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Translational Control of Germ Cell-Expressed mRNA Imposed by Alternative Splicing: Opioid Peptide Gene Expression in Rat Testis

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The three genes encoding the opioid peptide precursors (prodynorphin, proenkephalin, and proopiomelanocortin) are expressed in the rat testis. The sizes of the three opioid mRNAs in the testis differ from the sizes of the corresponding mRNAs in other rat tissues in which these genes are expressed. The smaller testicular proopiomelanocortin mRNA has previously been demonstrated to arise from alternative transcriptional initiation. In the present study, we found that the smaller testicular prodynorphin mRNA, expressed in Sertoli cells, results from alternative mRNA processing. Exon 2, which makes up 5′ untranslated sequence, is removed from the mature transcript. Polysome analysis of brain and testis RNA indicates that the alteration of the prodynorphin leader sequence in the testis-specific transcript does not affect the efficiency of translation of this mRNA. The larger testicular proenkephalin transcript, expressed in developing germ cells, also results from alternative mRNA processing. Alternative acceptor site usage in the splicing of intron A results in a germ cell-specific proenkephalin transcript with a 491-nucleotide 5′ untranslated leader sequence preceding the preproenkephalin-coding sequence. Polysome analysis indicates that this germ cell-specific proenkephalin mRNA is not efficiently translated. Mechanisms by which alternative mRNA splicing may serve to confer translational regulation upon the testicular proenkephalin transcript are discussed.

The endogenous opioid peptides comprise a family of neuroendocrine peptide hormones related by the presence of a Leu- or Met- enkephalin sequence motif. In all mammalian species examined, there are three known precursor proteins which are posttranslationally processed to give rise to the 20 to 25 different bioactive forms of the opioid peptides. Each of these three precursors—proopiomelanocortin (POMC), proenkephalin, and prodynorphin—is encoded by a corresponding gene. The opioid peptide genes are expressed at numerous sites within the central nervous system, as well as in several peripheral tissues. Translation of the opioid mRNAs gives rise to polyprotein precursors, which are proteolytically processed to the various smaller bioactive forms. In different tissues, a given precursor can be differentially processed to yield alternative forms of mature bioactive peptides. Once released from the cells in which they are synthesized, the opioid peptides exert their effects by binding to specific cell surface receptors on target cells. There are at least three known classes of opioid peptide receptors, separable by their affinity for the various opioid peptide ligands (for reviews, see references 1, 12, and 44).

The endogenous opioid peptides have been implicated in the modulation of the activities of a variety of seemingly diverse neuroendocrine processes, including nociception, adaptation to stress, and hypothalamic control of reproductive functions (9, 18, 31). From the diversity of sites at which the opioid peptides are synthesized, the number of different bioactive peptides generated, and the diversity of opioid receptors, it might be expected that these peptides function in an even greater number of physiological processes.

There are several reports that opioid peptides are present at locations throughout the mammalian reproductive tract in both males and females (13, 23, 43, 45). Opioid peptide receptors have been shown to be present at sites in the male reproductive tract (11, 15). Experimental evidence suggests that opioid peptide agonists and antagonists can exert effects upon reproductive functions (14, 16, 33). The demonstration that all three opioid peptide precursor genes are expressed at sites in the mammalian reproductive tract suggests that these tissues are in fact sites of de novo synthesis of the opioids. In particular, the POMC, proenkephalin, and prodynorphin genes are all expressed within the rat testis (7, 13, 25, 37).

The testes are the site of germ cell proliferation, meiotic division, and spermiogenesis. In addition, androgen synthesis by testicular Leydig cells is essential for the development of numerous male secondary sexual characteristics. The possible involvement of testicular opioid peptides in modulating these important processes prompted us to investigate aspects of opioid peptide gene expression in the testis. In the rat, the sizes of testicular transcripts from the three opioid peptide genes are different from sizes of transcripts from other tissues in which these genes are expressed. The shorter POMC transcript has been shown to arise from an alternative downstream transcriptional initiation site (19, 20), and it is unclear whether this mRNA can be translated to generate the POMC precursor protein product. In this report, we show that the variations in the size of the testicular proenkephalin and prodynorphin mRNAs result from alternative mRNA splicing. In both instances, the length of the 5′ nontranslated region is altered. The shorter 5′ leader sequence of the testicular prodynorphin mRNA does not affect translation of the message, and any functional consequence of the alternative splicing remains obscure. In contrast, the germ cell-specific proenkephalin mRNA is spliced to yield a 491-nucleotide (nt) 5′ leader sequence preceding the preproenkephalin-coding sequence. This leader region contains several short open reading frames (ORFs). Polysome analysis indicates that the germ cell-specific proenkephalin mRNA is not translated efficiently.

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MATERIALS AND METHODS

Plasmid constructions and probe synthesis. Restriction fragments used to generate antisense RNA probes were subcloned from previously isolated genomic or cDNA clones (10, 40, 48) into the plasmid vector pGem 3 (Promega Biotec, Madison, Wis.). All procedures for bacteriophage DNA preparation, restriction enzyme digestion, gel electrophoresis, fragment isolation, ligation, bacterial transformation, and plasmid purification were according to standard published methods (29). Radiolabeled ([α-^{32}P]UTP; 3,000 Ci/mmol; DuPont, NEN Research Products, Boston, Mass.) RNA probes were synthesized in vitro by using either SP6 or T7 RNA polymerase under published reaction conditions (30). Specific activities were routinely greater than 10^9 cpm/μg. For RNase protection mapping, template DNA was removed from the transcription reaction by treatment with RNase-free DNase I (RQ-1 DNase; Promega).

RNA preparation and Northern (RNA) blot analysis. Total cellular RNA was prepared by the guanidinium isothiocyanate lysis method (8), followed by either centrifugation through a CsCl pad (46) or LiCl precipitation (6). mRNA was enriched by oligo(dT) cellulose chromatography by standard methods (29). For Northern blot analysis, total RNA (20 μg) was fractionated on a 1.2% formaldahyde-agarose gel in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) electrophoresis buffer. After capillary transfer (in 10× SSC [1× SSC is 0.15 M NaCl plus 0.015 sodium citrate]) to Nytran membranes (Schleicher & Schuell, Keene, N.H.), membranes were dried and the RNA was fixed by exposure to long-wave UV light. The membranes were hybridized in a solution consisting of 50% formamide, 400 mM $NaPO_4$ (pH 7.2), 1 mM EDTA, 1 mg of bovine serum albumin per ml, and 5% sodium dodecyl sulfate. Hybridization was at 62°C, usually for 12 to 20 h. After a prehybridization period of 2 to 3 h, the probe was added to a concentration of 10^6 cpm/ml. Following hybridization, membranes were washed in 0.1× SSC–1 mM EDTA–0.5% sodium dodecyl sulfate at 70°C for 1 to 2 h and then exposed to film for periods ranging from 12 h to 2 days.

RNase protection mapping. Antisense RNA probe (50,000 cpm) was mixed with the RNA of interest (from 10 to 100 μg) and ethanol precipitated. The pellet was dried and then suspended in 35 μl of an annealling buffer consisting of 80% formamide, 40 mM PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid), pH 6.4], 400 mM NaCl, and 1 mM EDTA. The solution was heated at 90°C for 10 min to denature the RNA and then annealed at 60°C for 10 to 12 h. Paraffin oil was layered over the top of the annealling solution to prevent evaporation. Following annealling, the RNA solution was diluted into 300 μl of an RNase digestion buffer consisting of 300 mM NaCl, 10 mM Tris (pH 7.5), 5 mM EDTA, 40 μg of RNase A per ml, and 1,000 U of RNase T1 per ml; digestion was for 30 min at 30°C. Nuclease digestion was terminated by the addition of sodium dodecyl sulfate and proteinase K to final concentrations of 1% and 250 μg/ml, respectively, and incubation at 37°C for 15 min. After phenol-chloroform extraction, the protected hybrids were concentrated by ethanol precipitation, suspended in formamide, and size fractionated on 6% acrylamide-urea sequencing gels. Gels were dried and exposed to film for 1 to 3 days. Sizes of protected hybrids were determined by reference to DNA sequencing ladders of known templates run on the same gel.

DNA sequence analysis. Restriction fragments representing regions of proenkephalin intron A were subcloned into the M13 vectors mp18 or mp19 and sequenced by the chain termination technique (42). Analysis of nucleic acid and protein sequences was with the Intelligenetics (Mountain View, Calif.) package of software run on a MicroVax II computer (Digital Electronics Corp.).

Polysome analysis. Polysomes were prepared and analyzed by the protocol of Kleene et al. (26). Briefly, 0.82 g of adult rat striatum or 2.3 g of adult rat testis was homogenized in HNM buffer (20 mM HEPES [pH 7.45], 100 mM NaCl, 1.5 mM $MgCl_2$, 5% Triton X-100, 5 mM vanadyl ribonucleotide complex, 3 μM cycloheximide), and nuclei were removed by centrifugation at 10,000 × *g* for 5 min. Supernatants were loaded onto linear 10 to 35% (wt/vol) sucrose gradients dissolved in HNM buffer with a 0.5-ml cushion of 60% (wt/vol) sucrose and centrifuged for 115 min at 37,000 rpm and 4°C in a rotor (SW41; Beckman Instruments, Inc., Fullerton, Calif.). As a control, equal portions of each supernatant were adjusted to 20 mM EDTA and centrifuged through gradients also containing 20 mM EDTA but lacking $MgCl_2$. Gradients were manually fractionated into 1-ml aliquots. RNA from each fraction was prepared by digestion in 150 mM NaCl–50 mM Tris (pH 7.5)–0.5% sodium dodecyl sulfate–20 mM EDTA–100 μg of proteinase K per ml for 1 h at 45°C followed by a single extraction with phenol-chloroform (1:1). RNA was ethanol precipitated twice and then suspended in H_2O for subsequent Northern blot analysis.

RESULTS

Northern blot analysis of testicular opioid mRNAs. Northern blot analysis showed the rat testicular prodynorphin mRNA to be ~50 to 100 nt smaller than the 2,400-nt transcript seen in striatum, a brain region with an abundance of prodynorphin mRNA (Fig. 1A). The rat testicular proenkephalin mRNA is ~1,750 nt long, larger than the 1,400-nt transcript found in striatum and other brain regions (Fig. 1B). A 540-base-pair (bp) *Pst*I-*Sph*I fragment, consisting of 460 bp of intron A sequence and 80 bp of exon 2 sequence (Fig. 1D), was subcloned from a rat genomic proenkephalin clone into the vector pGem 3 and used to generate an antisense RNA probe. At high stringency, this probe failed to hybridize to striatum RNA but did hybridize to the 1,750-nt testicular proenkephalin transcript (Fig. 1C), indicating that the larger transcript contains at least some portion of intron A sequence.

RNase protection mapping of prodynorphin mRNA. In the rat, there are single genes for both prodynorphin and proenkephalin (10, 40). Therefore, the tissue-specific differences in transcript size must arise from alternative sites of transcription initiation or termination, alternative mRNA splicing patterns, or differing lengths of poly(A) tails. RNase protection mapping experiments were undertaken to precisely define the basis for the observed size differences.

The prodynorphin transcripts were mapped in striatum, the tissue from which rat prodynorphin cDNA clones were originally isolated, and testis RNA. Liver RNA, in which prodynorphin mRNA is undetectable by the Northern blot technique, was used as a negative control. The results of this analysis are presented in Fig. 2. A 595-bp *Sau*3AI fragment, extending from within exon 1 to ~500 bp 5′ to the putative transcriptional initiation site, was subcloned from a genomic prodynorphin clone into pGem 3. The resulting plasmid, pRDP10, was used to generate an antisense probe for mapping. With both striatum and testis RNA, a 88-nt band was protected (Fig. 2A). This maps the transcriptional initiation site to a position 25 bp downstream from a

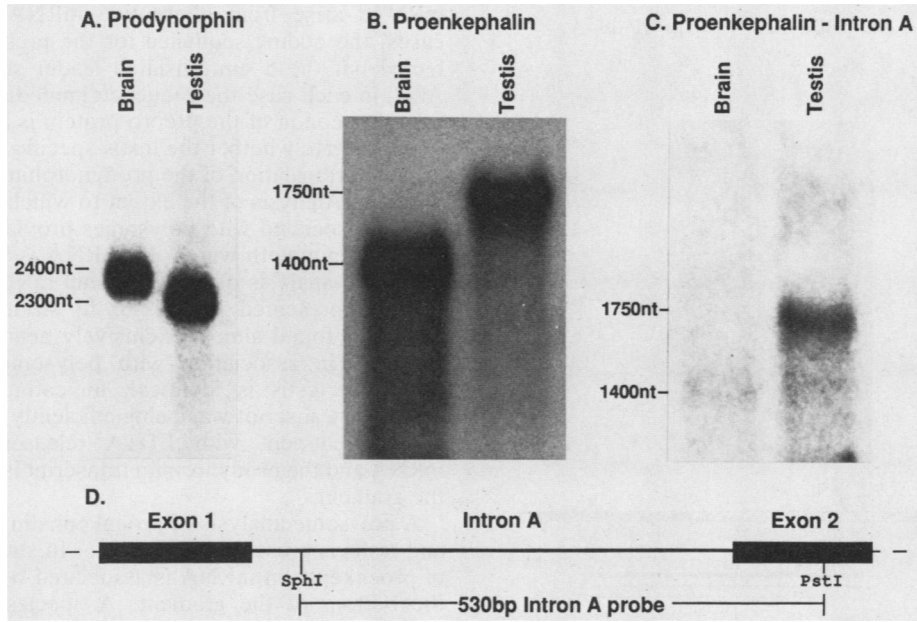

FIG. 1. Northern blot analysis of opioid transcript size. Total RNA (20 μg), isolated from striatum (brain) or testis, was hybridized with antisense RNA probes of prodynorphin (A), proenkephalin (B), or proenkephalin intron A sequence (C). A diagram of the intron A probe used in panel C is also shown (D); it comprises mainly intron A sequence but also contains 89 nt of exon 2 sequence. In vitro-generated RNA transcripts of defined size were used as size markers on the gels. The sizes of the hybridizing mRNAs are indicated.

TATAAA sequence on the gene, which corresponds to the previously mapped cap site for prodynorphin mRNA in striatum. No bands were protected by liver RNA. A 260-bp *Eco*RI-*Sac*I fragment, extending from within exon 2 to near the 5′ end of exon 1, was subcloned from a striatum cDNA clone to give the plasmid pRDP12. Mapping with this probe showed a 187-nt band protected by striatum RNA, as expected, and a 155-nt band protected by testis RNA (Fig. 2B). This size difference, 32 nt, corresponds to the length of exon 2-specific sequence represented on the pRDP12 antisense probe, suggesting that exon 2 is absent in the testicular mRNA. An antisense probe extending from a *Bgl*II site within exon 2 to a *Nco*I site within exon 4 (pRDP16) was used to map the exon 2-exon 3 and exon 3-exon 4 borders. A 280-nt band was protected by striatum RNA, as expected; a 245-nt band was protected by testis RNA (Fig. 2C). Again, the size difference of 35 nt corresponds to the length of exon 2-specific sequence represented on the probe. Mapping with additional probe fragments confirmed the absence of exon 2 in the testicular prodynorphin mRNA; the exon 3-exon 4 splicing is the same as that in striatum (data not shown). Thus, the smaller testicular prodynorphin transcript results from alternative mRNA splicing, with exon 2 absent. The size of this small exon, 65 bp, is in close agreement with the size difference observed on Northern blots (~50 to 100 nt).

RNase protection mapping of proenkephalin mRNA. Northern blot analysis indicated that the greater size of the testicular proenkephalin transcript was due to the presence of intron A sequences. RNase protection mapping was used to precisely define the nature of the size difference (Fig. 3). The 1,400-nt proenkephalin transcript was mapped by using either striatum or epididymis RNA, in which this transcript is present in great abundance. Liver RNA, in which proenkephalin mRNA is undetectable by Northern blot analysis, was used as a negative control. All subclones used for mapping were derived from rat genomic proenkephalin clones (40). A 140-nt antisense probe (pRenkS550), extending from a *Sac*I site within exon 1 to a *Pst*I site ~100 bp upstream from the previously identified cap site, was used to map the site of transcriptional initiation. In striatum, epididymis, and testis RNAs, a predominant band of 52 nt was protected (Fig. 3A). This corresponds exactly to the previously identified cap site for proenkephalin mRNA in brain (48). No bands were protected by liver RNA. An additional band of 55 nt was also observed consistently to be protected by the striatum, epididymis, and testis RNAs. Whether this is an artifact of the nuclease digestion or indicates variability in the exact site at which RNA polymerase II initiates transcription of the gene is unknown. A 163-bp *Sac*I-*Sph*I fragment (pRenkRS700) was used to map the exon 1-intron A border. A 93-nt band was protected by both epididymis and testis RNA (Fig. 3B), indicating that in the longer testicular transcript the previously identified exon 1-intron A splice junction is still being utilized. The intron A-exon 2 junction was mapped with a 540-bp *Sph*I-*Pst*I fragment (pRenkPS540). Epididymis RNA protected a 86-nt band, corresponding to the length of exon 2 sequence present on the antisense probe (Fig. 3C). Testis RNA protected a much larger band (>350 nt), although the gel system utilized prevented a precise size determination for this larger band. A 290-bp *Sph*I-*Alu*I probe (pRenkSH290), derived from within intron A, was used to more precisely define the region of intron A remaining in the testicular proenkephalin transcript. Epididymis RNA failed to protect any bands with this probe, confirming the expected absence of intron A. Testis RNA protected a predominant band of 170 nt (Fig. 3D). Fainter bands of 180 and 195 nt were also sometimes seen. The results indicate that the larger testicular proenkephalin transcript results from alternative mRNA splicing, in which 350 nt of the 3′ end of intron A remains in the mature transcript. This size difference is in close agreement with the ~350-nt larger testicular proenkephalin mRNA seen on Northern blots. The alternative mRNA splicing patterns of

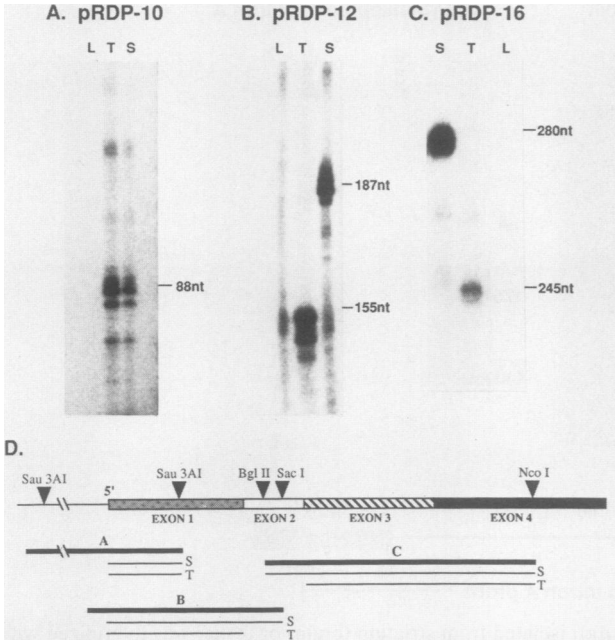

FIG. 2. RNase protection mapping of brain and testis forms of prodynorphin mRNA. Probes were obtained from genomic (pRDP10) or cDNA (pRDP12, pRDP16) clones of rat prodynorphin. The locations of these probe fragments are indicated in panel D (heavy lines) beneath a diagram of the prodynorphin cDNA structure. Autoradiograms showing the protected hybrid bands generated by each probe, analyzed on 6% acrylamide-urea gels, are shown. The lanes represent liver (L), testis (T), or striatum (S) RNA. Sizes of protected hybrid bands are indicated. The locations of the protected hybrids generated by each probe in striatum and testis RNA are indicated (D, light lines).

the testicular forms of proenkephalin and prodynorphin are schematically summarized in Fig. 4.

Nucleotide sequence of proenkephalin gene intron A. The presence of intron A sequence in the mature testicular proenkephalin transcript suggested that it may have some functional importance. The nucleotide sequences of the exon regions of the rat proenkephalin gene have been previously determined (40). DNA fragments representing intron A were subcloned from a rat proenkephalin genomic clone, λRE-4, into M13 vectors and sequenced. The nucleotide sequences of exon 1, intron A, and a portion of exon 2 are presented in Fig. 5. Intron A is 511 bp long. There is a good consensus 3′ splice site acceptor sequence (17) at the point where the RNase mapping indicates that the testis-specific splicing occurs. Comparison of the rat and human proenkephalin gene intron A sequences shows that this region where the testis-specific splicing occurs is more highly conserved than the remainder of the intron sequence (data not shown). The consequence of the testis-specific splicing is to generate a proenkephalin mRNA with a 491-nt 5′ leader sequence preceding the preproenkephalin ORF. Analysis of this leader sequence reveals no long uninterrupted ORFs. However, there are three short methionine-initiated ORFs encoded by the longer testis-specific leader. The longest of these encodes 46 amino acids; this small amino acid sequence has no significant homology with entries in the National Biomedical Research Foundation protein database. Each of these small ORFs terminates before the preproenkephalin ORF.

Translation of testicular opioid mRNAs. The size differences of both prodynorphin and proenkephalin testicular mRNAs arise from alternative mRNA splicing. In both cases, the coding sequence for the prepro-protein is unaffected but the 5′ untranslated leader sequence is altered. Also, in each case the sequence immediately preceding the initiation codon of the prepro-protein is altered. This led us to investigate whether the testis-specific splicing had effects upon the translation of the prodynorphin and proenkephalin mRNAs. Analysis of the extent to which an mRNA population is associated with polysomes provides an indication of the efficiency with which the mRNA is being translated. A polysome analysis of prodynorphin mRNA in striatum and testis is presented in Fig. 6. In striatum, prodynorphin mRNA is found almost exclusively near the bottom of the gradient, in association with polysomes. The polysome profile in testis is identical, indicating that the shorter testicular transcript was being efficiently translated. In both cases, treatment with EDTA releases ribosomes from mRNA and the prodynorphin transcript is found at the top of the gradient.

A polysome analysis of proenkephalin mRNA in striatum and testis is presented in Figure 6. In striatum, the majority of proenkephalin mRNA is associated with polysomes near the bottom of the gradient. A species of proenkephalin mRNA slightly smaller than the 1,750-nt testis-specific form can be seen in the upper fractions of the striatum polysome profile. We do not know what this low-abundance species of RNA represents. In contrast to the striatum polysome profile, most of the testicular proenkephalin mRNA tend to be found near the top of the gradient, indicating that it was not being efficiently translated. Some of the testicular proenkephalin mRNA is found lower in the gradient, and EDTA treatment released it into the upper fractions. However, the longer testicular proenkephalin mRNA clearly is not associated with polysomes to the extent that the 1,400-nt striatum mRNA is. Thus, the 1,750-nt testicular proenkephalin transcript appears to be inefficiently translated, while the 1,400-nt striatal proenkephalin mRNA is highly translated.

DISCUSSION

Opioid peptides are found in the testis, and the discovery that all three genes encoding the opioid peptide precursors are expressed in the testis suggested that this organ is a site of de novo synthesis of the endogenous opioids. However, in the rat, as well as other mammalian species, the sizes of the testicular opioid mRNAs are different from sizes of opioid mRNAs observed in the brain regions from which cDNA clones of the opioid precursors were originally obtained. There are examples of other peptide hormone genes in which alternative mRNA splicing generates different precursor proteins which give rise to novel bioactive products (2). Thus, we were prompted to investigate whether the mechanisms generating differing sizes of the testicular opioid gene transcripts had functional consequences.

The shorter prodynorphin mRNA found in testis is generated by alternative mRNA splicing. The small, 65-bp exon 2 is not present in the mature transcript, with exon 1 being directly spliced to exon 3. Exon 2 makes up 5′ untranslated sequence; thus, the prodynorphin-coding sequence is unaltered. However, the initiation codon of the preprodynorphin ORF lies just 15 bp 3′ to the exon 2-exon 3 splice junction, so the sequence environment in which the ribosomes will initiate translation of the testicular mRNA is dramatically altered. Additionally, the testicular prodynorphin mRNA has a leader sequence lacking 65 nt found on the prodynorphin mRNA in other tissues. There are a few examples of

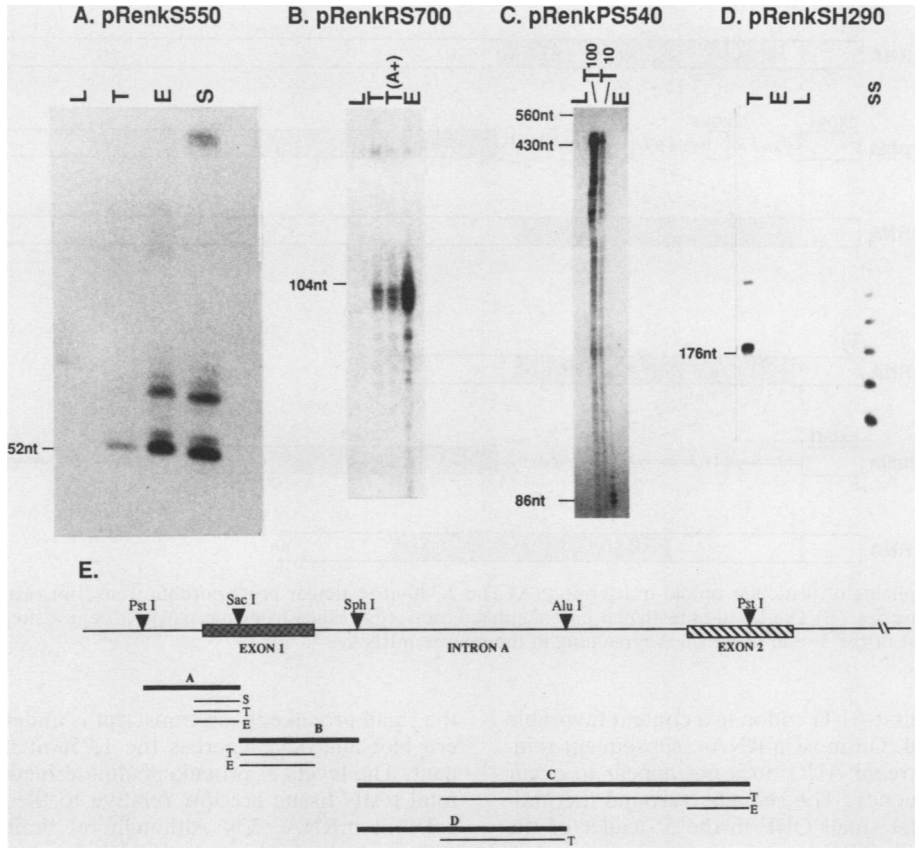

FIG. 3. RNase protection mapping of proenkephalin mRNA. The locations of restriction enzyme fragments used to generate antisense probes for the mapping are indicated by heavy lines in the diagram (E) beneath a map of the 5′ region of the rat proenkephalin gene. Autoradiograms showing the protected hybrid bands generated by each probe, analyzed on 6% acrylamide-urea gels, are shown. The lanes represent liver (L), testis (T), epididymis (E), and striatum (S) RNA. Protection experiments were performed with 20 μg of total RNA, except that T(A^+), T_{100}, and T_{10} indicate that 1 μg of poly(A^+) or 100 or 10 μg of total testis RNA was used. The sizes of hybrid-protected bands are indicated. The locations of the protected bands generated by each of the probes in striatum, testis, and epididymis RNA are indicated in the diagram (light lines).

mRNA sequence regions functioning in *cis* to regulate translation of the message on which they reside. Ferritin mRNA contains a region in the 5′ leader that interacts with a protein factor to confer translational regulation (3), and there is evidence that RNA secondary structure can affect the efficiency of translational initiation (21, 36, 41). The alteration of the 5′ leader of the prodynorphin mRNA by tissue-specific splicing suggested the possibility that similar mechanisms control dynorphin production in the testis. However, polysome profiles indicate that the testicular prodynorphin mRNA is being translated as efficiently as the larger brain form. Within the testis, the prodynorphin gene is expressed in Sertoli cells, and prodynorphin-derived peptides are present in these cells (M. Collard, unpublished results). This is consistent with the present finding that the testis-specific splicing of prodynorphin mRNA does not alter the coding or translation of this message. We cannot discount the possibility that the alternative splicing generates some other important functional consequence, such as altering the stability of the mRNA. The mechanism by which this cell-specific splicing occurs is unknown. There are numerous examples of similar alternative splicing, in which an exon in one form of a message is completely spliced out of an alternative form. The donor and acceptor splice sites of exons 1, 2, and 3 of the prodynorphin gene conform to the consensus sequences for these sites (17), so the prodynorphin gene does not appear exceptional in this regard.

As in the case of prodynorphin, the testis-specific splicing of proenkephalin mRNA gives rise to a transcript with an altered 5′ leader sequence. In this case, the testicular transcript arises from a specific splicing event in which only the 5′ 170 nt of intron A are spliced out, generating a mature transcript with a 491-nt leader sequence preceding the preproenkephalin ORF. Polysome profiles indicate that this larger mRNA is translated very poorly. Most eucaryotic mRNAs have a 5′ untranslated leader sequence on the order of 30 to 60 nt (27). A leader sequence the length of that found on the testicular proenkephalin transcript is relatively unusual. Nucleotide sequence analysis of intron A does not show any long regions capable of forming stable RNA secondary structures; only poorly matched hairpin regions 10 to 20 nt long with ΔG^0 of ~ -10 to -20 kcal/mol could potentially form. It does show, however, that the testis-specific message contains three small methionine-initiated ORFs preceding the preproenkephalin ORF. Evidence, both from experimental systems (28) and from naturally occurring examples (32, 34), indicates that the presence of methionine-initiated ORFs 5′ to a protein-coding region will severely inhibit translation of the downstream region. Presumably, ribosomes load onto the mRNA at the 5′ cap and scan

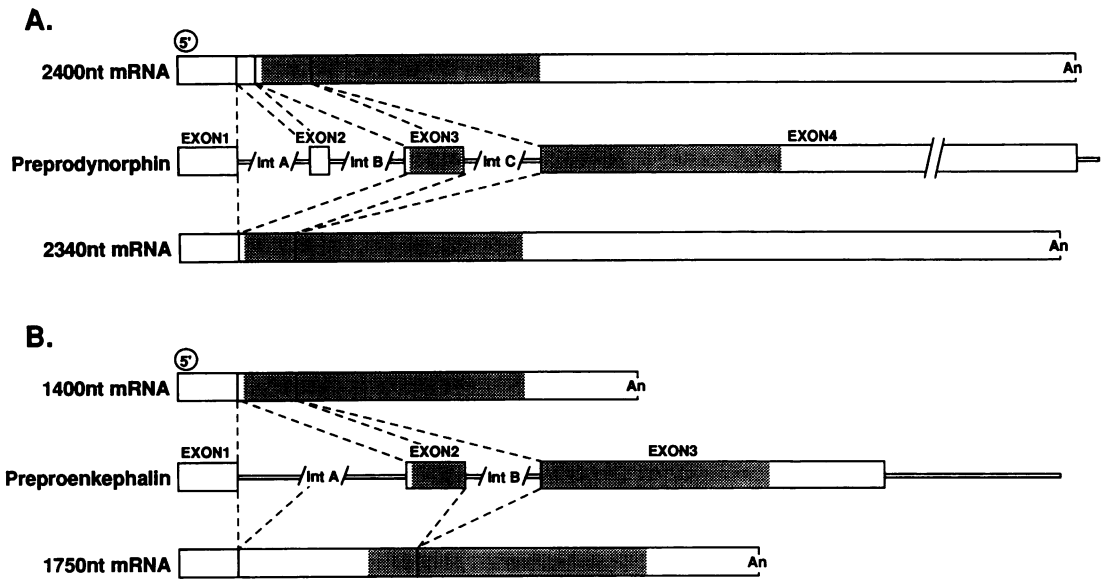

FIG. 4. Alternative splicing of testicular opioid transcripts. (A) The 2,340-nt testicular prodynorphin transcript results from removal of exon 2 in the mature transcript. (B) The 1,750-nt testicular proenkephalin transcript results from alternative acceptor site usage in the splicing of intron A, with ~350 nt of the 3′ end of intron A remaining in the mature mRNA.

downstream until the first AUG codon in a context favorable to initiation is reached. On most mRNAs, subsequent reinitiation at any downstream AUG does not appear to occur with appreciable frequency. The sequence around the initiation codon of the first small ORF in the 5′ leader of the testicular proenkephalin mRNA conforms to the consensus sequence of a strong translational initiation site compiled by Kozak (28), and it seems likely that initiation at this upstream ORF inhibits translation of the preproenkephalin precursor.

The 1,750-nt proenkephalin transcript found in adult testis is expressed in developing germ cells (24). The 1,400-nt proenkephalin transcript is also expressed in adult rat testis, specifically in the Leydig cell population (J. Garrett, manuscript submitted). Leydig cells make up a small proportion of the total testicular cells (<5%) relative to the germ cell population (>90%). In a total adult testis RNA population, the small proenkephalin transcript is undetectable by Northern blot analysis, whereas the 1,750-nt transcript is abundant. The levels of proenkephalin-derived peptides found in total testis tissue are low relative to the abundance of the 1,750-nt mRNA (22). Although rat brain and testis have comparable proenkephalin mRNA levels, the testis has less than 4% of the rat brain content of proenkephalin-derived peptides. This is in keeping with the present demonstration that the large proenkephalin transcript is not efficiently translated. The proenkephalin-derived peptides that are found in the testis could result from translation of the 1,400-nt transcript in Leydig cells.

The finding that the proenkephalin gene is abundantly transcribed in germ cells but spliced in such a manner that the resulting mRNA is not efficiently translated raises the question of what, if any, functional role the proenkephalin mRNA may have. One of the small ORFs on the germ

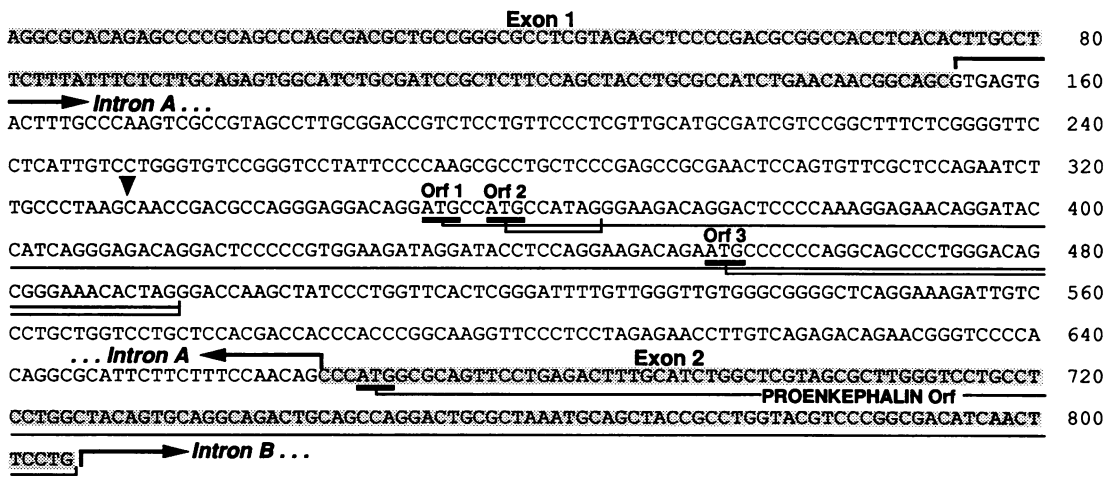

FIG. 5. Nucleotide sequence of proenkephalin gene intron A. The sequences of exon 1 and exon 2 are shaded. The arrow indicates the site of germ cell-specific splicing of intron A. The three small ORFs that precede the proenkephalin ORF in the 1,750-nt transcript are shown.

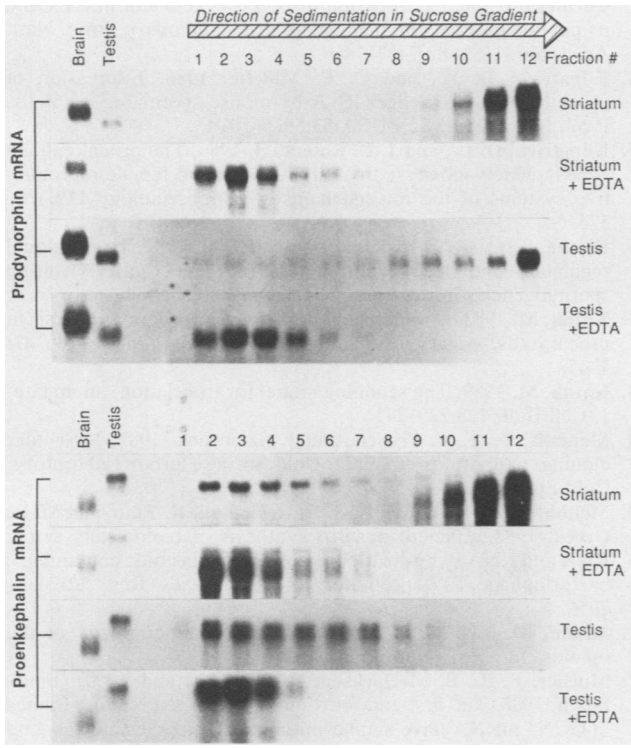

FIG. 6. Polysome analysis of testicular opioid transcripts. The association of prodynorphin and proenkephalin mRNA with polysomes in the testis and striatum was analyzed as described in Materials and Methods. The direction of sedimentation in the sucrose gradient is indicated. Lanes of rat brain and testis RNA were run on the gels along with RNA from the gradient fractions. The same four Northern blots were hybridized with prodynorphin and then proenkephalin probes.

cell-specific 5′ leader could potentially encode a small polypeptide of 46 amino acids. It is plausible that this is a functional product, although we have no evidence to suggest that it is.

The polysome analysis of proenkephalin mRNA in the testis revealed a slight tendency for the 1,750-nt mRNA to spread into the lower regions of the gradient, suggesting that it was associating with monosomes or polysomes to some extent. There are several mRNAs expressed in developing germ cells that are translationally regulated. For example, protamine mRNA is expressed at the round spermatid stage of development but stored for several days until translated in the elongating spermatids (26). There is evidence suggesting that these stored mRNAs are bound up in a ribonucleoprotein complex, the chromatoid body (35), and presumably each regulated mRNA possesses a region functioning to confer this translational regulation. Likewise, the germ cell-specific proenkephalin transcript could be subject to stage-specific translational regulation. If it is translated only at a specific stage of germ cell development, performing the polysome analysis on the total testicular cell population would tend to mask this. The small fraction of the message found in the lower regions of the gradient could represent translation at a specific spermatogenic stage. The intron A-specific sequence remaining on the mature mRNA could perhaps be functioning to provide signals for this translational regulation. However, for this to be true, it would still be necessary to postulate some mechanism to overcome the block to translation imposed by the upstream initiation codons.

A further possibility is that the production of proenkephalin-derived peptides in germ cells is being inhibited at the translational level by a mechanism involving alternative splicing and that the transcript itself is nonfunctional. A number of examples in which the on-off regulation of the production of a gene product is controlled at the level of mRNA splicing (for a review, see reference 4) have been discovered. The sex-specific expression of the product of the *transformer* locus in *Drosophila* spp. is controlled by a mechanism of alternative splicing quite similar to that seen with proenkephalin; alternative acceptor site usage in the first intron determines the production of functional versus apparently nonfunctional mRNAs (5). Thus, although the proenkephalin gene is abundantly transcribed in developing germ cells, the primary transcript is specifically spliced in such a manner that it can not be efficiently translated. Although such a mechanism seems "wasteful" in contrast to a mechanism of regulation operating at the level of transcription of the gene, it does appear to be a strategy that has evolved for several genes. The inhibition of translation by the presence of short ORFs in the leader sequence would prevent an excessive expenditure of resources on an unnecessary product.

A number of the genes known to be expressed in developing germ cells give rise to transcripts of sizes different from sizes of transcripts seen in somatic tissues (39, 47). These germ cell-specific transcript size differences have been shown to arise by several different mechanisms, either alternative splicing or different sites of transcriptional initiation of termination. Although in many cases the germ cell-specific message is still functional, for several genes it seems unclear whether they could encode their normal product or any protein product. For example, the other opioid peptide precursor gene, POMC, is abundantly expressed in germ cells (22; Garrett, submitted). However, it is a shortened transcript that initiates within the POMC protein coding region and cannot possibly serve as template for normal POMC synthesis (19, 20). The c-*mos* transcript expressed in mouse germ cells has a 5′ leader sequence containing four AUG codons preceding the *mos* ORF. This mRNA was not found associated with polysomes to a great extent, and attempts to find c-*mos* protein in germ cells were apparently unsuccessful (it should be noted, however, that c-*mos* protein is in very low abundance in all the tissues in which it has been found) (38). These observations suggest that perhaps there is a propensity for aberrant transcripts to be generated by germ cells. The determination of whether these germ cell-specific transcripts actually give rise to protein products seems warranted.

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