Pro-Opiomelanocortin mRNA and Peptide Co-Expression in the Developing Rat Pituitary

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KHACHATURIAN, H., S. P. KWAK, M. K.-H. SCHAFER AND S. J. WATSON. Pro-opiomelanocortin mRNA and peptide co-expression in the developing rat pituitary. BRAIN RES BULL 26:2 195-201, 1991. Pro-opiomelanocortin (POMC) is synthesized in both the pituitary gland and the brain. Various peptide products of this precursor, namely beta-endorphin, ACTH and alpha-MSH are co-localized in the anterior lobe corticotrophs, all intermediate lobe cells and in hypothalamic neurons. Messenger RNA (mRNA) for POMC has further been shown to exist in these tissues. In this study, we have shown that POMC mRNA, and peptide accumulation as detected by in situ hybridization and immunocytochemistry, respectively, occur simultaneously within the rat pituitary gland during ontogeny and that their maturation occurs in parallel during prenatal and early postnatal development.

METHOD

Adult male and female Sprague-Dawley rats were used to obtain timed-pregnant females. One male and three females were placed in a cage before 6 p.m., and vaginal smears were examined at 8 a.m. the following day for the presence of sperm. The first day of detection of sperm in vaginal smear was designated day zero of gestation. Pregnant (sperm-positive) rats were placed in individual marked cages, and at various time points they were sacrificed by cervical dislocation to obtain embryonic rat pups. These included embryonic days E14, E15, E17, and E19. Some pregnant rats were kept to term, and the male progeny were sacrificed at the following postnatal days: P1, P7, P14, and P21. The rat pups were flushed through the heart with 4°C normal saline, decapitated, and the head was fixed in 4°C neutral buffered paraformaldehyde. The postnatal rats, as well as an adult male rat, were sacrificed by intracardiac perfusion of the same fixative, and the pituitaries were extracted for further processing. After fixation, all tissues were immersed in 20% sucrose (4°C) for 24 hours, and frozen in liquid nitrogen. Ten micron cryostat sections of the pituitary gland were mounted onto polylysine-coated slides and stored at -80°C. Some sections were processed for peroxidase anti-peroxidase immunocytochemistry using a mouse POMC riboprobe (22).

Pro-opiomelanocortin (POMC) codes for several bioactive peptides, ACTH, alpha-MSH, beta-endorphin, that are important not only in the adult neuroendocrine function, but also during development (10, 15, 19, 20, 31, 34, 36, 48). It is synthesized in both the pituitary gland and the brain (1, 8, 13, 18, 24-26, 34). Furthermore, POMC gene expression has been shown to occur in the brain as well as the pituitary gland (5, 9, 17, 27-30, 43). Developmentally, POMC peptide products are seen very early during gestation in the pituitary and brain (2, 4, 7, 11, 21, 23, 31, 32, 41, 47). Given the known effects of POMC products on nerve cell maturation (45), as well as differential posttranslational processing of POMC during different developmental stages (21), it is of interest to investigate the expression of this important precursor and its mRNA during ontogenetic development. Thus, in the present study, we present evidence for the co-appear-

Immunocytochemistry

Sections were incubated (37°C) with an ACTH antiserum (1/2000), the cross-reactivities of which have been fully characterized (23,51). After incubation for 1 hour, the slides were placed in a moist-box and refrigerated for 24 hours. The sections were washed in 0.02 M phosphate-buffered saline (PBS) and incubated with goat anti-rabbit serum (1/1000, Arnel) for 30 minutes followed by refrigeration for 24 hours. After several PBS washes,
the sections were incubated successively with anti- horseradish peroxidase (anti-horseradish peroxidase (anti-HRP), and 4 μg/ml HRP enzyme (Sigma, type VI), each for 40 minutes. After PBS rinse, the sections were reacted in a solution of 0.125 mg/ml diamobenzidine (Sigma) and 0.03% H₂O₂, for 15 minutes at room temperature. The slides were then washed in distilled water, dehydrated through ethanol and xylenes, and coverslipped in Permount for microscopic observations.

In Situ Hybridization

Mouse POMC cDNA (gift of Dr. J. L. Roberts, Columbia University) was subcloned into SP6. A cRNA probe was prepared using the SP6 transcription system (Promega). The plasmid was linearized using restriction enzyme NcoI, and was transcribed in the presence of SP6 polymerase and [³⁵S]UTP yielding a probe of 436 bases (complementary to nucleotides 479-915 of the mouse POMC cDNA). Labeled cRNA (specific activity: 10.94 x 10⁶ Ci/mmol) was separated from free label on a Sephadex G50 column. Prior to hybridization, sections were deproteinized in 0.2 N HCl for 15 minutes and washed in 2X SSC (300 mM NaCl, 30 mM Na citrate). Hybridization buffer contained 50% formamide, 10% dextran sulfate, 3X SSC, 1 X Denhardts (0.02% ficoll, 0.02% polyvinyl pyrollidone, 10 μg/ml BSA), 100 μg/ml yeast RNA, and 100 μg/ml Salmon perm DNA. The [³⁵S]cRNA probe (2 million counts: 8.23 x 10⁶ mol/slide) was applied to each section, coverslipped, edges sealed with rubber cement, and incubated at 45°C for 24 hours. Control sections were incubated with an equal concentration of radiolabeled sense strand cRNA to the sections. No binding was observed with the sense strand. P2I, and in the adult, autoradiographic grain density over the anterior and intermediate lobes at embryonic days E17 and E19, also mirrored the pattern of ACTH staining in these lobes. Labeling became increasingly intense over both lobes from E17 to E19 (Fig. 1F, H). Postnatally, in situ labeling patterns became progressively less intense in the anterior lobe but not the intermediate lobe. At P1, grain density over the anterior lobe was lower than that seen at E19 (Fig. 2B). Likewise, at P7 (data not shown), P14, and P21, and in the adult, autoradiographic grain density over the anterior lobe was less intense when compared to embryonic patterns of labeling (Fig. 2D, F, H). This diminution of label with increasing age corresponded well with the progressively less intense ACTH staining observed under immunocytochemical conditions, a phenomenon attributable perhaps to general tissue expansion with increasing age.

RESULTS

The ACTH antiserum used in the present study has been extensively characterized under immunocytochemical conditions (23,51). The antiserum recognizes the ACTH-(20–24) region and was not blocked by alpha-MSH, beta-lipotropin, beta-endorphin, or Met-enkephalin. For in situ hybridization control, the specificity of labeling seen with the POMC riboprobe was confirmed by the application of equal concentrations of radiolabeled sense strand cRNA to the sections. No binding was observed with the sense strand probe; this result was expected because the sense strand POMC cRNA has the same sequence as that of the POMC mRNA. The mouse POMC riboprobe showed high specificity for POMC mRNA. It had distinct advantages over oligonucleotides (cDNA) under in situ hybridization conditions. Labeling one out of four nucleotides ([³⁵S]UTP) over the entire 436 bases of cRNA yielded a probe with higher specific activity when compared to the labeling of much shorter sequences of nick-translated cDNA (3). Since RNA-RNA bonds are more stable than DNA-RNA bonds, more stringent washing conditions were applied to reduce nonspecific background. Lastly, posthybridization digestion with RNase A was used to further reduce nonspecific signals.

Analysis of sections processed for ACTH immunocytochemistry revealed a pattern of staining in the anterior lobe corticotrophs and intermediate lobe cells as previously described by a number of investigators (21,41). At the earliest embryonic day analyzed, i.e., E14, no ACTH staining could be detected in either anterior or intermediate lobes of the pituitary gland (Fig. 1A). At embryonic day E15, a few ACTH immunoreactive cells were seen in the anterior lobe, with no immunoreactivity discernable in the intermediate lobe (Fig. 1C). By day E17, the pattern of ACTH staining changed drastically such that a large number of anterior lobe cells and numerous (but not all) intermediate lobe cells exhibited immunoreactivity (Fig. 1E). At E19, the pattern of ACTH immunoreactivity resembled that of the adult pituitary with the notable exception that the cells in both lobes were more densely packed such that the staining pattern was much denser than that normally seen in the adult pituitary (Fig. 1G).

From postnatal days P1 through P21 (data not shown for P7), ACTH immunoreactivity in the anterior and intermediate lobes matured to more adult-like patterns (Fig. 2A, C, E). The major change observed during this period was one of rapid tissue expansion resulting in an apparent “dilution” of the staining intensity in the later stages studied. Finally, the pattern of staining at P21 was identical to that seen in the adult, save for tissue expansion (Fig. 2E, G).
Sections A, C, E, and G were processed for ACTH immunocytochemistry. Sections B, D, F, and H, were processed for in situ hybridization for POMC synthesis. Since this peptide, along with alpha-MSH, is produced in the same time period, much shorter sequences of nick-translated cDNA (3) can be used, and therefore more stringent washing conditions can be applied to reduce nonspecific binding. Lastly, posthybridization digestion with RNase A can further reduce nonspecific signals due to single-stranded probe "stickiness" to tissue. The control conditions used in this study consisted of the application of equal concentrations of radiolabeled sense strand cRNA to the sections. Since the sense strand POMC cRNA has the same sequence as that of the POMC mRNA, no binding was observed, confirming the specificity of the in situ labeling seen with the POMC riboprobe.

Numerous studies have addressed the development of the POMC precursor and its peptides during prenatal and postnatal development (2, 4, 7, 11, 14, 17, 30, 31, 32, 41, 47). All of these studies are in agreement that POMC peptides occur very early in both pituitary and brain during development. For example, beta-endorphin, and ACTH immunoreactivity are seen in the rat hypothalamus as early as embryonic day E12 (21, 41). The appearance of POMC peptides in the pituitary, however, is comparatively delayed until embryonic day E15. Nevertheless, POMC peptides are among the earliest peptidergic systems to develop during ontogeny. Even among the opioid peptides, POMC products are seen earlier in both brain and pituitary when compared to either the pro-enkephalin or pro-dynorphin peptides (2, 4, 16, 21, 35, 37, 39, 12, 50). The significance of early POMC synthesis during ontogenetic development has been explored by several investigators. For example, alpha-MSH and other ACTH fragments have been shown to stimulate nerve cell maturation, and thus making the label per unit area appear less intense with advancing age. In the intermediate lobe, mRNA labeling continued to increase slightly in density throughout postnatal ages studied, consistent with the findings of other investigators (49).

Finally, the observation that both in situ hybridization histochemistry and immunocytochemistry detect mRNA and peptide labeling, respectively, on the same embryonic day (i.e., E15), suggests that POMC gene transcription and subsequent translation occur within a very short time in the pituitary gland during embryogenesis. It is important to note that the results of the present study were expected since POMC peptide expression and mRNA expression have already been shown independently to first occur in embryonic pituitary cells at approximately the same gestational age (21, 22, 40). Furthermore, this study does not specifically pinpoint the exact time of either POMC mRNA or peptide expression. Rather, this study confirms the functional status of peptide-synthesizing apparatus within POMC cells at the same time when the POMC gene is transcribed into mRNA.

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