Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 5672–5675, September 1984
Biochemistry

Expression of pro-opiomelanocortin-like gene in the testis and epididymis*

(RNA blot analysis/peptide hormones/male reproductive system)

CHING-LING C. CHEN, JENNIE P. MATHER, PATRICIA L. MORRIS, AND C. WAYNE BARDIN

The Population Council and The Rockefeller University, New York, NY 10021

Communicated by Roy Hertz, May 25, 1984

ABSTRACT **Adrenocorticotropin (ACTH), β-endorphin, and the melanocyte-stimulating hormones (MSHs), which are products of a common precursor, pro-opiomelanocortin (POMC), are present in a variety of tissues other than pituitary. The recent detection of immunoreactive POMC-derived peptides in the male reproductive tract raised the possibility that these hormones might regulate reproductive function. To determine whether the low concentrations of POMC-derived peptides in the male reproductive tract are synthesized locally and are not contaminants from blood, we have demonstrated POMC-like gene expression in both testis and epididymis. The identification of cells in testis capable of synthesizing POMC mRNA was established by showing the presence of this mRNA in mouse Leydig cell lines (TM_3 and I10A). The hybridizing species of POMC-like mRNA in the testis, epididymis, and Leydig cell lines (TM_3 and I10A) were approximately 150 bases shorter than those in the pituitary or hypothalamus but were similar in size to that in the amygdaloid nucleus of rat brain. The concentration of POMC-like mRNA in the testis is almost as high as that in the hypothalamus. This finding is quite unexpected because the concentrations of POMC-derived peptides in the testis were 2–3 orders of magnitude lower than those in the hypothalamus. The demonstration of a POMC-like gene expression in male reproductive tissues suggests that POMC-derived peptides are synthesized in Leydig cells and epididymis. These observations are consistent with the postulate that POMC-derived peptides may exert paracrine and/or autocrine effects in these organs.**

Pro-opiomelanocortin (POMC) is a precursor protein that contains the sequence for several bioactive peptides including adrenocorticotropin (ACTH), β-endorphin, and melanocyte-stimulating hormones (MSHs) (1, 2). These POMC-derived peptides have been detected in a variety of tissues. The *de novo* synthesis of POMC-derived peptides has been demonstrated in the pituitary (for a review, see ref. 2), hypothalamus (3–5), and human placenta (6, 7). Immunocytochemical studies have revealed the presence of POMC-derived peptides in the gastrointestinal tract (8, 9), pancreas (10), adrenal (11, 12), lymphocytes (13, 14), and the unicellular organism, *tetrahymena pyriformis* (15). Recently, immunoreactive POMC-derived peptides also have been described in both male and female reproductive tissues (11, 16–21). Materials similar to β-endorphin and α-MSH were characterized in human semen (16) and rat testicular extract (17–19) by a variety of physicochemical techniques. Immunostainable POMC-derived peptides were detected in the Leydig cells of the testis and epithelium of epididymis and other male reproductive tissues (11, 20). The concentrations of POMC-derived peptides in these tissues are very low, suggesting that they could only have local paracrine and/or autocrine functions (18, 19). The functional significance of these hormones as modulators of reproductive function would be clarified if it could be demonstrated whether such POMC-derived peptides could be locally synthesized. In this report, we present evidence that a POMC-like gene is expressed in the testis and epididymis. Furthermore, the POMC-like mRNAs found in male reproductive tissues differ in size from those in the pituitary or hypothalamus.

METHODS

Animals. Male Sprague–Dawley rats and male Golden hamsters were purchased from Charles River Breeding Laboratories and male NCS (Nelson Collins Swiss) mice were obtained from the Laboratory Animal Research Center of The Rockefeller University. The animals were decapitated, and pituitaries, brains, livers, testes, and epididymides were removed quickly. The anterior and neurointermediate lobes of rat pituitaries were separated by using a dissecting microscope. Hypothalami and amygdala were dissected from brains. All tissues were frozen in liquid nitrogen.

Preparation of Testicular Cell Lines. Established cell lines derived from testicular cells were routinely cultured on Ham's F-12/Dulbecco-modified Eagle's medium, 1:1 (vol/vol) (Irvine Scientific) supplemented with 15 mM Hepes buffer, 20 mg of gentamycin per liter, 5% horse serum, and 2.5% newborn bovine serum. Sera, Hepes, and gentamycin were purchased from GIBCO. Cells were subcultured every 4–6 days when subconfluent. To prepare mRNA, cells were grown to confluency in 100-mm tissue culture dishes (Corning), washed twice with phosphate-buffered saline, and scraped. The cells were pelleted by centrifugation, and the pellets were stored at −70°C until RNA extraction. The derivations of the Leydig cell line, TM_3, and the Sertoli cell line, TM_4, have been described (22). I10A cells, established from a mouse Leydig cell tumor by Shin (23), were obtained from the American Type Culture Collection.

Blot Hybridization Analysis. Total RNA from various tissues was isolated by the urea/lithium chloride precipitation method (24), and poly(A)-containing RNAs were obtained by oligo(dT)-cellulose column chromatography (25). The poly(A)$^+$ RNAs were denatured with 1 M glyoxal and 50% dimethylsulfoxide and were fractionated in 1.5% agarose gel as described by Thomas (26). The poly(A)$^+$ RNAs were then transferred to nitrocellulose paper, and POMC-like mRNAs were identified by hybridization with a radioactive POMC complementary DNA (cDNA) probe and exposed to x-ray film.

Dot-Blot Assay. Equivalent amounts of poly(A)$^+$ RNA from each sample were resuspended in 100 μl of 1.5 M NaCl/0.15 M sodium citrate, pH 7.2, and spotted on a nitrocellulose filter by using a Microfold Dot Blot (Schleicher &

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: POMC, pro-opiomelanocortin; MSHs, melanocyte-stimulating hormones.

*Preliminary results were reported at the Eighth Testis Workshop, October 14–17, 1983, Bethesda, MD.

Schuell) (27). The POMC mRNA on the filter was identified as described for blot hybridization analysis.

Preparation of Radioactive POMC cDNA Probe. Rat POMC cDNA was constructed from poly(A)$^+$ RNA of neurointermediate pituitary by reverse transcription and inserted in the *Pst* I site of plasmid pBR322 by dG·dC-tailing (28, 29). Clone pI13 used in these studies contains the nucleotide sequence coding from the midportion of the NH_2-terminal glycopeptide of POMC to the poly(A) tail (27). The pI13 insert was isolated and made radioactive by nick translation to a specific activity of 2–5 × 10^8 cpm/μg.

RESULTS

Identification of POMC-Like mRNA in the Testis and Epididymis. The existence of POMC-like mRNA in the male reproductive tissues was determined by using blot hybridization analysis. Poly(A)$^+$ RNAs were isolated from various rat tissues, and POMC-like mRNAs were identified by hybridization with a cloned rat POMC cDNA (pI13). With this probe, POMC-like mRNAs were detected in the testis and epididymis as well as in the pituitary and brain (Fig. 1). The hybridizable species of POMC-like mRNA in the testis and epididymis had the same apparent molecular size, which was approximately 150 bases shorter than those in the pituitary and hypothalamus. Civelli *et al.* (30) found that in some regions of the rat brain, such as amygdala and cerebral cortex, POMC-like mRNA was shorter than that from pituitary or hypothalamus. The results in Fig. 1 (lanes 3, 4, and 5) show that POMC-like mRNAs in the testis and epididymis were the same size as those detected in the amygdala.

The detection of immunostainable β-endorphin material in the testes of five species (11) suggested that POMC mRNA would be found in multiple species. Poly(A)$^+$ RNAs prepared from rat, mouse, and hamster testes contained POMC-like mRNAs as detected by blot hybridzation analysis (Fig. 2). The testicular POMC-like mRNAs of these species had similar apparent molecular sizes, all about 150 bases shorter than that of pituitary.

Localization of POMC-Expressing Cells in the Testis. To determine which testicular cell type expresses the POMC-like gene, the presence of POMC-like mRNAs in established cell lines derived from different cell types of the mouse testis was studied. The POMC-like mRNAs were identified in Leydig cell lines originating from normal testis (TM_3) and from a Leydig cell tumor (I10A). The POMC cDNA probe (pI13) did not hybridize to the mRNA from a mouse Sertoli-derived cell line (TM_4) (Fig. 3). These observations indicated that POMC-like mRNA was in the same cell type as the immunostainable POMC-like peptides detected with antibodies against β-endorphin, ACTH, α-MSH, or γ-MSH (11, 18, 20). We conclude that the POMC-like gene is expressed in the Leydig cells of testis.

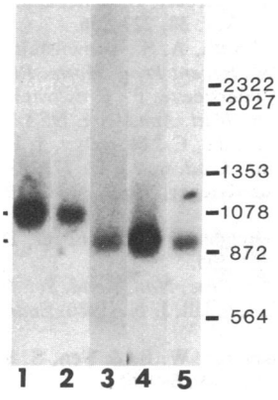

FIG. 1. POMC-like mRNAs in various tissues of rat. The poly(A)$^+$ RNAs were denatured with 1 M glyoxal and 50% dimethyl sulfoxide and fractionated in 1.5% agarose gel as described by Thomas (26). The RNAs were then transferred to a nitrocellulose paper, and the POMC-like mRNAs were identified by hybridization with ^{32}P-labeled POMC cDNA (pI13) probe. The poly(A)$^+$ RNA samples in lanes were: 1, neurointermediate pituitary (30 ng); 2, hypothalamus (5 μg); 3, amygdala (30 μg); 4, testis (25 μg); and 5, epididymis (25 μg). ^{32}P-end-labeled *Hea* III fragments of ϕX174 DNA and *Hin*dIII fragments of phage λ DNA used as molecular size markers (in bases) are shown on the right. Note that POMC-like mRNAs in lanes 3, 4, and 5 are smaller in size than those in lanes 1 and 2.

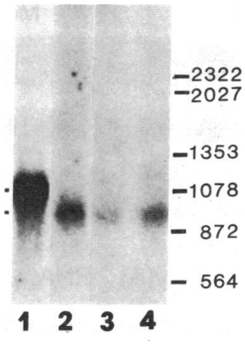

FIG. 2. POMC-like mRNA in the testis of different species. The testicular poly(A)$^+$ RNAs were isolated from three different species, and POMC-like mRNAs were identified as described in Fig. 1. The poly(A)$^+$ RNAs in lanes were: 1, rat neurointermediate pituitary (30 ng); 2, rat testis (15 μg); 3, hamster testis (10 μg); and 4, mouse testis (10 μg). Note that in all species, smaller POMC-like RNAs were found in the testes.

Determination of POMC-Like mRNA Concentration. Since the major forms of POMC-derived peptides present in rat testis are similar to those detected in the hypothalamus (19), it was of interest to compare the concentration of POMC-like mRNA in these organs. Equal amounts of poly(A)$^+$ RNAs from testis, hypothalamus, and liver were spotted on a nitrocellulose filter, and the concentration of POMC-like mRNA in each tissue was determined by hybridization to the POMC cDNA (pI13) probe (Fig. 4). The concentration of POMC-like mRNA in the testis was similar to that in the hypothalamus, which represents approximately 0.01–0.02% of total poly(A)$^+$ RNA. This observation was unexpected because the concentrations of immunoreactive β-endorphin and α-MSH present in the hypothalamus are 2–3 orders of magnitude greater than those in the testis (19). By contrast,

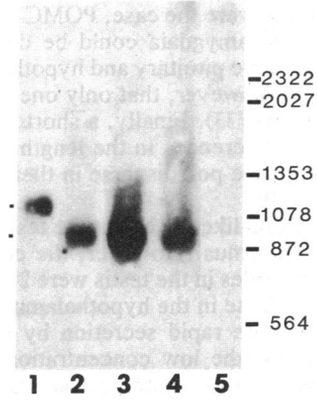

FIG. 3. POMC-like mRNA in testicular cell lines. The poly(A)$^+$ RNAs were isolated from various testicular cell lines, and POMC-like mRNAs were identified as described in Fig. 1. The RNAs in lanes were: 1, rat anterior pituitary total RNA (10 μg); 2, poly(A)$^+$ RNAs isolated from rat testis (5 μg); 3, mouse Leydig cell line TM_3 (25 μg); 4, mouse Leydig tumor cell line I10A (25 μg); and 5, mouse Sertoli cell line TM_4 (25 μg). Note that smaller POMC-like mRNAs were detected in the testis and in Leydig cell lines.

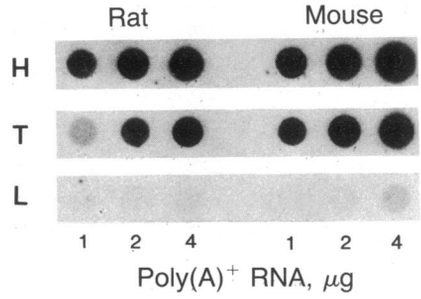

FIG. 4. Comparison of POMC-like mRNA concentrations by dot-blot analysis. Poly(A)$^+$ RNA (1, 2, and 4 μg) isolated from hypothalamus (H), testis (T), or liver (L) of rat and mouse were spotted on nitrocellulose filter, and the concentrations of POMC-like mRNA were determined by hybridization to ^{32}P-labeled pI13 probe.

the level of POMC-like mRNA in the epididymis was approximately 20% of that observed in the testis (Fig. 1, lanes 4 and 5), whereas the concentrations of POMC-derived peptides in these organs were similar (18).

DISCUSSION

In this report, we have demonstrated the expression of a POMC-like gene in testis and epididymis. The presence of POMC-like mRNA in Leydig cells suggests that this is the major site of POMC synthesis in the testis. The POMC-like mRNAs detected in these tissues were similar in size to that from the amygdala, but approximately 150 bases shorter than those in the hypothalamus or pituitary.

There are several possible explanations for the tissue-specific polymorphism of the mRNA observed in this study. The smaller POMC-like mRNAs detected in the amygdala, testis, and epididymis as compared to the pituitary may result from transcription of a single POMC gene with tissue-specific processing of nuclear precursor RNA to its mature mRNA. The tissue-specific splicing of the same nuclear RNA has been described in the hypothalamus and thyroid, where alternative processing of the calcitonin gene transcripts results in the production of two distinct mRNAs coding for different polypeptides (31). In the case of mouse α-amylase, differential use of two promoters and their 5′-untranslated regions of a single α-amylase gene generated two tissue-specific distinct mRNAs in the liver and salivary gland (32). Alternatively, it is possible that different POMC-like mRNAs may be transcribed from multiple nonallelic POMC genes. If this were the case, POMC mRNA from testis, epididymis, and amygdala could be derived from one gene, and mRNA in the pituitary and hypothalamus from another. It is of note, however, that only one POMC gene has been identified in rat (33). Finally, a shorter POMC mRNA could result from differences in the length of poly(A) tails, which are known to be polydisperse in their length within a specific mRNA (34).

The level of POMC-like mRNA in the testis was similar to that in the hypothalamus. However, the concentrations of POMC-derived peptides in the testis were 2–3 orders of magnitude lower than those in the hypothalamus (19). High protease activity or more rapid secretion by Leydig cells are possible reasons for the low concentrations of POMC-derived peptides in the testis. Alternatively, this also could be due to low translational activity of testicular POMC-like mRNA. Several studies indicate that the length of poly(A) affects the translational efficiency of a specific mRNA (35, 36). If the smaller size of POMC-like mRNA in the testis were due to a shorter poly(A) tail, this might account for the lower concentrations of POMC-derived peptides produced in this organ.

The low concentrations of POMC-derived peptides present in testis and epididymis as compared to those in the pituitary and the fact that the concentrations of these peptides decline in plasma (but not in testis or epididymis) to below physiological levels after hypophysectomy indicate that the reproductive tract does not contribute significantly to the blood levels of these hormones (18). In addition, a variety of studies suggested that POMC-derived peptides have paracrine effects in the testis and male-reproductive tract. For example, adrenocorticotropin has been shown to stimulate Sertoli cell growth *in vitro* (22), and adrenocorticotropin and α-MSH elevate cyclic AMP accumulation in these cells (37). By contrast, *in vivo* studies suggest that β-endorphin or another endogenous opiate in the testis inhibits Sertoli cell growth during early testicular development (38). These observations suggest that peptides derived from different portions of POMC may have differential effects on Sertoli cells. This is analogous to the opposing behavioral effects of POMC-derived peptides, which have been described in brain (39). Thus, the biological consequences of synthesis of POMC peptides in the testis are related not only to the amount of the precursor that is made but also to which of its component peptides are present in active form.

In a recent study, the [D-Ala^2-Met^5] analog of enkephalin was shown to inhibit forskolin-activated adenylyl cyclase in membranes prepared from rabbit corpora lutea (40). [D-Ala^2-Met^5]Enkephalin-induced inhibition of the rabbit luteal enzyme was ATP-dependent, supporting the suggestion that the corpus luteum contains a receptor for opiate peptides that is coupled to an inhibitory guanine nucleotide regulating component. These findings of a function for β-endorphin in the corpus luteum and other steroid-secreting cells (11) suggest that this or another endogenous opiate could also serve as a negative autocrine regulator in Leydig cells.

The excellent technical assistance of Ms. Marie Ambroskiewicz is gratefully acknowledged. We also thank Ms. Jean Schweis and Ms. Linda McKeiver for preparation of the manuscript. This work was supported by National Institutes of Health Grants HD13541 and HD16149.

1. Eipper, B. A. & Mains, R. E. (1980) *Endocr. Rev.* **1,** 1–27.
2. Roberts, J. L., Chen, C.-L. C., Eberwine, J. H., Evinger, M. J., Gee, C. E., Herbert, E. & Schachter, B. S. (1982) *Recent Prog. Horm. Res.* **38,** 227–256.
3. Krieger, D. T., Liotta, A. S., Brownstein, M. J. & Zimmerman, E. A. (1980) *Recent Prog. Horm. Res.* **36,** 277–344.
4. Liotta, A. S., Gildersleeve, P., Brownstein, M. J. & Krieger, D. T. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 1448–1452.
5. Liotta, A. S., Loudes, C., McKelvy, J. F. & Krieger, D. T. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1880–1884.
6. Liotta, A. S. & Krieger, D. T. (1980) *Endocrinology* **106,** 1504–1511.
7. Liotta, A. S., Houghten, R. & Krieger, D. T. (1982) *Nature (London)* **295,** 593–595.
8. Larsson, L. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 2990–2994.
9. Orwoll, E. S. & Kendall, J. N. (1980) *Endocrinology* **107,** 438–442.
10. Bruni, J. F., Watkins, W. B. & Yen, S. S. C. (1979) *J. Clin. Endocrinol. Metab.* **49,** 649–651.
11. Tsong, S. D., Phillips, D. M., Halmi, N., Krieger, D. T. & Bardin, C. W. (1982) *Biol. Reprod.* **27,** 755–764.
12. Evans, C. J., Erdelyi, E., Weber, E. & Barchas, J. D. (1983) *Science* **221,** 957–960.
13. Smith, E. M. & Blalock, J. E. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 7530–7534.
14. Smith, E. M., Meyer, W. J. & Blalock, J. E. (1982) *Science* **218,** 1311–1312.
15. LeRoith, D., Liotta, A. S., Roth, J., Shiloach, J., Lewis, M. E., Pert, C. B. & Krieger, D. T. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2086–2090.
16. Sharp, B. & Pekary, A. E. (1981) *J. Clin. Endocrinol. Metab.* **52,** 586–588.

17. Sharp, B., Pekary, A. E., Mayer, N. V. & Hershman, J. M. (1980) *Biochem. Biophys. Res. Commun.* **95,** 618–623.
18. Tsong, S. D., Phillips, D. M., Halmi, N., Liotta, A. S., Margioris, A. N., Bardin, C. W. & Krieger, D. T. (1982) *Endocrinology* **110,** 2204–2206.
19. Margioris, A. N., Liotta, A. S., Vaudry, H., Bardin, C. W. & Krieger, D. T. (1983) *Endocrinology* **113,** 663–671.
20. Shaha, C., Liotta, A. S., Krieger, D. T. & Bardin, C. W. (1984) *Endocrinology* **114,** 1584–1591.
21. Lim, A. T., Lolait, S., Barlow, J. W., Wai, S. O., Zois, I., Toh, B. H. & Funder, J. W. (1983) *Nature (London)* **303,** 709–711.
22. Mather, J. P. (1980) *Biol. Reprod.* **23,** 243–252.
23. Shin, S. (1967) *Endocrinology* **81,** 440–448.
24. Auffray, C. & Fougeon, F. (1980) *Eur. J. Biochem.* **107,** 303–314.
25. Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 1408–1412.
26. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 5201–5205.
27. Chen, C.-L. C., Dionne, F. T. & Roberts, J. L. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2211–2215.
28. Payvar, F. & Schimke, R. T. (1979) *J. Biol. Chem.* **254,** 7636–7642.
29. Chang, A. C. Y., Nunberg, J. H., Kaufman, R. J., Erlich, H. A., Schimke, R. T. & Cohen, S. N. (1978) *Nature (London)* **275,** 617–624.
30. Civelli, O., Birnberg, N. & Herbert, E. (1982) *J. Biol. Chem.* **257,** 6783–6787.
31. Rosenfeld, M. G., Mermod, J.-J., Amara, S. G., Swanson, L. W., Sawchenko, P. E., Rivier, J., Vale, W. W. & Evans, R. M. (1983) *Nature (London)* **304,** 129–135.
32. Young, R. A., Hagenbuchle, O. & Schibler, U. (1981) *Cell* **23,** 451–458.
33. Drouin, J. & Goodman, H. M. (1980) *Nature (London)* **288,** 610–613.
34. Vournakis, J. N., Gelinas, R. E. & Kafatos, F. C. (1974) *Cell* **3,** 265–273.
35. Deshpande, A. K., Chatterjee, B. & Roy, A. K. (1979) *J. Biol. Chem.* **254,** 8937–8942.
36. Jacobson, A. & Favreau, M. (1983) *Nucleic Acids Res.* **11,** 6353–6367.
37. Bardin, C. W., Shaha, C., Mather, J., Salomon, Y., Margioris, A. N., Liotta, A. S., Gerendai, I., Chen, C.-L. C. & Krieger, D. T. (1984) *Ann. NY Acad. Sci.*, in press.
38. Gerendai, I., Nemeskei, A. & Csevrus, V. (1983) *Andrologia* **15,** 398–403.
39. Krieger, D. T. (1983) *Science* **22,** 975–985.
40. Abramowitz, J. & Campbell, A. R. (1983) *Biochem. Biophys. Res. Commun.* **116,** 574–580.