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Identification and localization of secretin and secretin receptor mRNAs in rat testis

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Secretin is a well-conserved member of the growth hormone-releasing hormone (GHRH) family of peptides expressed in brain, gut and gonads. To determine whether secretin may also play a physiological role in testis, we examined the level and cellular distribution of secretin and secretin receptor gene expression in rat testis. RNAs from total testis, Sertoli, germ and Leydig cells were amplified by comparative reverse transcription-polymerase chain reaction (RT-PCR). Southern blot analysis of the PCR products indicated secretin and secretin receptor mRNA expression primarily in germ cells. Sequence comparisons of cloned secretin and secretin receptor PCR products showed 100% identity with the previously reported sequences. To localize secretin and secretin receptor mRNAs at the cellular level within testis, *in situ* hybridization was performed. Specific hybridization to secretin mRNA was observed in low abundance in many germ cell types, but was heaviest over step 19 spermatids in stages VII and VIII tubules. Secretin receptor mRNA was detected in approximately the same cell types as was secretin mRNA, except that labeling was greatest in round spermatids (steps 6–8). Since the patterns of gene expression of secretin and its receptor overlap, these data suggest that there may be an intrinsic secretin system in testis.

Keywords: Secretin; growth hormone-releasing hormone; testis; spermatogenesis; RT-PCR; *in situ* hybridization

Introduction

Secretin is a very well-conserved member of the growth hormone-releasing hormone (GHRH) family of gut and neuropeptide hormones. This family includes GHRH, pituitary adenylyl cyclase activating peptide (PACAP) and its related peptide (PRP), vasoactive intestinal peptide (VIP) and its related peptide histidine-isoleucine (PHI), glucagon and the glucagon-like peptides (GLP-1&2), gastric inhibitory peptide (GIP) and secretin. Some of these peptides have been localized to the brain, gut and recently to the gonads (Campbell & Scanes, 1992; Ohta *et al.*, 1992).

Secretin was the first hormone to be characterized; it is a 27-amino acid peptide originally isolated from the duodenum (Bayliss & Starling, 1902). Consistent with its known essential functions, secretin is highly conserved throughout evolution: canine secretin differs from that of human or porcine by only a single amino acid (Carlquist *et al.*, 1985; Shinomura *et al.*, 1987); rat and porcine secretins differ also by a single amino acid (Gossen *et al.*, 1989; Mutt *et al.*, 1970). Secretin mRNA has a wide tissue distribution in rat, including small intestine, kidney, heart, lung, brain and testis (Ohta *et al.*, 1992). Secretin is now known to act via specific cell surface receptors, one of which has been isolated to date. This receptor couples to $G_{s\alpha}$ to activate adenylyl cyclase (Ishihara *et al.*, 1991). Secretin receptor mRNA has been found in rat stomach, pancreas and heart (Ishihara *et al.*, 1991).

Other members of the GHRH peptide family, their receptors, or their messages have been localized to specific testicular cell types (Srivastava *et al.*, 1993b, 1994; Shioda *et al.*, 1994). Since little is known about secretin activity or localization in testis, we sought to determine the cellular site of transcription of testicular secretin and to identify and localize a secretin receptor mRNA, within rat testis.

Results

Production of secretin and secretin receptor mRNAs was localized within testis by comparative RT-PCR analysis of RNA from whole testis, Sertoli, germ and Leydig cells. Histone 3.3 was used as a control for the efficiency of the RT-PCR reactions.

Secretin receptor

A product of the expected size (122 bp) hybridizing to the secretin receptor oligonucleotide probe is detected primarily in whole testis and in germ cells (Figure 1). The cloned PCR product was sequenced and found to be identical to the corresponding segment of rat secretin receptor previously published (Ishihara *et al.*, 1991) (Figure 2).

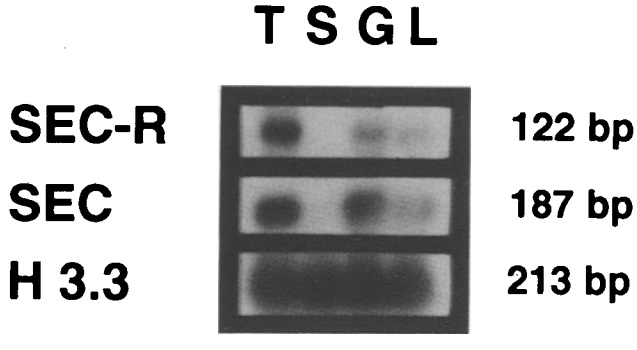

Figure 1 Analysis of secretin and secretin receptor mRNAs in separated testicular cells. Total RNAs from whole testis (T), Sertoli (S), germ (G) and Leydig (L) cells were amplified by comparative RT-PCR as described under Methods. The PCR products were separated by electrophoresis on 1.8% agarose gels, transferred to nylon membranes and hybridized to ^{32}P-labeled oligonucleotide probes from the sequences amplified. top, secretin receptor mRNA; center, secretin mRNA; and bottom, histone 3.3 mRNA, using the primers described under Methods. This figure is representative of three separate experiments, using tissue from at least three different rats

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Secretin receptor

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                .         .         .         .         .
Testis        1 ttgaggaagcagtaaaggacagctaccaccagaccctggaaggagcccag 50
                ||||||||||||||||||||||||||||||||||||||||||||||||||
Ishihara   1387 TTGAGGAAGCAGTAAAGGACAGCTACCACCAGACCCTGGAAGGAGCCCAG 1338
et al.,
1991

                .         .         .         .         .
             51 ggccagttcgaagaacagctggacctccatggcgtcctctggggagaagg 100
                ||||||||||||||||||||||||||||||||||||||||||||||||||
           1337 GGCCAGTTCGAAGAACAGCTGGACCTCCATGGCGTCCTCTGGGGAGAAGG 1288


                .         .
            101 cgaagacaatgtagtggatgcc 122
                |||||||||||||||||||||||
           1287 CGAAGACAATGTAGTGGATGCC 1266
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Secretin

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                .         .         .         .         .
Testis        1 gaaaatattccagagaacagcgtggcccgtcccaagccattagaggacca 50
                ||||||||||||||||||||||||||||||||||||||||||||||||||
Kopin      225 GAAAATATTCCAGAGAACAGCGTGGCCCGTCCCAAGCCATTAGAGGACCA 274
et al.,
1990

                .         .         .         .         .
             51 actctgcttgctgtggtcgaacactcaggccctacaggattggcttctgc 100
                ||||||||||||||||||||||||||||||||||||||||||||||||||
           275 ACTCTGCTTGCTGTGGTCGAACACTCAGGCCCTACAGGATTGGCTTCTGC 324


                .         .         .         .         .
            101 ccaggctgtccctggatgggtccctgtctctctggctgcctcctggacca 150
                ||||||||||||||||||||||||||||||||||||||||||||||||||
           325 CCAGGCTGTCCCTGGATGGGTCCCTGTCTCTCTGGCTGCCTCCTGGACCA 374


                .         .         .
            151 aggcctgctgtcgaccattcagagtggactgaaacaa 187
                |||||||||||||||||||||||||||||||||||||
           375 AGGCCTGCTGTCGACCATTCAGAGTGGACTGAAACAA 411
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Figure 2 Sequence comparisons of RT-PCR clones of testicular secretin and secretin receptor show 100% identity with published sequences

Secretin

A product of the expected size (187 bp) hybridizing to the secretin oligonucleotide probe is detected predominantly in whole testis and in germ cells (Figure 1). PCR primers for secretin were designed such that the sequence amplified spans an intron. Absence of genomic contamination of the RNA was confirmed by the appearance of only one band, of the expected size, on gels and blots of the PCR reaction mixtures. To verify that the secretin RT-PCR product was the same as that cloned from duodenum, the PCR product from whole testis was cloned and sequenced. Comparison with the published sequence shows 100% identity with the corresponding segment of rat duodenal secretin (Kopin *et al.*, 1990) (Figure 2).

We next used *in situ* hybridization histochemistry to determine the cellular localization of the secretin and secretin receptor messages within the testis.

Secretin receptor

A ^{35}S-labeled riboprobe generated from the secretin receptor PCR clone was hybridized to testis sections from adult rats. In low-power magnification darkfield microscopy, stage-specific expression of secretin receptor mRNA is evident, as specific signal is not seen equally in all tubules (Figure 3). Higher power brightfield examination reveals specific hybridization signals concentrated over round spermatids (steps 6–8) mainly in midcycle tubules (Figure 4). Detectable signal is seen over developing spermatids within tubules at all stages. No signal above background is discerned in Sertoli or interstitial cells.

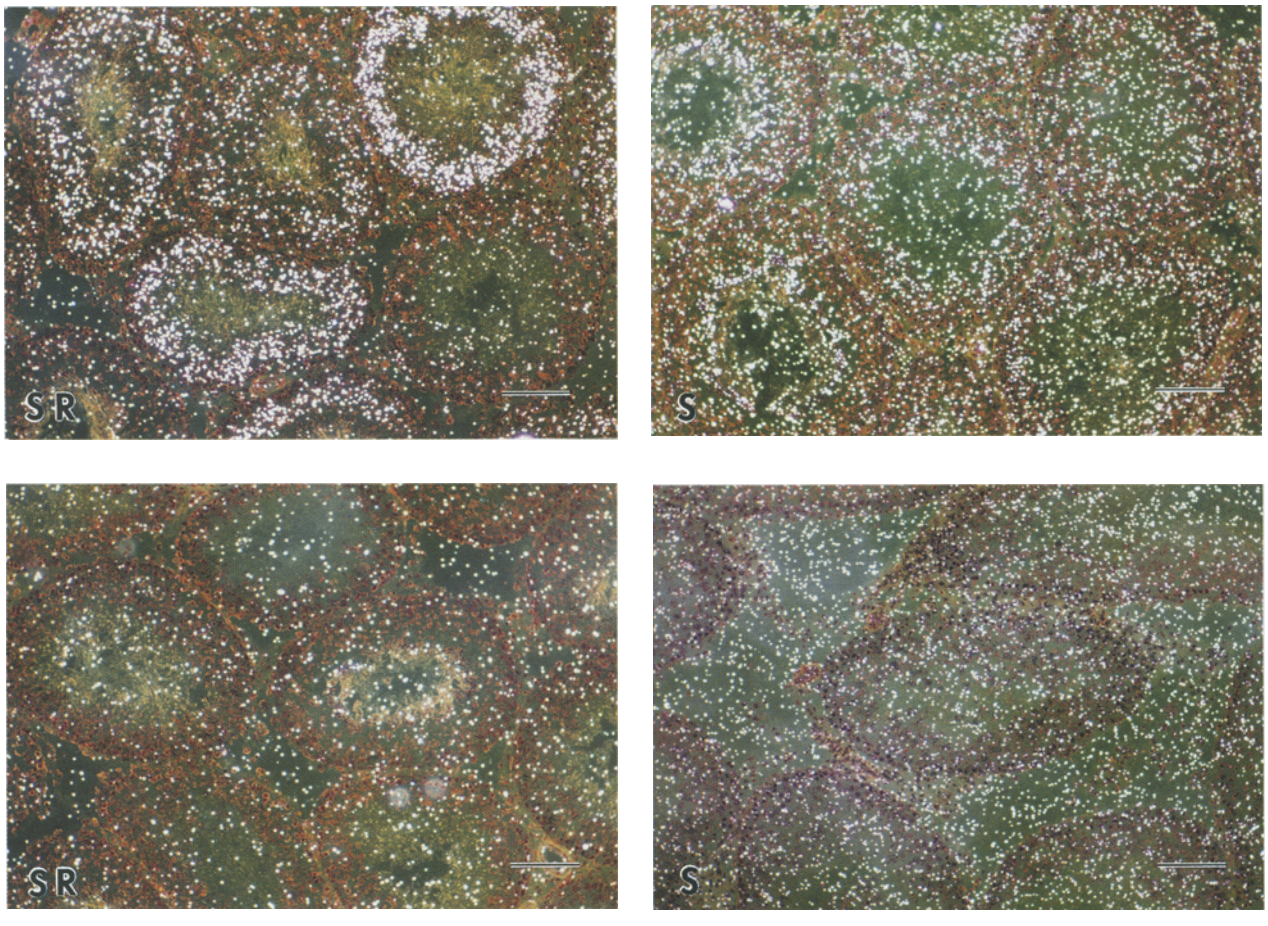

Figure 3 *In situ* hybridization of rat secretin (S) and secretin receptor (SR) riboprobes to rat testis sections, visualized by low-power darkfield microscopy. Upper panels, stage-specific hybridization to antisense secretin receptor (SR) and secretin (S) probes. Bottom panels, hybridization to sense strand probes (negative controls). This figure is representative of three separate experiments, using tissues from at least four different rats. Scale bars = 100 μm

Secretin

A ^{35}S-labeled riboprobe generated from the secretin PCR clone was then hybridized to adult rat testis sections. Stage-specific expression of secretin mRNA is evident (Figure 3), with most of the hybridization signal over elongating (step 19) spermatids in stages VII–VIII tubules (Figure 4). Low levels of secretin mRNA hybridization were seen over developing spermatids within tubules of all stages and over more advanced spermatocytes. Spermatocytes and other germ cells that resided near the base of the epithelium were small and thus it was difficult to determine if the cells were labeled.

Sense strand riboprobes used as negative controls revealed no signals above background (Figure 3). Localization of secretin and secretin receptor messages is summarized in Figure 5.

Discussion

We have confirmed earlier findings that the mRNA for secretin is present in rat testis (Ohta *et al.*, 1992), and have extended these findings to the localization of secretin mRNA to testicular germ cells. More importantly, a secretin receptor mRNA also was identified and localized to spermatogenic cells. We have demonstrated stage-specific expression of these mRNAs and determined that, in general, secretin mRNA is expressed in more mature spermatogenic cells than those that express secretin receptor mRNA.

The current results provide initial support for a functional role of secretin in testis. Spermatogenic cells contain the most abundant transcripts for secretin and secretin receptor. Since PCR is extremely sensitive, it is possible that there is some contamination of one cell type by another; however, the localization of these transcripts by *in situ* hybridization strongly supports the PCR results.

The presence of a secretin receptor mRNA in round spermatids suggests that secretin may act in these cells. Support for the concept that germ cell products have unique functions comes from the fact that several testicular transcripts are expressed in specific germ cells in highly constrained patterns. For example, certain c-*mos*, *pim*-1, c-*abl* and *Wnt*-1 transcripts are selectively expressed in spermatids, as reviewed (Kierszenbaum, 1994). Also, spermatids preferentially express c-*ras N* transcripts, whereas earlier-stage spermatogenic cells express c-*ras H* and c-*ras K* (Sorrentino *et al.*, 1988). Also, only post-meiotic spermatogenic cells express protamine (Kleene *et al.*, 1985).

Evidence for the testicular production of members of the GHRH family of peptides, as well as their receptors, has accumulated in recent years. Several members of this peptide family have been shown to affect testicular cell function. GHRH induces Sertoli cell expression of mRNAs for c-*fos* and stem cell factor (SCF) (Srivastava *et al.*, 1993a), which are known to be essential for normal spermatogenesis (Geissler *et al.*, 1988; Huang *et al.*, 1990; Johnson *et al.*, 1992). GHRH also potentiates hCG-stimulated steroidogenesis in rat Leydig cells (Ciampani *et al.*, 1992). There is evidence that PACAP, which is transcribed in germ cells (Shioda *et al.*, 1994), also has specific binding sites on germ cells (Shivers *et al.*, 1991). PACAP also stimulates secretion of lactate, estradiol and inhibin in rat Sertoli cells (Heindel *et al.*, 1992). Secretin, VIP, glucagon and PHM-27 stimulate progesterone and testosterone production in neonatal rat testicular cells (Kasson *et al.*, 1986) and VIP stimulates cAMP accumulation in cultured rat Leydig cells (Hueso *et al.*, 1987; Ciampani *et al.*, 1992). Further support for physiological significance comes from the developmentally regulated testicular expression demonstrated for some of these mRNAs (Berry & Pescovitz, 1990; Pescovitz *et al.*, 1994).

Some testis-specific mRNA isoforms have unique attributes that are thought to have functional significance in testis. Some of these include 3′ sequences that protect the mRNAs from degradation, allowing the delayed transcription typical of postmeiotic germ cells to take place (Braun *et al.*, 1989). Other testis-specific transcripts have unique 5′ sequences that may direct their transcription in response to signals relevant to testicular function. Precedence for tissue-specific transcrip-

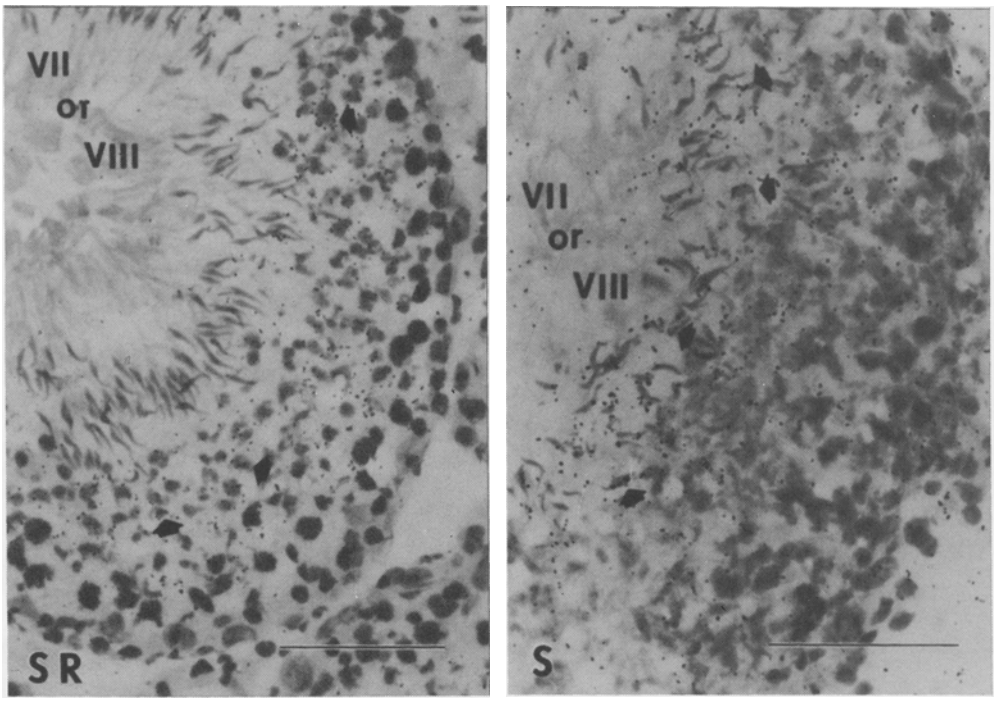

Figure 4 *In situ* hybridization of rat secretin and secretin receptor riboprobes to rat testis sections, visualized by high-power, brightfield microscopy. In both figures tubules are at stage VII or VIII of the spermatogenic cycle. Hybridization to antisense secretin receptor (SR) probe reveals round spermatids containing clusters of grains, indicated by arrows. Hybridization to antisense secretin (S) probe shows elongating (step 19) spermatids overlain by clustered grains, indicated by arrows. Scale bars = 50 μm

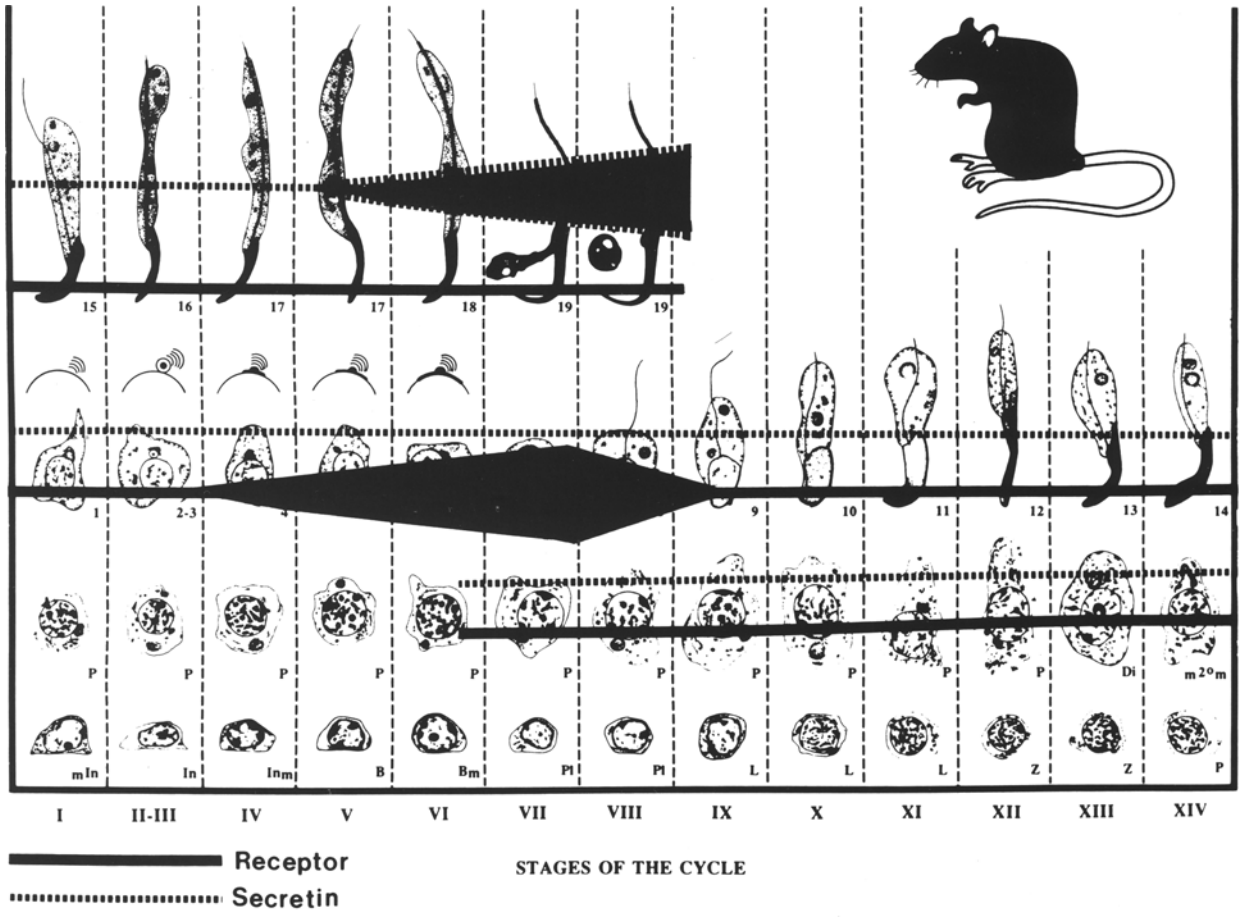

Figure 5 Map summarizing the labeling for secretin receptor message (solid line) and for secretin message (stippled line) in frozen sections. The cell types labeled are crossed by their respective lines. The thickness of the lines indicates the relative labeling intensity. Although both lines show labeling beginning at approximately stage VI of the spermatogenic cycle, the size of germ cells and the intensity of labeling in cells at stages earlier than step six prohibited us from determining if these cells were labeled

tional regulation comes from the example of GHRH, which has unique first exons transcribed in hypothalamus, placenta and testis (Mayo *et al.*, 1985; Gonzalez-Crespo & Boronat, 1991; Srivastava *et al.*, 1995).

The future characterization of testicular secretin and receptor peptide(s) should help to elucidate possible functional roles in testis. Meanwhile, the presence of both secretin and secretin receptor transcripts in testis suggests that an intrinsic secretin system may have a role in testicular function.

Materials and methods

Animals

Adult male Sprague-Dawley rats obtained from Harlan (Indianapolis, IN) were killed by decapitation, and testes were rapidly dissected and decapsulated. All animals were maintained in accordance with the guidelines set by the NIH Guide for the Care and Use of Laboratory Animals, as well as the Animal Use and Care Committee of Indiana University School of Medicine.

Cell fractionation and Sertoli cell culture

Germinal cells were isolated using sequential collagenase and trypsin treatment of adult testis essentially as described (Romrell *et al.*, 1976). Leydig cells were isolated by collagenase treatment of adult seminiferous tubules followed by ficoll gradient purification (Janszen *et al.*, 1976; Rommerts *et al.*, 1985) using modifications previously described (Garrett & Douglass, 1989). Cell purity was monitored by phase contrast microscopy and showed variable germ cell contamination that was usually $<5\%$. Sertoli cell cultures were prepared from 20–22 day-old rats (Dorrington & Fritz, 1975) and cultured at 32°C in Ham's F-12 medium supplemented with 9 mM HEPES, pH 7.5, 215 μg/ml L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamicin and 0.625 μg/ml fungizone. Medium was changed the third day after plating and RNA was isolated 24 h later.

Primers and probes

Secretin and secretin receptor PCR primers and oligonucleotides used for Southern blot analysis, were synthesized using an Applied Biosystems Oligonucleotide Synthesizer (Foster City, CA). For comparative RT-PCR analysis, secretin receptor primers were generated from membrane spanning domains 6 (5′-CGCTCTTTGGCATCCACTACAT-3′) and 7 (5′-TGAGGAAGCAGTAAAGGACAGC-3′). Secretin primers, generated from exons 3 (5′-GAAAATATTCCAGAGAACAGCGTGG-3′) and 4 (5′-TTGTTTCAGTCCACTCTGAATGGTC-3′) yield a 187 bp PCR product. Secretin and secretin receptor PCR products were cloned (see below) and used for probes. Histone 3.3 primers generate a 213 bp DNA fragment after PCR, as described previously (Kelley *et al.*, 1993), from which a probe was made for hybridization to Southern blots. Probes were ^{32}P-labeled by the hexanucleotide primer method (Feinberg & Vogelstein, 1983), using a kit from Stratagene (La Jolla, CA).

Reverse transcription (RT) and polymerase chain reaction (PCR) analysis

RNA was isolated by the guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987) subsequent procedures were performed as described (Wilson *et al.*, 1992; Kelley *et al.*, 1993). Three micrograms of total RNA were used for reverse transcription; five microliters of the reverse transcription reaction from each sample were incubated in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM$MgCl_2$, 200 μM each of deoxynucleotide triphosphate, 0.01% gelatin, 0.01% Tween-20, 0.01% Nonidet P-40, 2 μM of each primer, and 2 U Taq polymerase (US Biochemical, Cleveland, OH). The polymerase amplification was carried out in a PTC-100 Thermal Controller (Medical Research, Inc.), using 25 cycles of amplification (95°C, 30 s; 55°C, 1 min; 72°C, 2 min), followed by 10 min at 72°C. For negative controls, each RNA was treated as above, but with reverse transcriptase omitted; also, an RT-PCR reaction was performed with no RNA.

Southern blot analysis

After PCR, 15–20 μl of the reaction products were separated by electrophoresis using 1.8% agarose gels and transferred to MagnaGraph nylon membranes. (Micron Separations, Inc., Westboro, MA). The blots were hybridized to either histone 3.3, secretin or secretin receptor ^{32}P-labeled oligonucleotide probes under conditions previously described (Srivastava *et al.*, 1993b). Autoradiograms of the quantitative PCR were scanned on a Microtek Scanmaker II, and data were quantitated using Sigma Scan (Jandel Scientific, San Rafael, CA). Secretin and secretin receptor autoradiographic signals were normalized to that of histone 3.3 PCR products.

Cloning and sequencing of PCR products

After PCR, 15–20 μl of the reaction products were separated by electrophoresis using 1.5–1.8% agarose gels. Bands of the proper size were excised and purified using a Spin-X column (Costar, Cambridge, MA). The secretin receptor DNA fragments were cloned using the TA version 1.3 system (Invitrogen, San Diego, CA). The secretin DNA fragment was cloned using the Clone-Amp system (Gibco BRL, Gaitherburg, MD), as previously described (Kelley *et al.*, 1993). Cloned secretin and receptor products were used to transform *E. coli* HB101 cells. The resulting colonies were used for DNA sequencing, by the dideoxynucleotide method of Sanger *et al.* (Sanger *et al.*, 1977), which employed a Sequenase kit (US Biochemical, Cleveland, OH), [^{35}S]dATP and SP6 and T7 primers. Sequence analysis was performed using the University of Wisconsin Genetics Computer Group sequence analysis software (Devereux *et al.*, 1984).

In situ *hybridization*

Immediately after dissection, testes were snap frozen in isopentane and then stored at −70°C. Sections of 10 μm thickness were cut in a cryostat, thaw-mounted onto poly-L-lysine coated slides, and stored at −70°C until hybridization. Cloned rat secretin and secretin receptor PCR products were used for the synthesis of cRNA probes. ^{35}S-labeled antisense and sense riboprobes were synthesized as described (Bondy *et al.*, 1993). The average specific activity of probes generated by this protocol was 2.5×10^8 dpm/μg. Prior to hybridization, tissue sections were warmed to 25°C, fixed in 4% formaldehyde and acetylated in 0.25% acetic anhydride, 0.1 M triethanolamine hydrochloride, 0.9% NaCl. Tissue was dehydrated through an ethanol gradient, delipidated in chloroform, rehydrated and air dried. Hybridization buffer was added to sections, which were then covered with glass coverslips and placed in humidified chambers overnight (14 h) at 55°C. Slides were washed several times in 4 × saline-sodium citrate (SSC) to remove coverslips and hybridization buffer, dehydrated and immersed in 0.3 M NaCl, 50% formamide, 20 mM Tris HCl, 1 mM EDTA at 60°C for 15 min. Sections were then treated with RNase A (20 μg/ml; Boehringer Mannheim, Indianapolis, IN) for 30 min at 37°C and passed through graded salt solutions, followed by a 15 min wash in 0.1 × SSC at 50°C. Slides were dehydrated, air dried and apposed to Hyperfilm-beta Max (Amersham, Arlington Heights, IL) for 1–5 days. To generate emulsion autoradiograms slides were dipped in NTB3 nuclear emulsion (Kodak, Rochester, NY) and stored with dessicant at 4°C for 3–15 days. Slides were developed using Kodak D19 developer and fixer, and stained with hematoxylin and eosin for microscopic evaluation.

Staging criteria and methodology

The staging criteria of (Leblond & Clermont, 1952) as modified by (Russell *et al.*, 1990) were used to stage tubules from frozen sections. While acrosomal development was not visible in frozen sections, the tubules could be classified into three groups as follows: (1) those with two generations of spermatids and the spermatids were within crypts of the Sertoli cell (stages I–VI), (2) those with two generations of spermatids and the spermatids lined the seminiferous epithelium (stages VII–VIII), and (3) those with one generation of elongating spermatids (stages VIII–XIV). Tubules in these three categories were examined to determine the logical sequence of labeling and the pattern of labeling placed on a stage map for the rat.

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