Expression and Cell Type–Specific Processing of Human Preproenkephalin with a Vaccinia Recombinant

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The posttranslational maturation of a complex precursor polyprotein, human proenkephalin, was assessed by infection of a wide spectrum of cell types with a recombinant vaccinia virus that expressed human proenkephalin. The infected cells rapidly produced both cellular and secreted Met-enkephalin immunoreactivity. Although each cell line could secrete intact proenkephalin, only a mouse pituitary line was capable of processing proenkephalin to mature enkephalin peptides. The quantity of intact proenkephalin secreted from BSC-40 cells (derived from African Green monkey kidney) was sufficient to establish the value of vaccinia virus as a mammalian cell expression vector.

NEUROENDOCRINE PEPTIDE GENE expression has been examined by introducing prohormone genes into heterologous cell types (*1–7*). Stable integration of the cloned genes into the host cell genome leads to production of the prohormone and, in some recipient cell types, processing of the proprotein into mature peptides. Although these studies indicate that transformed cells can process a precursor to mature peptides, this type of DNA transfer yields low levels of transformation and the foreign genes are expressed at low rates. A better approach to the study of cell-specific processing and secretion of a precursor protein would be to transiently express the messenger RNA (mRNA) directly in the cytoplasm of a host cell. In this way one can circumvent the difficulties associated with incorporating precursor protein genes into the host cell genome. This approach is now possible because of the recent development of vaccinia virus (VV) as a cloning and expression vector (*8, 9*). We have used recombinant VV as an expression vector to assess the expression and posttranslational maturation of a polyfunctional protein, human proenkephalin (hPE), in a variety of heterologous cell types.

Like several other neuroendocrine peptide precursors, the proteolytic maturation of proenkephalin is tissue-specific (*10, 11*). Proenkephalin is cleaved at pairs of basic amino acid residues to yield free enkephalins in several brain regions, while processing in the adrenal medulla yields a variety of larger enkephalin-containing peptides (ECP's) and very little free enkephalin (*12, 13*). Our results now show that many types of cells infected with a recombinant VV containing an hPE gene, accurately express and secrete intact human proenkephalin. Indeed, an African Green monkey kidney line secretes sufficient quantities of intact hPE to use for processing studies with isolated endopeptidases. However, of all the cell types tested, only a mouse pituitary line processes proenkephalin to mature enkephalin peptides.

A 918–base pair (bp) complementary DNA (cDNA) insert encoding hPE (*14*) was inserted in the correct orientation into the VV insertion-vector plasmid pVV3 (Fig. 1). The resulting recombinant plasmid, pVV3:hPE, was used to introduce the hPE cDNA into the VV genome by marker transfer techniques (*15, 16*). A VV recombinant containing the hPE insert (VV:hPE) was subsequently isolated and purified by plaque hybridization techniques. DNA and RNA hybridization procedures were used to confirm that the hPE cDNA had been inserted at the desired location within the Hind III J fragment of VV and that it was actively transcribed during viral infection.

The VV expression vector system can infect a wide spectrum of cell types, resulting in the expedient production of a foreign protein. Four different cell lines were infected with VV:hPE. AtT-20 (mouse anterior pituitary) and GH4C1 (rat pituitary) contain the full complement of secretory organelles—endoplasmic reticulum, Golgi, and mature secretory granules. The other cell lines, BSC-40 (African Green monkey kidney) and Ltk$^-$ (mouse fibroblast), do not contain stored granules typical of secretory cells. Each cell type was infected with either wild-type vaccinia virus (VV:WT) or VV:hPE. After 24 hours, a small portion of the cell extract and culture medium was digested with trypsin and then with carboxypeptidase B to release free Met-enkephalin peptide from larger ECP's (*17*). The amount of free Met-enkephalin present in each extract was then quantitated by a Met-enkephalin–specific radioimmunoassay (Table 1). Infection of each cell type with VV:hPE led to the production of significant

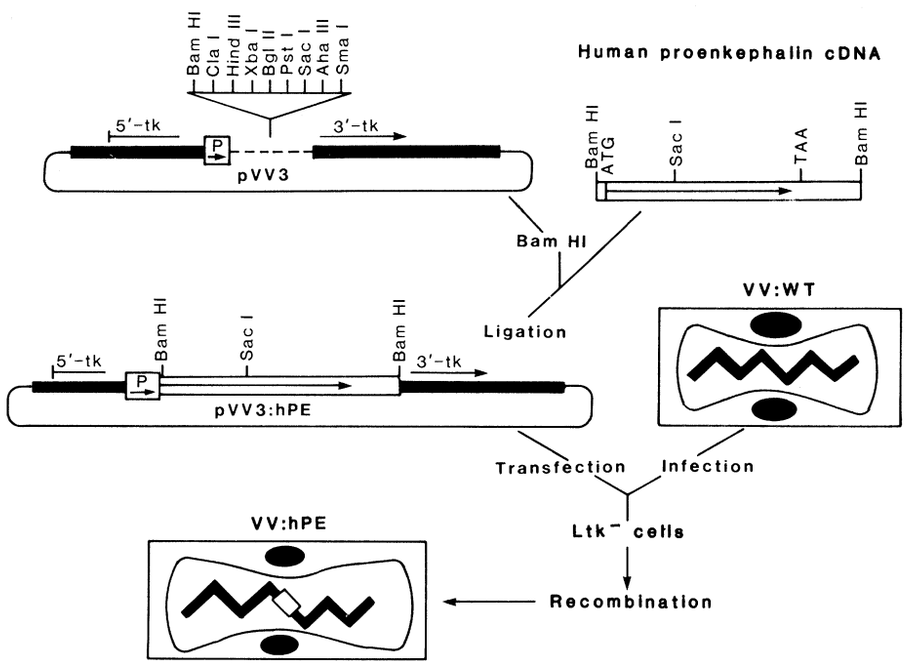

Fig. 1. Construction of VV:hPE. A 918-bp cDNA insert containing the entire coding region of human preproenkephalin (*14*) was isolated from plasmid pHR5 (*29*) and ligated into the Bam HI cloning site of the VV recombination plasmid pVV3. The plasmid pVV3 contains the VV thymidine kinase (tk) gene interrupted by the constitutive VV 7.5K promoter adjacent to a multiple cloning site. Insertion of a foreign DNA into any of the multiple cloning sites places it under control of the 7.5K promoter. Purified pVV3:hPE was then transfected into Ltk$^-$ cells infected with wild-type VV (WR strain, 0.05 PFU per cell). Homologous recombination between the pVV3:hPE and the VV:WT tk gene resulted in the insertion of the hPE DNA into the VV genome. Recombinant VV:hPE was purified by plaque hybridization techniques.

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amounts of Met-enkephalin immunoreactivity (IR) in the cells and medium.

To determine how effectively hPE was processed into smaller ECP's, acetic acid extracts of cells and culture medium from each cell type were resolved on a TSK-125 (high-performance liquid chromatography) sizing column. Each fraction was then assayed for Met-enkephalin IR after sequential digestion with trypsin and carboxypeptidase B. Analysis of BSC-40 cells revealed two prominent peaks of Met-enkephalin IR after infection with VV:hPE (peaks 1 and 2, Fig. 2A), which eluted with an apparent molecular size of 28 kilodaltons (kD) and 16 kD, respectively. Identical results were obtained for Ltk^- cells. To determine whether these forms are secreted, a small portion of the culture medium was also analyzed. Only one prominent peak of Met-enkephalin IR was detected and it coeluted with peak 1. The inability of BSC-40 and Ltk^- cells to process proenkephalin to small peptides is in agreement with studies demonstrating that other lines of African Green monkey kidney cells (*1, 18*) and L cells (*2*) are also unable to cleave the Lys-Arg sequences in proinsulin.

GH4C1 rat pituitary cells, which have stored secretory granules (*19*), exhibited an identical processing profile to that observed in BSC-40 cells (Fig. 2B). GH4C1 cells that secrete growth hormone and prolactin efficiently process human preproparathyroid hormone (preproPTH) to PTH by removal of the amino terminal hexapeptide sequence from the prohormone (*3*). The hexapeptide sequence terminates at a putative endopeptidase cleavage sequence, Lys-Lys-Arg. Perhaps GH4C1 cells cannot cleave hPE to small peptides because of the high degree of endopeptidase specificity for the basic amino acid cleavage sequences in the precursor protein.

In contrast, extracts of AtT-20 cells, which also have a stored population of secretory granules, contained five major peaks of Met-enkephalin IR after infection with VV:hPE (Fig. 2C). Peaks 1 and 2 coeluted with peaks 1 and 2 of Met-enkephalin IR from each of the other cell lines infected with VV:hPE. The presence of peaks 1 and 2 in each cell type studied shows that the modification responsible for their separation on the sizing column is common to many cell types and would therefore occur at a point in the secretory pathway before packaging of the precursor into secretory granules. Peaks 3 and 4 eluted with an apparent molecular size of 4.5 kD and 2.5 kD. The slowest migrating peak, 5, coeluted with purified Met-enkephalin. Analysis of the media from VV:hPE–infected AtT-20 cells revealed two major peaks of secreted Met-enkephalin IR. The faster migrating peak coeluted with peak 1 of the cell sample, and the slower migrating peak coeluted with peak 5 and purified Met-enkephalin. The amount of both cellular and secreted Met-enkephalin IR detected in peak 5 was not significantly changed when assayed prior to trypsin and carboxypeptidase B digestion; this indicates that the major immunoreactive component in peak 5 is mature Met-enkephalin.

AtT-20 cells express high levels of proopiomelanocortin (POMC) and process it to β-lipotropin and ACTH by proteolytic cleavage at paired Lys-Arg residues. In addition, AtT-20 cells transformed with the human proinsulin cDNA (*2*) or the human proenkephalin gene (*7*) cleave these precursor proteins at paired, basic amino acid sequences to release mature insulin and Met-enkephalin, respectively. The size distribution of Met-enkephalin immunoreactivity (Fig. 2C) is in agreement with the size distribution of Met-enkephalin IR in AtT-20 cells stably transformed with the human preproenkephalin gene. This suggests that the VV infection does not alter the cellular protein maturation pathway.

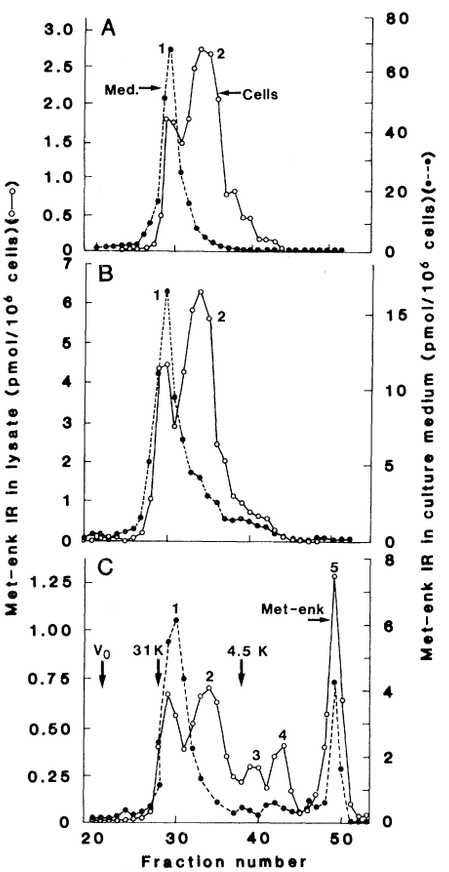

Fig. 2. Gel filtration chromatography of VV:hPE infected cell lines. An equivalent amount (by cell number) of each cell lysate and culture-medium sample (Table 1) were lyophylized and resuspended in 100 μl of 0.25*M* triethylammonium-formate (TEAF), *p*H 3.0. Samples were sonicated, applied to a Bio Sil TSK-125 HPLC gel filtration column, and then were eluted in TEAF buffer at a flow rate of 0.5 ml/min. Each fraction was lyophylized and assayed for Met-enkephalin IR as described in Table 1. (A) BSC-40 cells (B) GH4C1 cells (C) AtT-20 cells. (○), Cellular Met-enkephalin IR; (●)secreted Met-enkephalin IR.

The coelution of the cellular and secreted 28-kD protein in cultures of each cell type infected with VV:hPE indicates that this protein may be mature human proenkephalin. The secreted form from BSC-40 cells was, therefore, purified to homogeneity and the amino terminal sequence was determined for the first 20 amino acid residues (Fig. 3). The sequence corresponds exactly to that of amino acid residues 25 to 44 of human preproenkephalin as determined by cDNA sequence analysis. Thus, the infected cells are able to accurately cleave the signal peptide terminating at Ala^{24}. Furthermore, the purified hPE reacts with an antiserum specific for the carboxyl terminal heptapeptide sequence of proenkephalin, demonstrating that this protein also possesses the correct carboxyl terminus of hPE (*20*). The large amount of secreted proenkephalin from BSC-40 cells (1 μg hPE per 10^6 cells after 24 hours of infection) demonstrates that recombinant VV can be readily used as a mammalian cell expression vector. This method has recently been used for the production of human factor IX (*21*) and mouse POMC (*22*). The recombinant hPE produced in these experiments can be used as a substrate for in vitro processing experiments and structural determinations. Indeed, preliminary results demonstrate that a partially purified enkephalin-generating endopeptidase isolated from bovine adrenal medulla (*23*) can accurately cleave the recombinant hPE produced in these experiments to small ECP's (*24*).

It will be important to determine whether proenkephalin and Met-enkephalin peptides are secreted through the same or different pathways in AtT-20 cells (Fig. 2C). If there is a single pathway for secretion, the data indicate that the level of proenkephalin entering the pathway exceeds the capacity of the proteolytic enzymes, resulting in the secretion of both proenkephalin and Met-enkephalin. However, the absence of detectable levels of other enkephalin-processing intermediates in the culture medium (Fig. 2C, peaks 2 to 4) argues against their cosecretion through a single pathway. It is likely that the precursor is sorted through two distinct secretory pathways. Movement of the precursor through one pathway results in the cleavage of proenkephalin to small Met-enkephalin peptides. The secretion of intact hPE suggests that movement through the other pathway does not involve contact

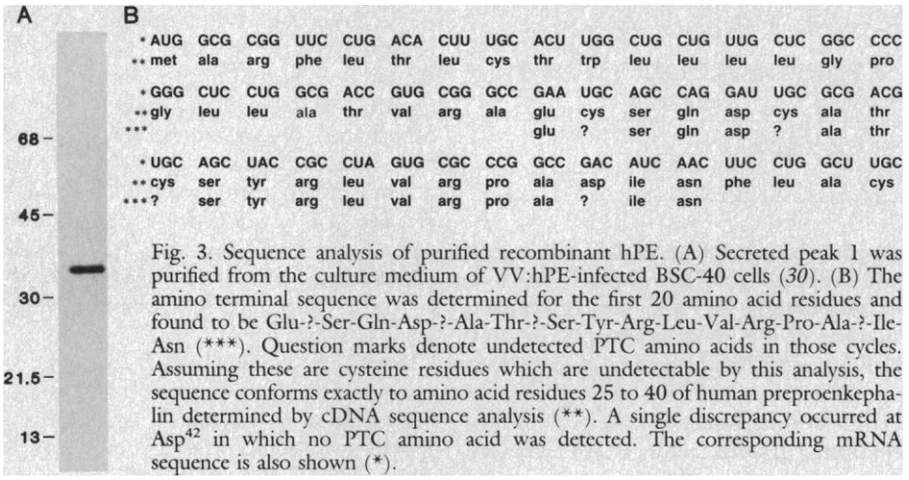

Fig. 3. Sequence analysis of purified recombinant hPE. (A) Secreted peak 1 was purified from the culture medium of VV:hPE-infected BSC-40 cells (*30*). (B) The amino terminal sequence was determined for the first 20 amino acid residues and found to be Glu-?-Ser-Gln-Asp-?-Ala-Thr-?-Ser-Tyr-Arg-Leu-Val-Arg-Pro-Ala-?-Ile-Asn (***). Question marks denote undetected PTC amino acids in those cycles. Assuming these are cysteine residues which are undetectable by this analysis, the sequence conforms exactly to amino acid residues 25 to 40 of human preproenkephalin determined by cDNA sequence analysis (**). A single discrepancy occurred at Asp^{42} in which no PTC amino acid was detected. The corresponding mRNA sequence is also shown (*).

with endoproteolytic enzymes. It is not known whether these two secretory pathways are identical to the constitutive and regulated pathways previously described in AtT-20 cells (*25*).

Thus, we have demonstrated that, in addition to its established use as a live vaccine (*26, 27*), recombinant VV can also be used as a transient expression vector for both studying the posttranslational maturation of complex precursor proteins and for producing large quantities of a foreign gene product.

Elucidation of the proenkephalin processing pathway has been hampered by the lack of a cultured cell line that produces high levels of proenkephalin-derived peptides. However, AtT-20 cells infected with VV:hPE contain several prominent processing intermediates of human proenkephalin (Fig. 2C). Purification and identification of the enkephalin-containing intermediates from established cell lines or primary cell cultures infected with VV:hPE will enable the tissue-specific proteolytic maturation pathway of human proenkephalin to be determined.

Sequence analysis of the proenkephalin secreted from BSC-40 cells demonstrates that the preproenkephalin signal peptide is accurately cleaved after Ala^{24} in BSC-40 cells to yield the mature precursor. The relative ease of recombinant VV construction, together with the efficient synthesis and secretion of mature hPE in BSC-40 cells, illustrates the usefulness of this system as a mammalian cell expression vector. The development of efficient mammalian expression vectors will become increasingly important as the need for proteins possessing correctly modified side groups (*21*) increases.

Table 1. Production of Met-enkephalin IR by different cell types. All cell lines were maintained in Eagle's minimum essential medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories), 2 m*M* L-glutamine and 10 μg of gentamicin sulfate per milliliter. Parallel cultures of BSC-40, Ltk^-, GH4C1, and AtT-20 cells were grown in 28-cm^2 plates and were either mock-infected (MI), or infected with VV:WT or VV:hPE for 24 hours. After infection, the cells were resuspended in 1*M* acetic acid, *p*H 1.9, heated to 100°C for 15 minutes, and sonicated. Culture media were immediately placed at −70°C. A proportionate amount (based on cell number) of each cell lysate was lyophylized and resuspended in 400 μl of 50 m*M* tris, *p*H 8.0, and 2 m*M* $CaCl_2$ containing trypsin TPCK (Cooper Biomedical, 20 μg/ml). Culture media were digested with an equal volume of the trypsin solution. After trypsin-digestion, samples were treated with carboxypeptidase B (Sigma, 2 μg/ml), heated to 100°C for 15 minutes, and assayed for Met-enkephalin IR with a Met-enkephalin–specific antiserum. Optimal virus titer and time of infection for each cell line were previously determined. BSC-40 cells were infected with 0.5 plaque-forming units (PFU) per cell; all other cell types were infected with 5 PFU per cell.

Infecting agent	Met-enkephalin immunoreactivity per 10^6 cells (pmol)			
	BSC-40	AtT-20	GH4C1	Ltk^-
MI	—	—	—	—
VV:WT	—	0.1	—	—
VV:hPE				
Cell	19.4	10.1	43.6	7
Medium	180	36	79	43

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29. The pHR5 contains the 918-bp Hinc II fragment of the human preproenkephalin cDNA flanked with Bam HI linkers and ligated into pUC13.
30. Parallel plates of BSC-40 cells were infected with 0.5 PFU per cell of VV:hPE for 24 hours. Fifty milliliters of the culture medium were diluted with 0.01% Triton X-100 and passed over an Amicon filtration membrane (YM-10) to lower the ionic strength to 0.03. The retentate was then adjusted to 25 m*M* histidine, *p*H 6.2, and applied to a PBE 94 chromatofocusing resin (Pharmacia). The sample was eluted with Polybuffer 74, *p*H 3.5. A single peak of Met-enkephalin IR eluted at *p*H 4.75. The fractions with Met-enkephalin IR were pooled and applied to an HPLC reversed phase column (Vydac, C4 resin 300A). The column was resolved with 95:5 acetonitrile and H_2O in 0.1% trifluoracetic acid. A single peak of Met-enkephalin IR eluted at 31% acetonitrile. The Met-enkephalin IR–containing fractions were diluted with five volumes of 0.2% heptafluorobutyric acid (HFBA) and reapplied to the same column. The column was resolved with 95:5 acetonitrile and H_2O in 0.2% HFBA. A single peak of Met-enkephalin IR eluted at 46% acetonitrile. The fractions containing the Met-enkephalin IR were next separated on a 12.5% polyacrylamide–sodium dodecyl sulfate gel and stained with silver (*28*) to determine purity. A single band of 35 kD was detected (Fig. 3A).

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