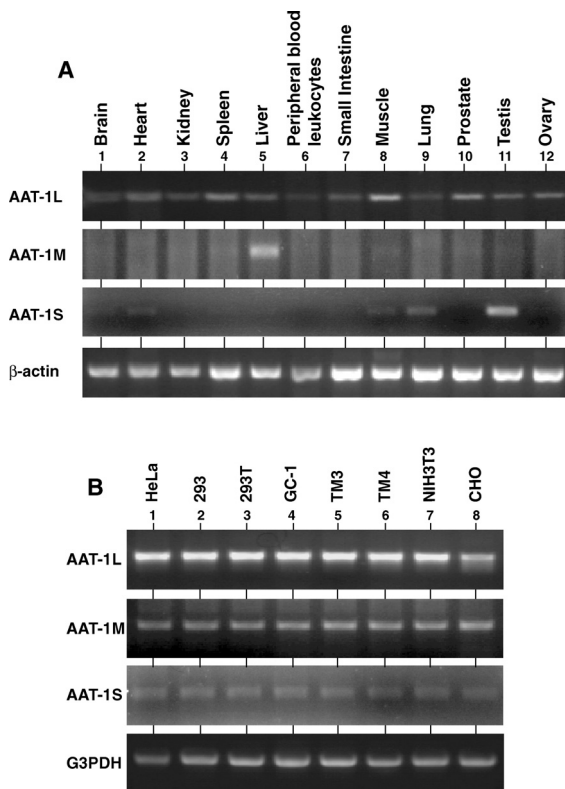


Fig. 2. Expression of AAT-1 mRNA in Human Tissues and in Cultured Cells

(A) Human total RNAs were obtained from OriGene Technologies, and the expressions of AAT-1L, AAT-M and AAT-1S were analyzed by RT-PCR with specific primers for each AAT-1 isoform and β -actin on total RNA as substrates. The amplified DNAs were separated in 1.4% agarose gels and visualized under UV-light. Primers used were AAT-1M (sense) and AAT-1L (antisense) for detection of AAT-1L, AAT-1M (sense) and AAT-1M-human (antisense) for that of AAT-1M, AAT-1 large (sense) and AAT-1S-human (antisense), and β -actin (sense) and β -actin (antisense) for that of β -actin, respectively. Sizes of PCR products corresponding to AAT-1L, AAT-1M and AAT-1S to be amplified are 446, 465 and 302 base pairs, respectively. (B) Total RNAs were extracted from HeLa, 293, 293T, GC-1, TM3, TM4, NIH3T3 and CHO cells, and expressions of AAT-1 isoforms were analyzed as described in A. Primers used were AAT-1M (sense) and AAT-1L (antisense) for detection of AAT-1L, AAT-1M (sense) and AAT-1M-mouse (antisense) for that of AAT-1M, AAT-1 large (sense) and AAT-1S-mouse (antisense), and G3PDH-up (sense) and G3PDH-low (antisense) for that of G3PDH, respectively. Sizes of PCR products corresponding to AAT-1L, AAT-1M and AAT-1S to be amplified are 446, 477 and 329 base pairs, respectively.

testis and moderately in the lung (Fig. 2A). Expression patterns of AAT-1L and AAT-1M are therefore different from that of AAT-1 α , which is specifically expressed in the testis, and the expression pattern of AAT-1S is similar to that of AAT-1 α . AAT-1L, AAT-1M and AAT-1S were found to be expressed in all of the cultured cells, including HeLa, 293, 293T, GC-1, TM3, TM4, NIH3T3 and CHO cells, the expression pattern of which is similar to that of AAT-1 α described previously (Fig. 2B).⁴⁾

To observe the complex formation of AAT-1 splicing isoforms with AMY-1 *in vivo*, expression vectors for FLAG-tagged AAT-1L, AAT-1M or AAT-1S together with HA-tagged AMY-1 were transfected into human 293T cells. Forty-eight hours after transfection, cell extract was prepared and the proteins in the extract were first immunoprecipitated with an anti-FLAG antibody or non-specific IgG. The anti-FLAG antibody precipitated AAT-1L-, AAT-1M- and AAT-1S-FLAG (Fig. 3, lower panel). AMY-1-HA, on the other hand, was detected in the immunoprecipitate from FLAG-tagged AAT-1 splicing isoforms-transfected cells with an

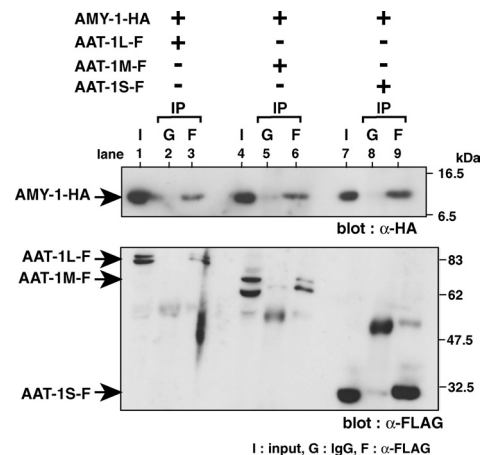


Fig. 3. Association of AAT-1 Isoforms with AMY-1 in Human Cells

anti-HA antibody but not with IgG (Fig. 3, upper panel). These results indicate that AMY-1 is associated with the AAT-1L, AAT-1M or AAT-1S in ectopic expressed 293T cells. Expression vectors for AAT-1L-, AAT-1M- or AAT-1S-FLAG along with AMY-1-HA were introduced into human 293T cells by the calcium phosphate precipitation technique.⁵⁾ Two days after transfection, cell extracts were prepared, and the proteins in the extracts were first immunoprecipitated (IP) with an anti-FLAG monoclonal antibody (M2, Sigma) (lanes 3, 6, 9) or non-specific IgG (lanes 2, 5, 8). The proteins in the precipitates were separated in a 10% polyacrylamide gel and analyzed by Western blotting with the anti-FLAG antibody (Fig. 3, lower panel) or an anti-HA polyclonal antibody (Y11, Santa Cruz) (Fig. 3, upper panel). I (lanes 1, 4, 7), G (lanes 2, 5, 8) and F (lanes 3, 6, 9) in the upper figure indicate input, IgG and the anti-anti-FLAG antibody that were used for immunoprecipitation.

anti-HA antibody but not with IgG (Fig. 3, upper panel). These results indicate that AMY-1 is associated with the AAT-1L, AAT-1M or AAT-1S in ectopic expressed 293T cells.

To determine the cellular localizations of AAT-1L, AAT-1M and AAT-1S, human HeLa cells were transfected with expression vectors for FLAG-tagged AAT-1L, AAT-1M or AAT-1S alone or together with HA-tagged AMY-1. Forty-eight hours after transfection, HeLa cells were stained with anti-FLAG and anti-HA antibodies, and the proteins were detected by FITC- and rhodamine-conjugated second antibodies, respectively, and then visualized under a confocal laser microscope (Fig. 4). In HeLa cells transfected with AMY-1-HA alone, AMY-1-HA was found to be localized both in the cytoplasm and nucleus (Fig. 4A). When HeLa cells were transfected with AAT-1L-, AAT-1M-, AAT-1S- or AAT-1 α -FLAG alone, all of the isoforms were found to be localized in the cytoplasm but distinct patterns of localization were observed (Fig. 4B). Both AAT-1L and AAT-1M and both AAT-1S and AAT-1 α were found to be localized in the cytoplasm diffusely and as dot-like structures that are indicated by arrows, respectively (Fig. 4B). The dot-like structures in the cytoplasm are likely to be mitochondria as described previously.⁴⁾ In HeLa cells cotransfected with AAT-1L-, AAT-1M, AAT-1S- or AAT-1 α -FLAG and AMY-1-HA, all of the AAT-1 isoforms were found to be still localized diffusely or as dot-like structures in the cytoplasm and colocalized with AMY-1-HA as shown by the yellow color (Fig. 4C). These results indicate that AAT-1 isoforms are bona-fide AMY-1-binding proteins.

In this study, we identified novel AAT-1 isoforms, AAT-1L, AAT-1M and AAT-1S, and we determined their expression profiles and cellular localization. These isoforms seem to be regulated by a promoter different from that for AAT-1 α in the same AAT-1 gene, and only about 60 amino acids located at the C-terminus are common among the isoforms. The results

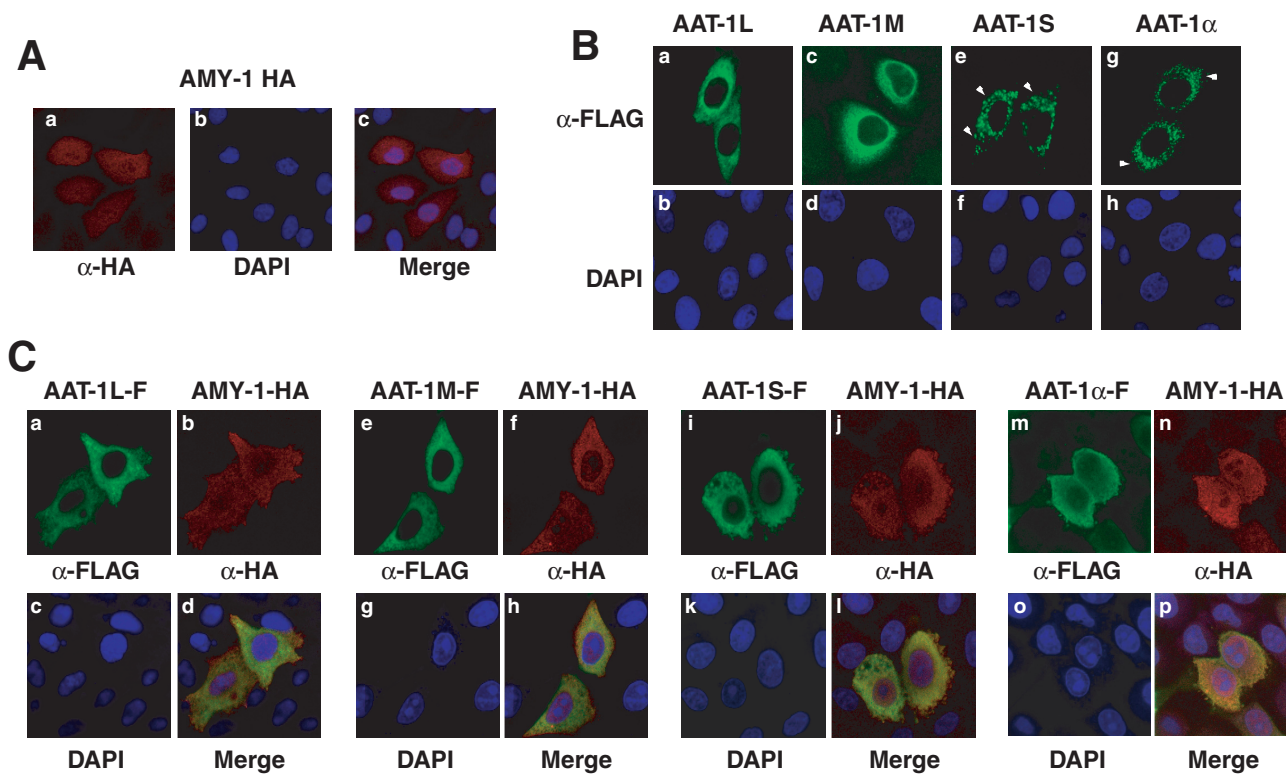


Fig. 4. Colocalization of AAT-1 Isoforms with AMY-1 in Human Cells

(A) An expression vector for AMY-1-HA was transfected into human HeLa cells by the calcium phosphate precipitation technique.⁵⁾ Two days after transfection, the cells were fixed, reacted with an anti-HA polyclonal antibody (Y11, Santa Cruz), and visualized with a rhodamine-conjugated anti-rabbit antibody (Fig. 4A-a). Cells were stained with DAPI, and both figures were merged (Figs. 4A, b and c, respectively).

(B) Expression vectors for AAT-1L-, AAT-M- or AAT-1S-FLAG were transfected into human HeLa cells. Two days after transfection, the cells were fixed, reacted with an anti-FLAG monoclonal antibody (M2, Sigma), and visualized with a FITC-conjugated anti-mouse antibody as described in A.

(C) Expression vectors for AAT-1L-, AAT-M- or AAT-1S-FLAG were cotransfected with AMY-1-HA. The cells were reacted with an anti-FLAG monoclonal antibody or an anti-HA polyclonal antibody and visualized with a FITC-conjugated anti-mouse antibody or rhodamine-conjugated anti-rabbit antibody (α -FLAG and α -HA, respectively) as described in A. Both figures were merged (Merge).

showed that AAT-1L, AAT-M and AAT-1S were bound to and colocalized with AMY-1 in *in vivo* cultured cells and were expressed distinctly in human tissues, indicating that the carboxyl 60 amino acids are sufficient for the isoforms to bind to AMY-1. Expression in tissues and cellular localization of AAT-S, but not AAT-1L and AAT-1M, are similar to those of AAT-1 α , suggesting that the N-terminal sequences of AAT-1L and AAT-1M contribute to a distinct phenotype compared to AAT-1S and AAT-1 α . These results also suggest that each isoform plays a distinct role in cells in association with AMY-1 or alone. AMY-1 was shown to be located in the mitochondria by making a ternary complex among AMY-1, AKAP84/149 and RII subunit of PKA,³⁾ thereby suggesting that it plays a role in spermatogenesis.³⁾ It is necessary to determine whether AAT-1 isoforms participate in this complex.

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