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Mouse Odf2 cDNAs Consist of Evolutionary Conserved as Well as Highly Variable Sequences and Encode Outer Dense Fiber Proteins of the Sperm Tail

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ABSTRACT The outer dense fibers (ODF) of the mammalian sperm tail comprise a unique, specialized, and very prominent structure, consisting of nine fibers surrounding the axoneme. The ODF may play an important but as yet undefined role in sperm morphology, integrity and function. Study of the ODF is hampered by insufficient knowledge of their protein composition and the genetic regulation of their synthesis. We report here on the characterization of cDNAs encoding the Odf2 proteins of outer dense fibers of mouse sperm. We isolated two cDNA clones with variable 5′ regions. Variability in sequence is restricted to specific regions in the N-terminal part of the encoded proteins, whereas the C-terminal part is highly conserved in Odf2 proteins both between species and within a species. This variability is confirmed at the protein level. The outer dense fibers could be detected immunologically in total sperm tails allowing a direct comparison of their length in relation to the length of the sperm tail. *Odf2* transcripts could be demonstrated in testicular RNA and are restricted to germ cells. The start of transcription is in step 5 spermatids of tubular stage V and the RNA could be detected in the cytoplasm of differentiating spermatids in all subsequent tubular stages. *Mol. Reprod. Dev. 51:167–175, 1998.*

Key Words: ODF; sperm tail; postmeiotic transcription; in situ hybridization

INTRODUCTION

A prominent component of the mammalian sperm tail is a structure composed of nine fibers, the outer dense fibers (ODF). These fibers accompany the axoneme on its outer side in parallel to the microtubuli doublets and are found in the middle and principal piece of the sperm tail. The outer dense fibers represent a very prominent cytoskeletal structure, unique for the tails of spermatozoa, which is not present in cilia and flagella. Despite their probable important function in sperm integrity, motility, and durability, knowledge about their protein composition as well as their formation and their function is only rudimentary.

The outer dense fibers of mammalian sperm consist of several proteins in the molecular mass range from about 11 kDa to about 87 kDa (Baccetti et al., 1973; Price, 1973; Olson and Sammons, 1980; Vera et al., 1984; Oko, 1988; Henkel et al., 1992). Analysis of the protein composition of outer dense fibers of rat spermatozoa revealed at least 14 polypeptides (Oko, 1988). The major ODF protein has a molecular mass of about 30 kDa and a high cysteine and proline content. It is also the main zinc-binding protein of the sperm tail (Calvin, 1979).

The gene encoding this major ODF protein (*Odf1*) has been isolated from rat, man, and mouse (van der Hoorn et al., 1990; Burfeind and Hoyer-Fender, 1991; Burfeind et al., 1993; Gastmann et al., 1993; Morales et al., 1994; Hoyer-Fender et al., 1995). The amino acid sequence of the mammalian Odf1 is similar in part to the deduced amino acid sequence of the male germ cell specific transcript *Mst87F* of *Drosophila melanogaster* (Schäfer, 1986). *Mst87F* may therefore encode a structural protein of insect sperm tails. Structures similar to the outer dense fibers of mammalian sperm tails have also been described in the sperm tails of insect species (Baccetti et al., 1973). These findings point to a conserved protein composition and morphology of sperm

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tails within the animal kingdom and to an important function of the proteins involved.

In order to gain an understanding of the molecular composition and the function of the outer dense fibers we undertook the identification and characterization of the genes encoding the ODF proteins and associated proteins. Recently, a group of highly related cDNAs were identified, encoding nearly identical and previously unknown proteins of the outer dense fibers of rat sperm, designated *Odf2* (Brohmann et al., 1997; Shao et al., 1997).

In the present study we describe the identification and characterization of mouse *Odf2* cDNAs, isolated by hybridization screening of mouse testis libraries. Sequencing of the cDNA clones revealed that variable transcripts are produced in the mouse that may yield variable proteins. Antibodies to Odf2 detected a heterogeneous group of bands that migrated at ~80 kDa and localized to specific regions along the sperm tail. *Odf2* transcripts and proteins were detected only in testis among the tissues examined. Detailed analysis of *Odf2* expression revealed that it is transcribed during spermiogenesis, with the start of transcription in step 5 spermatids of tubular stage V.

MATERIALS AND METHODS

Sources of Tissues

Normal tissues were obtained from Swiss Webster mice (Charles River, Wilmington, DE). Neonatal testes were obtained from mice at postnatal days 7 and 17, and adult tissues from mice at least 35 days old. The mouse mutant strains *atrichosis* (*at*; ATEB/LE *a/a d/d+at/eb+*) and *white spotting* (*W/Wv*; WBB6F1/J-*W/Wv*) were obtained from The Jackson Laboratory (Bar Harbor, ME).

Isolation of cDNA Clones and Sequence Analyses

Screening of the mouse testis cDNA library (Stratagene) was performed by standard methods (Young and Davies, 1983). DNA probes used were the rat *Odf2* cDNA and a PCR fragment of a protein kinase. Probes were labeled with 32P-dNTP by the random hexanucleotide primer method (Feinberg and Vogelstein, 1983). Hybridization was in 5× SSC, 5× Denhardt's solution, 0.5% SDS, 0.1% Lauroylsarcosine, 100 µg/ml denatured fish sperm DNA at 65°C overnight. Posthybridization washing was performed twice in 2× SSC and once in 1× SSC, 0.1% SDS and in 0.1× SSC, 0.1% SDS at hybridization temperature. DNA sequences were determined (Sanger et al., 1977; Chen and Seeburg, 1985) using Sequenase 2.0 (Amersham Corp., Braunschweig, Germany).

Northern Blot Hybridization Analysis

RNA was prepared by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) as described previously (Rhee and Wolgemuth, 1995). Total RNA was electrophoresed in a denaturing 0.85% agarose gel containing 2.2 M formaldehyde, transferred to a nitrocellulose membrane, and hybridized with a full length *Odf2/2* cDNA riboprobe, using standard protocols (Chapman and Wolgemuth, 1993). Ethidium bromide staining of the 18S and 28S RNAs was used to determine equal loading for each sample (data not shown).

Western-Blotting

Outer dense fibers were isolated from epididymal spermatozoa as described (Vera et al., 1984). ODF proteins were separated by SDS-PAGE (Laemmli, 1970) and transferred to Hybond-C (Amersham Corp.) (Towbin et al., 1979). The membrane was blocked in 5% dry milk in TBST (10 mM Tris/HCl pH 7.6, 150 mM NaCl, 0.05 % Tween 20), and incubated with the affinity purified anti-Odf2-antiserum, diluted 1:50 in blocking solution. Bound antibodies were detected via binding of anti-rabbit-IgG-antibodies linked to horseradish peroxidase (Sigma, Deisenhofen, Germany) and chemiluminescence (ECL-Western-Blotting, Amersham Corp., and Renaissance Western-Blot Chemiluminescence Reagent, DuPont/NEN, Boston, MA).

Affinity Purification of Anti-Odf2-Antibodies

An antiserum against total outer dense fiber proteins of rat sperm were raised in rabbits by Eurogentec. Rat *Odf2* cDNA was cloned into pGEX-3X (Pharmacia). Odf2 protein, fused to glutathione-S-transferase (GST), was expressed by induction with 1mM IPTG at 37°C and proteins were separated on SDS-polyacrylamide gels (Laemmli, 1970). The antiserum was first preabsorbed with total *E. coli* proteins including GST that were immobilized on nitrocellulose sheets. The preabsorbed antiserum was then incubated with total *E. coli* proteins containing the Odf2 protein fused to GST. Elution of bound antibodies was performed as described (Weinberger et al., 1985). In brief, elution of antibodies was performed by incubation in 5 mM glycine (pH 2.3), 150 mM NaCl, 0.5 % Triton X-100, and 100 µg/ml bovine serum albumine. Binding specificity of the eluted antibodies was tested on Western-Blots containing total ODF proteins as described previously.

Immunocytochemistry

Sperm were isolated from epididymis, air-dried onto slides, and fixed in acetone. Sperm were predigested with proteinase K (2 µg/ml) at 37°C for 30 min. Antibody incubation was performed with the affinity-purified anti-Odf2-antibody, diluted 1:400, for 2 hr at RT. For detection, a second antibody linked to alkaline phosphatase and color reaction with NBT/BCIP was used.

In Situ Hybridization

Testis samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4; PBS) and embedded in paraffin. 4–5 µm sections were cut and placed on pretreated slides. Digoxigenin-labeled in vitro transcripts were generated from the rat *Odf2* cDNA (Brohmann and Hoyer-Fender, 1997) in pBluescript. In vitro

transcription was performed essentially according to the instructions (RNA Labeling Kit, Boehringer/Mannheim, Germany). Both sense and antisense transcripts were hybridized at 37°C overnight in 50 % formamide, 4× SSC (20× SSC is 3.0 M NaCl, 0.3 M trisodium citrate), 1× Denhardts (Denhardt, 1966), 5 % dextran sulfate, 0.25 mg/ml yeast tRNA and 0.5 mg/ml salmon sperm DNA. Probe concentrations were between 100 ng/ml and 500 ng/ml. Posthybridization washes were performed in 50% formamide, 2× SSC for 30 min at room temperature, in 50 % formamide, 2× SSC for 15 min at 50°C, and in 1× SSC for 15 min at room temperature. After digestion with RNase labeled transcripts were detected by incubation with an anti DIG-antibody that was linked to alkaline phosphatase, and by subsequent color reaction using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrates.

RESULTS

Cloning of Mouse Odf2 cDNAs

Sequencing of clones isolated from mouse testis cDNA libraries revealed the existence of two slightly different DNA sequences that revealed homology to rat *Odf2* (Fig. 1A). Except for several minor nucleotide changes, the nucleotide sequences and the deduced amino acid sequences are completely identical in the 3′ region (Fig. 1A). In the 5′ region, one *Odf2* cDNA clone (*Odf2/1*) contains an insertion of 57 bp, encoding an additional 19 amino acids. The two clones differ in their most 5′ sequences (Fig. 1A). The open reading frame (ORF) of *Odf2/1* extends from nucleotide 310 to 2139, encoding a protein of 610 amino acids with a putative molecular weight of 70,764 Da and an isoelectric point of 6.22. The open reading frame of *Odf2/2* starts at nucleotide position 318 and ends at position 2231. The putative protein consists of 638 amino acids and has a molecular mass of 73,402 Da, with an estimated isoelectric point of 7.2. With respect to the N-terminal amino acids, Odf2/2 has an additional sequence of 45 amino acids as compared to Odf2/1 (Fig. 1B). The ORF of Odf2/1 starts with the sequence MKG, which is present at amino acid positions 46–48 of Odf2/2. The amino acid sequences are completely identical in both proteins except for the insertion in Odf2/1 at amino acid position 18 to 36 (Fig. 1B).

The N-terminal region and the start of translation of the mouse Odf2/1 are nearly identical to the putative amino acid sequence of the rat Odf2/1 (Brohmann et al., 1997), except for the insertion found in the mouse clone (Fig. 1B). An insertion of the same size could be detected by RT-PCR with sequence specific primers in rat testis cDNA (Brohmann and Hoyer-Fender, unpublished observations). The sequence variability therefore seems to be similar in mouse and rat *Odf2* cDNAs.

Odf2 Is Transcribed in Testis

Northern blot hybridization analysis was carried out to determine the distribution of *Odf2* expression in mouse tissues (Fig. 2). *Odf2* mRNA was detected only in testis among the tissues examined (Fig. 2A). Three transcripts hybridizing to *Odf2* were noted, 4.8 kb, 2.5 kb, and 2.0 kb in size. The 2.5 kb transcript was detected most predominantly.

To determine the developmental and lineage specificity of *Odf2* expression in the testis, Northern blot hybridization analysis was used with RNA isolated from testes from germ cell–deficient or immature mice. Mice homozygous for mutations in the *atrichosis* (*at*) or *white-spotting* (*W*) loci are virtually devoid of germ cells, but have the normal complement of somatic cell types, including Leydig, Sertoli, and peritubular myoid cells (Mintz, 1957; Hummel, 1964). The heterozygous littermates of both mutant strains have the normal somatic and germ cell complements and are fertile. Specific *Odf2* transcripts were detected in intact testes (*at*/+, *W*/+ and +/+ in Fig. 2B), but not in germ cell–deficient testes (*at*/*at*, *W*/*W* in Fig. 2B), indicating that *Odf2* is expressed specifically in germ cells.

The characteristic progression of spermatogenic differentiation in the postnatal mouse testis allows for the enrichment or elimination of particular germ cell types by using testes of mice at specific days of development (Bellvé et al., 1977). For example, testes from 7-day-old mice consist largely of Sertoli cells and spermatogonia. In testes from 17-day-old mice, germ cells have entered meiotic prophase and have progressed to spermatocytes. Both d7 and d17 testes lack postmeiotic spermatids (Bellvé et al., 1977). The Northern blot hybridization analysis showed that virtually no *Odf2* transcripts were detected in d7 testis and only a low level of *Odf2* expression was detected in d17 testis in comparison to the adult testis (Fig. 2C). This expression pattern suggested that *Odf2* is expressed at highest levels in postmeiotic germ cells.

Odf2 Is Transcribed in the Haploid Phase of Spermatogenesis

To determine exactly the germ cell stages in which *Odf2* is transcribed, we performed in situ hybridizations to mouse testis sections. Hybridization with the antisense probe revealed very specific localization, depending on the stage of the spermatogenic cycle (Fig. 3A,B).Transcripts were detected only in spermatids. Transcription of *Odf2* was observed in spermatids at approximately step 5 (in tubular stage V of the spermatogenic cycle; Fig. 3A) and increased in the following stages of spermiogenesis. *Odf2* transcripts were very abundant in the cytoplasm of elongating spermatids (tubular stages X/XI; Fig. 3B). In early tubular stages *Odf2* RNA is still present in the cytoplasm of elongating spermatids but transcript level decreases. In step 14/15 spermatids of tubular stage III/IV only minor amounts of *Odf2* RNA are detected (not shown). All other testicular cells as well as the early round spermatids were negative for *Odf2* transcripts. Hybridization with the sense RNA as control yielded no signals (Fig. 4).

Odf2/1

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1       GAAACCCGAGAGGCTCCGGGGGGGGGTTTGAACTGGCCAATGGGCGAGCTGCCGACCGGTTGTCGAAAAGGAGGCGGAACGGGGAGGAGCCGCCGCGGGAGCCGGACTGCATCCGCCGCGGGCTCTC
127     CATGGCACGACCCTGGCCTCCGACTTCAACGACTTCATAAGGAGACGGTTCTGGGCGCAGCCGTGCCGCTCTTGGTTTCCATCGTGTGGGAAGAACGGAGTAACGAGTCTCACACAGAAAAAAGTCTTG
256     AGAACACCTTGTGGCGCACCCAGTGTAACTGTTACGAAATCTCATAAGCGCGGAATGAAAGGGGACACCGTGAATGTACGGCGGAGTGTCCGGGTGAAAACCAAGGTACCTTGGATGCCCCCTGGAAAA
                                                                M K G D T V N V R R S V R V K T K V P W M P P G K
385     TCATCTGCCCGGCATGTGGGATGCAAGTGGGAGAATCCACCTCATTGCCTGGAGATCACACCACCATCTTCAGAAAAGCTGGTCTCGGTGATGCGATTAAGTGACCTTTCTACAGAAGACGACGATTCA
         S S A R H V G C K W E N P P H C L E I T P P S S E K L V S V M R L S D L S T E D D S
514     GGTCACTGTAAAATGAACCGTTACGATAAGAAGATTGACAGTCTAATGAACGCGGTCGGTTGTCTCAAATCTGAGGTCAAGATGCAGAAAGGTGAACGCCAAATGGCCAAAAGGTTCCTGGAGGAGAGG
         G H C K M N R Y D K K I D S L M N A V G C L K S E V K M Q K G E R Q M A K R F L E E R
643     AAGGAAGAGCTGGAGGAGGTGGCACACGAGCTTGCAGAGACGGAGCATGAGAACACGGTGCTCAGACACAACATCGAGCGTATCAAGGAGGAGAAGGACTTCACCATGCTTCAAAAGAAACACCTCCAG
         K E E L E E V A H E L A E T E H E N T V L R H N I E R I K E E K D F T M L Q K K H L Q
772     CAGGAGAAGGAATGCCTCATGTCCAAACTAGTGGAGGCTGAAATGGATGGAGCAGCTGCTGCCAAGCAGGTCATGGCCTTGAAGGATACCATCGGGAAGCTGAAAACCGAGAAACAGATGACTTGCACC
         Q E K E C L M S K L V E A E M D G A A A A K Q V M A L K D T I G K L K T E K Q M T C T
901     GACATCAACACCTTAACAAGGCAGAAGGAACTTCTCCTGCAGAAGCTGAGTACCTTTGAGGAGACCAATCGCACCCTCCGAGATCTGTTGAGGGAACAGCACTGCAAAGAGGATTCCGAGAGACTAATG
         D I N T L T R Q K E L L L Q K L S T F E E T N R T L R D L L R E Q H C K E D S E R L M
1030    GAGCAACAAGGTACATTACTGAAGCGTCTGGCAGAGGCAGACTCAGAGAAAGCGCGCCTGCTGTTACTACTGCAAGACAAGGACAAGGAGGTGGAAGAACTCCTCCAGGAGATACAATGTGAAAAGGCT
         E Q Q G T L L K R L A E A D S E K A R L L L L L Q D K D K E V E E L L Q E I Q C E K A
1159    CAAGCAAAGACAGCGTCTGAGCTTTCCAAGTCCATGGAGTCCCATGCGGGGGCATTTGCAGGCACAGCTTCGCTGCAAAGAGGCTGAGAACAGTCGCCTGTGCATGCAGATCAAGAACTTAGAGCGCAGT
         Q A K T A S E L S K S M E S M R G H L Q A Q L R C K E A E N S R L C M Q I K N L E R S
1288    GGGAACCAGCATAAGGCGGAAGTAGAGGCCATCATGGAGCAGCTAAAGGAACTGAAGCAGAAGGGAGACCGAGACAAAGAGACCCTAAAGAAGGCCATCCGAGCCCAGAAAGAGCGAGCCGAGAAGAGT
         G N Q H K A E V E A I M E Q L K E L K Q K G D R D K E T L K K A I R A Q K E R A E K S
1417    GAGGAGTATGCCGAGCAGCTGCATGTGCAACTGGCCGACAAGGACCTTTATGTTGCTGAAGCTTTATCTACTCTGGAGTCTTGGAGGAGCCGTTACAACCAGGTTGTGAAGGACAAAGGAGATCTTGAG
         E E Y A E Q L H V Q L A D K D L Y V A E A L S T L E S W R S R Y N Q V V K D K G D L E
1546    TTGGAGATTATCGTCCTAAATGACCGGGTGACAGATCTTGTAAACCAACAACAAAGCTTGGAGGAGAAGATGCGGGAAGACCGGGACAGCTTGGTAGAGAGGCTGCACCGGCAGACGGCTGAGTACTCT
         L E I I V L N D R V T D L V N Q Q Q S L E E K M R E D R D S L V E R L H R Q T A E Y S
1675    GCATTCAAGCTAGAGAACGAGAGGCTCAAGGCTAGCTTTGCCCCTATGGAGGACAAGCTCAACCAGGCTCACTTGGAGGTTCAGCAGCTGAAGGCGTCAGTGAAGAACTACGAGGGGATGATAGACAAC
         A F K L E N E R L K A S F A P M E D K L N Q A H L E V Q Q L K A S V K N Y E G M I D N
1804    TACAAGAGCCAGGTGATGAAAACAAGATTGGAGGCTGATGAAGTGGCTGCACAGCTAGAGCGCTGCGACAAAGAGAACAAGATGCTTAAAGACGAGATGAACAAAGAGATCGAGGCGGCCCGTCGGCAG
         Y K S Q V M K T R L E A D E V A A Q L E R C D K E N K M L K D E M N K E I E A A R R Q
1933    TTCCAGTCACAGCTGGCTGACCTACAACAGCTGCCTGACATCCTCAAGATCACGGAGGCCAAGCTGGCTGAGTGCCAAGACCAGCTGCAGGGCTACGAGAGGAAGAACATCGACCTCACAGCCATCATT
         F Q S Q L A D L Q Q L P D I L K I T E A K L A E C Q D Q L Q G Y E R K N I D L T A I I
2062    TCAGACCTGCGCAGCCGGGTAAGGGACTGGCAGAAGGGATCCCACGAACTGGCCCGAGXAGGGGCCCGCTTACCGAGATGAGCTGCACGCCCCCCCAAGGGAGGACCACTTCCTTTTTCTTGGCTGCCGA
         S D L R S R V R D W Q K G S H E L A R A G A R L P R stop

2191    TTTCTGAAAGGAGTGAGCTATCATCAGTGCTGTGAAATAAAAGTCTGGTGTGCC
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Odf2/2

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1       CCGGCCGCCTCCGTCCTCTCCGGGCCATCGGCAGCGCTCTTTCCCTCCTCTGCTGTCCTCGGGCAGGGCTGCGCCCTTCTATGGGCAGAACCCCGTCCCCTCTTGCCCGCTGGACAGTT
120     GCTGTGCGACTTGGACAGTAGAGGAGCGCCTCCCAAGTTTTCATCCAACTGCCACCCCCAAAGCTTCCACCCTCCTCCCCTCAGAGAGAGGACGTTTGATGCCCGGCCCCTGAGATTCTC
240     ATTGGCCAGCCCCCTCTGGGTCCCCCTGAGCAGAGCCTGCCGACCCAATTGCCCATCTTTGCGGCTTTGATGCTTAACCATGTCTGCCTCATCCTCAGGCGGCTCCCCCAGGTTTCCATCG
                                                                M S A S S S G G S P R F P S
360     TGTGGGAAGAACGGAGTAACGAGTCTCACACAGAAAAAAGTCTTGAGAACACCTTGTGGCGCACCCAGTGTAACTGTTACGAAATCTCATAAGCGCGGAATGAAAGGGGACACCGTGAAT
         C G K N G V T S L T Q K K V L R T P C G A P S V T V T K S H K R G M K G D T V N
480     GTACGGCGGAGTGTCCGGGTGAAAACCAAGAATCCACCTCATTGCCTGGAGATCACACCACCATCTTCAGAAAAGCTGGTCTCGGTGATGCGATTAAGTGACCTTTCTACAGAAGACGAC
         V R R S V R V K T K N P P H C L E I T P P S S E K L V S V M R L S D L S T E D D
600     GATTCAGGTCACTGTAAAATGAACCGTTACGATAAGAAGATTGACAGTCTAATGAACGCGGTCGGTTGTCTCAAATCTGAGGTCAAGATGCAGAAAGGTGAACGCCAAATGGCCAAAAGG
         D S G H C K M N R Y D K K I D S L M N A V G C L K S E V K M Q K G E R Q M A K R
720     TTCCTGGAGGAGAGGAAGGAAGAGCTGGAGGAGGTGGCACACGAGCTTGCAGAGACGGAGCATGAGAACACGGTACTCAGACACAACATCGAGCGTATCAAGGAGGAGAAGGACTTCACC
         F L E E R K E E L E E V A H E L A E T E H E N T V L R H N I E R I K E E K D F T
840     ATGCTTCAAAAGAAACACCTCCAGCAGGAGAAGGAATGCCTCATGTCCAAACTAGTGGAGGCTGAAATGGATGGAGCAGCTGCTGCCAAGCAGGTCATGGCCTTGAAGGATACCATCGGG
         M L Q K K H L Q Q E K E C L M S K L V E A E M D G A A A A K Q V M A L K D T I G
960     AAGCTGAAAACCGAGAAACAGATGACTTGCACCGACATCAACACCTTAACAAGGCAGAAGGAACTTCTCCTGCAGAAGCTGAGTACCTTTGAGGAGACCAATCGCACCCTCCGAGATCTG
         K L K T E K Q M T C T D I N T L T R Q K E L L L Q K L S T F E E T N R T L R D L
1080    TTGAGGGAACAGCACTGCAAAGAGGATTCCGAGAGACTAATGGAGCAACAAGGTACATTACTGAAGCGTCTGGCAGAGGCAGACTCAGAGAAAGCGCGCCTGCTGTTACTACTGCAAGAC
         L R E Q H C K E D S E R L M E Q Q G T L L K R L A E A D S E K A R L L L L L Q D
1200    AAGGACAAGGAGGTGGAAGAACTCCTCCAGGAGATACAATGTGAAAAGGCTCAAGCAAAGACAGCGTCTGAGCTTTCCAAGTCCATGGAGTCCATGCGGGGGCATTTGCAGGCACAGCTT
         K D K E V E E L L Q E I Q C E K A Q A K T A S E L S K S M E S M R G H L Q A Q L
1320    CGCTGCAAAGAGGCTGAGAACAGTCGCCTGTGCATGCAGATCAAGAACTTAGAGCGCAGTGGGAACCAGCATAAGGCGGAAGTAGAGGCCATCATGGAGCAGCTAAAGGAACTGAAGCAG
         R C K E A E N S R L C M Q I K N L E R S G N Q H K A E V E A I M E Q L K E L K Q
1440    AAGGGAGACCGAGACAAAGAGACCCTAAAGAAGGCCATCCGAGCCCAGAAAGAGCGAGCCGAGAAGAGTGAGGAGTATGCCGAGCAGCTGCATGTCCAACTGGCCGACAAGGACCTTTAT
         K G D R D K E T L K K A I R A Q K E R A E K S E E Y A E Q L H V Q L A D K D L Y
1560    GTTGCTGAAGCTTTATCTACTCTGGAGTCTTGGAGGAGCCGTTACAACCAGGTTGTGAAGGACAAAGGAGATCTTGAGTTGGAGATTATCGTCCTAAATGACCGGGTGACAGATCTTGTA
         V A E A L S T L E S W R S R Y N Q V V K D K G D L E L E I I V L N D R V T D L V
1680    AACCAACAACAAAGCTTGGAGGAGAAGATGCGGGAAGACCGGGACAGCTTGGTAGAGAGGCTGCACCGGCAGACGGCTGAGTACTCTGCATTCAAGCTAGAGAACGAGAGGCTCAAGGCT
         N Q Q Q S L E E K M R E D R D S L V E R L H R Q T A E Y S A F K L E N E R L K A
1800    ACCTTTGCCCCTATGGAGGACAAGCTCAACCAGGCTCACTTGGAGGTTCAGCAGCTGAAGGCGTCAGTGAAGAACTACGAGGGGATGATAGACAACTACAAGAGCCAGGTGATGAAAACA
         T F A P M E D K L N Q A H L E V Q Q L K A S V K N Y E G M I D N Y K S Q V M K T
1920    AGATTGGAGGCTGATGAAGTGGCTGCACAGCTAGAGCGCTGCGACAAAGAGAACAAGATGCTTAAAGACGAGATGAACAAAGAGATCGAGGCGGCCCGTCGGCAGTTCCAGTCACAGCTG
         R L E A D E V A A Q L E R C D K E N K M L K D E M N K E I E A A R R Q F Q S Q L
2040    GCTGACCTACAACAGCTGCCTGACATCCTCAAGATCACGGAGGCCAAGCTGGCTGAGTGCCAAGACCAGCTGCAGGGCTACGAGAGGAAGAACATCGACCTCACAGCCATCATTTCAGAC
         A D L Q Q L P D I L K I T E A K L A E C Q D Q L Q G Y E R K N I D L T A I I S D
2160    CTGCGCAGCCGGGTAAGGGACTGGCAGAAGGGATCCCACGAACTGGCCCGAGCAGGGGCCCGCTTACCGAGATGAGCTGCACGCCCCCCCAAGGGAGGACCACTTCCTTTTTCTTGGCTGC
         L R S R V R D W Q K G S H E L A R A G A R L P R stop

2280    CGATTTCTGAAAGGAGTGAGCTATCATCAGTGCTGTGAAATAAAAGTCTGGTGTGCCAAAAAAAAAAAAAAAA
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A

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MmOdf2/1:   . . . . . 1M K G D T V N V8
MmOdf2/2:   1M S A S S S G G S P R F P S C G K N G V T S L T Q K K V L R T P C G A P S V T V T K S H K M K G D T V N V53
RnOdf2/1:   . . . . . 1M K G Y T V N V8

MmOdf2/1:   R R S V R V K T K V P W M P P G K S S A R H V G C K W E N P P H C L E I T P P S S E K L V S V M R L S D L61
MmOdf2/2:   R R S V R V K T K62- - - - - - - - - - - - - - - - - - - -63N P P H C L E I T P P S S E K L V S V M R L S D L88
RnOdf2/1:   R R S V R V K T K17- - - - - - - - - - - - - - - - - - - -18N P P H C L E I T P P S S E K L V S V M R L S D L42
RnOdf2/2:   . . . . . 1M S G S S S Q A Q S9

MmOdf2/1:   S T E D D D S G H C K M N R Y D K K I D S L M N A V G C L K S E V K M Q K G E R Q M A K R F L E E R K E E114
MmOdf2/2:   S T E D D D S G H C K M N R Y D K K I D S L M N A V G C L K S E V K M Q K G E R Q M A K R F L E E R K E E141
RnOdf2/1:   S T E D D D S G H C K M N R Y D E K I D S L M N A V G C L K S E V K M Q K G E R Q M A K R F L E E R K E E95
RnOdf2/2:   S P I T F L G G V T W N S G S S G T S H P F G F P F L C P L P Q V K M Q K G E R Q M A K R F L E E R K E E62
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B

Figure 1.

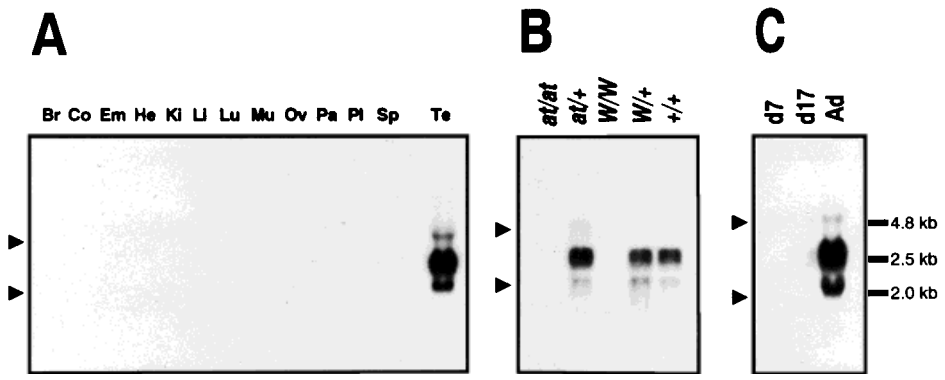


Fig. 2. Northern blot analyses of *Odf2* expression. Total RNA (10 µg) was isolated from various tissues (**A**), from testes of germ cell-deficient (*at/at, W/W*) or normal (*at/+, W/+, +/+*) mice, (**B**) or from testes of immature (d7, d17) or adult (Ad) mice (**C**). The sizes of the specific *Odf2* transcripts are indicated on the right side of the figures. Arrowheads mark positions of the 28S and 18S ribosomal RNA bands. Br, brain; Co, colon; Em, 12.5-day embryo; He, heart; Ki, kidney; Li, liver; Lu, lung; Mu, muscle; Ov, ovary; Pa, pancreas; Pl, placenta; Sp, spleen; Te, testis. Exposure of autoradiographic film: 6 hours.

Odf2 Sequence Variability Is Found at the Protein Level

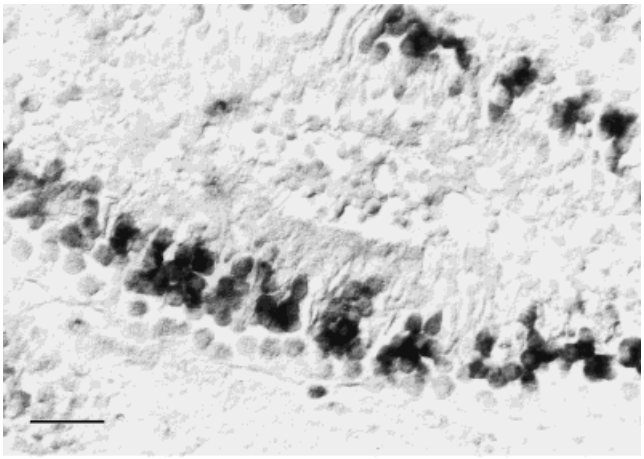
Affinity purified anti-Odf2-antibodies detect antigens exclusively in total protein extracts of mouse testis and in total ODF proteins but not in total protein extracts of mouse ovary, liver, kidney, and spleen (Fig. 5B). Moreover, the specificity of these antibodies was demonstrated by binding to bacterially expressed Odf2 protein (Fig. 5B, fp), but not to the protein extracts of bacteria containing only the plasmid vector pGEX-3X (Fig. 5B, p+). Expression of plasmid encoded proteins in both bacterial cultures were induced by IPTG (Fig. 5B, p+ and fp+). The plasmid pGEX-3X expresses GST at about 26 kDa (Fig. 5B, p+, arrowhead), which did not react with the antibodies. pGEX-3X linked to the 3′ region of *Odf2* cDNA expressed a fusion protein of about 75 kDa (Fig. 5B, fp+, arrowhead) composed of GST (26 kDa) and the C-terminal part of Odf2 with a molecular mass of about 49 kDa. Several minor proteins with lower molecular masses also react with the antibodies and are most probably degradation products of Odf2. Without IPTG induction a small amount of GST-Odf2-fusion protein was expressed (Fig. 5B, fp–). Staining of the immunoblot shown in Fig. 5A reflects the composition of proteins in the extracts of tissues as compared to total ODF proteins. The results therefore prove the specificity of the purified antibodies and demonstrate the testis-specific expression of Odf2 proteins.

Proteins of isolated outer dense fibers from rat and mouse sperm were separated on denaturing SDS-gels and incubated with affinity purified anti-Odf2-antibodies (Fig. 6). The antibody detected two or three proteins in the molecular mass range of approximately 80 kDa, which also corresponds to the main Odf2 proteins. Another protein present in relatively high amounts is in the molecular mass range of 66 kDa. As is obvious from Fig. 5B (Te), the Odf2 proteins of approximately 80 kDa can be separated into several individual protein bands using a high resolution gel. In addition to two major protein fractions, at least four minor fractions are present. For both rat and mouse, the same Odf2 pattern was obtained (Fig. 6). The protein composition of the outer dense fibers is shown in Fig. 6A. The immunoblot has been incubated with an antiserum against total outer dense fiber proteins after first incubation with anti-Odf2-antibodies. The Odf2 proteins are only a minor fraction in total outer dense fiber proteins (Brohmann et al., 1997). Figure 6A therefore does not represent the quantitative proportion of individual proteins. Instead, it demonstrates that the Odf1 protein of ~30 kDa, which is the major protein of outer dense fibers, as well as several minor proteins are not detected by the affinity purified anti-Odf2-antibodies.

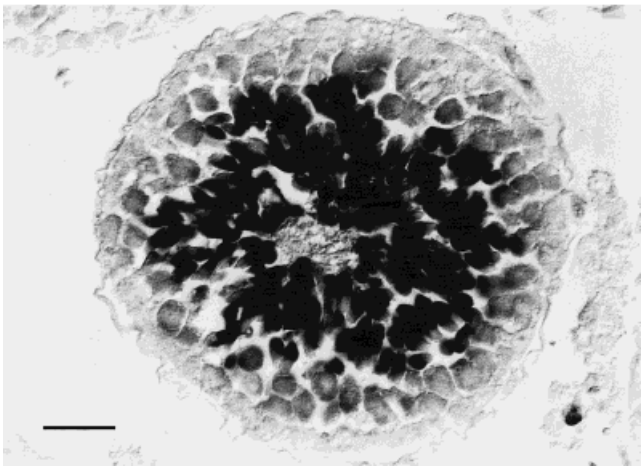
Detection of the Outer Dense Fibers in Sperm Tails

Epididymal sperm were digested with proteinase K, incubated with anti-Odf2-antibodies, and the antibodies detected by NBT/BCIP color reaction. Control experiments without the first antibody revealed no staining at all (Fig. 7d). As is evident form the dark staining in Fig.7b, the outer dense fibers are present in the middle piece of the sperm tail and extend for varying lengths into the principal piece of the sperm tails. Whereas the middle piece shows a nearly homogeneous staining, the principal piece has a more speckled appearance. The

Fig. 1. (**A**) Nucleotide sequences and deduced amino acid sequences of mouse *Odf2/1* and mouse *Odf2/2*. The nucleotide sequence of *Odf2/1* consists of 2244 bp and encodes a protein of 610 amino acids. The nucleotide sequence of *Odf2/2* consists of 2336 bp (without the A-tail) and encodes a protein of 638 amino acids. The translation initiation codons, the stop codons and the polyadenylation signals are in bold letters. (**B**) Comparison of the highly variable N-terminal amino acid sequences of the deduced Odf2 proteins of mouse and rat. For the rat only two sequences are shown (Brohmann et al., 1997). The figure demonstrates that variability is restricted to specific regions of the molecules and could be found in mouse as well as rat Odf2 proteins. Besides these variable regions all Odf2 proteins are almost completely identical especially in the C-terminal regions that are not shown in this figure.



A



B

Fig. 3. Postmeiotic transcription of *Odf2* in mouse testis. DIG-labeled *Odf2*-antisense RNA was hybridized to mouse testis sections and detected with NBT/BCIP as substrate for alkaline phosphatase that is linked to anti-DIG-antibodies. (**A**) Transcription of *Odf2* starts in step 5 round spermatids of tubular stage V. *Odf2* transcripts are detected in all subsequent tubular stages during spermatid differentiation. In the cytoplasm of elongating spermatids increasing amounts of *Odf2* transcripts are found that are clearly visible in tubular stage X/XI (in elongating spermatids of step 10/11) (**B**). The definition of the tubular stages follows that given by Oakberg (1956) and Russell et al. (1990) and depends solely on the overall appearance of the tubules. The bars represent 31 μm.

border between the middle piece and the principal piece, termed the annulus, is clearly visible as a constriction in phase contrast (Fig. 7a and b, arrow and arrowhead). A comparison of the staining of the sperm tails of mouse and rat revealed that the outer dense fibers of rat sperm detected by these antibodies extend over the greatest part of the principal piece, whereas the outer dense fibers of mouse sperm are essentially shorter. The results demonstrate that detection of cytoskeletal structures inside the whole sperm tail is clearly possible by immunological staining and permits

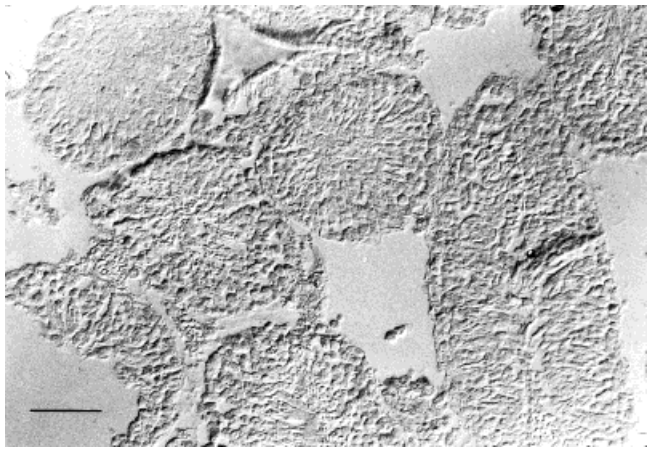


Fig. 4. Control in situ hybridization. DIG-UTP labeled *Odf2*-sense transcripts were hybridized to paraffin-embedded mouse testis sections. DIG-transcripts were detected via anti-DIG-antibodies linked to alkaline phosphatase and color reaction with NBT/BCIP. The bar represents 77 μm.

a direct comparison of different structures in a variety of species.

DISCUSSION

The main function of the differentiated male germ cell is the transport of the genetic material to the oocyte. For this purpose the sperm cells possess a specialized tail that consists of general as well as unique cytoskeletal structures. These unique structures that are not present in other flagella and cilia are the outer dense fibers (ODF), which surround the axoneme, and the fibrous sheath (FS), which surrounds the ODF in the principal piece of the sperm tail. Anteriorly, the ODF make close contact with the paracentriolar connecting piece and extend posteriorly for varying lengths into the principal piece. At the border between the middle piece and the principal piece of the sperm tail, the annulus, which is cytologically visible, two of the nine ODF terminate abruptly and are replaced by the two longitudinal columns of the fibrous sheath (Fawcett, 1975). Since no ATPase activity could be demonstrated in isolated ODF proteins, these fibers seem not to contribute to the active motility but instead may maintain the passive elastic structure and elastic recoil of the sperm tail during epididymal transport and ejaculation (Baltz et al., 1990).

Both structures, the ODF as well as the FS, consist of multiple proteins and are highly insoluble due to cross-linking via disulfide bonds (Calvin and Bleau, 1974; Olson et al., 1976; Calvin, 1979; Olson and Sammons, 1980; Vera et al., 1984; Oko, 1988). Both structures may play an important but as yet undefined role in sperm morphology, integrity, and function. The study of the protein composition of these structures as well as of their individual proteins is a first step toward understanding their function.

To date, only two genes encoding ODF proteins have been cloned. cDNAs corresponding to the major ODF

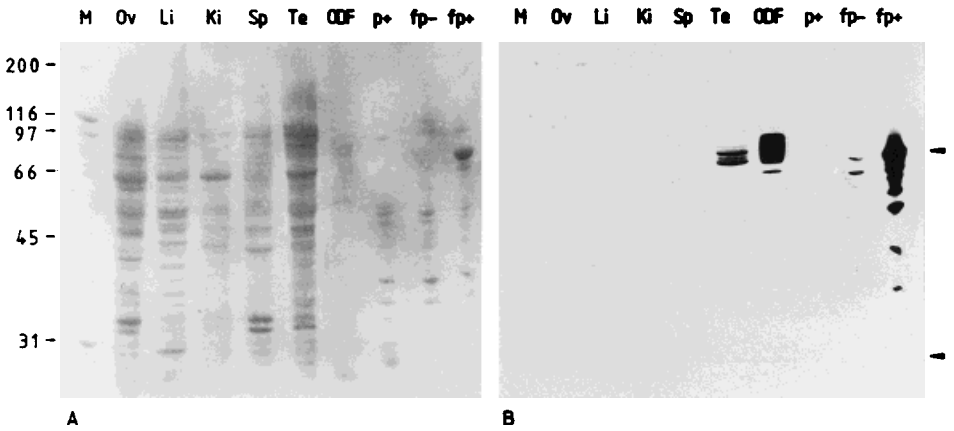


Fig. 5. Anti-Odf2-antibodies react specifically with Odf2 proteins that are expressed exclusively in testis. Total proteins were isolated from various mouse tissues (Ov, ovary; Li, liver; Ki, kidney; Sp, spleen; Te, testis), from isolated outer dense fibers of mouse spermatozoa (ODF), from bacteria transformed with the GST expression vector pGEX-3X and induced with IPTG (p+), and from bacteria transformed with pGEX-3X linked to the 3′ region of *Odf2* cDNA that encodes a GST-Odf2-fusion protein, without (fp–) and with (fp+) IPTG-induction. Proteins were separated on a denaturing SDS-gel, transferred to Hybond-C, and incubated with the affinity purified anti-Odf2-antibodies (**B**). Detection of bound antibodies was performed by chemiluminescence (Renaissance, DuPont/NEN). Staining of the blot with amido black is shown in (**A**). The molecular weights (in kDa) of the marker proteins (M) are indicated on the left.

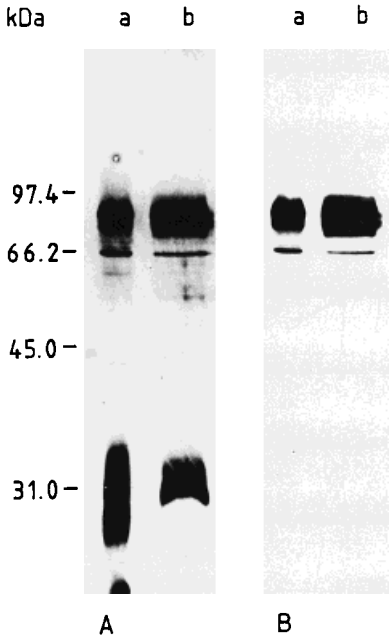


Fig. 6. Rat and mouse ODF exhibit the same pattern of proteins encoded by *Odf2*. Total proteins of isolated outer dense fibers of rat (a) and mouse (b) sperm were separated on a denaturing SDS-gel, transferred to Hybond and incubated with antibodies directed against total ODF proteins (**A**) following first incubation with affinity-purified anti-Odf2-antibodies (**B**). Antibodies were detected by chemiluminescence via secondary antibodies linked to peroxidase and Renaissance chemiluminescence detection (DuPont/NEN).

protein of about 30 kDa (*Odf1*) have been isolated from rat (Van der Hoorn et al., 1990; Burfeind and Hoyer-Fender, 1991; Morales et al., 1994), mouse (Hoyer-Fender et al., 1995), man (Gastmann et al., 1993), boar, and bull (Kim et al., 1995). Their protein sequences display greater than 80% similarity. A second class of cDNA has been isolated from rat and reported to encode ODF proteins of approximately 80 kDa (*Odf2*; Brohmann et al., 1997; Shao et al.,1997). It was shown that both ODF proteins can interact with each other (Shao et al., 1997). The Odf2 proteins have further been shown to be heterogeneous (Brohmann et al., 1997).

To address the issue of evolutionary conservation of the ODF proteins among species, we have compared the composition of Odf2 between mouse and rat. Sequence analysis of the mouse *Odf2* cDNA clones revealed that there exists a variability in sequence but that *Odf2* is nevertheless highly conserved between species.

The *Odf2* sequences can be subdivided into two parts: the very highly conserved 3′ region and the 5′ region that exhibits sequence variability. The C-terminal sequences not depicted in Fig. 1B are nearly completely identical in all Odf2 proteins of the mouse (Fig. 1A) and the rat (Brohmann et al., 1997). An exception is an insertion of 23 amino acids found only in some rat Odf2 proteins (Brohmann et al., 1997). The conserved C-terminal region contains the two leucine zipper motifs that are required for interaction with Odf1 (Shao et al., 1997). Sequence variability of the 5′ region yields (1) differences in the putative lengths of the N-terminal parts, (2) differences in the sequences downstream of the translation start, and (3) small insertions or deletions in the 5′ part. The variable N-terminal regions of the mouse Odf2 proteins and the rat Odf2 proteins are compared in Fig. 1B. An insertion of similar size and in the same region of the molecule as in MmOdf2/1 was also found in cDNA of rat testis (Brohmann and Hoyer-Fender, unpublished observations). It is not known whether the variability of the 5′ region results from alternative splicing or from transcription from different genes, although genomic Southern blot hybridizations do not indicate the presence of multiple *Odf2* genes (Brohmann et al., 1997).

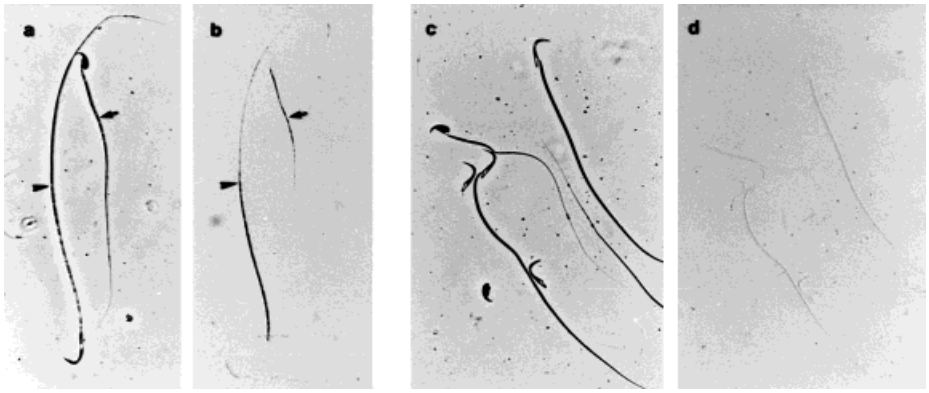


Fig. 7. Immunological detection of outer dense fibers in total sperm tails. Rat and mouse sperm were digested with proteinase, incubated with anti-Odf2-antibodies and bound antibodies were detected by NBT/BCIP color reaction as substrate for alkaline phosphatase that is linked to the secondary antibody (**a, b**). The control is shown in **c** and **d** where the incubation with the first antibody was omitted. a and c, phase contrast; b and d, bright-field microscopy. The annulus is marked (arrowhead in rat sperm, arrow in mouse sperm).

The heterogeneity in the Odf2 proteins suggested at the mRNA level was also observed at the protein level, where at least three proteins reacted with affinity purified anti-Odf2-antibodies. In both rodent species, proteins in the same molecular mass range were detected. In addition to the sequence variability of the N-terminal regions, heterogeneity of Odf2 proteins may reflect posttranslational modifications although phosphorylation has not been detected (not shown).

The Odf2 proteins could be detected in the tails of whole sperm after digestion with proteinase, which destroys the overlying structures and makes the antigens accessible for antibody recognition. The midpieces of the sperm tails were intensely but not completely uniformly stained. Since the axoneme and the accompanying outer dense fibers are in the midpiece surrounded by the mitochondrial sheath, the staining pattern may reflect an incomplete digestion and therefore a nonuniform accessibility of antigens. Alternatively, the punctuate staining pattern seen in the principal piece may not reflect nonuniform accessibility but instead could be caused by an overdigestion of antigens. Most probably it reflects the reduced quantity of ODF proteins in this part of the sperm tail due to the termination of these fibers. Comparison of the sperm tails of mouse and rat revealed that not only are the whole tails of rat sperm are longer than those of the mouse but that the midpiece as well as the ODF, which were decorated by the antibodies, are essentially longer in rat sperm. This observation is in agreement with the proposed function of ODF in the protection of sperm tails against shearing forces (Baltz et al., 1990). Both the diameter as well as the length of the fibers contribute to their tensile strength. The ODF are therefore likely an essential component for successful fertilization.

Whereas for a long time diagnosis of human male infertility has focused on malformations of sperm heads, attention has been given more recently to the significance of sperm tail abnormalities with respect to sperm motility. In this respect, the composition of ODF and the amino acid sequences of their individual proteins has to be taken into consideration in elucidation of the molecular reasons for male infertility.

ACKNOWLEDGMENTS

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