PROTEIN EXPRESSION AND PURIFICATION **10,** 80–88 (1997)
ARTICLE NO. PT960715

High-Level Expression of the Prohormones Proenkephalin, Pro-Neuropeptide Y, Proopiomelanocortin, and β-Protachykinin for *in Vitro* Prohormone Processing

Vivian Y. H. Hook,*[,1] Kim Moran,† Rama Kannan,† Andrea Kohn,† Mark O. Lively,‡ Anahit Azaryan,† Martin Schiller,† and Kurt Miller§

**Department of Medicine and Center for Molecular Genetics, University of California, San Diego; †Department of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda, Maryland; ‡Molecular Genetics Program, Bowman Gray School of Medicine, Winston-Salem, North Carolina 27157; and §Department of Molecular Biology, University of Wyoming, Laramie, Wyoming 82071*

Received September 26, 1996, and in revised form December 4, 1996

Prohormone substrates are required for investigation of the proteolytic processing of prohormones and proproteins into active peptide hormones and neurotransmitters. However, the lack of prohormone proteins has been a limiting factor in elucidating proteolytic mechanisms for conversion of prohormones into active peptides. Therefore, in this study, cloned cDNAs encoding the prohormones proenkephalin (PE), pro-neuropeptide Y (pro-NPY), proopiomelanocortin (POMC), and β-protachykinin (β-PT) were utilized to express recombinant prohormones in *Escherichia coli.* High-level expression of milligrams of prohormones was achieved with the pET3c expression vector utilizing the T7 promoter for production of PE, pro-NPY, and POMC, as demonstrated by SDS–PAGE gel electrophoresis, Western blots, and ^{35}S-methionine labeling. In addition, β-PT was expressed at high levels as fusion proteins with the maltose-binding protein and glutathione S-transferase by the pMAL-c and pGEX-2T expression vectors, respectively. Relative rates of processing by the established processing proteases "prohormone thiol protease" (PTP), 70-kDa aspartyl protease, and PC1/3 and PC2 (PC, prohormone convertase) were examined with purified PE, pro-NPY, and POMC. Distinct preferences of processing enzymes for different prohormones was demonstrated. PTP prefered PE and pro-NPY substrates, whereas little processing of POMC was detected. In contrast, the 70-kDa aspartyl protease cleaved POMC more readily than pro-NPY or PE. However, PC1/3 and PC2 prefer POMC as substrate. Demonstration of selectivity of processing enzymes for prohormone substrates illustrates the importance of expressing recombinant prohormones for *in vitro* processing studies.

The biosynthesis of peptide hormones and neurotransmitters requires proteolytic processing of inactive prohormone precursors into the smaller biologically active neuropeptides (1–3). Production of enkephalin, neuropeptide Y (NPY), ACTH and β-endorphin, and substance P requires proteolytic processing of their respective precursors proenkephalin (PE) (4,5), pro-NPY (6), proopiomelanocortin (POMC) (7,8), and β-protachykinin (β-PT) (9–11). It is noted that substance P may be derived from α-, β-, and γ-preprotachykinin precursors derived by differential splicing of the primary transcript from the preprotachykinin gene (9,10). Each precursor must undergo specific proteolytic processing at paired basic residues, or in some cases, at single basic residues, to generate the active peptides. Established processing endoproteases that participate in prohormone processing are the subtilisin-like PC1/3 and PC2 proteases (1–3,12–15), the cysteine protease "prohormone thiol protease" (PTP) (16–18), and a 70-kDa aspartyl protease (19–22) known as "proopiomelanocortin converting enzyme" (PCE) in pituitary (19,20).

Knowledge of the biochemical properties of processing proteases is essential for understanding regulation of prohormone processing. Characterization of the processing proteases requires use of recombinant prohormones as substrates for *in vitro* processing studies. However, adequate quantities of full-length prohor-

[1] To whom correspondence should be addressed at Department of Medicine, University of California, San Diego, 9500 Gilman Drive, No. 0822, La Jolla, CA 92093-0822. Fax: (619) 543-7717. E-mail: vhook@ucsd.edu.

1046-5928/97 $25.00

mone substrates have not been available for studies of prohormone processing. We therefore demonstrate in this study that high-level expression of proenkephalin, pro-NPY, and POMC prohormones is achieved in *Escherichia coli* using the pET3c T7 expression vector (23). These studies also illustrate expression of β-protachykinin as fusions with the maltose-binding protein and glutathione S-transferase, using the pMAL-c (24) and pGEX-2T (25) vectors, respectively. Comparison of the efficiencies of bacterial expression of these prohormones indicates some differences in their levels of expression. Importantly, use of purified recombinant prohormones for *in vitro* processing assays suggests selectivity of processing enzymes for different precursor substrates. Overall, this study demonstrates that expression of recombinant prohormones can provide model prohormone substrates for investigations of processing proteases.

MATERIALS AND METHODS

Prohormone expression constructs in the pET3c vector. The pET3c expression vector was used to generate recombinant prohormones in BL21(DE3) *E. coli* cells that contain the RNA polymerase of bacteriophage T7 (23). The proenkephalin/pET3c construct was generated by linearizing the rat preproenkephalin (PPE) cDNA (4) in the pSP65 vector with *Sma*I, followed by ligation (with T4 DNA ligase) with *Bam*HI linkers and digestion with *Bam*HI, and digestion with *Hpa*II to generate a 0.72-kb proenkephalin (PE) *Hpa*II/*Bam*HI DNA fragment. The PE *Hpa*II/*Bam*HI fragment was ligated to a 51-bp synthetic DNA fragment encoding the 5′-region of PE. This 51-bp fragment was generated by annealing two complementary oligonucleotides (kinased by T4 polynucleotide kinase) to generate a 51-bp *Nde*I/*Hpa*II DNA fragment; annealed oligonucleotides were 5′-TATGGACTGCAGCCAGGACTGCGCTAAATGCAGCTACCGCCTGGTACGTGG-3′ and 5′-CGGGACGTACCAGGCGGTAGCTGCATTTAGCGCAGTCCTGGCTGCAGTCCA-3′. Ligation of the 51-bp *Nde*I/*Hpa*II 5′-region of PE, the 0.72-kb *Hpa*II/*Bam*HI PE fragment, and the *Nde*I/*Bam*HI-digested pET3c vector generated the PE/pET3c expression construct, which was then amplified in DH5α *E. coli* cells.

Pro-NPY and POMC expression constructs in the pET3c vector were generated by PCR (polymerase chain reaction) of rat pre-pro-NPY cDNA (6) and porcine pre-POMC cDNA (7), respectively, with deletion of the NH_2-terminal signal sequence. Primers for PCR of pro-NPY were 5′-AAACATATGTACCCCTCCAAGCCG-3′ and 5′-AAAGGATCCCCATCACCACATGGAAG-3′. Primers for PCR of POMC were 5′-AAACATATGTGGTGCTTGGAGAGCAGCCAGTGTCAG-3′ and 5′-AAAGGATCCCCCTCACTGGCCCTTCTTGTGGGCGTTCTT-3′. These primers incorporated *Nde*I and *Bam*HI restriction sites at 5′- and 3′-ends of the DNAs. PCR reactions (according the protocol of the Perkin–Elmer PCR kit) were performed with 1 ng cDNA as template, primers at 0.2 or 0.1 μM, and 30 cycles of 1 min each at 94, 50, and 72°C. The PCR reaction for POMC included 10% glycerol. The pro-NPY (227 bp) and POMC (748 bp) DNAs generated by PCR were digested with *Nde*I and *Bam*HI, ligated (with T4 ligase) to the pET3c vector digested with *Nde*I and *Bam*HI, and transformed into DH5α *E. coli* cells for amplification of plasmid constructs.

The β-PT/pET3c expression construct was generated from the human β-preprotachykinin cDNA (11). The 854-bp *Ava*II/*Sma*I β-PPT DNA fragment was ligated to *Bam*HI linkers, and digested with *Bam*HI to form an *Ava*II/*Bam*HI DNA fragment. The *Ava*II/*Bam*HI fragment was ligated to the *Nde*I/*Ava*II 40-bp fragment corresponding to the 5′-region of β-PT. This 40-bp fragment was generated by annealing kinased (by T4 polynucleotide kinase) oligos 5′-TATGGAAGAAATAGGAGCCAATGATGATCTGAATTACTG-3′ and 5′-GACCAGTAATTCAGATCATCATTGGCTCCTATTTCTTCCA-3′. Ligation of the 40-bp *Nde*I/*Ava*II 5′-fragment of β-PT, the *Ava*II/*Bam*HI fragment, and the *Nde*I/*Bam*HI-digested pET3c vector generated the β-PT/pET3c construct. The β-PT/pET3c construct was amplified in DH5α *E. coli* cells.

β-Protachykinin expression constructs in pMAL-c and pGEX-2T expression vectors. Production of β-PT/pMAL-c and β-PT/pGEX-2T constructs for expression of fusions with the maltose-binding protein (MBP) and glutathione S-transferase (GST), respectively, was also generated. The pMAL-c vector (New England Biolabs, Beverly, MA) encodes a factor Xa proteolytic cleavage site at the junction of the MBP and β-PT segments of the fusion protein. The pGEX-2T vector contains a thrombin proteolytic cleavage site at the junction of GST and β-PT of the fusion protein.

The β-PT/pMAL-c construct was prepared by first ligating a 35-bp *Stu*I/*Ava*II DNA fragment corresponding to the 5′-region of β-PT with the 850-bp *Ava*II/*Hin*dIII DNA fragment of the human β-PPT cDNA (11); the 35-bp *Stu*I/*Ava*II fragment was formed by annealing kinased (by T4 polynucleotide kinase) oligos 5′-GAAGAAATAGGAGCCAATGATGATCTGAATTACTG-3′ and 5′-GACCAGTAATTCAGATCATCATTGGCTCCTATTTCTTC-3′. The resultant β-PT DNA fragment was then ligated to the pMAL-c vector (New England Biolabs) that was digested with *Stu*I and *Hin*dIII to generate the β-PT/pMAL-c construct that was expressed in PR722 *E. coli* cells.

For preparation of the β-PT/pGEX-2T construct, PCR of the β-PT DNA from the human β-PPT cDNA (11) was performed with sense and antisense primers 5′-AAGGATCCGAAGAAATAGGAGCC-3′ and 5′-AAGAATTCTTA-

TTAACGTCTTCTTTC-3′ that incorporated *Bam*HI and *Eco*RI sites at 5′- and 3′-ends of β-PT, respectively. The PCR reaction was performed according to the protocol by Perkin–Elmer with 1 ng β-PPT cDNA as template (linearized with *Hin*dIII), 0.5 μM of sense and antisense primers, and thermocycling consisting of 30 cycles each at 95°C for 1 min, 61°C for 2 min, and 72°C for 2 min. The PCR-generated 350-bp β-PT DNA was digested with *Bam*HI and *Eco*RI and ligated with the pGEX-2T vector digested with the same restriction enzymes to generate the β-PT/pGEX-2T construct. The construct was amplified and expressed in JM101 *E. coli* cells (Stratagene, La Jolla, CA).

All prohormone inserts in expression constructs were subjected to DNA sequencing by the dideoxy chain terminating method with the automated DNA sequencer (Applied Biosystems), as we have described previously (26), to confirm that authentic prohormone DNA sequences were subcloned into expression vectors.

Induction of prohormone expression and analysis by SDS–PAGE, Western blots, and ^{35}S-methionine labeling. Expression of prohormones by the pET3c vector was performed by IPTG (0.5 mM final concentration) induction in BL21(DE3) *E. coli* in M9ZB medium with 100 μg/ml ampicillin. Expression of β-PT in the pMAL-c vector was achieved in PR722 *E. coli* grown in LB broth with 100 μg/ml ampicillin, and expression of β-PT in pGEX-2T utilized JM101 *E. coli* cells in M9ZB medium containing 100 μg/ml ampicillin.

For expression of all prohormones, cells at an optical density of 0.15–0.20 at 600 nm were induced by addition of IPTG (0.5 mM final concentration) to the culture medium and incubation at 37°C for 1–2 h. At different incubation times with IPTG, 1 ml of control (no IPTG) and IPTG-induced cell cultures were collected by centrifugation (15,000*g* for 20 min at 4°C), and resuspended in 100 μl of SDS–PAGE sample buffer (16,22) per 1-ml cell aliquot of 0.4 optical density at 600 nm. Samples (20 μl) were analyzed on SDS–PAGE gels (12% polyacrylamide) and Western blots with antibodies specific for each prohormone. Antibodies used in Western blots were the PE-18 monoclonal antibody (27) (1:1000 final dilution) to detect proenkephalin, anti-NPY serum (Accurate Chemicals, Westbury, NY) to detect pro-NPY, anti-ACTH serum (National Pituitary Agency, Gaithersburg, MD) for detection of POMC, and an anti-β-PT serum (28) known as PM-3 to detect expression of β-PT. For expression of β-PT as fusion proteins with the maltose-binding protein (MBP) and glutathione S-transferase (GST), anti-MBP (New England Biolabs) and anti-GST (Pharmacia, Piscataway, NJ) sera were also used to detect fusion proteins. Western blots were performed as we have previously described (22,29).

Biosynthesis of prohormones was analyzed by incorporation of ^{35}S-methionine into ^{35}S-prohormones. BL21(DE3) cells were incubated without and with IPTG (0.5 mM) for 0–2 h, and 1-ml aliquots were incubated with ^{35}S-methionine at 10 μCi/ml medium (^{35}S-methionine was 800–1000 Ci/mmol, New England Nuclear) for 1 min at 37°C. Cells were immediately placed on ice and 100 μl ice-cold 100% TCA (trichloroacetic acid) was added with vortexing, and after 30 min at 4°C the sample was centrifuged at 15,000*g* (at 4°C) to collect ^{35}S-prohormones as the precipitate. The precipitate was resuspended in 50 μl 2× sample buffer (as described previously (16,18)) for SDS–PAGE gels (same total cpm for each sample was loaded onto gel) and autoradiography. It is noted that in uninduced cells, basal levels of *E. coli* proteins were being synthesized that were detected as minor ^{35}S-bands on SDS–PAGE gels. However, upon induction, the prohormones became the primary proteins produced, as indicated by the major bands of ^{35}S-prohormones on SDS–PAGE.

^{35}S-β-Preprotachykinin (^{35}S-β-PPT) generated by in vitro transcription and translation, and signal peptidase formation of ^{35}S-β-protachykinin (^{35}S-β-PT). ^{35}S-β-PPT generated by *in vitro* transcription with T7 RNA polymerase and *in vitro* translation with wheat germ extract (as described previously (16,30)) was digested with hen oviduct signal peptidase (31) to assess the apparently slower electrophoretic mobility of ^{35}S-β-PT (signal peptide removed) compared to ^{35}S-β-PPT. ^{35}S-β-PPT (55,000 cpm, based on trichloroacetic acid precipitation of the precursor (16,31)) from the translation reaction was incubated with hen oviduct signal peptidase (3 μl) in 0.20 M sucrose, 68 mM DTT, and 6.8 mg/ml phosphatidylcholine, in low salt buffer consisting of 50 mM triethanolamine-HCl, pH 7.5, 50 mM KCl, and 5 mM $MgCl_2$ at 24°C for 90 min. Samples were analyzed by SDS–PAGE gels and autoradiography.

Purified recombinant prohormones for in vitro processing assays by the processing enzymes PTP, 70-kDa aspartyl protease, and PC1/3 and PC2. Relative rates of processing by established processing enzymes (1–3) were assessed with purified PE, pro-NPY, and POMC. As described previously (22,29,32), PE, pro-NPY, and POMC were purified from 5- to 10-liter cultures of *E. coli*. The prohormone processing enzymes PTP, 70-kDa aspartyl protease, and PC1/3 and PC2 were purified from bovine adrenal medullary chromaffin granules as previously described (16,21,33).

To compare relative rates of *in vitro* processing of recombinant prohormones by four different processing enzymes, prohormones at 10^{-5} M were incubated (in a total volume of 20 μl) with purified PTP (1 ng), 70-kDa aspartic protease (1 μg), or PC1/3 or PC2 (1 μg) under optimum assay conditions previously established for each protease (16,21,33). These *in vitro* processing assays were conducted near estimated *in vivo* levels of

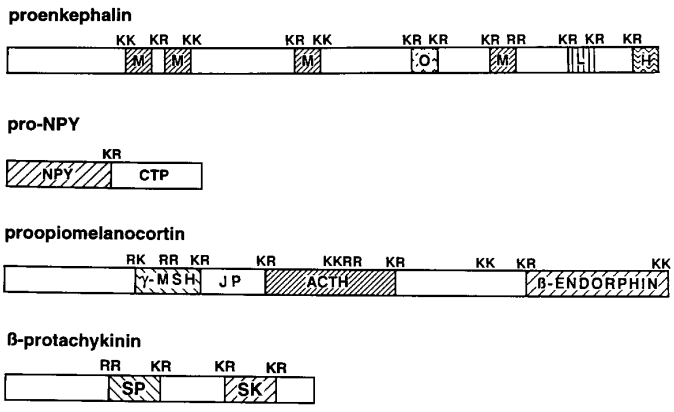

FIG. 1. Prohormone structures. The structures of proenkephalin (PE), pro-neuropeptide Y (pro-NPY), proopiomelanocortin (POMC), and β-protachykinin (β-PT) are schematically illustrated. Proenkephalin contains the opioid peptides (Met)enkephalin (M), (Met)enkephalin-Arg^6-Gly^7-Leu^8 (O), (Leu)enkephalin (L), and (Met)enkephalin-Arg-Phe (H). Pro-neuropeptide Y (pro-NPY) contains the single active neuropeptide Y, and a COOH-terminal peptide (CTP). POMC contains the hormone ACTH (adrenocorticotropin hormone), the opioid peptide β-endorphin, γ-MSH (γ-melanocyte stimulating hormone), a segment known as the joining peptide (JP), and several other peptide products (19,20). β-Protachykinin contains the tachykinin neuropeptides substance P (SP) and substance K (SK). Active peptides within prohormone molecules are typically flanked by basic residues Arg (R) or Lys (K).

prohormones (34) to provide information on relative rates of prohormone processing that may occur *in vivo*. In addition, the amount of processing proteases used in these *in vitro* assays was chosen to achieve partial cleavage of each prohormone within 1–8 h incubation at 37°C. *In vitro* assays were subjected to SDS–PAGE and the initial cleavage of prohormones was quantitated by densitometry of prohormone bands. Duplicate assays varied by less than 10–15%.

RESULTS

Expression of prohormones in the pET3c vector. Complementary DNAs encoding the prohormones (illustrated in Fig. 1) proenkephalin (PE) (5,6), pro-neuropeptide Y (pro-NPY) (6), proopiomelanocortin (POMC) (7,8), and β-protachykinin (β-PT) (9–11) were subcloned into *Nde*I and *Bam*HI sites, downstream of the T7 promoter, of the pET3c expression vector (Fig. 2) (23). Expression was induced in the host strain BL21(DE3) that contains the bacteriophage T7 RNA polymerase under the inducible control of the lac UV5 promoter (23). Prohormone induction by IPTG was assessed by Coomassie blue protein staining of SDS–PAGE gels, Western blots with antibodies directed against active peptide regions of the prohormones, and by ^{35}S-methionine labeling of recombinant prohormones.

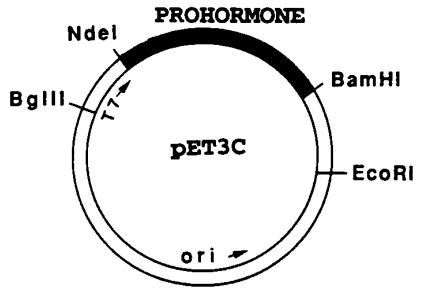

FIG. 2. pET3c expression vector. Prohormone cDNAs (with signal sequence deleted) were subcloned into *Nde*I and *Bam*HI sites of the pET3c vector, possessing the T7 promoter sequence upstream of the prohormone cDNA insert.

Expression of PE (Fig. 3) and pro-NPY (Fig. 4) at high levels in BL21(DE3) cells was induced by IPTG (0.5 mM for 1–2 h), as demonstrated by production of 35-kDa PE (Fig. 3a) and 8-kDa pro-NPY (Fig. 4a), detected by Coomassie blue protein staining of SDS–PAGE gels. The 35-kDa band was confirmed as PE by recognition of this band in Western blots by anti-PE monoclonal antibody directed against the midregion of the proenkephalin molecule (Fig. 3b). Western blots with anti-NPY serum also illustrated induction of 8-kDa pro-NPY (Fig. 4b). Biosynthesis of PE and pro-NPY was confirmed by IPTG-induced incorporation of ^{35}S-methionine into 35-kDa PE and 8-kDa pro-NPY (Figs. 3c and 4c). Based on the Coomassie blue-stained

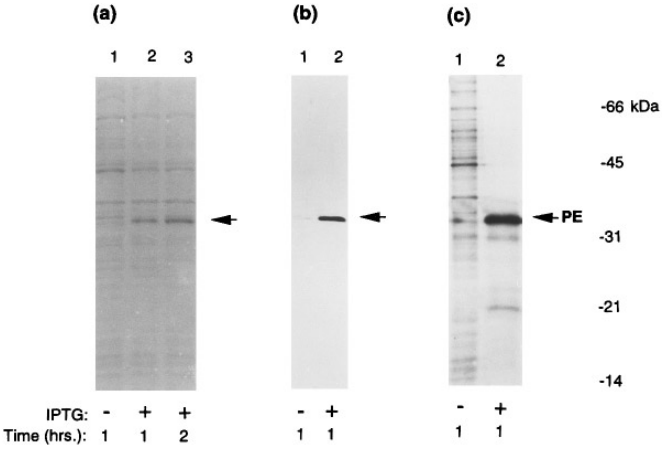

FIG. 3. Proenkephalin (PE) expression. (a) SDS–PAGE. PE expression was assessed without IPTG (lane 1), and with IPTG for 1 and 2 h (lanes 2 and 3) by Coomassie blue protein staining of a SDS–PAGE gel. The arrow indicates induction of the 35-kDa PE band. (b) Western blot. Western blot with monoclonal antibody (PE18) (27) against proenkephalin illustrates IPTG-induced expression of the immunoreactive 35-kDa PE band (lane 2). PE expression is not evident in uninduced cells (lane 1). (c) ^{35}S-Methionine labeling. ^{35}S-Methionine pulse-labeling of control (no IPTG, lane 1) and IPTG-induced (lane 2) cells indicates induction of a major 35-kDa ^{35}S-PE band (indicated by arrow) in cells incubated with IPTG for 1 h (lane 2).

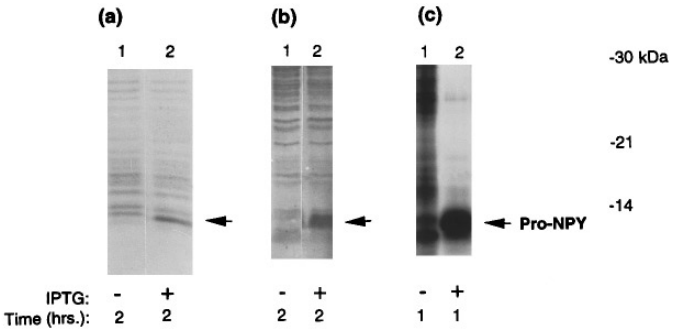

FIG. 4. Pro-NPY expression. (a) SDS–PAGE. Expression of pro-NPY detected by Coomassie blue staining of a SDS–PAGE is illustrated by the induction of the 8-kDa pro-NPY band after 2 h incubation with IPTG (lane 2), compared to control cells incubated without IPTG (lane 1). (b) Western blot. Use of anti-NPY serum for Western blot analysis indicated that the IPTG-induced 8-kDa band is immunoreactive for NPY (lane 2), compared to control cells incubated without IPTG (lane 1). (c) ^{35}S-Methionine labeling. ^{35}S-Methionine pulse-labeling indicates IPTG-induced expression of high levels of ^{35}S-pro-NPY (indicated by arrow) after 1 h (lane 2), compared to control cells incubated without IPTG (lane 1).

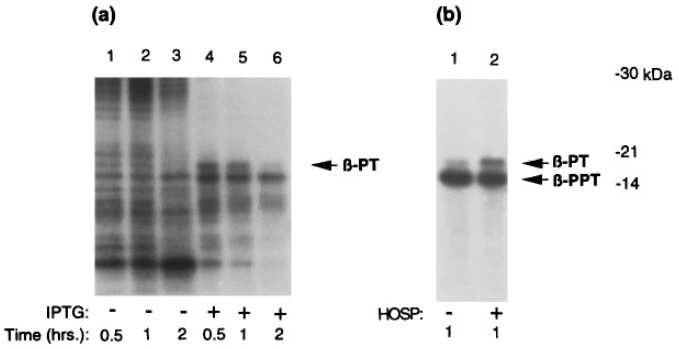

FIG. 6. β-Protachykinin expression: ^{35}S-methionine labeling and signal peptidase. (a) ^{35}S-Methionine labeling. ^{35}S-Methionine labeling in BL21(DE3) cells shows the production of ^{35}S-β-PT of 16 kDa, indicated by the arrow. Cells were incubated without (lanes 1–3) or with IPTG (lanes 4–6) for 30 min (lanes 1 and 4), 1 h (lanes 2 and 5), and 2 h (lanes 3 and 6). (b) Signal peptidase conversion of ^{35}S-preprotachykinin (^{35}S-β-PPT) to ^{35}S-β-protachykinin (^{35}S-β-PT). ^{35}S-β-PPT generated by *in vitro* translation was incubated without (lane 1) or with (lane 2) hen oviduct signal peptidase, and analyzed by SDS–PAGE and autoradiography to compare the electrophoretic mobilities of ^{35}S-β-PPT and ^{35}S-β-PT. The signal peptidase converts 14-kDa ^{35}S-β-PPT to ^{35}S-β-PT that appears as a 16-kDa band on SDS–PAGE.

SDS–PAGE gel, it is estimated that milligrams of PE and pro-NPY are expressed in liter cultures of *E. coli*. These results demonstrate high-level expression in *E. coli* of intact PE and pro-NPY by the pET3c expression vector.

Expression of POMC was evident, as detected by IPTG induction of a 35-kDa band detected by anti-ACTH serum in Western blots (Fig. 5a). The biosynthesis of POMC was also demonstrated by IPTG induction of ^{35}S-methionine labeling of the 35-kDa POMC band (Fig. 5b). Levels of POMC expression were lower than

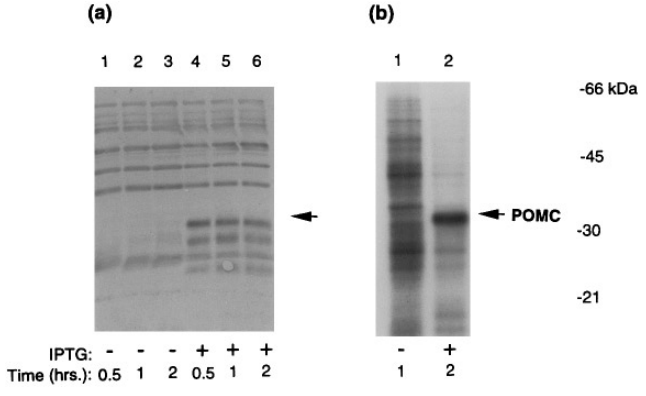

FIG. 5. Proopiomelanocortin expression. (a) Western blot. POMC (indicated by arrow) of 35 kDa was detected by anti-ACTH serum (lanes 4–6, respectively) in Western blots. POMC was not detected in control cells incubated without IPTG (lanes 1–3). (b) ^{35}S-Methionine labeling. ^{35}S-Methionine pulse-labeling demonstrates IPTG induction of ^{35}S-POMC (lane 2, ^{35}S-POMC indicated by arrow), compared to cells incubated without IPTG (lane 1).

that for proenkephalin or pro-NPY, since POMC was not detected on Coomassie-stained SDS–PAGE gels.

Expression of recombinant β-PT was detected by ^{35}S-methionine labeling of a 16-kDa band (Fig. 6a). ^{35}S-β-PT was induced with 30 and 60 min incubation with IPTG. However, by 2 h, lower levels of ^{35}S-(Met)-β-PT remained, indicating that ^{35}S-β-PT was somewhat unstable or was partially degraded. The observation that β-PT was not detected in Western blots or by Coomassie blue protein staining of SDS–PAGE gels (data not shown) also indicated a lower level of β-PT expression compared to the other prohormones expressed in this study.

It was noted that the 16-kDa ^{35}S-β-protachykinin (Fig. 6a) contrasts with the apparent molecular weight of ^{35}S-β-preprotachykinin (generated by *in vitro* translation (30)) of 14 kDa that includes the NH_2-terminal signal peptide. Removal of the signal peptidase from 14-kDa ^{35}S-β-PPT by hen oviduct signal peptidase to result in 16-kDa ^{35}S-β-PT was demonstrated (Fig. 6b). Thus, signal peptidase cleavage of the signal peptide of β-PPT results in β-PT with retarded mobility on SDS–PAGE. These data suggest that differences in protein conformation of β-PPT compared to β-PT may be involved in the apparently slower mobility of β-PT on SDS–PAGE.

Expression of β-protachykinin by the pMAL-c and pGEX-2T vectors. Since β-PT was expressed at low levels by the pET3c system, expression of β-PT as fusion proteins was examined to achieve higher levels of expression. Expression of β-PT as a fusion

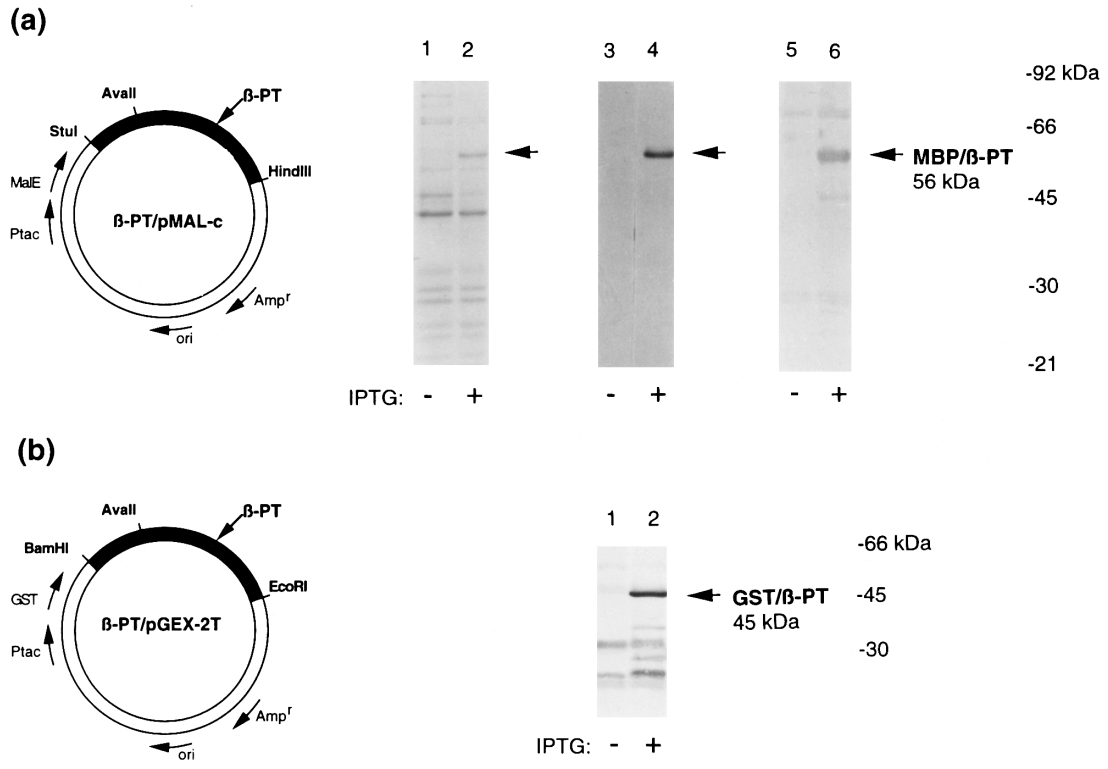

FIG. 7. Expression of β-protachykinin as fusions with maltose-binding protein (MBP) and glutathione S-transferase (GST). (a) MBP/β-PT fusion protein expression: analysis by SDS–PAGE and Western blots. IPTG-induced expression of MBP/β-PT was analyzed by SDS–PAGE gels stained with Coomassie blue (lanes 1, 2), and Western blots with anti-MBP (lanes 3, 4) or anti-PT sera (lanes 5, 6). Comparison of control cells incubated without IPTG (lanes 1, 3, 5) with those incubated with IPTG (lanes 2, 4, 6) for 1 h indicates expression of a 56-kDa MBP/β-PT fusion protein. (b) GST/β-PT fusion protein expression: analysis by Western blots. Expression of the GST/β-PT fusion protein in control cells incubated without IPTG (lane 1) and in cells incubated with IPTG (lane 2) was detected by Western blots with anti-protachykinin serum (PM-3). Expression is indicated by IPTG induction of the 45-kDa GST/β-PT band (shown by arrow).

protein with MBP or with GST using the pMAL-c and pGEX-2T vectors, respectively (Fig. 7), was excellent. Expression of the MBP/β-PT fusion was demonstrated by IPTG induction of a 56-kDa band that was recognized by Coomassie blue staining of a SDS–PAGE gel, and by anti-β-PT and by anti-MBP sera in Western blots (Fig. 7a). The apparent molecular weight of MBP/β-PT of 56 kDa is consistent with a fusion of 16-kDa β-PT and 40-kDa MBP. Expression of the GST/β-PT fusion was also very good (Fig. 7b), as demonstrated by IPTG induction of a 45-kDa band recognized by anti-β-PT serum; the molecular weights of 16-kDa β-PT and 27-kDa GST are consistent with the GST/β-PT fusion of approximately 45 kDa.

Comparison of the expression of prohormones in E. coli. Detection of prohormone expression by Coomassie blue protein staining, Western blots, and ^{35}S-methionine labeling indicated detection of differing levels of prohormone expression (Table 1). Expression of high levels of PE, pro-NPY, MBP/β-PT, and GST/β-PT was apparent since the induced prohormones were readily detected by Coomassie protein staining of SDS–PAGE gels. Moderate levels of POMC were expressed, since it was detected by Western blots, but not by protein staining of gels. Expression of β-PT was lowest since it was detected by only

TABLE 1

Expression of Prohormones in *E. coli*

	Method of detection of prohormone expression		
	SDS–PAGE gels and Coomassie blue	Western Blots	^{35}S-Met labeling
Proenkephalin	+	+	+
Pro-neuropeptide Y	+	+	+
Proopiomelanocortin	–	+	+
β-Protachykinin	–	–	+
MBP/β-PT	+	+	ND
GST/β-PT	+	–	ND

Note. ND, not determined; MBP, maltose-binding protein; GST, glutathione S-transferase; β-PT, β-protachykinin.

the most sensitive ^{35}S-Met-prohormone labeling approach. Overall, most prohormones examined showed excellent expression with the pET3c vector, or with the pMAL-c and pGEX-2T vectors.

Relative rates of prohormone processing by multiple processing proteases: Demonstration of precursor selectivity of processing enzymes. While it is known that the established prohormone processing enzymes—PTP (16–18), 70-kDa aspartyl protease (19–22), and prohormone convertase (PC) enzymes (12–15,33)—possess similar cleavage specificity at paired basic residue sites (Lys–Arg, Arg–Lys, Arg–Arg, and Lys–Lys), it is not known whether each processing protease possesses preference for particular prohormones. It is predicted that these proteases may possess selectivity for prohormone substrates, since each prohormone possesses a unique protein structure defined by its primary sequence.

Comparison of the relative rates of PE, pro-NPY, and POMC *in vitro* processing by the established prohormone cleaving proteases PTP, 70-kDa aspartyl protease, PC1/3, and PC2 demonstrated clear differences in the relative efficiencies of these proteases to cleave the different precursors (Fig. 8). Recombinant prohormones were purified after expression for *in vitro* processing by purified prohormone processing enzymes. PE and pro-NPY were most readily cleaved by PTP, with little processing by the 70-kDa aspartyl protease or PC1/3 and PC2 enzymes. In contrast, POMC was most readily processed by the 70-kDa aspartyl protease, with some processing evident with PC1/3 and PC2; however, almost no processing of POMC was detected with the PTP enzyme. It is of interest to note that PTP appears as the most efficient processing enzyme with respect to the low levels of enzyme (nanograms) needed for *in vitro* processing compared to the subtilisin and aspartyl proteases tested (using microgram amounts of enzyme in the assays). Overall, these studies indicate the significant property of selectivity of prohormone processing enzymes for different precursor substrates.

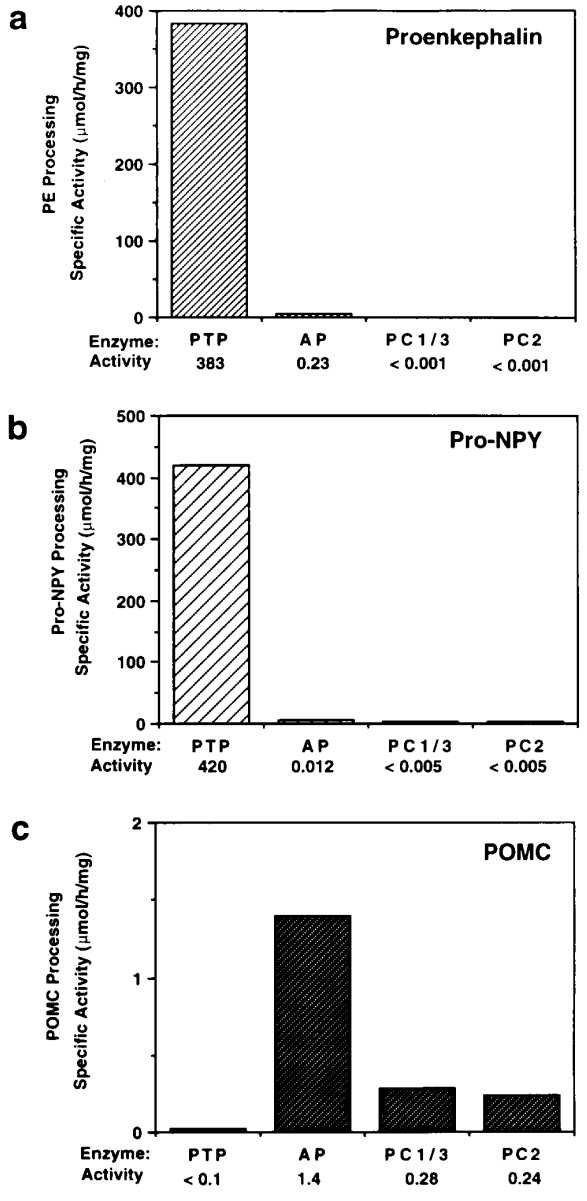

FIG. 8. In vitro processing of prohormones by processing proteases. (a) Proenkephalin processing by PTP, 70-kDa aspartyl protease (AP), PC1/3, and PC2. (b) Pro-NPY processing by PTP, 70-kDa aspartyl protease (AP), PC1/3, and PC2. (c) POMC processing PTP, 70-kDa aspartyl protease (AP), PC1/3, and PC2. The relative rates of processing recombinant PE, pro-NPY, and POMC by established prohormone processing enzymes—the cysteine protease "prohormone thiol protease" (PTP), the 70-kDa aspartyl protease (AP), and the subtilisin-like PC1/3 and PC2 proteases—were determined. Purified prohormones (10^{-5} M) were each incubated with purified processing proteases, as described under Materials and Methods, and the amount of prohormone cleaved was determined by densitometry of prohormone bands on SDS–PAGE gels. Specific activity of prohormone processing was calculated as μmol prohormone cleaved per hour per milligram enzyme protein (μmol/h/mg enzyme). The determined specific enzyme activities for each prohormone are indicated below each bar graph.

DISCUSSION

Development of recombinant prohormones expressed from cloned cDNAs is essential for *in vitro* studies of prohormone and proprotein processing. For this reason, effective expression of high levels of the prohormones proenkephalin, pro-NPY, POMC, and β-PT was characterized in this study utilizing the T7 expression vector pET3c in *E. coli*. Effective expression of recombinant prohormones was obtained, as assessed by SDS–PAGE, Western blots, and ^{35}S-methionine labeling. Expression of recombinant prohormones allowed comparison of the relative efficiencies of prohormone processing enzymes—PTP (16–18), 70-kDa aspartyl protease (19–22), PC1/3 and PC2 (12–15,33)—to process differ-

ent prohormone substrates. Significantly, results demonstrate selectivity of processing enzymes for prohormone substrates.

Characterization of prohormones expressed in *E. coli* showed that expression of recombinant proenkephalin, pro-NPY, and POMC prohormones was achieved with the pET3c vector containing the T7 promoter (23). These results demonstrate the effectiveness of the pET3c vector for generating recombinant prohormones. Expression may also be achieved with the pMAL-c and pGEX-2T expression vectors that generate fusion proteins with MBP (24) and with GST (25), as demonstrated in this study with expression of β-protachykinin as fusions with MBP or GST. Expression in *E. coli* generates milligrams of prohormones, as demonstrated for proenkephalin and pro-NPY, whereas *in vitro* translation only generates micrograms of these precursors (16,30). Therefore, expression in *E. coli* is more efficient than synthesis by *in vitro* transcription and translation. The efficient expression is based, in part, on the ribosomal binding domains engineered into the pET3c expression vector that allow efficient translation of prohormone mRNAs by the *E. coli* protein synthetic machinery (23). These studies show that expression of large quantities (milligrams) of prohormones in *E. coli* should be advantageous for *in vitro* processing studies.

It was noted that expression of prohormones occured at slightly different levels with the same pET3c vector. While intact PE and pro-NPY were expressed at high levels detectable by Coomassie staining of SDS–PAGE, expression of POMC and β-PT was lower since their detection required more sensitive analyses by Western blot or ^{35}S-methionine labeling. It is possible that the relative stability of different prohormone proteins, or their susceptibility to *E. coli* proteases, may influence the level of prohormone expression.

Importantly, this study demonstrates that recombinant prohormones may be used as model substrates for investigating proteolytic processing mechanisms. Comparisons of the ability of four distinct prohormone processing proteases—the cysteine protease PTP (16–18), the 70-kDa aspartyl protease known as POMC converting enzyme (19–22), and the subtilisin-like PC1/3 and PC2 (12–15,33)—to cleave proenkephalin, pro-NPY, and POMC indicated clear selectivity of each protease for a particular prohormone substrate. Further characterization of *in vitro* prohormone processing will be important in understanding the enzymology of prohormone processing. It will be advantageous to utilize recombinant prohormones to facilitate progress in understanding proteolytic processing mechanisms required for production of potent peptide hormones and neurotransmitters.

ACKNOWLEDGMENTS

The authors appreciate gifts of rat PPE and rat prepro-NPY cDNAs from Dr. Steve Sabol (National Institutes of Health, Bethesda, MD), porcine POMC cDNA from Dr. Guy Boileau (University of Montreal), and human β-PPT cDNA from Dr. Hans Urs Affolter (formerly at the National Institutes of Health, Bethesda, MD). In addition, kind gifts of monoclonal antibody against PE (PE-18) from Dr. Barbara Spruce (Imperial College of Science, UK) and anti-protachykinin serum from Dr. James Krause (Washington University, St. Louis, MO) are acknowledged. The authors thank Steve White for technical assistance in DNA sequencing. This work was supported by grants from the National Institute of Neurological Disease and Stroke (NINDS) of the National Institutes of Health (NIH), the National Institute of Drug Abuse (NIDA, NIH), and the National Science Foundation.

REFERENCES

1. Hook, V. Y. H., Azaryan, A. V., Hwang, S. R., and Tezapsidis, N. (1994) Proteases and the emerging role of protease inhibitors in prohormone processing. *FASEB J.* **8,** 1269–1278.
2. Seidah, N. G., Day, R., Marcinkiewicz, Benjannet, S., and Chretien, M. (1991) Mammalian neural and endocrine pro-protein and prohormone convertases belonging to the subtilisin family of serine proteinases. *Enzyme* **45,** 271–284.
3. Steiner, D. F., Smeekens, S. P., Ohagi, S., and Chan, S. F. (1992) The new enzymology of precursor processing endoproteases. *J. Biol. Chem.* **267,** 23435–23438.
4. Yoshikawa, K., Williams, C., and Sabol, S. L. (1984) Rat brain preproenkephalin mRNA. *J. Biol. Chem.* **259,** 14301–14308.
5. Comb, M., Seeburg, P., Adelman, J., Eiden, L., and Herbert, E. (1982) Primary structure of the human Met- and Leu-enkephalin precursor and its mRNA. *Nature* **295,** 663–666.
6. Higuchi, H., Yang, H. Y. T., and Sabol, S. L. (1988) Rat neuropeptide Y precursor gene expression. *J. Biol. Chem.* **263,** 6288–6295.
7. Boileau, G., Barbeau, C., Jeannotte, L., Chretien, M., and Drouin, J. (1983) Complete structure of the porcine pro-opiomelanocortin mRNA derived from the nucleotide sequence of cloned cDNA. *Nucleic Acids Res.* **11,** 8063–8071.
8. Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C. Y., Cohen, S. N., and Numa, S. (1979) Nucleotide sequence of cloned cDNA for bovine corticotropin-β-lipotropin precursor. *Nature* **278,** 423–427.
9. Krause, J. E., Chirgwin, J. M., Carter, M. S., Xu, Z. S., and Hershey, A. D. (1987) Three rat preprotachykinin mRNAs encode the neuropeptides substance P and neurokinin A. *Proc. Natl. Acad. Sci. USA* **84,** 881–5.
10. Nawa, H., Hirose, T., Takashima, H., Inayama, S., and Nakanishi, S. (1983) Nucleotide sequences of cloned cDNAs for two types of bovine brain substance P precursor. *Nature* **306,** 32–36.
11. Harmar, A. J., Armstrong, A., Pascall, J. C., Chapman, K., Rosie, R., Curtis, A., Going, J., Edwards, C. R., and Fink, G. (1986) cDNA sequence of human beta-preprotachykinin, the common precursor to substance P and neurokinin A. *FEBS. Lett.* **208,** 67–72.
12. Seidah, N. G., Gaspar, L., Mion, P., Marcinkiewicz, M., Mbikay, M., and Chretien, M. (1990) cDNA sequence of two distinct pituitary proteins homologous to Kex2 and furin gene products: Tissue-specific mRNAs encoding candidates for pro-hormone processing proteinases. *DNA Cell Biol.* **9,** 415–424.
13. Seidah, N. G., Marcinkiewicz, M., Benjannet, S., Gaspar, L., Beaubien, G., Mattei, M. G., Lazure, C., Mbikay, M., and Chretien, M. (1991) Cloning and primary sequence of a mouse candidate prohormone convertase PC1 homologous to PC2, furin, and Kex2: Distinct chromosomal localization and messenger RNA distribution in brain and pituitary compared to PC2. *Mol. Endocrinol.* **5,** 111–122.

14. Smeekens, S. P., and Steiner, D. F. (1990) Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease Kex2. *J. Biol. Chem.* **265,** 2997–3000.
15. Ohagi, S., LaMendola, J., LeBeau, M. M., Espinosa, R., Takeda, J., Smeekens, S. P., Chan, S. J., and Steiner, D. F. (1992) Identification and analysis of the gene encoding human PC2, a prohormone convertase expressed in neuroendocrine tissues. *Proc. Natl. Acad. Sci. USA* **89,** 4977–4981.
16. Krieger, T. K., and Hook, V. Y. H. (1991) Purification and characterization of a novel thiol protease involved in processing the enkephalin precursor. *J. Biol. Chem.* **266,** 8276–8383.
17. Azaryan, A. V., and Hook, V. Y. H. (1994) Unique cleavage specificity of "prohormone thiol protease" related to proenkephalin processing. *FEBS Lett.* **341,** 197–202.
18. Tezapsidis, N., Noctor, S., Kannan, R., Krieger, T. J., Mende-Mueller, L., and Hook, V. Y. H. (1995) Stimulation of "prohormone thiol protease" (PTP) and (Met)enkephalin by forskolin: Blockade of elevated (Met)enkephalin by a cysteine protease inhibitor of PTP. *J. Biol. Chem.* **270,** 13285–13290.
19. Loh, Y. P., Parish, D. C., and Tuteja, R. (1985) Purification and characterization of a paired basic residue-specific pro-opiomelanocortin converting enzyme from Bovine pituitary intermediate lobe secretory vesicles. *J. Biol. Chem.* **260,** 7194–7205.
20. Parish, D. C., Tuteja, R., Altstein, M., Gainer, H., and Loh, Y. P. (1986) Purification and characterization of a paired basic residue-specific prohormone-converting enzyme from bovine pituitary neural lobe secretory vesicles. *J. Biol. Chem.* **261,** 14392–14397.
21. Azaryan, A. V., Schiller, M., Mende-Mueller, L., and Hook, V. Y. H. (1995) Characteristics of the chromaffin granule aspartic proteinase involved in proenkephalin processing. *J. Neurochem.* **65,** 1771–1779.
22. Azaryan, A. V., Schiller, M. R., and Hook, V. Y. H. (1995) Chromaffin granule aspartic proteinase processes recombinant proopiomelanocortin. *Biochem. Biophys. Res. Commun.* **215,** 937–944.
23. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185,** 60–89.
24. Ausubel, F. M., Brant, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1990) *Current Protocols Mol. Biol.* **2,** 16.6.1–16.6.14.
25. Johnson, K. S., Harrison, G. B. L., Lightowlers, M. W., O'Hoy, K. L., Cougle, W. G., Dempster, R. P., Lawrence, S. B., Vinton, J. G., Heath, D. D., and Rickard, M. D. (1989) Vaccination against ovine cysticercosis using a defined recombinant antigen. *Nature* **338,** 585–587.
26. Hwang, S. R., Kohn, A. B., and Hook, V. Y. H. (1994) Molecular cloning reveals isoforms of bovine α_1-antichymotrypsin. *Proc. Natl. Acad. Sci. USA* **91,** 9579–9583.
27. Spruce, B. A., Jackson, S., Lowry, P. J., Lane, D. P., and Glover, D. M. (1988) Monoclonal antibodies to a proenkephalin A fusion peptide synthesized in Escherichia coli recognize novel proenkephalin A precursor forms. *J. Biol. Chem.* **263,** 19788–19795.
28. MacDonald, M. R., Takeda, J., Rice, C. M., and Krause, J. E. (1989) Multiple tachykinins are produced and secreted upon post-translational processing of the three substance P precursor proteins, α-, β-, and γ-preprotachykinin. *J. Biol. Chem.* **264,** 15578–15592.
29. Schiller, M. R., Mende-Mueller, L., Miller, K. W., and Hook, V. Y. H. (1995) "Prohormone thiol protease" (PTP) processing of recombinant proenkephalin. *Biochemistry* **34,** 7988–7995.
30. Krieger, T. K., and Hook, V. Y. H. (1992) Purification and characterization of a cathepsin D protease from bovine chromaffin granules. *Biochemistry* **31,** 4223–4231.
31. Lively, M. O., Newsome, A. L., and Nusier, M. (1994) Eukaryote microsomal signal peptidases. *Methods Enzymol.* **244,** 301–314.
32. Schiller, M. R., Kohn, A. B., Mende-Mueller, L., Miller, K., and Hook, V. Y. H. (1996) Expression of recombinant pro-neuropeptide Y, proopiomelanocortin, and proenkephalin: relative processing by "prohormone thiol protease" (PTP). *FEBS Lett.* **382,** 6–10.
33. Azaryan, A. V., Krieger, T. J., and Hook, V. Y. H. (1995) Purification and characteristics of the candidate prohormone processing proteases PC2 and PC1/3 from bovine adrenal medulla chromaffin granules. *J. Biol. Chem.* **270,** 8201–8208.
34. Ungar, A., and Phillips, J. H. (1983) Regulation of the adrenal medulla. *Physiol. Rev.* **63,** 787–843.