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Corticotropin-releasing factor (CRF)-like immunoreactivity in the vertebrate endocrine pancreas

(immunocytochemistry/hormones/islets of Langerhans/evolution)

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ABSTRACT **The light microscopic immunocytochemical localization of corticotropin-releasing factor (CRF) is described in the endocrine pancreas of several species representing the major classes of vertebrates: fishes (channel catfish, *Ictalurus punctatus*), amphibians (African clawed toad, *Xenopus laevis*), reptiles (chameleon, *Anolis carolinensis*), birds (chicken, *Gallus domesticus*), and several mammals (rat, mouse, cat, rhesus monkey, and man). The CRF-containing cells are scattered over the entire islet tissue in primates and cat, whereas in rat and mouse they are located at the periphery of the islets. In the chicken and catfish, the CRF-containing cells are found in a central location within islets and form larger clusters or cords. Single cells with CRF-like immunoreactivity are interspersed between acinar cells of the exocrine pancreas in all species studied. The CRF cells show a substantial topographical overlap with glucagon cells, but their precise identity and function remain to be determined.**

Corticotropin-releasing factor (CRF), a 41-amino acid peptide, has been isolated and characterized from extracts of ovine hypothalamus as a potent stimulator of the secretion of corticotropin (ACTH) and β-endorphin (1–3). Immunocytochemical studies have established that most of the CRF in the hypothalamus is located both in neurons of the paraventricular nucleus and in terminals around portal capillaries of the median eminence (4–7). CRF-like immunoreactivity has been identified in hypophysial portal blood (8). The neural pathways through which CRF reaches the median eminence also have been described (9). Like other neuropeptides, CRF is widely distributed in extrahypothalamic areas of the brain, determined by both radioimmunoassay (10) and immunocytochemistry (11–13). In addition to stimulating the release of ACTH and β-endorphin, CRF has a broad range of pharmacological effects, which include changes in behavior, heart rate, blood pressure, and in blood concentrations of epinephrine, norepinephrine, glucagon, and glucose (14–18). Thus, CRF appears to be an important regulatory peptide mediating stress-related physiological responses and predictably possessing other, hitherto unsuspected, hormone- or neurotransmitter-like activities. On the basis of current hypotheses concerning the common evolutionary origin of hormones and neurotransmitters (19–21), CRF may be expected to occur at peripheral sites, such as autonomic and sensory ganglia or nerve fibers and terminals, or in endocrine cells scattered in a variety of organs.

This report describes the immunocytochemical localization of CRF-like immunoreactivity in the endocrine pancreas in representative species of the major classes of vertebrates, including man.

MATERIALS AND METHODS

Sources of Tissues. Five adult male Sprague–Dawley rats were obtained from ARS/Sprague–Dawley (Madison, WI). One Sprague–Dawley rat and one White Leghorn chicken (*Gallus domesticus*) were sacrificed at approximately 1 day of age. Three adult female mice (C57BL/6J) were obtained from Blue Spruce Farms (Altamont, NY). One African clawed toad (*Xenopus laevis*) and pancreatic tissue from two channel catfish (*Ictalurus punctatus*) were gifts from L. J. Haverkamp (Neurobiology Program, University of North Carolina) and J. E. Brinn (Department of Anatomy, East Carolina University), respectively. Three lizards (*Anolis carolinensis*) were purchased from Carolina Biological Supply (Burlington, NC). Pancreatic tissue from two adult cats and two adult rhesus monkeys (*Macaca mulatta*) also was studied. Human pancreas (courtesy of E. K. MacRae and F. D. Dalldorf) was from a 1-year-old infant who died in an accident.

Preparation of Tissues. Rat, mouse, cat, monkey, chicken, toad, and lizard tissues were fixed by perfusion with 4% paraformaldehyde as described (22). The pancreata from both the human and the catfish were fixed by immersion, the former in 10% formalin and the latter in Bouin's solution. Fixation was followed by washing, dehydration, and embedding in paraffin. Serial sections were cut at 5 or 8 μm.

Antisera. All primary antisera (except the antiinsulin serum) were prepared in rabbits. The antiserum to CRF (SV-22) was made by coupling synthetic ovine CRF (gift from D. H. Coy, Department of Medicine, Tulane University) to bovine serum albumin with glutaraldehyde (7). The glucagon antiserum (R-32) against highly purified porcine glucagon was a gift from K. P. Lund (Department of Physiology, University of North Carolina) and D. J. Sanders (Department of Physiology, Medical School, Newcastle upon Tyne, England). The antiserum recognizes the midportion of the glucagon molecule and binds both pancreatic glucagon and its precursors. The antiserum (6-F) to somatostatin (somatotropin release-inhibiting factor, SR-IF) was made by using synthetic SR-IF-14 coupled to bovine thyroglobulin with glutaraldehyde and was a gift from E. R. Perl (Department of Physiology, University of North Carolina). The insulin antiserum (no. 7) was raised in a guinea pig against purified porcine insulin and was a gift from L. E. Underwood (Department of Pediatrics, University of North Carolina). The antiserum to bovine pancreatic polypeptide (PP) was purchased from Accurate Chemical (Westbury, NY). Optimal dilutions for all antisera ranged between 1:2,000 and 1:10,000.

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Abbreviations: ACTH, corticotropin; CRF, corticotropin-releasing factor; PP, pancreatic polypeptide; SR-IF, somatotropin release-inhibiting factor or somatostatin.

Immunocytochemistry. Slides were deparaffinized and stained by using the peroxidase-antiperoxidase complex (Sternberger–Meyer Immunocytochemicals, Jarrettsville, MD) according to the "double PAP" method (23). The distribution of CRF was compared to that of other pancreatic hormones by localizing two antigens in the same section with contrasting chromogens (24).

Controls. Method specificity (25) was tested by a series of increasing dilutions of the primary antiserum, resulting in a gradual decrease and eventual disappearance of the immunostaining. Antiserum specificity was tested by absorption of the anti-CRF serum with synthetic CRF, bovine insulin (Sigma), glucagon (Sigma), synthetic SR-IF-14 (Beckman Bioproducts, Palo Alto, CA), pentagastrin (Bachem Fine Chemicals, Marina Del Rey, CA), γ-endorphin (Peninsula Laboratories, San Carlos, CA), bovine PP (gift from R. E. Chance, Lilly Research Laboratories, Indianapolis, IN), and synthetic human gastrin I, motilin, secretin, gastric inhibitory peptide, and vasoactive intestinal polypeptide (gifts from D. H. Coy, Tulane University). The anti-CRF serum also has been characterized by immunodiffusion and radioimmunoassay (7) and by immunocytochemistry on brain tissue (9, 11).

Sections stained for the simultaneous localization of two antigens, and adjacent sections stained alternatively for CRF and either insulin, glucagon, SR-IF, or PP, provided additional controls of specificity. These techniques also were used in attempts to determine whether CRF-like immunoreactivity occurred in

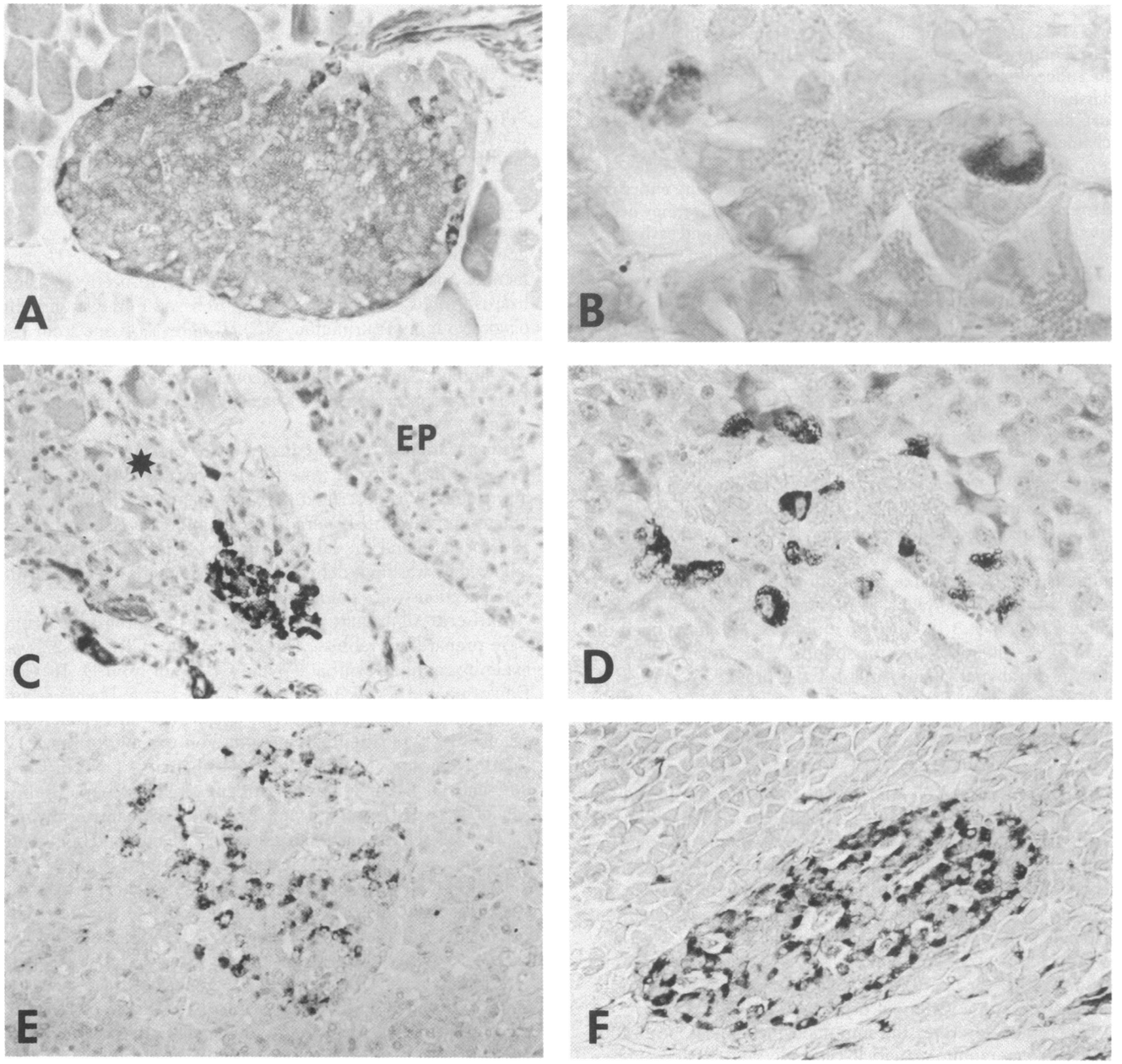

FIG. 1. CRF-like immunoreactivity in pancreatic tissue. (*A*) Islet of Langerhans from the mouse pancreas. Note peripheral localization of CRF cells. (×250.) (*B*) CRF cell in the exocrine pancreas of the toad (*X. laevis*). The cell is wedged between exocrine cells at the periphery of an acinus. (×1,000.) (*C*) Cat pancreas. A cluster of CRF cells is attached to a nerve ganglion (*) located in the interlobular connective tissue. EP, exocrine pancreas. (×250.) (*D*) Islet of Langerhans from the cat pancreas. CRF cells are present both at the periphery and in the central regions of the islet. (×400.) (*E*) Islet of Langerhans from the pancreas of a 1-year-old human. The CRF cells are scattered in all regions of the islet. (×250.) (*F*) Islet of Langerhans from the pancreas of a rhesus monkey. The CRF cells are distributed evenly over the entire islet. (×250.)

the same cells together with any of the known pancreatic hormones. The results were analyzed with a Zeiss Universal microscope equipped with a drawing attachment (camera lucida).

RESULTS

Immunoreactive CRF was detected in pancreatic cells in all of the species studied (Figs. 1, 2, and 3). There was substantial CRF-like immunoreactivity in the pancreas of the 1-year-old human and the 1-day-old rat and chicken. Absorption of the anti-CRF serum with synthetic CRF (7.5 $\mu g/\mu l$) abolished all immunostaining, whereas absorption with excess insulin, glucagon, SR-IF, PP, gastrin, γ-endorphin, motilin, secretin, gastric inhibitory peptide, or vasoactive intestinal polypeptide had no effect on the intensity or distribution of the reaction product. Cells containing CRF-like immunoreactivity were generally small, oval, or polygonal and were only rarely stellate. The immunostaining was granular and was localized over the cytoplasm.

In the mouse and the rat, CRF cells were located at the periphery of the islets of Langerhans (Figs. 1*A* and 3*A*). Some of these cells were attenuated and often appeared to constitute the outermost boundary of the islet. In all of the species studied, CRF cells were found scattered in the exocrine tissue, often wedged between acinar cells like endocrine cells in the gastrointestinal epithelium (Fig. 1*B*). In the cat, a large cluster of CRF-positive cells was found closely attached to a nerve ganglion in the interlobular connective tissue, entirely separate from the pancreatic parenchyma (Fig. 1*C*). The same cluster contained a large number of glucagon cells as well. No CRF-containing cells were seen in the epithelium of the pancreatic ducts, although SR-IF cells in such locations were readily detected, confirming the observations of Hökfelt *et al.* (26). In the human, the monkey, and the cat, the cells with CRF-like immunoreactivity were scattered throughout the islets, with only a slight tendency for greater numbers to occur at the periphery (Fig. 1 *D–F*). However, larger areas (sometimes as much as one-third) of certain islets were completely free of CRF cells. A small proportion of the islets did not appear to contain any CRF at all. The CRF cells were especially numerous in the chicken (Fig. 2*A*) and the catfish (Fig. 2*B*). In both species, they were present in the central regions of certain islets, whereas they seemed to be absent from others. In both chicken and catfish, several neighboring CRF cells formed cords or columns in close apposition to capillaries. Only the duodenal portions of the lizard and toad pancreas were studied. No islets were present in this region, but individual cells with CRF-like immunoreactivity were seen dispersed within the endocrine parenchyma (Fig. 2 *C* and *D*). In the lizard, CRF cells were more frequent in the vicinity of small intralobular ducts.

Immunostaining of adjacent sections from rat, mouse, cat, chicken, and human pancreata and dual staining for two antigens in the same section from rat pancreas established that the CRF-containing cells in these species were distinct from insulin-containing (B) cells (for the classification of gastroenteropancreatic endocrine cells, see ref. 27). The same tests, together with the nonstellate morphology of the CRF cells, indicated that the latter also were distinct from SR-IF-containing (D) and PP-containing (F) cells (cf. refs. 28 and 29). However, an extensive, although incomplete, topographical overlap was seen between the cells containing CRF and those containing glucagon (A cells; Fig. 3).

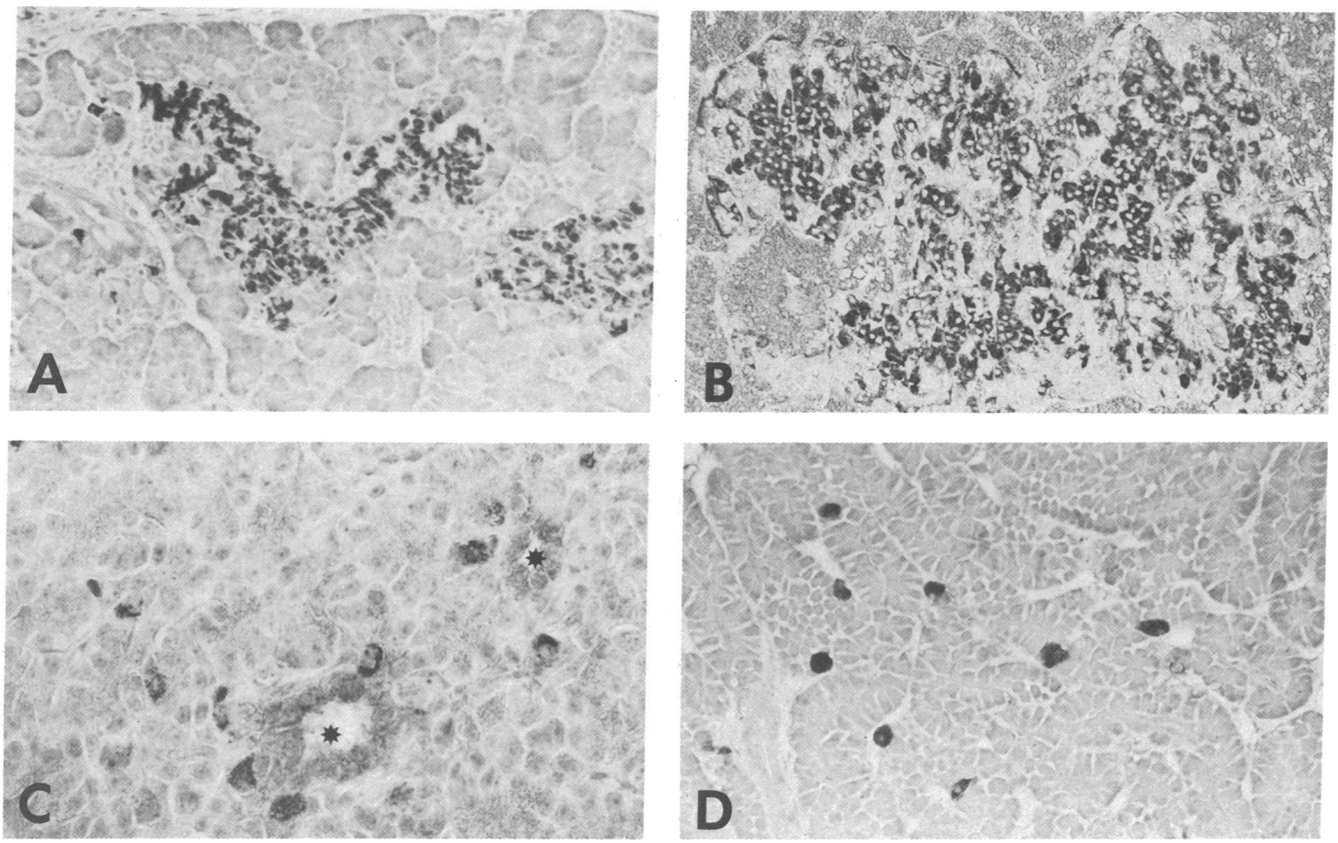

FIG. 2. CRF-like immunoreactivity in pancreatic tissue. (*A*) Pancreas from a 1-day-old chicken. Numerous CRF cells form clusters and cords in the central regions of the islets. (×200.) (*B*) Pancreas from the catfish (*I. punctatus*). Dense clusters of CRF cells are located centrally in the islet. (×160.) (*C*) Duodenal lobe of the lizard (*A. carolinensis*) pancreas. Note scattered CRF cells in the exocrine parenchyma in the vicinity of two small ducts (*). (×400.) (*D*) Duodenal lobe of the toad (*X. laevis*) pancreas. CRF cells are scattered in the exocrine parenchyma. (×200.)

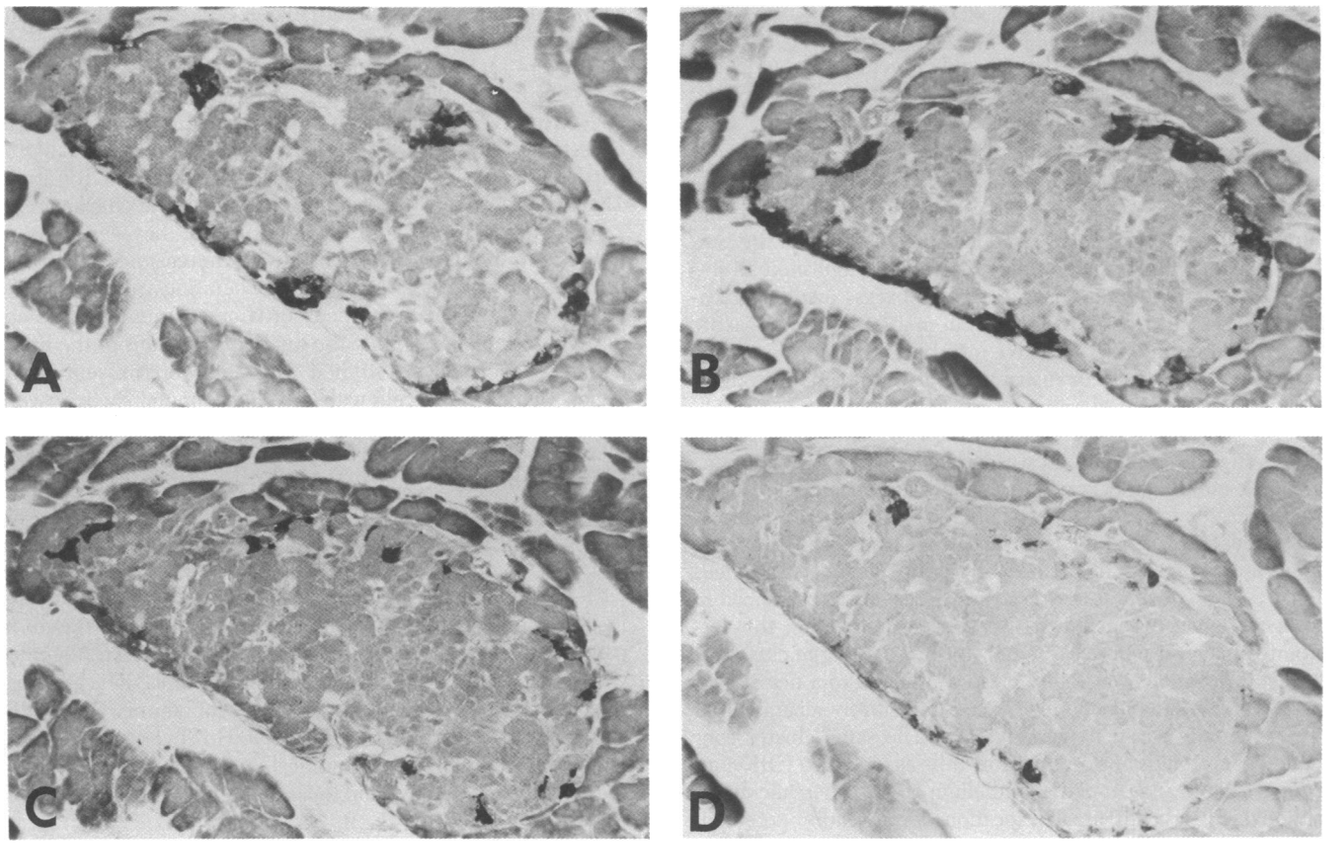

FIG. 3. Adult rat pancreas. Near-adjacent 5-μm paraffin sections showing the distribution of immunoreactive CRF (*A*), glucagon (*B*), SR-IF (*C*), and PP (*D*). For detailed explanation, see the text. (×200.)

DISCUSSION

Our results demonstrate that cells with CRF-like immunoreactivity can be detected with immunocytochemistry in the endocrine pancreas of representative species of the major classes of vertebrates—i.e., fishes, amphibians, reptiles, birds, and mammals (including man). Extensive tests of specificity indicate that the antigen we have localized shares major determinants with synthetic ovine CRF but is different from a large number of known pancreatic and gastrointestinal hormones, including the recently described preproglucagon molecule (30). The precise relationship of the pancreatic CRF-like material to brain CRF and to other CRF-like peptides (cf. ref. 1) can be established only by future extraction and sequence analysis studies.

The majority of the cells with CRF-like immunoreactivity in the rat and the mouse are located at the periphery of the islets of Langerhans, like glucagon-, SR-IF-, and PP-containing cells. In the human, monkey, and cat pancreas, CRF cells are distributed over the entire islet tissue, whereas in the chicken and the catfish they are located centrally within the islets. In addition, scattered individual cells with CRF-like immunoreactivity are present in the exocrine pancreas in all of the species studied. Observations on adjacent serial sections and on dual stainings for two antigens in the same section from rat, cat, mouse, and chicken pancreatic tissue did not provide evidence for colocalization of CRF with insulin, SR-IF, or PP. However, a substantial, although still incomplete, overlap was seen between the cells containing CRF and those containing glucagon. Thus, it appears that the pancreatic CRF cell represents either a distinct cell type closely associated with glucagon cells or a subpopulation of the glucagon cells. Additional light and electron microscopic studies will be required to fully characterize the pancreatic CRF cell.

The presence of CRF-containing cells in the human pancreas is particularly interesting. In the human, islet cell tumors accompanied by an "ectopic ACTH syndrome" have been described and have been shown to contain bioassayable CRF-like activity (31). The presence of CRF in such tumors can be better understood in the light of our findings. It may be noted that another recently discovered hypothalamic peptide, growth hormone-releasing factor, was isolated from a human pancreatic tumor (32).

Although little is known about the peripheral role(s) of CRF, several interesting hypotheses may be proposed with regard to the functional significance of our results. Various forms of stress may be associated with decreased glucose tolerance, hyperglycemia, and elevated glucagon concentration (cf. ref. 33). These effects are generally regarded as neurogenic and are thought to be mediated through the rich autonomic innervation of islet cells (34). Local "paracrine" (35) regulation of glucagon release by CRF may represent a complementary mechanism, which may be the stimulatory counterpart of the inhibitory effects of SR-IF (36). Intracerebroventricular administration of CRF is followed by a significant increase in blood glucagon and glucose concentrations (14).

The blood circulation of the pancreas is organized as an insulo-acinar portal system, so that long veins which drain the capillaries of the islets divide into a secondary capillary network to supply the exocrine (acinar) tissue (37). This anatomical arrangement would permit pancreatic CRF to influence the secretory activities of the exocrine pancreas. Similar interactions have been described for other islet hormones (38, 39). The consistent pres-

ence of CRF cells scattered among the acinar cells in all species studied reinforces this possibility.

It also has been shown that CRF as well as the related peptides sauvagine and urotensin I possess significant vasomotor activities (16, 40–42). Thus, pancreatic CRF may play a role in the local regulation of blood flow through the endocrine or the exocrine pancreas. Finally, it is possible that pancreatic CRF, like insulin, glucagon, and PP, enters the general circulation to function as a "hormone" in the classical sense. However, its peripheral target sites are currently unknown.

In summary, we have described the presence of CRF-like immunoreactivity in endocrine cells of the pancreas of catfish, toad, lizard, chicken, mouse, rat, cat, monkey, and man. The cells with CRF-like immunoreactivity occur in close association with glucagon cells, but their precise identity remains to be established. The functional significance of these findings is not known at this time. However, the presence of a CRF-like antigen in the pancreas over a wide phylogenetic range points to considerable physiological importance for this recently discovered neuropeptide and provides further support for the concept of a close evolutionary and embryonic relationship between the nervous system and the endocrine system.

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1. Vale, W., Spiess, J., Rivier, C. & Rivier, J. (1981) *Science* **213**, 1394–1397.
2. Spiess, J., Rivier, J., Rivier, C. & Vale, W. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6517–6521.
3. Rivier, C., Brownstein, M., Spiess, J., Rivier, J. & Vale, W. (1982) *Endocrinology* **110**, 272–278.
4. Bugnon, C., Fellmann, D., Gouget, A. & Cardot, J. (1982) *C.R. Hebd. Seances Acad. Sci. Ser. D* **294**, 279–284.
5. Paull, W. K., Schöler, J., Arimura, A., Meyers, C. A., Chang, J. K., Chang, D. & Shimizu, M. (1982) *Peptides* **3**, 183–191.
6. Bloom, F. E., Battenberg, E. L. F., Rivier, J. & Vale, W. (1982) *Regul. Pept.* **4**, 43–48.
7. Vigh, S., Merchenthaler, I., Torres-Aleman, I., Sueiras-Diaz, J., Coy, D. H., Carter, W. H., Petrusz, P. & Schally, A. V. (1982) *Life Sci.* **31**, 2441–2448.
8. Gibbs, D. M. & Vale, W. (1982) *Endocrinology* **111**, 1418–1420.
9. Merchenthaler, I., Vigh, S., Petrusz, P. & Schally, A. V. (1983) *Regul. Pept.*, in press.
10. Fishman, A. J. & Moldow, R. L. (1982) *Peptides* **3**, 149–153.
11. Merchenthaler, I., Vigh, S., Petrusz, P. & Schally, A. V. (1982) *Am. J. Anat.* **165**, 385–396.
12. Olschowka, J. A., O'Donohue, T. L., Mueller, G. P. & Jacobowitz, D. M. (1982) *Neuroendocrinology* **35**, 305–308.
13. Cummings, S., Elde, R., Ells, J. & Lindall, A. (1982) *Abstr. Soc. Neurosci.* **8**, Part 1, 110.
14. Brown, M. R., Fisher, L. A., Spiess, J., Rivier, C., Rivier, J. & Vale, W. (1982) *Endocrinology* **111**, 928–931.
15. Fisher, L. A., Rivier, J., Rivier, C., Spiess, J., Vale, W. & Brown, M. R. (1982) *Endocrinology* **110**, 2222–2224.
16. Kalin, N. H., Shelton, S. E., Kraemer, G. W. & McKinney, W. T., Jr. (1982) *Lancet* **ii**, 1041.
17. Britton, D. R., Koob, G. E., Rivier, J. & Vale, W. (1982) *Life Sci.* **31**, 363–368.
18. Morley, J. E. & Levine, A. S. (1982) *Life Sci.* **31**, 1459–1464.
19. Pearse, A. G. E. & Takor Takor, T. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 2288–2294.
20. Acher, R. (1981) *Trends Neurosci.* **4**, 225–229.
21. Roth, J., LeRoith, D., Shiloah, J., Rosenzweig, J. L., Lesniak, M. A. & Havrankova, J. (1982) *N. Engl. J. Med.* **306**, 523–527.
22. Ordronneau, P. & Petrusz, P. (1980) *Am. J. Anat.* **158**, 491–506.
23. Ordronneau, P., Lindström, P. B.-M. & Petrusz, P. (1981) *J. Histochem. Cytochem.* **29**, 1397–1404.
24. Joseph, S. A. & Sternberger, L. A. (1979) *J. Histochem. Cytochem.* **27**, 1430–1437.
25. Petrusz, P., Ordronneau, P. & Finley, J. C. W. (1980) *Histochem. J.* **12**, 333–348.
26. Hökfelt, T., Efendič, S., Hellerström, C., Johansson, O., Luft, R. & Arimura, A. (1975) *Acta Endocrinol.* **80**, Suppl. 200, 1–41.
27. Solcia, E., Capella, C., Ruffa, B., Frigerio, B., Usellini, L. & Fiocca, R. (1980) in *Gastrointestinal Hormones*, ed. Glass, G. B. J. (Raven, New York), pp. 1–17.
28. Rahier, J. & Wallon, J. (1980) *Cell Tissue Res.* **209**, 365–370.
29. Munger, B. L. (1981) in *The Islets of Langerhans*, eds. Cooperstein, S. J. & Watkins, D. (Academic, New York), pp. 3–34.
30. Lund, K. P., Goodman, R. H., Dee, P. C. & Habener, J. F. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 345–349.
31. Amatruda, T. T., Jr., & Upton, G. V. (1974) *Ann. N.Y. Acad. Sci.* **230**, 168–180.
32. Guillemin, R., Brazeau, P., Böhlen, P., Esch, F., Ling, N. & Wehrenberg, W. B. (1982) *Science* **218**, 585–587.
33. Bloom, S. R. (1976) *Horm. Metab. Res. Suppl. Ser.* **6**, 85–90.
34. Bloom, S. R., Edwards, A. V. & Vaughan, N. J. (1974) *J. Physiol. (London)* **236**, 611–623.
35. Fujita, T. & Kobayashi, S. (1979) *Trends Neurosci.* **2**, 27–30.
36. Unger, R. H., Raskin, P., Srikant, C. B. & Orci, L. (1977) *Recent Prog. Horm. Res.* **33**, 477–517.
37. Fujita, T., Yanatori, Y. & Murakami, T. (1976) in *Endocrine Gut and Pancreas*, ed. Fujita, T. (Elsevier, Amsterdam), pp. 347–357.
38. Fujita, T. & Watanabe, Y. (1973) in *Gastro-Entero-Pancreatic Endocrine System. A Cell-Biological Approach*, ed. Fujita, T. (Igaku Shoin, Tokyo), pp. 164–173.
39. Taylor, I. L., Solomon, T. E., Walsh, J. H. & Grossman, M. I. (1979) *Gastroenterology* **76**, 524–528.
40. Brown, M. R., Fisher, L. A., Spiess, J., Rivier, J., Rivier, C. & Vale, W. (1982) *Regul. Pept.* **4**, 107–114.
41. MacCannel, K. & Lederis, K. (1977) *J. Pharmacol. Exp. Ther.* **203**, 38–46.
42. Melchiorri, P. & Erspamer, V. (1981) in *Gut Hormones*, eds. Bloom, S. R. & Polak, J. M. (Churchill Livingstone, Edinburgh), pp. 38–42.