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Sequence and expression of the rat prodynorphin gene

(dynorphin/α-neoendorphin/genomic DNA/gene evolution/gene expression)

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ABSTRACT **We report here the isolation of a λ genomic clone that contains the nucleotide sequence coding for the main exon of the rat prodynorphin (proenkephalin B) gene. This exon codes for the majority of the translated region of prodynorphin mRNA including the opioid peptides α-neoendorphin, dynorphin A, and dynorphin B. The entire 3′ untranslated region is also contained on the λ clone. Nucleotide sequence comparison with the main exon of the human prodynorphin gene reveals both structural and sequence homology. RNA blot analysis reveals that prodynorphin transcripts can be seen in numerous regions of the rat brain and in the adrenal gland, spinal cord, testis, and anterior pituitary.**

Prodynorphin is the most recently characterized opioid peptide precursor molecule. Molecular cloning has revealed that three copies of [Leu]enkephalin are present within the precursor molecule and that all of the bioactive opioid peptides that are proteolytically processed from the precursor are COOH-terminal extensions of these [Leu]enkephalin sequences (1). α-Neoendorphin and β-neoendorphin (2), dynorphin A and dynorphin A-(1–8) (3–6), dynorphin B (7, 8), and leumorphin (9, 10) (a COOH-terminal extension of dynorphin B) all appear to be stable biologically active peptides that are generated from the prodynorphin precursor.

A large body of evidence suggests that prodynorphin-derived peptides may serve to modulate a variety of responses such as pain perception (3, 11, 12), intestinal peristalsis (13), feeding (14), sleep, and general activity (15). Thus, prodynorphin-derived peptides may act as neurotransmitters or neuromodulators in the brain or as neurohormones in the endocrine system.

The nucleotide sequence of porcine prodynorphin mRNA (16) and the human prodynorphin gene (17) have been reported. The latter study revealed that the human prodynorphin gene is present on four exons separated by intronic regions varying in length from 1 to 10 kilobases (kb). The human proopiomelanocortin, proenkephalin, and prodynorphin genes are very similar in structure, and it has been suggested that these genes may have evolved via similar evolutionary mechanisms (17). These three genes comprise the family of known opioid peptide genes.

Since the rat has been used almost exclusively in experiments aimed at determining various aspects of synthesis, processing, transport, secretion, and regulation of prodynorphin-related peptides, it is important to determine the structure of rat prodynorphin and its corresponding gene. This is especially true for nucleic acid-related studies. For example, the degree of heterogeneity between mammalian prodynorphin genes is sufficiently high that radiolabeled porcine prodynorphin cDNA fails to detect bovine prodynorphin mRNA (18).

In this paper, we report the isolation and characterization of the main exon of the rat prodynorphin gene. A comparison of amino acid and nucleic acid sequences of the main exon of the rat and human prodynorphin gene reveals that the opioid peptide coding sequences have been highly conserved, while the remaining regions have diverged during evolution. Rat prodynorphin genomic DNA has also been used as a molecular probe to analyze the expression of the prodynorphin gene in a variety of rat tissues. This work provides a basis for further studies on the regulation of the prodynorphin gene in the rat.

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MATERIALS AND METHODS

Isolation of a Porcine Prodynorphin cDNA Clone. RNA was prepared from supraoptic nuclei of porcine hypothalami by the guanidinium thiocyanate/CsCl procedure. Poly(A) mRNA was purified by chromatography on oligo(dT)-cellulose. Oligodeoxynucleotides were synthesized by the triester method. The oligomer used to prime first strand cDNA synthesis was 17 bases long, had the sequence 5′ C-A-T-T-G-A-T-C-G-G-T-A-A-A-C-T-T 3′, and was complementary to the sequence of porcine prodynorphin mRNA between bases 1112 and 1128 (16). The tetradecamer (14-mer) pool used to screen the specifically primed cDNA library represented all possible cDNA sequences predicted from the COOH-terminal pentapeptide sequence of porcine dynorphin A, Lys-Trp-Asp-Asn-Gln.

Supraoptic nuclei poly(A) mRNA (25 μg) was incubated with 1000 pmol of the 17-mer, and cDNA synthesis was performed by reverse transcriptase and the Klenow fragment of DNA polymerase I. After treatment with S1 nuclease and size fractionation by acrylamide gel electrophoresis, the cDNA fragments were electroeluted, d(C)-tailed, annealed to d(G)-tailed pBR322, and transformed into *Escherichia coli*. Approximately 15,000 tetracycline-resistant cDNA clones were transferred to nitrocellulose filters, lysed, and immobilized. After hybridization with the 14-mer pool, which was ^{32}P-labeled, DNA from three hybridization-positive clones was isolated and sequenced. One of the clones, pSSP3-5, was shown to be a partial porcine prodynorphin cDNA clone, as it coded for residues 656–1125 of porcine prodynorphin mRNA (16).

Isolation of Rat Prodynorphin Genomic DNA Fragments. For screening, we used a rat λ genomic library (kindly provided by G. Scherer), which was constructed by partial *Sau*3A digestion of rat liver DNA, followed by cloning into the λ phage vector, EMBL-3. Plaques (500,000) were screened in duplicate with nick-translated pSSP3-5 insert. DNA from two positively hybridizing plaques, λRD1 and λRD2, was subjected to restriction mapping. This analysis revealed that the two λ clones contained a common *Hin*dIII/*Eco*RI fragment that hybridized to the pSSP3-5 probe. A 5.1-kb *Eco*RI fragment was isolated from λRD2 and subcloned into *Eco*RI-cut pBR322 (pλRD2-19) and was further characterized by mapping with additional restriction enzymes. The appropriate restriction fragments were then subcloned into the

Abbreviations: kb, kilobase(s); bp, base pair(s).

replicative form of M13 mp8, mp9, mp10, or mp11 DNA for nucleotide sequence analysis, or pUC8 and pUC9 for use as hybridization probes in RNA and Southern analysis.

Transfer Blotting of Rat mRNA and Genomic DNA. Tissues were dissected from adult male Sprague–Dawley rats. Poly(A) mRNA was denatured in 3 M glyoxal and subjected to electrophoresis on a 1.5% agarose gel in 10 mM phosphate buffer. Transfer to nitrocellulose, baking, and hybridization were performed according to Thomas (19). Three subclones covering a 2700-base-pair (bp) *Bgl* II/*Pst* I fragment of pλRD2-19 were isolated and used as a hybridization probe. These fragments contain the entire 3′ main exon of the rat prodynorphin gene.

Analysis of genomic DNA isolated from a single rat liver by hybridization to radiolabeled DNA fragments from the subclones described above was carried out according to Southern (20).

RESULTS

Isolation and Nucleotide Sequence of the Main Exon of the Rat Prodynorphin Gene. A two-step strategy was used to isolate the rat prodynorphin gene. The first step involved the isolation of a cDNA clone specific to a portion of porcine prodynorphin mRNA. The second step involved the use of this cDNA clone as a hybridization probe to isolate the rat prodynorphin gene from a rat λ genomic library.

Six positively hybridizing λ plaques were isolated and purified. Restriction analysis revealed that two clones (λRD2 and -4) were identical and that part of their sequence was common with λRD1. The three remaining λ clones were unique, but upon further analysis they were determined to be unrelated to the prodynorphin gene (data not shown). After *Eco*RI digestion, λRD2 was shown to contain a 5100-bp fragment that hybridized with the pSSP3-5 cDNA insert. This fragment was subcloned into the *Eco*RI site of pBR322 and will be referred to as pλRD2-19.

The complete nucleotide sequence of the main exon of the human prodynorphin gene (17) led us to propose the following organization of the pλRD2-19 insert (Fig. 1). The first (5′) 390 bases are part of an intron that terminates in the sequence C-T-T-C-A-G, consistent with the consensus splice junction sequence. The next 12 bases are identical to those found at

FIG. 1. Nucleotide sequence of the main exon of the rat prodynorphin gene. The direction of transcription (5′ to 3′) is indicated. The partial amino acid sequence encoded by rat prodynorphin mRNA is shown below the corresponding DNA sequence. The [Leu]enkephalin moieties and the three opioid domains (α-neoendorphin, dynorphin A, and dynorphin B) are outlined by boxes. The poly(A) recognition sites (A-A-T-A-A-A) are underlined and the putative site of poly(A) addition is indicated by a vertical arrow.

the 5′ end of the main exon of the human prodynorphin gene and thus allow us to predict the exact position of an intron/exon splice junction in the rat prodynorphin gene. The next 612 bases constitute the major part of the coding region of rat prodynorphin mRNA, including the nucleotides that code for α-neoendorphin and β-neoendorphin, dynorphin A and dynorphin A-(1–8), leumorphin, and dynorphin B. The 3′ untranslated region of rat prodynorphin mRNA begins after the translational termination codon at nucleotide 1006. As with porcine and human prodynorphin mRNA, rat prodynorphin mRNA appears to have an unusually long 3′ untranslated region. Although there is not a high degree of nucleotide sequence homology between this region of rat, porcine, and human prodynorphin mRNA, it is possible to draw some conclusions concerning the length and organization of the 3′ untranslated region. In the rat prodynorphin gene, two poly(A) addition sites overlap each other (residues 2441–2450), while the third site is located ≈300 bases upstream. In human and porcine prodynorphin mRNA, three poly(A) addition sites are situated at identical locations with respect to the translation termination codon and the poly(A) tail. The conserved positioning of the three poly(A) addition sites in the rat prodynorphin gene allows us to predict that poly(A) addition occurs at residue 2462.

To determine whether λRD1 and -2 represent a unique prodynorphin gene, rat genomic DNA was digested with a variety of restriction endonucleases and subjected to Southern analysis (data not shown). Several DNA fragments representing various portions of pλRD2-19 served as hybridization probes. In all cases, only a single hybridizing band was visualized after autoradiography. This result and the fact that all positively hybridizing λ genomic clones shared common restriction patterns led us to the conclusion that there is a single prodynorphin gene present in the rat genome.

Sequence Homology Between the Rat, Porcine, and Human Prodynorphin Gene. Fig. 2 highlights the regions of homology among the rat, porcine, and human prodynorphin gene at both the nucleic acid and peptide levels. At the amino acid level (Fig. 2*A*), the three species of prodynorphin share perfect homology in the sequence of α-neoendorphin, dynorphin A, and dynorphin B, as well as in the basic amino acids that flank these domains. It can also be seen that the peptide region between α-neoendorphin and dynorphin A is two or four amino acids longer in the rat precursor than in the porcine or human precursor, respectively. Conversely, the COOH-terminal extension of dynorphin B in the rat is one amino acid shorter than its human or porcine counterpart, indicating that there must be a size difference in leumorphin among these species. Rat prodynorphin is also unique in that it contains an additional pair of basic amino acid residues upstream from α-neoendorphin, which could serve as a cleavage site for the generation of the dipeptide Gln-Ala.

Nucleic acid sequence homology comparison between the main exon of the rat and human prodynorphin gene (Fig. 2*B*) reveals that the highest degree of homology (>90%) is present in the regions that code for α-neoendorphin (nucleotides 742–813) and dynorphin A/B (nucleotides 834–973). Two additional regions within the translated portion of the mRNAs from the two species (nucleotides 398–480 and 544–608) also share a high degree of homology (75–90%).

Four regions showing 75–90% nucleotide sequence homology were also observed in the 3′ untranslated region of the human and rat prodynorphin genes (nucleotides 1319–1399, 1753–1861, 2077–2141, and 2306–2456). The putative poly(A) addition recognition sequence for the rat prodynorphin transcript (A-A-U-A-A-A-U-A-A-A) is contained within one of these regions (2306–2456).

Tissue-Specific Expression of the Rat Prodynorphin Gene. Numerous groups have used immunocytochemical methods to show that prodynorphin-related peptides are present in widespread regions of the brain and in several nonbrain tissues (6, 21–30). To determine whether prodynorphin-derived peptides are synthesized at these sites or transported to these sites from other regions of synthesis, RNA blot

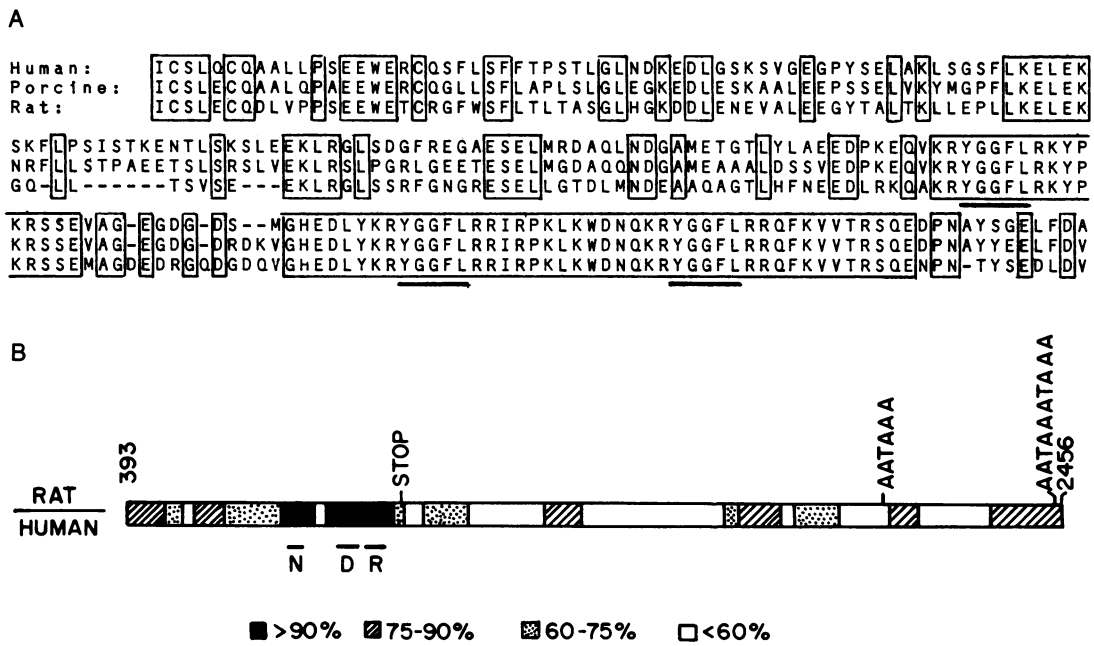
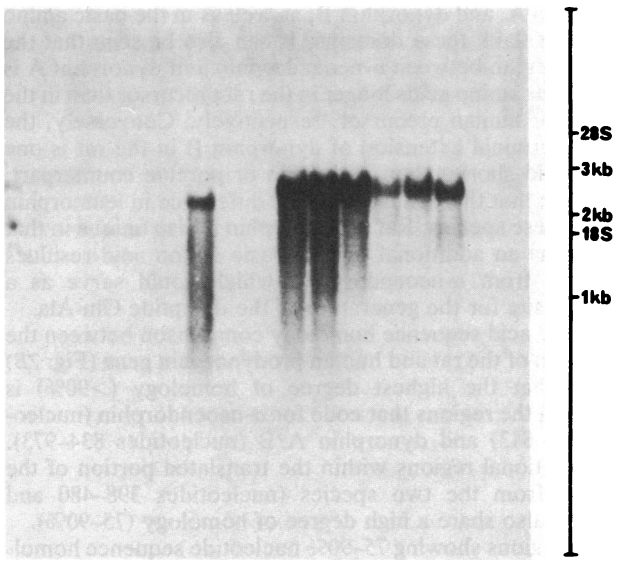


FIG. 2. Amino acid comparison and nucleic acid sequence homology between human, porcine, and rat prodynorphin. (*A*) Alignment of the amino acid sequences of human (21), porcine (22), and rat prodynorphin. The single letter amino acid notation is used. Boxed areas represent residues that are identical between the three species. Gaps (−) have been introduced to achieve maximum homologies. The locations of the enkephalin sequences are indicated by a solid line below the sequences. (*B*) Schematic representation of the nucleotide sequence homology between the main exon of the human and rat prodynorphin gene. The residue numbers indicated correspond to the rat sequence. The translation termination codon (STOP) and the poly(A) recognition sites (A-A-T-A-A-A; A-A-T-A-A-A-T-A-A-A) are indicated. The three [Leu]enkephalin-containing domains (N, α-neoendorphin; D, dynorphin A; R, dynorphin B) are underlined. Percentage homology is as noted in the figure.

analysis was used to detect the presence of prodynorphin mRNA in a variety of tissues (Fig. 3).



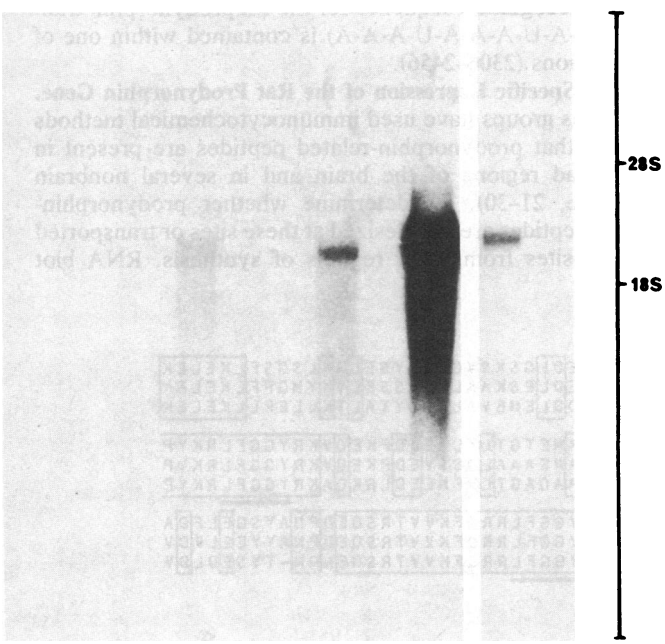


FIG. 3. Localization of prodynorphin mRNA in different rat tissues. Prodynorphin mRNA sequences were detected by hybridization to the 2700-bp *Bgl* II/*Pst* I restriction fragment of pλRD2-19 (entire 3′ main exon). The fragment was radiolabeled with [α-^{32}P]dCTP to a specific activity of 10^9 cpm per μg of DNA. Hybridization was performed at 42°C overnight. The poly(A) mRNA samples (amounts noted) that were analyzed were derived from the following rat tissues: A, thymus, 4 μg; B, spleen, 20 μg; C, kidney, 20 μg; D, lung, 20 μg; E, pancreas, 20 μg; F, intestine, 20 μg; G, adrenal gland, 8 μg; H, pituitary gland, 2 μg; I, cerebral cortex poly(A)$^-$ RNA, 2 μg; J, hypothalamus, 10 μg; K and KK, striatum, 10 μg; L, hippocampus, 10 μg; M, nucleus tractus solitarius region of brainstem, 10 μg; N, midbrain, 10 μg; O, cerebral cortex, 20 μg; P, cerebellum, 20 μg; Q, thalamus, 6 μg; R, muscle, 6 μg; S, liver, 7 μg; T, submaxillary gland, 12 μg; U, spinal cord, 15 μg; V, heart, 21 μg; W, tongue, 23 μg; X, testis, 26 μg; Y, anterior pituitary, 14 μg; and Z, neurointermediate pituitary, 2 μg.

The highest concentrations of prodynorphin mRNA are found in the hypothalamus, striatum, and hippocampus. Prodynorphin mRNA is also detected in the midbrain, nucleus tractus solitarius region of the brainstem, and cerebral cortex. After a longer exposure of the autoradiogram shown in Fig. 3, prodynorphin mRNA sequences could also be detected in the thalamus and cerebellum. The prodynorphin mRNA content of brain regions calculated as percentage of the total poly(A) mRNA content may thus be represented as follows: striatum = hippocampus > hypothalamus >> midbrain > nucleus tractus solitarius = cortex >>> thalamus > cerebellum. In the nonbrain tissues analyzed, prodynorphin transcripts were detected in the adrenal gland (whole adrenal), spinal cord, testis, and anterior pituitary. In the adrenal gland and testis, dynorphin mRNA concentrations are of the same order of magnitude as that in the cerebral cortex, while the spinal cord and anterior pituitary dynorphin mRNA levels are similar to that found in the cerebellum.

Prodynorphin mRNA in the striatum, hippocampus, and hypothalamus is ≈2400 bases long. Interestingly, prodynorphin mRNA in the cerebral cortex, cerebellum, and testis is ≈100 bases shorter than in the hypothalamus. Moreover, the adrenal prodynorphin mRNA is ≈350 bases shorter than prodynorphin transcripts in the hypothalamus.

DISCUSSION

To determine the structure of rat prodynorphin and to develop tools to study the regulation of expression of the rat prodynorphin gene, we have used a partial porcine prodynorphin cDNA clone as a hybridization probe to isolate and characterize the rat prodynorphin gene from a rat λ genomic library. Two unique but overlapping λ genomic clones were isolated, and the nucleotide sequence of the main exon of the rat prodynorphin gene was determined. This exon codes for 204 amino acids of rat prodynorphin. The precursor shows the same organization as its human and porcine counterpart, containing three [Leu]enkephalin sequences corresponding to α-neoendorphin, dynorphin A, and dynorphin B. The peptide sequence of these three opioid domains and the basic amino acid residues flanking them are identical in the three species.

Nucleic acid sequence comparison between the major exon of the rat and human prodynorphin gene reveals, as expected, that the regions sharing the highest degree of homology code for the bioactive peptides. Two additional segments in the translated region appear to share a high level of homology but do not code for any known prodynorphin-derived peptide. There are also four distinct regions within the 3′ untranslated portion of rat and human prodynorphin mRNA that appear to be conserved, as well as the unusually long length of 3′ untranslated sequence. These multiple regions of homology between the rat and human prodynorphin gene suggest that this gene has been highly conserved through evolution.

Southern analysis also reveals that there is a single prodynorphin gene in the rat genome. Hybridization of rat genomic DNA with the DNA fragment present in pλRD2-19 revealed a single band after autoradiography (data not shown). This result and the fact that screening of the rat λ genomic library resulted in the isolation of a single DNA fragment containing prodynorphin-coding sequences suggest the presence of a single rat prodynorphin gene.

We have also determined the sites of synthesis of rat prodynorphin by screening for the presence of prodynorphin mRNA in a variety of tissues. Prodynorphin mRNA can be detected in virtually all brain regions analyzed. In general, the concentration of prodynorphin mRNA in various brain regions corresponds to the level of dynorphin-related peptides in those tissues (21–26). However, there are some differences. In the brain, dynorphin-related peptides are most

highly concentrated in the hypothalamus, yet levels of prodynorphin mRNA are significantly higher in the striatum and hippocampus than in the hypothalamus. Several factors such as tissue-specific proteolytic processing, secretion, transport, or rate of turnover of dynorphin-related peptides may account for this finding (27, 28). The relative concentrations of prodynorphin mRNA in the various rat brain regions are quite different from the levels of proopiomelanocortin mRNA (31) and proenkephalin mRNA (32) seen in the same regions. Thus, transcription of the three opioid peptide genes in the rat brain is unique for each brain region.

In the nonbrain tissues tested, rat prodynorphin mRNA was detected in the adrenal gland, spinal cord, testis, and anterior pituitary. In general, in these tissues the levels of prodynorphin mRNA do not parallel the levels of prodynorphin-derived peptides (29). For example, prodynorphin mRNA is moderately abundant in the rat testis and adrenal gland, while little, if any, detectable immunoreactive dynorphin is present in these tissues (29). On the other hand, high levels of immunoreactive dynorphin are present in the rat anterior pituitary and spinal cord (29), while low levels of prodynorphin mRNA are present in these tissues. In addition, dynorphin-related peptides have been detected in rat lung (30), kidney, intestine, muscle, liver, and heart (29), while no detectable levels of prodynorphin mRNA are present in these tissues. These data suggest that the sites of synthesis of the prodynorphin precursor may not always correspond to the sites where dynorphin-related peptides are found. Factors such as transport and uptake of dynorphin-related peptides, peptide stability, tissue-specific protein processing, and sensitivity of detection techniques may all play a role in the apparent discrepancy between dynorphin mRNA and peptide levels in these nonbrain tissues.

In a porcine tissue survey, prodynorphin mRNA was detectable in the spinal cord and ileum but was not seen in the adrenal gland and anterior pituitary (18). These results suggest that in some tissues prodynorphin gene expression is species specific.

RNA blot analysis also revealed that the size of prodynorphin mRNA was variable. Prodynorphin mRNA in the cerebral cortex and testis is $\approx$100 bases shorter than in the hypothalamus. Interestingly, this size difference has also been reported for proopiomelanocortin mRNA in the same tissues (31). Also, rat adrenal prodynorphin mRNA is at least 300 bases smaller than the brain prodynorphin transcript. This difference in length may be due to a variability in the size of the 3′ untranslated region or to aberrant initiation of transcription. The latter has been observed for transcription of the proopiomelanocortin gene in bovine adrenal medulla and a variety of other nonbrain tissues (18).

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