Two Novel Odorant Receptor Families Expressed in Spermatids Undergo 5′-Splicing*

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We report the identification of two novel families of odorant receptor (OdR)-like proteins, termed spermatid chemoreceptors (SCRs), in rat spermatids of the testis. The full-length genomic clones encode seven transmembrane domain receptors that share 35–40% identity with certain OdRs and are among the most divergent members of the OdR superfamily based on phylogenetic analysis. RNase protection assays and in situ hybridization studies confirmed the expression of SCRs in spermatids, the post-meiotic, differentiating cell population in the testis. SCR transcripts were undetectable in the prepubertal testis but were readily identified in spermatids of sexually maturing and mature testis. Rapid amplification of cDNA end-polymerase chain reaction and genomic clone sequencing led to the discovery that SCRs are spliced upstream of their presumptive starting methionines. 5′-Splicing of OdRs may regulate the expression of functional chemoreceptors.

In an effort to understand the molecular basis of olfaction, Buck and Axel (1) cloned a family of putative odor receptors (OdRs)† predicted to contain up to 1000 distinct gene products (2). The number and diversity of putative receptors is consistent with the ability of mammals to differentiate thousands of odors (2). Some of these receptors have been directly localized to olfactory neurons by in situ hybridization (3–6). Despite the compelling circumstantial evidence that this family of receptors functions in mammalian odor discrimination, definitive data documenting ligand-receptor binding and functional receptor expression are lacking. The possibility that these orphan receptors may serve a more general function in chemodetection comes from the discovery that OdR-like proteins are expressed in the testis (7). Subsequently, we (8) and others (9) demonstrated the localization of putative OdRs in spermatids of the testis and on the midpiece of mature spermatozoa. Moreover, mammalian sperm tails possess proteins involved in olfactory signal transduction including G-protein receptor kinase 3 (8), β-arrestin2 (8), inositol 1,4,5-trisphosphate receptors (10), adenylyl cyclase (11), and cyclic nucleotide-gated channels (12).2 In this report, RNase protection assays likewise detect OdR transcripts in the spleen. These findings suggest that sperm tails, olfactory cilia, and immune cells may share common mechanisms to detect exogenous chemical signals.

The hypothesis that OdRs function in mammalian sperm to regulate motility in response to exogenous signals derives from the existence of sperm-egg chemotaxis in invertebrates. The small peptides, speract and resact, are secreted by sea urchin eggs and attract spermatozoa in a species-specific manner by stimulating sperm motility and respiration (13–15). Recent evidence indicates that sperm-egg chemotaxis may likewise occur during mammalian fertilization (16, 17).

We were interested in clarifying the role of OdR expression in the testis. During the meiotic phase of spermatogenesis, gene promoters may become exposed as a result of altered chromatin structure, potentially leading to spurious transcription of a host of gene products lacking physiologic function. To avoid this confounding issue in studying whole testis, we restricted our investigation of OdR expression to round spermatids, the post-meiotic, differentiating cell population in the testis. We report the identification of two novel families of OdR-like proteins, termed spermatid chemoreceptors (SCRs), in spermatids of rat testis. In situ hybridization studies illustrate the selective localization of SCRs to spermatids in the testis and demonstrate that SCR expression is developmentally regulated, with transcripts first detectable during sexual maturation. RACE-PCR and sequencing of genomic clones led to the discovery that SCRs undergo 5′-splicing, which may regulate receptor expression during spermatogenesis. The 5′-splicing of OdR transcripts may influence the stability of OdR mRNAs and the expression of functional OdR proteins in olfactory, reproductive, immunologic, and experimental systems.

EXPERIMENTAL PROCEDURES

RT-PCR of Putative SCRs—Total RNA was prepared from testis, whole brain, and olfactory tissue using TRIzol reagent (Life Technologies, Inc.) and Oligotex poly(A)⁺ selection (Qiagen) according to manufacturer protocols. Round spermatids were purified from the testis by elutriation (18); the cell pellet was homogenized in guanidinium isothiocyanate and RNA pelleted through a cesium chloride gradient according to established procedures (19). RNA was treated with 10 units of RNase-free DNase (Life Technologies, Inc.) per μg of RNA. Five μg of RNA from each source was reverse-transcribed using oligo(dT) or ran-

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† The abbreviations used are: OdR, odorant receptor; RACE, rapid amplification of cDNA ends; RPA, RNase protection assay; SCR, spermatid chemoreceptor; pSCR, partial spermatid chemoreceptors; TMD, transmembrane domain; TBS, Tris-buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair(s).

2 I. Weyand and U. B. Kaupp, personal communication.

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RESULTS

Two Novel Spermatid Chemoreceptor (SCR) Families Are Identified in Purified Round Spermatids—In a search for OdR-like proteins that may serve functional roles in reproduction, we reasoned that the post-meiotic and actively differentiating cells of the testis would be most likely to express chemoreceptors physiologically relevant to mature spermatozoa. Accordingly, we purified round spermatids from rat testis (Fig. 1a) to isolate total RNA from this select population of cells, and conducted RT-PCR experiments using degenerate primers (OdR 1 and 2) designed against highly conserved regions of the OdR family. A PCR product of 520 bp was generated using cDNA derived from the round spermatid total RNA (Fig. 1b). The 520-bp product corresponded in size to the band generated from PCR using the control template F2, an originally cloned Buck and Axel OdR.

To determine the origin of the novel sequence identified upstream of the rat OdR-like sequences, cloning of genomic clones containing SCR G was performed. A putative phylogenetic tree relating SCRs to previously cloned full-length OdR sequences was constructed using the un-weighted pair group method with arithmetic mean analysis (24, 25) of the MacVector program (Oxford Molecular Group). Two sets of PCR primers were designed for pSCRs D and G to generate probes corresponding to the two halves of each PCR product. The probes were used to screen 2.5 million plaques, and labeled using a nick translation kit (Boehringer Mannheim). The probes were run on 6% polyacrylamide, 8 M urea precast TBE gels and were gel-purified. RNase protection assays were performed using mock dC-tailed control cDNA, were gel-purified (QiAEX, Qiagen) and reamplified by PCR with the adapter primer and primer D6. To confirm the presence of SCR D sequence in the RACE products, gel-purified bands were checked by PCR with D5 (MTS/VNCS, 5'-ATGCGATGCGATGTTCGTCTT), a 5'-primer to the presumptive amino terminus of SCR D and D6. If the appropriate 93-base pair D5-D6 product could be generated, the RACE product was subcloned into pCR II vector (Invitrogen) and sequenced with sp6 and t7 primers. The identical procedure was followed using the SCR G-specific 3'-primer G2 to generate first strand cDNA, a nested SCR G-specific primer G6 (YLALSMG, 5'-GCCCATGATGCGACGGCCGGAATG) for RACE-PCR with the anchor primer and primer D6. To determine if SCR transcripts in spleen and olfactory tissue like-wise underwent 5'-splicing, 1 µg of spleen poly(A)⁺ RNA and olfactory epithelium total RNA were reverse-transcribed as described above using primer G2 to generate first strand cDNA. PCR was then conducted using a 5'-primer to the SCR G RACE sequence (5'-ACACTCCAGATTGCCAATGCCAAGTA) and the 3'-G6 primer; the generated products were TA-cloned and sequenced.

To investigate the origin of the novel sequence identified upstream of the spermatid mRNAs of SCRs, genomic clones containing SCRs G-14, G-15, and D-9 were sequenced in the 5’ direction. PCR using primers designed against SCR D RACE sequence (5’-CTGAGTGATGCCATGGCAATG) and upstream SCR D genomic sequence (3’-AGATGCATACACGTTTCATG) was also performed on SCR D-9 phase DNA to identify intervening sequence.

To determine if SCR transcripts in spleen and olfactory tissue likewise underwent 5'-splicing, 1 µg of spleen poly(A)⁺ RNA and olfactory epithelium total RNA were reverse-transcribed as described above using primer G2 to generate first strand cDNA. PCR was then conducted using a 5'-primer to the SCR G RACE sequence (5’-ACCACCTCAAAGATTGCCAATGCCAAGTA) and the 3'-G6 primer; the generated products were TA-cloned and sequenced.

RESULTS

Two Novel Spermatid Chemoreceptor (SCR) Families Are Identified in Purified Round Spermatids—In a search for OdR-like proteins that may serve functional roles in reproduction, we reasoned that the post-meiotic and actively differentiating cells of the testis would be most likely to express chemoreceptors physiologically relevant to mature spermatozoa. Accordingly, we purified round spermatids from rat testis (Fig. 1a) to isolate total RNA from this select population of cells, and conducted RT-PCR experiments using degenerate primers (OdR 1 and 2) designed against highly conserved regions of the OdR family. A PCR product of 520 bp was generated using cDNA derived from the round spermatid total RNA (Fig. 1b). The 520-bp product corresponded in size to the band generated from PCR using the control template F2, an originally cloned Buck and Axel OdR. Whereas a 520-bp product was also obtained from testis cDNA, no product was obtained using whole brain cDNA. No products were obtained using specific genomic clones containing SCRs G-14, G-15, and D-9 were sequenced in the 5’ direction. PCR using primers designed against SCR D RACE sequence (5’-CTGAGTGATGCCATGGCAATG) and upstream SCR D genomic sequence (3’-AGATGCATACACGTTTCATG) was also performed on SCR D-9 phase DNA to identify intervening sequence.

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were generated when PCR was conducted using negative control samples from each tissue that were mock reverse-transcribed in the absence of reverse transcriptase. The spermatid PCR product was TA-subcloned, and subsequent EcoRI digestion of 14 clones revealed two recurrent restriction patterns, with the exception of clone B which did not contain an insert of the appropriate size (Fig. 1c). DNA sequencing of 13 clones identified two distinct and unique sequences (Fig. 1d) that aligned with members of the OdR family. All inserts were either identical to the sequence of clone D, which contained an internal EcoRI site, or matched the sequence of clone G. In contrast, sequencing of seven subcloned PCR products from the testis yielded seven different partial receptor sequences (data not shown), indicating that a multitude of receptor transcripts are generated in whole testis.

To obtain full-length SCRs, we screened a rat genomic library using probes generated from the partial SCR clones (Fig. 1d). Three distinct clones containing the pSCR D sequence were isolated and encode 321 amino acid proteins sharing >86% amino acid identity (Fig. 2a). Three distinct clones containing the pSCR G sequence were also identified and encode 327 amino acid proteins sharing >86% amino acid identity (Fig. 2b). The highly homologous clones within each family were determined to be individual gene products based on their unique 3′-genomic DNA sequence (Fig. 2, a and b). Hydropathy plots generated by Kyte-Doolittle analysis (23) predict that the receptors contain seven hydrophobic transmembrane domains (TMDs) (Fig. 2, a and b).

Multiple sequence alignment with previously cloned OdRs reveals that SCRs share sequence characteristics of the superfam-

ily, with conserved motifs in TMDs 2, 6, and 7 and in the extra-
cellular loop between TMDs 3 and 4.

To conceptualize the relationship of SCRs to other full-length OdR proteins, we constructed a putative phylogenetic tree using the Geneworks program (Fig. 3). Based on the program’s unweighted pair group method with arithmetic mean analysis (24, 25), the SCR G family is the most evolutionary divergent of all members of the OdR superfamily. The clustering of OdRs does not appear to correlate with tissue source or species of origin. For example, the SCR D family is only 35% identical to SCR G receptors and is more closely related to select mouse OdRs (OR3 and OR37) than to chemoreceptors expressed in dog and human testis (DTMT and HGMPO71, -E, and -J).
**Fig. 2.** Amino acid sequences of SCR families. Amino acid sequences of members of the SCR D (a) and SCR G (b) families are aligned. Regions of amino acid identity are boxed and shaded and conservative amino acid substitutions are shaded only. TMDs as predicted by hydrophathy plots are indicated above the corresponding sequence. The receptors are believed to be distinct gene products based upon differences in their 3’ genomic sequence present beyond the stop codons (marked with asterisks). SCR sequence data (including an additional D-7 receptor) are available from GenBank™ (AF034897-AF034903).
FIG. 3. A predicted phylogenetic tree demonstrates the relatedness of SCRs D and G with other full-length members of the OdR superfamily. The unweighted pair group method of the Genewoks program was used to construct a potential phylogenetic tree of SCRs and select full-length OdRs (F3, -5, -12 and I3, -7, -8, -9, -I4, and -15 (1); OR3 (41); HGMP07E, -I, -J, and DTM (7); OR5, 12.18,37 (4); TB534,567,641 (33); OL1 (34)). The calculated genetic distances between pairs of OdRs are presented numerically. The analysis indicates that members of the SCR G family are the most genetically distant OdRs. The clustering of OdRs does not appear to correlate with tissue source or species of origin.
sponding to pSCR D (D.1-D.4) and pSCR G (G.1-G.4) were employed in RNA protection assays using 10 μg of total RNA from pachytene spermatocytes, round spermatids, and olfactory tissue and 0.5 μg of poly(A)⁺ RNA from testis, lung, brain, liver, and spleen. Hybridization of the antisense riboprobe with the corresponding sense cRNA served as a marker for full-length protection. Full-length protection of both probes was observed with total RNA from round spermatids and olfactory tissue and with poly(A)⁺ RNA from testis and spleen. Prominent smaller bands representing partial riboprobe detection were generated from testis poly(A)⁺ RNA served as a negative control for the experiment.

**Tissue Localization of SCR D and G Transcripts—**RNA protection assays were used to confirm the presence of SCR D and G transcripts in spermatids of the testis. Full-length protection of SCR D and G riboprobes was observed with total RNA from round spermatids but not with RNA purified from spermatocytes in the pachytene stage of meiosis (Fig. 4). Full-length RNase protection was likewise achieved using total RNA from olfactory tissue, with multiple partially protected products generated by the SCR G riboprobe. Hybridization of antisense probes with the corresponding sense cRNAs served as markers for full-length protection. The integrity of template RNA was confirmed using a 125-bp β-actin riboprobe. In experiments utilizing poly(A)⁺ RNA, we observed full-length protection of SCR riboprobes in testis, with a less robust but detectable product in spleen. No RNase protection occurred with the yeast control RNA or with poly(A)⁺ RNA from brain, lung, or liver.

**In situ** hybridization was used to localize SCR D and G in rat testis (Fig. 5). The expression of SCR D and G is developmentally regulated, with the absence of detectable RNA message at age 2 weeks, when the seminiferous epithelium contains spermatogonia and spermatocytes but no spermatids (Fig. 5, a and b). SCR D and G transcripts were detected in select seminiferous tubule cross-sections of 6-week-old sexually maturing rats; by this stage of puberty, round spermatids are being produced by the second meiotic division (Fig. 5, c–f). Prominent hybridization with SCR D and G riboprobes was observed in 10-week-old rat testis (Fig. 5, g and h). The specific labeling seen with the antisense riboprobes was absent when sections were incubated with the corresponding sense riboprobes (Fig. 5, i and j). In rat, the cellular composition of seminiferous tubule epithelium varies along the length of the tubule according to a 14-stage growth cycle (27). Thus, different cross-sectional segments contain distinct groupings of spermatogonia, spermatocytes, and spermatids at a characteristic stage of rat spermatogenesis. High power images of **in situ** hybridization experiments with SCR D and G riboprobes demonstrated the absence of RNA message in spermatogonia and spermatocytes located peripherally to spermatids in the seminiferous tubule cross-sections (Fig. 5, e and f). In contrast, the more adluminal round and elongating spermatids showed robust hybridization with SCR riboprobes (Fig. 5, e and f). The SCR mRNA pattern is consistent with the localization of OdR protein to elongating spermatids in rat testis using antibodies generated against conserved OdR peptide sequences (8) and in dog testis using fusion protein antibodies directed against the DTMT receptor (9).

5’-Splicing of SCRs D and G—Several observations prompted us to pursue a comparative analysis of SCR cDNA and 5’-genomic sequence. 1) The splice acceptor sequence (28), TTTCTTTCAGG, occurred immediately upstream of the presumptive starting methionine in the genomic sequence of SCRs D-2, -8, and -9. 2) **In situ** hybridization studies comparing the localization of SCR D and G transcripts in serial sections of sexually mature testis revealed overlapping but also non-overlapping mRNA patterns among seminiferous tubule cross-sections (data not shown). This finding, coupled with the observed developmental expression of SCRs, suggested that distinct transcriptional and/or translational controls may govern the production of SCRs. 3) The introduction of constructs containing OdR coding sequences into transgenic mice has failed to produce detectable expression of OdR proteins.³ We reasoned that the functional expression of OdR proteins may require 5’-translated or -untranslated spliced sequence for message stability, membrane targeting, and/or receptor function.

**RACE-PCR experiments were conducted by employing SCR D- and SCR G-specific 3’-primers to generate first strand cDNAs from testis poly(A)⁺ RNA. RACE-PCR using the anchor primer and nested SCR 3’-primers yielded products for both SCRs D and G. SCR D RACE products contained sequence upstream of the SCR D-8 presumptive starting methionine that was distinct from the genomic clone, providing evidence of 5’-splicing (Fig. 6a). Interestingly, the novel sequence encodes an upstream open reading frame of 20 amino acids starting with a methionine at nt 60 which is preceded by a stop codon at nt −75. Theoretically, the RACE cDNA sequence could encode a 5’-MREPIPQSRHVL-KEEGFCLE amino-terminal extension, which is hydrophilic by Kyte-Doolittle analysis (23). Sequences of the SCR G RACE prod-

³ R. Reed, personal communication.
ucts are identical to the genomic SCR G-14 clone up to nt ~73, at which point novel upstream sequence was identified (Fig. 6b). At position ~90, a stop codon occurs, and no alternative methionine is encoded downstream. As could also be the case for SCR D, the novel SCR G RACE sequence likely represents 5’-untranslated sequence.

To confirm that the clones obtained by RACE-PCR represent 5’-splice products, SCR genomic clones were restriction digested with EcoRI/BamHI or HindIII/EcoRV, and Southern analysis was performed using probes corresponding to the novel 5’-sequence. The SCR G RACE probe, for example, recognized bands in SCR G-14 and G-15 digests, indicating that the novel 5’-RACE sequence was contained in the phage insert (data not shown). Sequencing of the SCR G-14 genomic clone identified the identical 140-bp SCR G RACE sequence 1475 bp upstream of the starting methionine (Fig. 7). In the SCR G-15 clone, sequence 88% identical to SCR G RACE was found 3503 bp upstream of the starting methionine (Fig. 7). In both cases, a splice acceptor site precedes the starting methionine by 73 bp. 5’-Sequencing and PCR experiments also led to identification of homologous SCR D RACE sequence in the upstream genomic DNA of SCR clone D-9 (data not shown). In contrast to SCR G receptors, the SCR D splice acceptor sequence occurs immediately adjacent to the presumptive starting methionine.

To determine if splicing of SCR transcripts likewise occurs in spleen and olfactory epithelium, PCR experiments were conducted using the 5’-SCR G RACE and 3’-nested G.6 primers on first strand cDNAs generated from both tissues with primer G.2. The identically spliced SCR G-14 product originally obtained from testis was detected in both spleen and olfactory epithelium, indicating that 5’-splicing of this SCR is a common phenomenon in these tissues.

To localize the spliced RNA sequences in rat testis, we conducted in situ hybridization studies using probes corresponding to the novel 5’-RACE sequences (Fig. 8b and data not shown). The 5’-spliced sequences were detected in adluminal spermatids, with robust hybridization occurring in seminiferous tubule profiles throughout the testis. Whereas in situ hybridization with a full-length SCR D-8 probe-labeled spermatids of select tubule profiles (Fig. 8a), a serial section incubated with a probe to the novel 5’-SCR D RACE sequence displayed a strong signal in many more profiles (Fig. 8b). This finding indicates that the 5’-spliced sequence is present in multiple mRNAs, in addition to the subset of SCR D-8 mRNAs localized by the

FIG. 5. SCR D and G in situ hybridization. Digoxigenin-labeled antisense riboprobes corresponding to full-length D-2 and G-14 receptors identified chemoreceptor transcripts in spermatids of sexually maturing and mature testis only. a and b, low power images of fresh-frozen testis from a 2-week-old rat are negative for SCR transcripts. c and d, SCR mRNAs are detected in select seminiferous tubule cross-sections in the testis of a sexually maturing 6-week-old rat. e and f, higher power images demonstrate the absence of RNA message in spermatogonia and spermatocytes located peripherally to spermatids in the seminiferous tubule cross-section (region demarcated by black arrowheads). The more adluminal round and elongating spermatids show robust hybridization with SCR riboprobes (white arrows). g and h, sexually mature testis from a 10-week-old rat contains high levels of SCR mRNAs in spermatids. i and j, the specific labeling seen with the antisense probes is completely absent when sections are incubated with the corresponding sense probes. Black bar (a–d and g–j) = 150 μm; white bar (e) = 75 μm; checkered bar (f) = 60 μm.
specific probe. This conclusion is consistent with our identification of conserved RACE sequence in the upstream genomic DNA of distinct members of a given SCR family. Although a search of the National Center for Biotechnology Information database did not identify the RACE sequences in other known cDNAs, our results do not exclude the possibility that the spliced sequences may also be present in other testis transcripts unrelated to SCRs.

**DISCUSSION**

In the present study, we have identified two families of putative OdRs in spermatids of rat testis, termed spermatid chemoreceptors (SCRs) D and G. The two classes of chemoreceptors are only 35% identical to each other, suggesting that they recognize disparate ligands and thereby subserves distinct functions. Within each family, the individual chemoreceptors are highly homologous, displaying at least 86% amino acid identity. The existence of nearly identical receptors may have resulted from gene duplication and may provide a biological back-up mechanism that ensures detection of signals important to the reproductive process. In contrast to the exclusive expression of one or at most a small number of OdRs by individual olfactory neurons (3, 6, 29), SCR D and G transcripts are abundant and widely detected among spermatids of the rat testis.

Whereas the identical pair of degenerate primers repeatedly identifies only two classes of chemoreceptors in rat spermatid cDNA, a much wider variety of partial OdR-like products are generated from whole testis cDNA. The physiologic relevance of OdRs in the testis has been questioned, because changes in chromatin structure during meiosis may generate spurious genetic transcripts. The identification of many partial receptor transcripts in whole testis compared with spermatids may lend support to this hypothesis or represent the requirement for multiple receptors to serve ecletic functions in spermatogenic cells. Nevertheless, the identification of SCRs D and G in the post-meiotic differentiating cells of the testis by RT-PCR, RNase protection assays, and in situ hybridization implies a physiologically relevant role for SCRs in mature spermatozoa. We previously used antibodies to conserved OdR peptide sequences to identify 64-kDa proteins in the membrane fractions of rat, hamster, and human spermatozoa (8). The molecular weights observed are roughly double the ~35-kDa mobility predicted from SCR amino acid sequence. Whereas glycosylation can account for some of the molecular weight disparities (30), others have detected multimeric forms of OdRs by immunoblot analysis (31). The latter observation may account for the approximate molecular weight doubling we observe in testis tissue and is believed to arise from the inability to completely disrupt inter-receptor hydrophobic interactions by denaturing gel electrophoresis. The localization of OdR protein to spermatids of the testis and to the midpiece of mature spermatozoa provides further support for the physiological relevance of such chemoreceptors in male reproductive function (8, 9). The localization of desensitization proteins (8) and inositol 1,4,5-trisphosphate receptor (10) to the tail midpiece, the respiratory center of mature sperm, provides additional intriguing support for the existence of a signaling mechanism to regulate mammalian sperm motility. Sengupta et al. (32) recently established an explicit functional role for Odr-10, a *Caenorhabditis elegans* olfactory receptor, in recognizing the molecule diacetyl, which they are to olfactory tissue-derived OdRs from the same or species unrelated to SCRs.

**FIG. 6. RACE-PCR provides evidence for 5‘-splicing of SCRs.** a, alignment of 5‘-flanking sequence obtained from genomic clone SCR D-8 and SCR D RACE-PCR demonstrates sequence identity beginning at the presumptive starting ATG (numbered +1 and marked by a black arrowhead) but completely different sequences immediately upstream. The novel 5‘-sequence obtained by RACE-PCR (underlined) has a potential translation initiation ATG at nt –60 (gray arrowhead) which is preceded by a stop codon at nt –75 (asterisk) and is in frame with the downstream ATG (+1, black arrowhead). b, alignment of genomic SCR G-14 with SCR G RACE sequence reveals novel 5‘-sequence (underlined) beginning at nt –73. A stop codon appears at nt –90 (asterisk) of the RACE product and is in frame with the presumptive starting ATG (numbered +1 and marked by a black arrowhead). The sequence data are available from GenBank™ (AF034896, AF034897, and AF034901).
different species. Vanderhaeghen et al. (36) have recently completed a thorough analysis of partial olfactory receptor transcripts identified by PCR from human, rat, dog, and mouse whole testis cDNA. Across the different species evaluated, the testicular clones show no specific sequence characteristics compared with OdRs identified in olfactory tissue; testicular receptors within a given species likewise do not cluster. However, the investigators do find highly homologous and possibly orthologous receptors in two species, with three of the receptor couples both detected in testis by RPA. Theoretically, the diversity of testicular receptors between species may enable species-specific positive and negative regulation of sperm-egg signaling, whereas orthologous receptors may serve roles common to spermatogenesis and sperm function across species.

The location of splice acceptor sites upstream of the presumptive starting methionine in genomic SCR clones led to the discovery that SCRs undergo 5′-splicing. Novel 5′-SCR D and G sequences were identified by RACE-PCR and localized in corresponding SCR genomic clones by upstream sequencing. Excluding a 2-kilobase pair gap in the genomic sequence of SCR G-14, alignment of SCRs G-14 and G-15 reveals stretches of homologous intron sequence (represented as wide-striped boxes in the schematic). The sequence data are available from GenBank™ (AF034901 and AF034902). Scale bar = 0.5 kilobase pairs.

**FIG. 7.** Identification of conserved SCR G RACE sequence in two distinct SCR G genomic clones. 5′-Genomic sequencing of SCRs G-14 and G-15 identified the SCR G RACE sequence (represented as black boxes labeled RACE G in the schematic and underlined in the sequence alignment below) at variable distances upstream of the respective starting methionines (arrowheads). In the SCR G-14 cDNA, the RACE sequence adjoins 73 nt upstream of the starting ATG (represented as a continuation of the black box in the schematic and marked with an asterisk in the alignment). The splicing of SCR G-14 is demonstrated by the arrow in the schematic. Excluding a 2-kilobase pair gap in the genomic sequence of SCR G-14, alignment of SCRs G-14 and G-15 reveals stretches of homologous intron sequence (represented as wide-striped boxes in the schematic). The sequence data are available from GenBank™ (AF034901 and AF034902). Scale bar = 0.5 kilobase pairs.
sequences in distinct members of a given family. Whereas the novel SCR G RACE sequence likely represents spliced 5′-untranslated DNA, SCR D RACE could theoretically encode an alternate translation initiation site. Use of this site could produce a hydrophilic 20 amino acid amino-terminal extension, potentially affecting SCR function. In general, splicing of OdR transcripts may play important and perhaps tissue-specific roles in regulating mRNA stability, translation, membrane targeting, and functional receptor expression.

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Note Added in Proof—Feinstein and co-workers recently showed functional expression of mammalian odorant receptors (Zhao, H., Otaki, J. M., Hadimoto, M., Mikoshita, K., and, Feinstein, S. (1998) Science 279, 237–242).

REFERENCES

1. Buck, L., and Axel, R. (1991) Cell 65, 175–187
2. Buck, L. B. (1992) Curr. Opin. Neurobiol. 2, 282–288
3. Ressler, K. J., Sullivan, S. L., and Buck, L. B. (1993) Cell 73, 597–609
4. Raming, K., Krieger, J., Strotmann, J., Boekhoff, I., Kubick, S., Baumbast, C., and Breer, H. (1993) Nature 363, 355–356
5. Kishimoto, J., Cox, H., Reverne, E. B., and Emson, P. C. (1994) Brain Res. Brain. Res. Rev. 23, 33–39
6. Vassar, R., Ngai, J., and Axel, R. (1993) Cell 74, 309–318
7. Parmentier, M., Libert, F., Schurmans, S., Schifflman, S., Lefort, A., Eggerickx, D., Ledent, C., Mollereau, C., Gerard, C., Perret, J., Grootegesel, A., and Vassart, G. (1992) Nature 355, 453–455
8. Walseny, I. D., Boskma, A. J., Keijerwitz, R. J., Snyder, S. H., and Ronnitz, G. V. (1995) Mol. Med. 1, 130–141
9. Vanderhaeghen, P., Schurmans, S., Vassart, G., and Parmentier, M. (1993) J. Cell Biol. 123, 1441–1452
10. Walseny, I. D., and Snyder, S. H. (1995) J. Cell Biol. 130, 857–869
11. Tash, S. J., and Means, A. R. (1983) Biol. Reprod. 25, 78–104
12. Weyand, I., Godde, M., Frings, S., Muller, F., Altenhofen, W., Hatt, H., and Kaupp, U. B. (1994) Nature 369, 859–863
13. Suzuki, N., and Garbers, D. L. (1984) Biol. Reprod. 30, 1167–1174
14. Suzuki, N., Normura, K., Ohkote, H., and Isaka, S. (1981) Biochem. Biophys. Res. Commun. 109, 1238–1244
15. Hanbrough, J., and Garbers, D. L. (1982) J. Biol. Chem. 257, 1447–1452
16. Cohen-Dayag, A., Tur-Kaspa, I., Dori, J., Mashiah, S., and Eisenbach, M. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 11039–11043
17. Eisenbach, M., and Rahl, D. (1992) Am. J. Physiol. 263, C1055–C1101
18. Shaper, N., Wright, W., and Shaper, J. (1990) Proc. Natl. Acad. Sci., U.S.A. 87, 791–795
19. MacDonald, R., Swift, G., Przybyla, A., and Chirgwin, J. (1987) Methods Enzymol. 152, 219–227
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 2.82–2.107 Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. McCombie, W. R., Heiner, C., Kelly, J. M., Fitzgerald, M. G., and Gocayne, J. D. (1992) DNA Seq. 2, 289–296
22. Smith, L. M., Sander, J. Z., Kaiser, R. J., Hughes, P., Dodd, C., Connel, C. R., Heiner, C., Kent, S. B., and Holm, L. L. (1988) Nature 331, 674–679
23. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132
24. Nei, M. (1987) Molecular Evolutionary Genetics, pp. 287–326, Columbia University Press, New York, NY
25. Fitch, W. M., and Margoliash, E. (1967) Science 155, 279–284
26. Blackshaw, S., and Snyder, S. H. (1997) J. Neurosci. 17, 8074–8082
27. Dym, M., and Clermont, Y. (1970) Ann. N. Y. Acad. Sci. 18, 205–206
28. Mouni, S. M. (1982) Nucleic Acids Res. 10, 459–472
29. Ngai, J., Chess, A., Dowling, M. M., Neces, N., Macagno, E. R., and Axel, R. (1993) Cell 72, 667–680
30. Krieger, J., Schleicher, S., Strotmann, J., Wanner, I., Boekhoff, I., Raming, K., De Geus, P., and Breer, H. (1994) Eur. J. Biochem. 220, 829–835
31. Nekrašová, E., Sausinaskaja, A., Natochkin, M., Lacant, D., and Gutiérrez (1996) Eur. J. Biochem. 238, 38–37
32. Sengupta, P., Chou, J. H., and Bargmann, C. I. (1996) Cell 84, 899–909
33. Thomas, M. B., Haines, S. L., and Akesson, R. A. (1996) Gene (Amst.) 178, 1–5
34. Drutel, G., Arrang, J. M., Dijz, J., Wennekes, C., Schwartz, K., and Schwartz, J. C. (1992) Receptors Channels 3, 33–40
35. Mombaerts, P., Wang, F., Dural, C., Chao, S. K., Nemes, A., Mendolsohn, J., and Axel, R. (1996) Cell 87, 675–686
36. Vanderhaeghen, P., Schurmans, S., Vassart, G., and Parmentier, M. (1997) Genomics 39, 239–246
37. Howard, T. E., Shai, S. Y., Langford, K. G., Martin, B. M., and Bernstein, K. E. (1990) Mol. Biol. Cell. 1, 4304–4309
38. Kilpatrick, D., Zinn, S. A., Fitzgerald, M., Higuchi, H., Sabol, S. L., and Meyerhardt, J. (1990) Mol. Cell. Biol. 10, 3717–3726
39. Asai, H., Kashi, H., Matsuda, Y., Yamazaki, N., Nagawa, F., Sakano, H., and Tsuboi, A. (1996) Biochem. Biophys. Res. Commun. 221, 240–247
40. Gu, W., Morales, C., and Hecht, N. B. (1995) J. Biol. Chem. 270, 236–243
41. Neff, P., Hermans-Borgmeyer, I., Artieres-Pin, H., Beasey, L., Dionne, V. E., and Heinemann, S. F. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 8948–8952

FIG. 8. Comparison of SCR D-8 and 5′-SCR D RACE localization by in situ hybridization. a, in situ hybridization using a full-length SCR D-8 antisense riboprobe labels spermatids of select seminiferous tubule profiles (marked by +) in the testis of a 10-week-old rat. b, a serial section treated with a probe to the novel 5′-SCR D RACE sequence detects a strong signal in all of the tubule profiles. Scale bar = 110 μm.
