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Structure of *msj-1* gene in mice and humans: A possible role in the regulation of male reproduction ☆

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Abstract

Msj-1 gene encodes a DnaJ protein highly expressed in spermatids and spermatozoa of both rodents and amphibians, possibly involved in vesicle fusion and protein quality control by means of interaction with heat shock proteins.

We isolated and characterized the entire murine *msj*-1 gene and searched for putative *msj-1*-like genes into the human genome. Furthermore, ultrastructural localization of MSJ-1 was analyzed in mouse germ cells by immunogold electron microscopy. The analysis of murine *msj-1* genomic sequence reveals that it is an intron less gene. Putative promoter region was predicted within the 600 bp upstream the transcription start site. In mouse, *msj-1* maps on chromosome 1, into an intronic region of UDP glucuronosyl-transferase 1 family cluster. At ultrastructural level, MSJ-1 marks the developing acrosomic vesicle and the sperm centriolar region. A blast search against the human genome database revealed two closed regions (*Ha* and *Hb*) on human chromosome 2 having high nucleotide identity with murine *msj-1* coding region. Similarly to mouse, in human both regions map into an intronic region of UDP glycosyl-transferase 1 family polypeptide A cluster (*ugt1a@*). A significant ORF encoding a putative DnaJ protein of 145 aa was predicted from *Ha*. Finally, expression analysis, conducted by RT-PCR in human sperm cells, demonstrated that *Ha* mRNA is effectively present in humans; by Western blot, a specific MSJ-1 band of approximately 30 kDa was detected in human sperm. Taken together, these data suggest that *msj-1* gene might be conserved among vertebrates and might exert fundamental functions in reproduction.

Keywords: DnaJ protein; Human; Mouse; *msj-1*; Testis; UDP glucuronosyltransferase

1. Introduction

DnaJ proteins are molecular co-chaperones modulating Hsp70 protein family member activity. Several fundamental cellular processes, as protein folding, degradation of misfolded proteins and vesicular trafficking, are modulated by heat shock protein (Hsp)70/DnaJ system. The main features of DnaJ proteins are: (1) a J domain responsible for Hsp70 recruitment and activation; (2) a glycine/phenylalanine (G/F)-rich domain; (3) a cystein string motif involved in vesicular trafficking (see for example auxilin, cystein string protein) (Ungewickell et al., 1995; Hartl, 1996; Buchner and Gundensen, 1997; Flink, 1999; Lemmon, 2001). The DnaJ MSJ-1 (Mouse Sperm Cell Specific DnaJ first homologue), also known as HSJ-3 or DnaJB3 [DnaJ(Hsp40) homologue, subfamily B, member 3], consists of a conserved J domain, a G/F-rich domain, and an unique carboxyl ter-

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minal domain (Berruti et al., 1998a,b). Despite other members of the DnaJ protein family, MSJ-1 lacks the cystein string motif.

For several years *msj-1* has been considered a testis-specific gene due to its high expression restricted to testicular post meiotic cells in mice (spermatids and spermatozoa) (Berruti et al., 1998a,b); recently, using more sensitive techniques, a scanty expression of *msj-1* has also been reported in the central nervous system (CNS) (Boilleè et al., 2002). MSJ-1 protein has a cytoplasmic localization: in mature spermatozoa it is located in the peri-acrosomal and peri-centriolar regions and it is diffusely distributed in the cytoplasm of motoneurones in the ventral hornes of the spinal cord. In addition, the gene is down regulated in wobbler mice, a natural mutant characterized by aberrant spermiogenesis and degeneration of motoneurones in brain stem and cervical spinal cord (Boilleè et al., 2002; Meccariello et al., 2002). Wobbler phenotype is due to a missense mutation in *vps54* (vacuolar–vesicular protein sorting) encoding a member of the Golgi associated retrograde protein (GARP) complex, involved in vesicular trafficking (Schmitt-John et al., 2005). Histological signs of wobbler mutants indicate a clear defect in the endomembrane system; in fact, during spermiogenesis, acrosome vesicles do not fuse (Heimann et al., 1991) and in motoneurones abnormal dilatation and vesicle formation by endoplasmic reticulum together with intermediate neurofilament accumulation occur (Andrews, 1975; Pernas-Alonso et al., 2001; Boilleè et al., 2002). In this respect, MSJ-1 may exert a fundamental role in vesicle fusion, an event that finely characterizes the process of acrosomogenesis, even if other functions related to protein folding and misfolding might be postulated. Indeed, in testicular germ cells, MSJ-1 immunoprecipitates and co-localizes with Hsp70-2, one of the testis specific variant of Hsp70 (Dix et al., 1996; Eddy, 1999 and references therein), and mUBPy (Berruti and Martegani, 2005), a novel de-ubiquitinating enzyme, highly expressed in testis and brain (Gnesutta et al., 2001). Interestingly, deposits of ubiquitinated proteins are often located in the cell peri-centriolar region (Kopito and Sitia, 2000), just where in sperm cells proteasome 26S, mUBPy and MSJ-1 have been shown to be (Berruti and Martegani, 2005).

Recently, it has been identified by molecular cloning in *Macaca fuscata mfsj-1* (*Macaca fuscata* spermatogenetic cell specific DnaJ 1 homolog), a gene, close to *msj*, with a similar testicular expression pattern (Yu and Takenaka, 2003). This suggests that in mammals there is a *msj-1* like gene also in primates besides mice. In addition, genomic sequences related to *dnaJB3/mfsj-1* from *Canis familiaris, Bos Taurus, Pan troglodytes and Papio anubia* have been recently deposited in GenBank.

The fundamental role exerted by MSJ-1 in both spermiogenesis and CNS activity is strongly suggested by its expression in a lower vertebrate. In fact, in the anuran amphibian, the frog *Rana esculenta*, the gene has partially been sequenced (Meccariello et al., 2004) and it shows the same expression pattern observed in mice; moreover, the protein is expressed in post-meiotic cells and in the spinal cord (Meccariello et al., 2002, 2004). Thus, MSJ-1 activity is highly conserved in evolution and, therefore, it might exert fundamental functions. Therefore, it would be interesting to characterize the entire *msj*-1 gene and to extend the investigations to humans.

In this work, we have isolated the murine *msj-1* gene and studied its structure. Furthermore, we have determined the putative chromosomal localization and a minimal promoter region. In addition, by immunogold electron microscopy we analyzed MSJ-1 localization at ultrastructural level during the spermiogenesis and in epididymal spermatozoa. Finally, we have found putative *msj-1* like genes in human genome and we have detected by RT-PCR and by Western blot analysis a MSJ-1-like mRNA/protein in human spermatozoa.

2. Materials and methods

2.1. Mouse tissue sampling and sperm cell collection from mouse and human

Sixty-day-old CD1 mouse ($n = 3$) was provided from Arvel (Napoli, Italy). Testes were removed from mice under asphyxiation in CO_2 and stored at −80 °C until used for total RNA extraction or cut in small slices and processed as reported in immunoelectron microscopy section. Mouse spermatozoa were collected in phosphate-buffered saline (PBS) pH 7.4 by cutting epididymis in small pieces and centrifuged at 1000*g* for 15 min at 4 °C. Mouse sperm pellet was processed for protein extraction or for immunogold electron microscopy.

Human whole blood and semen samples were provided from healthy volunteer donors. Following 30 min at 37 °C for liquefaction, sperm was diluted 5-fold in 0.9% NaCl and centrifuged at 500*g*; pellet was re-suspended in 0.9% NaCl and centrifuged for additional 10′. This washing step was repeated at least three times. Human spermatozoa pellets ware processed for total RNA/protein extraction.

This research was approved by the Italian Ministry of University and Scientific and Technological Research (MIUR).

2.2. msj-1 Gene isolation

We screened a phagic genomic library derived from 129/Sv mouse tissues using a *msj-1* cDNA (see below for details). Once extracted DNA from positive clones, the inserts were excised from phagic DNA by digestion with NotI and sub-cloned in pBluescriptII (SK−). Maxi preparations of plasmidic DNA were carried out by Quiagen Plasmid kit (Qiagen, Ltd. UK). Two micrograms of DNA were digested with restriction enzymes (BamHI, EcoRI, EcoRV, HindIII, SacII, StuI) (Amersham Biosciences Europe GmbH, Freiburg Germany) and analyzed by electrophoresis on 0.8% agarose gel in Tris–borate EDTA buffer (TBE). Digested DNA was transferred and fixed onto nylon filter membranes (Hybond N+, Amersham Biosciences Europe GmbH). Fragments containing *msj-1* gene were identified by hybridization with the cDNA probe as described below. Then, 10 μg of plasmid DNA were digested with appropriate digestion enzymes and analyzed by electrophoresis; fragments containing *msj-1* gene were cut from gel, purified by QIAquick Gel Extraction kit (Qiagen), sub-cloned in pBluescriptII (SK−) and lastly sequenced using a Big Dye terminator strategy, and an ABI Prism 3000 Automatic Sequentior (Applied Biosystems).

2.3. Probes and hybridizations

A *msj-1* cDNA fragment of 514 bp (nt 1–514) obtained from pUC21 by digestion with EcoRI (Berruti et al., 1998b) was used as probe for library screening and Southern blot hybridizations. Probe was labelled by random priming with ^{32}P-CTP using the Ready Prime II labelling kit (Amersham Biosciences Europe GmbH). Hybridization was carried out in Church buffer (0.5 M Phosphate buffer pH 7.4, 7% SDS, 0.5 mM EDTA, 100 μg/ml sonicated salmon sperm DNA) containing 10^6 cpm/ml at 65° overnight. Filters were washed twice 30 min in 2× SSC, 1% SDS at 65 °C, and then 30 min in 0.4× SSC, 1% SDS at 65 °C. Filters were then exposed to X-ray film (Kodak) for suitable time.

2.4. cDNA preparation and PCR analysis

Total RNA was extracted from mouse testis and human spermatozoa pellets using Trizol Reagent (Invitrogen Life Technologies, Paisley, UK). After treatment with DNase, purity and integrity of the RNA preparations were determined by densitometry analysis at 260/280 nm and by electrophoresis.

Three micrograms of total RNA were reverse transcribed using 0.5 μg oligo dT, 0.5 mM dNTP, 10 mM dithiothreitol (DTT), 1× First Strand Buffer, 40 U RNAase OUT (Invitrogen Life Technologies) and 200 U SuperScript-III RNaseH$^-$ Reverse Transcriptase (RT) (Invitrogen Life Technologies) in a final volume of 20 μl, following the manufacturer's instructions. As negative control, total RNA not treated with RT was used. In order to check the quality of cDNA preparations, amplification for glyceraldehyde-3-phosphate dehydrogenase (*gadph*) was carried out using 1 μl of cDNA, 10 pMol specific primers (see Table 1 for details) in PCR mix [0.25 mM dNTP, 1× PCR buffer, 1.5 mM $MgCl_2$, 1.25 U Taq Polymerase (Invitrogen Life Technologies)] using an Applied Biosystem Thermocycler. Possible contaminations among samples were evaluated using, as negative controls, samples prepared without cDNA. PCR reaction was carried out using 1 μl of cDNA or genomic DNA, the latter extracted from a 2700 bp EcoRI sub-cloned fragment containing *msj-1* promoter region, 5′UTR and half coding region. The reaction was carried out using 25 pMol *msj-1a* and *msj-1b* primers, respectively, located 53 bp downstream the putative TATA box and 33 bp upstream the transcription start site (see Table 1 for details) in PCR mix.

Finally, 20 μl of PCR amplification mixture were analyzed by electrophoresis on 1.2% agarose gel in 1× TBE buffer and stained with 0.5 μg/ml EtdBr.

2.5. Immunogold electron microscopy

Mouse spermatozoa pellets and testis were fixed in a mixture of 0.5% glutaraldehyde and 2% paraformaldehyde in 10 mM phosphate buffer pH 7.4, respectively, for 1 and 2 h. The samples were embedded in Epon resin and ultrathin sections (60 nm) were collected on 200 mesh nickel grids. Slices were treated 2 × 15 min with 3% sodium metaperiodate, quickly rinsed in distilled water and treated 3 × 5 min in citrate buffer (19 mg citric acid/10 ml) pH 6 at room temperature; lastly, an additional incubation in citrate buffer at 95 °C for 10 min was carried out. After 3 × 5 min washing in distilled water, sections were incubated 15 min in blocking solution [tris buffered saline (TBS) pH 7.4, 1% BSA] and lastly incubated overnight with primary antibody diluted 1:250 in blocking solution. Grids were then washed 5 × 2 min in TBS, 5 × 2′ in TBS 0,5% BSA and lastly 15 min in TBS 1% BSA. Immunocomplexes were visualized by incubation in goat anti rabbit immunoglobulins conjugated to colloidal gold (30 nm diameter) diluted 1:250 in TBS for 1 h at room temperature. After a new series of washings, sections were stained with uranyl acetate (5 min for spermatozoa and 15 min for tissues) and analysed using a Leo 912 Zeiss electron microscope. Controls were incubated with primary antibody preadsorbed with 10^{-6} M of the corresponding peptide (1 V peptide/10 V TBS).

2.6. Genomic human DNA extraction and analysis

Genomic DNA was extracted from blood buffy coat using the hypotonic lyses method. In brief, whole blood was treated with hypotonic solution (Tris base 10 mM, EDTA 10 mM) (2 ml blood/8 ml lysis solution) and centrifuged at 800*g* to lyse eritrocytes; treatment with hypothonic solution was repeated 3-4 times until a cleared supernatant was obtained. Lymphocyte pellet was then re-suspended in 20 mM Tris base, 20 mM EDTA, 1% SDS, 150 mM NaCl, 200 μg Proteinase K and lysis was carried out overnight at 45 °C. Sample was then treated with 1 V phenol:isoamilic alcohol:chlorophorm (25:24:1), centrifuged 10 min at 10,000*g*; DNA located in the aqueous phase was precipitated in 2 V absolute ethanol, washed in 70% ethanol and re-suspended in a suitable volume of TE 1× (10 mM Tris base, 1 mM EDTA).

Two hundred nanograms of genomic DNA obtained from blood and 1 μl of cDNA obtained from human spermatozoa were used for PCR amplification using PCR mix, 0.1% deionised formamide, 1 μm specific primer *Ha up/down* and *Hb up/down* (see Table 1 for details) in a final volume of 25 μl. Amplificates were cut from agarose gel, subcloned in pGemTEasy vector (Promega) and sequenced.

2.7. Bioinformatics

We used the BLAST network service at NCBI (National Centre for Biotechnology Information) database (http://www.ncbi.nlm.mih.gov/BLAST); multiple alignments were carried out using ClustalW software at Biology Workbench (http://workbench/sdsc.edu/). Transcription factor binding site searching and prediction of promoter region were performed using MatInspector software (http://genomatix.gsf.de/mat/fam).

Homo sapiens chromosome 2 sequence (GenBank Accession No. NT_005120) was obtained from NCBI database (http://www.ncbi.nlm.mih.gov).

Chromosomal localization was determined by searching on the mouse and human genome draft at NCBI. ORF prediction was carried out at Biology Workbench SIXFRAME Generate & Import 6 Frame Translations on a NS-tools.

Table 1
Primer sequences for RT-PCR and PCR analyses

Primers	Sequence 5′ → 3′	PCR program	Size (bp)	Accession Number in GenBank	Source
Gadph up	CTACCCACGGCAAGTTCAAT	94 °C, 5 min, 1 cycle; 94 °C 1 min, 58 °C 45 s,	150	NM_ 007393.1	Rat
Gadph down	ACGCCAGTAGACTCCACGAC	72 °C 1 min 30 cycles; 72 °C 15 min 1 cycle			Rat
msj-1a	GCACCGCCCGCCTCCGCCAA	94 °C, 5 min, 1 cycle; 94 °C 1 min, 53 °C 45 s,	518	NM_008299	Mouse
msj-b	GGTGCTCCTCCGATCCCCAAAAA	72 °C 1 min 30 cycles; 72 °C 15 min 1 cycle			Mouse
Ha up	CGCTGTGCCAGTGCCTGGTG	94 °C, 5 min, 1 cycle; 94 °C 30 s, 62 °C 45 s,	908	NT_005120	Human
Ha down	CCCAACGATAAGAACAGGTG	72 °C 180 s 30 cycles; 72 °C 15 min 1 cycle			Human
Hb up	CGCTGTGCCAGTGCCCCGCA	94 °C, 5 min, 1 cycle; 94 °C 30 s, 62 °C 45 s,	906	NT_005120	Human
Hb down	CCCAACAATAAAGACAGGCA	72 °C 180 s 30 cycles; 72 °C 15 min 1 cycle			Human

2.8. Protein extraction from spermatozoa and immunoprecipitation

Mouse and human spermatozoa pellets were lysed in cold 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS containing protease inhibitors [0.5 mM phenylmethylsulfonylfluoride (PMSF), 4 μg/ml leupeptine, 4 μg/ml chymostatin, 4 μg/ml pepstatin A, and 5 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)]. The lysate was sonicated twice for 15 sec and shaken 1 h at 4 °C; cleared protein extract was collected after centrifugation at 10,000*g* for 15 min at 4 °C. Protein concentration was evaluated by Lowry methods (Lowry et al., 1955).

Three hundred micrograms total protein were processed for immunoprecipitation. Sample was diluted by adding 10 vol RIPA buffer [PBS, pH 7.6 (9.1 mm Na_2HPO_4, 1.7 mm NaH_2PO_4, and 150 mM NaCl), 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS] and incubated with 2 μg of MSJ-1 antiserum for 1 h at 4 °C. The immunoprecipitation was carried out at 4 °C on a rocker platform overnight, adding 20 μl of the appropriate immunoprecipitation reagent (0.25 μg/μl; Protein G-Plus-Agarose, sc-2002, Santa Cruz Biotechnology, Inc.). Immunoprecipitates were pulled down by centrifugation at 1000*g* for 5 min at 4 °C and washed four times with PBS, pH 7.6, each time repeating the centrifugation step. Finally, the pellet was dissolved in 30 μl electrophoresis sample buffer and boiled for 5 min. The negative control was obtained as described above using RIPA buffer instead of primary antibody or instead of total protein extract.

2.9. Western blot

Total proteins from spermatozoa and immunoprecipitates were resolved by 12% SDS–PAGE and blotted to PVDF membranes (Amersham Pharmacia Biotech) for 2.5 h at 280 mA at 4 °C. Membranes were then rinsed in PBS pH 7.6 (20 mM NaH_2PO_4, 80 mM Na HPO_4,100 mM NaCl) and treated for 2 h with blocking solution [5% non-fat powdered milk in TBS-T (10 mM Tris–HCl pH 7.6, 150 mM NaCl, 0.25% Tween-20)]. Hybridization was performed using anti-MSJ-1 antibody diluted 1:3000 in PBS 4% non-fat powdered milk overnight at 4 °C. The membranes were washed 3 × 15 min in TBS-T and incubated 1 h at room temperature with a horseradish-conjugated anti-rabbit immunoglobulin-G antibody (Dako Cytomation, Glostrup, Denmark) diluted 1:1200 in TBS, 1% Normal Swine Serum (NSS) (Dako Cytomation). After an additional series of washes, immunocomplexes were detected using the ECL Western blotting detection system (Amersham Pharmacia Biotech). Filters were stripped in stripping buffer (62.5 mM Tris pH 6.8, 2% SDS, 100 mM β mercaptoethanol) at 60 °C for 30 min and re-probed with primary antibody pre-adsorbed with a large excess (10^{-6} M) of the respective antigen to check antibody specificity.

2.10. Antibody

The MSJ-1 antibody has been raised against a recombinant protein glutathione *S*-transferase (GST)/MSJ-1 (amino acids 149–242) corresponding to a fusion between GST and the C-terminal portion of MSJ-1 that does not contain the highly homologous J domain (Meccariello et al., 2002).

3. Results

3.1. Structure of msj-1 gene

The sequences of the genomic clones were found to be overlapping with the sequence of the cDNA (GenBank Accession No. NM_008299), suggesting that in mice *msj-1* gene lacks introns. In fact, alignment of *msj-1* cDNA and *msj-1* genomic sequence reveals a complete cDNA/genomic overlap in 5′UTR, coding region and 3′UTR, indicating that *msj-1* gene structure consists of a unique exon of 1016 bp. However, the two sequences overlap starting from cDNA position +3.

3.2. 5′ Flanking region sequence analysis

A TATA box was observed upstream (from −125 to −109) the putative transcription start site, according to the program MatInspector (Quandt et al., 1995). In addition two high confidence binding sites for CAAT are located both downstream (from −41 to −30) and upstream (from −162 to −152) to the putative TATA box. Additional binding sites for GATA-1 (from −68 to −56) and Tax/CREB (from −63 to −42) are close to the transcription start site, while other binding sites, such as GABP (GA binding protein) and a GAGA box, are observed further upstream, respectively, from −253 to −237 and from −279 to −259. In particular, an ERE (Estrogen Response Element) and a CREB site (cAMP Responsive Element) span, respectively, from −358 to −340 and from −479 to −458 (Fig. 1).

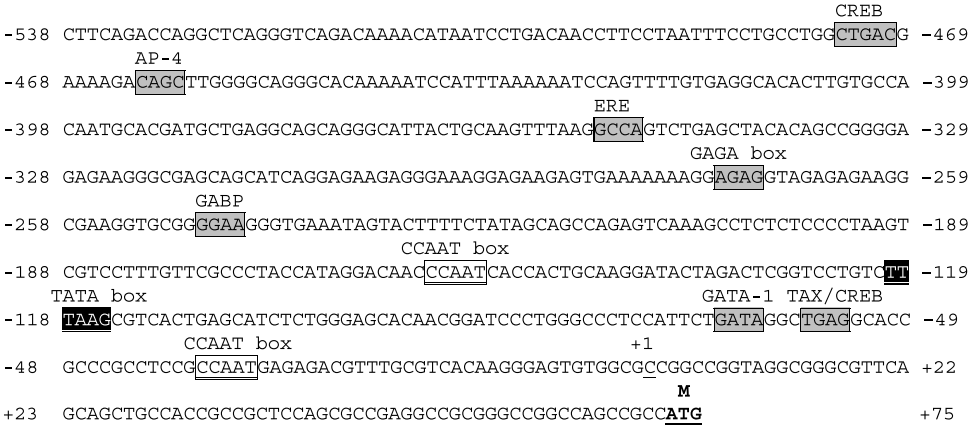

Fig. 1. Promoter region analysis. A promoter region was identified within the 600 bp upstream *msj-1* gene. Grey boxes denote core sequence potential transcription factor binding site with high identity to authentic core and matrix sequences. Putative TATA box is black; putative CAAT box is in white. Transcription factor binding sites searching and prediction of promoter region were performed using MatInspector software (http://genomatrix.gsf.de/mat/fam). +1 = transcription start site; bold italic characters = ATG start codon.

Due to the minimal difference in length observed in the 5′UTR between mouse *msj-1* genomic sequence and the cDNA sequence deposited in GenBank, together with the prediction of a putative TATA box located 109 bp upstream the transcription start site, we assayed the possibility of a *msj-1* transcript with a longer 5′UTR. Therefore, we selected a sense primer in the region between the putative TATA box (spanning from −53 to −33) and an anti sense primer in *msj-1* coding region (nt 465–485). PCR analysis, carried out on both genomic DNA and cDNA, indicates the presence of an amplificate of the predicted size of 518 bp only in genomic samples, excluding the possibility of a longer transcript (Fig. 2). One band of the predicted size of 150 bp was obtained from GAPDH amplification (not shown).

3.3. MSJ-1 localization in adult mouse testis and spermatozoa

In order to characterize MSJ-1 localization at ultrastructural level, immunogold electron microscopy analysis was carried out in testis of 60 day old mice and in the epididymal spermatozoa. As expected, only post meiotic stages, spermatids and spermatozoa display MSJ-1 immunolabelling. The transformation of spermatids into spermatozoa involves a complex sequence of events collectively termed spermiogenesis. Acrosome biogenesis is one of the earlier step of spermiogenesis and starts when sorted Golgi derived proacrosomal vesicles fuse with each other to form an acrosome sac tightly bound to the nuclear envelope. Nuclear changes, development of flagellum, reorganization of cytoplasm and cell organelles subsequently take place. In the earlier phases of acrosomogenesis, the Golgi phase, a scattered MSJ-1 immunolabelling marks the cytoplasmic area close to proacrosomic granule and Golgi apparatus (Fig. 3A, arrows); no labelling emerged inside the proacrosomic granules or Golgi apparatus. Interestingly, gold particles are also located behind the acrosomic region, nearby some large vesicular structures and in few cases inside themselves, structure that resemble the features of mitochondria (Fig. 3B, dotted arrows). In fact, in primary and secondary spermatocytes mitochondria show dilated intracristal spaces that result in the crystal membrane being pushed to the periphery of the organelle. Such peripheral margination of the cristae persists in spermatids and, as a consequence, a central clear zone appears in the mitochondria and these organelles appear as "vacuolated" (De Krester and Kerr, 1988). As acrosomogenesis proceeds to the cap and acrosome phase, in the anterior part of the spermatids, MSJ-1 labelling follows the contour of the developing acrosomic vescicle (Fig. 3C and D, arrows); isolated gold particles are also scattered in the lateral and posterior region of the spermatids (Fig. 3D, dotted arrows). When testis section were incubated with primary antibody preadsorbed with the corresponding peptide, only several non-specific gold particles were observed in the nucleus (Fig. 3E, asterisks). During the maturation phase, anti MSJ-1 immunogold labels the outer (Fig. 3F, arrow head) and the inner acrosomic membrane and also the subacrosomal space (Fig. 3G, arrows). Thirty nanometer gold particles mark the proximal centriolar region and are retained in the cytoplasm residual droplet (Fig. 3F, arrows). In control section, no labelling in elongating spermatids head or tail emerged (Fig. 3I). In spermatozoa collected from epididymus, MSJ-1 immunolabelling is restricted to the outer, the inner acrosomic vesicle and to subacrosomic space (Fig. 4A and B). Few gold particles also mark the connecting piece (Fig. 4C). Signals completely disappears in sections incubated with the primary antibody preadsorbed with a large excess of the corresponding antigen (Fig. 4D and E).

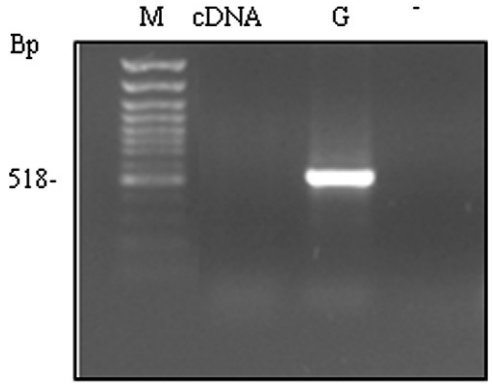

Fig. 2. PCR analysis carried out on genomic DNA (G) (an EcoRI fragment of 2700 bp containing promoter region, *msj-1* 5′UTR and half coding sequence), and cDNA prepared from mouse testis. Upper primer matches 53–33 bp upstream the putative transcription start site, lower primer into the coding sequence. A single band of 518 bp is obtained only in genomic DNA preparation. Negative control: PCR analysis carried out without DNA or cDNA (−). The presence of an amplificate of the predicted size of 518 bp, only in genomic samples, excludes the possibility of a longer transcript. *M* = 100 bp Ladder Plus Marker (Fermentas).

3.4. Is there a msj-1 like gene in human?

The nucleotide sequences of the murine *msj-1* gene were used to query the human genome using the BLAST search programs in GenBank. Interestingly, six blast hits, that had greater than 80% identity with several region in the coding region of murine *msj-1* gene, were identified on human chromosome 2 (NT_005120 *Homo sapiens* chromosome 2 genomic contig region from 585317 to 596666 nt). These hits outline two regions on chromosome 2, far away 10,685 bp each other and spanning a region, respectively, of 669 bp and 664 bp.

At NCBI human genome database we downloaded the two regions of interest adding upstream approximately 150 bp and downstream approximately 400 bp, in that *msj-1* murine gene has 5′ and 3′UTR, respectively, of 72 and 212 bp; therefore we obtained a sequence of 1200 bp that we named *Ha* and a region of 1192 bp that we named *Hb*. Finally, by Clustal W program, we globally aligned *Ha* and *Hb* to mouse *msj-1* genomic region and to *mfsj-1* cDNA (GenBank Accession No. AB095737), the recently

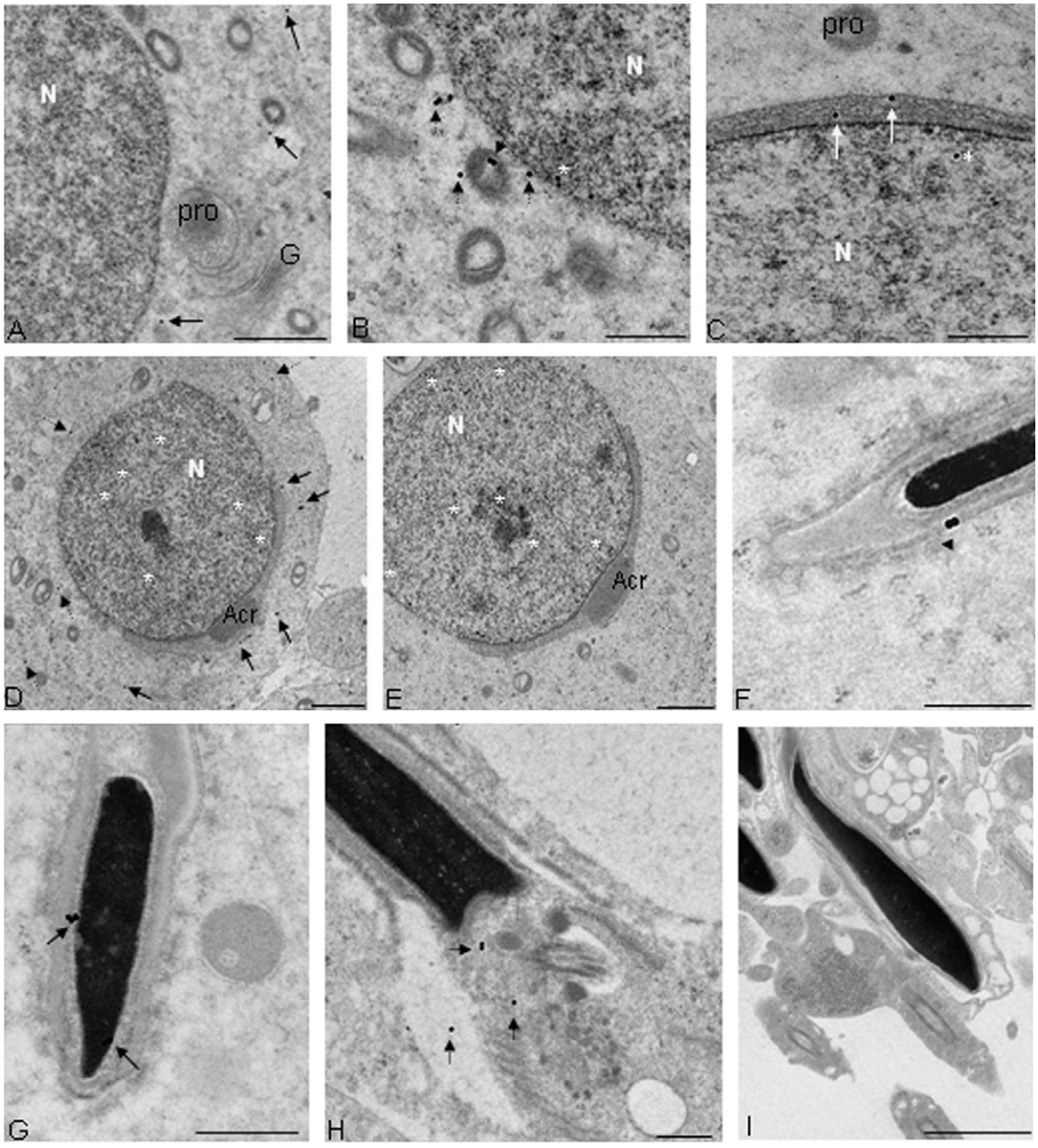

Fig. 3. Immunogold electron microscopy analyses of adult mouse testis. MSJ-1 immunolabellig marks the cytoplasmic area close to proacrosomic granule and Golgi apparatus (A) and to mitochondria clusters (B). MSJ-1 labelling follows the contour of the developing acrosomic vescicle (arrows) and is also detected behind the developing acrosome (dotted arrows) (C and D) Control section obtained incubating the primary antibody with a large excess of the corresponding antigen (E). In elongating spermatids anti MSJ-1 immunogold labels the outer (arrow head) and the inner acrosomic membrane (arrows) (F and G). Thirty nanometer gold in the proximal centriolar region and in the cytoplasm residual droplet (arrows) (H). Control section obtained incubating the primary antibody with a large excess of the corresponding antigen (I). (*) Indicates aspecific nuclear labelling; Acr, acrosomic vesicle; Cd, cytoplasm residual droplet; Pro, proacrosomic vescicle; G, Golgi apparatus; N, nucleus. Scale bars = 1 μm in A,D,E = 0.5 μm in B,C,F,G,H and =2 μm in F.

cloned *msj-1*-like gene identified in *Macaca fuscata* (Yu and Takenaka, 2003) (Fig. 5). Comparison of human and murine genomes indicates the identity summarized in Table 2.

3.5. Genes or pseudogenes?

Alignments of *Ha* and *Hb* reveal 92% identity (Fig. 5), the difference consisting in several randomly spanning point mutations. To asses whether *Ha* and *Hb* are functional genes, we searched for a putative ORF in both *Ha* and *Hb*. HA forward frame 1 reveals an ORF of 483 nt encoding a putative protein of 160 amino acid (aa) residues (Fig. 6A). Interestingly, global alignments report an aa identity of 35% and 44,6%, respectively, with MSJ-1 and MFSJ-1, and no conserved domain results in this putative product. Nevertheless, local alignments report aa identity

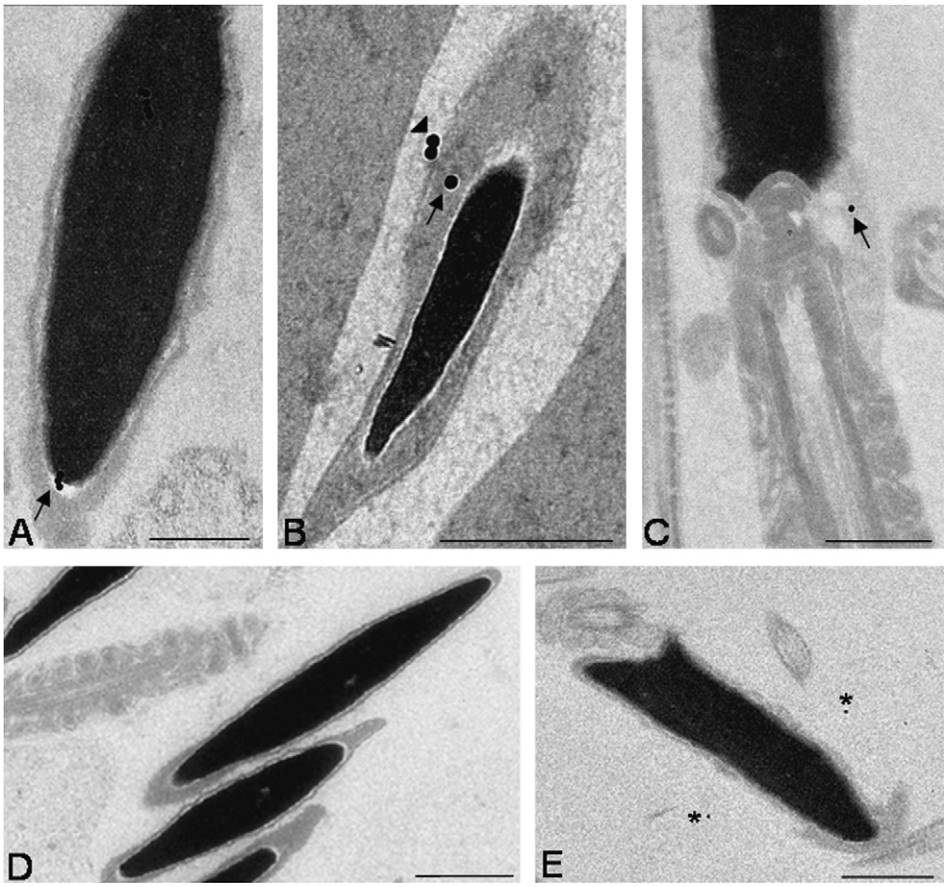

Fig. 4. Immunogold electron microscopy analyses conducted on spermatozoa collected from mouse epididymus. Inner and outer acrosomic membrane are MSJ-1 immunolabelled (A and B). A 30 nm gold particle marks the connecting piece (C). No signal is detected using the primary antibody with a large excess of the corresponding antigen (D and E). Scale bars = 0.5 μm in A–C and =1 μm in D and E. (*) Indicates aspecific labelling.

of 66.7% and 90.1%, respectively, with MSJ-1 and MFSJ-1 C terminal domain, in a 101 aa residues overlap.

While forward frame 2 does not contain a significant ORF, *Ha* forward frame 3 reveals an ORF of 438 bp encoding a putative protein of 145 aa residues. This protein consists of a canonical J domain (aa 2–75), a G/F rich region, and has 69% and 74% identity with MSJ-1 and MFSJ-1, respectively, in a 145 aa overlap (Fig. 6B). Worth of note is that most of the C-terminal domain of MSJ-1 and MFSJ-1 is missing in HA. This 145 aa residue protein is accounted in NCBI database as the product of *hgc3* gene (NM_001001394). Interestingly, starting from *ha* forward frame 3/*HGC3* G_{126} aa residue, identity with MSJ-1 and MFSJ-1 is quite poor. At nucleotide level, insertion of a guanosine just in codon 126 is observed (Fig. 5 bold character), so a frame shift responsible for the formation of a premature stop codon may be postulated.

Lastly, *Hb* reveals the presence of multiple short ORF in all six frames, but translational analysis reveals the presence of multiple stop codons and no significant products, except for forward frame 3, where a putative product of 131 aa, with no significant identity with all protein sequences deposited in database, is evinced by blast. HB forward frame 2 ORF analysis reveals the absence of a significant AUG start codon and the presence of several stop codons, indicating that no significant product is produced. Nevertheless, when the whole deduced aa sequence was aligned with MSJ-1 and MFSJ-1, identity of 70% and 85%, respectively, was obtained (Fig. 6C).

3.6. Chromosomal localization of mouse msj-1, and human Ha and Hb

An analysis carried out at NCBI mouse genome Map view database allowed to determine the putative location of *msj-1* gene on chromosome 1 (1C5), in the cluster of UDP glucuronosyl-transferase 1 family (*ugt1a*). In particular *msj-1* and *ugt1a* genes are transcribed in opposite orientation, and *msj-1* is located into an intron region.

An analysis conducted at NCBI human genome Map view database allowed to determine the putative location of both *Ha* and *Hb* on chromosome 2, 2q37, in the cluster of UDP glycosyl-transferase 1 family polypeptide A (*ugt1a@*). Also *Ha* and *Hb* are located into an intron region of *ugt1*A@ cluster gene and the latter are transcribed with opposite orientation respect *Ha* and *Hb*.

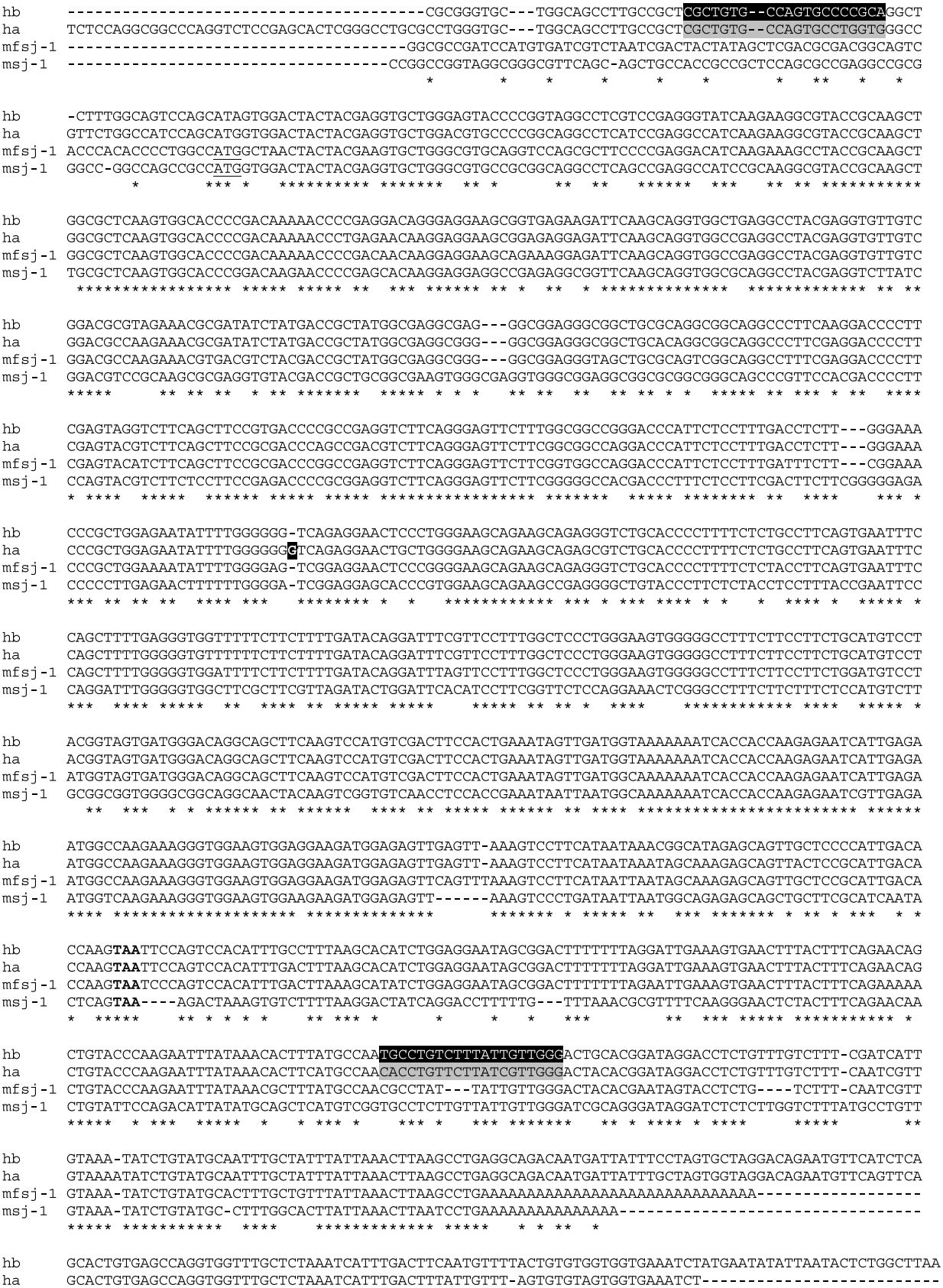
hb
ha
mfsj-1
msj-1

Table 2
Nucleotide and amino acidic identity (%)

	msj-1	*msj-1* (*cds*)	*mfsj-1*	*ha*	*hb*	MSJ-1	MFSJ-1	HA	HB	
msj-1	100					100				MSJ-1
msj-1(*cds*)	100	100				71	100			MFSJ-1
mfsj-1	72	77.8	100			69	74	100		HA
Ha	74	78	83	100		—	—	—	100	HB
Hb	74	77	82	92	100					

Italic and capital letters indicate gene and protein data, respectively.

3.7. Expression analyses of Ha and Hb

Since in mice *msj-1* is mainly expressed at haploid stages, spermatids and spermatozoa, we assayed the expression of both *Ha* and *Hb* by RT-PCR using cDNA preparations obtained from human spermatozoa and specific primers designed upon *Ha* and *Hb* sequences. Amplificates of the predicted size of 908 and 906 nt were obtained from human genomic DNA, used as positive control; cDNA analysis revealed the presence of a signal for *Ha* and not for *Hb*. No signal for *Ha* and *Hb* was observed in negative controls (Fig. 7). Data were confirmed analyzing the sequence of both *Ha* and *Hb* genomic amplication products, and of *Ha* cDNA amplification products.

3.8. Protein analyses and immunoprecipitation

By Western blot, we have detected a band of approximately 30 kDa in human spermatozoa. A stronger band has been detected also in mouse spermatozoa used as positive control. Specificity of the reaction has been assayed by using MSJ-1 antiserum previously bound *in vitro* with a large excess of the corresponding antigen (10^{-6} M); in fact the signal of 30 kDa disappeared in human samples and strongly attenuates in mouse spermatozoa (Fig. 8A). To further assess antibody specificity, immunoprecipitation (Fig. 8B) was carried out. A band of 30 kDa was also immunoprecipitated from whole sperm protein tool (lane 1); no signal (lane 2) was detected when MSJ-1 antiserum was not added in immunoprecipitation reaction.

4. Discussion

In this work *msj-1* gene primary structure together with MSJ-1 protein ultrastructural localization were characterized in mice. Interestingly, *msj-1* is an intron-less gene, confirming our preliminary previous results (Meccariello et al., 2005); although once considered a histone gene characteristic, several functional genes are intron-less (i.e. c-jun, ADAM1a and ADAM1b) (Hattori et al., 1988; Nishimura et al., 2002). A weak difference among cDNA and genomic sequence, consisting in GG/CC substitutions, appears just at the transcription start site. In this respect, it is important to stress that, in the present work, *msj*-1 5′-flanking region was sequenced several times, on both strands and from different overlapping fragments, obtained from restriction analysis. Therefore, previous data, showing discrepancies in *msj-1* transcription start site (Berruti et al., 1998a), might be due to vector contamination in cDNA cloning and sequencing. In addition, being *msj-1* an intron-less gene, the detection of transcripts of 1 and 1.2 kb (Berruti et al., 1998a; Meccariello et al., 2002) might be explained by differential poly-adenylation rates.

Using bioinformatics, a putative promoter region was predicted in the immediate 5′-upstream region of *msj-1*. It is worth of note that a TATA box was predicted further upstream the transcription start site (−125 to −109) previously described by 5′ RACE (Berruti et al., 1998b). Therefore, we assayed the possibility of *msj-1* transcript with a longer 5′UTR, carrying out a PCR analysis on cDNA and genomic DNA using a sense primer in the region downstream the putative TATA box (−53 to −33) and an anti sense primer in *msj-1* coding region. PCR analysis got no product in cDNA, clearly excluding the possibility of a longer 5′UTR. TATA less promoters often characterize genes expressed at haploid stages and we cannot exclude that *msj-1*, a gene mainly expressed in spermatids, has a non-functional TATA box. By contrast, two high confidence CAAT boxes are predicted at −41/−30 and at −162/−152. Interestingly, no androgen responsive element is detected upstream *msj-1* gene, confirming that testosterone does not directly influence *msj-1* expression (Meccariello et al., 2002). Furthermore, the presence of CREB binding sites in the putative promoter region is consistent with *msj-1* down regulation in $CREM^{-/-}$ mice (Berruti et al., 1998a). Therefore, further experiments, such as luciferase vector construction and mutagenesis, have to be carried out to better characterize a minimal functional promoter.

In mouse, *msj-1* maps to chromosome 1, into an intron region of *ugt1a* cluster. Interestingly, blast search of murine *msj-1* gene against the human genome draft sequence identified several blast hits onto chromosome 2, into an intron region of *ugt1a@*, a region of demonstrated homology as

Fig. 5. ClustalW (1.81) alignment of *msj-1* gene *vs Ha*, *Hb* and *mfsj-1* cDNA. Nucleotide identity: *msj-1* coding region *vs Ha* 78%; *msj-1* coding region *vs Hb* 77%; *msj-1 vs Ha* 74%; *msj-1 vs Hb* 74%; *Ha vs Hb*: 92%; *mfsj-1* cDNA *vs Ha* 83%; *fsj-1* cDNA *vs Hb* 82%. Start codons are underlined; stop codons are in bold characters; (*) single, fully conserved residue; (-) no consensus. *Ha up/down* and *Hb up/down* primers used for the expression analyses are, respectively, in light grey and black boxes.

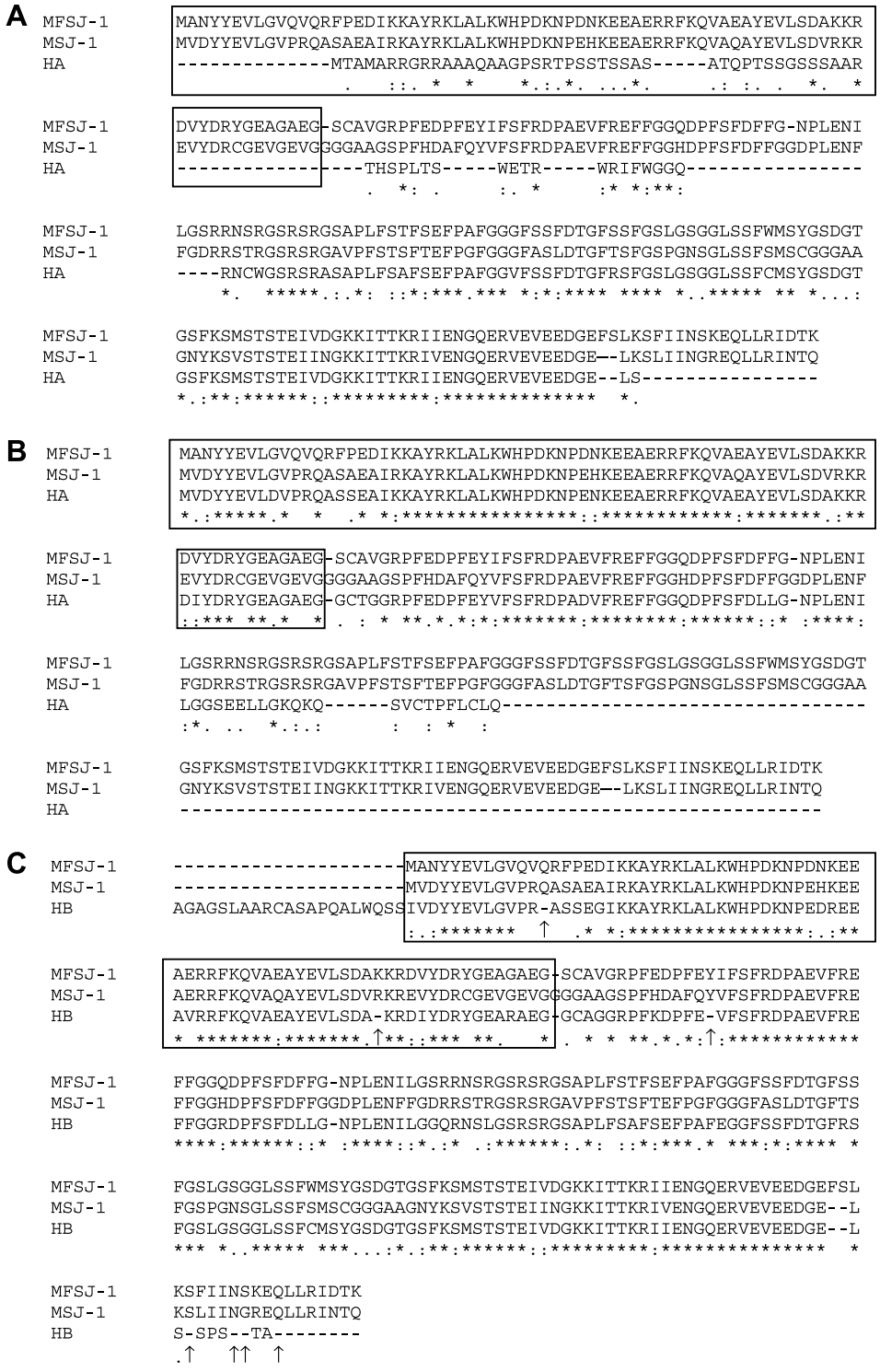

Fig. 6. ORF prediction in *Ha*. (A) A significant ORF of 483 bp, encoding a 160 aa protein was predicted from *Ha* frame 1. The predicted protein does not contain a J domain (boxes) and has 66.7 and 90.1% amino acid identity, respectively, with MSJ-1 ed MFSJ-1 C-terminal domains. (B) A significant ORF of 438 bp, encoding a 145 aa protein was predicted from *Ha* frame 3. The predicted protein contains a J domain (boxes) and has 69% and 74% amino acid identity, respectively, with MSJ-1 ed MFSJ-1. (C) alignments among HB frame three and MSJ-MFSJ-1. Arrows indicates the presence of stop codons in HB protein predicted from frame two. (*) Single, fully conserved residue; (:) conservation of strong groups; (.) conservation of weak groups; (-) no consensus.

compared to mouse chromosome 1 (Sato et al., 1992). In human, all thirteen UDP glucuronosyl-transferase genes of family 1A (UGTs) are derived from a single gene locus extending over 100 kb of chromosome 2 and containing several exons. Although all UGTs have an own TATA box and exon 1, the last four exons are common; as a result, mRNAs encoding UGTs have identical 3′ ends resulting in transferase isozymes with an identical carboxyl

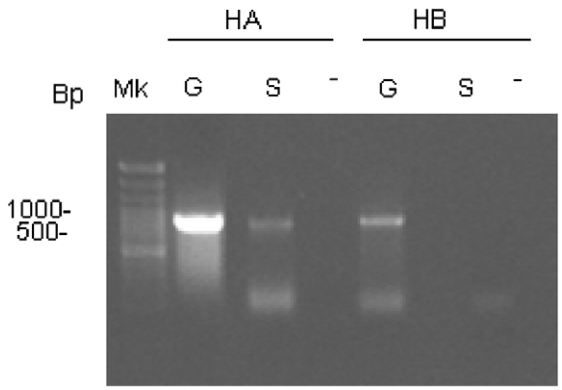

Fig. 7. Expression analysis of *Ha* and *Hb* in human spermatozoa. By RT-PCR a band of 908 nt is observed for *Ha*: no signal is observed for *Hb*. G, genomic DNA used as positive control; S, spermatozoa cDNA; (-) negative control; Mk = 100 bp Ladder Plus (Fermentas).

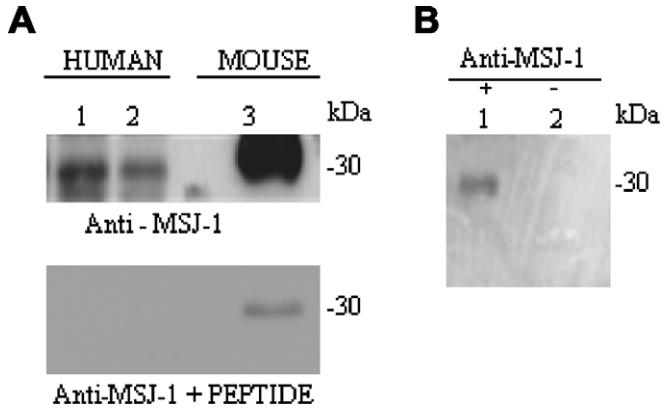

Fig. 8. (A) Western blot analyses conducted on human and mouse sperm. A specific band of 30 kDa is detected in human and mouse spermatozoa (proteic extract loaded on gel: 1 = 80 μg; 2 = 40 μg; 3 = 25 μg). Specificity of the reaction was tested by incubating the same filter with a large excess of the corresponding peptide (10^{-6} M). (B) The specific band of 30 kDa was immunoprecipitated with 2 μg of murine MSJ-1 antiserum (lane 1 + MSJ antiserum; lane 2 − MSJ antiserum).

terminus and a unique amino-terminal domain deriving from alternative splicing of upstream exons (Gong et al., 2001).

Blast search in human database, RT-PCR and Western blot analysis conducted in this study on human sperm, and the analysis of *Ha* and *Hb* strongly support the presence of two *msj-1*-like genes in human genome and the expression of a MSJ-1-like protein in sperm cells. In particular, *Ha* and not *Hb* is really expressed in human spermatozoa, cells where MSJ-1 expression in mouse is well known (Berruti and Martegani, 2001, 2005; Berruti and Aivatiadou, 2006). It is worth of note that *Ha* region may contain an optimal *msj*-like gene; in fact, a putative significant ORF encoding a DnaJ protein, similar to MSJ-1/MFSJ-1, was predicted from frame 3 but, due to a premature stop codon, this putative protein is truncated and lacks most part of the canonical C-terminal domain characteristic of MSJ-1/MFSJ-1; nevertheless, possibility that the stop codon UGA may also be translated as a selenocysteine, should be considered (Copeland, 2003). Lastly, also *Ha* frame 1 has a putative significant ORF encoding a protein similar to MSJ-1/MFSJ-1 in the C-terminal domain, but unfortunately devoid of a complete J domain. In addition, our results candidate *Hb* as a remnant *msj*-like gene. In fact, a DnaJ protein highly similar to MSJ-1/MFSJ-1 was predicted from *Hb* forward frame 2, but unfortunately no significant ORF could be associated to this frame, due to several stop codons and the loss of an AUG start codon. Species-specific gene inactivation, modification or loss may occur during evolution when particular roles become obsolete or subjected to regulation by different biochemical pathways. Hypothesis of remnant genes in human, mouse and rat genomes are reported in literature, the best example being represented by GnRH II receptor gene, whose presence in humans is still matter of debate (Pawson et al., 2003). In fact, besides the well established GnRH I receptor gene, located on human chromosome 4, an apparent GnRH II receptor on chromosome 1 and a sterile GnRH receptor-like homolog gene encoded on the antisense DNA strand of the 3′UTR of *rbm8* gene on chromosome 14 have been described. Remnant GnRH II receptor genes have also been located on rat chromosome 18 and mouse chromosome 2 (for review Neill, 2002; Millar, 2003; Pawson et al., 2003). Although GnRH II receptor immunoreactivity is described in human brain and pituitary, a full-length, appropriately processed GnRH II transcript, has not yet been identified, due to the presence of frame shift and stop codons in all transcripts analyzed (Millar, 2003).

Partial cloning of a *msj*-1 like cDNA has been reported in the frog, *Rana esculenta* testis (Meccariello et al., 2004), and MSJ-1 protein, with a similar expression pattern as in mice, is widely described in this anuran (Meccariello et al., 2002, 2004), extending *msj-1* gene presence also to lower vertebrates. The detection of MSJ-1 protein in human spermatozoa and putative *msj-1* like genes in human genome, with a similar organization to murine gene (location into intron region, no interruption in putative CDS and opposite orientation regards UGTs), clearly suggests that *msj-1* is highly conserved in evolution from lower vertebrates to primates. Therefore, a fundamental function can be predicted and previous evidences suggest MSJ-1 involvement in vesicle traffic (including acrosomogenesis) and/or protein folding (Meccariello et al., 2002). In this respect, immunogold electron microscopy analysis conducted in this study confirms most of the subcellular localization of MSJ-1 previously described by immunofluorescence microscopy and immunoelectron microscopy conducted in epididymal spermatozoa (Berruti and Martegani, 2002,2005; Berruti and Aivatiadou, 2006). Interestingly, the extension of MSJ-1 ultrastructural localization in testis reveals that, during spermiogenesis, MSJ-1 is close to the cellular membranous-vesicular system. As soon as spermiogenesis proceeds, this protein follows the contour of the developing acrosomic vesicle and in mature sperm marks the outer acrosome membrane and the subacrosomic space, regions involved in acrosome reaction and egg-sperm inter-

action. No association with any cytoskeleton counterpart, *i.e.* the acroplaxome, a cytoskeletal plate that anchors the developing acrosomic vesicle to nuclear lamina (Kierszenbaum et al., 2003), emerged. Lastly, during the spermiogenesis also remarkable movements of organelles and cytoplasm toward the caudal end of the spermatids take place: the greater part of the spermatid cytoplasm is shed as residual body which undergoes phagocytosis by Sertoli cells. In this respect, beside acrosomic region, MSJ-1 follows all the cytoplasmic reorganization, is partially retained in the cytoplasmic droplets and has a final localization also in the centriolar region, where proteasome 26S resides (Mochida et al., 2000 and references therein). To date, during the spermiogenesis vesicular trafficking has a key role in acrosome formation from trans Golgi network and also in the sorting of structural proteins to the centrosome and the developing sperm tail (Kierszenbaum, 2002), processes that require folding control. Interestingly, MSJ-1 immunoprecipitates and colocalizes with m-UBPy (Berruti and Martegani, 2005), a deubiquitinating enzyme associated to the centrosomal γ-tubulin (Berruti and Aivatiadou, 2006). The control of ubiquitination rate may represent an intriguing check point in sperm cell functionality. Just to cite an example, constitutive ubiquitination of sperm mitochondrial proteins, such as prohibitin, a putative chaperone, is suggested to be a death signal for paternal mitochondria at fertilization, thus promoting the maternal inheritance of mtDNA in mammals; by contrast further ubiquitination in mitochondrial membrane proteins is a tag for the degradation of defective spermatozoa during sperm descent down epididymus (Sutovsky, 2003; Thompson et al., 2003). Thus, MSJ-1 together with mUBPy may also represent a fundamental molecular switch in the control of the ubiquitinating rate, responsible in turn for differential signalling pathways.

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