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Expression of the Neurotransmitter-Synthesizing Enzyme Glutamic Acid Decarboxylase in Male Germ Cells

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The gene encoding glutamic acid decarboxylase (GAD), the key enzyme in the synthesis of the inhibitory neurotransmitter γ-aminobutyric acid, is shown to be expressed in the testis of several different species. Nucleotide sequence analysis of a cDNA clone isolated from the human testis confirmed the presence of GAD mRNA in the testis. The major GAD mRNA in the testis was 2.5 kilobases. Smaller amounts of a 3.7-kilobase mRNA with the same size as GAD mRNA in the brain was also detected in the testis. In situ hybridization using a GAD-specific probe revealed GAD mRNA expressing spermatocytes and spermatids located in the middle part of rat seminiferous tubules. Studies on the ontogeny of GAD mRNA expression showed low levels of GAD mRNA in testes of prepubertal rats, with increasing levels as sexual maturation is reached, compatible with GAD mRNA expression in germ cells. In agreement with this, fractionation of cells from the rat seminiferous epithelium followed by Northern (RNA) blot analysis showed the highest levels of GAD mRNA associated with spermatocytes and spermatids. Evidence for the presence of GAD protein in the rat testis was obtained from the demonstration of GAD-like immunoreactivity in seminiferous tubules, predominantly at a position where spermatids and spermatozoa are found. Furthermore, GAD-like immunoreactivity was seen in the midpiece of ejaculated human spermatozoa, the part that is responsible for generating energy for spermatozoan motility.

Neurotransmitter-synthesizing enzymes are predominantly expressed in the nervous system, where their enzymatic products function in synaptic transmission. A similar restricted pattern of gene expression has also been inferred for neuropeptides, and a large amount of evidence suggests that these peptides also function in neurotransmission. However, in recent years, several neuropeptides have also been shown to be expressed in nonneuronal tissues, suggesting a broader function for these peptides than heretofore anticipated. Thus, oxytocin and vasopressin have been shown to be expressed in the thymus (12). Preprotachykinin A mRNA, the common precursor for substance P and neurokinin A, is also expressed in the thymus (A. Ericsson, V. Geenen, F. Robert, J.-J. Legros, Y. Vrindts-Gevaert, P. Franchimont, S. Brené, and H. Persson, Mol. Endocrinol., in press). In the rat, neuropeptide Y mRNA and peptide have been detected in megacaryocytes and lymphocytes, respectively (10, 33). Furthermore, mRNAs encoding the opioid precursor proteins proopiomelanocortin (POMC) and preproenkephalin are expressed in mitogen-stimulated B and T lymphocytes, respectively (43, 46, 51).

In particular, several different neuropeptides have been shown to be expressed in reproductive tissues. POMC-derived peptides were first observed in the testis by using immunocytochemistry (30, 45). Subsequently, Northern (RNA) blot analysis and in situ hybridization were used to document that the POMC gene is locally expressed in the testis, predominantly in interstitial Leydig cells associated with discrete tubule stages of the seminiferous epithelium (4, 14, 37). Although the overall role of opioid peptides in the testis is poorly understood, β-endorphin has been reported to modulate testosterone secretion by Leydig cells and to suppress Sertoli cell growth in neonatal rats (13).

The two other opioid peptide precursors, preproenkephalin and prodynorphin, have also been shown to be widely expressed in both male and female reproductive tissues from many different species (8, 23). Proenkephalin mRNA is mainly associated with male germ cells with an enrichment in spermatocytes and spermatids, and enkephalin-derived peptides have been suggested to function as germ cell-associated hormones or autocrine-paracrine factors (22). On the other hand, prodynorphin-derived peptides are mostly present in interstitial Leydig cells (8). Also, corticotropin-releasing factor has been detected in rat Leydig cells and suggested to modulate Leydig cell function (44, 49).

More recently, the gene encoding the neurotransmitter-neuromodulator cholecystokinin (CCK) has been shown to be expressed in male germ cells of many different species in which peptide accumulates in the acrosomal granule (34–36). This, together with the recent finding of functional CCK receptors on *Xenopus laevis* oocytes (31), has led to the suggestion that CCK may be one of the components that activate the oocyte for fertilization through a receptor-mediated pathway (36).

In this study, we report on the expression of mRNA encoding glutamic acid decarboxylase (GAD), the key enzyme in the synthesis of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (39, 47, 48), in male germ cells of several different species. GAD-like immunoreactivity (GAD-LI) was detected in seminiferous tubules of rat testes, and GAD-LI was also found in the midpiece of ejaculated human spermatozoa, where mitochondria which power the flagellum are located. A preliminary report describing the detection of GAD mRNA in the rat testis has recently appeared (34).

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MATERIALS AND METHODS

Preparation of rat testicular cells. Testicular cells were prepared from five pooled testes from 60-day-old Sprague-Dawley rats by enzymatic digestion and centrifugal elutriation on the basis of a method described by Bucci et al. (3) with several modifications.

Testes were decapsulated, weighed, and placed in McCoy 5A medium containing 1.0 mM sodium pyruvate, 0.6% (wt/vol) sodium lactate, and collagenase (type 1; Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 150 U/ml. The tissue was incubated for 15 to 20 min at 32°C with continuous shaking until the interstitial connective tissue was degraded. The seminiferous tubules were washed three times by sedimentation at 1 × *g*. The washing medium was pooled and centrifuged to collect the released interstitial cells. After two washings, the interstitial cells were pelleted and frozen at −70°C as described below. To release tubular cells, the collected seminiferous tubules were further incubated for 5 min at 32°C with DNase (type 1, 100 U/ml; Sigma) followed by trypsin (50 U/ml; Worthington Diagnostics, Freehold, N.J.) for 15 to 20 min with continuous shaking. A mixture of fetal calf serum (final concentration, 10% [vol/vol]), DNase (type 1, 100 U/ml), and soybean trypsin inhibitor (1.25 mg/ml; Sigma) was then added, and the tubular fragments were dispersed with a Pasteur pipette to release the cells. To remove cell aggregates, the cell suspension was passed through three nets with pore sizes of 315, 80, and 25 μm and then pelleted before suspension in phosphate-buffered saline containing 1.0 mM sodium pyruvate, 0.6% sodium lactate, 2% fetal calf serum, 0.5% bovine serum albumin, soybean trypsin inhibitor (0.25 mg/ml), and DNase (type 1, 100 U/ml) at 4°C.

Cell separation. Cell separation was carried out at 4°C in elutriation buffer (phosphate-buffered saline without Ca^{2+} and Mg^{2+} containing 0.1% glucose, 0.6% sodium lactate, 1.0 mM sodium pyruvate, 5 mM EDTA, 1% fetal calf serum, and 0.5% bovine serum albumin) by using an elutriation rotor (model JE-G; Beckman Instruments, Inc., Fullerton, Calif.) as previously described (42). Cells (4.5×10^8) in elutriation medium were loaded, and the first fraction was collected at a rotor speed of 2,200 rpm and a flow rate of 15 ml/min. Fraction 2 was obtained at a rotor speed of 1,800 rpm, and fraction 3 was obtained after further increasing the flow rate to 25 ml/min. Fraction 4 was obtained by stopping the rotor

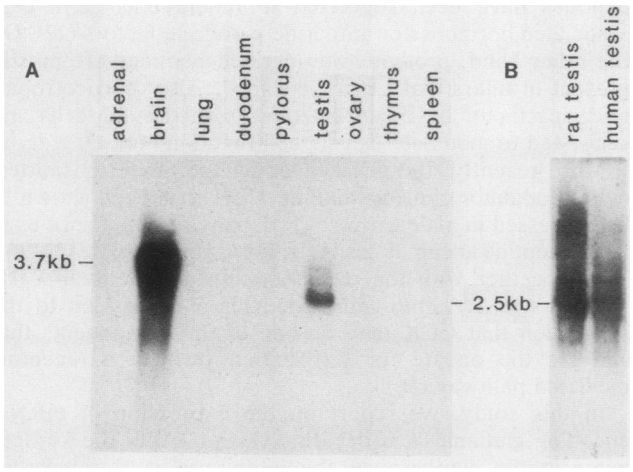

FIG. 1. Detection of GAD mRNA in various rat organs. (A) Poly(A)$^+$ RNA (20 μg) prepared from the indicated organs of adult male or female (for ovary) Sprague-Dawley rats was electrophoresed in a 1% agarose gel containing 0.7% formaldehyde and transferred to a nitrocellulose filter. The filter was then hybridized to a nick-translated feline GAD cDNA fragment. Filters were washed at high stringency and exposed to Kodak XAR-5 film for 2 days. (B) Same analysis as described for panel A, using poly(A)$^+$ RNA from either the rat testis or the human testis.

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A
human      1 CAGACAGCCCTCAACGACGGGAAAAGCTACACAAGGTGGCTCCAAAAATC
             |||| |||||| |||||||||||||| ||||||| |||||| || ||||||
feline  1694 CAGATAGCCCTGAACGACGGGAAAAACTACACAGGGTGGCCCCCAAAATC
                                                          .  *
           51 AAAGCCCTGATGATGGAGTCAGGTACGACCATGGTTGGCTACCAGCCCCA
              |||||||||||||||||||| || ||||||||||||||||||||| ||||
         1744 AAAGCCCTGATGATGGAGTCTGGCACGACCATGGTTGGCTACCAG.CCCA
                .    .    .    .    .    .    .    .    .
          101 AGGGGACAAGGCCAACTTCTTCCGGATGGTCATCTCCAACCCAGCCGCTA
              |||||||||||||||| |||||||||||||||||| |||||||| ||||
         1793 GGGGGACAAGGCCAACTTTTTCCGGATGGTCATCTCGAACCCAGCTGCTA
                .    .    .    .    .    .    .    .    .
          151 CCCAGTCTGACATTGACTTCCTCATTGAGGAGATAGAAAGACTGGGCCAG
              | |||||| || |||||||||||||| |||||||||||||||||||||||
         1843 CACAGTCCGATATTGACTTCCTCATCGAGGAGATAGAAAGACTGGGCCAG
                .    .    .    .    .    .    .    .    .
          201 GATCTGTAA.TCATCTCGCAAGAACATGAGTTTATGGGAATGCCTTTCCT
              ||||||||| |   |||  ||||||||||||||||||||||| || || |
         1893 GATCTGTAATTGCCCTCCATAGAACATGAGTTTATGGGAATCCCCTTTCC
                .    .    .    .    .    .    .    .    .
          250 CT...GCACTCCAGAACAACCTCTATATGTTGCTGAAACACACAGGCCAT
               |   ||| || |||| |||||||||  ||||  ||||||| |||| ||
         1943 TTCTGGCATTCTAGAATAACCTCTATAAATTGCCAAAACACATAGGCTAT
                .    .    .    .    .    .    .    .    .
          297 TTCATTGA.GGAAAACATAATATCTTGAAGAATATTGTTAAAACCTTACT
              |||| ||| |||||| |||||||||||||| || |||| |||| |||||
         1993 TTCACTGAGGGAAAATATAATATCTTGAAGACTACTGTTTAAACTTTACT
                .    .    .    .    .    .    .    .    .
          346 TGCTTGTTTGTTCTAGTTAGCAGGAAATAGTGTTCTTTTTAAAAAGTTGC
              |    | ||||||||||       | |||| ||||||||||||||||||||
         2043 T..AAGCTTGTTCTAGTATAGTAGGAATAATGTTCTTTTTAAAAAGTTGC
                .    .    .    .    .    .    .    .    .
          396 ACATTAGGAACAGAGTATATATGTACAGTTATACATACCTCTCTC.....
              |||||||||||| |||||||||||||||||||||| ||||||||||
         2091 ACATTAGGAACACAGTATATATGTACAGTTATATATACCTCTCTCTGTAT
                .    .    .    .    .    .    .    .    .
          441 .TATATATACATGTATAGTGAGTGTGGCTTAGTAATAGATCACGGGCAAT
               | |||||  ||||||||||||||||||||| || ||||||||| |   ||
         2141 ATGTATATGTATGTATAGTGAGTGTGGCTTGGTGATAGATCACAG..CAT
                                  Eco RI
          490 GTTTCCCGATCCAAGAGAATTCACTTTACCTTCAGCAGTTAC..CGGGAG
              || ||||  |||||||||||| |||||||||||||||| |||   |||
         2189 GTGTCCCCCTCCAAGAGAATTAACTTTACCTTCAGCAGCTACTGAGGGGC
                .    .    .    .
          538 CTAACATGCTGCCAACCAGC 557
              | |||||||||| |||| ||
         2239 CAAACATGCTGCAAACCTGC 2258

B
human      1 DSPQRREKLHKVAPKIKALMMESGTTMVGYQPQGD
             |||:|||||:||||||||||||||||||||||||||
rat      525 DSPERREKLHRVAPKIKALMMESGTTMVGYQPQGD
             |||||||||||||||||||||||||||||||||| |
feline   526 DSPERREKLHRVAPKIKALMMESGTTMVGYQPRGT

            36 KANFFRMVISNPAATQSDIDFLIEEIERLGQDL*
               |||||||||||||||||||||||||||||||||
           560 KANFFRMVISNPAATQSDIDFLIEEIERLGQDL*

           561 RPTFSGWSSRTQLLHSPILTSSSRR*
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FIG. 2. (A) Alignment of the nucleotide sequences of a human testis GAD cDNA clone and GAD cDNA from the feline brain. The upper line shows the sequence of the human testis GAD cDNA clone, and the lower line shows the feline brain GAD cDNA sequence from Kobayashi et al. (24). The human clone starts at position 1694 in the feline sequence. The first internal *Eco*RI site in the human clone is indicated. The extra cytosine residue at position 97 in the human sequence is indicated by an asterisk. Termination codons for human and feline GAD are underlined. (B) Comparison of the deduced amino acid sequences of human, rat, and feline GAD protein. The human sequence deduced from the isolated cDNA clone starts at amino acids 525 and 526 in the rat and feline GAD protein, respectively. Sequence identity is shown by vertical lines, and colons indicate two conservative amino acid replacements between the human and rat proteins. The carboxy-terminal amino acid in each protein is indicated by an asterisk.

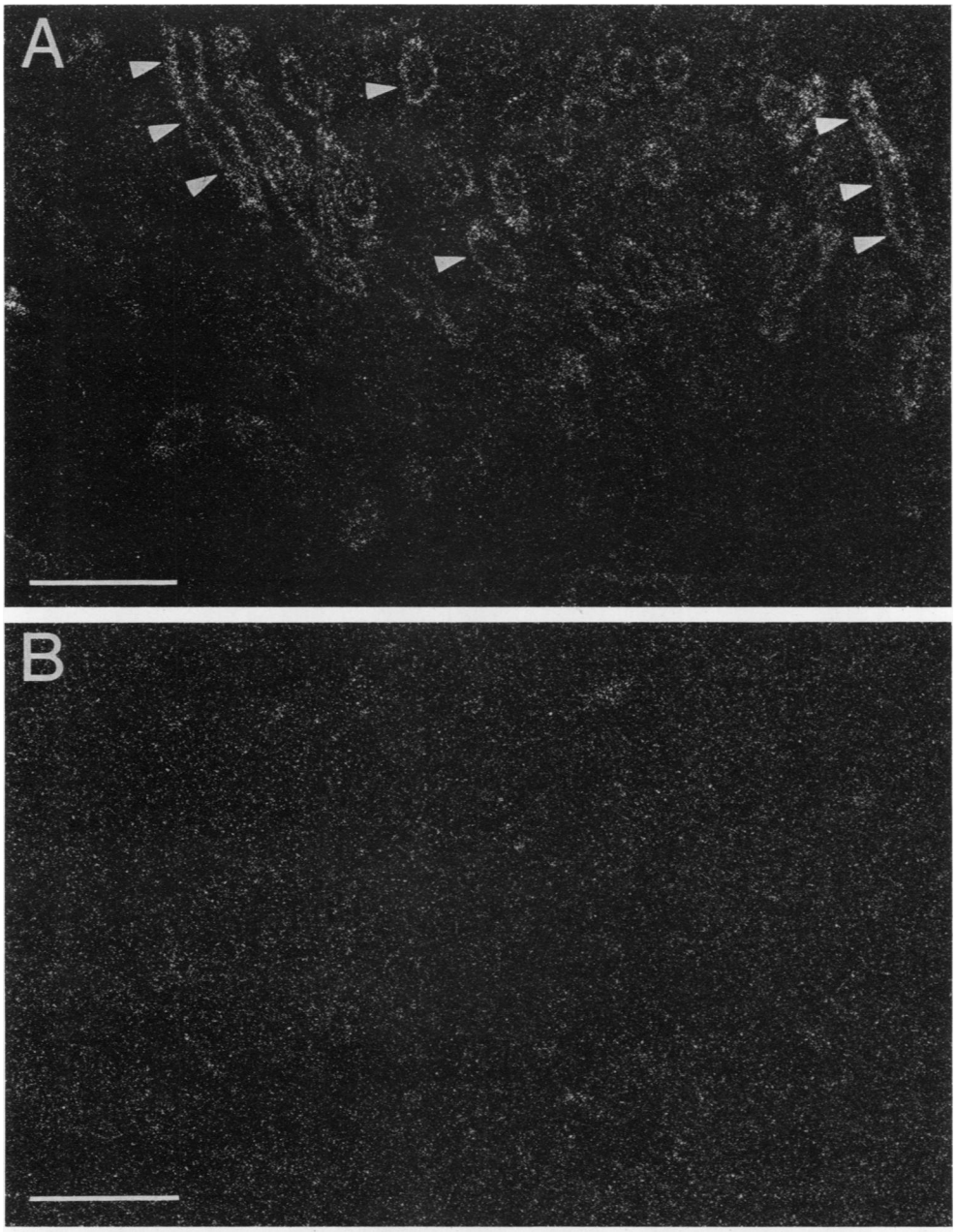
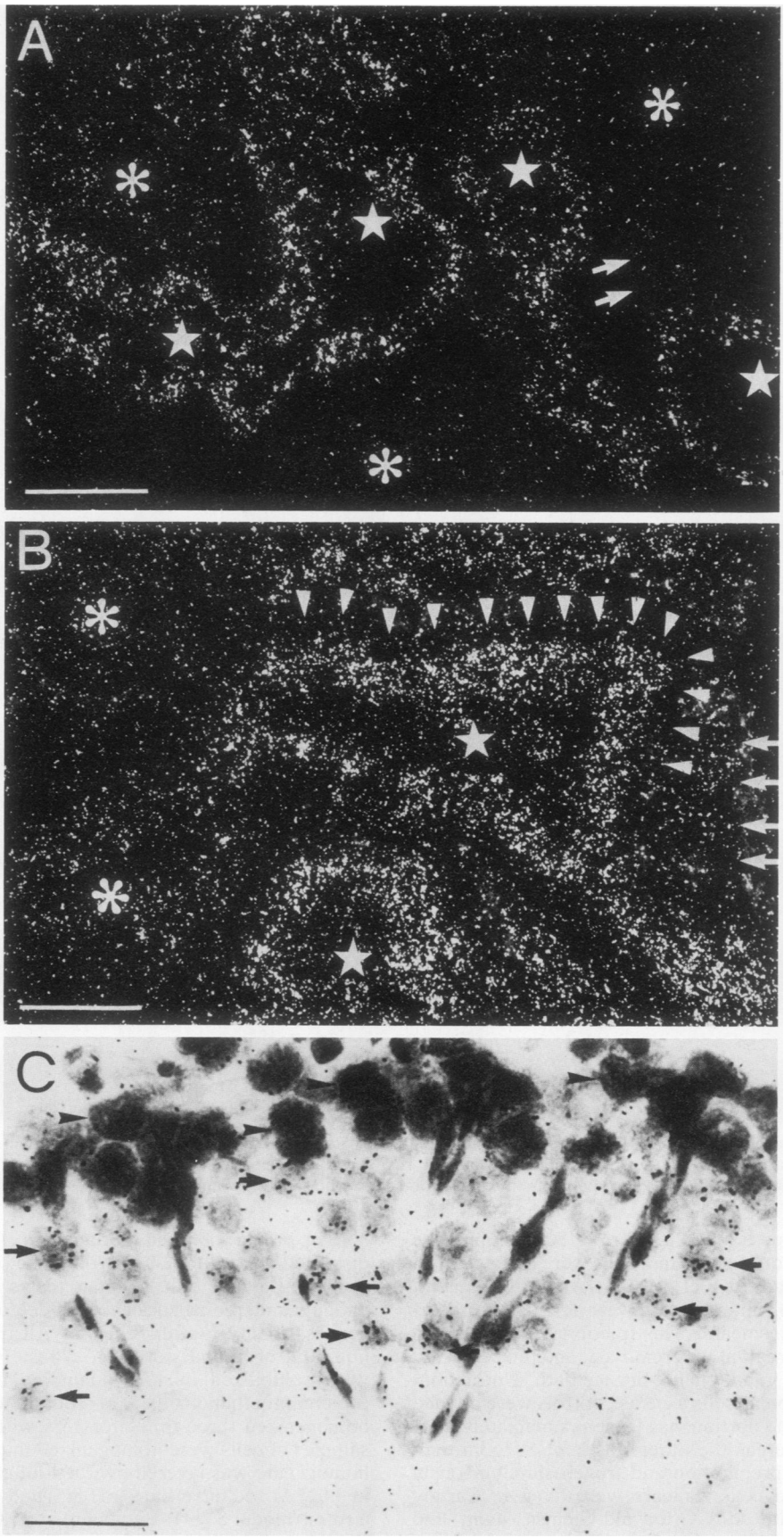


FIG. 3. X-ray film autoradiograms of the rat testis after hybridization with GAD and preprotachykinin oligonucleotide probes. (A) GAD mRNA is present in part of the tubules (arrowheads), while the rest of the tubules lack detectable GAD mRNA. (B) Adjacent section hybridized under identical conditions with a preprotachykinin probe shows no labeling. Bars, 150 μm.

and flushing the separation chamber at 80 ml/min. The volume of each fraction was 100 ml. Cells were counted in an electronic cell counter (model AI 134; Analysinstrument, Stockholm, Sweden), and samples were taken for preparfation of cytological smears. The fractionated cells were spun down, the supernatant was removed, and the cell pellet was frozen at −70°C in an ethanol-dry ice bath. Unfractionated seminiferous tubular cells used as controls were divided into two portions. One portion was frozen immediately after cell preparation, whereas the other was kept at room temperature during cell preparation and frozen simultaneously with the fractionated cells. Smears were fixed in Carnoy medium, stained with May-Grunwald-Giemsa stain, and examined under a light microscope.

RNA preparation and blot analysis. Adult rat tissues were dissected from 60- to 70-day-old male or female (for ovaries) Sprague-Dawley rats. Human testes were obtained from patients undergoing surgical orchidectomy for prostatic cancer less than 2 h after removal. All tissues were frozen in liquid nitrogen and stored at −70°C. Frozen tissue samples were solubilized in 4 M guanidine isothiocyanate–0.1 M β-mercaptoethanol–0.025 M sodium citrate (pH 7.0) and homogenized three times for 15 s with a Polytron. Frozen samples of cells were homogenized in the same buffer. Each homogenate was layered over a 4-ml cushion of 5.7 M CsCl in 0.025 M sodium citrate (pH 5.5) and centrifuged at 15°C in a rotor (model SW41; Beckman) at 35,000 rpm for 16 h (11). Poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chroma-


A
B
C

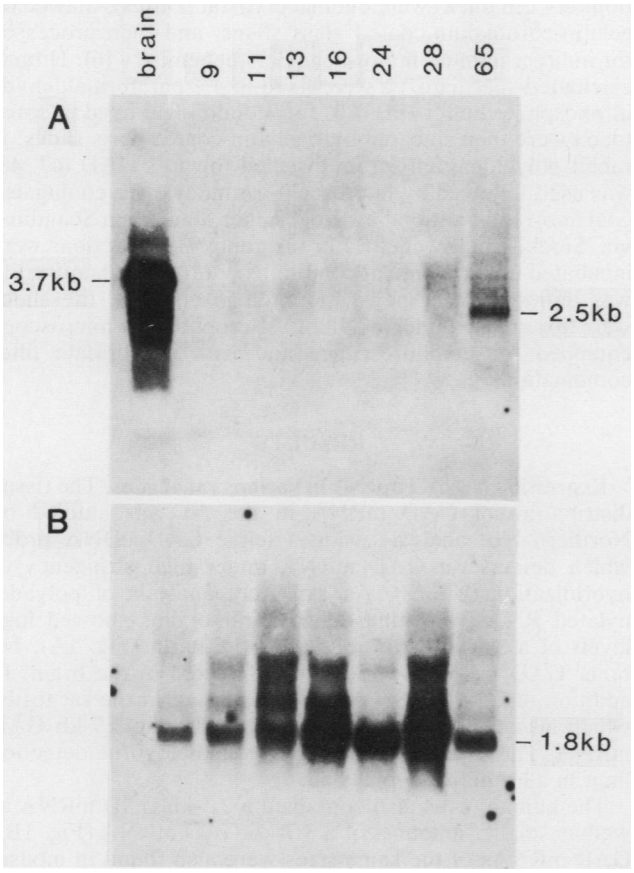


FIG. 5. Ontogeny of GAD mRNA expression in the rat testis. (A) Total RNA (40 μg per lane) prepared from testes of rats at the indicated days of age was separated on a formaldehyde-containing agarose gel, transferred to a nitrocellulose filter, and hybridized to a feline GAD cDNA probe. The left lane contained 5 μg of poly(A)$^+$ RNA from the adult rat brain. (B) The same filter as that used in panel A was boiled and rehybridized to a rat ABP cDNA probe.

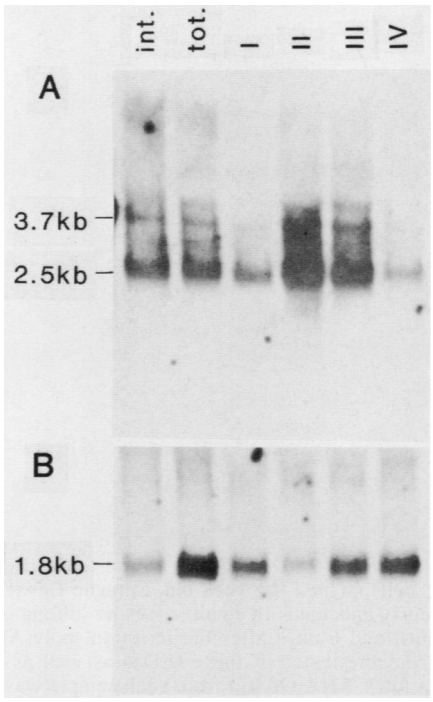


FIG. 6. Detection of GAD mRNA in fractionated testicular cells. Testicular cells from five 60-day-old Sprague-Dawley rats were fractionated by centrifugal elutriation as described in Materials and Methods. Total RNA was prepared from each fraction, and 40 μg of each sample was separated by electrophoresis, blotted onto nitrocellulose, and hybridized to a feline GAD probe. The filter was washed at high stringency and exposed to Kodak XAR-5 film for 2 days. (B) The same filter as that used in panel A was boiled and rehybridized to a rat ABP cDNA probe. The following fractions were analyzed: int., fraction enriched for interstitial cells but also containing a significant number of germ cells; and tot., total testicular cells before fractionation. Fractions were enriched for spermatogonia and Sertoli cells (I), spermatocytes (II), spermatids (but also containing a significant number of Sertoli cells) (III), and Sertoli cells (IV).

tography (1), and the recovery of RNA was quantified spectrophotometrically before use in RNA blot analysis. Poly(A)$^+$ RNA (20 μg) or, where indicated (Fig. 5 and 6), total cellular RNA from each sample was electrophoresed in a 1% agarose gel containing 0.7% formaldehyde and transferred to a nitrocellulose filter. The filter was then hybridized to the indicated DNA probes. The probes were labeled with α-[^{32}P]dCTP by nick translation to a specific activity of approximately 5×10^8 cpm/μg. For GAD, a 2.3-kilobase (kb) *Eco*RI fragment from a feline GAD cDNA (21) was used as a hybridization probe. The probe used to detect rat androgen-binding protein (ABP) mRNA was a 735-base-pair rat ABP cDNA insert (19). Hybridization was carried out in 4× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate, pH 7.0)–40% formamide–1× Denhardt solution–10% dextran sulfate at 42°C. Filters were washed at high stringency (0.1× SSC–0.1% sodium dodecyl sulfate at 54°C) and exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.).

cDNA cloning and nucleotide sequence analysis. Polyadenylated RNA (5 μg) from the human testis was used as a template for the construction of a cDNA library in phage λgt10 by using a cDNA synthesis kit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) according to the manual from the manufacturer. From a primary library of 1.2×10^6 independent clones, 4×10^5 clones were screened with a 2.3-kb feline GAD cDNA probe (21) under stringent hybridization conditions by using conventional procedures (29). One hybridization-positive phage clone containing a 1.5-kb cDNA insert was isolated and mapped with restriction enzymes. In the cDNA insert, three *Eco*RI fragments of 600, 500, and 400 nucleotides were identified and subcloned in pBSKS (Stratagene, La Jolla, Calif.). The nucleotide sequence of the three *Eco*RI fragments was determined by the dideoxy-chain termination method of Sanger et al. (40). The established nucleotide sequence was aligned with the

FIG. 4. Emulsion autoradiography of rat testis hybridization with GAD probe. (A) GAD mRNA is localized to the middle part of the seminiferous epithelium (stars). (B) Higher magnification demonstrates the localization of GAD mRNA in the labeled tubules (stars). The position of the basement membrane is indicated by arrowheads. Nonlabeled tubules (asterisks) and interstitial cells (arrows) are seen in both panel A and panel B. (C) Bright-field micrograph of a cresyl violet-stained section showing labeling over spermatocytes and round spermatids (arrows). Spermatogonia and Sertoli cells (arrowheads) lack labeling. Bars, 60 μm (A), 40 μm (B), and 20 μm (C).

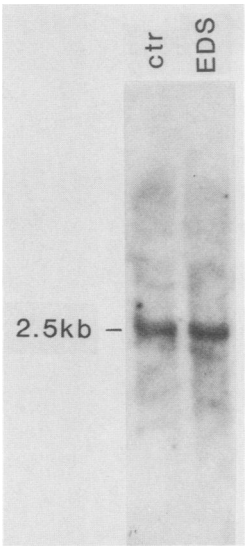


FIG. 7. Levels of GAD mRNA in the rat testis after destruction of Leydig cells. Three 10-week-old Sprague-Dawley rats were subcutaneously injected with ethane methane sulfonate (75 mg/kg of body weight), and 6 days after the injection, poly(A)$^+$ RNA was prepared from the testes of these (EDS) as well as age-matched control rats (ctr). RNA (20 μg) from each sample was analyzed by Northern blot using a feline GAD cDNA probe.

published feline GAD sequence (24) by using an algorithm as described by Devereux et al. (7).

In situ hybridization. Rat testes were collected, immersed in 4% paraformaldehyde in phosphate buffer containing 0.3% picric acid (50) for 2 to 3 h, and transferred to 10% sucrose in 0.1 M phosphate buffer and left overnight. Sections (14 μm) were cut in a cryostat (Dittes, Heidelberg, Federal Republic of Germany) and thawed on glass slides dipped in poly-L-lysine (100 mg/liter). The sections were then hybridized to a 48-mer oligonucleotide complementary to feline GAD mRNA coding for amino acids 389 to 405 (24), as previously described (41). As a control, adjacent sections were also hybridized to a 48-mer oligonucleotide complementary to rat preprotachykinin A mRNA coding for amino acids 49 to 64 (25). The oligonucleotides were synthesized on a DNA synthesizer (model 381 A; Applied Biosystems, Foster City, Calif.), purified with oligonucleotide purification cartridges (Applied Biosystems), and labeled with α-[^{35}S]dATP (New England Nuclear Corp., Boston, Mass.) at their 3′ end by using terminal deoxynucleotidyltransferase (International Biotec Inc., New Haven, Conn.) to a specific activity of 7×10^8 to 10×10^8 cpm/μg.

Following hybridization, sections were washed at 45°C in 1× SSC for 60 min, transferred through distilled water and 60% and 95% ethanol, air-dried, and exposed to X-ray film. Subsequently, NTB2 (Eastman Kodak Co.) nuclear track emulsion was applied, and finally the sections were stained with cresyl violet. The nuclear track emulsion was developed in D 19 (Eastman Kodak Co.) for 2.5 min and fixed in G333 (Agfa Gefvert, Leverkusen, Federal Republic of Germany) for 4 min. The autoradiograms were analyzed under a Nikon Mikrophot-FX microscope using dark-field illumination.

Immunocytochemistry. Rat testes were collected, immersed in 4% formaldehyde in phosphate buffer containing 0.3% picric acid (50) for 2–3 h, and transferred to 10% sucrose in 0.1% phosphate buffer and left overnight. Sections (14 μm thick) were cut in a cryostat (Dittes), thawed on gelatin-chromalum-coated glass slides, and then processed for indirect immunofluorescence histochemistry (6). Human ejaculated spermatozoa were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.3) for 30 min. The fixed spermatozoa were then smeared onto gelatin-coated glass slides. A rabbit polyclonal antiserum directed towards GAD (47, 48) was used followed by fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies (Boehringer Mannheim Scandinavia, Stockholm, Sweden). For the control, the sections were incubated under identical conditions but the GAD antiserum was omitted. After being mounted in glycerol, the slides were examined under a Nikon Microphot-FX microscope equipped for epifluorescence and with appropriate filter combinations.

RESULTS

Expression of GAD mRNA in various rat organs. The tissue distribution of GAD mRNA in the rat was studied by Northern blot analysis using a feline GAD cDNA probe which detects rat GAD mRNA under high stringency of hybridization (27, 28). As expected, analysis of polyadenylated RNA from nine different rat organs showed high levels of a 3.7-kb GAD mRNA in the brain (Fig. 1A). No other GAD mRNA species were detected in the brain. In addition, a 2.5-kb GAD mRNA was found in the rat testis, which also contained smaller amounts of the 3.7-kb GAD mRNA. The level of GAD mRNA was below the detection limit in all other organs tested.

The human testis also contained a 2.5-kb GAD mRNA as well as smaller amounts of a 3.7-kb GAD mRNA (Fig. 1B). GAD mRNAs of the same sizes were also found in mouse, guinea pig, pig, and monkey testes (data not shown).

Cloning and nucleotide sequence of a GAD cDNA from the human testis. In order to confirm that the transcripts in the testis that hybridized to the feline GAD cDNA probe were transcribed from the GAD gene, a cDNA clone was isolated from the human testis by using the feline GAD cDNA probe (21). From a primary screen of 400,000 clones, one hybridization-positive clone was isolated. The insert contained three *Eco*RI fragments of approximately 600, 500, and 400 nucleotides. All fragments were subcloned in plasmid pBSKS and sequenced by the chain termination method (40). The nucleotide sequence (Fig. 2A) revealed that the 5′ end of the isolated cDNA clone was located at position 1694 in the sequence of feline GAD cDNA (24). An overall nucleotide sequence homology of 87% was found between the human and feline GAD sequences starting at position 1694 and ending at position 2258 in the feline GAD sequence (24). The predicted amino acids 1 to 32 in the human GAD sequence and amino acids 526 to 557 in the feline GAD sequence are identical with the exception of two conservative amino acid replacements (Fig. 2B). However, an additional cytosine residue at position 97 in the human sequence causes a frameshift, compared with the feline GAD sequence, that results in no amino acid homology in the 36 most carboxy-terminal amino acids of human and feline GAD. Also, the predicted human GAD protein includes eight additional carboxy-terminal amino acids, compared with the feline protein. However, nucleotide sequence analysis of a recently isolated rat GAD cDNA clone also revealed an extra nucleotide (thymidine), compared with the feline sequence (20), at the same position as the extra cytosine in the human GAD sequence. The deduced carboxy-terminal amino acid sequence of the rat GAD protein is identical to

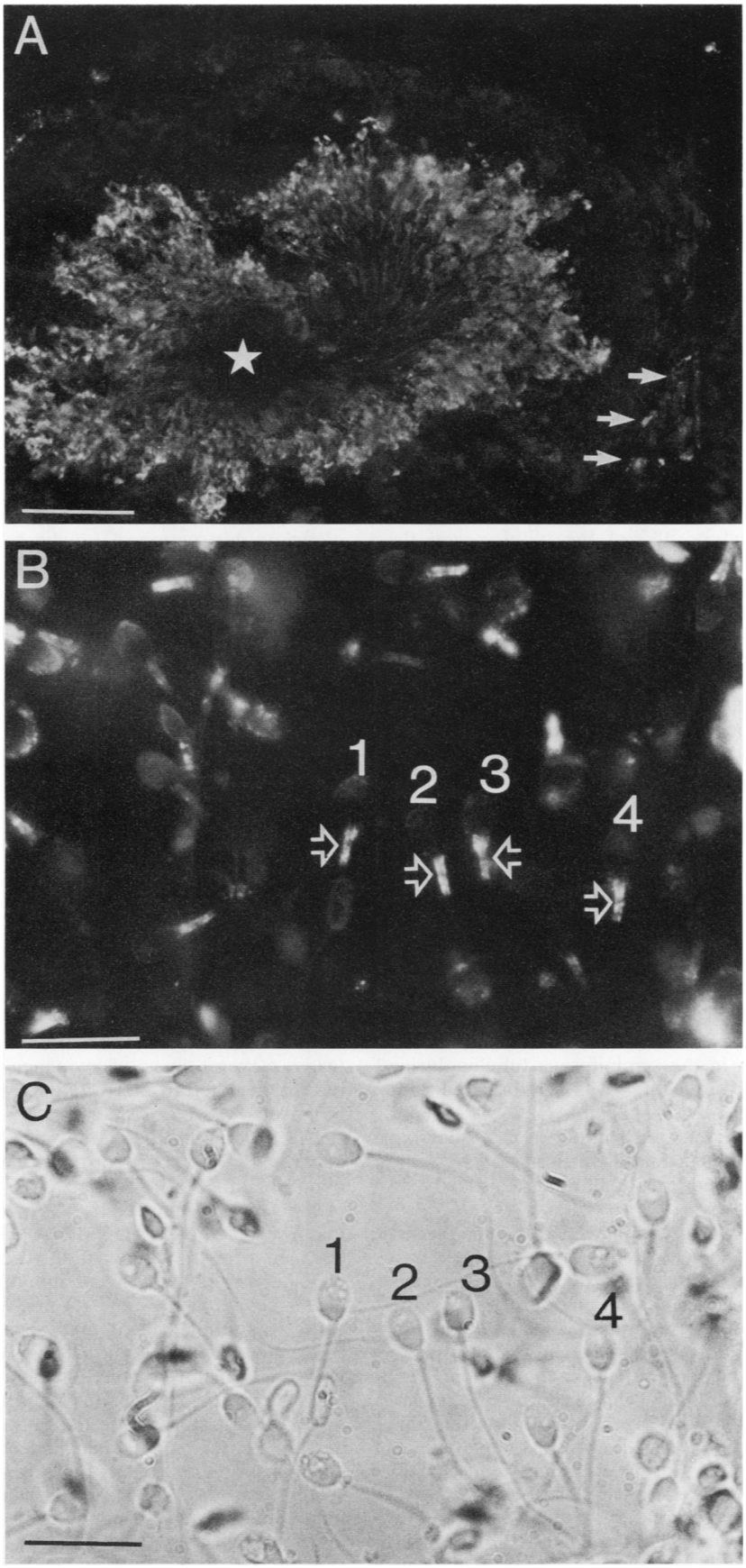


FIG. 8. Immunocytochemical demonstration of GAD-LI in the rat testis and in human ejaculated spermatozoa. (A) Intense immunofluorescence is present in the middle part of the rat seminiferous tubule. Star indicates the lumen. Arrows indicate a nonimmunoreactive Leydig cell. (B) In human ejaculated spermatozoa, GAD-LI is localized in the middle piece (open arrows). (C) The spermatozoa, some of which are indicated with numbers, can be identified by phase-contrast microscopy. Bars, 30 μm (A) and 10 μm (B and C).

that predicted for the human GAD protein (Fig. 2B). The discrepancy between the carboxy-terminal sequences of the human and feline GAD protein might be the result of a cloning artifact or sequencing error, most likely in the feline sequence, given the identity between the carboxy-terminal sequences of the human and rat GAD protein (Fig. 2B).

Localization of GAD mRNA-synthesizing cells in the rat testis by in situ hybridization. Cells expressing GAD mRNA in the rat testis were identified by in situ hybridization using a 48-mer oligonucleotide complementary to feline GAD mRNA. This oligonucleotide probe has previously been shown to specifically label GAD mRNA-expressing cells in several different regions of the rat brain (27, 28). On sections from the rat testis, labeling was found in the middle part of the seminiferous tubules where spermatocytes and spermatids are found (Fig. 3A and 4A and B). Hybridization using a similar-length oligonucleotide probe specific for rat preprotachykinin mRNA showed no labeling over any cells in the rat testis (Fig. 3B). Labeling with the GAD probe was restricted to approximately 50% of the tubules, and the remaining tubules lacked labeling altogether. Examination of the sections at a higher magnification revealed that the labeling was primarily found over spermatocytes and round spermatids (Fig. 4C). No labeling was found over interstitial tissue or over the central parts of the seminiferous tubules containing more mature germ cells (Fig. 4A and B).

Ontogeny of GAD mRNA expression in the rat testis. In the rat, sexual maturation occurs sequentially after birth and, as a result of this, the somatic and germ cell types in the seminiferous tubules undergo both qualitative and quantitative changes. In the rat, postmeiotic germ cells appear at about 25 days of age, whereas somatic cells, including Sertoli cells, make up the bulk of the seminiferous epithelium during the early postnatal development of the testis (2). To further differentiate between somatic and germ cell expression of GAD mRNA, Northern blot analysis was performed by using mRNA prepared from testes of rats of different ages. In the prepubertal rat, GAD mRNA was first detected at 28 days of age (Fig. 5A). However, the level of GAD mRNA in testes of 28-day-old rats was significantly lower than the level found in adult rat testes. As a marker for somatic cell expression in the seminiferous tubules during sexual maturation, the same filter was rehybridized to a cDNA probe for the Sertoli cell-specific ABP (19). A major 1.8-kb ABP mRNA was detected already in the 9-day-old testis, the earliest day tested, and a transcript of the same size was found in testes from rats of all other ages analyzed (Fig. 5B).

Levels of GAD mRNA in fractionated cells from the rat seminiferous epithelium. Isolated seminiferous tubules from adult rats were enzymatically dissociated into single cells and fractionated by centrifugal elutriation (3). Samples of the different fractions were subjected to fixing and counterstaining followed by examination under a light microscope to determine the cellular composition of each fraction. This analysis revealed enriched populations of spermatogonia and spermatocytes in fractions 1 and 2, respectively. Sertoli cells were also found in fraction 1 together with spermatogonia. Fraction 3 was enriched for spermatids but also did contain Sertoli cells that otherwise predominated in fraction 4. Next, preparation of RNA from each of these fractions followed by Northern blot analysis using either a feline GAD cDNA or a rat ABP cDNA as a hybridization probe was carried out. The highest levels of GAD mRNA were found in fractions 2 and 3, which were enriched for spermatocytes and spermatids, respectively (Fig. 6A). Fraction 4 (enriched for Sertoli cells) contained significantly smaller amounts of GAD mRNA than fraction 2 or 3. The fraction enriched for interstitial cells also showed relatively high levels of GAD mRNA. However, the cellular origin of GAD mRNA in this fraction is uncertain, since it also contained a significant number of germ cells. The relative proportion of the 2.5- and 3.7-kb GAD mRNAs was the same in all fractions, indicating that they are both primarily derived from spermatocytes and spermatids. The 1.8-kb ABP mRNA predominated in fractions 3 and 4, which were enriched for Sertoli cells (Fig. 6B).

The possibility that GAD mRNA detected in the interstitial tissue fraction was, to a significant extent, due to expression in interstitial Leydig cells was studied by measuring the level of GAD mRNA in the rat testis after Leydig cells had been specifically destroyed by a single injection of ethane dimethane sulfonate. As previously reported (17), a single injection of ethane dimethane sulfonate resulted in an approximately 30-fold decrease in the level of testosterone in the testis 6 days after the injection, indicating an almost complete destruction of Leydig cells. However, this treatment had no effect on the level of GAD mRNA in the rat testis (Fig. 7).

Immunohistochemical localization of GAD-LI in rat testes and human spermatozoa. Immunohistochemical staining of sections from the rat testis with a GAD-specific antiserum showed GAD-LI in the middle and central parts of the seminiferous tubules where spermatids and spermatozoa are located (Fig. 8A). Staining with the secondary antibody only showed no positive structures in the seminiferous tubules. Immunohistochemical staining of ejaculated and fixed human spermatozoa with the same antiserum revealed strong immunoreactivity in the midpiece of the spermatozoa (Fig. 8B and C). The majority of the ejaculated spermatozoa contained GAD-LI, but some nonimmunoreactive spermatozoa were also observed.

DISCUSSION

In this study, we show that mRNA encoding GAD, the key enzyme in the synthesis of the inhibitory neurotransmitter GABA, is expressed not only in the brain but also in the testis. Several lines of evidence suggest that the GAD mRNA detected in the testis is derived from expression in germ cells.

The possibility that interstitial Leydig cells, known to express POMC, corticotropin-releasing factor, and prodynorphin mRNA (4, 8, 14, 37, 44, 49), were a major source of GAD mRNA in the testis was made unlikely by the finding that the level of GAD mRNA in the rat testis was not changed after the chemical destruction of Leydig cells. To distinguish between expression in germ cells and Sertoli cells, the ontogeny of GAD mRNA expression in the rat testis was studied. This analysis did not show detectable levels of GAD mRNA until 28 days of age, with higher levels in sexually mature 65-day-old rats. The late onset of GAD mRNA expression is in sharp contrast to the onset of ABP mRNA synthesis in the testis. In the rat testis, ABP mRNA, encoding a Sertoli cell-specific secretory protein that binds and transports androgens (38), was detected already at day 9, the first day of analysis. The level of ABP mRNA increased up to day 16 and reached a plateau thereafter, with lower levels in the sexually mature rat. The temporal expression of ABP mRNA in the rat testis closely matches the volume density of Sertoli cells, which peaks at day 15 with a proportionally lower number in the adult testis, where the Sertoli cells are diluted by the increased number of germ

cells. The late onset of GAD mRNA expression in the testis suggests that GAD mRNA is not expressed in Sertoli cells but rather in germ cells, more specifically, in round and condensing spermatids which appear in the testis at this time (2, 15). In agreement with this, in situ hybridization revealed GAD mRNA-positive spermatocytes and round spermatids in the middle part of the seminiferous tubules with no labeling over interstitial tissues. Furthermore, fractionation of testicular cells followed by Northern blot analysis showed the highest level of GAD mRNA in fractions enriched in spermatocytes and spermatids. Hence, expression of GAD mRNA in the rat testis appears to coincide with postmeiotic spermatogenesis.

The major GAD mRNA species detected in the testis of all species tested (rat, mouse, guinea pig, monkey, and human) had a size of approximately 2.5 kb, significantly smaller than the 3.7-kb GAD mRNA species found in the brain. However, lower levels of the 3.7-kb mRNA species were also found in the testis. The major opioid peptide transcripts detected in the testis also differed in size from their respective transcripts in the brain. In the case of POMC, the predominating transcript in the testis is 250 nucleotides shorter than that in the pituitary (4, 14), because of the use of an alternative transcription initiation site within the gene (16, 18, 26).

Nucleotide sequence analysis of a 1.5-kb cDNA clone isolated from a human testis library showed an overall 87% sequence homology to the 3′ end of a previously isolated feline GAD cDNA clone (24). This high degree of homology strongly suggests that the isolated cDNA clone was derived from GAD mRNA expressed in the human testis. The predicted feline GAD protein consists of 586 amino acids, and the 5′ end of the isolated 1.5-kb GAD cDNA from the human testis is located at a position corresponding to that of amino acid 526. Provided that the isolated cDNA clone was derived from the 2.5-kb GAD mRNA, it could be predicted to encode approximately 400 amino acids of the GAD protein. If this were the case, the major 2.5-kb GAD mRNA in the testis could originate from a transcriptional start site within the GAD gene and therefore encode a truncated GAD protein. However, the 500-base-pair *Eco*RI fragment located at the 3′ end of the isolated cDNA clone did hybridize only to the 3.7-kb GAD mRNA in both the brain and the testis, suggesting that the isolated cDNA clone was derived from the 3.7-kb GAD mRNA. Hence, the 2.5-kb GAD mRNA in the testis may encode a complete GAD protein.

Direct evidence for the presence of GAD protein in the rat testis was obtained from the demonstration of GAD-LI in seminiferous tubules. The GAD-LI was located mainly in the middle and central parts of the seminiferous tubules where spermatids and spermatozoa are found. In agreement with the possibility that GAD protein is also expressed in the human testis, GAD-LI was detected in ejaculated human spermatozoa. Interestingly, the GAD-LI in ejaculated spermatozoa was not evenly distributed but concentrated to the midpiece of the cells. The GAD protein in the midpiece of ejaculated human spermatozoa appears to be functional and synthesize GABA, since GABA-LI was detected in this part of ejaculated human spermatozoa and these cells also contained authentic GABA identified by reverse-phase high-pressure liquid chromatography (M. Pelto-Huikko et al., unpublished data).

Previous studies have shown the presence of the neurotransmitter-neuromodulator CCK in the acrosome of spermatids and spermatozoa of several different species (32, 36). Similarly, in humans, the CCK-related peptide gastrin has been detected in the acrosome of these cells as well as in the acrosome of ejaculated spermatozoa (M. Schalling, H. Persson, M. Pelto-Huikko, L. Ödum, T. Hökfelt, and J. F. Rehfeld, J. Clin. Invest., in press). As previously discussed (35, 36), the acrosomal location of these peptides indicates that they are released during the acrosome reaction to participate in the fertilization process. The location of GAD-LI in the midpiece of spermatozoa suggests that the GAD enzyme and its product GABA have a function in spermatozoa different from that of CCK and gastrin.

The midpiece of the spermatozoa contains a sheaf of mitochondria which provides ATP required for flagellum movements produced by sliding of microtubules formed by polymerization of tubulin. The confinement of GAD-LI to this region suggests that GAD and GABA may be important for spermatogenesis and sperm motility. It is of considerable interest in this context that the GABA agonist sodium valproate (used as an anticonvulsive agent) has been noted to decrease sperm content and motility in rats (5). Moreover, in a recent review article, Erdö and Wolff (9) quoted unpublished observations that semen possesses a high-affinity uptake system for GABA and that GABA, depending on its concentration, may both increase and decrease the motility of the spermatozoa. Although the functional roles of GAD and GABA in the male reproductive system are poorly understood, the results of the present study together with results from previous studies with regard to CCK and gastrin (32, 34–36) show that neurotransmitters and neuromodulators are locally synthesized within male germ cells where they may play an important role in reproductive function.

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