

Olfactory Receptors Are Displayed on Dog Mature Sperm Cells

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Abstract. Olfactory receptors constitute a huge family of structurally related G protein-coupled receptors, with up to a thousand members expected. We have shown previously that genes belonging to this family were expressed in the male germ line from both dog and human. The functional significance of this unexpected site of expression was further investigated in the present study. We demonstrate that a few dog genes representative of various subfamilies of olfactory receptors are expressed essentially in testis, with little or no expression in olfactory mucosa. Other randomly selected members of the family show the expected site of expression, restricted to the olfactory system. Antibodies were generated against the deduced amino acid sequence of the most abundantly expressed olfactory receptor gene in dog testis. The purified serum was able to detect the gene product

(DTMT receptor) in late round and elongated spermatids, as well as in the cytoplasmic droplet that characterizes the maturation of dog sperm cells, and on the tail midpiece of mature spermatozoa. Western blotting further confirmed the presence of a 40-kD immunoreactive protein in the membrane of mature sperm cells. Altogether, these results demonstrate that the main expression site of a subset of the large olfactory receptor gene family is not olfactory mucosa but testis. This expression correlates with the presence of the corresponding protein during sperm cell maturation, and on mature sperm cells. The pattern of expression is consistent with a role as sensor for unidentified chemicals possibly involved in the control of mammalian sperm maturation, migration, and/or fertilization.

OLFACTORY receptors constitute the largest subfamily of G protein-coupled receptors with up to a thousand members expected in mammalian species. This diversity represents the molecular basis allowing the olfactory system to discriminate among thousands of odorants. Signal transduction in olfactory receptor cells is known to involve either adenylyl cyclase (Pace et al., 1985; Sklar et al., 1986; Bakalyar and Reed, 1990) (through the specific G protein G_{olf} , Jones and Reed, 1989) and a cyclic nucleotide-gated ion channel (Dhalan et al., 1990; Ludwig et al., 1990), or phospholipase C (Huque and Bruch, 1986; Boekhoff et al., 1990; Ronnett et al., 1993). Putative olfactory receptor genes have been cloned recently from rat (Buck and Axel, 1991), human (Parmentier et al., 1992; Selbie et al., 1992), dog (Parmentier et al., 1992), mouse (Ressler et al., 1993), and catfish (Ngai et al., 1993a). Functional expression was only achieved for rat receptors in the baculovirus Sf9 cell system, showing coupling to the IP_3/Ca^{2+} cascade (Raming et al., 1993). All receptors cloned so far display common sequence characteristics that make them clearly belong to a common subfamily of G protein-coupled receptors. From a structural viewpoint, they can be divided into subgroups, but the correlation of this structural classification with the func-

tional interaction of the receptors with specific classes of odorants and/or with different intracellular cascades has not been demonstrated. The distribution of transcripts corresponding to cloned receptor genes has been studied in mouse (Ressler et al., 1993) and catfish (Ngai et al., 1993b) olfactory epithelium, demonstrating that individual receptor genes are expressed in a relatively small number of cells distributed in the epithelium, according to a pattern reflecting most probably a functional specialization. Each olfactory neuron is believed to express a single (or a small number of) olfactory receptor gene(s).

We reported previously that transcripts encoding putative olfactory receptors were present in the male germ line, particularly in spermatocytes and spermatids (Parmentier et al., 1992). The olfactory receptor genes expressed in the male germ line do not constitute a structural cluster within the olfactory family. On the contrary, they belong to quite distantly related subgroups. Although the presence of the corresponding proteins was not demonstrated, this finding led to the hypothesis that members of the olfactory receptor family could be implicated in sperm chemotaxis during fertilization. Sperm chemotaxis has been demonstrated in many invertebrate species (Yoshida et al., 1993; Ward and Kopf, 1993), and was extensively studied at the molecular level in sea urchin (Garbers, 1989; Ward and Kopf, 1993). Experimental evidence for a similar phenomenon in protochordates and vertebrate species has been provided recently

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(Villanueva-Diaz et al., 1990; Ralt et al., 1991; Yanamigashi et al., 1992; Yoshida et al., 1993). Since the involvement of olfactory receptors in sperm-egg interactions would have direct implications in the fields of fertility and contraception, we decided to explore further the physiological relevance of our findings. Two questions were addressed in the present study. First, we determined whether individual olfactory receptor genes are expressed in both olfactory mucosa and germ line, or if their expression is mutually exclusive. Second, we generated antibodies directed against the recombinant protein encoded by the major olfactory receptor transcript found in dog testis (DTMT) and determined the developmental pattern of expression of this receptor during spermatogenesis and sperm maturation. Our results indicate that a few members of the olfactory receptor gene family are preferentially expressed in dog testis, with little or no expression in olfactory mucosa. Antibodies directed against the protein encoded by one of these testis-expressed genes allowed detection of the receptor during the latest stages of germ cell maturation, and on mature sperm cells. This distribution pattern is consistent with a role as sensor for unidentified chemical signals potentially implicated in the regulation of sperm maturation, migration, and/or fertilization.

Materials and Methods

RNase Protection Assay

Olfactory receptor gene fragments obtained by reverse PCR from dog testis (DTMT, DTPCRH02, and DTPCRH09) and olfactory mucosa (DOPCRH02 and DOPCRH07) (Parmentier et al., 1992) were subcloned in pBluescript SK+ (Stratagene, La Jolla, CA) and antisense radiolabeled probes were obtained by *in vitro* transcription as described (Sambrook et al., 1988). Total RNA was prepared by the guanidinium thiocyanate/cesium chloride gradient method (Sambrook et al., 1988). RNase protection assays (RPA)¹ were performed as described (Sambrook et al., 1988) with RNA (10–50 μ g) from dog testis, olfactory mucosa, and liver. For the collection of olfactory mucosa, care was taken to harvest all olfactory tissue from each animal. RNA samples were hybridized with 1–2 ng of the ³²P-labeled probe (0.5–5 \times 10⁵ cpm), for 15 h at 48°C. Free probe was further digested with 40 μ g/ml RNase A and 2 μ g/ml RNase T1 and protected fragments were run on a 6% acrylamide/7 M urea sequencing gel.

E. coli Expression of DTMT Receptor Fragments

cDNA fragments encoding peptides corresponding to the NH₂-terminal extracellular domain (NDT: amino acids 2–26), the second extracellular loop (EDT: amino acids 164–195), and the COOH-terminal intracellular domain (CDT: amino acids 289–313) of DTMT were generated by PCR (see Fig. 1 A). These fragments, each of which terminated by a stop codon, were inserted into the polylinker of the pMALcR1 vector (New England Biolabs, Beverly, MA), downstream of the *malE* gene, which encodes maltose-binding protein (MBP), resulting in the synthesis of MBP fusion proteins (Fig. 1 B). TBI cells were transformed with pMALcR1, pMALcR1NDT, pMALcR1EDT, and pMALcR1CDT, and the insert of the selected transformants was verified by sequencing. Production and purification of the fusion proteins was performed as described by the manufacturer (New England Biolabs) with the following modifications. Cells were grown in SOB medium, and, when the OD (600 nm) reached 0.3, fusion protein synthesis was induced by the addition of isopropylthiogalactoside (IPTG, 1 mM final concentration). After 4 h at 37°C, cells were harvested and resuspended in lysis buffer (20 mM Tris.HCl pH 7.4, 1 mM EDTA, 200 mM NaCl). The cell suspension was frozen overnight at –20°C, thawed in cold water, and digested with lizozyme (1 mg/ml) for 30 min at 0°C. After sonication, the

1. *Abbreviations used in this paper:* MBP, maltose-binding protein; PAF, paraformaldehyde; PAP, peroxidase anti-peroxidase; RPA, RNase protection assays.

lysate was centrifuged at 9,000 *g* for 30 min. The supernatant was passed several times through a 21-gauge syringe needle and loaded on a 25-ml amylose column (New England Biolabs). After extensive washing, the fusion protein was eluted with lysis buffer containing 10 mM maltose. Protein content was measured with the Bradford method (Bradford, 1976). Samples from uninduced and induced cells, transformed with recombinant or wild-type pMALcR1 vectors as well as samples from each purification step were analyzed by SDS-PAGE according to standard methods (Laemmli, 1970).

Rabbit Immunization, Immunoglobulin Purification, and Elisa

Each purified fusion protein (pMal-NDT, pMal-EDT, pMal-CDT, 0.2 mg) was injected intradermally to three rabbits with complete Freund adjuvant. Two booster injections were given 2 and 6 wk later (0.2 mg per rabbit with incomplete Freund adjuvant) and blood was collected 2 wk later. A 2-ml sample from each of the nine sera (NI-3, EI-3, CI-3) were sequentially affinity purified according to standard protocols (Harlow and Lane, 1988). Briefly, anti MBP antibodies were first removed from the sera by passing them several times through a column of an irrelevant MBP fusion protein coupled to CNBr Sepharose (Pharmacia, Brussels). The flow-through was then loaded on a column containing the specific fusion protein. After extensive washing, antibodies were eluted with 100 mM glycine pH 2.5, neutralized immediately, and dialyzed against PBS. A volume of 10 ml was recovered after dialysis, corresponding to a fivefold dilution of the crude sera. All dilutions of the purified sera reported in the text have been modified accordingly in order to represent equivalent crude sera dilutions. The activity and specificity of each preimmune, immune, and purified serum were analyzed by ELISA, performed as described (Coligan et al., 1992). Briefly, microwells coated with the purified fusion protein were successively incubated with the various sera and alkaline phosphatase-labeled goat anti-rabbit antibodies (Promega Corp., Madison, WI). The substrate *p*-nitrophenyl phosphate was added (1 mg/ml in 10% diethanolamine, 1 mM MgCl₂, pH 9.8) and absorbance read at 405 nm in a multiscan spectrophotometer.

Extraction of Sperm Proteins, Western Blotting

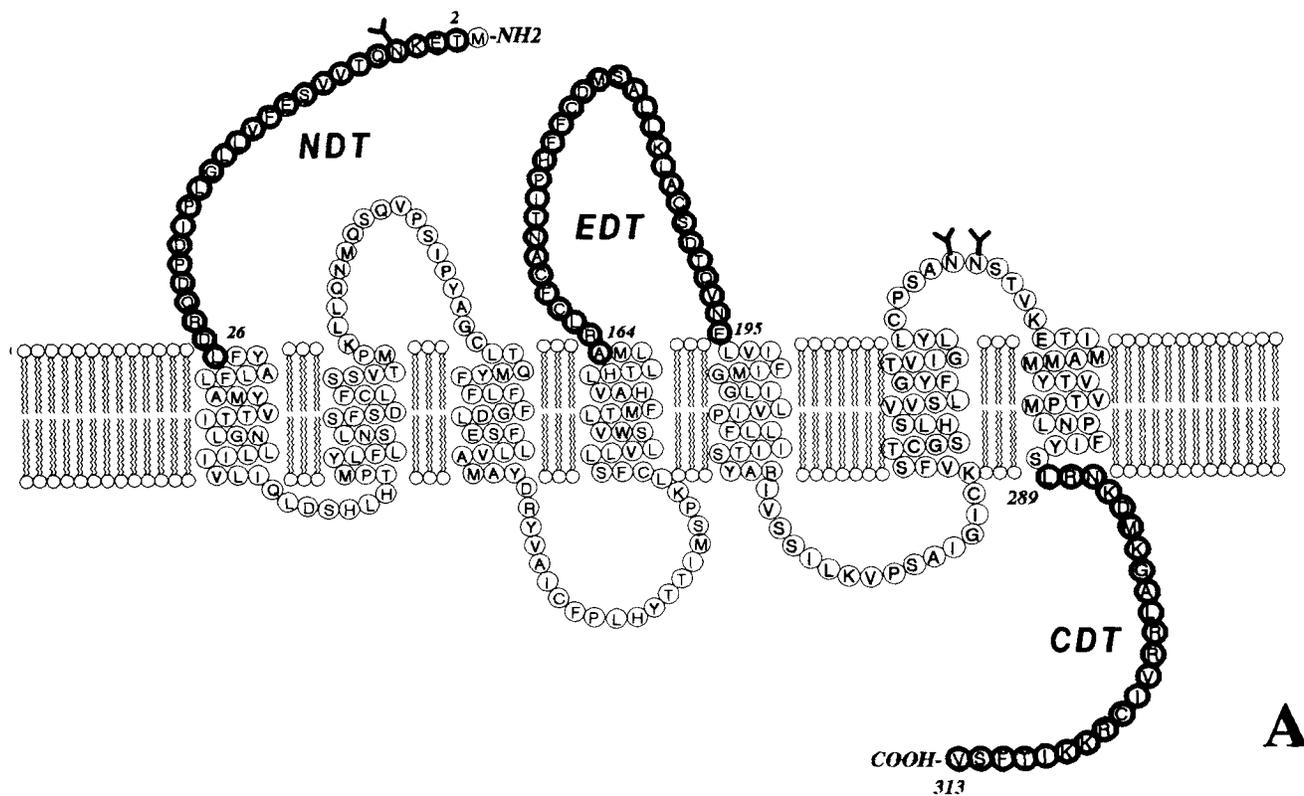
Membrane proteins were prepared from sperm cells with a procedure modified from Arboleda and Gerton (1988) and Snow and Ball (1992). Spermatozoa from a single dog ejaculate (2–4 ml semen) were washed twice with TBS (50 mM Tris.Cl pH 7.5, 150 mM NaCl), resuspended in 20 mM Tris.Cl pH 7.6 containing 1% Triton X-100, 1% deoxycholate, 50 mM NaCl, 1 mM EDTA, 5 μ g/ml leupeptin and 1 mM PMSF, left on ice for 40 min, and homogenized in a glass/glass homogenizer. The suspension was centrifuged at 20,000 *g* for 30 min, and the protein content of the supernatant was determined with the Bradford assay (Bradford, 1976). Samples were separated on a 10% SDS-PAGE gel and proteins were electrophoretically transferred to nitrocellulose membranes (Towbin et al., 1979). Blots were incubated for 1 h at room temperature in TBS supplemented with 0.05% Tween 20, 50% nonfat dry milk and 0.02% Na azide, then with the purified antibody, overnight at 4°C. Blots were further incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega) for 1 h at room temperature, and revelation was performed with Nitroblue tetrazolium (NBT) and 5-Bromo 4-Chloro 3-Indolyl Phosphate (BCIP) as substrates (Promega). Controls of immunodetection included incubation with preimmune sera, preincubation of the immune sera with an excess of the corresponding fusion protein (at a micromolar concentration) or preincubation with an unrelated fusion protein.

Transient Expression of DTMT Receptor in Cos-7 Cells

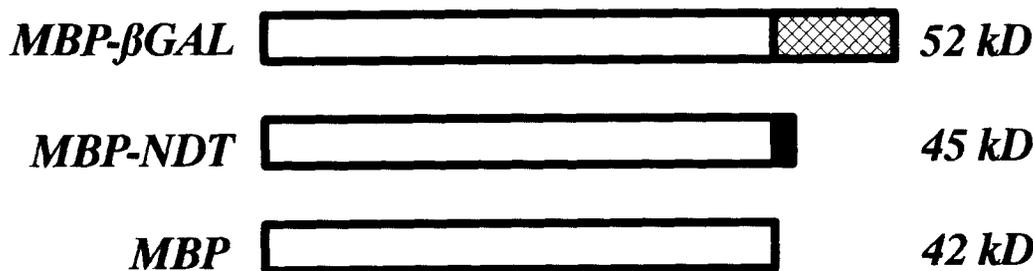
The DTMT full-length coding sequence was inserted into the polylinker of the pSVL expression vector (Pharmacia, Brussels). Cos-7 cells (2 \times 10⁷/ml) were electroporated (320 V, 500 μ F) as described (Callis et al., 1987; Chu et al., 1987) with pSVL-DTMT (40 μ g/ml), or with pSVL-hTSHr (encoding the human thyrotropin receptor, Libert et al., 1989) as a control, and grown on coverslips for 72 h in DME medium.

Tissue Sections/Cell Smears/Immunohistochemistry

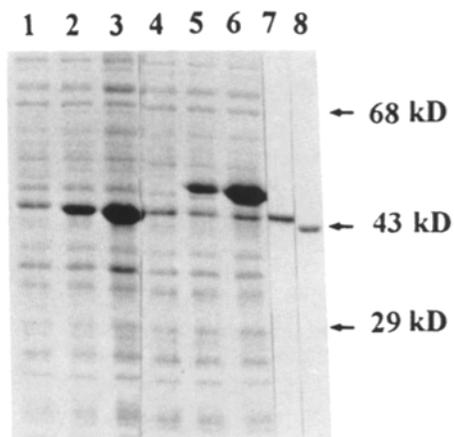
Dog olfactory mucosa, testis, ovary, and brain (cortex and striatum) were dissected and fixed overnight at 4°C in 4% paraformaldehyde (PAF). Tissues were then sequentially transferred to 10, 20, and 30% sucrose solutions at 4°C, and frozen at –80° in isopentane. Cryosections (15 μ m) were taken



A



B



C

Figure 1. *E. coli* expression of DTMT receptor fragments as MBP fusion proteins. (A) Schematic representation of the putative transmembrane organization of the DTMT receptor. The three peptides chosen to be produced as MBP fusion proteins are shown as bold circles (*NDT*, *EDT*, and *CDT*). The three potential sites for N-linked glycosylation are indicated. (B) Schematic structure of the recombinant MBP-NDT fusion protein, as compared with the pMALcR1 encoded fusion protein (MBP-βGAL) and wild-type MBP. Expected molecular weights are shown on the right. (C) Coomassie blue staining of a 10% SDS-PAGE gel showing induction and purification of the MBP-NDT fusion protein. *E. coli* strains recombinant for pMALcRINDT (lanes 1-3) or wild-type pMALcR1 (lanes 4-6) were grown in absence (lanes 1 and 4) or presence of IPTG for 1 (lanes 2 and 5) or 3 h (lanes 3 and 6), showing induction of fusion proteins with the expected molecular weights. After affinity purification on an amylose column, the purified fusion protein was obtained, corresponding to a single band (lane 7), well distinct from wild-type MBP (lane 8). Positions of the molecular size markers are indicated on the right.

up on poly-L-Lysine-coated slides. Coverslips covered with transfected COS-7 cells were rinsed twice with PBS and fixed for 15 min at 4°C with 4% PAF. Epididymal sperm cells obtained by squeezing the epididymis tail, or ejaculated sperm cells, were washed twice with PBS, resuspended at a final concentration of 10⁶ cells/ml, and smeared on poly-L-Lysine-coated slides. After 15 min, the slides were washed with PBS, fixed in 3% PAF for 15 min at 4°C, and washed again twice.

Peroxidase anti-peroxidase (PAP) immunostaining was performed on the various preparations as previously described (Sternberger et al., 1970). Endogenous peroxidases were inactivated by a 15-min incubation in 0.2% (2% for Cos-7 cells) hydrogen peroxide, and cells were permeabilized with 0.1% Triton X-100 (1% for Cos-7 cells). Preparations were incubated overnight in primary antibodies, then in swine anti-rabbit IgG and rabbit PAP (DAKOPATTS, Copenhagen) (30 min, each). 3,3'-diaminobenzidine was used as chromogenic substrate.

Immunogold staining followed by silver enhancement was performed with slight modifications of previously described protocols (Holgate et al., 1983; Springall et al., 1984). Sections were incubated for 1 h in solution 1 (50 mM Tris.Cl pH 7.4, 300 mM NaCl, 0.1% Triton X-100, 0.8% BSA, 0.1% gelatin) containing 20% normal goat serum, and overnight at room temperature with primary antibodies. They were further washed twice in washing solution 1 and twice in washing solution 2 (50 mM Tris.Cl pH 8.2, 150 mM NaCl, 0.8% BSA, 0.1% gelatin), and incubated for 4 h with gold-labeled goat anti-rabbit IgG (AuroProbe One GAR, Amersham, Buckinghamshire), diluted 1:50 in solution 2. Sections were washed twice in solution 2, three times in 50 mM Tris.Cl pH 8.2, 50 mM NaCl, and five times in bidistilled water. Silver enhancement (IntensEM, Amersham, Buckinghamshire) was performed according to the manufacturer's instructions and revelation was monitored under the microscope. Sperm cells were lightly counterstained with hematoxylin before mounting. Immunohistochemical controls included incubation with the preimmune serum, and preadsorption of the purified immune serum with either the corresponding immunogenic fusion protein or with an unrelated MBP fusion protein (both at a micromolar concentration).

Results

RNase Protection Assay Analysis

We investigated the expression level of specific members of the olfactory receptor gene family in both olfactory mucosa and testis. Given the relatively low sensitivity, and the lack of absolute specificity of Northern blotting (the olfactory receptor family includes closely related genes (Buck and Axel, 1991; Parmentier et al., 1992), this question was addressed by RNase protection assay, using five dog receptor genes. Two of these (DOPCRH02 and DOPCRH07) were initially obtained by reverse PCR amplification from olfactory mucosa RNA, while three others (DTMT, DTPCRH02, and DTPCRH09) were amplified from dog testis (Parmentier et al., 1992). RNA probes (in the range of 300 bp) were used to detect specific transcripts in dog RNA from olfactory mucosa and testis, and from liver and yeast tRNA as negative controls. With the two olfactory mucosa-derived probes, a protected fragment was detected only in olfactory mucosa, no signal being obtained in the testis (Fig. 2). Each of the three testis-derived probes however detected a strong signal in the testis (Fig. 2). The protected band was particularly strong for the DTMT probe, confirming the relative abundance of the corresponding transcripts in this organ, as previously reported by Northern blotting experiments (Parmentier et al., 1992). For two of these probes (DTMT and DTPCRH02), a weak signal was also obtained in the olfactory mucosa, while the third receptor (DTPCRH09) turned out to be strictly testis specific (Fig. 2). For all probes, controls including liver RNA and yeast tRNA were negative. With one of the testicular probes (DTPCRH02), three distinct protected fragments were obtained with the olfactory

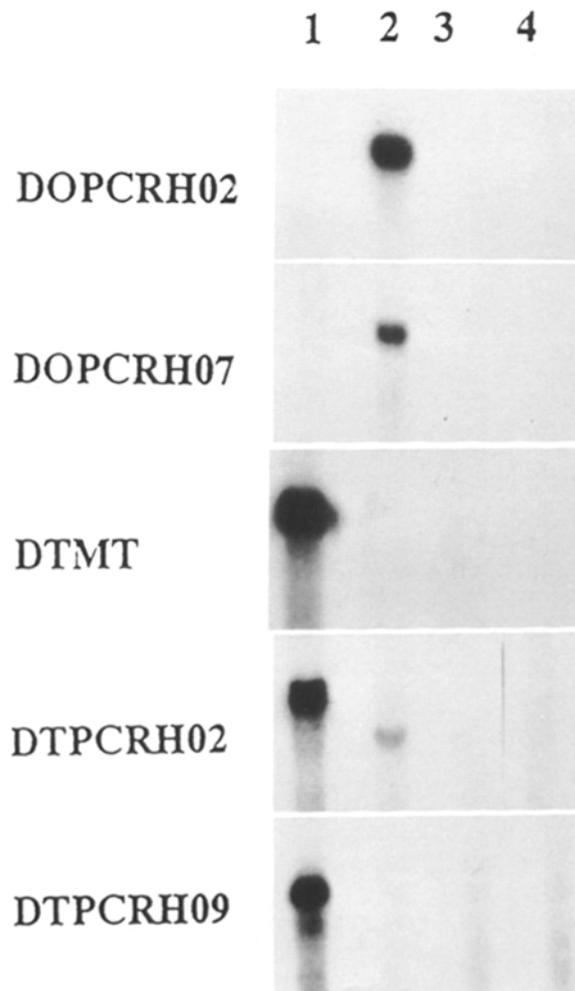


Figure 2. Expression of olfactory receptor genes as determined by RNase Protection Assay. Total RNA (20 μ g) from dog testis (lane 1), olfactory mucosa (lane 2), liver (lane 3), and yeast tRNA (lane 4), were hybridized with radiolabeled RNA probes corresponding to olfactory receptor genes cloned from either dog olfactory mucosa (DOPCRH02 and DOPCRH07), or testis (DTMT, DTPCRH02, and DTPCRH09). Note the selective expression of DOPCRH02, DOPCRH07, and DTPCRH09, and strong expression of DTMT in testis, and the three protected fragments obtained with DTPCRH02 on olfactory mucosa.

mucosa RNA, possibly reflecting a sequence polymorphism of this receptor gene (the mucosa of six dogs were pooled) or the existence of multiple genes with nearly identical sequences.

Production of Antibodies Directed against the DTMT Receptor

PCR amplification and Northern blotting analysis identified DTMT as the major transcript found in the testis (Parmentier et al., 1992). RNase protection assays confirmed the abundance of DTMT transcripts in the testis (see above). This prompted us to select the DTMT gene in order to assay the presence of the corresponding protein in the male germ line. The amino acid sequence of olfactory receptors is highly hydrophobic, most of the extra- and intracellular loops being short, as compared to other G protein-coupled receptors.

Three peptides representing the most hydrophilic regions of DTMT were selected, and expressed in *E. coli* as fusion proteins with the MBP. These three peptides correspond to the NDT (including a putative N-linked glycosylation site), the EDT, and the CDT. The limits and location of these peptides in the DTMT structure are given in Fig. 1, together with the structure of the fusion proteins.

When *E. coli* strains recombinant for one of the constructs (pMALcRINDT, pMALcRIEDT, or pMALcRICDT) were grown in presence of IPTG, a 44–45 kD protein was induced, representing the major protein of the soluble fraction (Fig. 1). This size corresponds to the predicted molecular weight of the fusion proteins, and clearly differs from the molecular weights expected for MBP (42 kD) and for the MBP- β GAL fusion protein, encoded by the wild-type pMALcR1 vector (52 kD) (Fig. 1). After affinity purification on amylose column, ~20 mg of each fusion protein were obtained from 1 l of culture, corresponding to a single band on SDS-PAGE.

The three purified fusion proteins were used as immunogens for the productions of antibodies directed against the DTMT-encoded receptor. When screened by ELISA, all immune sera, in contrast to the corresponding preimmune sera, showed reactivity toward their respective immunogenic fusion protein at dilutions ranging from 1:10⁴ to 1:10⁶. Affinity purified sera (called N1-3G, E1-3G, C1-3G), still showed reactivity toward their corresponding immunogens, whereas all reactivity toward the MBP- β GAL fusion protein was abolished. One of the purified antibodies, N1G, showed stronger reactivity toward its immunogen at dilutions up to 1:5 \times 10⁵.

Immunohistochemistry

From the nine purified immune sera tested in immunohistochemistry, only one, N1G, directed against the NH₂-terminal extracellular domain of DTMT, gave positive results. The corresponding unpurified immune serum, N1, gave similar results but with unsatisfactory background. As a positive control, the N1G purified antibody was assayed on COS-7 cells transfected with the recombinant expression plasmid pSVL-DTMT. PAP immunostaining resulted in the strong labeling of a small percentage (estimated to 5%) of the cells (Fig. 3 A). The staining appeared heterogenous, involving both the plasma membranes and granular structures in the cytoplasm. COS-7 cells transfected with the pSVL-hTSHR plasmid used as a negative control showing no staining at all (Fig. 3 B). pSVL-DTMT transfected cells, incubated with either the preimmune serum or N1G preincubated with the immunogenic fusion protein (pMal-NDT), revealed no staining (Fig. 3 C). The staining was however conserved when the antibody was incubated with an irrelevant fusion protein (pMal-CDT) (not shown).

PAP immunostaining was performed on testis and epididymis cryosections with the N1G purified antibody (Figs. 5 and 6). It revealed the selective staining of one type of cells in the testis seminiferous tubules (Fig. 4 A): these cells, located next to the lumen, presented a condensed birefringent nucleus, a large cytoplasm, and an emerging flagellum, and corresponded therefore to late stages of round spermatid maturation. The staining was cytoplasmic and heterogeneous with a granular appearance. Nuclei were negative and the plasma membrane did not appear as the main immunoposi-

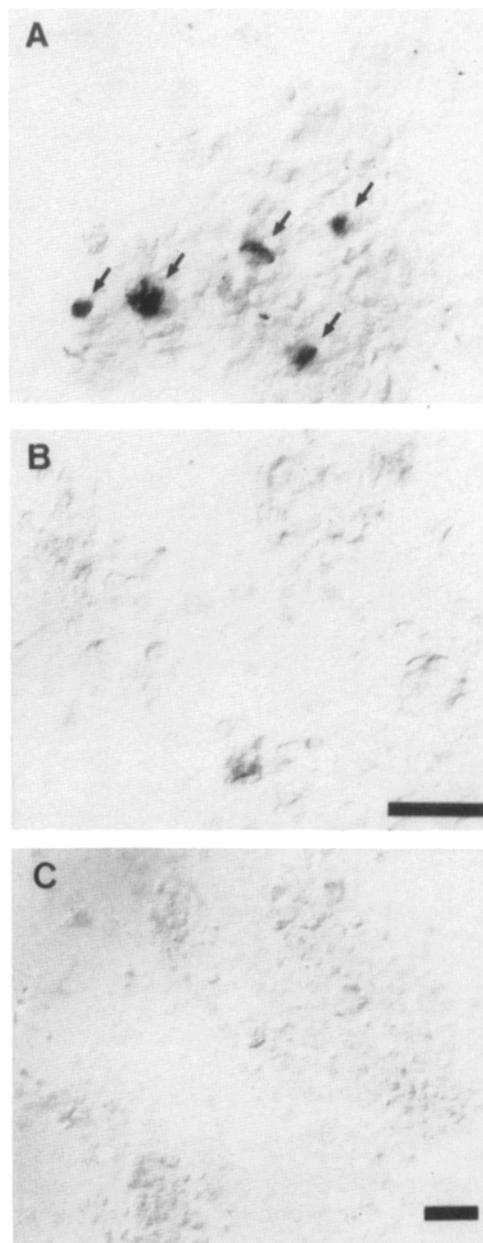


Figure 3. Demonstration of DTMT receptor immunoreactivity in transfected Cos-7 cells. PAP immunohistochemistry was performed with the N1G affinity purified antibody (1:500 dilution) on Cos-7 cells transfected with pSVL-DTMT (A) or pSVL-hTSHR as a negative control (B). Arrows indicate the immunoreactive cells. When an equivalent dilution of the corresponding preimmune serum was used on Cos-7 cells transfected with pSVL-DTMT, no staining was obtained (C). Bars, 50 μ m.

tive structure. Elongated spermatids, found in fewer tubules, showed similar staining. Germ cells at earlier stages of spermatogenesis, such as early round spermatids, spermatocytes or spermatogonia remained unstained, as did Sertoli cells and interstitial tissue. In the lumen of some tubules, additional staining could be seen within the cytoplasmic droplet of spermatozoa, which is located at this stage of maturation at the head-tail junction (Fig. 6 A). Similar staining pattern was prominent in the rete testis (data not shown). PAP immu-

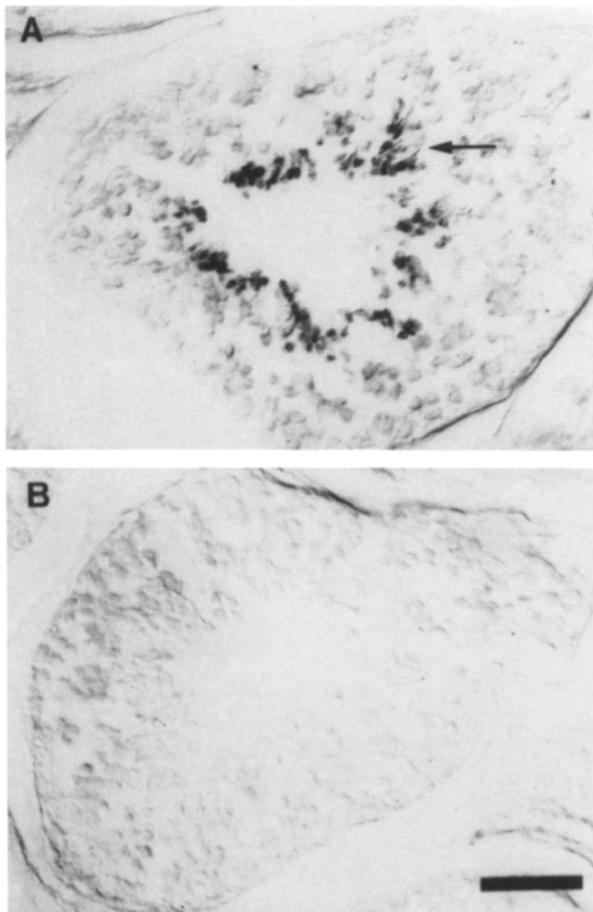


Figure 4. Demonstration of DTMT receptor immunoreactivity in late round spermatids. (A) PAP immunohistochemistry was performed on testis cryosections with the N1G affinity purified serum (1:500 dilution). The arrow points to the birefringent nucleus of one of the stained round spermatids. (B) The use of the preimmune serum at an equivalent dilution revealed no staining. Bar, 50 μ m.

nostaining on epididymal cryosections showed intense labeling of the content of the epididymal tubes, while their epithelial wall and the surrounding tissue remained unstained (Fig. 5, A and C). This labeling was observed throughout the epididymal length down to the vas deferens. Higher magnification revealed a selective staining of the cytoplasmic droplet of epididymal spermatozoa (Fig. 6 B), mostly located at this stage at the junction between tail midpiece and principal piece, as has been previously described (Herms et al., 1988). A faint staining was also found on the midpiece of some spermatozoa, but the head and principal piece remained reproducibly negative (Fig. 6 B).

The specificity of the testicular and epididymal staining was assessed by staining adjacent testis and epididymis sections with the N1 preimmune serum (Figs. 4 B and 5 B) or N1G purified serum preadsorbed with the immunogenic fusion protein (pMal-NDT) (Fig. 5 D). Absence of staining was consistently obtained under these conditions. Staining was however conserved when the N1G antibody was preadsorbed with an excess of an irrelevant fusion protein (pMal-CDT) (Fig. 5 C). N1G was also used for PAP immunostaining of olfactory mucosa, brain (cortex and striatum) and ovary,

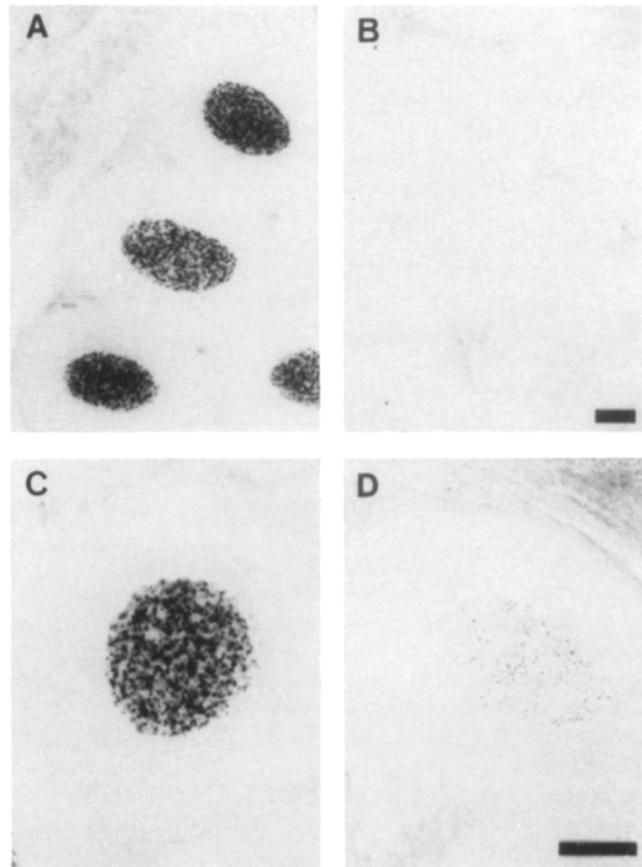


Figure 5. Demonstration of DTMT immunoreactivity in epididymal tubes. (A) PAP immunohistochemistry was performed on epididymal cryosections with the N1G affinity purified serum (1:500 dilution). (B) The preimmune serum at an equivalent dilution revealed no staining. (C) Preincubation of N1G with an irrelevant fusion protein (pMAL-CDT) did not prevent the specific staining. (D) Preincubation of N1G with the immunogenic pMAL-NDT fusion protein abolished all staining, while birefringent sperm cell nuclei are still visible in the lumen. Bars, 50 μ m.

processed in a similar way as testis cryosections. No staining was obtained in any of these tissues.

The faint labeling of the midpiece present on some epididymal spermatozoa prompted us to perform immunogold labeling with silver enhancement as a more sensitive immunodetection method, and to use cell smears instead of cryosections in order to obtain a better resolution of the sperm structure. The intense labeling of the cytoplasmic droplet of all sperm cells confirmed the PAP staining results. Besides, a heterogeneous staining along the midpiece of the flagella was also found, in continuity with the droplet staining (Fig. 6, C and E). To assess whether this additional flagellar staining was maintained during the final spermatozoa maturation, we analyzed ejaculated sperm cells for DTMT immunoreactivity.

Immunogold staining of ejaculated sperm smears labeled the midpiece of the flagella with variable intensity, while the principal piece remained unstained (Fig. 7). A considerable heterogeneity was seen among the sperm cells: ~5% were heavily stained (Fig. 7, E and F), 80% were moderately stained (Fig. 7 A), whereas 10–15% showed no significant

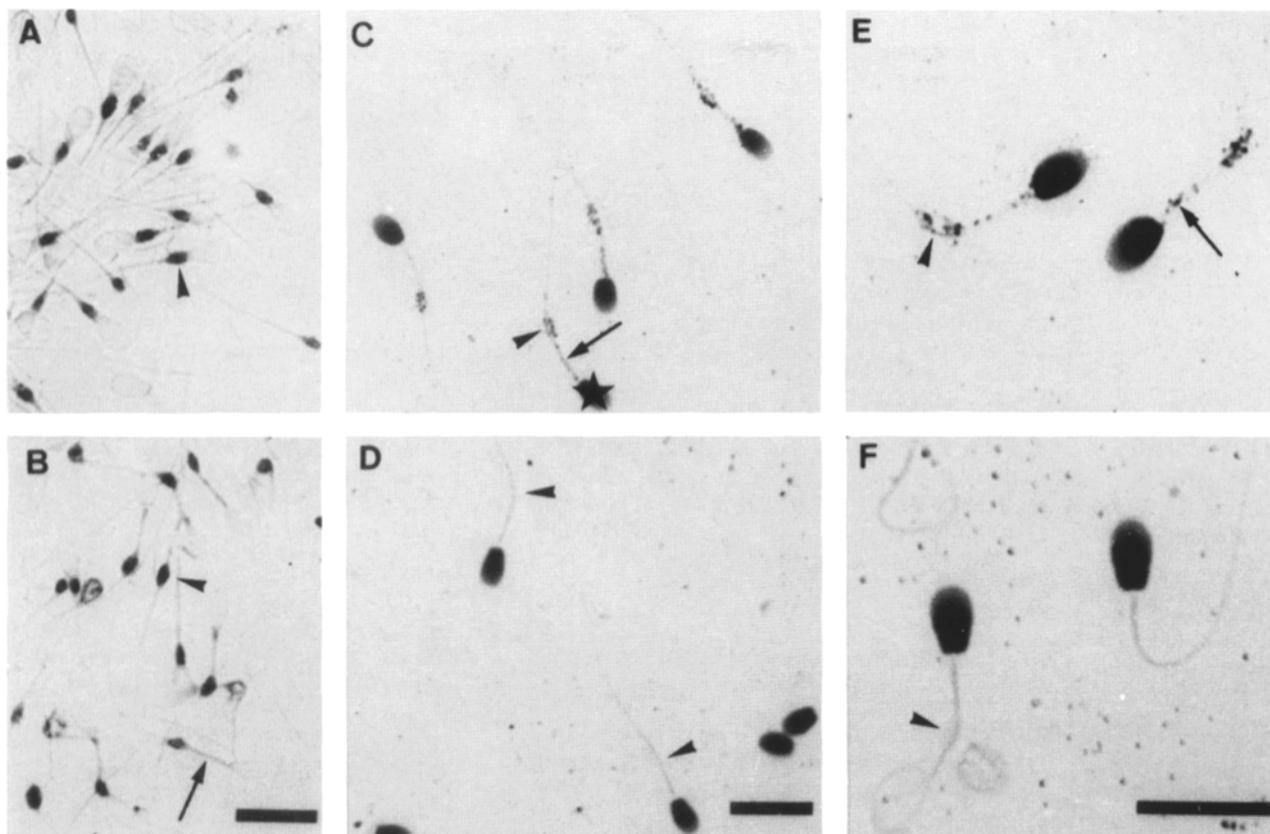


Figure 6. Demonstration of DTMT immunoreactivity on immature spermatozoa. PAP immunohistochemistry was performed on testis (A) and epididymal (B) cryosections with the NIG affinity purified serum (1:500 dilution). Arrowheads point to the intense staining of the cytoplasmic droplets of spermatozoa. Note the different location of the cytoplasmic droplet, which is located at the head-tail junction in A, and at the midpiece-principal piece junction in B. The arrow in B shows the faint staining that could be detected on the midpiece of some spermatozoa after migration of their cytoplasmic droplet. Immunogold labeling with silver enhancement was performed on epididymal sperm smears (C-F). A light hematoxylin counterstaining was performed after the immunogold procedure (a star indicates one of these colored sperm heads). Arrowheads show the location of the cytoplasmic droplets, while arrows point to sperm midpieces. The NIG purified serum (C), or NIG preincubated with an irrelevant fusion protein (pMAL-CDT) (E) revealed specific staining of sperm cytoplasmic droplets and flagellum midpieces. The preimmune serum at an equivalent dilution (D), or NIG preincubated with the immunogenic fusion protein (pMAL-NDT) (F) revealed only the self-nucleation background. Bars, 10 μ m.

staining. Once again, preincubation of the serum with pMAL-NDT abolished the staining, with the exception of the non-specific background, resulting from silver self nucleation on the poly-L-Lysine coating of the slides (Fig. 7).

Western Blotting

Unpurified and purified sera were tested systematically on Western blots prepared from olfactory mucosa, whole testis, and dog sperm membrane preparations. As for immunohistochemical procedures, only the NIG purified antibody gave reliable results. It detected a specific band of 40 kD in Triton/deoxycholate extracts of ejaculated sperm (Fig. 8). The size of the band is consistent with the calculated molecular weight of DTMT (34 kD), taking into account that the receptor contains three potential sites of N-linked glycosylation (Fig. 1). This specific band was observed up to a dilution of 1:250 for the purified serum. Preimmune serum or NIG preincubated with its immunogenic fusion protein (pMAL-NDT) at a micromolar concentration did not stain the 40-kD band (Fig. 8). The staining was conserved when the antibody

was preincubated with an irrelevant fusion protein (pMAL-CDT) at the same concentration (data not shown).

Discussion

Selected Olfactory Receptors Are Preferentially Expressed in the Testis

The first aim of this study was to determine whether the same olfactory receptor genes would be expressed in both olfactory mucosa and germ line, or if their expression was mutually exclusive. Using RPA, we demonstrated the strong and specific expression in olfactory mucosa of the two olfactory receptor genes previously cloned from this tissue (DOPCRH02 and DOPCRH07) (Fig. 2). In contrast, despite the RPA sensitivity, olfactory mucosa expression could hardly be detected for two (DTPCRH01 and DTPCRH02) out of three genes cloned from the testis, while the third one was completely silent in this tissue (DTPCRH09) (Fig. 2). These results clearly demonstrate that some of the so-called

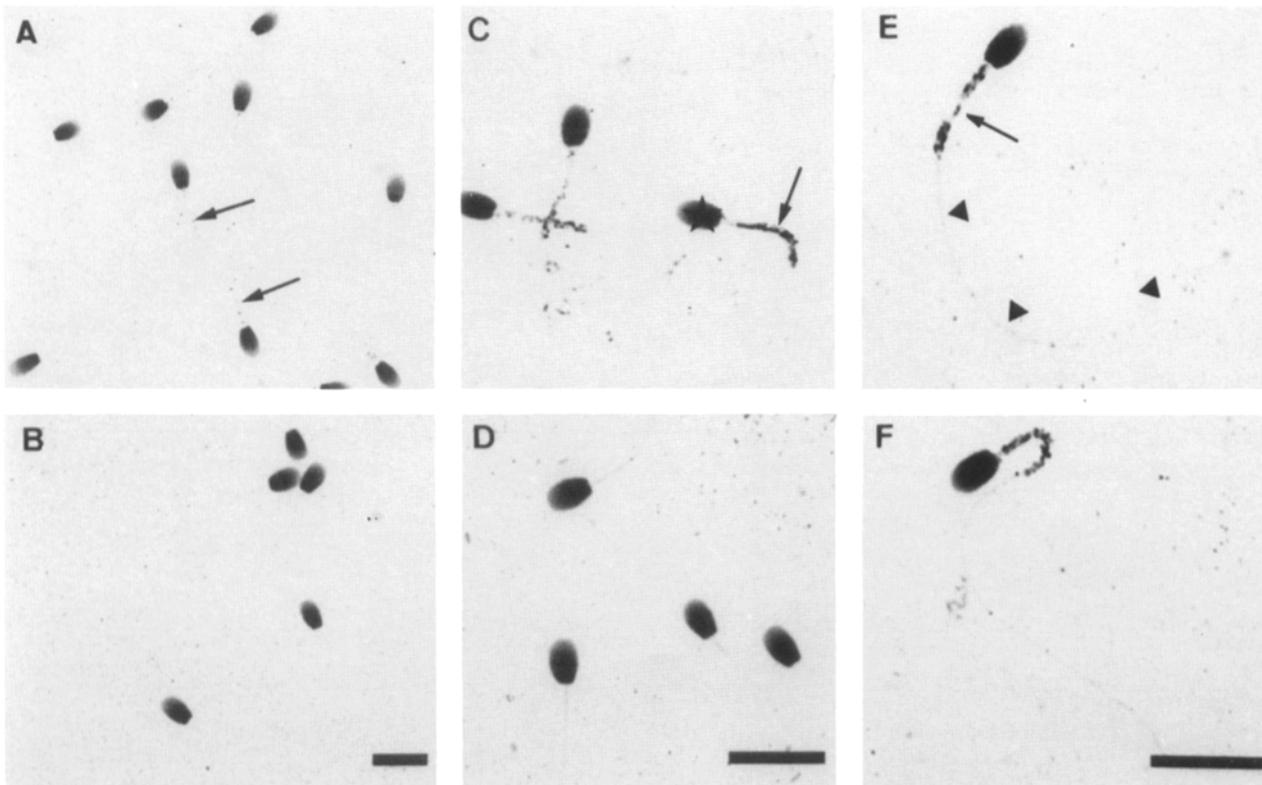


Figure 7. Demonstration of DTMT immunoreactivity on mature spermatozoa. Immunogold labeling with silver enhancement was performed on ejaculated sperm smears. A light hematoxylin counterstaining of sperm heads (*star*) was performed after the immunogold procedure. The NIG affinity purified serum (1:500 dilution) (*A*, *C*, and *E*) or NIG preincubated with an irrelevant fusion protein (*F*) revealed a specific staining of sperm midpiece (*arrows*). The principal pieces (*arrowheads*) are not labeled. The use of the preimmune serum at an equivalent dilution (*B*), or preincubation of NIG with the immunogenic fusion protein (pMAL-NDT) (*D*) abolished the staining. Bars, 10 μ m.

olfactory receptor genes are barely expressed at their expected physiological site of expression (i.e., olfactory mucosa) while their major site of expression resides in the testis. They also demonstrate that testis expression is not the consequence of a leaky control of gene expression in this tissue, that would be shared by the whole olfactory receptor gene family. The limited number of olfactory receptor genes that could be amplified from dog testis mRNA (Parmentier et al., 1992) therefore constitute a subset of this gene family characterized by a specific (although unexpected) expression in testis. As stated earlier, this subset of putative olfactory receptors do not display specific sequence characteristics: they belong to quite different structural subgroups of the olfactory receptor family. Though not conclusive on its own, this specificity of expression is obviously consistent with the physiological relevance of the olfactory receptor expression in testis. In situ hybridization in mouse (Ressler et al., 1993) and catfish (Ngai et al., 1993b) have shown that each olfactory neuron expresses a few, if not a single olfactory receptor gene. It is not known at this time if the low level of transcripts detected for DTMT and DTPCRH02 in olfactory mucosa is broadly distributed (but at very low levels) or restricted to a very small number of cells. In this latter situation only would these be endowed with a function in the olfactory epithelium.

A Putative Olfactory Receptor Is Displayed by Late Spermatids and Mature Sperm Cells

The second aim of this study was to explore whether the expression of olfactory receptor genes in germ cells correlates effectively with the presence of the corresponding proteins on their cell surface, and, if so, to characterize their pattern of expression during sperm maturation. Since DTMT represents the most abundantly expressed receptor gene in dog testis (Parmentier et al., 1992, Fig. 2), we focused on this gene and raised antibodies against different parts of the receptor, expressed in *E. coli* as fusion proteins (Fig. 1).

One of the purified antibodies, NIG, raised against the NH₂-terminal extracellular domain of DTMT (Fig. 1), gave consistent results in ELISA, immunohistochemistry, and Western blotting. This antibody was used therefore in all subsequent experiments. The specificity toward the recombinant part of the immunogenic fusion protein was demonstrated in each case. The NH₂-terminal extracellular domain of DTMT constitutes one of the least conserved segments among olfactory receptors and among G protein-coupled receptors in general. The NIG antibody is therefore expected to be reasonably specific for DTMT with little cross-reactivity with other olfactory receptors. The ability of NIG to recognize the full size DTMT receptor was demon-

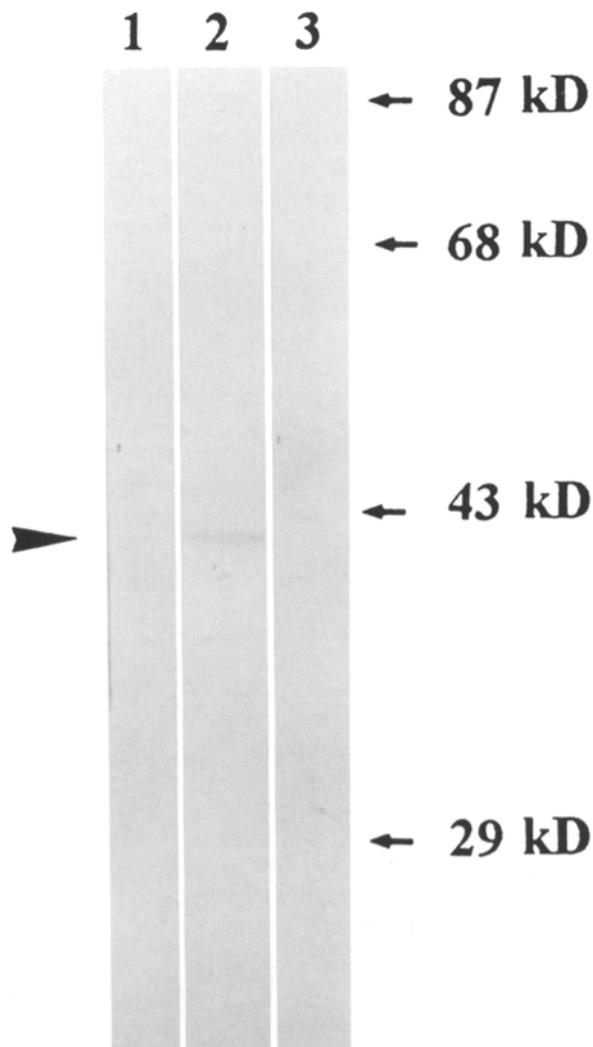


Figure 8. Western blotting of ejaculated sperm detergent extracts. (lane 1) Preimmune serum (1:250 dilution). (lane 2) NIG affinity purified serum at an equivalent dilution. (lane 3) NIG affinity purified serum preincubated with the pMAL-NDT fusion protein. Molecular size markers are indicated on the right. The arrowhead on the left points to the 40-kD band.

strated on Cos-7 cells transiently expressing the protein (Fig. 3).

From the immunohistochemical and immunoblotting experiments, a clear pattern of expression emerges, that should be correlated with the peculiarities of sperm maturation in dog. In all mammals, transition between spermatid and sperm cell is characterized by the condensation of the nucleus and the delimitation of the major part of the cytoplasm as a residual body. This residual body, which includes most of the cell organelles and proteins that are useless for the remaining lifespan of sperm cells, is eliminated through resorption by Sertoli cells. However, in several species including dog and rat, part of this cytoplasm, together with poorly characterized membrane structures (Hermo et al., 1988), remains in early sperm cells as a cytoplasmic droplet, located at the head-tail junction. This droplet then migrates along the flagellar midpiece during the epididymal transit

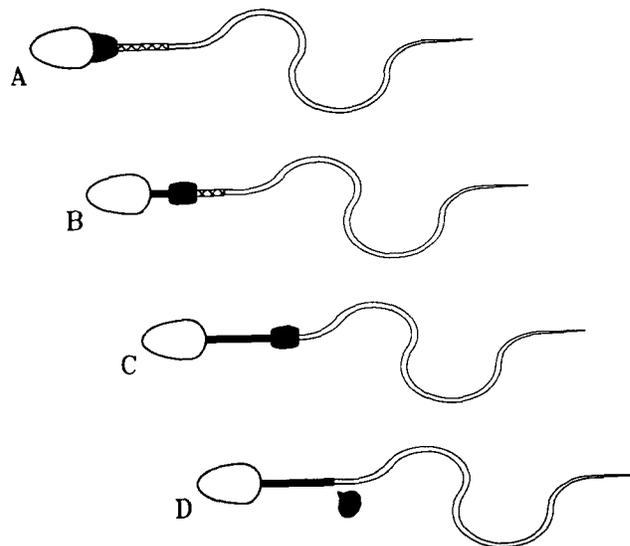


Figure 9. Diagram illustrating the location of the DTMT immunoreactivity (represented in black), during the migration of the cytoplasmic droplet along the midpiece of dog epididymal sperm. In the testis and rete testis, the immunostained cytoplasmic droplet surrounds the neck region of the flagellum, the flagellar midpiece (cross-hatched) being unstained (A). During the proximal epididymal transit, the droplet migrates rapidly along the flagellum (B), and remains at the junction of flagellum midpiece and principal piece up to the vas deferens (C). Simultaneously, DTMT immunoreactivity becomes detectable on the midpiece (B and C). The droplet is then eliminated, while ejaculated spermatozoa retain a heterogenous but sometimes strong immunoreactivity on the flagellum midpiece (D).

and is eliminated at this site (Fig. 9). As will be seen below, all the detected immunoreactivities find a coherent pattern of expression in this developmental scheme (Fig. 9). DTMT immunoreactive material is first detected in late round and elongated spermatids (Fig. 4). This correlates well with the Northern blotting experiments previously reported on fractionated testis cells, which showed selective DTMT expression in two fractions, one enriched in elongated spermatids, the other in round spermatids and spermatocytes (Parmentier et al., 1992). The immunoreactive material further seems to concentrate in the cytoplasmic droplet, intensely stained in testicular and epididymal spermatozoa (Figs. 5 and 6). After migration and elimination of the droplet, a heterogenous but sometimes strong immunoreactivity persists on the midpiece of mature sperm flagellum (Fig. 7). Western blotting performed on sperm membrane extracts confirmed the presence of a DTMT receptor immunoreactivity, corresponding to a protein of 40 kD, in accordance with the expected size for a glycosylated receptor (Fig. 8).

Immunohistochemistry or Western blotting did not allow the detection of DTMT in olfactory mucosa. This is not surprising, given the low level of DTMT transcripts in this tissue. These negative results favor the hypothesis that DTMT transcripts are spread at very low levels all over the olfactory neurons, although a few positive cells expressing DTMT as the sole receptor of the family (see above) could have been overlooked. Tissues known by reverse PCR (Buck and Axel, 1991; Parmentier et al., 1992) to express no olfactory recep-

tor genes did not show any immunoreactivity. The good correlation between the Northern blotting, immunohistochemical, and Western blotting experiments makes the possibility of a cross-reaction with a protein other than DTMT-encoded receptor highly unlikely.

Potential Physiological Significance of Olfactory Receptors on Sperm Cells

We have thus demonstrated the presence of a receptor of the olfactory family on dog spermatids and spermatozoa. High expression of a protein in a specific tissue could, in some cases, be devoid of physiological meaning, as has been recently hypothesized (Erickson, 1993). This possibility cannot be formally excluded in the present context. Nevertheless, the presence of a protein on the mature sperm cell strongly reinforces the hypothesis that DTMT gene expression in germ cells is endowed with physiological significance. The selective expression of a limited number of olfactory receptor genes in dog testis, as well as in man (Parmentier et al., 1992), and probably in fish (Ngai et al., 1993a), is also in favor of a specific role for the corresponding proteins. This role could be part of the complex paracrine or endocrine networks that regulate spermatid final differentiation, spermiogenesis, spermiation, or sperm maturation. Alternatively, DTMT-encoded receptor could be involved in mature sperm physiology, as suggested by its presence on mature flagella.

In the frame of this latter hypothesis, observation that the immunoreactivity was located on the cytoplasmic droplet and flagellum is worth discussing. The fact that most of the immunoreactive material is eliminated at the same time as the droplet, leaving only small amounts of detectable protein on the sperm cell tail could militate against a functional role of DTMT in mature spermatozoa. However, several reports have described a pattern of developmental expression comparable to that of DTMT, including a strong cytoplasmic droplet immunoreactivity, for a number of proteins known to be present and/or functional on mature sperm. These proteins include flagellar structural components (Herms et al., 1988), regulatory proteins (Tash and Means, 1982, 1983), and uncharacterized antigens (Toshimori et al., 1992). It was also suggested that the cytoplasmic droplet played a role in sperm membrane final organization (Shlegel et al., 1986; Magargee et al., 1988; Bains et al., 1992). The labeling heterogeneity we found among individual sperm could also question the physiological significance of our finding. Again, this has been reported for several other sperm antigens (O'Rand and Romrell, 1981; Rotem et al., 1990; Benoff et al., 1993), and in some instances, this heterogeneity could be correlated with sperm functional parameters (Rotem et al., 1990; Benoff et al., 1993). Thus, although it cannot be excluded that DTMT immunoreactivity only represents a non-functional remnant of a protein endowed with functions at earlier stages, its pattern of expression is compatible with a function in mature sperm. It is tempting to speculate that such a function could include regulation of sperm motility, chemotaxis, or possibly both. Sperm chemotaxis is a general phenomenon among invertebrates (Yoshida et al., 1993; Ward and Kopf, 1993), and its molecular substrates have been well delineated in sea urchin, involving receptors of the guanylate cyclase family and specific peptides synthesized by the oocyte (Garbers, 1989; Ward and Kopf, 1993). Notewor-

thy, these receptors and the proteins involved in the subsequent transduction cascade have been unambiguously localized to the tail of the sea urchin sperm (Toowicharanont and Shapiro, 1988; Harumi et al., 1991). Recently, experimental evidence for sperm chemotaxis in protochordates and vertebrate species was also provided (Villanueva-Diaz et al., 1990; Ralt et al., 1991; Yanamigashi et al., 1992; Yoshida et al., 1993), but the underlying molecular mechanisms have so far remained elusive. On the other hand, sperm motility regulation has been extensively studied at the molecular level, involving the stimulatory effects of cyclic AMP, and the bipolar effects of calcium, mainly through the modulation of flagellar proteins phosphorylation by cAPK, calcineurin, and PKC (Garbers and Kopf, 1980; Tash, 1989; Rotem et al., 1990; Suarez et al., 1993). G proteins have not so far been implicated formally in the regulation of motility, despite the facts that various ligands of well known G protein-coupled receptors (bradykinin and platelet-activating factor) have been shown to stimulate mammalian sperm motility (Sato and Schill, 1987; Ricker et al., 1989), and that G_o, G_i, and G_z have been reported in mouse and rat sperm cells (Glassner et al., 1991; Haugen et al., 1992). Transduction in olfactory neurons has also long been considered to be mediated by cAMP (Pace et al., 1985; Sklar et al., 1986; Bakalyar and Reed, 1990) but recent evidence also suggested the involvement of calcium (Boekhoff et al., 1990; Ronnet et al., 1993). This has recently been confirmed by the expression of recombinant rat receptor genes using baculovirus-Sf9 cell system (Raming et al., 1993). The analogy between sperm cell and olfactory mucosa is not limited to the expression of a common family of olfactory receptors. Interestingly, it was demonstrated that the same β -ARK and β -arrestin isoenzymes were to be found in both rat olfactory neurons and spermatids (Dawson et al., 1993).

DTMT may thus be involved in sperm motility regulation or chemotaxis. In this regard, it is noteworthy that one of the prominent features in experimental mammalian chemotaxis is the fact that only a fraction of sperm cells are responsive to a given chemoattractant (Ralt et al., 1991; Eisenbach and Ralt, 1992). The heterogenous distribution of DTMT among sperm cells could account for such an inter-gametic selection phenomenon.

As a conclusion, we have demonstrated in the present study that a subset of the olfactory receptor gene family is specifically expressed in the male germ line. For at least one of these genes, the protein itself can be found on mature sperm cells with a pattern of expression compatible with a functional role in the regulation of sperm maturation, motility, and/or fertilization. Additional research will focus on the function of the DTMT receptor by determining the nature of the corresponding ligand and its site of production, and by delineating the transduction cascade activated by the receptor. The corresponding receptors expressed in other species, particularly in human, will be isolated as well, since the potential role of these proteins in sperm chemoattraction has obvious implications in the fields of male and female fertility and contraception.

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