Structure and Expression of the Guinea Pig Preproenkephalin Gene: Site-Specific Cleavage in the 3' Untranslated Region Yields Truncated mRNA Transcripts in Specific Brain Regions

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We isolated the guinea pig preproenkephalin gene from a genomic library by hybridization to a rat cDNA probe. The entire nucleotide sequence of the gene was determined. Genomic Southern blot hybridization demonstrated that the gene exists in a single copy within the genome. On the basis of RNase protection transcript mapping and homology comparisons with known preproenkephalin sequences from other species and assuming a poly(A) tail length of 100 residues, we predicted an mRNA transcript of approximately 1,400 nucleotides encoded by three exons. Northern (RNA) blot analysis of total RNA from several brain regions showed high levels of preproenkephalin mRNA in the caudate putamen, nucleus accumbens, and hypothalamus, with detectable levels in the amygdala, ventral tegmental area, and central gray and also in the pituitary. Unexpectedly, in several brain regions, the mRNA appeared not only in the 1,400-nucleotide length but also in a shorter length of approximately 1,130 bases. Significant amounts of the shorter mRNA were found in the caudate putamen, nucleus accumbens, and amygdala. The longer, but not the shorter, transcripts from the caudate putamen were found to be polyadenylated, but the difference in size was not due solely to the presence of poly(A) tails. Northern gel analysis of total RNA from the caudate putamen with probes from each exon, together with RNase protection mapping of the 3' end of the mRNA demonstrated that the 1,400-base preproenkephalin mRNA transcripts are cleaved in a site-specific manner in some brain regions, yielding a 1,130-base transcript and a 165-base polyadenylated fragment derived from the terminal end of the 3' untranslated region of the mRNA. This cleavage may serve as a preliminary step in RNA degradation and provide a mechanism for control of preproenkephalin mRNA abundance through selective degradation.

The preproenkephalin gene is one of three genes, along with the preprodynorphin and preproopiomelanocortin genes, that encode precursor proteins from which are derived all endogenous opioid peptides. These peptides contain the methionine-enkephalin ([Met⁵]enkephalin) or leucine-enkephalin ([Leu⁵]enkephalin) sequence motif and exhibit a diversity of functions as neurotransmitters, neuromodulators, and hormones. Preproenkephalin is widely expressed in the brain and peripheral nervous system as well as in the pituitary, male and female reproductive tracts, adrenal medulla, gastrointestinal tract, lymphocytes, and other tissues. The preproenkephalin gene sequences from humans, rats, and golden hamsters have been previously reported (46, 52, 77), as have the cDNA sequences from humans, rats, mice, cattle, and *Xenopus laevis* (17, 26, 37, 40, 45, 76, 79). Characteristic features of preproenkephalin from mammalian species in which the gene or mRNA structure has been determined include a signal sequence, six cysteine residues in the amino-terminal region of the propeptide, and seven enkephalin-containing peptide sequences flanked by basic amino acid residues. Proteolytic processing of proenkephalin gives rise to Met-enkephalin (four copies per propeptide), Leu-enkephalin (one copy), and two Met-enkephalin sequences with additional carboxy-terminal residues. Several longer peptide fragments with opioid properties, such as peptide E, have also been identified (67).

Proenkephalin-derived peptides bind primarily to μ - and δ -opioid receptors and have a role in the modulation of pain

perception (29). Complex interactions between the μ - and κ -opioid receptor systems in opiate-induced analgesia, tolerance, and dependence also occur (1, 62, 66). Drugs of abuse, including opiates and cocaine, act upon the endogenous opioid system in the setting of addictive disease. Many animal models have been developed to study these interactions. Species-specific differences occur in the production and processing of the endogenous opioid ligands and in the distribution of opioid receptors and their subtypes. The κ -opioid receptor distributions in the brain and gastrointestinal tract of humans and guinea pigs are similar, with respect to both overall receptor distribution and the prevalence of the high-affinity receptor subtype. For some aspects of the opioid system, the guinea pig may therefore prove to be a better animal model than the rat, which has a lower overall abundance of κ -opioid receptors that are primarily of the low-affinity subtype (18, 63, 78).

We report here on the isolation and sequencing of the guinea pig preproenkephalin gene and its expression in the brain. Unexpectedly, we have also identified a site-specific cleavage in the 3' untranslated region (3' UTR) of preproenkephalin mRNA which yields 3'-truncated transcripts in specific brain regions, including the caudate putamen, nucleus accumbens, and amygdala. These regions are constituents of the nigrostriatal and mesolimbic dopaminergic pathways, which are known to be important for the reinforcing and locomotor actions of drugs of abuse. Site-specific cleavage in the 3' UTR of specific mRNA species has been identified in a small number of systems and is generally postulated to be a significant step in mRNA degradation (5, 60, 61, 71). This is the first report of the phenomenon occurring in the endogenous opioid system.

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MATERIALS AND METHODS

Isolation and sequencing of the guinea pig preproenkephalin gene. A guinea pig genomic library constructed from adult male Hartley guinea pig liver DNA was obtained from Clontech. Library screening and plaque purification were performed essentially as described previously (39). A total of 1.2×10^6 recombinant phage were screened with a ^{32}P -labeled 940-bp restriction endonuclease fragment from a rat cDNA clone for preproenkephalin supplied by Steven Sabol (76). All DNA probes used in library screening and other hybridization experiments were labeled with $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ by the random primer method (20). Using conditions of moderate stringency to ensure cross-species hybridization, we identified and plaque purified six clones which hybridized to the rat cDNA. Plate stocks of these clones were prepared (39), and bacteriophage DNA was isolated. We used multiple restriction endonuclease digests, Southern blotting (58), and the method of Smith and Birnstiel (56) to obtain a physical map of one clone, lambda gpenk-16, which contained the most complete sequence of the preproenkephalin gene, as determined by hybridization to the rat probe. Fragments of up to 4 kb were subcloned into the pBluescript vector (Stratagene). Plasmids with nested deletions for each of the cloned fragments were prepared by using exonuclease III and exonuclease VII. Single-stranded phage DNA was prepared from the deletions, and the phage DNA was sequenced by the dideoxy method (53). DNA sequence comparisons were made by using the FASTA algorithm of Pearson and Lipman (49). Analysis of the sequences showed that we isolated and sequenced the last two exons of the guinea pig preproenkephalin gene containing the entire coding region of the prepropeptide and 335 bp following the UAG termination codon. To obtain additional 5' sequences, we screened 1.6×10^6 clones from the lambda library exactly as described above, except that the probe was a restriction endonuclease fragment from the 5' end of the lambda gpenk-16 clone and stringent hybridization conditions were employed. Twelve clones were isolated and plaque purified. DNA from these clones was isolated and used to map the insert DNA. A 1.8-kb restriction fragment from clone lambda gpenk-3A, which contained sequences 5' with respect to those determined previously, was sequenced as described above.

Isolation and chromatography of guinea pig brain RNA. Male Hartley guinea pigs (Camm Laboratories), aged 3 to 4 months, were housed in groups of two to four animals per cage for at least 1 week prior to sacrifice. Animals were anesthetized with CO_2 and decapitated. Brains were removed and dissected into regions on ice. The entire dissection procedure was completed within 5 min following decapitation. Total RNA was isolated by the guanidinium thiocyanate-phenol method (14). Total RNA from guinea pig liver, kidney, adrenal gland, testis, and gastrointestinal tract was isolated by the same method. Poly(A)⁺ and poly(A)⁻ RNA fractions were isolated by two rounds of chromatography on small oligo(dT)-cellulose columns (3). RNA concentrations were determined by measuring optical density at 260 nm.

Northern (RNA) blot analysis of RNA. Gel electrophoresis of RNA was performed in denaturing formaldehyde gels (39). Molecular weight markers for agarose gels were ^{32}P -labeled RNA transcripts of known lengths produced with T7 RNA polymerase. RNA from the gels was transferred to nitrocellulose filters and hybridized overnight to a 680-bp *HindIII*-*PvuII* restriction endonuclease fragment from the coding region of the guinea pig gene labeled with $[\alpha\text{-}^{32}\text{P}]\text{dATP}$. Filters were exposed to film for 1 to 4 days with intensifying screens at -80°C .

Isolation and Southern blot analysis of guinea pig spleen DNA. High-molecular-weight DNA was isolated from a male Hartley guinea pig spleen as described previously (39). Spleen DNA (15 μg) was digested with the restriction endonucleases *HindIII* and *EcoRI*, electrophoresed in a 0.7% agarose gel, and transferred to a nitrocellulose filter by capillary action (58). The filter was hybridized to a ^{32}P -labeled 680-bp *HindIII*-*PvuII* restriction endonuclease fragment from the guinea pig preproenkephalin coding region. A *HindIII* digest of bacteriophage lambda DNA labeled with $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ was used for molecular weight determination.

Construction of template plasmids for RNA transcription. For construction of templates for 5' RNase protection analyses, a 748-bp *BamHI*-*NcoI* restriction endonuclease fragment from the guinea pig preproenkephalin gene was cloned into the polylinker region of the pSP65 RNA transcription vector (Promega). The *BamHI* site of this fragment is 114 bp 5' with respect to the presumptive TATA sequence of the gene. *NcoI* cleaves at the AUG initiation codon of the prepropeptide. Orientation of the gene fragment in the vector was such that RNA transcripts made with SP6 polymerase would hybridize to mRNA transcribed from the gene. The restriction endonuclease *BamHI* or *BsrFI* (which cleaves the sequence 10 bp 3' with respect to the TATA sequence) was used to generate linear templates for RNA transcription. To produce transcripts of shorter lengths, the enzymes *SacI* and *SacII* were used to produce plasmids in which sequences between those sites and the *BamHI* site at the 5' end of the gene sequence were deleted. The deleted plasmids were linearized with the restriction endonuclease *XbaI*, which cleaves in the polylinker sequence of pSP65. For 3' RNase protection analysis, a single construct was prepared by cloning a 1,050-bp *HindIII*-*PstI* restriction endonuclease fragment from the guinea pig preproenkephalin gene into the pSP65 plasmid in the antisense orientation. The *HindIII* site of this fragment is located 40 bp 3' with respect to the UAG termination codon of the prepropeptide, and the *PstI* site is located 795

bp following the second of two polyadenylation signals identified by DNA sequence analysis. This plasmid was linearized by using *HindIII*.

Preparation of RNA transcript probes. Antisense RNA transcripts for RNase protection experiments were prepared with 0.5 μg of template DNA, $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ (3,000 Ci/mmol), and SP6 RNA polymerase (42). Following transcription, template DNA was digested with 1 U of RNase-free DNase I (Promega), the reaction mixture was phenol extracted, and single-stranded RNA probes were purified by CF11 cellulose (Whatman) chromatography (21).

RNase protection transcript mapping. Conditions for hybridization and RNase digestion were essentially as previously described (6). Antisense RNA probes (80,000 cpm) were hybridized with 2.5 μg of total or poly(A)⁻ RNA. For analysis of poly(A)⁺ RNA fractions, 0.25 μg RNA was hybridized with the probe, with 4 μg of RNase-free tRNA included in the hybridization mixture. Following hybridization for 4 h at 75°C , samples were treated with RNases A and T₁ as described previously (6). RNase was then removed from the samples by treatment with 250 μg of proteinase K per ml in the presence of 0.1% sodium dodecyl sulfate (SDS) for 15 min at 37°C , followed by phenol-chloroform extraction and ethanol precipitation. Protected RNA transcripts were then resuspended in a buffer containing 99% formamide, 0.5 mM EDTA (pH 8.0), and xylene cyanol. Samples were heated for 2 min in a boiling water bath and electrophoresed in 6% acrylamide-50% urea sequencing gels (39). A radiolabeled restriction endonuclease digest of bacteriophage ϕX174 DNA was used as molecular weight markers. Gels were dried and autoradiographed for up to 2 days with intensifying screens at -80°C . Density analysis of bands in autoradiograms was performed with a Micro Computer Imaging Device (Imaging Research Inc., St. Catharines, Ontario, Canada).

Removal of the poly(A) tail from poly(A)⁺ RNA. Guinea pig caudate putamen poly(A)⁺ RNA purified by oligo(dT)-cellulose chromatography was deadenylated with RNase H and oligo(dT) exactly as described by Vournakis et al. (70). Following removal of poly(A) tails, RNA no longer bound to an oligo(dT)-cellulose column under the conditions in which it had previously bound (0.5 M NaCl, 10 mM Tris HCl [pH 7.2], 0.1% SDS), demonstrating the absence of significant poly(A) tails.

Preparation of regional hybridization probes. Four restriction endonuclease fragments of the preproenkephalin gene which were derived from sequences in exons I, II, and III (coding region and 3' UTR of exon III) were isolated. The probe from exon I was a 577-bp *BamHI*-*BglII* fragment encompassing the entire exon (168 bp). The exon II probe was a 546-bp *BsrXI* restriction endonuclease fragment containing the entire exon (141 bp). From exon III, two probes were prepared: a 264-bp *BglII*-*BglII* fragment from within the preproenkephalin coding sequence and a 975-bp *HincII*-*PstI* fragment which began 115 bp following the UAG termination codon and extended 795 bp beyond the second polyadenylation signal. The positions of the polyadenylation signals in the gene sequence allowed us to predict that approximately 165 to 215 bp of this probe would hybridize to the full-length polyadenylated preproenkephalin mRNA, depending on the polyadenylation signal selected for RNA cleavage and poly(A) addition.

Nucleotide sequence accession number. The complete nucleotide sequence of the guinea pig preproenkephalin gene, including all intron sequences, is available from GenBank under accession number U16138.

RESULTS

The guinea pig preproenkephalin gene. The nucleotide sequence and deduced amino acid sequence of the guinea pig preproenkephalin gene is given in Fig. 1. A total of 6,534 nucleotides were sequenced, including 1,165 bp 5' with respect to the transcription start site and 335 bp following the UAG termination codon of the prepropeptide. Sequence analysis, RNase protection transcript mapping (see Fig. 4), and sequence homology comparisons between the guinea pig gene and the rat, human, and golden hamster preproenkephalin genes allowed us to conclude that the guinea pig gene is organized into three exons separated by a short (434-bp) first intron and a long (3,622-bp) second intron. This organization is similar to that of the rat gene and differs from those of the human and hamster genes, which contain four exons. A comparison of the first exon of the rat and guinea pig genes with the first two exons of the human and hamster genes explains this species difference: the human and hamster genes contain a splice donor and splice acceptor sequence within the sequence corresponding to the first exon of the rat and guinea pig genes, which gives rise to a short intron (80 bp in the hamster, 87 bp in the human) in these species. Although a splice acceptor sequence is conserved at the same position among all four species, in the rat and guinea pig genes, the GT splice donor

FIG. 1. Nucleotide sequence and predicted amino acid sequence of the guinea pig preproenkephalin gene. Nucleotide and amino acid numbering is given to the left of the sequence. Positive integers in the nucleotide sequence begin at the major transcription start site. Derivative enkephalin peptides are given in bold type. Intron-exon junctions are indicated by horizontal arrows. The predicted site of signal sequence cleavage is between the amino acid residues Ala-25 and Glu-26, as shown by the vertical arrow. The TATA sequence at nucleotide position -37 and two polyadenylation signals at positions 5294 and 5320 are underlined.

flanking sequence required for RNA splicing is not present, which results in a single, longer exon in the rat and guinea pig.

The guinea pig preproenkephalin sequence contains a 100-bp region highly homologous to previously sequenced preproenkephalin genes located approximately between nucleotide positions -20 and -120 with respect to the major transcription start site. In the human gene, this region has been shown to contain several *cis*-acting elements required for treatment-specific transcriptional regulation (15, 16). In this region of high homology, between the human and guinea pig sequences, there are five mismatches and two gaps of 1 or 2 nucleotides; between the hamster and guinea pig sequences,

there are 10 mismatches and one gap; between the rat and guinea pig sequences, there are 13 mismatches and two gaps. The ENKCRE-1 (-105 to -99) and ENKCRE-2 (-93 to -87) cyclic AMP-phorbol ester regulatory elements identified in the human gene (16) are perfectly conserved among all four species (numbering in parentheses refers to the guinea pig sequence positions). The previously identified human AP-2 site is identical to that in the guinea pig and has a 1-base mismatch in the hamster and a 2-base mismatch with the rat sequence. Homology searches of the 1,165-bp 5'-flanking sequence of the guinea pig gene failed to identify a site homologous to the estrogen regulatory element (ERE) consensus

FIG. 2. Genomic Southern blot of guinea pig DNA hybridized to a preproenkephalin gene probe. High-molecular-weight DNA was isolated from a guinea pig spleen and digested with either *EcoRI* or *HindIII* restriction exonuclease. Digests were electrophoresed in an 0.7% agarose gel, denatured, and transferred to a nitrocellulose filter. The filter was hybridized to a 680-bp restriction endonuclease fragment from the coding region of exon III of the guinea pig preproenkephalin gene labeled with [α - 32 P]dATP. The molecular weight markers (MW) resulted from a 32 P-labeled *HindIII* digestion of bacteriophage lambda DNA.

sequence (4) or rat preproenkephalin ERE (51). No sequences homologous to the glucocorticoid regulatory element (GRE) core sequence (4) or to the glucocorticoid receptor binding sequence identified by Payvar et al. (48) were found. A sequence in the guinea pig gene at nucleotide positions -172 to -184 which shows partial homology to the canonical NF- κ B binding site (54) and the B2 or NF- κ B and BETA binding site (50) of the rat preproenkephalin gene was found.

The preproenkephalin polypeptide predicted from the nucleotide sequence contains 268 amino acid residues and has a calculated molecular mass of 30,423 Da. The initiator methionine is followed by a signal sequence. The method of von Heijne et al. (69) was used to determine that the most probable site of signal sequence cleavage is between alanine (amino acid position 24) and glutamic acid (amino acid position 25). Six cysteine residues in the N-terminal region of the propeptide are conserved between the guinea pig proenkephalin peptide and all other known proenkephalin sequences determined to date, as is the potential asparagine-linked glycosylation site (asparagine-X-serine) at amino acid positions 52 to 54 in the guinea pig sequence. The predicted guinea pig preproenkephalin peptide is highly homologous to those of other species, showing an 82 to 84% identity and 91 to 93% similarity to previously sequenced mammalian enkephalin prepropeptides. The seven enkephalin moieties predicted to be derived from the processing of the guinea pig propeptide, as well as their relative positions in the propeptide sequence, are perfectly conserved in the guinea pig, rat, human, bovine, and hamster propeptides. In each case, the predicted enkephalin peptides are flanked by two basic amino acid residues usually required for cleavage by converting enzymes (23).

The genomic Southern blot in Fig. 2 demonstrates that the guinea pig preproenkephalin gene exists in one copy within the genome. A single band position for each restriction endonuclease digest of genomic DNA hybridized to the preproenkephalin coding region probe (3.3 kb for the *HindIII* digest, and 6.6 kb for the *EcoRI* digest). The molecular weight of each hybridizing band is identical to the molecular weight of the hybridizing band in corresponding digests of the preproen-

FIG. 3. Northern blot analysis of total RNA isolated from guinea pig brain regions and pituitary. Total RNA was isolated from a guinea pig brain dissected into regions, and 6 μ g was electrophoresed in a 1.5% agarose gel, blotted onto a nitrocellulose filter, and hybridized to a 32 P-labeled 680-bp restriction endonuclease fragment from the coding region of the guinea pig preproenkephalin gene. Regions analyzed included pituitary (PIT), frontal cortex (FCX), nucleus accumbens (NAC), hypothalamus (HYP), caudate putamen (CPU), hippocampus (HIP), amygdala (AMY), substantia nigra (SN), ventral tegmental area (VTA), central gray (CG), and cerebellum (CER). The molecular weight markers (MW) were T7 RNA polymerase transcripts of the indicated lengths. In several brain regions, particularly the nucleus accumbens, caudate putamen, and amygdala, two distinct transcript sizes are apparent: the upper transcript length is approximately 1,400 bases and the lower transcript length is approximately 1,130 bases. The longer transcript is the predominant species in the pituitary, hypothalamus, ventral tegmental area, and central gray.

kephalin lambda clone, gpenk-16, used to determine the sequence of the gene (data not shown).

Expression of preproenkephalin mRNA in the guinea pig brain. Analysis of preproenkephalin mRNA expression in the guinea pig brain and pituitary by Northern blot hybridization is shown in Fig. 3. Preproenkephalin mRNA transcripts were detected in total RNA isolated from the pituitary, nucleus accumbens, hypothalamus, caudate putamen, amygdala, ventral tegmental area, and central gray. In 6 μ g of total RNA, we were unable to detect preproenkephalin mRNA in the frontal cortex, hippocampus, or cerebellum, and barely detectable amounts were observed in the substantia nigra. Six micrograms represents the amount of total RNA obtained from the ventral tegmental area of a single animal.

Of particular interest is the unexpected finding that preproenkephalin mRNA in several brain regions appeared in two transcript sizes of approximately 1,400 bases and approximately 1,130 bases. Moreover, the abundance of mRNA in the two transcript sizes differed in the various brain regions examined, with substantial amounts of the smaller transcripts found primarily in three areas of the nigrostriatal and mesolimbic dopaminergic systems: the nucleus accumbens, caudate putamen, and amygdala. This phenomenon does not appear to represent degradation due to nonspecific RNase activity: when the same Northern blots of guinea pig total RNA that yield two preproenkephalin mRNA transcript sizes were hybridized to cDNA probes for either 18S rRNA (19) or glyceraldehyde-3-phosphate dehydrogenase mRNA (65), only a single transcript size was detected (data not shown). To date, we have analyzed RNA from six individual animals by Northern blot hybridization, and in each case, the heterologous transcripts were observed in the caudate putamen and nucleus accumbens. Also, the shorter transcript appears to be found only in the brain: preproenkephalin mRNA was detected in Northern blots of guinea pig total RNA from testis, adrenal gland, pituitary, antrum, duodenum, jejunum, ileum, and colon in a single and expected transcript size of approximately 1,400 bases (data not shown). We further analyzed RNA from the guinea pig caudate putamen to determine the nature of the heterologous transcript size.

FIG. 4. RNase protection analysis of exon I of the guinea pig preproenkephalin gene. (A) Experimental design and interpretation of the RNase protection transcript mapping experiment shown in the autoradiogram (B). A 748-bp *Bam*HI-*Nco*I restriction endonuclease fragment of the guinea pig preproenkephalin gene was cloned into the RNA polymerase transcription vector pSP65. The *Nco*I site is at the AUG initiation codon of the enkephalin prepropeptide, and the *Bam*HI site is 114 bases 5' with respect to the TATA sequence. Transcription templates were prepared by cleavage at *Bam*HI and *Bsr*FI restriction endonuclease sites, with positions indicated by the vertical arrows in panel A. Deletions between the *Sac*I and *Sac*II sites and the *Bam*HI site were used to generate plasmids for transcription templates corresponding to the *Sac*I and *Sac*II sites indicated in the diagram. SP6 polymerase was used to transcribe ³²P-labeled RNA probes complementary to preproenkephalin mRNA for each transcript length. These probes were then hybridized to total RNA isolated from the guinea pig caudate putamen or liver, subjected to digestion by RNases A and T₁ to remove any nonprotected single-stranded RNA, denatured, and electrophoresed in a 6% polyacrylamide–50% urea sequencing gel. The gel was dried and autoradiographed, as shown in panel B. The full-length transcript is shown for each probe (P), as well as the protected bands observed in transcripts hybridized to RNA from the caudate putamen (B). No protected bands were observed in the liver (L), which contains little or no preproenkephalin mRNA. The molecular weight markers (MW) resulted from a ³²P-labeled restriction endonuclease digestion of bacteriophage ϕ X174 DNA. The lengths of the protected fragments hybridizing to the brain RNA are depicted in panel A, along with the deduced length and position of the first exon of the gene.

Transcription initiation site of guinea pig preproenkephalin mRNA. RNase protection mapping of the 5' end of the guinea pig preproenkephalin mRNA was used to determine if the transcript heterogeneity observed in the caudate putamen results from differences in transcription initiation, since in the rat and mouse testis and in the rat brain, multiple transcript sizes result from initiation at different sites (34, 72). The results of 5' RNase protection transcript mapping shown in Fig. 4 confirm the site of transcription initiation and the intron-exon organization of the 5' UTR of the guinea pig preproenkephalin gene predicted by sequence homology to other known preproenkephalin genes. A restriction endonuclease fragment from the 5' end of the guinea pig gene was cloned into the pSP65 transcription vector. Restriction endonucleases were used to produce transcription templates for antisense RNA probes of different lengths. RNA transcripts from these templates were used to localize the 5' UTR of preproenkephalin mRNA isolated from the guinea pig caudate putamen. All transcript probes began at the AUG initiation codon of the enkephalin prepropeptide. The longest probe, extending upstream to the *Bam*HI site 114 bp 5' with respect to the TATA sequence, yielded only a single protected band of 168 bases, indicating that the guinea pig gene has a single exon in the 5' UTR, as predicted from the sequence homology comparisons. Longer exposures of the gel shown in Fig. 4 resulted in the appearance of several faint bands above the 168-base position (data not shown) indicating that multiple minor transcription initiation sites may exist for the guinea pig gene. The appearance of a single major band in the protection experiment indicates either that mRNA transcription begins predominantly at a single site or that transcripts initiated at other sites are significantly less stable. However, each explanation would lead to the same net result: mRNA which begins at a single predominant site. The different mRNA transcript sizes observed in the Northern blot do not therefore arise as a result of differential start site selection.

Localization of the 5' exon of the guinea pig preproenkephalin gene was determined with the truncated antisense probes produced by cleaving the transcription template with *Bsr*FI, *Sac*I, or *Sac*II restriction endonuclease as shown in Fig. 4. The enzyme *Bsr*FI cleaves the template 10 bp following the TATA sequence, *Sac*II cleaves within the predicted 5' exon, and *Sac*I cleaves 18 bp following the exon I splice donor sequence predicted from homology comparisons. The *Bsr*FI probe yielded a single protected 168-base fragment identical in length to that produced by the longer *Bam*HI probe, the *Sac*II probe produced a protected band of 113 bases, and the *Sac*I probe resulted in no protected sequences. The small protected fragment in the sample hybridized to the *Sac*I probe is most likely an artifact of the technique. In our experience, a limited number of specific RNA transcript probes, particularly those beginning or terminating within a protected sequence give rise to additional small protected bands, presumably through anomalous hybridization. The 5' RNase protection mapping results confirm the transcription initiation site and the position of the 5' exon indicated in Fig. 4 and Fig. 1.

Polyadenylation analysis of preproenkephalin mRNA from the caudate putamen. Since the transcript size heterogeneity observed in the guinea pig brain preproenkephalin mRNA was not attributable to multiple gene copies or to differences in transcription start site selection, we examined the possibility that differences in poly(A) tail length might explain the observed heterogeneity. A Northern blot of guinea pig caudate putamen RNA fractionated on oligo(dT)-cellulose is shown in Fig. 5. The poly(A)⁺ fraction contained only the longer, 1,400-base preproenkephalin transcripts. In the poly(A)[−] fraction, only the shorter, 1,130-base transcripts were found. The difference in transcript lengths was not due solely to the presence or absence of poly(A) tails, however: when poly(A)⁺ RNA was deadenylated with oligo(dT) and RNase H, its size was approximately 1,300 bases (Fig. 5), still larger than the 1,130-base form observed in the caudate putamen. The absence of

FIG. 5. Polyadenylation analysis of preproenkephalin mRNA from the guinea pig caudate putamen. Total RNA isolated from the caudate putamen of a single guinea pig was purified by oligo(dT)-cellulose chromatography. RNA from the polyadenylated fraction (200 μ g) was digested with RNase H in the presence of oligo(dT) to remove poly(A) tails. This material was then passed over an oligo(dT) column under conditions in which it had previously bound, and the nonbinding fraction was collected. Total RNA from the caudate putamen, poly(A)⁺ [A(+)] and poly(A)⁻ [A(-)] fractions, and poly(A)⁺ RNA digested with RNase H in the presence of oligo(dT) were electrophoresed in a 1.5% agarose gel, transferred to a nitrocellulose filter, and hybridized to a 680-bp ³²P-labeled restriction endonuclease fragment from exon III of the guinea pig preproenkephalin gene. The molecular weight markers (MW) were T7 RNA polymerase transcripts of the indicated lengths. The size of the longer transcript from the poly(A)⁺ fraction following removal of poly(A) tails is approximately 1,300 bases.

poly(A) tails from the shorter RNA form isolated from the caudate putamen suggested to us that the difference in transcript size might be caused by sequence differences at the 3' end of the preproenkephalin message, since the signals for polyadenylation are located there.

Northern blot analysis of caudate putamen preproenkephalin mRNA with regional hybridization probes. To further examine the source of preproenkephalin transcript heterogeneity, we hybridized four Northern blots of total RNA isolated from the guinea pig caudate putamen with gene fragment probes from exon I, exon II, the coding region of exon III, and a region predicted to code for the terminal portion of the preproenkephalin mRNA 3' UTR. As shown in Fig. 6, the restriction endonuclease fragment probes for exon I, exon II, and the coding region of exon III all hybridized with both the longer and shorter preproenkephalin transcripts found in the caudate putamen, indicating that the difference in preproenkephalin transcript size is not due to differential splicing at the intron-exon level. The probe from the terminal portion of the preproenkephalin 3' UTR, however, hybridized only to the longer, 1,400-base transcript, supporting the conclusion that sequences at the 3' terminus of the preproenkephalin message are not present in the shorter transcripts found in the guinea pig caudate putamen.

RNase protection analysis of the 3' UTR of preproenkephalin mRNA. We used RNase protection analysis to localize the sequence differences predicted to lie in the 3' UTR of the guinea pig preproenkephalin mRNA. These results are shown in Fig. 7. A 1,050-bp *HindIII*-*PstI* restriction endonuclease fragment from the 3' end of the gene was cloned into the pSP65 RNA transcription vector. The *HindIII* site is located 40 bp following the UAG termination codon, and the fragment extends 795 bp beyond the second polyadenylation signal of the gene. Following cleavage with *HindIII*, this template vector was used to produce RNA transcripts which protected sequences in the 3' UTR of preproenkephalin mRNA isolated

FIG. 6. Northern blot analysis of total RNA from the guinea pig caudate putamen with probes from each exon. Eight micrograms of total RNA was electrophoresed in a 1.5% agarose gel and blotted onto a nitrocellulose filter. Four separate aliquots of RNA were run adjacent to lanes containing ³²P-labeled T7 polymerase-generated RNA transcripts of known lengths. The nitrocellulose filter was cut into four strips for hybridization to probes from different regions of the guinea pig preproenkephalin gene. Restriction endonuclease fragments were prepared for each exon and for a terminal fragment from the 3' UTR beginning 115 bp following the termination codon. These four restriction fragments were labeled with [α -³²P]dATP by the random primer method and hybridized to the nitrocellulose strips. MW, molecular weight markers.

from the caudate putamen, as shown in the gel in Fig. 7B and diagrammed in Fig. 7A. Protection analysis was performed on total RNA, as well as poly(A)⁺ and poly(A)⁻ fractions isolated by oligo(dT)-cellulose chromatography. The poly(A)⁻ fraction, which contains the shorter, 1,130-base, but not the longer, 1,400-base, preproenkephalin transcript, produced a single protected fragment of 115 bases. The poly(A)⁺ fraction, which contains the 1,400-base preproenkephalin mRNA but not the 1,130-base transcripts, protected two bands and two fragment sizes: one of 280 bases and another, weaker band of 165 bases. The 115-base protected fragment was not present in the poly(A)⁺ fraction. All three protected fragments were present in the total RNA. These results, together with the previous experimental results, allow us to propose that in those regions of the guinea pig brain which contain the two transcript sizes, initial transcripts of 1,400-base polyadenylated preproenkephalin mRNA are subsequently cleaved at a specific site in the 3' UTR, yielding a 1,130-base, nonadenylated, truncated transcript and a 165-base polyadenylated 3'-terminal fragment. This specific cleavage site is located 155 bases 3' with respect to the UAG termination codon. The presence of a single band at nucleotide position 280 has also allowed us to conclude that the second, but not the first, AAUAAA polyadenylation signal is utilized in cleavage and addition of poly(A) tails to primary transcripts.

Density measurements of the protected bands in the RNase protection autoradiogram (Fig. 7) were compared to density measurements of the two transcript sizes in Northern blot hybridizations (Fig. 3). The two experiments were performed with RNA isolated from the caudate putamen of the same animal. In the Northern blot analysis, the shorter form was present in a slightly larger amount than that of the longer form (approximately a 1.1:1.0 ratio). In contrast, when the RNase protection autoradiogram was analyzed for the relative quantitation of the two, after correcting for expected signal strength differences due to the different protected transcript lengths, the shorter form was found to be protected in smaller amounts than that of the longer form (approximately a 1:0.7 ratio). This apparent discrepancy might be explained by additional non-specific endo- or exonuclease degradation at either the 3' or 5' end of the RNA. The smearing of the lower band in the

FIG. 7. RNase protection analysis of the 3' UTR of guinea pig preproenkephalin mRNA from the caudate putamen. (A) Gene structure, experimental design, results, and deduced mRNA structure of the experiment shown in the autoradiogram (B). A 1,050-bp *Hind*III-*Pst*I restriction endonuclease fragment of the guinea pig preproenkephalin gene was cloned into the RNA transcription vector pSP65. The *Hind*III site is 40 bp following the UAG termination codon of the preproenkephalin coding sequence. Transcription of this template plasmid linearized with *Hind*III using [α - 32 P]GTP yielded probe RNA complementary to the 3' UTR of the preproenkephalin mRNA, as shown in panel A. This probe RNA was hybridized with total RNA isolated from a guinea pig caudate putamen, as well as poly(A)⁺ [A(+)] and poly(A)⁻ [A(-)] fractions of caudate putamen RNA purified by oligo(dT)-cellulose chromatography. Total RNA from the guinea pig liver was used as a negative control. Following hybridization, samples were treated with RNases A and T₁, denatured, and electrophoresed in a 6% polyacrylamide sequencing gel. The gel was dried and autoradiographed. The lengths of the protected fragments seen in the gel are indicated in the diagram in panel A, along with the deduced structure of the preproenkephalin mRNA fragments found in each experimental fraction. The site of specific RNA cleavage which gives rise to these fragments is indicated.

Northern gel (Fig. 3) suggests that this form may have undergone subsequent degradation. If this degradation were caused by nonspecific cleavage or exonuclease activity, it would not appear as a protected band in the RNase protection experiment. In addition, in the RNase protection autoradiograph, the truncated transcripts and the 165-base fragment derived from the 3' end do not match on a molar basis, with the small terminal fragment present in significantly lower amounts. This result might be caused by loss of the small fragment during ethanol precipitation of the RNA during isolation; however, it may represent an actual difference in molar ratio of the two mRNA fragments in the brain.

DISCUSSION

The guinea pig preproenkephalin gene and deduced peptide structure conform to patterns identified in other species. Functional elements at both the nucleotide and peptide levels are highly conserved. Like the rat gene, the guinea pig gene is organized into three exons with a short first intron and a longer second intron. RNA splice sites situated within the sequence corresponding to the first exon of the rat and guinea pig genes give rise to a short intron in human and hamster preproenkephalin genes. The peptide structure of guinea pig preproenkephalin is highly homologous to those of other mammalian species. The signal sequence, potential asparagine-linked glycosylation site, and six cysteine residues in the amino-terminal region of the propeptide are present in all mammalian species studied to date. The one Leu-enkephalin, one Met-enkephalin-Arg⁶-Phe⁷, one Met-enkephalin-Arg⁶-Gly⁷-Leu⁸, and four Met-enkephalin sequences predicted to be derived from the propeptide are completely conserved among the guinea pig and other mammals with the exception of the mouse, in which

a glycine-to-serine substitution results in the octapeptide Met-enkephalin-Arg⁶-Ser⁷-Leu⁸ (79). The propeptide sequence between Leu-enkephalin and the following Met-enkephalin gives rise to the longer opioid peptides BAM 12, BAM 22, and peptide E (67) and is also conserved among mammalian species studied.

In the guinea pig brain, preproenkephalin mRNA is found in regions which have been shown to express the gene in the rat and hamster (6, 22). The relative abundance of mRNA expression in the guinea pig is also similar to that in these species, with the exception of the rat caudate putamen, in which preproenkephalin mRNA is over three times more abundant than in the nucleus accumbens. In contrast, these regions in the guinea pig contain roughly equivalent amounts. Highly unusual, however, is the finding that guinea pig preproenkephalin mRNA in some brain regions is cleaved in a site-specific manner in the 3' UTR. This cleavage appears not to be caused by a nonspecific RNase degradation, since both 18S rRNA and glyceraldehyde-3-phosphate dehydrogenase mRNA were found in only one size in all brain regions studied. This cleavage is also sequence or structure specific, since it occurs at a distinct site. Previous researchers have observed a lower-molecular-weight form of preproenkephalin mRNA in occasional preparations of RNA from rat ovary (32) and striatum (76) and concluded that these forms were the product of RNA degradation. However, since poly(A)⁺ RNA was examined in these cases, the generation of lower-molecular-weight forms must have occurred by mechanisms other than that in the guinea pig brain, which removes the poly(A) tails from the truncated mRNA transcripts.

The 3' UTR of many mRNAs in both procaryotes and eucaryotes is recognized to contain important determinants of mRNA stability. Labile proto-oncogene and cytokine mRNAs

contain AU-rich destabilizing elements in the 3' UTR (10, 30, 55, 73). Cleavage of both 9E3 mRNA in chicken fibroblasts and *gro* α mRNA in human fibroblasts at a conserved specific site in the 3' UTR is believed to initiate the degradation of these mRNAs (60, 61). Endonucleolytic cleavage in the 3' UTR has been observed for the *Xenopus Xlhb2B* and *Xoo1* mRNAs in *Xenopus* oocytes and *Drosophila* embryo extracts (8, 9), as well as in the estrogen-regulated apolipoprotein II mRNA in chickens (5). In *Saccharomyces cerevisiae*, the rate-limiting step of *PGK1* mRNA degradation is an endonucleolytic cleavage in the 3'-terminal part of the coding region (71). A similar role for the specific cleavage in the 3' UTR of the IGF-II gene in human 293 cells has been proposed, although a stable 3'-terminal fragment of undetermined function is also produced in this case (41). The guinea pig preproenkephalin mRNA 3' UTR is rich in A and U residues and contains a large amount of predicted secondary structure consistent with that observed in or predicted for other systems of regulated mRNA stability (5, 41, 47, 64, 71). The cleavage site in the guinea pig preproenkephalin mRNA (AUUUCA) differs slightly from the AUUUA consensus sequence observed in the labile proto-oncogene and cytokine mRNAs (30, 55, 73) and is also distinct from previously identified cleavage sites in other 3' UTR-cleaved mRNAs.

Although the preproenkephalin gene has not generally been thought of as a rapid response gene, some stimuli have been shown to modulate its expression in 1 to 2 h (38, 51). Our comparison of the Northern blot and RNase protection measurements of the preproenkephalin mRNA from the caudate putamen suggests that both the 3'-truncated transcripts and the 3'-terminal fragment may be more labile than the full-length polyadenylated transcripts and may therefore be degraded at different rates following cleavage. The endonucleolytic cleavage in the 3' UTR of the guinea pig preproenkephalin mRNA may serve as a mechanism for controlling mRNA abundance through specific degradation.

The existence of a population of highly complex nonpolyadenylated mRNA in the mouse and rat brain was observed over 15 years ago. This poly(A)⁻ mRNA appears postnatally in the brain, accounts for up to 50% of adult brain mRNA, and was reported to primarily contain sequences distinct from those in poly(A)⁺ mRNA (12, 13, 68). Some eucaryotic mRNAs, such as certain histone mRNAs, are known to exist in an exclusively nonadenylated form (2), while several other genes give rise to a bimorphic distribution of mRNA transcripts both containing and lacking poly(A) tails (31, 43, 44). The adult brain is the only tissue in which there has been identified an abundant class of complex poly(A)⁻ mRNA. The origins of this nonadenylated mRNA have remained controversial. The early reports of this phenomenon concluded that little sequence overlap existed between the poly(A)⁺ and poly(A)⁻ polysomal mRNA fractions (12, 13, 68). However, attempts to clone unique cDNAs from this class have met with little success. To date, only a few cDNA clones have been isolated which apparently correspond to unique, brain-specific, nonadenylated mRNAs (7, 27). Other investigators were unable to identify cDNAs which were not represented in the poly(A)⁺ fraction of mRNA and have concluded that deadenylation in vivo, contamination of cytoplasmic mRNA with nuclear RNA, or nicking during RNA isolation were more likely origins of the observed complexity in the poly(A)⁻ fraction (24, 28, 57).

Our observations of 3' truncation (and resultant deadenylation) of the preproenkephalin mRNA may shed light on the potential source of nonadenylated mRNA in the brain. Our data suggest that mechanisms which regulate the degradation

of specific mRNAs through endonucleolytic cleavage may play a role in determining brain mRNA levels. The resultant deadenylated transcripts would appear as complex mRNA and may be the source of some of the RNA complexity observed earlier, although this would not explain the finding that poly(A)⁻ sequences were distinct from those in the poly(A)⁺ fraction. This phenomenon may well be more common than has yet been reported. If the site of cleavage of an mRNA is near the site of polyadenylation, the difference in transcript size would not be apparent on Northern blots of total RNA, leading to a failure to identify a population of transcripts as poly(A)⁻. Also, poly(A)-enriched mRNA is frequently used in Northern blot analysis of mRNA, particularly for measuring low-abundance transcripts, which would again fail to identify a subpopulation of nonadenylated transcripts. Future studies to assess the role of endonucleolytic cleavage in the 3' UTR and its importance in mRNA stability and degradation should provide insight into this potential mechanism of the control of specific mRNA abundance.

Multiple transcription initiation sites have been observed for the preproenkephalin gene in rats and mice, but not in hamsters. The predominant form of preproenkephalin mRNA from haploid spermatogenic cells in these species is approximately 300 bases longer than somatic transcripts, both neuronal and peripheral (32). These longer transcripts result from initiation at several sites within the first intron of the gene as it is expressed elsewhere in the body (34, 75). Similarly to the testicular preproenkephalin mRNA in the hamster (33), we found guinea pig testicular preproenkephalin mRNA to be the same size as the somatic form. In the rat, in both neuronal and adrenal tissues, multiple transcription start sites located within 30 bp of the TATA sequence of the preproenkephalin gene have been reported. Initiation at these distinct sites varied in a tissue- and treatment-specific manner (72), although the functional significance of this phenomenon is unknown. Transcription of the preproenkephalin gene in the guinea pig caudate putamen begins predominantly at one site, although our data suggest several minor start sites up to approximately 30 bases upstream from the primary start site may also exist.

Several *cis*-acting transcriptional regulatory elements have been identified in a 100-bp sequence of the 5'-flanking sequence of the human preproenkephalin gene which is very highly conserved among all species studied. These elements include the ENKCRE-1 and ENKCRE-2 cyclic AMP-phorbol ester response elements, as well as an AP-2 consensus site, all of which are present in the guinea pig sequence. This region of the preproenkephalin promoter is responsible for regulation of expression by both the cyclic AMP-phorbol ester and the calcium-dependent signal pathways (15, 16). Several proteins and transcription factor complexes have been shown to interact at this site including AP-1, AP-2, AP-4, ATF/CREB proteins, and the novel enkephalin transcription factor ENKTF-1 (16, 59). The virtually complete identity among the human, guinea pig, rat, and hamster sequences in the 30-bp region which contains these enhancer elements suggests that regulation of the preproenkephalin expression mediated by these sequences occurs by similar mechanisms in these species.

The transcription factor NF- κ B mediates the induction of preproenkephalin mRNA in activated T lymphocytes by binding to a sequence in the 5'-flanking region of the rat preproenkephalin gene (B2) with partial homology to the NF- κ B binding site (κ B) (50). We have identified a 13-bp sequence in the 5'-flanking region of the guinea pig gene with partial sequence homology to both the κ B and B2 sites at nucleotide positions -172 to -184. This sequence (5'-GGGGGAGTCACCC-3') also shows the partial inverted dyad symmetry characteristic of

these elements. Functional studies will be necessary to confirm this site as an NF- κ B-inducible element.

Glucocorticoids modulate the expression of the preproenkephalin gene in several tissues and cell types (11, 25, 36, 74). Putative GRE sequences have been located in the first intron of the rat (36) and hamster (77) preproenkephalin genes. However, functional demonstration of the glucocorticoid responsiveness of these sites has yet to be established. Searches of 1,165 bp of the guinea pig preproenkephalin gene 5'-flanking region failed to identify any positive GRE sites with either the GRE core consensus sequence (4), GRE binding sequence (48), or preliminary negative GRE consensus sequence (4). However, two potential positive GRE elements were located within the first intron of the guinea pig gene: a sequence homologous to the GRE core sequence is located at bp +344 to +349, and a sequence corresponding to the GRE binding sequence is located at bp +478 to +484.

Gonadal steroid hormones have also been shown to regulate preproenkephalin mRNA expression in the ventromedial nucleus of the hypothalamus in a gender-specific manner (51). A 15-base sequence partially homologous to the EREs of several estrogen-responsive genes is located in the 5'-flanking region of the rat preproenkephalin gene (51). In the guinea pig, however, no sequences homologous to the rat ERE or the ERE consensus sequence (35) were found in 1,165 bp of the 5'-flanking region, suggesting that if estrogen regulates the gene in this species, its regulatory element is located further upstream or its regulatory sequences differ from the ERE sequences previously identified.

The successful cloning of the guinea pig gene will now allow additional studies of preproenkephalin expression in this species. Further investigation into the unanticipated and novel finding of cleavage in the 3' UTR of guinea pig preproenkephalin mRNA and its potential role in determining mRNA stability and therefore abundance may also lead to a better understanding of gene regulation in the brain. The fact that this cleavage occurs in regions which are known sites of action of drugs of abuse may be important in future studies of the neurobiology of addiction.

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