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Differential expression of opioid peptide genes by testicular germ cells and somatic cells*

(proenkephalin/pro-opiomelanocortin/RNA/paracrine factors)

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ABSTRACT **Spermatogenic cells have been previously shown to be a major site of testicular proenkephalin gene expression. Using RNA gel-blot analysis of purified mouse and hamster germ cells and of testes from prepuberal and germ cell-deficient mutant mice, we now have demonstrated that, in addition to its previously described expression by somatic (Leydig) cells, the gene for a second opioid peptide precursor, pro-opiomelanocortin (POMC), is also expressed by spermatogenic cells. Of particular significance is the finding that the RNAs for proenkephalin and POMC are differentially regulated during spermatogenesis. Two forms of POMC RNA were detected in mouse testis, a larger component 675- to 750-nucleotides (nt) in size common to somatic and spermatogenic cells and a smaller 625-nt RNA found only in pachytene spermatocytes. Two distinct, cell-specific proenkephalin RNAs were also shown to be present in mouse testis: a 1700-nt transcript previously shown to be expressed by spermatogenic cells and a 1450-nt form associated with somatic cells. These data suggest that (*i*) proenkephalin- and POMC-derived peptides are produced by both somatic cells and germ cells in the testis and (*ii*) in germ cells these two families of opioid peptides may function at different stages of spermatogenesis.**

Paracrine mechanisms play an important role in the maintenance and regulation of spermatogenesis within the testis (1–3). Several factors appear to mediate interactions between different testicular cell types, including P Mod-S, a protein produced by peritubular cells that modifies Sertoli cell secretion (4); transport proteins and mitogenic factors produced by Sertoli cells that seem important for germ cell proliferation (3–5); and testosterone produced by Leydig cells and required for normal spermatogenesis (2). The recent demonstration that each of the three opioid peptide precursors, pro-opiomelanocortin (POMC), proenkephalin, and prodynorphin, are expressed in the testis (6–9) suggests that their peptide products also locally regulate testicular function. We have previously shown that spermatogenic cells are a major site of proenkephalin RNA expression in the mouse testis (10), results suggesting that proenkephalin-derived peptides function as germ cell-associated paracrine factors. The gene for the opioid peptide precursor POMC has already been shown to be expressed by Leydig cells (6, 7). In this report the POMC gene is demonstrated to be expressed also by testicular germ cells and in a distinct manner during spermatogenesis from that described for the proenkephalin gene. Multiple proenkephalin and POMC transcripts are further shown to be produced in the rodent testis in a cell-type-specific manner.

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MATERIALS AND METHODS

Guanidine thiocyanate and *N*-lauroylsarcosine were from Fluka Chemical (Happauge, NY), ^{32}P-labeled deoxynucleotides (3000 Ci/mmol; 1 Ci = 37 GBq) were purchased from New England Nuclear, cesium chloride was from Bethesda Research Laboratories, collagenase was obtained from Worthington, and trypsin (pancreatic) and DNase I were from Sigma. Immature and adult male CD-1 mice, adult Syrian hamsters, and adult and 6-day-old CD-1 rats were from Charles River Breeding Laboratories; inbred male mice either homozygous for the gene mutations *at* (atrichosis) and W^v (viable dominant spotting) or normal controls were obtained from The Jackson Laboratory.

Spermatogenic cells were prepared from adult mouse testes by unit gravity sedimentation on linear gradients of bovine serum albumin as described (10). These isolated cell populations represent meiotic prophase (pachytene spermatocytes) as well as postmeiotic stages of development (round and condensing spermatids and cytoplasts). Cytoplasts are defined as the anucleate cytoplasmic remnants generated from condensing spermatids during the isolation process. Purities of round spermatids, pachytene spermatocytes, and cytoplasts ranged from 87 to 94% using Nomarski optics. Major contaminants were condensing spermatids (round spermatid and cytoplast fractions) and binucleated spermatids (pachytene spermatocyte fractions). Condensing spermatids (94% pure) were prepared by an additional step of Percoll density gradient centrifugation (11, 12). The procedures for preparing spermatogenic cells from adult hamster testes were essentially identical to those used for mouse except for the initial collagenase digestion conditions: 4–6 decapsulated testes incubated for 10 min in Krebs–Ringer buffer containing 0.1 mg of collagenase per ml (Cooper Biomedicals; Malvern, PA) and 2 μg of DNase I per ml with shaking at 100 cycles/min. Purities of hamster germ cell preparations were similar to those from mouse.

To confirm the absence of spermatogenic cells in testes from sterile mutant mice, tissues were fixed in Karnovsky's fixative, embedded in plastic, and stained with a hematoxylin/eosin mixture.

All procedures for RNA analysis were essentially as previously described (10). Total RNA was prepared from spermatogenic cells by guanidinium thiocyanate extraction and cesium chloride gradient centrifugation (13). RNA samples were separated on either 1% or 2% agarose/6% formaldehyde gels (16 cm) and transferred onto nylon membranes (GeneScreen*Plus*, New England Nuclear). Membranes were prehybridized and hybridized at 42°C using either ^{32}P-labeled

Abbreviations: POMC, pro-opiomelanocortin; nt, nucleotide(s).

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rat POMC (pI13) (14) or rat proenkephalin [pRPE-1(165–600)] (15) complementary DNAs (cDNAs) as probes. DNA probes were labeled by random primer synthesis (16) using a Pharmacia oligolabeling kit. Filters were washed and then exposed to x-ray film (Kodak XAR-5) for 12–96 hr at −80°C with intensifying screens (Lightning Plus, DuPont).

RNase H digestions to remove poly(A)$^+$ tails were done essentially as described by Vournakis *et al.* (17). Poly(A)$^+$ RNA samples (15 μg) were incubated with 1.5 μg of oligo-(dT)$_{12-18}$ cellulose (Pharmacia) before the addition of RNase H (Bethesda Research Laboratories). Following digestion for 30 min at 37°C, samples were ethanol precipitated in the presence of carrier tRNA (40 μg) and subjected to RNA gel-blot analysis.

RESULTS

Our previous observations of proenkephalin RNA in mouse spermatogenic cells suggested that these cells might also contain RNA for POMC. Gel-blot analysis of total RNA from purified mouse germ cells (pachytene spermatocytes, round spermatids, and cytoplasts) revealed that this was, in fact, the case (Fig. 1). A broad band of POMC RNA was readily detected in pachytene spermatocytes, which showed a significant enrichment of this transcript per μg of RNA over that seen in adult mouse testis. Round spermatids contained markedly lower concentrations, whereas in cytoplasts the levels of POMC RNA were below the detection limits. Comparison of proenkephalin and POMC RNA concentrations in testicular germ cells showed that the genes for these two opioid peptide precursors are differentially expressed during spermatogenesis. As previously reported (10), proenkephalin RNA was present in meiotic pachytene spermatocytes and postmeiotic round spermatids and cytoplasts—with the highest levels occurring in round spermatids (Fig. 1). In contrast, the POMC RNA concentration was highest in pachytene spermatocytes and was much reduced in postmeiotic cells. Testicular germ cells thus regulate the expression of these two opioid peptide precursor genes in quite distinct fashions.

As is evident in Fig. 1, testicular POMC RNA exhibits a marked size heterogeneity on denaturing agarose gels, a feature also observed by Chen *et al.* (7). In some of our experiments, the presence of at least two distinct size classes of this transcript was evident. Analysis using higher-resolution (2% agarose) gels confirmed this (Fig. 2). POMC RNA from mouse testis consisted of two components, a heterogeneous "band" that on this gel system ranged in size from 675–750 nucleotides (nt), and a more distinct, lower-M_r

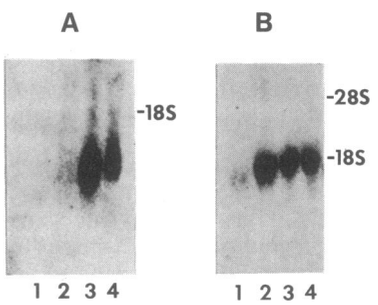

FIG. 1. Relative contents of POMC and proenkephalin RNAs in mouse spermatogenic cells. Total RNA was separated on a 1% agarose denaturing gel. RNA samples were prepared from (lanes 1) cytoplasts (15 μg); (lanes 2) round spermatids (18 μg); (lanes 3) pachytene spermatocytes (24 μg); (lanes 4) adult mouse testis (30 μg). The same membrane was sequentially probed for (*A*) POMC and (*B*) proenkephalin RNA as described. The positions of ribosomal RNAs are indicated.

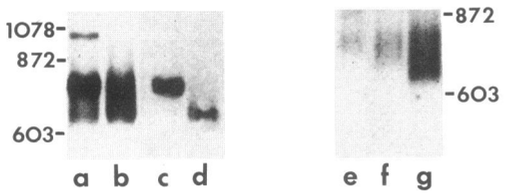

FIG. 2. Analysis of POMC RNA in spermatogenic cells on higher-resolution agarose denaturing gels. Poly(A)$^+$ RNA was examined from (lane a) hamster testis (20 μg), (lane b) mouse testis (20 μg), (lane c) hamster round spermatids (11 μg), (lane d) hamster pachytene spermatocytes (27 μg), (lane e) mouse condensing spermatids (10 μg), (lane f) mouse round spermatids (30 μg), and (lane g) mouse pachytene spermatocytes (30 μg). Samples were separated on 16-cm formaldehyde gels composed of 2% agarose and then subjected to RNA gel-blot analysis as described. The positions of ^{32}P end-labeled *Hae* III fragments of φX174 DNA are shown.

species of ≈625 nt. This estimated size range for testicular POMC RNA is smaller than that previously reported using glyoxal gels (7) and may reflect differences in mobility in the two gel systems.

The two size classes of POMC RNA were more evident in hamster testis in which the higher M_r component migrated as a more prevalent and distinct band of ≈750 nt (Fig. 2). In addition, a third RNA species was often detected in hamster testis that was similar in size to the transcript previously reported for pituitary (≈1000 nt) (6, 7). When POMC RNA was examined in purified mouse and hamster spermatogenic cells on higher-resolution gels, a differential pattern of germ cell expression was seen (Fig. 2). In the mouse, pachytene spermatocytes contained similar amounts of both the high- and low-M_r forms, whereas in round spermatids and condensing spermatids, only the higher-M_r component was detected. The 625-nt POMC RNA is thus expressed in a stage-specific manner during spermatogenesis in the mouse. A similar distribution was seen in hamster germ cells, with two differences: (*i*) The 625-nt transcript was much more predominant relative to the higher-M_r band in hamster pachytene spermatocytes than in those from mouse. (*ii*) The abundance of POMC RNA was greater in hamster round spermatids than in hamster pachytene spermatocytes (the reverse of that seen in the mouse). In the experiment shown in Fig. 2, the 1000-nt POMC RNA was not detected in significant amounts in hamster germ cells; however, in other germ cell preparations, it was readily identified in these cells (data not shown). The reason for this variability is not known.

The stage-specific nature of POMC RNA expression by spermatogenic cells was also evident from the developmental variation in POMC RNA content in the prepuberal mouse testis (Fig. 3). During the period from day 16 to day 21 there was a dramatic increase in the concentration of both the high- and low-M_r forms of testicular POMC RNA. In particular, the smaller 625-nt RNA associated with pachytene spermatocytes first appeared at this time—precisely the period during which pachytene spermatocytes are the predominant germ cell population in the developing mouse testis (18). At later ages, both size classes of POMC RNA showed a corresponding decline in abundance as early and late spermatids appeared and ultimately became the major spermatogenic cell types.

In immature mouse testes that contain few or no meiotic cells (days 4–12), a small but detectable amount of POMC RNA corresponding solely to the more heterogeneous, higher-M_r form was seen in mouse testis. These data could represent expression of POMC RNA by early spermatogenic stages (e.g., spermatogonia), by somatic cells, or by both. To address this, POMC RNA expression was examined in testes from germ cell-deficient, atrichosis mutant mice (Fig. 4). Testes from these mutants have previously been shown to be

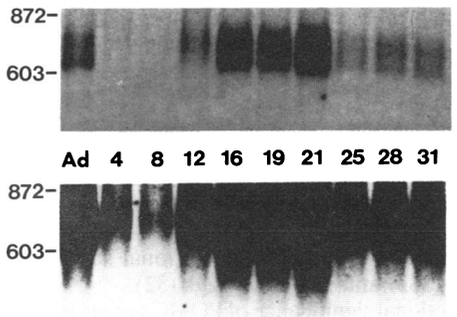

FIG. 3. Developmental regulation of POMC RNA in mouse testis. Poly(A)$^+$ RNA (25 μg each) from prepuberal and adult mouse testes was subjected to RNA gel-blot analysis as described for Fig. 2. Ad, adult mouse testis. Numbers refer to the age of immature mice (in days) from which testicular RNA was prepared. (*Upper*) Twelve-hour exposure; (*Lower*) 96-hr exposure that more clearly shows the higher-M_r POMC RNA band at the earliest ages (days 4 and 8).

completely devoid of germ cells but appear to contain normal Sertoli and Leydig cells (19–21). The absence of spermatogenic cells in the atrichosis mutants used in the present studies was also confirmed by light microscopy of histological sections (data not shown). In *at/at* mutants, a low concentration of only the 675- to 750-nt form of POMC RNA was seen. While this result shows that the higher-M_r class of POMC RNA is present in somatic cells of the mouse testis, it is still possible that this RNA is also present in spermatogonia and early spermatocytes in the *normal* testis. In this regard, Cheng *et al.* (22) have provided immunohistochemical evidence for *N*-acetyl-α-endorphin-like immunoreactivity in rat spermatogonia and spermatocytes. The absence of the lower M_r class of POMC RNA in testes from immature mice and atrichosis mutants suggests that it is not expressed by testicular somatic cells. However, the expression of POMC RNA by Leydig cells appears to be influenced by spermatogenic cells (23). Thus the pattern of POMC transcripts in testicular somatic cells could differ in the normal adult testis.

The heterogeneity of testicular POMC RNA raised the question of whether poly(A)$^+$ tail length might contribute to the observed differences in transcript size. RNA from mouse and hamster testis was therefore treated with RNase H to remove poly(A)$^+$ sequences and then analyzed by RNA gel-blot analysis (Fig. 4). Such treatment converted both the 675- to 750-nt POMC RNA as well as the smaller 625-nt form to essentially a single RNA species of ≈550 nt. In contrast, the 1000-nt pituitary-like POMC RNA in hamster testis was converted to a distinct species (900 nt) by RNase H. These results are similar to those previously reported by Chen *et al.* (7) for rat testis and pituitary. It thus appears that the heterogeneity in the higher M_r POMC RNA seen in mouse testis (which is contributed mainly by pachytene spermatocytes), as well as the specific size of the 625-nt pachytene-spermatocyte form are due to poly(A)$^+$ tail-length polymorphism.

Examination of proenkephalin RNA in the developing mouse testis revealed the presence of multiple forms of this RNA as well (Fig. 5). It was previously shown that rat and mouse testis as well as mouse spermatogenic cells contain a specific form of proenkephalin RNA that is considerably larger than the 1450-nt species detected in brain and other tissues that express proenkephalin (8, 10, 24). This larger transcript, which migrated as a 1700-nt RNA in the gel system employed for Fig. 4, was not detectable until 16 days in mouse testis, when pachytene spermatocytes begin to proliferate. The transcript increases in amount with age as round spermatids increase in number (18). Before day 16, a smaller proenkephalin RNA is exclusively seen in mouse testis. On day 16 and later, this lower-M_r form is present together with the larger 1700-nt RNA and exhibits a gradual decrease in abundance with increasing age. This shorter transcript is identical in size to the 1450-nt mRNA detected in brain (Fig. 5). We have previously reported evidence for the existence of a smaller proenkephalin RNA, similar in size to the 1450-nt species, as a minor component in the testis of the adult rat (24). As for the mouse, a 1450-nt proenkephalin RNA was the only form of this transcript detected in the early postnatal 6-day rat testis (Fig. 5). Mouse and rat testes thus contain two distinct proenkephalin RNAs that are differentially expressed during development.

The 1700-nt proenkephalin RNA appears to be the only form expressed by mouse pachytene spermatocytes, round and condensing spermatids, and cytoplasts (Fig. 6). The reduced amount of the 1450-nt RNA with increasing age in the mouse testis is therefore likely due to its dilution within the testicular RNA pool as spermatogenic cells become predominant, as substantiated by RNA gel-blot experiments in which testicular RNA pool changes with increasing age were taken into account (data not shown). To determine whether the 1450-nt transcript was expressed by earlier spermatogenic cell types or somatic cells, sterile mutant mice were examined. In testes from both W/W^v (25) and *at/at* mutants, the 1450-nt form alone was detected (Fig. 6). Somatic cells thus are at least one source of this smaller proenkephalin RNA in the mouse testis. Earlier germ cell types (e.g., spermatogonia and primary spermatocytes) may also contain this transcript, although preliminary evidence indicates that primitive type A spermatogonia do not (D.K. and C. Millette, unpublished observations).

In contrast to testicular POMC RNA, variation in poly(A)$^+$ tail length does not account for the size difference of the two proenkephalin RNAs in mouse testis. Removal of poly(A)$^+$ converted the 1700-nt RNA from mouse testis and the 1450-nt RNA from mouse brain to smaller, but distinct, species of ≈1500 and 1200 nt, respectively (Fig. 6). The 1200-nt poly(A)$^+$ form was also detected in RNase H-treated samples

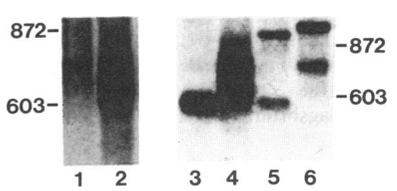

FIG. 4. Testicular POMC RNA from sterile mutant mice and the effects of RNase H digestion. RNA gel-blot analysis was done on testicular poly(A)$^+$ RNA (25 μg each) from mice either homozygous for the atrichosis mutation (*at/at*) (lane 1) or from normal controls (?/+) (lane 2). Lanes 3–6 show the effects of RNase H treatment of POMC RNA from mouse (lanes 3 and 4) and hamster (lanes 5 and 6) testis. Samples (15 μg) were either treated with RNase H (lanes 3 and 5) as described or were untreated (lanes 4 and 6).

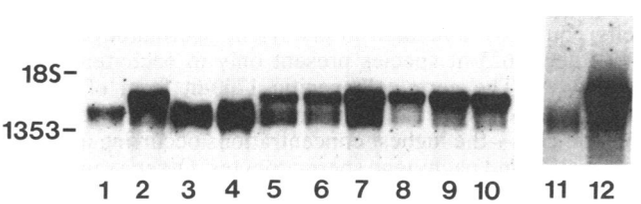

FIG. 5. Developmental regulation of proenkephalin RNA from mouse testis. Analyses were done essentially as described in the legend of Fig. 3 for POMC RNA. Poly(A)$^+$ RNA (25 μg unless othewise indicated) was prepared from the following: (lane 1) mouse brain (10 μg); (lane 2) adult mouse testis; (lanes 3–10) mouse testis from postnatal days 8, 12, 16, 19, 21, 25, 28, and 31; (lane 11) 6-day rat testis; and (lane 12) adult rat testis.

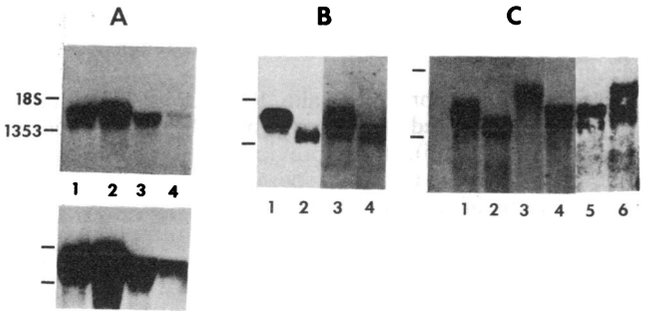

FIG. 6. RNA gel-blot analysis of proenkephalin RNA on 2% agarose denaturing gels. (*A*) Proenkephalin RNA content in poly(A)$^+$ RNA from mouse spermatogenic cells: (lanes 1) pachytene spermatocytes (30 μg); (lanes 2) round spermatids (30 μg); (lanes 3) condensing spermatids (10 μg); and (lanes 4) residual bodies (5 μg). (*Upper*) Twelve-hour exposure; (*Lower*) 48-hr exposure. (*B*) Analysis of testicular proenkephalin RNA in sterile mutant mice and heterozygous control mice: (lane 1) +/+ control; (lane 2) w/w^v mutants; (lane 3) ?/+ controls; (lane 4) *at/at* mutants. Twenty micrograms of poly(A)$^+$ RNA was examined in each case. (*C*) The effect of RNase H treatment on the size of proenkephalin RNA in brain and testis. Poly(A)$^+$ RNA samples (15 μg) were treated and analyzed as described for Fig. 4. (Lane 1) Untreated mouse brain; (lane 2) mouse brain treated with RNase H; (lane 3) untreated mouse testis; (lane 4) mouse testis treated with RNase H; (lane 5) day-19 mouse testis treated with RNase H; and (lane 6) untreated day-19 mouse testis.

from adult and 19-day mouse testis (Fig. 6)—providing further support for the identity between the 1450-nt proenkephalin RNAs in mouse testis and mouse brain.

DISCUSSION

A greater complexity to opioid peptide gene expression in the testis is revealed than was previously appreciated. Mouse testis contains multiple forms of proenkephalin and POMC RNA that are expressed in a cell-specific manner by somatic and spermatogenic cells. Earlier studies on POMC expression in the testis suggested that the RNA for this precursor was present mainly, or exclusively, in Leydig cells (6, 7). However, the existence of POMC RNA in spermatogenic cells is supported by *in situ* hybridization studies by Gizang-Ginsberg and Wolgemuth (23), who reported labeling of seminiferous tubules as well as Leydig cells in mouse testis using a POMC cDNA probe. In addition, the developmental regulation of POMC RNA is consistent with spermatogenic cells—in particular, pachytene spermatocytes—being a major source of this transcript in mouse testis.

For both opioid peptide precursors, specific transcripts have been detected in mouse germ cells that are developmentally regulated during spermatogenesis. Two classes of germ-cell POMC RNA were found in mouse testis, one a heterogeneous mixture of 675- to 750-nt common to meiotic and postmeiotic spermatogenic cells (as well as somatic cells), but most abundant in pachytene spermatocytes, and the other a 625-nt species present only in pachytene spermatocytes. The germ-cell-specific 1700-nt form of proenkephalin RNA is detected in both meiotic as well as postmeiotic cells—the highest concentrations occurring in round spermatids and pachytene spermatocytes. Other examples of germ-cell-specific mRNAs that undergo developmental regulation have been identified, including those for α-tubulin (26), the protooncogene c-*abl* (27), β- and γ-actin (28), protamines (29), and the presumptive gene product from the mouse homebox gene MH-3 (30, 31).

Whereas proenkephalin and POMC gene expression in mouse testis are both characterized by the presence of multiple RNA species, the mechanisms that produce this size heterogeneity differ. Poly(A)$^+$ tail length differences probably account for all forms of POMC RNA, whereas other processes are involved for proenkephalin RNA. The marked increase in poly(A)$^+$ tail length of POMC RNA seen in the transition from pachytene spermatocytes to round spermatids in the hamster is noteworthy. Whether this size shift is due to adenylylation of preexisting POMC RNA (32) or to the appearance of new transcripts in hamster round spermatids is unknown. Changes in polyadenylylation have been correlated with changes in mRNA translational status in spermatogenic cells (33) and fertilized eggs (32).

The functional significance of POMC and proenkephalin gene expression by both testicular somatic cells and germ cells is unclear. These observations may mean that testicular opioid peptides function in a manner specific to each cell type. The apparently selective somatic cell expression of these two opioid peptide genes in the immature testis may indicate a role for this source in early testicular development. A role for endogenous opioids in regulating Sertoli cell growth and Leydig cell steroidogenesis in the developing rat testis has already been suggested by studies using opiate antagonists (34). Differential expression of proenkephalin and POMC RNAs by spermatogenic cells could indicate that different families of opioid peptides function at specific stages as germ cell hormones or paracrine/autocrine factors. Opioid peptides from different precursor proteins exhibit differing selectivities for the various opioid receptor subtypes (35). Data from several laboratories suggest that spermatogenic cells can alter the functioning of neighboring Sertoli and Leydig cells in a stage-specific manner (1, 3, 36); conceivably, germ cell-associated opioid peptides could be involved in mediating such interactions. Functional opioid receptors have been detected on Sertoli cells (37), indicating the latter may be one site of action for putative germ-cell opioid peptides. However, actual production of proenkephalin and POMC-derived peptides by spermatogenic cells must be demonstrated before a role as germ cell factors can be established. A complete analysis of potential proenkephalin and POMC peptide products in spermatogenic cells is now warranted.

Since the completion of ths work, POMC RNA has been detected in mouse spermatogenic cells by Gizang-Ginsberg and Wolgemuth (38).

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