

## RESEARCH ARTICLE

# Generation of expression constructs that secrete bioactive $\alpha$ MSH and their use in the treatment of experimental autoimmune encephalomyelitis

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$\alpha$  Melanocyte-stimulating hormone ( $\alpha$ MSH) is a 13 amino acid peptide with potent anti-inflammatory effects. We created two DNA expression constructs (miniPOMC and pACTH1–17) that encode bioactive versions of the  $\alpha$ MSH peptide, and tested these constructs for therapeutic effects in experimental autoimmune encephalomyelitis (EAE). Each construct contained the sequences for  $\alpha$ MSH, as well as the sequences that are involved in the secretion and processing of the POMC gene with the assumption that these sequences would promote processing and release of the encoded  $\alpha$ MSH peptide. The differences between the two constructs lie at the C-terminal end where amino acids

necessary for amidation of  $\alpha$ MSH were included in only the pACTH1–17 construct. These two constructs were tested *in vitro* in bioassays, and *in vivo* in a mouse model of EAE. The results show that although bioactive peptides are secreted from cells transfected with either construct, there appears to be a significant therapeutic effect only with the pACTH1–17 construct which contains the extra C-terminal amino acids. The data suggest that it is possible to engineer DNA expression vectors encoding small secreted peptides such as  $\alpha$ MSH, and that similar type constructs may be useful as therapeutics for the treatment of inflammatory diseases. Gene Therapy (2003) 10, 348–355. doi:10.1038/sj.gt.3301902

**Keywords:**  $\alpha$ MSH; inflammation; gene therapy; NF- $\kappa$ B; EAE

## Introduction

$\alpha$ -Melanocyte-stimulating hormone ( $\alpha$ MSH) is a 13 amino acid peptide derived from pro-opiomelanocortin (POMC). POMC is a pro-hormone that is post-translationally processed at paired basic residues to yield several biologically active peptide hormones, including adrenocortico-tropin (ACTH),  $\alpha$ MSH,  $\beta$ -endorphin and  $\gamma$ MSH.<sup>1–3</sup> The process that results in the generation of  $\alpha$ MSH begins when POMC is directed to the lumen of the endoplasmic reticulum (ER) by the POMC signal peptide (amino acid residues 1–26). The sorting peptide (amino acid residues 27–52) subsequently directs POMC to secretion granules where it is further processed by pro-hormone convertase 1 (PC1) into the 39 amino acid peptide, ACTH (Figure 1). After the initial cleavage of POMC that creates ACTH, the first 17 amino acids of the ACTH peptide are then liberated by pro-hormone convertase 2 (PC2) to form the backbone of what will become native  $\alpha$ MSH (Figure 1).<sup>2,4–6</sup> The final steps in the production of native  $\alpha$ MSH involve cleavage of ACTH1–17 to ACTH1–13, followed by amidation of the carboxyl terminus and acetylation of the amino terminus. The amidation modification requires a signal, located at amino acids 14–16 of ACTH (Gly, Lys, Lys; Figure 1). Like most other hormones and neuropeptides, the subsequent release of  $\alpha$ MSH from the cell occurs in

response to certain stimuli, although there is some level of constitutive secretion.<sup>2,7</sup>

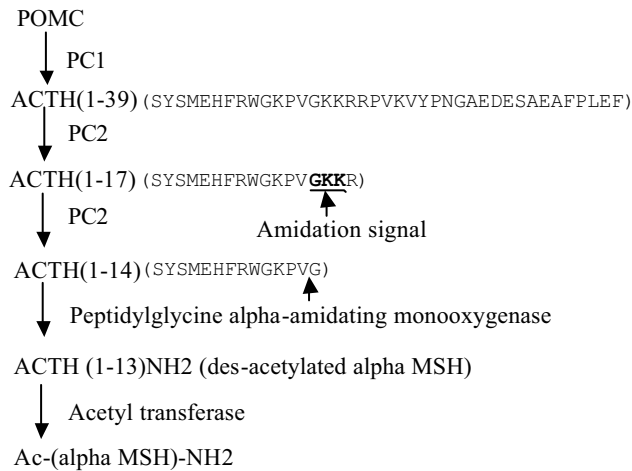
Originally, it was thought that  $\alpha$ MSH was produced primarily in the pituitary gland. More recently, the peptide has been found in several other regions of the brain and various peripheral organs including the skin.<sup>8,9</sup> In fact, many cells are now known to produce  $\alpha$ MSH, including keratinocytes, melanocytes, Langerhans cells, monocytes, macrophages, endothelial cells, fibroblasts and mast cells.<sup>8,9</sup>  $\alpha$ MSH has several known activities including the ability to stimulate melanogenesis, regulate body weight, and inhibit inflammation.<sup>10–15</sup> These effects are mediated by cell surface, G-protein-coupled, melanocortin receptors (MC-R).<sup>16–19</sup>  $\alpha$ MSH binding to MC-R1 and MC-R5 activates adenylyl cyclase and results in a significant increase of intracellular cAMP.<sup>20,21</sup> In melanocytes this event up-regulates expression of tyrosinase, an enzyme that converts tyrosine to melanin.<sup>22,23</sup> The anti-inflammatory activity of  $\alpha$ MSH is due in part to inhibition of I $\kappa$ B degradation,<sup>24,25</sup> an effect that prevents the transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B) from entering the nucleus. NF- $\kappa$ B is required for the production of several pro-inflammatory factors, such as IL-1 and TNF- $\alpha$ <sup>11,12,14,26–28</sup>, and has been shown to have a role in the pathogenesis of many inflammatory/autoimmune diseases.<sup>25</sup>

The ability of  $\alpha$ MSH to inhibit NF- $\kappa$ B translocation makes it an interesting target for therapeutic intervention. In fact,  $\alpha$ MSH and its analogs have shown therapeutic activity in many animal disease models<sup>11–14</sup> such as inflammatory bowel disease,<sup>26</sup> arthritis<sup>29</sup> and hepatitis.<sup>27</sup> However, the inherent instability of the

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**Figure 1** Schematic diagram of POMC processing. ACTH 1–39 is generated from POMC by PC1 cleavage. It is further cleaved at basic residues to yield ACTH1–17 and then to ACTH 1–14 by PC2. Peptidylglycine alpha-amidating monooxygenase converts ACTH 1–14 to  $\alpha$ MSH by removing Gly14 and amidating the C-terminus. Finally, the peptide is acetylated at the amino terminus to produce native  $\alpha$ MSH.

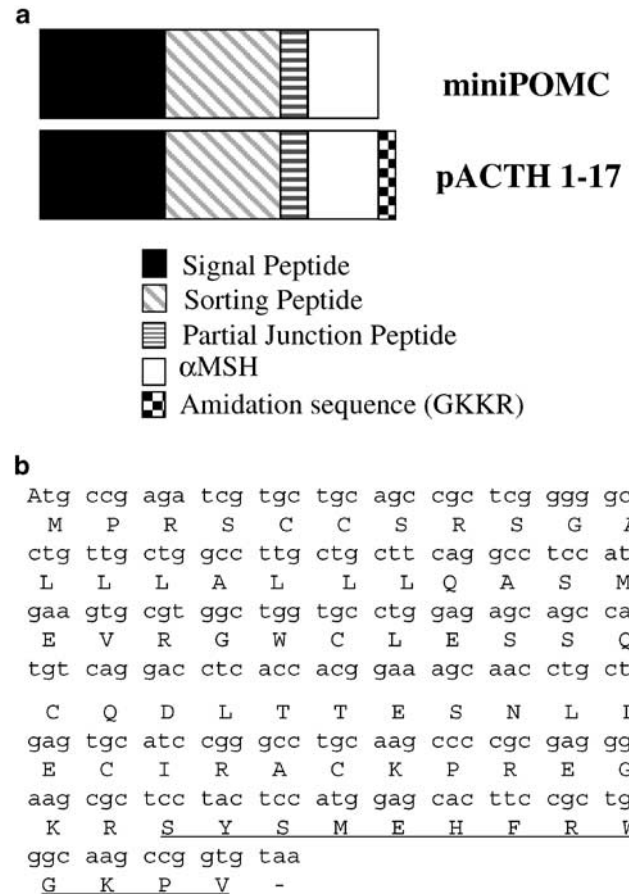
peptide does not make it suitable for use as a therapeutic agent in humans.<sup>30</sup>

In an effort to recognize the potential of  $\alpha$ MSH as a drug for the treatment of disease, we have applied a DNA approach and have generated two DNA expression constructs that secrete bioactive peptides with  $\alpha$ MSH activity. The POMC cDNA was not utilized to produce  $\alpha$ MSH, since this would have resulted in the production of other bioactive peptides such as ACTH. Rather, sequences from the POMC gene involved in the secretion, processing, and post-translational modification of the pre-pro-hormone were included in two constructs (miniPOMC and pACTH1–17) in order to promote production solely of biologically active  $\alpha$ MSH.<sup>28,31</sup> Transfection of cells with either of the constructs resulted in the production of secreted peptide with  $\alpha$ MSH activities that could be detected by a variety of assays. When the constructs were tested in mice with EAE, there were observable changes in the kinetics and severity of the disease that reinforced the observation that these constructs produced bioactive peptides. These data also support the utility of a DNA-based approach for the therapeutic use of  $\alpha$ MSH in inflammatory disease.

## Results

### Generation of $\alpha$ MSH expression constructs

The goal for the first part of these studies was to create DNA expression constructs that produce a secreted peptide with  $\alpha$ MSH bioactivity. Creating a construct to secrete a single small peptide in the absence of a pro-peptide backbone is inherently difficult for reasons of RNA stability as well as the lack of normal post-translational processing and intra-cellular trafficking signals. Inclusion of the entire pro-peptide POMC coding sequence was not a viable solution as this would result in the unwanted production of additional neuropeptides. In an effort to optimize expression and secretion of peptides with  $\alpha$ MSH activity, a DNA construct was generated that

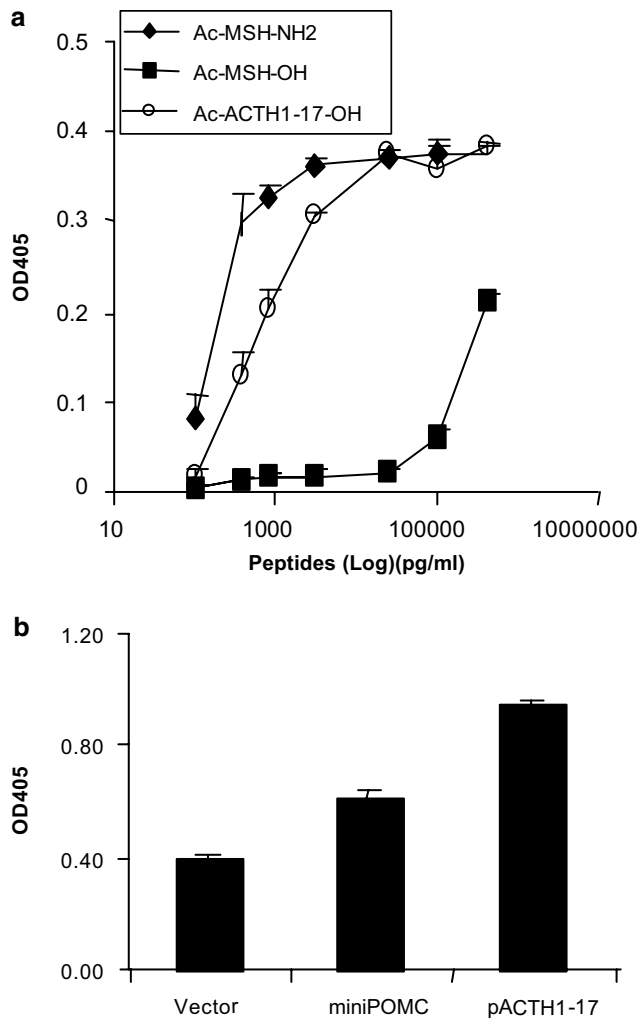


**Figure 2** The  $\alpha$ MSH expression constructs. (a) Schematic diagram of various  $\alpha$ MSH expression constructs. Segments of the encoded protein are as indicated. (b) The nucleotide and amino acid sequences of the miniPOMC coding sequences including the signal peptide, sorting peptide, partial junction peptide and  $\alpha$ MSH sequences (underlined). For the extended  $\alpha$ MSH expression construct, pACTH1–17, the sequence encoding GlyLysLysArg was added before the stop codon.

contains elements of the native POMC responsible for intra-cellular trafficking and processing. These include a signal sequence, sorting sequence, partial junctional peptide and sequences that encode  $\alpha$ MSH. This basic construct was named miniPOMC (Figure 2). The ACTH1–17 construct includes all of the miniPOMC sequences and an additional three amino acid residues that are essential for the amidation of ACTH1–13 and the production of native  $\alpha$ MSH (Figure 1).

### Bioactive $\alpha$ MSH is secreted by cells transfected with $\alpha$ MSH encoding constructs

One activity associated with  $\alpha$ MSH is the induction of melanin synthesis by melanocytes, and we have used an established assay to measure this with the B16/F10 melanoma cell line. When B16/F10 cells are cultured in the presence of increasing concentrations of native synthetic  $\alpha$ MSH peptide, the amount of melanin production is increased due to a darkening of the cell supernatant which is detected by absorbance reading at 405 nm (Figure 3a). Non-amidated forms of the synthetic  $\alpha$ MSH peptide are not as effective as the amidated version in promoting melanin production, although



**Figure 3** Activities of synthetic  $\alpha$ MSH and ACTH peptides or the peptides generated from  $\alpha$ MSH expression vectors in the melanin assay. (a) Synthetic peptides. The x-axis indicates the concentration (pg/ml) of synthetic peptides used in the melanin assay. The y-axis represents absorbance reading at 405 nm. Synthetic peptides are indicated as follows:  $\blacklozenge$ , native  $\alpha$ MSH;  $\blacksquare$ ,  $\alpha$ MSH acetylated, non-amidated;  $\circ$ , ACTH1-17 acetylated non-amidated. Plotted values are the mean of triplicate readings and error bars represent standard deviations. The data are representative of multiple experiments. (b) Peptides generated from  $\alpha$ MSH expression vectors are bioactive. The x-axis represents supernatants from the cells transfected with empty vector (vector) or the  $\alpha$ MSH constructs as indicated. The y-axis represents the  $OD_{405}$  reading obtained from untransfected cells stimulated by the indicated supernatants. Experiments were repeated three times. Plotted values are the average readings from one of the experiments with duplicated samples and error bars represent standard deviations.

inclusion of the amino acid amidation signal sequence in a synthetic peptide appears to boost activity (Figure 3a). A melanin assay was also performed to determine if the B16/F10 cells transfected with either of the two  $\alpha$ MSH DNA constructs produced peptides with  $\alpha$ MSH bioactivity. Supernatants from transfected cells were added to untransfected B16/F10 cells and melanin production was measured. The supernatants from cells transfected with miniPOMC or pACTH1-17 expression constructs promoted significant melanin production in untransfected cells as compared to the supernatants from cells transfected with vector alone (Figure 3b). Interestingly,

the supernatants from cells transfected with the pACTH1-17 construct were more active than those from cells transfected with the miniPOMC construct.

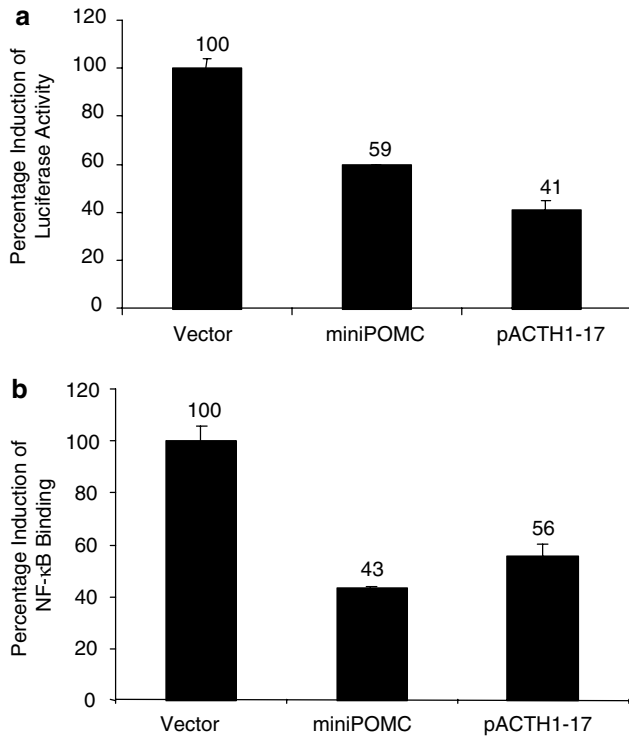
#### Products generated from $\alpha$ MSH expression constructs can inhibit NF- $\kappa$ B activation

NF- $\kappa$ B is a major transcription factor that up-regulates expression of several pro-inflammatory factors, such as TNF- $\alpha$  and interferon- $\gamma$ .  $\alpha$ MSH has been shown to inhibit the translocation of NF- $\kappa$ B to the nucleus to result in decreased expression of pro-inflammatory cytokines.<sup>24</sup> Two separate assays were performed to test whether the peptides generated from the  $\alpha$ MSH expression constructs were able to inhibit NF- $\kappa$ B translocation. In the first assay, vector control or either of the two  $\alpha$ MSH expression constructs were co-transfected into RAW cells together with an NF- $\kappa$ B-dependent luciferase reporter construct. RAW, rather than B16/F10 cells, were used because NF- $\kappa$ B is not effectively and reproducibly stimulated by LPS or TNF- $\alpha$  in melanocytes (data not shown). Following activation of NF- $\kappa$ B by LPS treatment of transfected RAW cells, cell lysates were prepared and assayed for luciferase expression. Luciferase expression is reported as the fold increase in luciferase activity in LPS treated *versus* untreated cells, as measured for cells transfected with each DNA construct. The fold induction of NF- $\kappa$ B in the RAW cells transfected with the empty vector control was set at 100 for purposes of comparison. Co-transfection of cells with the reporter construct and either of the  $\alpha$ MSH expression constructs resulted in a significant reduction in luciferase activity relative to cells transfected with vector control (Figure 4a). These data provide further evidence that both of the  $\alpha$ MSH expression constructs produce peptides with  $\alpha$ MSH activity.

The second assay used to detect a reduction in NF- $\kappa$ B activity was the Trans-AM™ NF- $\kappa$ B ELISA. This assay measures binding of NF- $\kappa$ B from cell lysates to an immobilized oligonucleotide containing the NF- $\kappa$ B binding site. RAW cells, transfected and activated as above, were lysed and the amount of bound NF- $\kappa$ B activity measured from cells transfected with vector control was set to 100 for purposes of comparison. Co-transfection of cells with the reporter construct and either of the  $\alpha$ MSH expression constructs resulted in a significant reduction in NF- $\kappa$ B binding relative to cells transfected with vector. The results of these two assays demonstrate that peptides generated from both  $\alpha$ MSH expression constructs are able to inhibit NF- $\kappa$ B activation.

#### Intra-muscular injection of the constructs leads to delayed onset and reduced severity of EAE

Groups of 10 PLJ  $\times$  SJ/1 F1 mice immunized with guinea pig myelin basic protein (gpMBP) to induce experimental autoimmune encephalomyelitis (EAE) were injected with 100  $\mu$ g of either vector, miniPOMC, or pACTH1-17. The plasmids were injected intra-muscularly into the tibialis, once per week, for 4 weeks on days 0, 7, 14, and 21. The EAE induced in these experiments is a relapsing-remitting form that results in a relatively severe form of the disease, and disease in the mice was scored daily starting on day 8 through day 28 (7 days after final DNA injection). The results show that both the miniPOMC and pACTH1-17 constructs did reduce the mean clinical

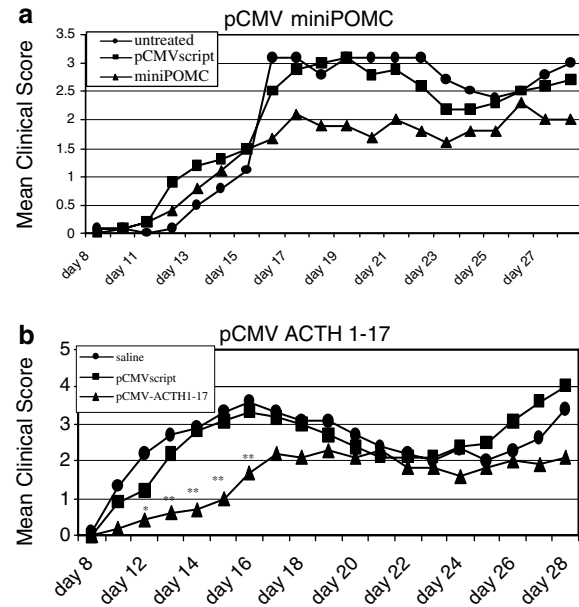


**Figure 4** Inhibition of NF- $\kappa$ B activation by  $\alpha$ MSH generated from  $\alpha$ MSH expression constructs. Lysates from RAW cells transfected with  $\alpha$ MSH expression constructs were tested for their ability to inhibit NF- $\kappa$ B activation. The x-axis represents cell lysates transfected with empty vector, miniPOMC or the pACTH1-17 expression construct as indicated. The y-axis in (a) demonstrates the percentage fold induction of luciferase activity in cells transfected with expression constructs relative to the activity in the cells transfected with empty vector. The y-axis in (b) represents the percentage fold induction of NF- $\kappa$ B binding in cells transfected with the miniPOMC or the pACTH1-17 expression constructs relative to that in cells transfected with empty vector.

score of the disease. The data shown in Figure 5a are representative of three separate experiments performed with miniPOMC and they show a slight, but statistically insignificant reduction of clinical scores from day 16 to day 28. The lower clinical scores observed in these experiments are likely due to a reduction in mortality observed in mice treated with miniPOMC (Table 1). The data shown in Figure 5b are representative of one of five separate experiments performed with pACTH1-17, and they indicate statistically significant reductions in the clinical scores of treated mice on days 12–16. This early reduction in clinical score was due to the fact that the disease onset was delayed in these mice. As shown in Table 1, the onset of disease was significantly delayed in five experiments by the treatment of mice with the pACTH1-17 plasmid. The treated mice also show a reduced mortality relative to mice injected with saline or empty vector.

## Discussion

The experiments described herein were performed with an intent to develop DNA expression constructs that permit secretion of peptides with  $\alpha$ MSH bioactivity and then to test them in an animal model of autoimmunity.



**Figure 5** Effect of  $\alpha$ MSH plasmids on a course of EAE. Mice (10/group) were treated with saline, pCMV-Script (empty vector), or  $\alpha$ MSH plasmids on days 0, 7, 14, and 21. Each data point represents the mean clinical score of the ten mice in each group (y-axis) as measured on the indicated day (x-axis). (a) Effect of treatment with the miniPOMC plasmid. (b) Effect of treatment with the pACTH1-17 plasmid. P values were generated by ANOVA (\*= $P < 0.05$ , \*\*= $P < 0.01$ ).

The data demonstrate that inclusion of the minimal endogenous POMC trafficking signals in frame with sequences that encode ACTH1-13 or ACTH1-17 can direct expression and secretion of peptides with  $\alpha$ MSH activity. The peptides are expressed by melanocytes (B16/F10) and macrophages (RAW) *in vitro* and have the expected melanin stimulating activity and effect on NF- $\kappa$ B activation. Furthermore, the supernatant of cells transfected with expression vectors that encode the minimal trafficking signals in frame with ACTH1-17 demonstrated enhancement of both these activities.

The front end of the miniPOMC sequence, including the signal, sorting and junctional peptides, may be cleaved from the encoded peptides to liberate peptides including, but not necessarily limited to, the ACTH1-13 sequence. The signal sequence, which is expected to direct all versions of the miniPOMC peptides to the ER is likely cleaved to liberate a peptide that includes the sorting signal, junctional peptide and ACTH1-13 sequences. The sorting signal, which directs the peptide to the secretory vesicles, and the junctional peptide should be removed in the vesicles to liberate ACTH1-13. The fact that muscle cells produce a bioactive peptide when transfected with the miniPOMC vectors (data not shown) indicates that non-secretory cells either have the capacity to perform processing of the sorting and junctional peptides, or that this event is not necessary for activity.

Signals in the ACTH1-17 peptide, at positions 14–16 (Gly, Lys, Lys), are responsible for the amidation of ACTH1-13. The peptides secreted by the miniPOMC vector are unlikely to be amidated since they do not have this signal. However, the peptide expressed from the pACTH1-17 construct is more likely to be processed and

**Table 1** Effect of  $\alpha$ MSH plasmids on day of disease onset and mortality<sup>a</sup>

	Saline	pCMVscript	miniPOMC	
<i>miniPOMC</i>				
Mean day onset	14.3±3.1	15.9±3.8	15.0±4.1	P=0.246 (ANOVA)
Incidence	30/30 (100%)	28/30 (93%)	29/30 (97%)	
Mortality	15/30 (50%)	11/30 (37%)	4/30 (13%)	
<i>ACTH17</i>				
Mean day onset	13.5±3.2	14.7±3.6	15.7±4.5	P=0.026 (ANOVA)
Incidence	46/48 (96%)	47/48 (98%)	46/48 (96%)	
Mortality	15/48 (31%)	16/48 (33%)	9/48 (19%)	

<sup>a</sup>Mean day onset was calculated by averaging the experimental day the first symptoms appeared in each mouse within the treatment group. Mortality is shown as the number of mice that died by day 28. Incidence is at or near 100% for all groups.

amidated to produce a peptide with a C-terminus identical to native  $\alpha$ MSH. Amidated  $\alpha$ MSH is more active than the non-amidated peptide (Figure 3a). Since the peptide generated from pACTH1-17 seems to have enhanced  $\alpha$ MSH activity in the melanin bioassay and *in vivo* models, this suggests that the expressed peptide may well be amidated. However, we cannot rule out other possibilities for enhanced activity, such as stability differences between peptides generated from miniPOMC and pACTH1-17.

Efforts to pinpoint the exact nature of the peptide secreted by cells expressing these constructs have been unsuccessful. Tested methods include Western blot analysis, HPLC and mass spectrometry. Western blot analysis with synthetic  $\alpha$ MSH required nanogram levels for detection (data not shown), and it is likely that the peptide concentration in supernatants of transfected cells was not high enough for detection by this assay. Concentration of the supernatant by column chromatography and detection by mass spectrometry also proved difficult as the active peptide degraded and oxidized during the purification process.

The anti-inflammatory properties of  $\alpha$ MSH are numerous. The peptide acts through cell surface receptors to reduce production of pro-inflammatory mediators, such as TNF- $\alpha$ , nitric oxide, IL-1 and IL-6.<sup>27,32</sup> Such down-regulation is due to the inhibition of NF- $\kappa$ B activity,<sup>24,25</sup> and this activity suggests that a therapeutic opportunity may exist for  $\alpha$ MSH. The peptide is safe when given in large and consistent doses to animals or humans.<sup>26,27,29</sup> However, it is very unstable *in vivo* and therapeutic use would require daily administration.<sup>26,27,29</sup> Researchers have been developing  $\alpha$ MSH analogs, such as [Cys4, Cys10]- $\alpha$ MSH<sup>33</sup> and [Nle4,D-Phe7]- $\alpha$ MSH<sup>34</sup>, to increase activity and stability relative to native  $\alpha$ MSH. Some of these peptides are currently being tested in clinical trials.<sup>35,36</sup>

A DNA-based therapeutic could reduce the need for daily administration and provide for long-term production of  $\alpha$ MSH. The first step towards this goal was to test these constructs in an animal model of inflammation/autoimmunity. Interestingly, the construct that had the strongest and the most consistent therapeutic effect *in vivo* was pACTH1-17. We are intrigued by the possibility that the enhanced effect of this plasmid may be due to

amidation, but we have not yet been able to detect peptides expressed from these constructs to test this theory. We are currently continuing in these efforts.

In summary, two  $\alpha$ MSH expression constructs have been generated and express peptides with  $\alpha$ MSH activities in cell lines following transfection with plasmid DNA. The encoded peptides inhibit NF- $\kappa$ B activation, a transcription factor essential for the production of inflammatory cytokines. One of the constructs, pACTH1-17, was shown to decrease disease severity in a mouse model of autoimmunity. Collectively, these data support the notion that DNA expression vectors can be created to direct the expression and secretion of small peptides and that their activities can have therapeutic effects. It will be interesting to try this approach for other peptides with inherent stability issues. One possible candidate is vasoactive intestinal peptide (VIP), a peptide recently shown to inhibit symptoms in an animal model of rheumatoid arthritis.<sup>37</sup>

## Materials and methods

### Cell culture

B16/F10 mouse melanocyte cells and RAW 267. 4, a mouse macrophage cell line, were obtained from ATCC (ATCC, Manassas, VA, USA). The cells were grown at 37°C in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS) in a humidified incubator at 5% CO<sub>2</sub>. Cells were split every other day.

### Construction of $\alpha$ MSH expression vectors

All oligonucleotides were purchased from Operon (Alameda, CA, USA). Complementary oligonucleotides encoding the human POMC signal peptide, sorting peptide, partial junction peptide and  $\alpha$ MSH were created based upon the human POMC cDNA sequence (NM\_000939, Genbank; Figure 2). The restriction enzyme sites, HindIII, BssHIII upstream of the start codon and BamHI, XhoI downstream of the stop codon, were added for cloning purposes. The synthesized oligonucleotides were annealed and cloned into the HindIII and XhoI sites of pCMV-Script (Stratagene, La Jolla, CA, USA), a mammalian expression vector containing a CMV

immediate early promoter and SV40 polyadenylation sequence, to generate miniPOMC.

A pair of oligonucleotides was utilized to generate the extended miniPOMC construct, pACTH1-17. The top strand oligonucleotide contains the HindIII site and sequence corresponding to the 5' end of the miniPOMC sequence. The bottom strand oligonucleotide has complementary sequences to the 3' end of the miniPOMC sequence, which adds a four amino acid extension before the stop codon ACTH1-17 (GGC AAG AAG CGG). The extended miniPOMC insert was generated by PCR using the pair of oligonucleotides indicated above and miniPOMC as a template. The newly synthesized insert was subcloned into pCMV-Script using HindIII and XhoI to generate pACTH1-17. All constructs were sequenced to ensure correctness.

#### Melanin assay

B16/F10 cells were plated into 6-well plates at a concentration of  $1 \times 10^5$  cells/ml, 2 ml per well. Cells were incubated at 37°C overnight. The following day, 3.5  $\mu$ g of the  $\alpha$ MSH expression construct or vector control and 0.5  $\mu$ g of secreted embryonic alkaline phosphatase (SEAP) expression construct as a transfection efficiency control (Gene Therapy Systems, San Diego, CA, USA) were mixed with 15  $\mu$ l lipofectamine (Gibco, Carlsbad, CA, USA) and added to the cells. Mock-transfected samples received lipofectamine and no DNA. After 24 h, transfection reagents were removed and replaced with fresh media. Cells were incubated for two additional days. The supernatants were collected and tested in the SEAP and melanin assays.

The SEAP assay was performed according to the manufacturer's instructions (Gene Therapy Systems, San Diego, CA, USA). Supernatants from transfected cells were heated at 65°C for 30 min to inactivate endogenous alkaline phosphatase activity. The SEAP activity was quantitatively determined by using a colorimetric assay based on hydrolysis of the chromogenic substrate paranitrophenyl phosphate (PNPP; Phospha-light assay system, TROPIX, Foster City, CA, USA). To start the enzymatic reaction, 200  $\mu$ l of the PNPP substrate was added to each sample. The reaction was allowed to stand at room temperature for 30 min prior to analysis. Absorbance at 405 nm of each sample was then determined.

For the melanin assay, B16/F10 cells seeded in 96-well plates at  $2.5 \times 10^4$  cells/ml, 100  $\mu$ l per well were incubated at 37°C overnight. Supernatants containing synthetic peptides or from DNA-transfected cells were then added. The synthetic peptides included acetylated, amidated  $\alpha$ MSH, acetylated, non-amidated  $\alpha$ MSH or acetylated, non-amidated ACTH1-17 (BACHEM, Torrance, CA, USA) at different concentrations. Then, cells were incubated at 37°C for an additional 4 days. Melanin production (which turned the cells and the supernatant dark to black) was measured by absorbance at 405 nm. These numbers were then normalized based upon transfection efficiency using SEAP as a control.

#### Luciferase assay

A total of  $8 \times 10^6$  RAW cells in 400  $\mu$ l DMEM medium were electroporated with pNF- $\kappa$ B-Luc plasmid (Clontech, Palo Alto, CA, USA), an  $\alpha$ MSH expression vector or vector control, and the SEAP expression vector at a ratio

of 1.5  $\mu$ g:1.5  $\mu$ g:0.5  $\mu$ g, respectively. The electroporation was performed with a Bio-Rad gene pulser at a setting of 200 V and 960  $\mu$ F. Cells were incubated for 24 h in phenol-red-free DMEM (Gibco, Carlsbad, CA, USA) after electroporation. Cells were then stimulated with LPS at a concentration of 10  $\mu$ g/ml (Sigma, St. Louis, MO, USA) for 4 h to activate NF- $\kappa$ B. Culture medium was collected and the SEAP assay was performed as described above. Cells were lysed with  $1 \times$  reporter lysis buffer and assayed for luciferase activity according to the manufacturer's directions (Luciferase assay kit; Promega, Madison, WI, USA). For each expression vector, fold induction of luciferase expression was calculated as the ratio of SEAP normalized luciferase activity from LPS-treated cells to SEAP normalized luciferase activity from untreated cells. Then, fold induction of luciferase expression in the cells transfected with empty vector was set as 100%. By comparing with this value, the percentage fold induction in the cells transfected with expression constructs were calculated accordingly.

#### Trans-AM NF- $\kappa$ B ELISA

The protein concentration of cell lysates in 1X reporter lysis buffer was measured by Bradford assay. Equal amounts of protein from lysates were used to determine the binding activity of NF- $\kappa$ B using the Trans-AM NF- $\kappa$ B ELISA kit (Active Motif; Carlsbad, CA, USA). The assay was carried out as described. Briefly, cell lysates were incubated in wells of a 96-well plate that had been coated with oligonucleotides containing the NF $\kappa$ B consensus binding site. The plates were washed, anti-p65 antibody specific for NF $\kappa$ B bound to DNA was added, and the samples were incubated at room temperature for 1 h. The secondary antibody coupled with horse radish peroxidase was then added and the samples were incubated for 1 h at room temperature. Following color development, the samples were quantified by spectrophotometry at OD450. Fold induction of NF $\kappa$ B binding was calculated as the ratio of spectrophotometric reading from LPS-induced cells to that of un-induced cells for each expression vector. Then, fold induction of NF $\kappa$ B binding in the cells transfected with empty vector was set as 100%. By comparing with this value, the percentage fold induction in the cells transfected with expression constructs were calculated accordingly.

#### Induction of EAE

On day 0, female PL/J  $\times$  SJL mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were immunized subcutaneously at the base of the tail with 300  $\mu$ g guinea pig myelin basic protein emulsified in incomplete Freund's adjuvant containing 2 mg/ml *Mycobacterium tuberculosis* H37 RA (all reagents were from Sigma, St Louis, MO, USA). On days 0, 2 and 7 400 ng of Pertussis Toxin (List Biologicals, Campbell, CA) was administered intraperitoneally. Groups consisted of 10 mice per group and disease was scored daily beginning on day 8 using the following standard scale:<sup>38</sup>

- 0 normal mouse, no overt signs of disease;
- 1 limp tail or hind limb weakness, but not both;
- 2 limp tail and hind leg weakness;
- 3 partial hind leg paralysis;

- 4 complete hind limb paralysis;
- 5 moribund state.

#### Treatment with $\alpha$ MSH producing plasmid

Plasmid DNA, 100  $\mu$ g, containing either vector alone (pCMV-Script) or vector containing the miniPOMC or pACTH1–17 constructs were injected intra-muscularly on days 0, 7, 14 and 21. The total volume injected was 100  $\mu$ l. The muscle used was the tibialis (50  $\mu$ l/ muscle). Experiments were performed under a protocol approved by the Animal Care and Use Committee.

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