Identification of proopiomelanocortin neurones in rat hypothalamus by *in situ* cDNA-mRNA hybridization

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Ardrenocorticotropic hormone (ACTH), β-endorphin and the melanotropins (MSHs) are all derived from a single large precursor molecule, proopiomelanocortin (POMC)[1–5] by individual processing through a series of co- and post-translational modifications. Although the primary site of synthesis is in the pituitary, POMC-derived peptides have been identified in various tissues, notably the brain (see refs 6, 7 for review). A major question concerning brain POMC is whether it is synthesized within the central nervous system (CNS) itself or whether it is taken up from plasma flowing in a retrograde fashion from the pituitary. POMC peptides have been detected immunohistochemically and biochemically in the medial basal hypothalamus, the amygdala and throughout the brain stem. POMC peptide-containing cell bodies have been identified only in two cell groups, however, principally in the periarcuate region of the hypothalamus and to a lesser extent in the nucleus of the tractus solitarius[8–10]. These and other[11,12] observations have suggested that POMC peptides are synthesized locally in the medial basal hypothalamus and reach other regions of the CNS by axonal transport. Civelli *et al.* identified POMC mRNAs in nucleic acid extracts of rat and bovine hypothalami by solution hybridization as well as Northern gel blot analysis[12], but because of the close proximity of the hypothalamus to the pituitary and the extremely low amounts of POMC mRNA being measured in the hypothalamus, the possibility of tissue contamination during dissection could not be ruled out. We report here the anatomical co-localization of POMC-related peptides and POMC-specific mRNAs to a single major cell group in the medial basal hypothalamus. The presence of POMC-specific mRNA in a POMC peptide-containing cell in the brain is strong support for POMC biosynthesis within brain tissue.

To localize the POMC-specific mRNA in single cells, we used the refined technique of *in situ* cDNA–mRNA hybridization on frozen 10-μm rat brain sections. This technique is conceptually very similar to immunohistochemistry, except that a DNA or RNA probe is used to detect the presence of a given nucleic acid sequence[13–15]. In this study, a cDNA probe is used to identify any nucleic acid sequences complementary to the POMC gene, principally POMC mRNA and heteronuclear RNA (hnRNA) in the cytoplasm and nucleus of cells. Tissues were fixed by perfusing the animal, through the aorta, with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. This fixation method preserves both the peptides and mRNAs in the cytoplasm of cells for subsequent analysis. The fixed brain was partitioned into three blocks by two coronal cuts, one rostral and one caudal to the hypothalamus. The blocks of tissue were placed in 15% sucrose in PBS at 4 °C overnight to rinse out excess fixatives, and then embedded in OCT compound (a water-soluble supporting medium from Tissue Tek) and frozen in liquid nitrogen. Ten-micrometre sections were cut with a cryostat microtome onto gelatin-coated glass microscope slides. The hybridization and autoradiographic procedure was as described previously[15], using a tritium-labelled nick-translated POMC cDNA.

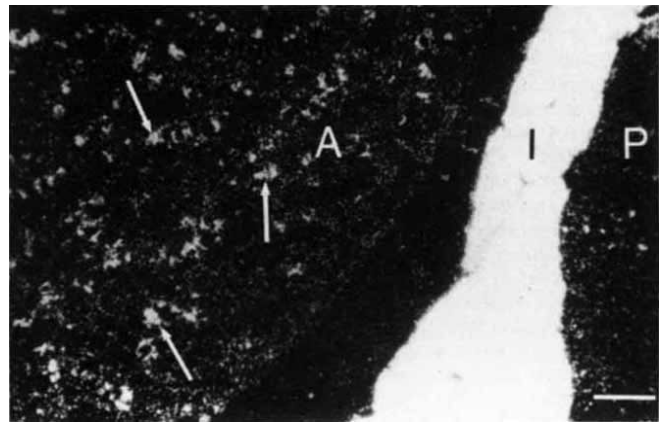

Fig. 1 *In situ* cDNA–mRNA hybridization of POMC-producing cells of the anterior and intermediate lobes of rat pituitary. The anterior lobe (A) corticotrophs can be identified by the dense patches of autoradiographic grains (arrows indicate three of the many such cells). The intermediate-lobe (I) melanotrophs all contain POMC mRNA and can be seen as a very dense streak of white autoradiographic grains. The posterior lobe (P) shows background staining. The I-13 rat POMC cDNA clone was constructed from a poly(A)$^+$ RNA preparation obtained from neurointermediate lobe, by reverse transcription and (dG-dC)n tailing[19,20]. The resulting 550-base pair probe (I-13) was isolated, sequenced and shown to code for the amino acid sequences of POMC from the middle of the NH_2-terminal glycopeptide to the carboxy terminus as well as a segment of the 3′-untranslated region not including the poly(A) tail. The I-13 sequence is identical to that published by Drouin and Goodman[3]. The I-13 insert was restriction-cut from the pBR322 plasmid and made radioactive by nick-translation[21], using all four tritiated triphosphates, to a specific activity of $2–5 \times 10^6$ c.p.m. per μg DNA. After perfusing the rat with 4% neutral buffered formalin, the tissue was removed, kept in 15% sucrose overnight, immersed in OCT mounting medium, frozen and sectioned. Tissue sections were thawed at 25 °C and rinsed in diethyl pyrocarbonate (DEP)-treated water (50 μl DEP per 250 ml water). The radioactive I–13 POMC probe was heat-denatured for 5 min at 100 °C in hybridization buffer[15] and immediately cooled in ice water. $2–5 \times 10^4$ c.p.m. of labelled probe in 5 μl of hybridization buffer was pipetted onto the section and reacted for 18 h at 28 °C. The section was washed in 2×SSC at room temperature for 4–5 h, changing wash every 15 min. The washed sections were then dehydrated through graded alcohols and air-dried. They were then dipped in Ilford L-4 nuclear emulsion (Polysciences, Pennsylvania), liquified 1:2 with water, air-dried for 1 h in the dark and exposed (10 days) in light-proofed boxes, with desiccant. Development was carried out in Kodak D-19 (diluted 1:1 in water) for 2–4 min at room temperature and fixed in Kodak rapid-fixer for 2–3 min, and washed in water for 20 min. Scale bar, 100 μm.

Validation of the *in situ* method and the ^{3}H-POMC cDNA probe is shown for rat pituitary (a tissue known to synthesize POMC) in Fig. 1. Silver grains are localized over the cytoplasm of cells in both the intermediate and anterior lobes of the pituitary, paralleling the distribution previously established by immunohistochemistry for POMC cells. Serial sections staining with an anti-ACTH antibody have confirmed these cells to be POMC peptide-producing cells. There is no labelling of other cells in the tissue section. The signals have been shown to represent specific POMC cDNA–mRNA hybrids[15].

Using identical procedures, coronal brain sections were hybridized with tritium-labelled POMC cDNA. Figure 2 shows the cytoplasmic localization of silver grains in specific cells in the medial basal hypothalamic region. Similar distributions of silver grains were seen in sections rostral and caudal to the section in Fig. 2, paralleling the known distribution of POMC

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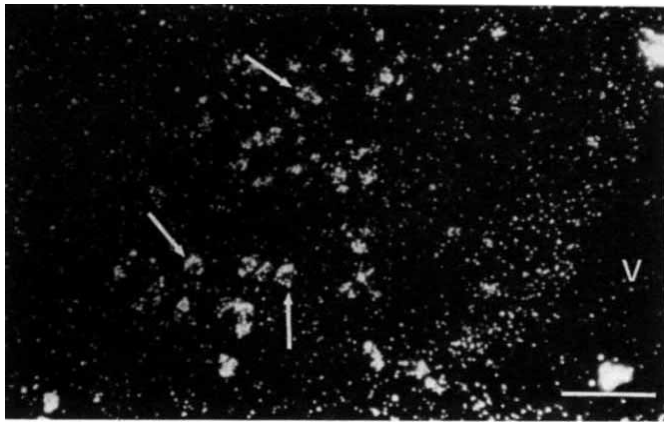

Fig. 2 *In situ* cDNA–mRNA hybridization of the POMC cells in the periarcuate region of medial basal hypothalamus. Autoradiographic exposure was for 5 weeks. Arrows indicate three of the several autoradiographically marked POMC cells. The third ventricle is seen at the right side of the photograph (V). The method is as described in Fig. 1 legend. Scale bar, 100 μm.

neurones in the hypothalamus. There was no similar cytoplasmic localization of POMC mRNA anywhere else in the brain. Treatment of the tissue sections with 25 $\mu g\ ml^{-1}$ pancreatic ribonuclease A at 42 °C for 1 h, before hybridization with POMC cDNA, gave no autoradiographic signal, showing that the positive signal obtained was derived from cDNA–mRNA hybrids (data not shown). When we hybridized brain sections with either nick-translated rat growth hormone cDNA or plasmid pBR322, there was no detectable localization above background in other cells or cells in the periarcuate region of the rat brain (for example, see Fig. 2). These observations show that our method is specifically identifying POMC mRNA in individual hypothalamic cells.

To characterize further the cytoplasmic localization, serial sections on either side of those labelled with ^{3}H-POMC-cDNA probes were stained with anti-ACTH antibody. Figure 3 shows three serial sections of the rat medial basal hypothalamic region, stained with anti-ACTH sera (*a*, *c*) or hybridized *in situ* with a ^{3}H-labelled POMC-specific DNA probe (*b*). Many of the cells in the periarcuate region were labelled with both POMC cDNA and anti-POMC antibodies, thus providing evidence for POMC biosynthesis in brain (that is, localizing POMC mRNAs and its corresponding peptides in the same neurone). A few cells showed labelling for only POMC mRNAs or peptides. In such serial section analysis, some cells stain with antisera alone and others react with cDNA probes alone. Often a cell is present in one 10-μm section but not present in the next. Only data in which the same cell could be clearly identified in two (or more) serial sections were interpreted. In those conditions we can conclude that most (if not all) POMC cells in the arcuate region are marked by both the antisera and the cDNA probe.

We have used the *in situ* hybridization technique to localize a specific mRNA within individual cells in the mammalian brain. Although the presence of a given mRNA in a cell does not necessarily mean that the mRNA is being actively translated, the concomitant presence of its corresponding protein product in the same cell is *prima facie* evidence for the endogenous biosynthesis of that peptide. Thus, the co-localization of the POMC mRNA by *in situ* cDNA–mRNA hybridization and the POMC protein products by immunocytochemistry within the same cells in the arcuate region of the rat hypothalamus provides the strongest evidence so far for the actual site of synthesis of a mammalian neuropeptide.

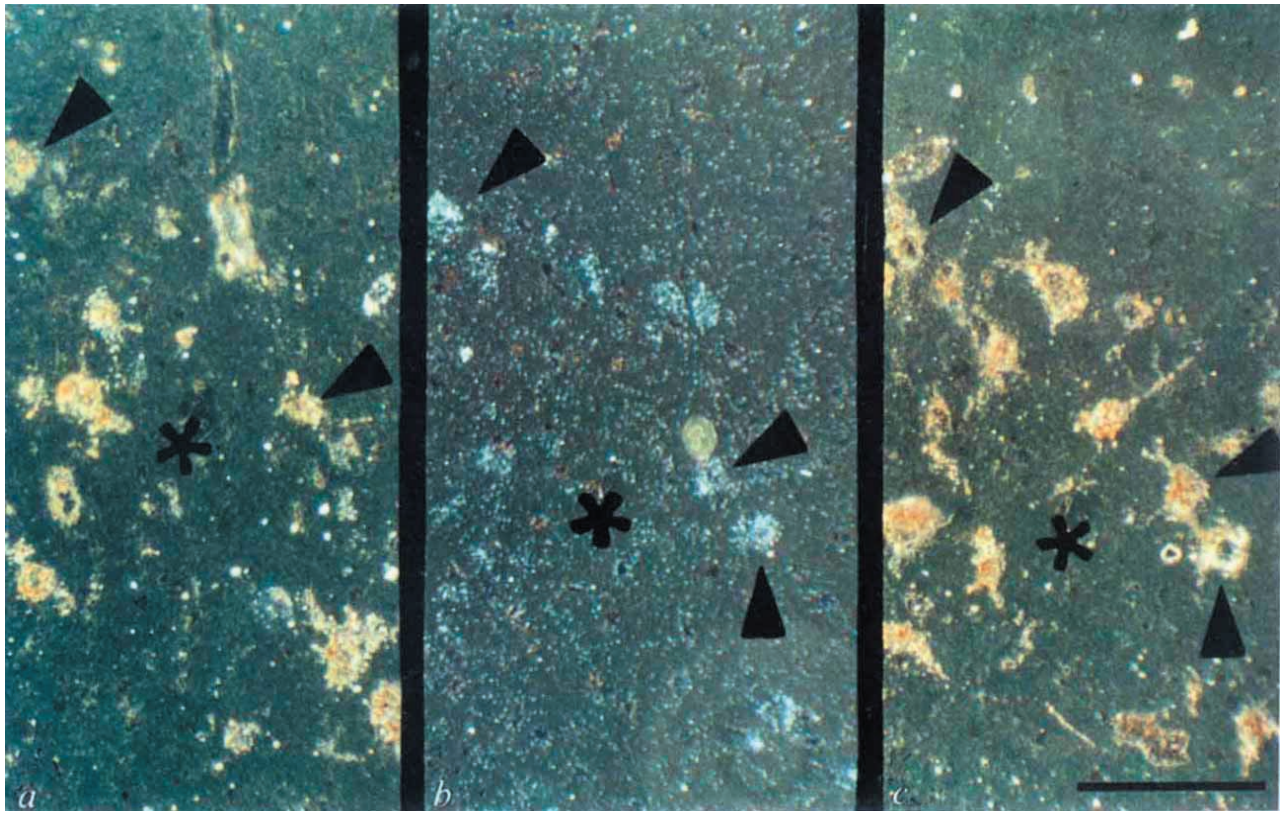

Fig. 3 Co-localization of ACTH immunoreactivity and POMC mRNA in the same cells of rat hypothalamus. *In situ* cDNA–mRNA hybridization of brain POMC neurones (*b*) was carried out as described for Fig. 1 using the POMC-cDNA I-13 probe. In 10-μm serial sections, immunocytochemistry of brain POMC neurones was carried out using the peroxidase–antiperoxidase method and antibody directed against ACTH (*a*, *c*). In unpublished studies this serum has been shown to be blocked by ACTH(1–39) and -(1–24), but not by α-MSH, β-endorphin or other known peptides. Arrows indicate several cells which both stained with anti-ACTH immunoperoxidase reaction and hybridized to the ^{3}H-labelled I-13 probe. Asterisks lie over an artery common to all three sections. Note the tight distribution of autoradiographic grains over the POMC cells. Scale bar, 100 μm.

Civelli *et al.* have found POMC-like mRNA in other regions of the brain[12]. There are several possible reasons why we did not find any *in situ* cell-body localization outside the hypothalamus. One is that the *in situ* cDNA–mRNA hybridization method we used was not sensitive enough to detect a POMC mRNA level much below that found in the hypothalamus. The data reported by Civelli *et al.* indicated that there is 10 times less POMC-like mRNA in the rat amygdala and cerebral cortex relative to the hypothalamus[12]. On the other hand, if the POMC mRNA in the amygdala was present at the same level per cell as that of the hypothalamus, and therefore in fewer cells, there would be a total of only 50–100 POMC cells in the whole amygdala. This small number of cells spread over such a large brain region may not have been detectable by our analysis. Furthermore, immunocytochemical studies using a variety of POMC-cross-reacting antisera have not been able to demonstrate any POMC cell bodies in either the amygdala or cortex, which is in agreement with our *in situ* mRNA studies. An alternative explanation may be that the POMC-like mRNA found outside the hypothalamus is not the authentic POMC mRNA. It may consist of a collection of cross-reacting species of mRNAs present in a large number of cells. If this were the case, the signal would be too dilute to be detectable over background in an *in situ* cDNA–mRNA hybridization experiment.

Since the *in situ* cDNA–mRNA hybridization technique can detect a specific mRNA within a single cell, when optimized, it will be especially useful in the study of the regulation of gene expression in complex systems such as the CNS. For example, there are about 10,000 POMC neurones in the periarcuate region of the hypothalamus, and they may not all respond identically to the same hormone or neurotransmitter modulators. Indeed, only a small percentage of the periarcuate POMC neurones can concentrate oestrogen[16], which is known to modulate the distribution and content of hypothalamic POMC peptides. Experiments measuring rat hypothalamic POMC mRNA levels during various endocrine states including ovariectomy have been done in this laboratory. Total RNAs were extracted from whole hypothalami, covalently bound to nitrocellulose paper and hybridized to POMC-specific cDNA probes. No reproducible variation of more than 20% from the control level was found. This may be explained by the differential regulation of the POMC gene in these POMC neurones. Since only a small percentage, about 10%, of these neurones concentrate oestrogen, that is, have primary responsiveness towards the steroid, then even if oestrogen has a dramatic effect on POMC gene expression in these cells it may be masked by the pool of POMC mRNA contributed by non-responsive neurones. This type of difficulty was encountered by Nakanishi *et al.* when they studied the glucocorticoid regulation of pituitary POMC gene expression[5]. Glucocorticoids suppress strongly the anterior-lobe POMC mRNA level but leave the intermediate-lobe POMC mRNA level apparently unaffected. Fortunately, the two lobes of the pituitary gland can be readily dissected, so that subsequent studies were able to show a more exact pattern of regulation of the expression of the POMC gene[17,18]. However, to dissect out neurones which are regulated differently would be a formidable task. An alternative method would be to look at these neurones individually or in endocrine-responsive subgroups by *in situ* cDNA–mRNA hybridization, so that one may obtain a more accurate account of the hormonal regulation of gene expression.

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1. Roberts, J. L. & Herbert, E. *Proc. natn. Acad. Sci. U.S.A.* **74,** 4826–4830 (1977).
2. Roberts, J. L. & Herbert, E. *Proc. natn. Acad. Sci. U.S.A.* **74,** 5300–5304 (1977).
3. Drouin, J. & Goodman, H. M. *Nature* **288,** 610–613 (1980).
4. Mains, R. E. & Eipper, B. A. *J. biol. Chem.* **254,** 7885–7894 (1979).
5. Nakanishi, S. *et al. Nature* **278,** 423–427 (1979).
6. Akil, H. & Watson, S. *Hbk Psychopharmac.* **16,** 209–253 (1983).
7. Krieger, D. T. & Liotta, A. S. *Science* **205,** 366–372 (1979).
8. Watson, S. H., Barchas, J. D. & Li, C. H. *Proc. natn. Acad. Sci. U.S.A.* **74,** 5155–5158 (1977).
9. Zimmerman, E. A., Liotta, A. & Krieger, D. T. *Cell Tissue Res.* **186,** 393–398 (1978).
10. Schwartzberg, D. G. & Nakane, P. K. *Abstr. 75.5 Mtg Neurosci.* (1981).
11. Liotta, A. S., Gildersleeve, D., Browstein, M. J. & Krieger, D. T. *Proc. natn. Acad. Sci. U.S.A.* **76,** 1448–1452 (1979).
12. Civelli, O., Birnberg, N. & Herbert, E. *J. biol. Chem.* **257,** 6783–6787 (1982).
13. Brahic, M. & Haase, A. T. *Proc. natn. Acad. Sci. U.S.A.* **75,** 6125–6129 (1978).
14. Hudson, P. *et al. Endocrinology,* **108,** 353–356 (1981).
15. Gee, C. E. & Roberts, J. L. *DNA* **2,** 155–161 (1983).
16. Morrell, J. & Pfaff, D. *J. Histochem. Cytochem.* Abstr. 1A (1983).
17. Schachter, B. S., Johnson, L. K., Baxter, J. D. & Roberts, J. L. *Endocrinology* **110,** 1442–1444 (1982).
18. Herbert, E., Birnberg, N., Lisitsky, J. C., Civelli, O. & Uhler, M. *Neurosci. Newslett.* **12,** 16–27 (1981).
19. Payvar, F. & Schimke, R. T. *J. biol. Chem.* **254,** 7636–7642 (1979).
20. Chang, A. C. Y. *et al. Nature* **275,** 617–624 (1978).
21. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. *J. molec. Biol.* **113,** 237–251 (1977).

Recognition of myelin-associated glycoprotein by the monoclonal antibody HNK-1

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Myelin-associated glycoprotein (MAG) is a quantitatively minor component in both peripheral and central myelin sheaths that is thought to have a role in cell–cell interactions within the nervous system[1]. We show here that a mouse monoclonal antibody, HNK-1, which is directed against human natural killer cells also recognizes an antigenic determinant of human central and peripheral nervous system white matter by immunoperoxidase staining of tissue sections. Immunoblot analysis of myelin proteins and purified extracted MAG indicates that the antigen recognized is MAG.

HNK-1 (Leu 7) is a monoclonal mouse IgM antibody which was produced against a membrane antigen from the cultured T-cell line, HSB-2. HNK-1 recognizes a differentiation antigen present on approximately 15% of normal peripheral blood lymphocytes[2,3]. These cells are principally large granular lymphocytes and have been shown to contain most of the natural killer cell activity of the peripheral blood[2].

Sections of human nervous tissue were cut using standard histological technique. Immunoperoxidase staining[4] was performed on these to detect recognition of the tissues by HNK-1. Control sections included those incubated with normal mouse serum (1:10) in place of specific antibody or the use of an irrelevant monoclonal mouse IgM at equivalent protein concentrations to HNK-1. Myelinated tissue from both central and peripheral nervous systems were strongly stained by HNK-1 (Fig. 1). Myelinated tissues from cerebral white matter, optic nerve, spinal cord and peroneal nerve all were specifically stained with HNK-1 (data not shown). Sections of kidney, adrenal, lymph node, liver and thymus were all negative when stained with HNK-1.

Crude myelin was prepared from fresh, normal human brain essentially by the technique of Norton and Poduslo[5], and delipidated by extraction with either ether or ether:ethanol (3:2). The ether-insoluble fraction was termed myelin protein[6]. Myelin proteins were separated by SDS-polyacrylamide gel electrophoresis[7], on 10 or 12% gels followed by either Coomassie blue staining or blotting onto nitrocellulose essentially by the technique of Towbin *et al.*[8]. On reacting the blots with HNK-1, a major band was observed with a mean molecular weight (MW) of 108,000 ± 3,500 (three determinations) (Fig. 2*a*, lane 3). A

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