

## Expression of proenkephalin messenger RNA by mouse spermatogenic cells

(opioid peptides/testis/paracrine factors)

DANIEL L. KILPATRICK\*<sup>†</sup> AND CLARKE F. MILLETTE<sup>‡</sup>

\*Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545; and <sup>‡</sup>Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, Boston, MA 02115

Communicated by Mahlon Hoagland, March 17, 1986

**ABSTRACT** The presence of proenkephalin mRNA in germ cells purified from adult mouse testis was examined using RNA gel- and dot-blot analyses. Both pachytene spermatocytes and round spermatids were shown to contain concentrations of this transcript that are 2- to 3-fold greater than that found for whole mouse testis, on a per  $\mu\text{g}$  of polyadenylated RNA basis. The detection of proenkephalin mRNA in purified spermatocytes and spermatids could not be accounted for by contamination by either Leydig or Sertoli cells. No proenkephalin mRNA was detectable in extracts of mature sperm. These data suggest that developing germ cells may be a major site of proenkephalin synthesis in the adult testis and that proenkephalin-derived peptides may function as germ cell-associated hormones or autocrine/paracrine factors.

The essential role of pituitary gonadotrophic hormones in testicular development and function has long been recognized. However, peptides and proteins produced locally within the testis are receiving increasing attention as potential paracrine and autocrine factors mediating interactions between different testicular cells (1, 2). For example, Sertoli cells produce androgen-binding protein, Sertoli-derived growth factor, transferrin, and other proteins that are important for germ-cell development (3). They also appear to be the source of a peptide similar to gonadotropin-releasing hormone that directly affects testosterone synthesis by Leydig cells (1, 2). In addition, peritubular cells secrete a protein factor (PMod-S) that stimulates androgen-binding protein and transferrin synthesis by Sertoli cells (4). Studies have suggested that opioid peptides are also among this class of intratesticular regulators. Opioid peptides are synthesized as part of larger precursor proteins, three of which are currently known [pro-opiomelanocortin (POMC), proenkephalin (proenkephalin A), and prodynorphin (proenkephalin B) (5-8)]. The mRNAs for each of these precursors and their respective opioid peptide products have been identified in rat testis as well as that of other species (9-12, §). The expression of all three opioid peptide gene products in the testis suggests that they play an important role in spermatogenesis. An important step in defining the function(s) of testicular opioid peptides is the determination of their sites of synthesis. Sastry *et al.* (13) have reported the detection of enkephalin immunoreactivity in spermatozoa, suggesting that testicular germ cells might express the proenkephalin gene. In this report, the distribution of proenkephalin mRNA in germ cells isolated from adult mouse testis is reported.

### METHODS

**Preparation of Germ Cells.** Following enzymatic digestion of decapsulated 8-wk-old adult mouse testes, pachytene

spermatocytes, round spermatids, and residual bodies were isolated on linear gradients of bovine serum albumin using a modification (14) of the method of Romrell *et al.* (15). These three fractions represent the most highly purified germ-cell components recovered from dissociated seminiferous epithelium with this procedure as assessed by Nomarski and phase microscopy (15). Mature sperm were isolated from the vas ductus of the mouse and rat as described by Wolfe *et al.* (16). In three separate experiments, the purity of pachytene spermatocyte preparations ranged from 88 to 91%, with binucleated spermatids being the major contaminants. The round spermatid fractions were between 91 and 94% pure, with large condensing spermatids and residual bodies as the main contaminants. In both fractions, contamination by somatic cells (Leydig and Sertoli cells) was generally less than 1%. Residual bodies were 88-91% pure, with condensing spermatids being the major contamination.

**Analysis of Proenkephalin mRNA Content.** Total RNA was prepared from isolated germ cells, whole testis, and liver from adult mice by the guanidinium thiocyanate/cesium chloride gradient procedure (17). Polyadenylated RNA was prepared by oligo(dT) chromatography (18). RNA samples were separated on 1.5% agarose/methyl mercury hydroxide gels (19) and then transferred to nylon membranes (Gene-ScreenPlus, New England Nuclear) (20). Membranes were prehybridized for 4-16 hr at 42°C, hybridized with nick-translated rat proenkephalin cDNA, pRPE-1 (165-600) (21) ( $2-4 \times 10^8$  cpm/ $\mu\text{g}$ ) and then washed as described (12, 21). Autoradiography was performed at -80°C for 16-24 hr. RNA blots were as described (12).

### RESULTS AND DISCUSSION

Total RNA and poly(A)<sup>+</sup> RNA prepared from mouse germ cells and whole mouse testis, respectively, were examined by RNA gel-blot analysis for the presence of proenkephalin mRNA. Like rat testis (12), mouse testis expresses a proenkephalin mRNA species that is similar in size to 18S ribosomal RNA (approximately 1900 nucleotides) (Fig. 1). This testicular proenkephalin mRNA is significantly larger than the 1450-nucleotide species commonly observed in other tissues that express proenkephalin (12). As shown in Fig. 1, total RNA fractions from both round spermatids and pachytene spermatocytes contain significant amounts of testicular proenkephalin mRNA. The relative abundances of this transcript per  $\mu\text{g}$  of total RNA were similar in the two germ-cell types, with round spermatids being slightly higher. To quantitate the concentration of proenkephalin mRNA in mouse germ cells relative to whole testis, RNA slot blots of poly(A)<sup>+</sup> RNA from round spermatids and mouse testis were com-

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.

Abbreviation: POMC, pro-opiomelanocortin.

<sup>†</sup>To whom reprint requests should be addressed.

<sup>§</sup>Margioris, A. N. & Sasaki, A. (1985) 67th Annual Meeting of the Endocrine Society, June 19-21, 1985, Baltimore, MD, abstr. 511.

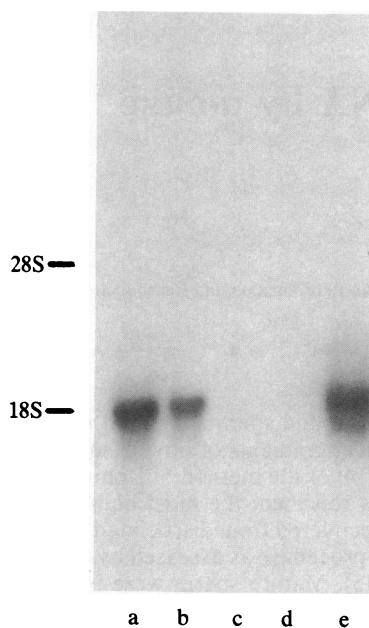


FIG. 1. RNA gel-blot analysis of mouse testicular germ-cell and whole-mouse testis RNA. Pachytene spermatocytes and round spermatids were purified from mouse testis using linear gradients of bovine serum albumin as described by Bellvé *et al.* (14). The positions of the 28S and 18S ribosomal subunits are indicated. RNA samples were as follows: mouse round spermatid total RNA from  $2.7 \times 10^7$  cells (26  $\mu\text{g}$ , lane a); mouse pachytene spermatocyte total RNA from  $0.5 \times 10^7$  cells (25  $\mu\text{g}$ , lane b); mouse liver total RNA (25  $\mu\text{g}$ , lane c); mouse liver poly(A)<sup>+</sup> RNA (20  $\mu\text{g}$ , lane d); mouse testis poly(A)<sup>+</sup> RNA (20  $\mu\text{g}$ , lane e).

pared along with poly(A)<sup>+</sup> RNA from rat testis and rat brain (Fig. 2). As shown, proenkephalin mRNA is 2- to 3-fold more abundant in round spermatids than in whole mouse testis when expressed per  $\mu\text{g}$  of poly(A)<sup>+</sup> RNA.

The germ-cell fractions employed in these studies are 90% or more pure, with the majority of contaminating cells being other germ-cell types. In light of the enrichment of proen-

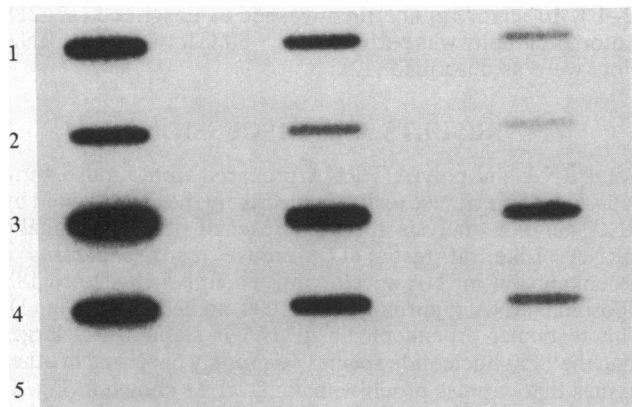


FIG. 2. Relative abundance of proenkephalin mRNA in poly(A)<sup>+</sup> RNA samples from round spermatids and mouse testis. Equivalent amounts of poly(A)<sup>+</sup> RNA from mouse round spermatids (row 1), mouse testis (row 2), rat brain (row 3), rat testis (row 4), and mouse liver (row 5) were denatured by heating to 60°C for 15 min in the presence of 7.4% (vol/vol) formaldehyde/0.9 M NaCl/0.09 M sodium citrate (denaturing buffer). Each sample was then dot-blotted onto nitrocellulose in 1:3 serial dilutions (blots: Left, 1.4  $\mu\text{g}$ ; Center, 0.42  $\mu\text{g}$ ; Right, 0.14  $\mu\text{g}$ ) using a slot blot apparatus (Schleicher & Schuell). Wells were then rinsed with denaturing buffer. The filter was baked, prehybridized, hybridized with nick-translated pRPE-1 (165-600), and then washed. Rat testis and brain samples were included for an interspecies comparison.

kephalin mRNA in these fractions, it seemed unlikely that contamination by nongerm-cell types (e.g., Leydig or Sertoli cells) accounted for the above observations. To further rule out this possibility, the distribution of proenkephalin mRNA throughout the bovine serum albumin gradient was examined relative to the various cell types in the gradient (Fig. 3). It is clear that the profile of proenkephalin mRNA coincides with that of the two major germ-cell types (pachytene spermatocytes and round spermatids) and is distinct from the profiles for either Sertoli or Leydig cells. Round spermatids represent the major proenkephalin mRNA-containing fraction on these gradients due to their significantly higher number. Due to the manner in which gradient fractions were pooled, it is not possible to determine from these experiments whether other germ-cell components (residual bodies and condensing spermatids) also contain significant concentrations of proenkephalin mRNA (see below).

The above results indicate that proenkephalin mRNA is present in germ cells both during meiotic prophase (spermatocytes) and following meiosis (spermatids). It is not clear at this time whether the presence of this mRNA in post-meiotic cells reflects haploid gene transcription or carry-over of unusually stable transcripts synthesized prior to cell division (22, 23). Proenkephalin mRNA was also detected in total RNA isolated from purified residual bodies (Fig. 4). However, it should be noted that the abundance of this transcript in residual bodies was significantly lower than that seen in round spermatids or whole testis. It is, therefore, possible that the mRNA observed in residual bodies is due to contamination by round and/or condensing spermatids (see also Fig. 3). Further fractionation will be necessary to resolve this question. If correct, these data would indicate that germ-cell expression of proenkephalin is maintained through the later stages of spermiogenesis (condensation/elongation).

To determine whether mature sperm might also contain proenkephalin mRNA, total RNA was prepared from sperm isolated from rat and mouse vas ductus. No detectable hybridization to nick-translated proenkephalin cDNA was observed in spermatozoa from either species, even when 50-fold more sperm cells than round spermatids were examined (Fig. 3). Thus, on a per cell basis, the levels of proenkephalin mRNA are markedly reduced in mature sperm. This is not entirely unexpected in light of the low content of total RNA isolated from these cells (see Fig. 3). It is, therefore, difficult to estimate the actual abundance of proenkephalin mRNA per  $\mu\text{g}$  of RNA in mature sperm.

Pachytene spermatocytes and round spermatids together account for greater than 50% of the poly(A)<sup>+</sup> RNA present in mouse seminiferous tubules (24). Furthermore, since the latter compartment makes up by far the majority of total testicular cell volume in the rat and mouse [90% in the rat (25)], these two germ-cell populations contribute a major fraction of total testicular poly(A)<sup>+</sup> RNA. The 2- to 3-fold enrichment of proenkephalin mRNA in mouse pachytene spermatocytes and round spermatids relative to whole testis, therefore, indicates that these germ cells are a major source of this mRNA in the mouse testis. However, this does not exclude the possibility that other testicular cell types, including somatic cells and germ cells at other stages of development, also express proenkephalin mRNA. *In situ* hybridization should help to clarify this question.

Studies by Pintar *et al.* (26) indicated that testicular POMC mRNA is exclusively expressed by Leydig cells in the rat (although see below). The present data implicate developing germ cells as a major site of proenkephalin biosynthesis within the adult mouse testis. These results are important in that germ cells are generally considered to be targets of hormone action as opposed to sites of hormone synthesis. However, indirect evidence suggests that germ cells can

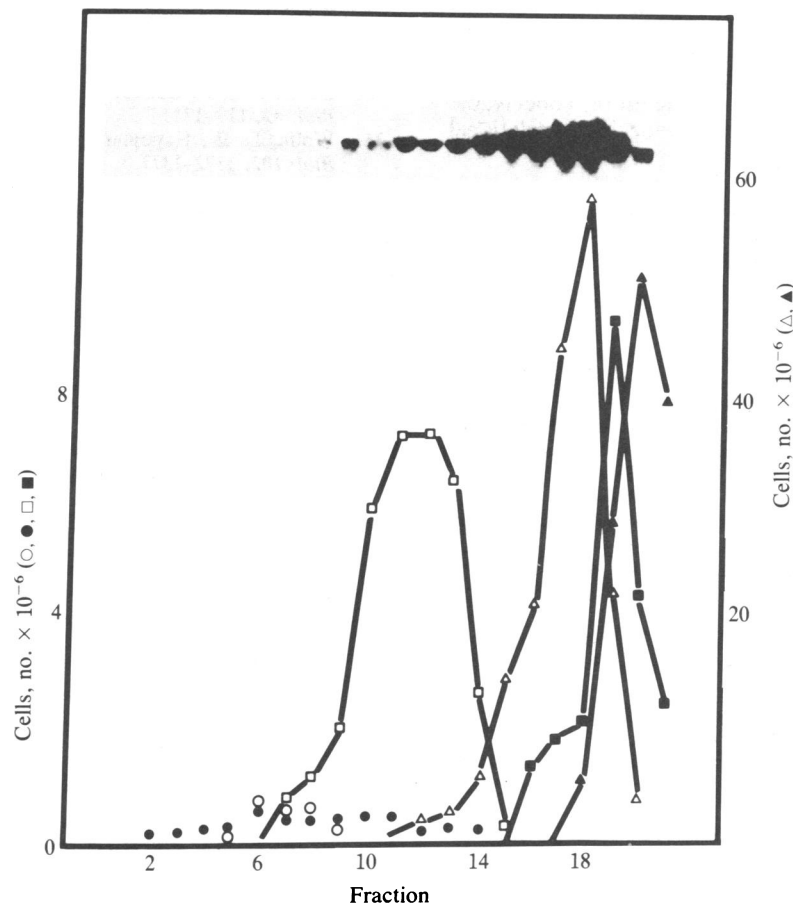


FIG. 3. Comparative distributions of proenkephalin mRNA and testicular cell types on linear bovine serum albumin gradients. Germ cells prepared from 24 mouse testes were separated by unit gravity sedimentation using a STA-PUT system as described (15). Collected fractions (10 ml) were pooled into consecutive groups of four, and the distribution of various cell types was determined by Nomarski light microscopy (15). Pelleted cells were stored at  $-80^{\circ}\text{C}$  for preparation of total RNA. Twenty micrograms of yeast tRNA was added as carrier prior to ethanol precipitation. Fifty percent of the total RNA from each pooled fraction was analyzed by RNA gel-blot analysis for proenkephalin mRNA. The numbers and types of cells identified are indicated on either vertical side. Note the difference in the scale in cell number between the two sides. (Inset) The relative content of proenkephalin mRNA is shown directly above the corresponding pooled cell fractions. ○, Sertoli cells; ●, Leydig cells; □, pachytene spermatocytes; ■, condensing spermatids; Δ, round spermatids; ▲, residual bodies.

exert a regulatory effect on other cell types in their local environment. For example, Sertoli cells and Leydig cells

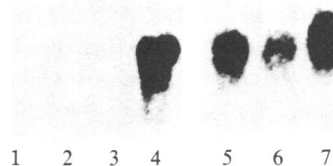


FIG. 4. RNA gel-blot analysis of total RNA from mouse residual bodies and mature sperm from the rat and mouse. The purity of the residual body fraction was 88.5%, with condensing spermatids being the major contaminant. Mature sperm were obtained from the vas ductus of the mouse or rat, and total RNA was prepared. Cesium chloride-gradient pellets were resuspended in distilled  $\text{H}_2\text{O}$ , and RNA concentration was determined by the absorbance at 260 nm. The yield of total RNA for each fraction was as follows: rat sperm,  $0.1 \mu\text{g}$  from  $5.4 \times 10^7$  cells; mouse sperm,  $0.4 \mu\text{g}$  from  $4 \times 10^8$  cells; residual bodies,  $10 \mu\text{g}$  from  $9.0 \times 10^7$  "cells". To ensure adequate recovery,  $20 \mu\text{g}$  of mouse liver total RNA was added to the RNA samples prior to concentration by ethanol precipitation. The entire sample in each case was then examined for the presence of proenkephalin mRNA by RNA gel-blot analysis. Lane 1, rat sperm total RNA; lane 2, mouse sperm total RNA; lane 3, mouse liver total RNA; lane 4, round spermatid RNA from  $8 \times 10^6$  cells (approximately  $5 \mu\text{g}$ ) together with  $15 \mu\text{g}$  of mouse liver total RNA; lane 5, mouse testis total RNA ( $10 \mu\text{g}$ ); lane 6, mouse residual body total RNA ( $10 \mu\text{g}$ ); lane 7, mouse round spermatid total RNA ( $7 \mu\text{g}$ ).

exhibit changes in protein secretory pattern and cell morphology, respectively, that are dependent on the stage of spermatogenesis of neighboring germ cells (1, 3, 27, 28). While cell-cell contact may mediate certain of these effects (29), it is possible that specific factors released from germ cells are also important. Consistent with this, protein secretion by cultured pachytene spermatocytes has been detected (30). Stimulation of Sertoli cell adenylcyclase by a germ cell-associated factor has also been reported (31). In addition, *N*-acetyldorphins, peptides derived from POMC, have been reported to be present in rat spermatogonia and primary spermatocytes (32). Evidence of POMC mRNA within mouse testicular germ cells was found using *in situ* hybridization (33). It is, therefore, possible that testicular germ cells express multiple opioid peptide precursors. The significance of proenkephalin mRNA expression by testicular germ cells remains to be determined. Studies by Gerendai *et al.* (34, 35) suggest that endogenous opioid peptides might exert effects on Sertoli cell growth and Leydig cell steroidogenesis. An autocrine function is also possible. Further analysis of the nature, secretion, and specific actions of germ cell-associated proenkephalin-derived peptides are necessary before their role as putative germ cell autocrine and/or paracrine factors can be established. Another important aspect to be examined is the developmental regulation of proenkephalin gene expression during spermatogenesis. Certain germ cell mRNAs are translationally regulated, being actively trans-

lated only at specific stages of the spermatogenic cycle (36). Preliminary experiments in this laboratory indicate that a significant fraction of proenkephalin mRNA in adult rat testis exists in an untranslated state. It is, therefore, conceivable that this transcript also undergoes stage-specific translational regulation during spermatogenesis.

We thank Dr. C. O'Connor for assisting in the preparation of purified mouse testicular germ cells and Dr. N. Hecht for his helpful comments. We also thank J. Rosenthal for her excellent technical assistance and L. Hager for preparing this manuscript. This work was supported by Grants AM-35855 and AM-36468 from the National Institutes of Health to D.L.K.

1. Ritzén, E. M. (1983) *J. Steroid Biochem.* **19**, 499–504.
2. Sharpe, R. M. (1984) *Biol. Reprod.* **30**, 29–49.
3. Mather, J. P., Gunsalus, G. L., Musto, N. A., Cheng, C. Y., Parvinen, M., Wright, W., Perez-Infante, V., Margioris, A., Liotta, A., Becker, R., Krieger, D. T. & Bardin, C. W. (1983) *J. Steroid Biochem.* **19**, 41–51.
4. Skinner, M. K. & Fritz, I. B. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 114–118.
5. Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C. Y., Cohen, S. N. & Numa, S. (1979) *Nature (London)* **278**, 423–427.
6. Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Hirose, T., Inayama, S., Nakanishi, S. & Numa, S. (1982) *Nature (London)* **295**, 202–206.
7. Gubler, U., Seeburg, P. H., Gage, L. P. & Udenfriend, S. (1982) *Nature (London)* **295**, 206–208.
8. Kakidani, H., Furutani, Y., Takahashi, H., Toyosato, M., Notake, M., Nakanishi, S. & Numa, S. (1982) *Nature (London)* **297**, 431–434.
9. Chen, C. L. C., Mather, J. P., Morris, P. L. & Bardin, C. W. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5672–5675.
10. Shu-dong, T., Phillips, D. M., Halmi, N., Krieger, D. & Bardin, C. W. (1982) *Biol. Reprod.* **27**, 755–764.
11. Civelli, O., Douglass, J., Goldstein, A. & Herbert, E. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4291–4295.
12. Kilpatrick, D. L., Howells, R. D., Noe, M., Bailey, L. C. & Udenfriend, S. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7467–7469.
13. Sastry, B. V. R., Janson, V. E., Owens, L. K. & Tayeb, O. S. (1982) *Biochem. Pharmacol.* **31**, 3519–3522.
14. Bellvé, A. R., Millette, C. F., Bhatnagar, Y. M. & O'Brien, D. A. (1977) *J. Histochem. Cytochem.* **25**, 480–494.
15. Romrell, L. J., Bellvé, A. R. & Fawcett, D. W. (1976) *Dev. Biol.* **49**, 119–131.
16. Wolfe, D. E., Hagopian, S. S. & Ishijima, S. (1986) *J. Cell Biol.* **102**, 1372–1377.
17. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299.
18. Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 1408–1412.
19. Bailey, J. M. & Davidson, N. (1976) *Anal. Biochem.* **70**, 75–85.
20. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5201–5205.
21. Howells, R. D., Kilpatrick, D. L., Bhatt, R., Monahan, J. J., Poonian, M. & Udenfriend, S. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7651–7655.
22. Monesi, V. (1964) *J. Cell Biol.* **22**, 521–532.
23. Erickson, R. P., Erickson, J. M., Betlach, C. J. & Meistrich, M. L. (1980) *J. Exp. Zool.* **214**, 13–20.
24. Kleene, K. C., Distel, R. J. & Hecht, N. B. (1983) *Dev. Biol.* **98**, 455–464.
25. Roosen-Runge, E. C. (1955) *Anat. Rec.* **123**, 385–398.
26. Pintar, J. E., Schachter, B. S., Herman, A. B., Durgerian, S. & Krieger, D. T. (1984) *Science* **225**, 632–634.
27. Ritzén, E. M., Boitani, C., Parvinen, M., French, F. C. & Foldman, M. (1982) *Mol. Cell. Endocrinol.* **25**, 25–33.
28. Bergh, A. (1983) *Int. J. Androl.* **6**, 57–65.
29. Russell, L. (1977) *Am. J. Anat.* **148**, 301–312.
30. Galdieri, M. & Monaco, L. (1983) *Cell Differ.* **13**, 49–55.
31. Welsh, M. J., Ireland, M. E. & Treisman, G. J. (1984) *Ann. N.Y. Acad. Sci.* **438**, 576–578.
32. Cheng, M. C., Clements, J. A., Smith, A. I., Lolait, S. J. & Funder, J. W. (1985) *J. Clin. Invest.* **75**, 832–835.
33. Gizang-Ginsberg, E. & Wolgemuth, D. J. (1985) *Dev. Biol.* **111**, 293–305.
34. Gerendai, I., Nemeskeri, A. & Csernus, V. (1983) *Andrologia* **15**, 398–403.
35. Gerendai, I., Shaha, C., Thau, R. & Bardin, C. W. (1984) *Endocrinology* **115**, 1645–1647.
36. Kleene, K. C., Distel, R. J. & Hecht, N. B. (1984) *Dev. Biol.* **105**, 71–79.