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# *Tsga*10 Encodes a 65-Kilodalton Protein That Is Processed to the 27-Kilodalton Fibrous Sheath Protein[1]

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## Abstract

We had previously reported the isolation of the testis-specific human gene *Tsga*10, which is not expressed in testes from two infertile patients. To study its role and function, we cloned the mouse homologue *Mtsga*10. *Mtsga*10 localizes to mouse chromosome 1, band B. This region is syntenic with human chromosome 2q11.2, where *Tsga*10 is located. We demonstrate that Mtsga10 mRNA is expressed in testis, but not in other adult tissues, and in several human fetal tissues and primary tumors. We uncovered that different species use different first exons and, consequently, different promoters. Using several antibodies, we discovered that, in mouse testis, *Mtsga*10 encodes a 65-kDa spermatid protein that appears to be processed to a 27-kDa protein of the fibrous sheath, a major sperm tail structure, in mature spermatozoa. Mtsga10 protein contains a putative myosin/Ezrin/radixin/moesin (ERM) domain. Transfection of fibroblasts with GFP-Mtsga10 fusion protein results in formation of short, thick filaments and deletion of the myosin/ERM domain abolished filament formation. Our results suggest the possibility that Tsga10 plays a role in the sperm tail fibrous sheath.

### Keywords

developmental biology; spermatid; spermatogenesis; testis

## INTRODUCTION

Mammalian spermatozoa are composed of specialized cytoskeletal elements, which appear to have no structural counterparts in somatic cells [1]. Several of these structures are associated with the sperm axoneme and might be involved in sperm motility [1, 2]. In the midpiece of the sperm tail, the axoneme is surrounded by nine outer dense fibers (ODFs), which in turn are covered by the mitochondrial sheath. In the principal piece, which is distal to the midpiece, the mitochondrial sheath is replaced by the fibrous sheath (FS). The FS is

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made up of two longitudinal columns replacing two ODFs, which are bridged by numerous transverse ribs. Evidence is building that the FS plays a structural, metabolic, and signal transduction role in sperm motility, which involves phosphorylation events [3].

Several FS proteins have been identified, but how the FS assembles or functions is not understood. Two identified FS components are members of the A-kinase anchoring protein (AKAP) family: AKAP4 (originally named AKAP82 or FSC1 in mouse) and AKAP3 (originally named AKP95T, FSP95, or AKAP110) [4–7]. AKAPs control targeting of PKA to the cytoskeleton and have domains for targeting to various subcellular compartments, including the actin cytoskeleton [8]. An intermediate filament-related protein, called FS39, has also been localized to the FS [9]. FS75 is another 75-kDa FS protein: the presence of a potential N-myristoylation site in this protein suggested that it covalently binds to the inner leaflet of the plasma membrane [10]. A member of the thioredoxin family, spermatid-specific thioredoxin-1, associates with the FS during development. However, this protein does not become a permanent structural component of mature FS [11].

Previous work from our laboratory had identified a novel gene *Tsga*10 [12]. It has been demonstrated that human *Tsga*10 is expressed in normal testis but not in a variety of tissues, nor is it expressed in fetal testis or testes of two infertile patients who suffered from nonobstructive infertility [12]. The histopathology of these testes showed that seminiferous tubules at various stages of maturation contained lower numbers of sperm in comparison with normal testis. In addition to the testicular expression, *Tsga*10-specific expressed sequence tags (ESTs) have been reported from several cancer cells, including adenocarcinoma (Accession no. AW057728, AI696619, and AW591313), acute myelogenous leukemia (Accession no. BF243403), and germ cell tumors (Accession no. BE047007).

In this study, we have cloned and characterized a cDNA encoding mouse Mtsga10. Mtsga10 mRNA is first detected in the postmeiotic phase of spermatogenesis. We demonstrate that Mtsga10 mRNA is translated to a 65-kDa protein, which appears to be processed to a major FS protein. *Tsga*10 is also expressed in actively dividing and fetal differentiating tissues and in various primary tumors.

## MATERIALS AND METHODS

### Isolation of Mouse cDNA and Genomic Clones Encoding *Mtsga*10

Primers GSP5 (5′ gag ttc cct agt aga aga 3′) and R6 (5′ tca ttg ctg aga tgg aac ag 3′), selected from the human *Tsga*10 sequence, were used for reverse transcription-polymerase chain reaction (RT-PCR) using mouse testis cDNA at an annealing temperature of 38°C. Resulting RT-PCR products were purified and labeled using the Rediprime 2 random prime labeling system (Amersham Pharmacia Biotech, Baie d'Urfe, PQ, Canada) and $^{32}$P-dCTP. Pharmacia NICK columns were used to remove unincorporated $^{32}$P-labeled nucleotides and the probe was denatured before use. To obtain full-length cDNA approximately $2 \times 10^5$ Pfu of a mouse testis cDNA library made from purified spermatocytes [13] were plated out on two 22-× 22-cm plates and duplicate filter lifts were taken. Replica filters were screened with the radioactively labeled RT-PCR product described above, which represents

nucleotides 1450–2070 of mouse *Tsga*10. Positive plaques were isolated, replated, and rescreened and one was sequenced (Accession no. AF530050). The sequence of *Mtsga*10 reported here is 99.5% identical to the sequence published in GenBank with accession no. XM 136734 (NCBI Annotation Project) and 99% identical to the corresponding mouse genomic sequence as determined by a Mouse Genomic Blast analysis (http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html).

A mouse genomic library consisting of seven gridded filters ($128 \times 10^3$ PAC clones, $1.5 \times 10^5$ base pair [bp] insert size provided by the UK HGMP Resource Center) was screened with the same probe as described above and positive clones were identified.

## Chromosome Mapping of *Mtsga*1

PAC 618H14 containing the mouse *Tsga*10 gene was labeled (Random Prime Kit; Gibco BRL, Gaithersburg, MD) with Avidin-conjugated fluorescein isothiocyanate and hybridized to male mouse metaphase spreads as described [14]. FISH signals and the DAPI (4,6-diamidine-2-phenylindole dihydrochloride) banding pattern were merged for figure preparation. Images of metaphase preparations were captured by a cooled CCD camera using the Cyto Vision Ultra image collection and enhancement system (Applied Imaging Int., Ltd., Newcastle, UK).

## Extraction of RNA and Analysis

RNA was extracted from all tissues using RNAzol B reagent (Biogenesis, Kingston, NH) following the manufacturer's instructions with minor modifications. Contaminating DNA was removed using DNase I (Pharmacia).

**RT-PCR analysis—**Single-stranded cDNA was prepared from 1–5 μg total RNA of various rat and mouse tissues using MMLV Reverse Transcriptase (Invitrogen, Burlington, ON, Canada) and oligo-$dT_{(12–18)}$ primer. A DNA polymerase (Clontech Advantage cDNA polymerase) with proof reading capability was used for the PCR amplification. Primers and annealing temperatures were as follows: β-actin primers (5′ CAA-CACCCCAGCCATGTACG3′ and 5′ AGGAAGAGGATGCGGCAG-TGG 3′) anneal at 55°C. R6 and GSP5 primers anneal at 48°C. Human cDNAs were checked for cDNA quality using primers designed from exon 10 5′ TCCGACTGAGCGGCACTGGGAGTGC3′ and 11 5′ GCC-CGCAGGTCCTCTTTCCCTCACA3′ of the housekeeping gene *phosphoglucoMutase-1*.

**Northern blotting analysis—**Fifteen micrograms total RNA isolated from indicated tissues was analyzed by Northern blotting using a $^{32}$P-radiolabeled MSTGA10 cDNA probe and a β-actin cDNA probe, as described before [15].

## Germ Cell Extract

For these experiments, we used rats because the procedure for elutriation is better established for rat than mouse in our lab, and larger amounts of germ cells can be obtained. All work involving animals was carried out according to the Guidelines of the Canadian Council on Animal Care. Elutriated germ cells were prepared as described previously [16,

17] with minor modifications. Fractions were obtained of 90% pure pachytene spermatocytes (fraction 220), 90% pure round spermatids (fraction 90), and 50% pure late spermatids (fraction 50). The elutriated cells were extracted in lysis buffer (50 mM Hepes [pH 7.0], 1% v/v Nonidet-P40 [Sigma, St. Louis, MO], 1 μg/ml of aprotinin, and 100 μg/ml of PMSF), and protein was quantified by spectrophotometry.

## DNA Sequencing and Computer Analysis

Both strands of cloned DNA or PCR products were sequenced using $\alpha$-[$^{33}$P]-ddNTP and a ThermoSequenase cycle sequencing kit (Amersham). Computer analysis was carried out using programs and software at the following sites: http://ca.expasy.org/, http://www.ensembl.org/, and http://www.sanger.ac.uk.

## Generation of Antibodies

Peptides from three Mtsga10 regions were synthesized: N-terminus (amino acids 2–15), middle region (amino acids 206–219), and C-terminus (amino acids 679–691). Peptides were mixed and conjugated to keyhole limpet hemocyanin and used to immunize two rabbits by Eurogenetec (Seraing, Belgium). Resulting antisera thus contain antibodies against the three Mtsga10 peptides. In indicated experiments, we used antibodies that were affinity purified against individual peptides.

## Isolation of ODF and FS from Sperm Tails

Sperm was collected from rat epididymis because of much larger amounts of material that can be isolated in comparison with mouse, washed twice in TBS, and sonicated at 4°C with a sonicator (XL; Scientific Company, Ltd., Guelph, ON, Canada). Efficient sonication was verified by phase contrast microscopy. After sonication, tails were isolated and used to purify ODF and FS following the protocol developed by Oko [18].

## Immunoblot Analysis of Sperm Proteins

Western blot assays were done using protein extracts to identify mouse Tsga10 as follows: protein extracts were boiled in SDS sample buffer and separated on 12% SDS-PAGE gels. After electrophoresis, proteins were transferred in buffered transfer systems (Bio-Rad protein II, Mississauga, ON, Canada) onto PVDF membranes (Amersham). The blots were blocked with 5% w/v fat-free milk, 0.1% w/v Tween, and 0.1% Nonidet-P40 in PBS and then incubated with Mtsga10 antisera raised against a mix of three Mtsga10 peptides or with antibody affinity purified against individual Mtsga10 peptides for 1 h at room temperature. Primary antibodies were detected using a probe with peroxidase-conjugated goat anti-rabbit IgG (Sigma, Oakville, ON, Canada), processed, and developed using Lumi-GLO kit (KPL, Gaithersburg, MD).

## Plasmid Constructs

The complete open reading frame region of the mouse *Tsga*10 gene was cloned into *Hind*III and *Eco*RI sites of pEGFP-c2 (Clontech, Palo Alto, CA). The plasmids pEGFP-Mtsga10 (Δ1Mtsga10), pEGFP-Mtsga10 (Δ2Mtsga10), and pEGFP-Mtsga10 (Δ3Mtsga10), containing different deletions of *Mtsga*10, were generated by PCR using pEGFP-Mtsga10.

The plasmid pEGFP-Δ1Mtsga10 was created by standard PCR using 5′ AAA-GATCTTCTGATACTCAGCG3′ primer and 5′ GGAGATGTTTACAC-CATACCGAC3′, a pEGFP vector primer. The PCR product was cloned into *Bgl*II and *Eco*RI sites of pEGFP-c2. PCR products from reverse primers 5′ GCTTTTCATCATTTCTCGTCG3′ and 5′ AAGGAGGAAGGTCT TGTCT3′ with a forward primer 5′ TGCACAGGACCTGGAGTGTA3′ were used for cloning of pEGFP-Δ2Mtsga10 and pEGFP-Δ3Mtsga10, respectively.

## Cell Culture, Transfection, and Fluorescent Staining

Mouse NIH3T3 cells were transiently transfected using FuGENE6 (Roche, Mississauga, ON, Canada) according to manufacturer's instructions. Transfected cells were harvested and analyzed for GFP signals after 48 h directly or after fixation and immunostaining with Phalloidin-TRITC (Sigma) to detect filamentous actin.

## Indirect Immunofluorescence Analysis of Sperm

Mature spermatozoa were collected from rat epididymis and washed in PBS according to the method described by Oko [18]. Spermatozoa were pelleted and resuspended in demembranization buffer (5 mM 1,4-dithiothreitol [DTT], 50 mM Tris-HCl [pH 9.0], and 2% v/v Triton) followed by fixation in 4% formaldehyde. The cells were spread on cover slips treated with poly-L-lysine (Sigma) and incubated with primary antibody diluted 1:25 (v/v) in blocking solution for 1 h at 37°C and washed in PBS. Sperm were incubated for 1 h at 37°C with secondary antibody (Cy3-conjugated goat anti-rabbit IgG; Jackson Immunoresearch Laboratory, Inc., West Grove, PA) diluted 1:500 (v/v) in blocking solution and washed in PBS before staining with DAPI (as described above). Cover slips were mounted on glass slides with Permount SP15-100 (Fisher Scientific, Edmonton, AB, Canada). Slides were viewed with a Zeiss confocal fluorescent microscope.

## Cytoplasmic and Nuclear Extract Protein Purification

Nuclear and cytoplasmic protein extracts were prepared essentially as described [19] with some modifications. Briefly, germ cells obtained by elutriation ($10^7$ cells) were washed twice with ice-cold PBS and suspended in 400 μl ice-cold buffer A (10 mM Hepes [pH 8.0], 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA [pH 8.0], 30 mM sucrose, 1 mM dithiothreitol, 0.4 mM PMSF, 0.3 μg/ml leupeptin, 5 μM pepstatin A, and 0.5% Nonidet P-40) and incubated on ice for 1 min. Nuclei were collected by centrifugation at 1500 × *g* for 10 min at 4°C, the supernatant (cytoplasmic fraction) was aliquoted and kept at −80°C. The nuclear pellet was washed twice with ice-cold buffer B (buffer A plus 10 mM $CaCl_2$) and collected by centrifugation at 1500 × *g* for 10 min at 4°C. The nuclear pellet was resuspended in 100 μl ice-cold buffer C (50 mM Hepes [pH = 7.8], 100 mM KCl, 0.1 M EDTA [pH 8.0], 15% glycerol, 1 mM dithiothrietol, 0.5 mM PMSF, and 5 μM pepstatin), mixed gently at 4°C for 20 min, and centrifuged for 5 min at 14 000 rpm in a microfuge at 4°C. The supernatant (nuclear extract) was transferred to a new microfuge tube, frozen in liquid nitrogen, and stored at −80°C.

# RESULTS

## Cloning of the Mouse Homologue of *Tsga*10

To understand in detail the expression profile of this gene as a clue to its possible function, we have isolated and characterized the mouse homologue. Mouse cDNA was obtained by screening a spermatocyte-specific cDNA library. Comparison with human *Tsga*10 revealed 92% similarity between mouse and human sequences.

## Variations in the First Exon Structure of *Tsga*10 in Different Species

The 5′ flanking region of mouse exon 1 was sequenced from PAC 618H14. It was aligned with the 5′ upstream region of human *Tsga*10 (sequenced from human clone AC019097). Surprisingly, rather than having homology to human exon 1 and its 5′ upstream region, mouse exon 1 is 89% identical to nucleotides 1976–2098 of the human clone AC019097, which are located more than 8 kb upstream of human *Tsga*10 exon 1 (Fig. 1). The mouse 5′ upstream region is 65% identical to the corresponding region of human sequence (Fig. 1). Analysis of partial rat *Tsga*10 sequences show that they align with mouse exon 1. Thus, mouse, rat, and human *Tsga*10 genes use different exon 1 sequences and different promoters.

A computer search for transcription binding sites in the mouse sequence showed a match with the Broad-Complex Z2 (BR-C Z2) binding sequence ATTGTTACTAGTTTTC at position −72 (Fig. 1).

## *Tsga*10 Maps to Mouse Chromosome 1, Band B

The PAC618H14 genomic clone of *Tsga*10 was used to localize the gene and confirm that it is a single-copy gene. PAC 618H14 obtained from the library was labeled and hybridized to male mouse metaphase spreads. The results of fluorescence in situ hybridization localized the gene to mouse chromosome 1, band B (Fig. 2). This location is expected given the mouse-human chromosome homologies (International Human Genome Sequencing Consortium, 2001) and confirmed the prediction of this location by an Ensembl genomic analysis of the entire mouse genome (www.ensemble.org).

## Mouse *Tsga*10 Is Transcribed in Testis, Fetal Tissues, and Cancer Cells

RNA extracted from different mouse tissues was first analyzed by Northern blotting. The result, shown in Figure 3, indicates that *Mtsga*10 expression is restricted to testis in which a single major transcript was observed. Rehybridization of filters with an actin probe confirmed presence of intact RNA in all instances (Fig. 3A). The observed size of Mtsga10 mRNA is 3.2 kb, similar to the human Tsga10 transcript. A faint band above the major mRNA was visible, possibly resulting from splice intermediates. To obtain detailed information on the expression in testis, we next analyzed rat testes at different developmental stages. Testicular RNA was converted to cDNA and used for PCR using primers from *Tsga*10 and β actin (Fig. 3B; note that mouse and rat *Tsga*10 [accession no. XM 237065, XM 237066] are over 95% identical, and larger numbers of rat germ cells can readily be obtained by this procedure). The results show that the Tsga10 transcript becomes detectable in rat testis between Days 15 and 21 after birth (Fig. 3B). Because of the well-characterized developmental stages of rat spermatogenesis [20], this corresponds to the

presence of the transcript in pachytene cells during meiosis, in agreement with our cloning of Mtsga10 cDNA from spermatocytes.

PCR amplification of cDNA made from fetal (16–20 wk) and tumor tissues was used to analyze fetal human *Tsga*10 expression. We detected *Tsga*10 expression in all actively dividing fetal tissues, including sternum, intestine, limb, kidney, and stomach (Fig. 4A). We also detected Tsga10 mRNA in tumors of the thyroid, parotid, and scalp (squamous cell carcinoma) (Fig. 4B). A database further showed that ESTs homologous to *Tsga*10 have been cloned from adenocarcinomas (Accession no. AW057728, AI696619, and AW591313), acute myelogenous leukemia (Accession no. BF243403), and germ cell tumors (Accession no. BE047007).

## Translation of *Mtsga*10 Protein in Late Spermatids

To characterize Mtsga10 protein and analyze its temporal expression pattern during spermatogenesis, germ cells were elutriated and analyzed. Three fractions were obtained containing pachytene spermatocytes, round spermatids, and elongating spermatids. Antisera raised against a mixture of three Mtsga10 peptides (see *Materials and Methods*) recognized one band in germ cells of the expected size (65 kDa). This analysis also showed that Tsga10 protein is most abundant in elongating spermatids (Fig. 5A). A small amount of Tsga10 protein is visible in round spermatids extract, which may be the result of presence of elongating spermatids in that cell fraction, as observed previously [15]. Because Tsga10 RNA but not protein is detectable in spermatocytes, as evidenced by the present developmental study and the original cloning of Tsga10 cDNA from a spermatocyte cDNA library, these results suggest the possibility of translational control. To define the location of Tsga10 in cells, nuclear extracts and cytoplasmic extracts were prepared from germ cells. Protein loading was analyzed using Coomassie Blue staining of gel-separated proteins. The same amount of protein was transferred to blots and probed with anti-Tsga10 antiserum. The result of immunoblotting revealed that the protein is present in the cytoplasm, not in the nucleus (Fig. 5B, lanes 2 and 3). A control demonstrates that, as expected, the CREMτ transcription factor is predominantly present in the nuclear extract (Fig. 5B, lanes 4 and 5) [21].

## *Mtsga*10 Protein May Be Processed

To investigate if Mtsga10 protein might be a component of one of the unique sperm tail structures, epididymal sperm tails as well as pure ODF and FS were isolated, extracted, and used for immunoblotting. Antisera raised against a mixture of the three Mtsga10 peptides were used to probe Western blots (Fig. 5C). The result demonstrates that, in tails of spermatozoa, only a small amount of 65-kDa Mtsga10 is detectable (lane 1) and that two proteins of 32 and 27 kDa reacted (lane 1). Using purified ODF and FS, we next found that Mtsga10 is present in purified FS (lane 3), not ODF (lane 2) and that FS purified from epididymal spermatozoa contain the 27-kDa Mtsga10 protein. Because the 65-kDa Mtsga10 translation product can be detected in total testis, in all testicular germ cells, as well as in epididymal sperm tails, whereas the 27-kDa Mtsga10 peptide is only detected in the FS of epididymal spermatozoa, our data suggest the possibility of processing of the 65-kDa Mtsga10 primary translation products. To determine which portion of the 65-kDa Mtsga10

protein is present in the 27-kDa FS protein, we affinity-purified antibodies against each one of the three different Mtsga10 peptides (Fig. 5D). The 27-kDa FS Mtsga10 protein was detectable using antibodies affinity purified against the first and second peptide (lanes 2 and 4) but not against the third (C-terminal) peptide (lane 6). Thus, the N terminus of the protein up to at least amino acid number 219 (the second peptide site) must be present in the 27-kDa FS protein. The size of this protein predicts that it contains approximately 240 amino acid residues. The origin of the 32-kDa band is not known at present.

### *Mtsga*10 Is Present in the Sperm Tail Principal Piece

To confirm the observation of the 27-kDa Mtsga10 protein in purified FS, indirect immunofluorescence microscopy of epididymal sperm was carried out using antiserum against a mixture of the three Mtsga10 peptides. The result shows that Mtsga10 is present only in the principal-piece region of the tail, not in the midpiece (Fig. 6). Because the midpiece contains ODF, axoneme, and mitochondria but not FS, which is confined to the principal piece, this result confirms our characterization of Mtsga10 as an FS protein. Sperm incubated without primary antibody did not show any staining and nonspecific secondary antibody binding to sperm was also undetectable (not shown).

### *Mtsga*10 Contains a Putative Myosin Domain

We analyzed the 65-kDa Mtsga10 protein sequence to gain insight regarding the structure and possible functional domains and to verify the possibility of its assignment to an existing gene family. Results revealed a putative domain near the middle of the protein (Mtsga10 amino acids 125–551) with significant sequence similarity to the myosin tail (pfam 01576) and also a high degree of similarity to ERM, the Ezrin/radixin/moesin family domain (pfam00769 [proMtsga10 amino acids 268–618]).

Transfection of mouse fibroblasts was carried out to analyze the possible functionality of the predicted coiled-coil myosin/ERM-like sequence in Mtsga10: Can it assemble into macromolecular filaments? Fibroblasts were transfected with pEGFP-Mtsga10 DNA, which produces a GFP-Mtsga10 fusion protein, or with pEGFP vector DNA and were fixed and stained with DAPI to visualize nuclei 24 or 48 h after transfection. In indicated experiments, cells were also treated with Phalloidin to detect actin stress fibers. As seen in Figure 7A, GFP alone is expressed diffusely in the entire cell. In contrast, GFP-Mtsga10 protein is exclusively cytoplasmic (Fig. 7B) and appears to form thick, short filaments (Fig. 7D). No colocalization with actin was detected (Fig. 7C). This result suggested that the putative myosin/ERM domain might be functional and assist in the formation of filaments in elongating spermatids.

To analyze if the Mtsga10 putative myosin/ERM domain is important for filament formation, we tested Mtsga10 deletion mutants, schematically presented in Figure 8. As shown, removal of the entire putative myosin domain (Δ1Mtsga10) or of the domain plus upstream sequences (Δ3Mtsga10) abolished filament production by Tsga10. Instead, cytoplasmic local accumulation of Tsga10 was observed at both 24 and 48 h after transfection (panels A, B, and E). Deletion of an N-terminal fragment (aa residues 6–212),

which removes the N-terminal portion of the myosin domain, also prevented filament formation at these time points (panels C and D).

## DISCUSSION

The *Tsga*10 gene, which we show here encodes the mature 27-kDa FS protein and forms filaments in transfected cells, is expressed in germ cells as well as in some fetal tissues and cancer cells. The gene is highly conserved among different species. The mouse sequence has 89% identity to the human homologue, and 94% identity at the amino acid level. EST clones with over 90% identity to the mouse sequence have been reported from testis of rat and pig. Mouse and human genes both have 19 exons, but we demonstrate here that the first exon in rat and mouse are different from those in human and pig. Multiple sequence alignment results and comparison of the 5′ flanking regions of the mouse and human genes indicate that the homologue of the first exon of the mouse gene is located at 8.3 kb upstream of human exon 1. The region upstream of mouse exon 1 and its homologous human sequence are 65% identical. There is no significant match to the human exon 1 5′ region in the mouse. This result may indicate the presence of an alternative promoter in human and pig *Tsga*10 genes compared with mouse and rat genes.

The FS is composed of several proteins, of which the 14-, 27-, 63-, and 75-kDa polypeptides are major proteins [18]. We demonstrate that Mtsga10 is predominantly translated in the postmeiotic phase of spermatogenesis, is synthesized as a 65-kDa Mtsga10 protein, and is processed to a 27-kDa FS protein. The localization of the 27-kDa protein was further supported by the immunoreactivity seen only in the rat sperm principal piece. It is unclear how Mtsga10 is processed and where. Clues come from the following observations: The predominant Mtsga10 protein in testis is the 65-kDa form, whereas the predominant form in mature sperm is the 27-kDa FS protein. In addition, Northern blot analysis shows the presence of one major Mtsga10 transcript, suggesting that the 65-kDa Mtsga10 protein is the primary translation product in elongating spermatids. This makes it less likely that the 27-kDa Mtsga10 protein is encoded by a separate mRNA. We suggest the possibility that the 65-kDa primary translation product is processed proteolytically at an unknown time point outside the testis. In support, our results of immunoblotting using antibodies affinity purified against different regions of the protein demonstrated that such a processing event likely occurs in the middle region, between aa residues 219 and 240. Modification of a protein in the sperm tail has also been shown for another FS protein. Human and mouse pro-AKAP82 are proteolytically processed to AKAP82 [5, 22]. The processed AKAP82, which remains in the FS, is made up of the C terminal part of proAKAP82.

We demonstrate that Mtsga10 is a structural protein, which suggests that defects could be implicated in infertility. We previously have shown that human *Tsga*10 is not expressed in some infertile patients [12]. However, the basis for the absence of Tsga10 protein in these patients is not known and the possibility that mutations or deletions in the 121-kb *Tsga*10 gene are involved is currently under investigation. Studies involving protein-protein interaction will help elucidate the possible role of the FS in normal sperm motility and of flagellar abnormalities that lead to male infertility, in particular in men with dysplasia of the FS (DFS). DFS is characterized by male infertility, asthenozoospermia, and morphologically

abnormal flagella [23]. It appears to be familial, making it likely that a genetic component is involved [23].

Recent models suggest that FS proteins can also serve as scaffolds for protein complexes involved in regulating signal transduction processes [24, 25]. It was suggested that components of the Rho signaling pathway associated with the FS might be involved in regulating flagellar function. These include the Rho-binding protein rhophilin [26] and ropporin, a spermatogenic cell-specific protein that binds to rhophilin and to the FS [27]. Rhophilin is highly expressed in testis and is localized specifically in sperm flagella. The presence of a PDZ domain suggested that rhophilin works as an adaptor molecule [28]. PDZ domains bind to a C-terminal motif with the sequence-X-Ser/Thr-X-Val/Leu-COOH, where X represents any residue. This motif is present between aa numbers 255 and 259 in Mtsga10, the region that may be involved in cleavage of pro-Mtsga10. Further investigation will confirm whether Mtsga10 is involved in regulating signal transduction and/or can bind rhophilin.

Bioinformatics analysis of the 65-kDa protein revealed the presence of regions with significant similarity to the myosin tail and ERM domain. It is not clear whether myosin is present within the sperm tail. However, there is some evidence for the possibility of myosin involvement in sperm function [29, 30]. A number of unconventional myosins, including myosin-X, contain the Myosin Tail Homology 4 and ERM domains [31]. The major function of ERM proteins is to act as cross-linkers between plasma membrane and actin filaments and they may also function in signaling cascades that regulate the assembly of actin stress fibers [32]. We provide some evidence that the myosin domain in Mtsga10 might be functional: In preliminary experiments, we observed that anti-Mtsga10 antibodies raised against all three Mtsga10 peptides recognize the maltose-binding protein-paramyosin protein, a commonly used molecular weight marker protein (not shown). Note that MBP-paramyosin and Mtsga10 share only 18% sequence similarity. Furthermore, Mtsga10 filaments are formed in fibroblasts transiently transfected with GFP-Mtsga10 fusion protein. Deletion analysis demonstrated that removal of the putative Mtsga10 myosin tail domain and C-terminus or of the Mtsga10 N-terminus plus the N-terminal portion of the myosin domain prevents filament formation. These experiments suggest that the putative myosin domain is required for filament formation. However, the possibility remains that both the N- and C-termini are required for this activity. Further experimentation to delineate the crucial residues is in progress. We propose that this domain may play a role in the formation of the FS during spermiogenesis. Our transfection results are similar to the result of immunofluorescence localization of E-cadherin-CD44/ERM fusion protein that binds moesin [33, 34].

We previously showed that the gene for human *Tsga*10 maps to 2q11.2 [12]. In the current study, the mouse gene, Mtsga10, was mapped to the B region of the mouse chromosome, one that is syntenic to the human 2q11.2 chromosome region. Although the homologues of several human genes and genetic diseases have been mapped to mouse B chromosome 1, the reported phenotypes are not suggestive of a defect in a sperm FS protein. However, a significant number of neighboring genes, including M*GAT4, LAF4,* and *REV1L,* are involved in cancer. For example, the highest expression of M*GAT4* is in the promyelocytic leukemia cell line HL-60 and the lymphoblastic leukemia cell line [35]. Another example is

*LAF4,* which has been suggested to function as nuclear transcription factor in lymphoid development and oncogenesis [36, 37]. *REV1L,* a transferase, another neighboring gene, may play a critical role during mutagenic translesion DNA synthesis [38]. We show here that *Tsga*10 is expressed in several cancer cells, including cancers originating from bone marrow, thyroid, germ cell, stomach, and uterus. Investigation of the expression and function of *Tsga*10 in malignant cells may help elucidate a possible role in transformation and production of metastatic cancers.

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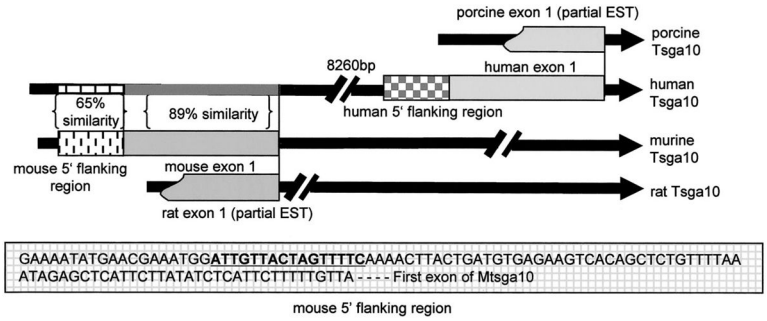


**FIG. 1.**

Location of *Tsga*10 exon 1 in different species. Schematic drawing showing an alignment of *Tsga*10 sequences containing exon 1 from four species, porcine, human, mouse, and rat. The 5′ flanking region of the mouse gene was sequenced from mouse PAC clone 618H14. Exon 1 is indicated by gray boxes and 5′ flanking regions of human and mouse are denoted by patterned boxes. Note that mouse exon 1 is 89% identical to a region in the human genome located more than 8 kb upstream of human *Tsga*10 exon 1. A putative Broad-Complex Z2 (BR-C Z2) transcription factor binding site in the mouse *Mtsga*10 5′ flanking region is indicated in bold underlined lettering (see box).

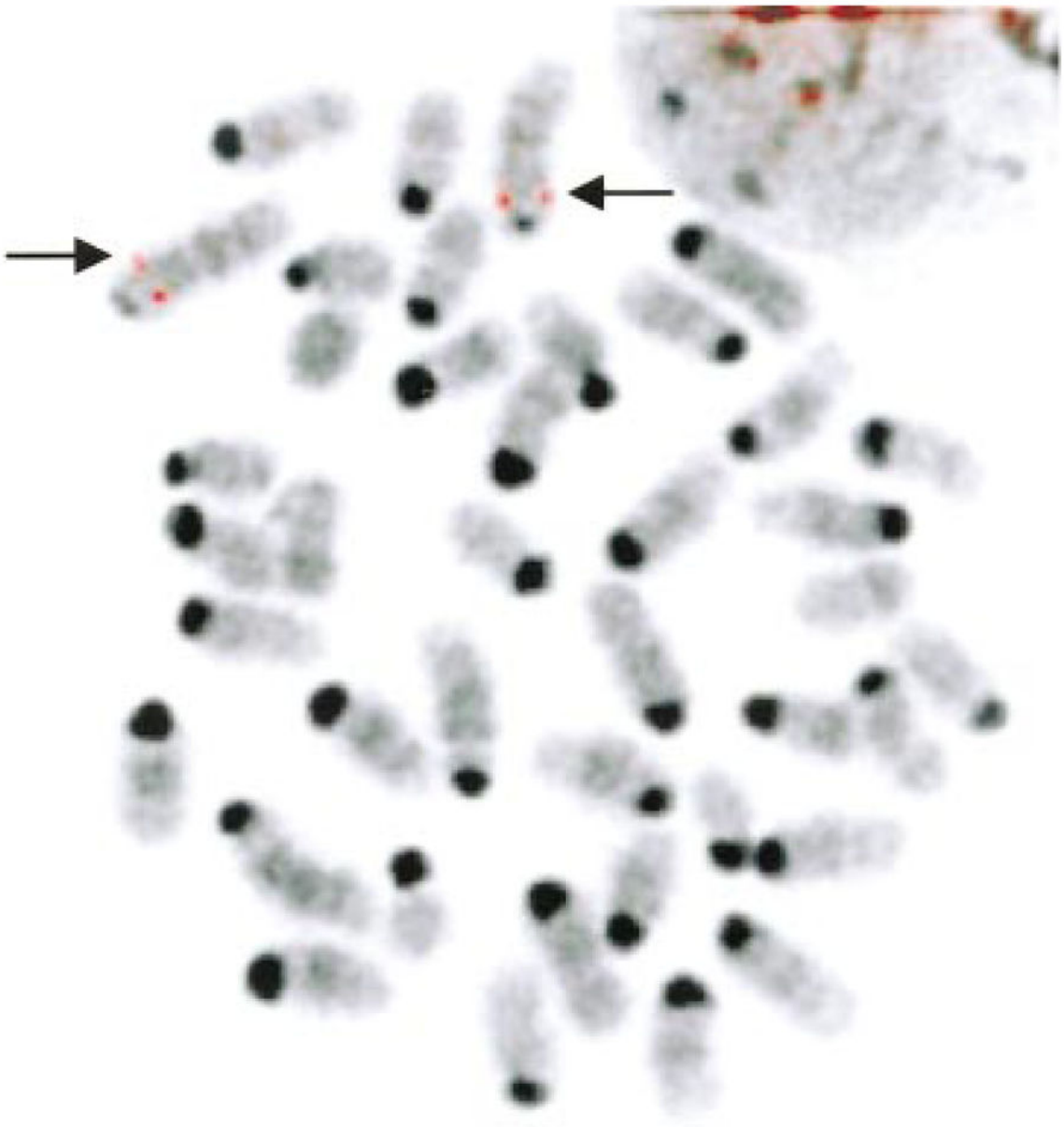

**FIG. 2.**

Localization of *Mtsga*10 on mouse chromosome 1, band B. The mouse *Mtsga*10 gene was localized to chromosome 1, band B, using fluorescence in situ hybridization to mouse metaphase chromosomes. The positions of hybridization signals (red dots) are marked by arrows. Original magnification ×100.

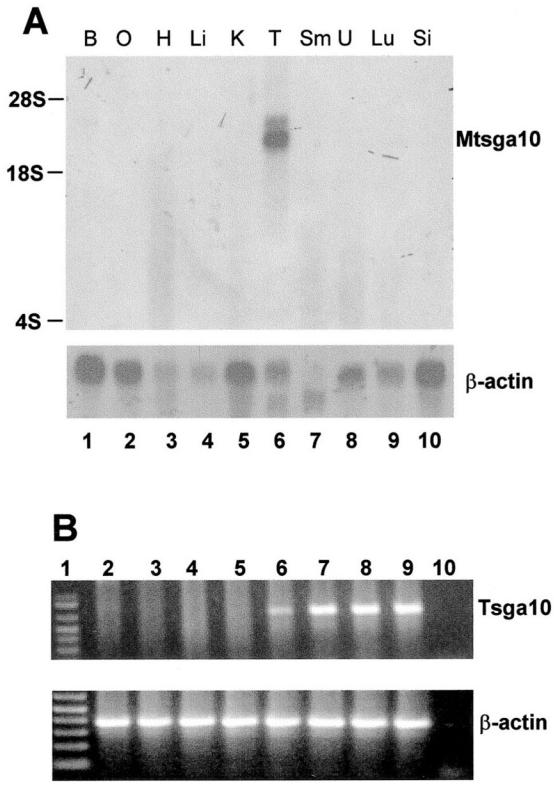


**FIG. 3.**

Mouse Tsga10 mRNA is expressed in testis. **A**) RNA was isolated from indicated adult mouse tissues and analyzed by Northern blotting, using as probes Mtsga10 cDNA (top panel) and β-actin (bottom panel). Lanes 1–10: brain (B), ovary (O), heart (H), liver (Li), kidney (K), testis (T), skeletal muscle (Sm), uterus (U), lung (Lu), and small intestine (Si) RNA, respectively. Indicated are 28S and 18S ribosomal marker RNAs and 4S tRNAs. **B**) To examine *Tsga*10 expression during male germ cell development, RNA was isolated from rat testes and used in RT-PCR with *Tsga*10-specific primers (top panel). A control RT-PCR amplification using β-actin primers was also done (bottom panel). The expected Tsga10 PCR product size is 632 bp. Lane 1: 100-bp molecular weight ladder. RNAs for RT-PCR were isolated from fetal testis (lane 2) and postnatal testes of 5, 10, 15, 21, 25, and 31 days and adult (lanes 3–9, respectively). Lane 10: a negative control for RT-PCR (no RT reaction, but otherwise identical to other PCR-reaction conditions).

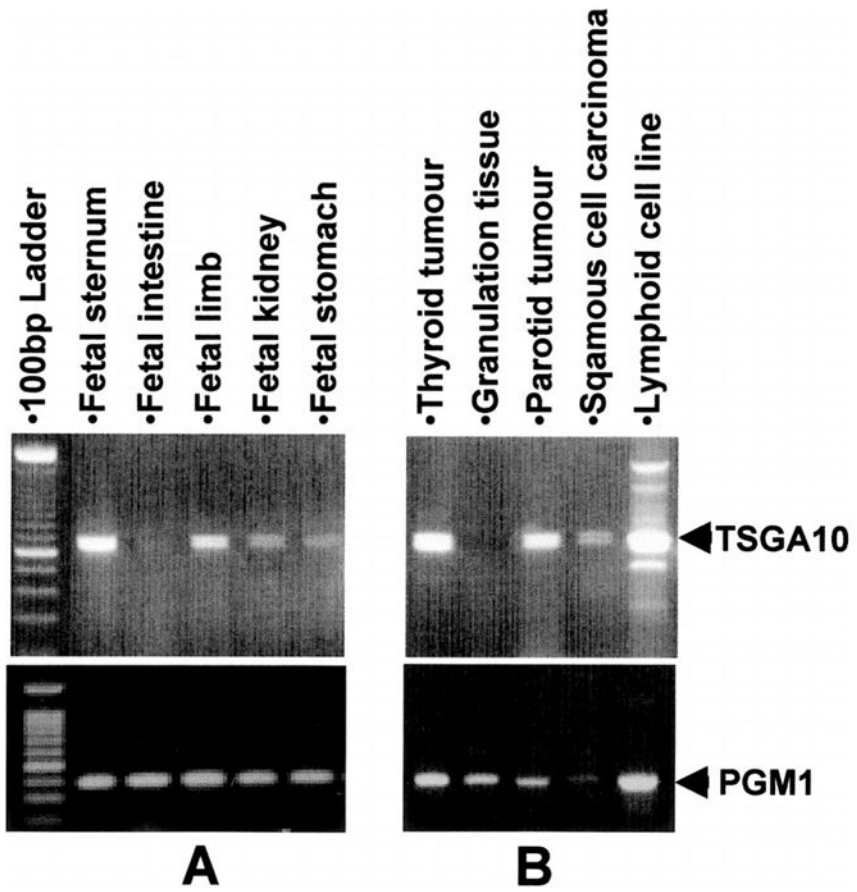


**FIG. 4.**

Expression of Tsga10 in fetal tissues and cancer cells. **A**) RT-PCR using *Tsga*10-specific primers was used to detect expression in the indicated fetal human tissues (top panel). **B**) RT-PCR analysis of Tsga10 mRNA from indicated human primary tumors shows the presence of Tsga10 in several tumors as well as in a lymphoid cell line (top panel). Two bands are seen in both sets of RT-PCR reactions (**A** and **B**), which derive from alternatively spliced Tsga10 mRNAs (one with an additional 44 bp in the 5′ UTR) [12]. Control RT-PCR reactions (bottom panels in **A** and **B**) were carried out with PGM1 primers to demonstrate the presence of intact mRNA in the samples prepared from adult and fetal human tissues.

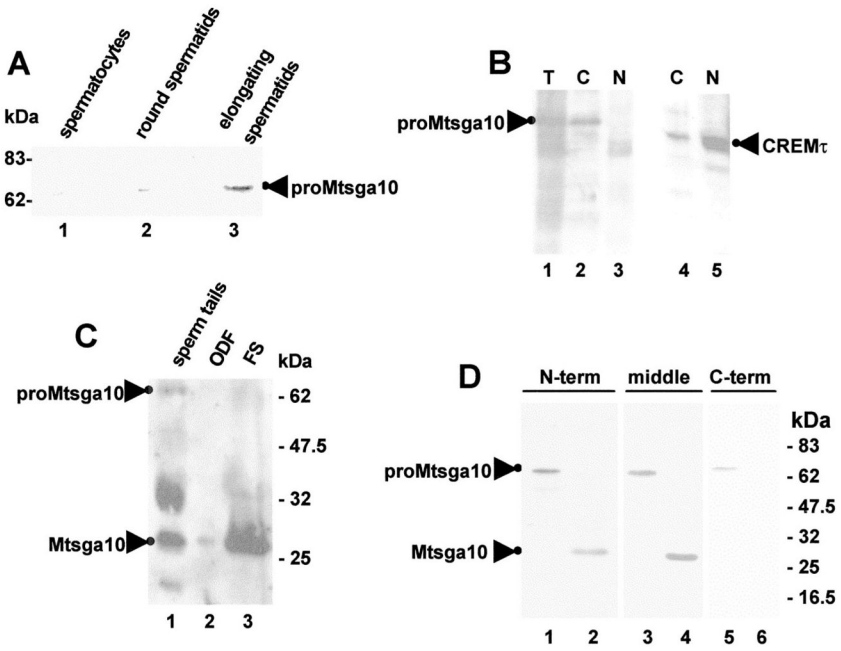


**FIG. 5.**

Immunoblot analysis of Mtsga10 protein. **A**) Indicated germ cell fractions (lanes 1–3) were obtained from adult rat testes using centrifugal elutriation. Cells were lysed and used for Western blot analysis using anti-Mtsga10 antiserum raised against all three peptides. Lanes 1–3: spermatocytes, round spermatids, and elongating spermatids, respectively. Note that full-length 65-kDa Mtsga10 is predominantly expressed in elongating spermatids. **B**) Western blot analysis of total testis (T), cytoplasmic (C), and nuclear (N) fractions of testicular germ cells using anti-Mtsga10 antiserum raised against all three peptides (lanes 1–3, respectively). Loading of equal amounts of cytoplasmic and nuclear samples was analyzed by Coomassie Blue staining before immunoblotting (not shown). As a control, cytoplasmic and nuclear proteins were also analyzed using anti-CREMτ antibody, which detects the testicular CREMτ transcription factor (lanes 4 and 5, respectively). Note the presence of Mtsga10 in cytoplasmic fractions. **C**) Analysis of Mtsga10 in sperm tail. Extracts from rat sperm tails (lane 1) and two major components of rat sperm tails, ODF and FS (lane 2 and 3, respectively), were gel separated and used for the immunoblot analysis using anti-Mtsga10 antiserum raised against all three peptides. Note that FS, but not in ODF, contain a prominent 27-kDa immunoreactive Mtsga10 protein. **D**) Immunoblot analysis of 65-kDa Mtsga10 and mature 27-kDa FS Mtsga10 was carried out using affinity-purified antibodies against peptides located in three different regions of Mtsga10 protein. Lanes 1, 3, and 5: cytoplasm fraction from testicular germ cells. Lanes 2, 4, and 6: FS proteins isolated from sperm tails. Lanes 1 and 2: filters were probed with affinity-purified antibody against a peptide located at the N-terminus (amino acids 2–15: N-term). Lanes 3 and 4: filters were probed with affinity-purified antibody against a peptide located at amino acids 206–219 (middle). Lanes 5 and 6: filters were probed with affinity-purified antibody against a peptide located at the C-terminus (amino acids 679–691: C-term).

**FIG. 6.**

Indirect immunofluorescence analyses of Mtsga10 in epididymal sperm. Epididymal spermatozoa were fixed on poly-L-lysine coated glass cover slips and stained with anti-Mstga10 antiserum followed by secondary antibody conjugated to Cy3. The principal piece of sperm tails, which is distal to the midpiece, strongly fluoresced. Sperm heads, showing very weak staining, are indicated with arrows. Bar = 20 μm.

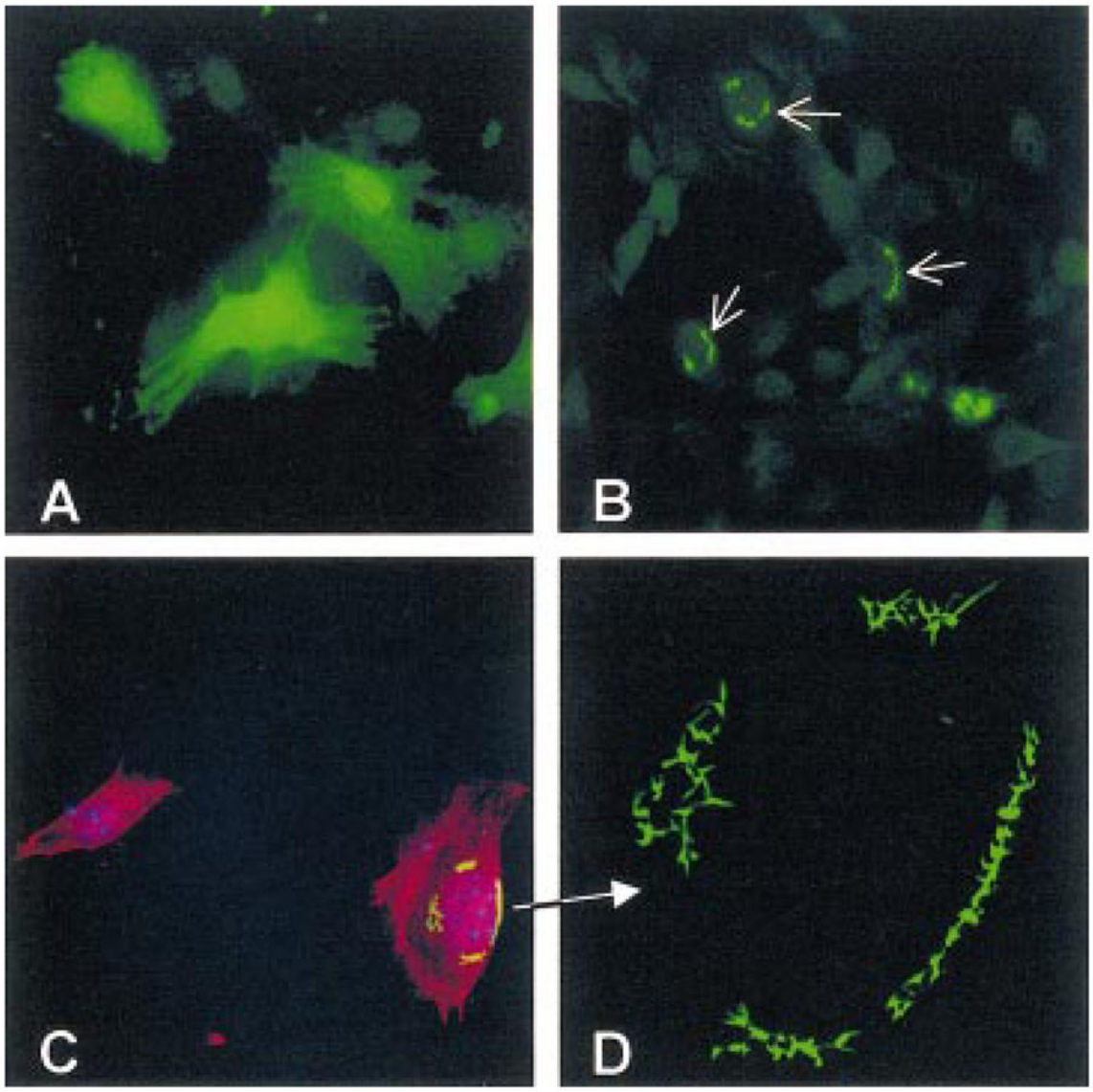


**FIG. 7.**

Mtsga10 forms filaments in transiently transfected cells. NIH3T3 mouse fibroblasts were transfected with empty pEGFP vector DNA and with pEGFP-Mtsga10 using FuGENE6. **A**) Control transfection showing general localization of GFP in transfected NIH3T3 cells. **B**) Cells expressing the GFP-Mtsga10 protein show formation of cytoplasmic short, thick filaments (arrows). **C**) Actin was visualized by fluorescence microscopy using TRITC-coupled phalloidin (red) in transfected cells expressing GFP-Mtsga10 protein (green). Nuclei were stained with DAPI (blue). **D**) Higher magnification of a cell in (**C**) expressing GFP-Mtsga10 fusion protein to better visualize the thick filaments. **A**–**C**) Original magnification ×20; (**D**) original magnification ×100.

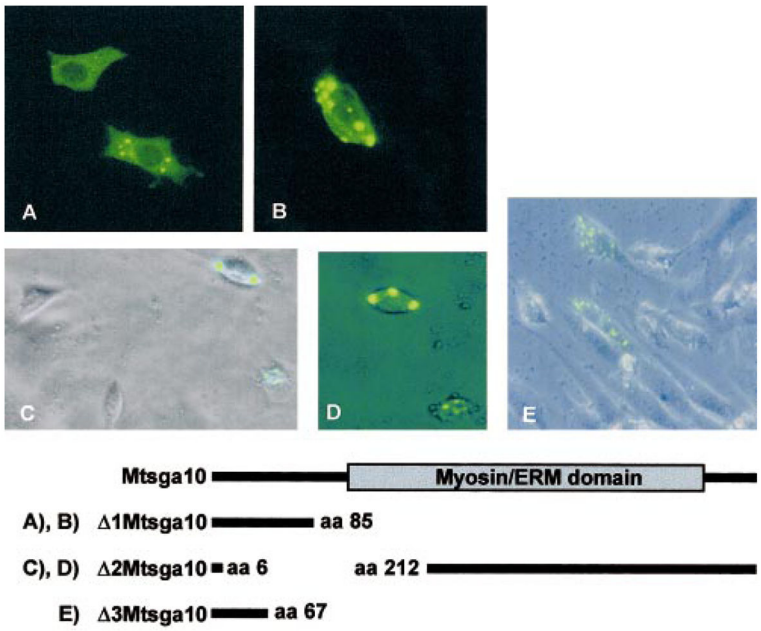


**FIG. 8.**

The myosin tail domain of Mtsga10 is important in formation of filaments. NIH3T3 cells were transfected with plasmids pEGFP-Δ1Mtsga10 Δ(**A** and **B**), pEGFP-Δ2Mtsga10 (**C** and **D**), and pEGFP-Δ3Mtsga10 Δ(**E**). Cells were examined 24 h (**A** and **C**) and 48 h (**B**, **D**, and **E**) after transfection. **A**, **B**, and **D**) Immunofluorescence images, (**C** and **E**) overlays of immunofluorescent and phase-contrast images. The bottom figure shows a schematic representation of Mtsga10, indicating the position of the putative myosin/ERM domain, and the deletion fragments tested. Note that the mutants tested fail to form filaments. **A**–**E**) Original magnification ×20.