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Molecular cloning of a novel putative G-protein coupled receptor expressed during rat spermiogenesis

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A cDNA clone encoding a novel putative G-protein coupled receptor has been isolated from a rat testis cDNA library using a PCR-amplified cDNA fragment as a hybridization probe. Northern blot analysis reveals that a corresponding 1.5 kb mRNA is exclusively expressed in testis. By in situ hybridization experiments this mRNA has been localized in spermatocytes and spermatids but not in spermatogonia, Leydig or sertoli cells. Ontogenic studies show that expression of the receptor-encoding mRNA and sexual maturation are correlated reaching highest levels during the second and third months. Although the ligand for this receptor has not yet been identified, this receptor may play a role during reproduction.

G-protein coupled receptor; Rat testis; Spermiogenesis

1. INTRODUCTION

A variety of hormones, neurotransmitters and neuropeptides exert their functions via a family of receptors which are coupled to regulatory GTP-binding proteins transducing the extracellular signal to certain intracellular effectors (reviewed in [1,2]). Sequence comparisons of a number of receptors of this family including muscarinic, adrenergic, dopaminergic, serotonergic receptors as well as neuropeptide receptors and receptors for glycoprotein hormones have revealed that they all possess a typical hydropathy profile displaying seven stretches of hydrophobic amino acids believed to span the plasma membrane ([3–8], and references therein). Moreover, within the putative trans-membrane regions there is a degree of sequence conservation which has allowed the deduction of degenerate oligonucleotides. These were successfully employed in the polymerase chain reaction (PCR) to isolated new types of G-protein coupled receptors [9]. Using this approach a novel putative G-protein coupled receptor has been identified which is expressed exclusively in the mature testis of rats.

2. MATERIALS AND METHODS

Isolation of poly(A)+RNA, cDNA synthesis and Northern blot analysis were carried out using standard procedures [10].

The G-protein coupled receptor cDNA sequence reported here has been assigned the accession number X59249 by the EMBL Data Library.

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Receptor-encoding cDNA fragments were isolated by amplification with polymerase chain reactions essentially according to the protocol described in [9]. A phage λgt11 rat testis cDNA library (Stratagene, Heidelberg) was screened using one of the receptor-encoding cDNA fragments as ^{32}P-labelled hybridization probe. The cDNA inserts of positive phages were subcloned into M13 mp18 vectors. The nucleotide sequence was determined from both complementary DNA strands using universal sequencing primers as well as internal primers and the dideoxy chain termination protocol [11].

For in situ hybridization the PCR-amplified cDNA fragment was subcloned into pBluescript in order to synthesize ^{35}S-labelled RNA

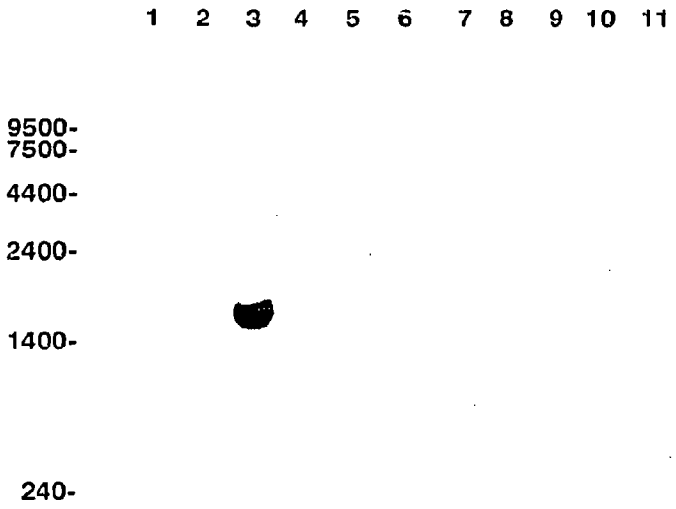

Fig. 1. RNA blot analysis of tgpcr1 mRNA expression. Poly(A)+RNA purified from GH3 cells [1], urinary bladder [2], testis [3], liver [4], spleen [5], adrenal gland [6], kidney [7], lung [8], heart [9], brain [10] and cerebellum [11] was glyoxylated, size separated on an agarose gel (each lane containing 30 μg of the respective poly(A)+RNA), transferred to nylon membrane and hybridized to ^{32}P-labelled tgpcr1 cDNA. Numbers refer to the length (in nucleotide residues) of RNA size standards run in parallel.

probes in antisense and, for control, in sense orientation. 15 μm cryostat sections of rat testis were acetylated [12] to reduce background-binding of the probe to the positively charged protamines and fixed with 4% paraformaldehyde and after prehybridization for 6 h incubated with 2×10^6 cpm of the respective RNA probes for 16 h in 50% formamide, 5× SSC, 5× Denhardt's solution, 0.2% SDS, 10 mM DTT, 10% dextran sulfate and 250 μg/ml of yeast carrier tRNA. Following hybridization tissue sections were incubated with RNase A, washed in 2×SSC and exposed for two weeks after dipping in Kodak NTB3 emulsion. Finally, the processed sections were stained with hemalaun. Hybridization specificity was controlled using sense RNA or tissue sections predigested with RNase A.

3. RESULTS AND DISCUSSION

In order to analyse neuropeptide or transmitter action in certain brain regions, cDNA from rat cerebellum

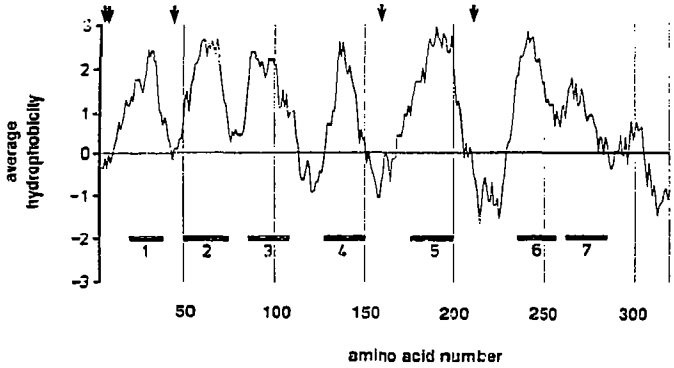

Fig. 3. Hydropathy profile of tgpcr1. The average hydrophobicity of a pentadecapeptide (n-7 to n+7) is plotted versus the amino acid number. Positive values indicate hydrophobic regions. Putative transmembrane domains are indicated by bars and numbered 1-7. Arrows point to potential N-linked glycosylation sites.

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gaattctccggactgttgctaccttctaacttctgggcagatgtctgtcaagagctaggt  -69
ccactggcccatacacatcctgctgaagaagcaacagaagtttccagctgaagcttctct  -9
                 *  *
        M  K  A  N  N  T  T  T  S  A  L  W  L  Q  I  T  Y  I   18
gagacagcatgaaagccaacaataccacgacgagtgccttgtggttgcaaatcacctaca   51
 T  M  E  A  A  I  G  L  C  A  V  V  G  N  M  L  V  L  W  V   38   TM I
tcaccatggaggctgccattggtctctgtgctgtagtgggcaacatgctggtcatctggg  111
    *
 V  K  L  N  R  T  L  R  T  T  T  F  Y  F  I  V  S  L  A  L   58
tggtcaagctgaaccgcactctgaggaccaccaccttctatttcatcytctccctagcac  171
                                                                    TM II
 A  D  I  A  V  G  V  L  V  I  F  L  A  I  A  S  A  W  R  S   78
tggctgacattgctgttggggtgctggtcataccccttggccatcgcgtcagcctggaggt  231

 R  C  T  S  M  A  C  L  F  M  S  C  V  L  L  V  F  T  H  A   98
ccagatgcacttctatggcctgccttttcatgtcctgtatgcttctggtcttcacccacg   291
                                                                    TM III
 S  I  M  S  L  L  A  I  A  V  D  R  Y  L  R  V  K  L  T  V  118
cttccatcatgtccttgctggccattgctgtagaccgatacctgcgagtcaagctgacag   351

 R  Y  R  T  V  T  T  Q  R  R  I  W  L  F  L  G  L  C  W  L  138
tcagatatagaacggttaccactcaaagaagaatatggctattcctgggcctctgctggc   411
                                                                    TM IV
 V  S  F  L  V  G  L  T  P  M  F  G  W  N  R  K  V  T  L  E  158
tagtgtccttctggtgggactgaccccatgtttggctggaatagaaaagtgaccttag     471
    *
 L  S  Q  N  S  S  T  L  S  C  H  F  R  S  V  V  G  L  D  Y  178
agctctctcaaaacagctccacctctcatgccacttccgttccgtggtcggcttggatt    531

 M  V  F  F  S  F  I  T  W  I  L  I  P  L  V  V  M  C  I  I  198
acatggtcttcttcagcttcatcacctggatcctcatccccctggttgtcatgtgcatca   591
                                                                    TM V
 Y  L  D  I  F  Y  I  I  R  N  K  L  S  Q  N  L  T  G  F  R  218
tctatctggacatcttctacatcatccgaaacaaactcagtcaaaatctgactggcttca   651

 E  T  R  A  F  Y  G  R  E  F  K  T  A  K  S  L  F  L  V  L  238
gagagacgcgtgcattttacggtcgggagttcaagaccgctaagtcgctgtttctggttc   711
                                                                    TM VI
 F  L  F  A  L  C  W  L  P  L  S  I  I  N  F  V  S  Y  F  N  258
tcttcttgtttgccttgtgctggctgcctttgtccatcatcaattttgtttcctacttta   771

 V  K  I  P  E  I  A  M  C  L  G  I  L  L  S  H  A  N  S  M  278
atgtgaagataccagagattgcaatgtgcctgggcatcctgttgtcccatgcgaactcca   831
                                                                    TM VII
 M  N  P  I  V  Y  A  C  K  I  K  K  F  K  E  T  Y  F  V  I  298
tgatgaaccctattgtctacgcctgcaaaataaaaaagttcaaagaaacctactttgtga   891
          ▼
 L  R  A  C  R  L  C  Q  T  S  D  S  L  D  S  N  L  E  Q  T  318
tcctcagagcttgcaggctctgtcagacctcggattctttggactcaaaccttgaacaga   951
 T  E
ctactgagtagttaccatgacagataaagagccagctcatttaccttcacagttcgcatc  1011
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gggctggttgccacagagctcccttccctcctccctccgctcctcccctccactcctccct 1131
ccagtcttccctccactcctccctcccgcttcatttatttttcttgagcttctctcattca 1191
attctgtggaggtctgacatgaaggcaatgcattcctggttaccacagacttcgccctttc 1251
cttcccagacacaagaagtaatggagtgaacgttgagggagtctcctctcaccaaagaag  1311
actctagtggggctggatgtacagaacctgcgttgaaggatccctaggatgttgggaaca  1371
cagggagtgaatttgaatttaaagagggctaaattcacctgtgtgggtgcatttgagcaaa 1431
taaaagatggcgcccaaaaaaaaaaccggaattc
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Fig. 2. Nucleotide and deduced amino acid sequence of tgpcr1 cDNA. Putative transmembrane regions are indicated by lines and designated as TM I to TM VII on the right. Stars denote potential N-linked glycosylation sites. The triangle points to a conserved cysteine residue, a potential site for palmitoylation.

poly(A)+RNA was used to PCR-amplify novel members of the G-protein coupled receptor family. Nucleotide sequence determination of cloned amplified cDNA fragments revealed that adrenergic and serotonergic receptors as well as the rat homologue of the dog receptor RDC7 [9] have been amplified. In addition several novel putative receptor-encoding fragments were identified (data not shown). One of the cDNA

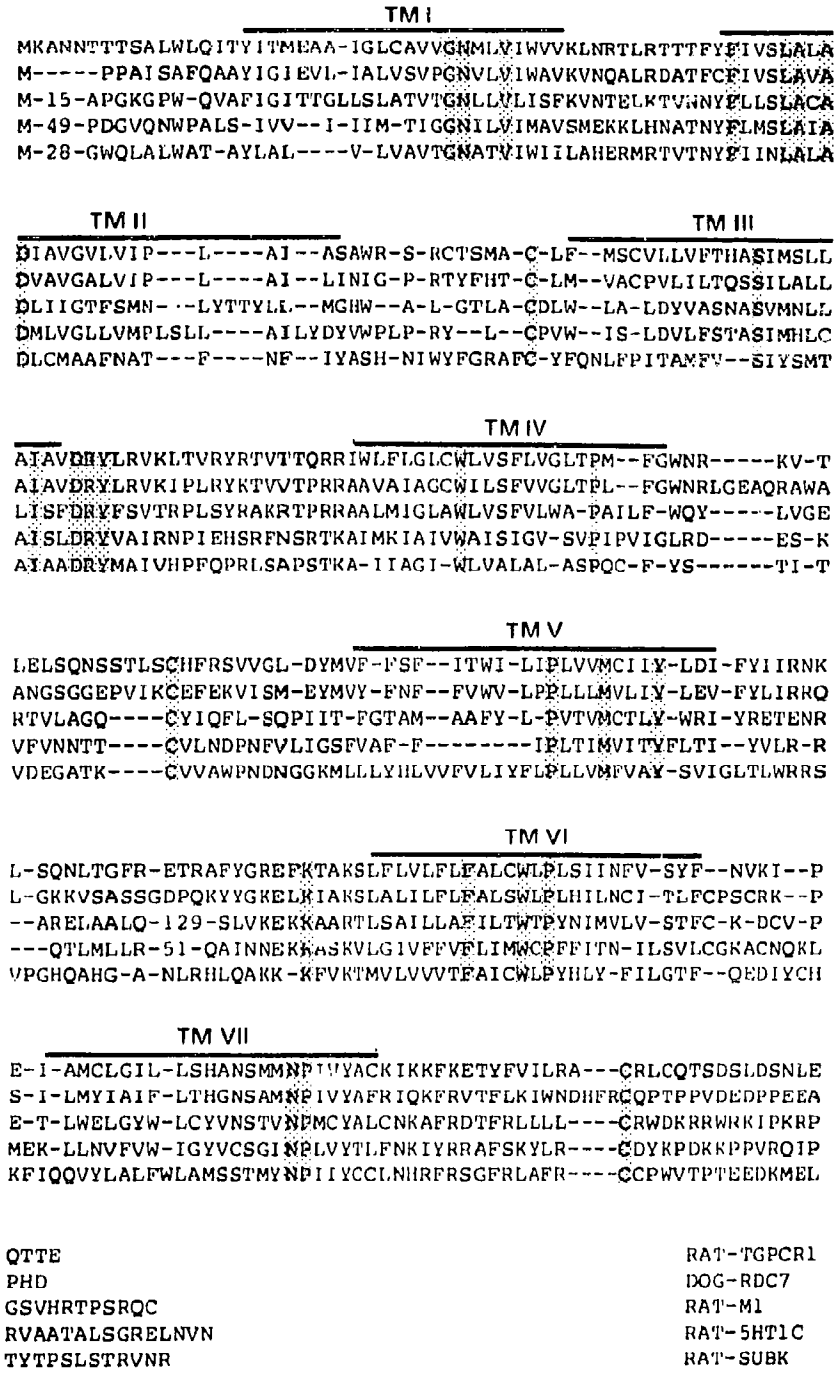

Fig. 4. Alignment of the amino acid sequences of tgpcr1 and of various G-protein coupled receptors. Gaps, indicated by hyphens, have been introduced to obtain maximal homology. Numbers designate the deleted amino acid sequences. Amino acid residues conserved in all of the 5 proteins are underlayed by dots. The sequences are taken from [6] (rat 5-HT1C, rat serotonin HT1c receptor), [9,15] (dog-RDC7, dog adenosine A_1 receptor, G. Vassart, Bruxelles, personal communication), [14] (rat-SUBK, rat substance K receptor) and [21] (rat-M1, rat muscarinic M1 receptor). Analysis of nucleotide and amino acid sequences were carried out using the DNASTAR (Wisconsin, USA) software package.

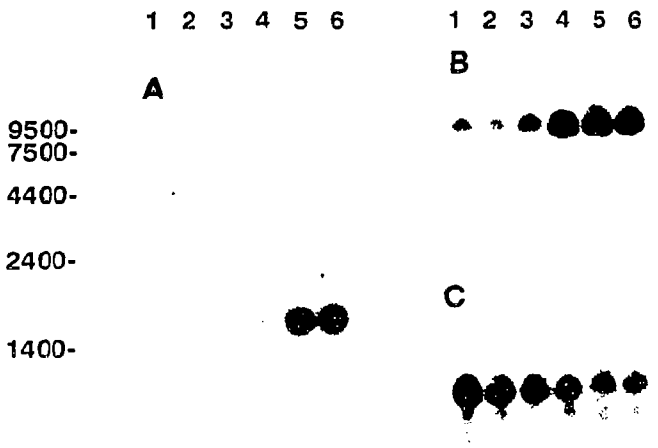

Fig. 5. tgpcr1 mRNA expression during postnatal development. Total RNA was extracted from rat testis of postnatal days 1(1), 7(2), 14(3), 28 (4), 60(5) and 90 (6), and analysed by Northern blotting (20 μg RNA/lane) as indicated in the legend to Fig. 1 using ^{32}P-labelled tgpcr1 cDNA as hybridization probe (A). After removal of the hybridized DNA, the blot was rehybridized using ^{32}P-labelled cDNA probes encoding either the immunoglobulin heavy chain binding protein (BiP; kindly provided by Dr. I. Haas, Cologne) (B) or chicken β-actin (C) demonstrating that all lanes contain comparable amounts of RNA.

fragments, termed tgpcr1, hybridized strongly and, exclusively to a 1.5 kb transcript present in poly(A)+ RNA from rat testis but not to poly(A)+RNA from other tissues (Fig. 1) including ovary and uterus (data not shown).

Screening of a rat testis cDNA library with the tgpcr1 cDNA fragment led to the isolation of a phage λ clone with a 1594 bp *Eco*RI fragment (Fig. 2) displaying at its 3′ terminus 10 A residues preceded by the consensus polyadenylation signal AATAAA. The sequence specifies an open reading frame coding for 320 amino acids suggesting a protein with a molecular weight of 36.6 kDa. The presence of in frame stop codons 5′ to the initiator ATG indicates that tgpcr1 contains the entire coding region. Part of the 5′ untranslated region seems to be missing in tgpcr1 since it is terminated by an endogenous *Eco*RI site rather than by an *Eco*RI linker sequence. The deduced amino acid sequence shows seven stretches of 20–25 hydrophobic amino acids embedded in more hydrophilic sequences thus displaying a hydrophobicity profile typical for the class of G-protein coupled receptors (Fig. 3). Furthermore this protein shares other receptor-typical features including potential N-linked glycosylation sites close to the N-terminus (Fig. 2, stars), a conserved cysteine residue (Fig. 2, residue number 302, triangle) known to comprise an acceptor site for palmitoylation [13], the presence of several serine and threonine residues that may provide potential phosphorylation sites as well as a number of highly conserved amino acid residues in transmembrane segments II, III, IV, VI and VII (Fig. 4, indicated by dotted areas).

Searching Gen-Bank data base revealed highest homology of tgpcr1 to the canine receptors RDC7 and RDC8 (Fig. 4, 47% and 42% identity, respectively, [9]) followed by muscarinic M1 [3] and β2-adrenergic receptors [4] and the serotonin HT1c receptor (30% identity, [6]) as well as the substance K receptor (27% identity, [14]). The sequence divergence of tgpcr1 and RDC7/RDC8 is on the one hand, by far too extensive to account for interspecies differences of the same receptor types. The rat homologue of the canine receptor RDC7 has also been cloned indicating an amino acid sequence homology of more than 90% (data not shown). On the other hand, the homology may suffice to group tgpcr1 with the adenosine receptors RDC7 (G. Vassart, Bruxelles, personal communication) and RDC8 [15] into a small receptor subfamily. This idea is supported by the observation that the degree of sequence homology between tgpcr1 and RDC7/RDC8 receptors is in the range of that for human β1- and β2-adrenergic receptors (54%, [16]) or porcine muscarinic M1 and M2 receptors (43%, [16]) and is considerably higher than that for serotonin HT1a and serotonin HT2/HT1c receptors (35%, [17–19]).

Northern blot analysis using rat testis RNA from various postnatal developmental stages (Fig. 5) indicates that expression of tgpcr1 mRNA is first detected at postnatal day 28 but undetectable at postnatal day 14. Expression further increases during the second and third months after birth. Thus tgpcr1 expression seems to coincide with sexual maturity which is reached at postnatal day 24. In situ hybridization was carried out in order to determine which testicular cell type expresses tgpcr1 receptor mRNA. Fig. 6 clearly demonstrates that silver grains are present almost exclusively within the central luminal regions of seminiferous tubules in ring-like structure apart from the basement membrane. Since sperm maturation is accompanied by cell movement from outer to the inner regions of seminiferous tubules, this result indicates that only first or second order spermatocytes, or preferably spermatides or mature sperm are labelled by the probe. Neither sertoli cells nor spermatogonia, both forming an epithelial-like cell layer which coat the basement membrane (for review of the seminiferous epithelium see [19]), are labelled. Leydig cells present in the interstitium between the seminal tubules also lack any hybridization signals. Thus the tgpcr1 expression pattern suggests that appearance of this receptor is temporally and spatially coinciding with sperm development. Although its ligand has not yet been identified it appears to be an attractive hypothesis that the tgpcr1 receptor is involved either in cell proliferation/differentiation or in binding to a chemoattractant by which sperm may be guided.

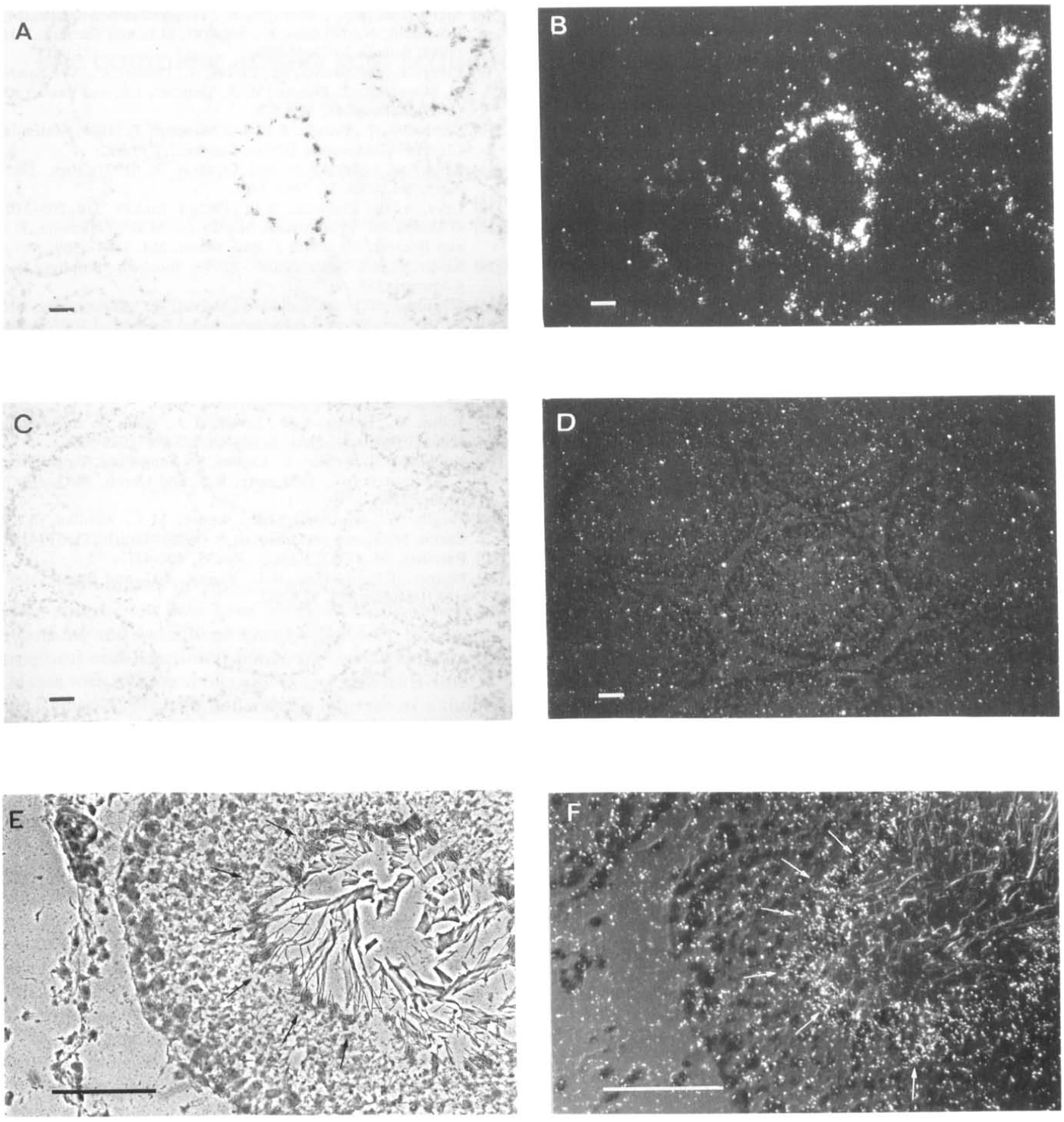

Fig. 6 In situ hybridization of ^{35}S-labelled tgpcr1 antisense cRNA to cross-sections of rat testis. (A) Bright field micrograph shows the typical topography of the seminiferous tubules: a ring of peripherally located spermatogonia and sertoli cells and the mature spermatids being localized within the central part of the lumen. Two of the tubules show the presence of silver grains in ring-like structures close to the inner lumen. (B) Corresponding dark-field image indicating the hybridizing structures by rings of grains. (C) and (D) Bright- and dark-field micrographs of in situ hybridization control using labelled tgpcr1 cRNA in sense orientation. (E) Phase contrast micrograph showing in higher magnification a part of a seminiferous tubule. The large peripheral cell nuclei represent spermatogonia and sertoli cells. Silver grains are colocated with a ring of spermatids (arrows). The intensely stained long nuclei are shown with their sperm tails (largely aggregated) pointing towards the lumen of the tubule. (F) A corresponding interference contrast image shows that most of the silver grains are concentrated around the central lumen of the tubule (arrows). Bars, 40 μm.

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