ORIGINAL

# Expression analysis of a mouse orthologue of HSFY, a candidate for the azoospermic factor on the human Y chromosome

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**Abstract : Heat shock transcription factor on Y (HSFY) is located in one of three candidate regions for azoospermic factor (AZF), AZFb on the Y chromosome. We and others have already revealed that some azoospermic males are missing the regions of the Y chromosome including HSFY. Previously, we showed that murine HSFY-like sequence [mHSFYL (Riken cDNA 4933413G11Rik)], which is the mouse orthologue of HSFY, is exclusively expressed in testis. The sequences encoding the presumed DNA-binding domain in HSFY and mHSFYL were found in other mammals such as dogs, cows and chickens. To elucidate mHSFYL expression in the testes in detail, we carried out in situ hybridization. mHSFYL was predominantly expressed in round spermatids. Furthermore, we clarified the intracellular distribution of mHSFYL in COS1 cells with HA- or GFP-tagged proteins. Both HA-mHSFYL and GFP-mHSFYL were located in the nucleus. Our results suggest that HSFY/mHSFYL may have evolutionarily conserved functions for spermatogenesis.**
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***Keywords*** **:** *HSFY, mHSFYL, HSF, Y chromosome, spermatogenesis*

## INTRODUCTION

Idiopathic azoospermia is an important cause of male infertility. Around 10% of males with azoospermia are known to have interstitial deletions on the long arm of the Y chromosome (1, 2). So far, there are three major candidate regions for the azoospermic factor (AZF), AZFa, AZFb and AZFc, on the Yq (1). Each AZF region contains several candidate genes predominantly expressed in testes (3).

Since HSFY (heat shock factor on the Y chromosome), which is located in AZFb (4), is involved in deletions found in azoospermic patients, it is suggested to be a good candidate for the AZF (5-7). The most striking structural feature of HSFY is a HSF-type DNA-binding domain (DBD) in the middle portion of the protein (5, 6). However, the presumed DBD shows only 30% homology to the DBD of classical HSFs such as HSF1 and HSF2 (5). Moreover, the putative DBD of HSFY seems to lack a structure needed for making contact with the heat shock element which is found upstream of the genes encoding heat shock proteins (5, 8). Therefore, HSFY is postulated to have functions different from those of the classical HSFs (5). In the

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previous report, we showed that HSFY is expressed in Sertoli cells and spermatogenic cells (5). The intracellular distribution of HSFY in spermatogenic cells varied depending on the spermatogenic stage, while HSFY seemed to be constitutively expressed in the cytoplasm in Sertoli cells .

We have already revealed that the putative mouse orthologue of HSFY, murine HSFY-like sequence 〔mHSFYL (Riken cDNA 4933413G11Rik)〕, whose HSF-type DBD has 70% homology to that of HSFY, is exclusively expressed in mouse testis (5). However, the type of cells that express mHSFYL in testis remains unknown.

Here we show that sequences similar to the HSFY/mHSFYL DBD are conserved among mammals and chickens and that mHSFYL is predominantly expressed in round spermatids. Moreover, we also show that mHSFYL has potential for translocation from the cytoplasm to nucleus in mammalian cells.

## MATERIALS AND METHODS

*Multiple alignment of HSFY-related sequences*

To compare HSFY/mHSFYL-related sequences among different species, Genetyx-SV/R version 7.08 or CLUSTALW was employed.

*In situ hybridization*

cDNA derived from mouse testes was used as a template for RT-PCR (5). The primers used in this RT-PCR were followed : mHSFYL-ISHF1 5'-GATACGATGGATGTCATCAG-3' and mHSFYL-ISHR1 5'-TTCTAATCTCTGCTATGATG-3' The products were separated with an agarose gel-based electrophoresis and extracted with a QIAXII kit (QIAGEN GmbH, Germany). The purified PCR products were cloned into a pT7 blue-T vector (Novagen, Darmstadt, Germany) that had a T7 promoter. The authenticity of the sequences was confirmed by sequencing. In situ hybridization was conducted with specimens of testes derived from 10 weeks old C57BL/6N mice according to the standard protocol (9). Labeled RNA probes were generated by transcribing with T7 RNA polymerase and digixigenin-UTP. This study conformed to guidelines for the Management of Laboratory Animals in Fujita Health University.

*Immunofluorecence assay of mHSFYL*

The plasimids expressing HA- or EGFP-tagged mHSFYL were generated by cloning an entire open reading frame (ORF) of mHSFYL into pCMV-HA (Clontech, Palo Alto, CA, USA)and pEGFP-C2(Clontech), respectively. COS1 cells established from a kidney of African green monkey were transfected with the plasmids using Fugen6 (Roche, Mannheim, Germany). One microgram of plasmid DNA which encodes EGFP- or HA-mHSFYL was transferred into the cells using Fugene 6, according to the manufacturer's instructions. For detection of HA-mHSFYL, as the primary antibody, a polyclonal antibody raised against HA-epitope (MBL, Nagoya, Aichi, Japan) was used at a dilution of 1 : 50 or 1 : 100. As the secondary antibody, anti-rabbit IgG derived from goat (Sigma Aldrich co, St.Louis, MO, USA) was employed (1:200). To confirm the location of the nuclei, propidium iodide (Sigma) was used. The immunolabeled cells were mounted with Vectashield (Vector laboratories, CA). The cells were analyzed with a fluorescence microscope (Olympas, Tokyo, Japan) and a confocal laser scanning microscope (CLSM, Leica TCS-NT mounted on a Leica light microscope DMRB, Leica AG, Germany).

## RESULTS

*HSFY/mHSFYL family is conserved among various vertebrates*

To elucidate whether species other than human and mouse have genes similar to HSFY/mHSFYL, we searched the database using the presumed DBD of mHSFYL as an electric probe. Sequences similar to the HSFY/mHSFYL DBD were found in some vertebrates including mammals such as the cow and dog, chicken, suggesting that the homologues of HSFY/mHSFYL have important roles among many species (Fig. 1). On the other hand, the orthologues varied in their N- and C- terminal portions (data is not shown). In chicken, we found six genes harboring sequences similar to the presumed DBD of HSFY/mHSFYL, although it remains unclear whether those genes are all expressed or functional.

Since mHSFYL is an intronless gene, we searched for intronless genes for HSFY in the human genome, but failed to find any. Multicopy genes in AZFs on the human Y chromosome and their homologues are summarized in Table 1(3, 10-16). HSFY differs from other Y-linked genes located in AZFs in evolutional conservation.

*mHSFYL is predominantly expressed in round spermatids*

To analyze the types of the cells expressing mHSFYL in testes, we carried out in situ hybridization. The mHSFYL transcript was detected in the seminiferous epithelium. It was predominantly expressed in round spermatids for spermatogenic cells, but not expressed

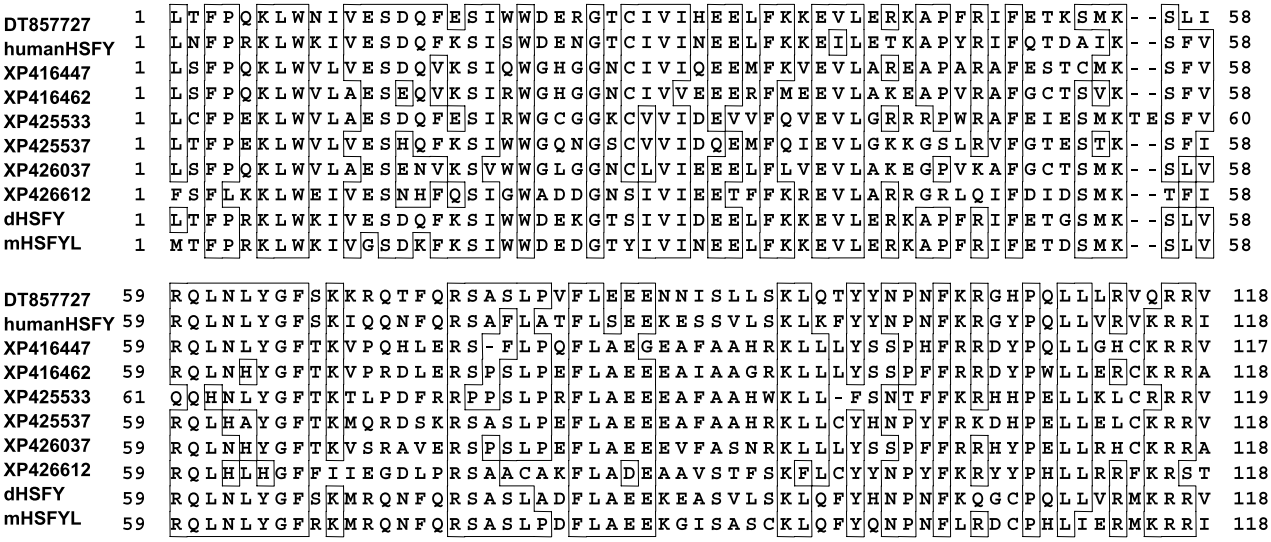


**Fig.1.** A comparison of the amino acid sequences of presumed HSFY/mHSFYL-related proteins among various species. The accession numbers in the database deposited at the NCBI web site head the amino acid sequences. Amino acids conserved among more than six proteins are boxed. DT 857727, cow homologue ; XP 416447, XP 416462, XP 425533, XP 425537, XP 426037, XP 426612, chicken homologues ; dHSFY, dog homologue.

**Table 1.** homologues andothrologues of the AZF candidate genes located in the palindromic regions on the human Y chromosome

| | Y-linked | | X-linked | | autosome-linked | | retrotransposon | |
|---|---|---|---|---|---|---|---|---|
| candidates | human | mouse | human | mouse | human | mouse | human | mouse |
| HSFY | + | − | + | − | −#1 | +#2 | − | +#2 |
| RBMY | + | + | + | + | + | + | + | + |
| CDY | + | − | − | − | + | + | + | + |
| VCY 2 | + | − | + | − | − | − | − | − |
| DAZ | + | − | − | − | + | + | − | − |

#1, HSFY has a presumed pseudogene on the chromosome 22.
#2, only mHSFYL gene (Riken cDNA 4933413G11) is known so far.

in Sertoli cells (Fig. 2). Lydig cells and myoid cells were also negative for mHSFYL expression.

A database analysis with the sequences deposited at the NCBI site revealed that the 2Kb promoter region of the mHSFYL gene has no significantly homologous sequence in the human genome.

*mHSFYL is localized in the nucleus*

To address the intracellular distribution of mHSFYL in mammalian cells, GFP- or HA-tagged mHSFYL was expressed in COS 1 cells. Both HA-mHSFYL and GFP-mHSFYL were detected in the nucleus, suggesting that mHSFYL has the potential to be translocated from the cytoplasm to the nucleus, although no apparent nuclear localization signals (NLSs) were found (Fig. 3) (5).

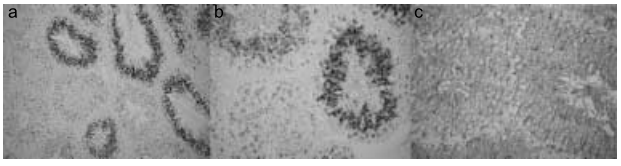


**Fig. 2 :** In situ hybridization analysis of mHSFYL in testes. Different stages of spermatogenesis were observed in the same section. Round spermatids in the inner side of seminiferous tubles were detected with the antisense probe for mHSFYL. a, antisense probe×100 ; b, antisense probe×400 ; c, sense probe×400. Seminiferous tubules positive and negative for mHSFYL are though to be in different spermatogenic cycles.

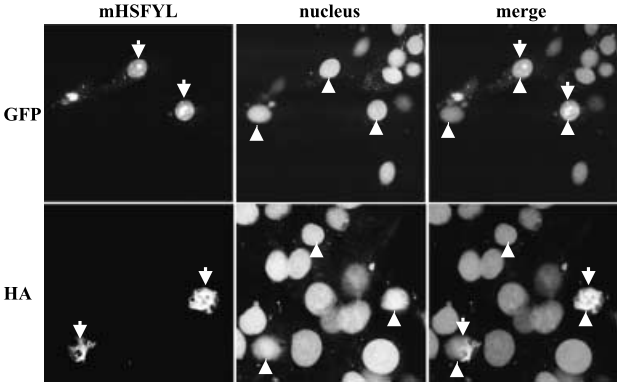


**Fig. 3:** Intracellular distribution of GFP-mHSFYL and HA-mHSFYL. Arrows show HA- or GFP- tagged mHSFYL. Arrow heads correspond to the nuclei. Tagged-mHSFY located in the nucleus is sandwiched between arrows and arrowheads.

## DISCUSSION

We have previously demonstrated that HSFY is expressed in spermatogenic cells from spermatogonia to round spermatids and Sertoli cells using an antibody against HSFY (5). However, by using in situ hybridization, the present study showed that mHSFYL is predominantly expressed in round spermatids. Recently, based on a serial analysis of gene expression (SAGE), Wu *et al.* reported a list of the transcripts expressed in mouse spermatogonia, spermatocytes, and round spermatids (17). They showed that Riken cDNA 4933413 G11Rik (mHSFYL) is predominantly expressed in round spermatids (see supplemental data of ref.17). Therefore, it is certain that mHSFYL is predominantly expressed in round spermatids. Expression patterns of HSFY and mHSFYL are likely to partially overlap for round spermatids, although the former and the latter were detected in testes by immunohistochemistry and in situ hybridization, respectively. This result may suggest that HSFY/mHSFYL have some role in round spermatids. HSFY/mHSFYL may be involved in the maturation of spermatogenic cells after meiosis.

In this study, HA-or GFP-tagged mHSFYL was found in the nucleus. We previously showed that the intracellular localization of HSFY changes between the cytoplasm and nucleus dependent on the stage of spermatogenesis (5). Moreover, epitope-tagged HSFY is located in the cytoplasm in NT 2/D 1 cells. It is noteworthy that the C-terminal portions of mHSFYL and HSFY differ considerably (5). Recently, several proteins interacting with the C-terminal portion of HSFY have been identified (unpublished data). Therefore, the C-terminal portions of HSFY and mHSFYL may differ in the proteins they interact with. The difference in intracellular localization between HSFY and mHSFYL may be related to their partner proteins. It is possible that regulation of the intracellular translocation of mHSFYL is different from that of HSFY. Since mHSFYL has a HSF-type DBD, it is presumed to act as a transcriptional regulator in the maturation of spermatogenic cells after meiosis. An antibody against mHSFYL will unveil intracellular distribution of the mHSFYL protein in detail.

The mHSFYL gene has no introns, suggesting that it was integrated into the genome with retrotransposition during evolution. Therefore, its promoter is postulated to be independent of the ancestral HSFY. Actually, we found this prediction to be correct. As shown in the present study, mHSFYL is expressed in a stage -specific manner during spermatogenesis. In mice, the orthologue of the HSFY gene with introns seems to have been lost. We could not conclude whether the retrotransposon of the gene arose only in the rodent lineage or whether the ancestor of mammals had it. The HSFY gene has a presumed pseudogene on chromosome 22 (5, 6). It is possible that" the original HSFY "on the autosome became a pseudogene after the ancestral gene was transposed to the Y chromosome. Relationship between the Y-linked genes and their homologues on the autosomes has variations for their evolutionary conservation. CDY, which is located on the human Y chromosome, is a retrotransposon of a autosomal gene CDYL (10, 11). However, for mice, CDYL has two alternative transcripts for testis-specific and ubiquitous expression (11). RBMY has intronless homologues on the autosomes (12). Another testis-specific gene on the Y chromosome, TSPY, has functional retrotransposons on the autosome (18).

In some cases, retrotransposon genes have crucial functions. In mice, Utp14b, an autosomal retrotransposon gene is a causative gene for a mouse jsd mutant that shows male infertility (19, 20). In human, TSPYL, which is derived from Y-linked TSPY, is mutated in sudden infant death with dysgenesis of the testes syndrome (21).

Even for RBMY, whose genomic structure is conserved between mouse and human, the stage of expression is different between humans and mice, although some overlap was observed (22, 23). Since HSFY and its orthologues are found across species regardless of genomic structure, they may have important roles in spermatogenesis.

In conclusion, we showed that mHSFYL is expressed in round spermatids. HSFY/mHSFYL may have some role in spermatogenesis.

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