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Primary structure of rat chromogranin A and distribution of its mRNA

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The primary structure of rat chromogranin A has been deduced from a rat adrenal cDNA clone. A comparison of rat and bovine chromogranin A reveals several similar features: clusters of polyglutamic acid, similar amino acid composition, position of seven of 10 pairs of basic amino acids, identical placement of the only two cysteine residues, a highly conserved N- and C-terminus, and a sequence homologous to porcine pancreastatin 1–49 [(1986) Nature 324, 476–478]. Unique features of rat chromogranin A are an eicosaglutamine sequence and two potential *N*-linked glycosylation sites. Chromogranin A mRNA is detectable in adrenal medulla, anterior pituitary, cerebral cortex, and hippocampus, as well as tumor cell lines derived from pancreas, pituitary, and adrenal medulla.

cDNA cloning; Sequence homology; mRNA; Chromogranin A; (Rat adrenal)

1. INTRODUCTION

Chromogranin A, an acidic glycoprotein [2,3], was the first-discovered constituent of adrenomedullary chromaffin granules [4–6]. This protein has been found in many endocrine and neural tissues [7–11]. Recently, the primary structure of bovine chromogranin A was determined [12,13]. It is a 431-amino-acid protein with an apparent molecular mass of 75 kDa and an actual molecular mass of 48 kDa. Several structural features of bovine chromogranin A have been noted: it contains regions of homology to two calcium-binding proteins, clusters of polyglutamic acid, an RGD sequence [13] which in fibronectin subserves attachment to the cell surface [14], eight dibasic amino acid residues which may be sites of specific proteolytic processing, and potential phosphorylation sites [12,13,15,16]. The biological actions of chromogranin A remain unknown, partly due to lack of structural information about chromogranin A in rat, the most widely studied and accessible animal model for endocrine function. Here, we describe the cloning and primary sequence of rat prechromogranin A, compare it to the bovine sequence, and describe the distribution of its mRNA in rat endocrine tumors and tissues.

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2. MATERIALS AND METHODS

2.1. *Isolation of rat chromogranin A cDNA clones*

A cDNA library of rat adrenal mRNA was constructed in pcDV1 vector according to Okayama and Berg [17]. The library contains 1×10^7 recombinant transformants. Approx. 2×10^5 transformants were screened by colony hybridization with a ^{32}P-labeled RNA probe mixture. Four complementary RNA probes to different portions of the bovine chromogranin A mRNA coding region were generated by in vitro transcription of linearized pGem plasmid containing fragments of the cDNA, pCHRG4A [17]. The probe hybridized to three clones. The longest clone, pCHRG12B, was determined to be incomplete in length by restriction and sequence analysis. The library was plated again and hybridized with a nick-translated 995 bp *Pst*I fragment of pCHRG12B. Ten positive clones were obtained after screening 5×10^5 colonies. The clone pCHRG2H was determined to be the longest. Overlapping fragments of the cDNA were isolated, subcloned into M13, and sequenced by the method of Sanger.

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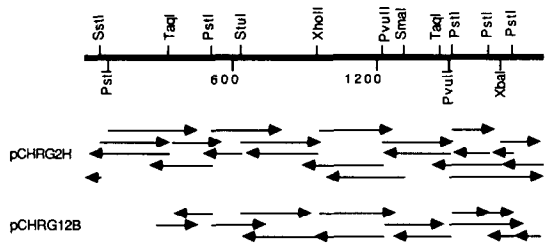

Fig.1. DNA sequencing strategy of pCHRG2H and pCHRG12B. Restriction map of cDNA shows sites used to subclone fragments into M13 for sequencing. Solid arrows below map indicate the direction and extent of sequencing.

2.2. *Northern blot analysis*

Total RNAs were extracted from tissues and cell lines with phenol after digestion with proteinase K or guanidinium hydrochloride. RNAs were denatured and electrophoresed on 1% agarose gel containing 2.2 M formaldehyde. Following electrophoresis, the RNAs were stained with ethidium bromide, transferred onto GeneScreen (NEN) and baked for 2 h at 80°C. The Northern blot was pre-hybridized at 45°C for 24 h, then hybridized at 45°C for 20 h with a nick-translated 995 bp *PstI* fragment of pCHRG2H. The blot was washed in 0.2 × SSC (1 × SSC = 150 mM NaCl/15 mM sodium citrate)/0.1% SDS at 55°C, then autoradiographed for 12–48 h at −70°C with Dupont Lightning Plus intensifying screens.

2.3. *In situ hybridization histochemistry*

Tissue preparation, hybridization, and autoradiography were performed according to Siegel and Young [18]. ^{35}S-labeled messenger and complementary RNA probes were produced by in vitro transcription of linearized pGEM plasmid containing 995 bp *PstI* fragment of pCHRG12B.

3. RESULTS AND DISCUSSION

A rat prechromogranin A cDNA was obtained from a rat adrenal clone bank probed with the bovine cDNA, pCHRG4A [12]. The rat clone, pCHRG2H, is 1871 nucleotides in length, and encodes a protein of 466 amino acids (fig.2). A hydrophobic signal sequence of 18 amino acids is 83% homologous to the corresponding bovine signal peptide (fig.3). The actual molecular mass of rat chromogranin A deduced from this sequence is 50.2 kDa. The apparent molecular mass of rat chromogranin A on SDS-PAGE is 86 kDa [19]. Rat chromogranin A is therefore similar to the bovine protein in its anomalous behavior upon denaturing gel electrophoresis [12,13]. The greatest homology between rat and bovine chromogranins lies in the N-terminal 76, and the C-terminal 108 amino acids (86 and 89% homology, respectively). Only two cysteines are found in rat chromogranin A; they are positioned 20 amino acids apart in the N-terminus, exactly as in the bovine sequence. Benedum et al. [20] have noted a similarly placed pair of cysteines in the sequence of human chromogranin B (secretogranin I), an acidic sulfated glycoprotein co-stored with chromogranin A in adrenomedullary chromaffin granules. Rat chromogranin A contains 10 pairs of basic amino acid residues, which are potential signals for proteolytic processing of hormone precursors [12,13]. Seven of these are similarly positioned in the bovine protein, and six of the invariant pairs are clustered at the C-terminus. In rat and bovine chromogranin A, a peptide designated WE-14 (fig.3) is flanked by pairs of basic amino acids, and is identical in both proteins except for a single conservative amino acid substitution (K for R) at the third position (fig.2). The sequence of this peptide is probably highly conserved in human, cow, rat, chicken, frog, sheep, and fish, since specific WE-14 immunoreactivity is found in chromaffin tissue of these species (Rieker, S. et al., unpublished). We suggest that the strong sequence conservation at the N-terminus of chromogranin A may be related to its intracellular role, including targeting to secretory vesicles, while sequence conservation at the C-terminus may be due to the presence of biologically active peptides for which chromogranin A is a prohormone. A prohormone function for chromogranin A is suggested by the homology to pancreastatin, a 49-amino acid polypeptide isolated from porcine pancreas and reported to inhibit insulin and somatostatin secretion from pancreas at nanomolar concentrations [1]. Porcine pancreastatin is 71% homologous to bovine chromogranin A 251–294 (prechromogranin A 269–312). This region of rat chromogranin A (residues 264–314; fig.2) possesses equal homology with the corresponding bovine sequence and porcine pancreastatin (59%). The homology between porcine pancreastatin 33–49, the region in which all the biological activity of the molecule resides, and the corresponding regions of bovine and rat chromogranins A is 76%. The C-terminal GK dipeptide which would be required for proteolytic cleavage and amidation is conserved in rat. However, the rat and bovine sequences differ N-terminally adjacent to the pancreastatin-like region. Identification of the cleavage sites of this

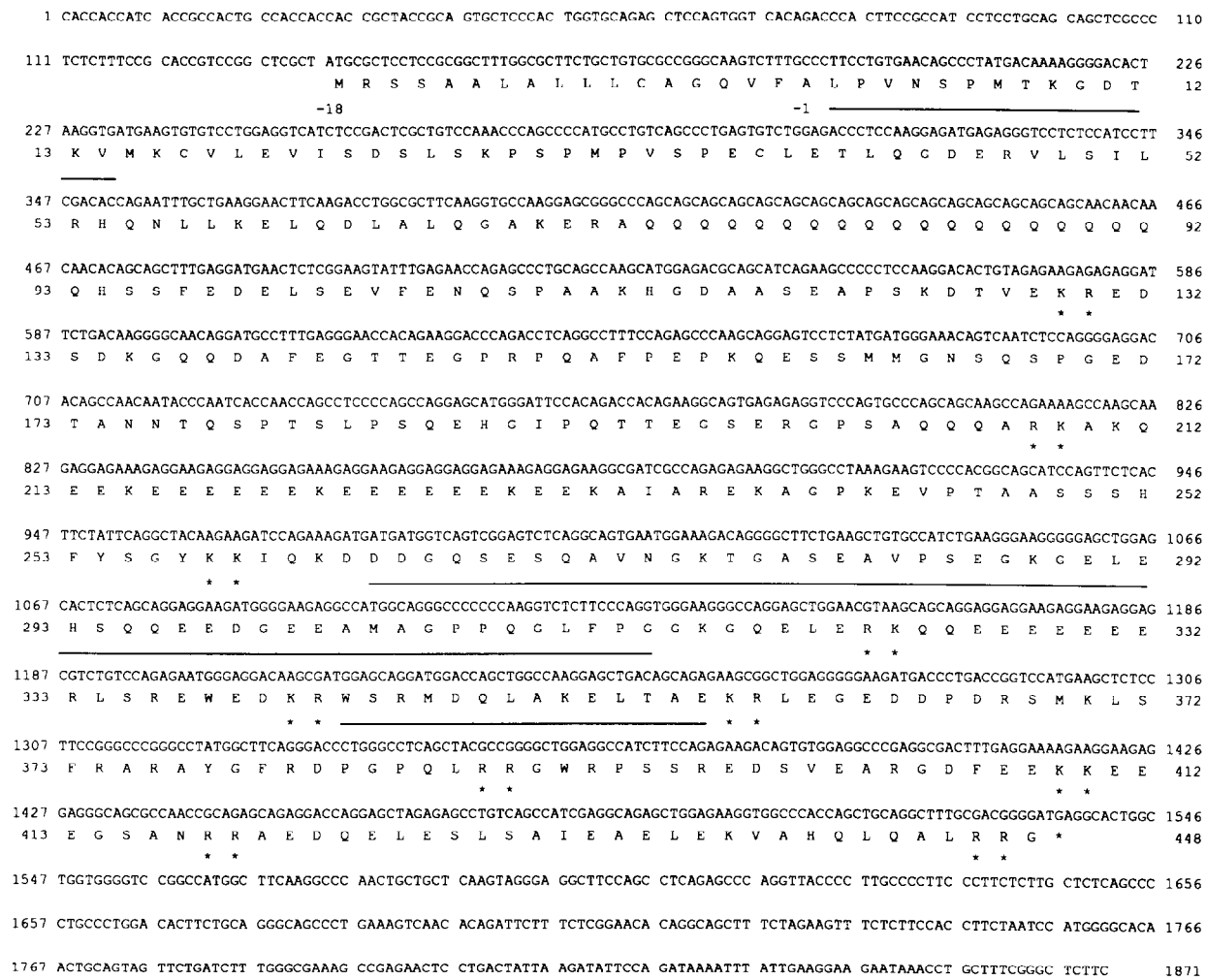

Fig.2. Primary structure of rat prechromogranin A mRNA. Negative numbers below the amino acid sequence indicate signal peptide. Numbers at the beginning and end of each sequence line refer to nucleotide and amino acid residues. Paired asterisks indicate dibasic amino acids. The underlined areas are: the N-terminus of rat chromogranin A 1–14 identical to that reported for β-granin [21], the region (residues 264–314) homologous to porcine pancreastatin, and the WE-14 region (residues 343–356).

peptide in rat, if indeed chromogranin A is its prohormone, must await processing studies employing rat-specific antibodies.

A peptide homologous to the N-terminus of bovine chromogranin A has been purified and sequenced from an insulinoma passaged in rats [21]. The N-terminus of this peptide, called β-granin, is identical to the sequence of rat chromogranin A [21]. Since β-granin is identical in known sequence to rat chromogranin A (fig.2), chromogranin A is probably the precursor for β-granin, as suggested by Hutton and co-workers [22,23] based on pulse-chase experiments in insulinoma tissue, demonstrating that β-granin derives from a chromogranin A-immunoreactive precursor. Rat chromogranin A 1–128 is most likely the primary structure of β-granin, since this fragment precedes a conserved KR processing sequence (fig.2). Hutton and co-workers have already postulated that a dibasic processing signal analogous to bovine chromogranin A KR114/115 is responsible for the generation of β-granin in pancreatic tissue [22,23]. They suggest that β-granin is a terminally processed product of chromogranin A in insulinoma and

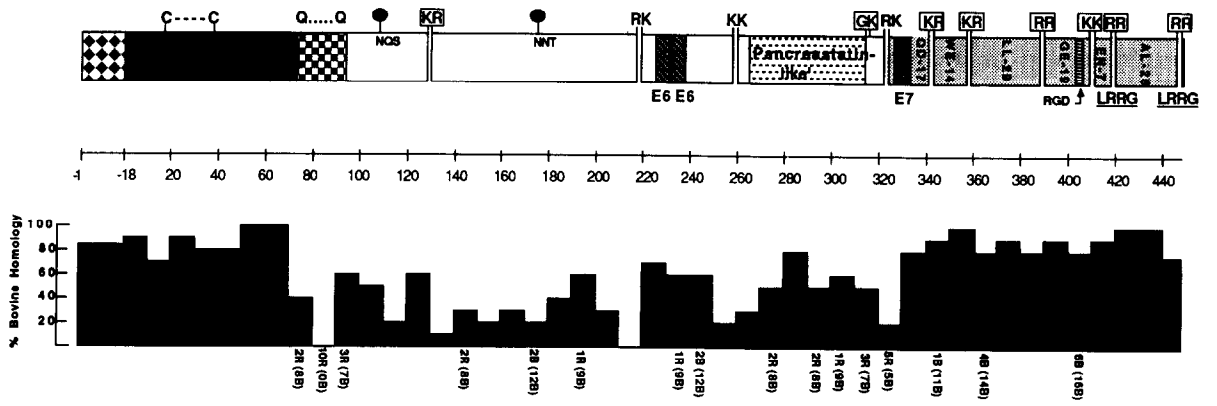

Fig.3. Sequence of rat prechromogranin A depicted schematically, with homology to bovine chromogranin A shown below. Percent homology of the 18-amino-acid signal sequence, and successive 10-amino-acid lengths of rat chromogranin, to the sequence of bovine prechromogranin A was calculated. Whenever the bovine or rat sequences were gapped to maximize alignment, the number of amino acids in the gap, and the species of the sequence gapped are indicated by a number and a letter R (rat) or B (bovine). Numbers in parentheses, followed by a B, indicate the number of residues in the corresponding region of the bovine sequence compared, when that number was not ten. Residues in the bovine sequence gapped were excluded from the homology calculation. Residues in the rat sequence gapped were included as 'zero homology' in the calculation of overall homology for the 10-amino-acid sequence. From left to right, diamond-patterned, signal sequence; dark-shaded, N-terminal sequence containing cysteine residues (C); checkered, polyglutaminyl sequence (Q...Q); hatched circles, potential glycosylation sites; hatched, polyglutamyl sequences; dotted, region homologous to porcine pancreastatin; light-shaded, peptide regions bounded by pairs of basic amino acids. Each peptide fragment is designated by the single-letter code for its N- and C-terminal amino acid residues, and the number of residues in the fragment. Positions of pairs of basic amino acids are shown above; boxed pairs are conserved between rat and bovine sequences. The position of the sequence RDG is indicated, as are the repeated sequences LRRG.

endocrine pancreas [22,23]. If so, patterns of chromogranin A processing are quite different in pancreas and adrenal medulla [24].

Chromogranin A immunoreactivity has been detected by histochemical and biochemical methods in bovine and rat endocrine tissues as well as in bovine brain [7–11]. In rat and cow, it is present in adrenal medulla, pituitary, parathyroid, thyroid, endocrine gut and pancreas [7–11]. A 2.1 kb chromogranin A mRNA is present in anterior pituitary, abundant in adrenal medulla, and not detectable in adrenal cortex of rat (fig.4). In bovine brain, chromogranin A immunoreactivity is found in cerebral cortex, hippocampus, amygdala, basal ganglia, and spinal cord by im-

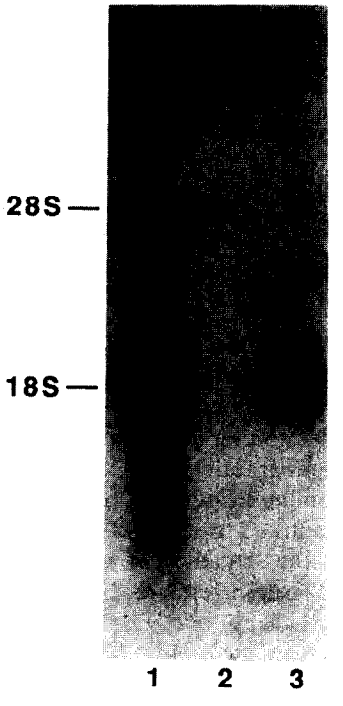

Fig.4. Distribution of chromogranin A mRNA in rat adrenal medulla (lane 1), rat adrenal cortex (lane 2), and rat anterior pituitary (lane 3) by Northern blot hybridization with clone pCHRG2H. All lanes contain 1 μg total RNA. Exposure was for 48 h.

munohistochemistry and radioimmunoassay [7,9]. Chromogranin A mRNA is detectable in bovine striatum [12]. In brain stem, chromogranin A immunoreactivity is co-localized to the major catecholaminergic cell groups [9]. The immunoreactivity was found mainly in the Golgi region of cells in these areas, although nerve terminals were stained in brain stem and spinal cord [9]. In the rat central nervous system (CNS), chromogranin A mRNA is detected in cerebral cortex and hippocampus (fig.5). The distribution of chromogranin A mRNA in the rat CNS is currently under investigation. Since chromogranin A immunoreactivity in bovine brain is visible mainly in cell bodies [9], chromogranin A may be processed to smaller non-immunoreactive peptides in axons and nerve terminals.

Several rat and mouse cell lines of neuroendocrine origin express chromogranin A mRNA (fig.5). These include PC12 pheochromocytoma [25], AtT-20/D16v-16 pituitary adenoma [26], and RIN-m insulinoma [27]. Chromogranin A mRNA is also expressed abundantly in 5F and 14B cell lines (fig.5), subclones of RIN-m that secrete insulin and somatostatin [28]. The presence of chromogranin A mRNA in insulinoma cell lines further supports the suggestion of Hutton and coworkers that chromogranin A is the precursor of β-granin [22,23]. Insulinoma, pheochromocytoma and pituitary adenoma cells may be excellent models for studying tissue specificity of chromogranin processing and secretion.

Fig.6 depicts chromogranin A mRNA in rat adrenal gland detected by in situ hybridization histochemistry. Messenger RNA expression is confined to the medulla, and is evenly distributed in it.

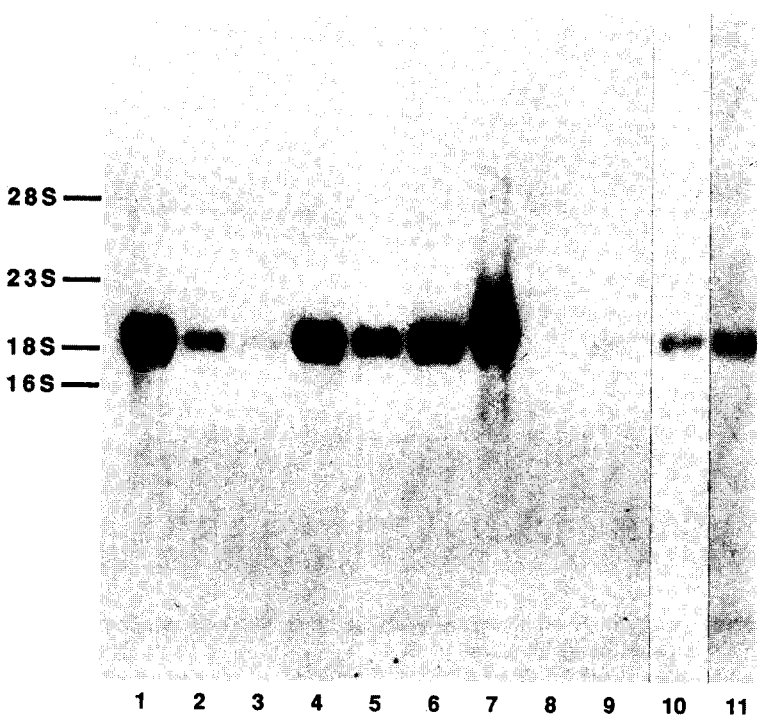

Fig.5. Northern blot analysis of chromogranin A mRNA in rat neuroendocrine tissues and tumor cell lines. RNA preparation and hybridization were performed as described in section 2. Positions of calf liver and *E. coli* ribosomal RNA markers are indicated. Rat adrenal medulla (1), rat cerebral cortex (2), rat hippocampus (3), 5F, an insulin-secreting subclone of the RIN-m cell line (4), 14B, a subclone of RIN-m that secretes somatostatin (5), RIN-m, a rat insulinoma cell line (6), AtT-20/D16v-16, a mouse pituitary tumor cell line (7), RAT-2, a fibroblast line (8), PC12, a rat pheochromocytoma cell line (9), PC12 (10), and rat hippocampus (11). All lanes contain 1 μg total RNA except for lane 2 which has 2.5 μg poly(A)$^+$ RNA. Lanes 1–9 are for a 12 h exposure. Lanes 10,11 are a 32 h exposure of lanes 3,9.

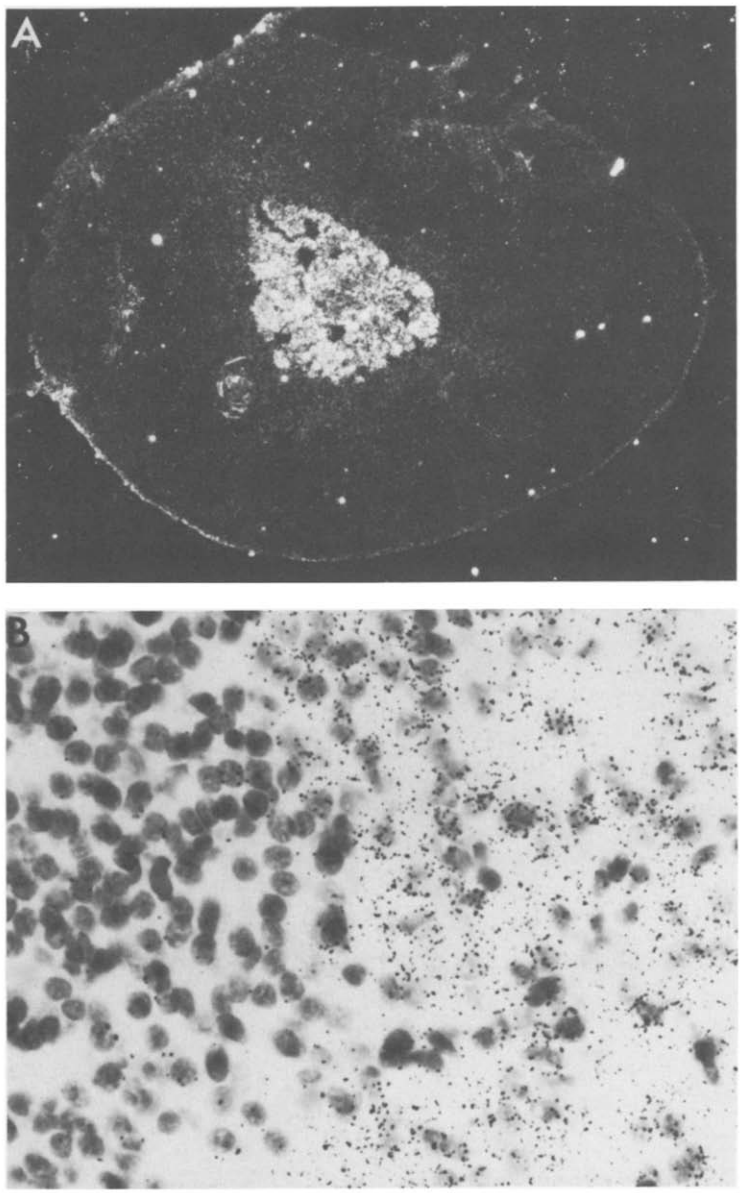

Fig.6. Localization of chromogranin A mRNA in the rat adrenal gland by in situ hybridization histochemistry. (A) Dark-field view showing heavy labeling in the medulla after hybridization with a complementary RNA probe (× 31). (B) Bright field view of chromogranin A mRNA positive cells in the medulla of the adrenal gland (× 500).

In bovine adrenal gland, chromogranin A mRNA is more abundantly distributed in the outer medulla, similar to phenylethanolamine *N*-methyltransferase (PNMT) mRNA (Siegel, R.E. et al., unpublished). Sietzen et al. have recently noted that chromogranin A in rat adrenal medulla, like PNMT, is decreased by hypophysectomy and restored by treatment with dexamethasone [29,30]. Chromogranin A mRNA in rat adrenal medulla is also decreased by hypophysectomy, demonstrating glucocorticoid regulation of chromogranin expression at a pretranslation locus (Fischer-Colbrie, R.

et al., unpublished). In cultured PC12 cells, chromogranin A mRNA is increased significantly in response to dexamethasone (Rausch, D. and Eiden, L.E., unpublished). Thus, the distribution of both chromogranin A and PNMT in rat and bovine adrenal medulla may be a function of different amounts of glucocorticoids, or differential sensitivity to glucocorticoid stimulation, from the outer to the inner portions of the gland.

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REFERENCES

[1] Tatemoto, K., Efendic, S., Mutt, V., Makk, G., Feistner, G.J. and Barchas, J.D. (1986) Nature 324, 476–478.
[2] Smith, A.D. and Winkler, H. (1967) Biochem. J. 103, 483–492.
[3] Smith, W.J. and Kirshner, N. (1967) Mol. Pharmacol. 3, 52–62.
[4] Banks, P. and Helle, K. (1965) Biochem. J. 97, 40C.
[5] Blaschko, H., Comline, R.S., Schneider, F.H., Silver, M. and Smith, A.D. (1967) Nature 215, 58–59.
[6] Kirshner, N., Sage, H.J., Smith, W.J. and Kirshner, A.G. (1966) Science 154, 529–531.
[7] O'Connor, D.T. and Frigon, R.P. (1984) J. Biol. Chem. 259, 3237–3247.
[8] Cohn, D.V., Elting, J.J., Frick, M. and Elde, R. (1984) Endocrinology 114, 1963–1974.
[9] Somogyi, P., Hodgson, A.J., DePotter, R.W., Fischer-Colbrie, R., Schober, M., Winkler, H. and Chubb, I.W. (1984) Brain Res. Rev. 8, 193–230.
[10] Nolan, J.A., Trojanowski, J.Q. and Hogue Angeletti, R. (1985) J. Histochem. Cytochem. 33, 791–798.
[11] Lassmann, H., Hagn, C., Fischer-Colbrie, R. and Winkler, H. (1986) Histochem. J. 18, 380–386.
[12] Iacangelo, A., Affolter, H.-U., Eiden, L.E., Herbert, E. and Grimes, M. (1986) Nature 323, 82–86.
[13] Benedum, U.M., Baeuerle, P.A., Konecki, D.S., Frank, R., Powell, J., Mallet, J. and Huttner, W.B. (1986) EMBO J. 5, 1495–1502.
[14] Pierschbacher, M.D. and Ruoslahti, E. (1984) Proc. Natl. Acad. Sci. USA 81, 5985–5988.
[15] Settleman, J., Fonseca, R., Nolan, J. and Hogue Angeletti, R. (1985) J. Biol. Chem. 260, 1645–1651.
[16] Rosa, P., Hille, A., Lee, R.W.H., Zanini, A., De Camilli, P. and Huttner, W.B. (1985) J. Cell Biol. 101, 1999–2011.
[17] Okayama, H. and Berg, P. (1983) Mol. Cell. Biol. 3, 280–289.
[18] Siegel, R.E. and Young, W.S. iii (1985) Neuropeptides 6, 573–580.
[19] Fischer-Colbrie, R. and Schober, M. (1987) J. Neurochem. 48, 262–270.
[20] Benedum, U.M., Lamouroux, A., Konecki, D.S., Rosa, P., Hille, A., Baeuerle, P.A., Frank, R., Lottspeich, F., Mallet, J. and Huttner, W.B. (1987) EMBO J. 6, 1203–1211.
[21] Hutton, J.C., Hansen, F. and Peshavaria, M. (1985) FEBS Lett. 188, 336–340.
[22] Hutton, J.C., Davidson, H.W., Grimaldi, K.A. and Peshavaria, M. (1987) Biochem. J. 244, 449–456.
[23] Hutton, J.C., Davidson, H.W. and Peshavaria, M. (1987) Biochem. J. 244, 457–464.
[24] Fischer-Colbrie, R., Hagn, C. and Schober, M. (1987) Ann. NY Acad. Sci. 493, 120–134.
[25] Greene, L.A. and Tischler, A.S. (1976) Proc. Natl. Acad. Sci. USA 73, 2424–2428.
[26] Yasumura, Y. (1968) Am. Zool. 8, 285–305.
[27] Gazdar, A.F., Chick, W.L., Oie, H.K., Sims, H.L., King, D.L., Weir, G.C. and Lauris, V. (1980) Proc. Natl. Acad. Sci. USA 77, 3519–3523.
[28] Bhathena, S.J., Oie, H.K., Gazdar, A.F., Voyles, N.R., Wilkins, S.D. and Recant, L. (1982) Diabetes 31, 521–531.
[29] Wurtman, R.J. and Axelrod, J. (1966) J. Biol. Chem. 241, 2301–2305.
[30] Sietzen, M., Schober, M., Fischer-Colbrie, R., Scherman, D., Sperk, G. and Winkler, H. (1987) Neuroscience 22, 131–139.