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Tissue-specific expression of the rat galanin gene

(neuropeptide/cDNA cloning/gene expression/gastrointestinal peptide/mRNA)

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ABSTRACT **We have isolated and characterized cDNAs encoding rat galanin from a cDNA library prepared from rat hypothalamic tissue. Analysis of these clones reveals that rat galanin is synthesized initially as part of a 124-amino acid precursor that includes a signal peptide, galanin (29 amino acids), and a 60-amino acid galanin mRNA-associated peptide. In the precursor, galanin includes a C-terminal glycine and is flanked on each side by dibasic tryptic cleavage sites. The deduced amino acid sequence of rat galanin is 90% similar to porcine galanin, with all three amino acid differences in the C-terminal heptapeptide. The predicted galanin mRNA-associated peptide includes a 35-amino acid sequence that is 78% similar to the previously reported porcine analogue. This sequence is set off by a single basic tryptic cleavage site and includes a 17-amino acid region that is nearly identical to the porcine counterpart. The high interspecies conservation suggests a biological role for this putative peptide. Blot hybridization analysis using rat genomic DNA is consistent with a single galanin-encoding gene. RNA blot analysis of total RNA prepared from rat tissues reveals a single band of hybridizing mRNA that is ≈900 nucleotides long. Rat galanin mRNA is located predominantly in the central nervous system and gastrointestinal tract. Highest central nervous system concentrations are found in the hypothalamus, with lower levels in the cortex and brainstem. Gastrointestinal rat galanin mRNA is most abundant in the duodenum, with progressively lower concentrations in the stomach, small intestine, and colon.**

Galanin is a 29-amino acid peptide, initially isolated from porcine intestine, that shares little homology with other known peptides (1). Galanin-like immunoreactivity is widely distributed in central and peripheral neurons of several mammalian species including humans, nonhuman primates, dog, cat, cow, and various rodents (2). In the central nervous system, high concentrations are found in several discrete areas including the median eminence, hypothalamus, arcuate nucleus, septum, neurointermediate lobe of the pituitary, and spinal cord (3–6). In the periphery, galanin-like immunoreactivity is found within neuronal fibers in the gastrointestinal tract, pancreas, adrenal medulla, genitourinary tract, and respiratory tract of several species (2). Its localization in neurosecretory granules suggests that galanin may function as a neurotransmitter (7, 8); galanin has been shown to coexist with a variety of other peptide and amine neurotransmitters within individual neurons (2).

Although the physiologic role of galanin has not been established, it has been shown to have a number of pharmacologic properties in whole animal and isolated tissue preparations. Direct injection of porcine galanin (pGal) into the third ventricle of rats stimulates food intake (9) and leads to increased plasma growth hormone and prolactin levels and decreased dopamine levels in the median eminence (10–12). Intravenous pGal infusion in dogs and humans leads to hyperglycemia and glucose intolerance (2), and it inhibits the pancreatic release of insulin, somatostatin, and pancreatic polypeptide (13, 14). pGal also appears to modulate smooth muscle contractility within the gastrointestinal and genitourinary tracts (2). The specific effects described, as well as the interspecies conservation of galanin-like immunoreactivity (2), suggest that this peptide may play an important role in the nervous modulation of endocrine and smooth muscle function.

Rokaeus and Brownstein (15) isolated and characterized cDNA clones encoding pGal from a library prepared from adrenal medulla mRNA. They determined that pGal is initially synthesized as part of a large precursor peptide (prepro-pGal). The structure of this peptide suggests that posttranslational cleavage of glycine-extended galanin and C-terminal amidation lead to the mature 29-amino acid peptide. The structure of the prepro-pGal cDNA also predicts a 59-amino acid C-terminal peptide (galanin mRNA-associated peptide; pGal MAP), although it is not known whether this peptide is present *in vivo*.

To understand the nature and extent of interspecies conservation of galanin, as well as to determine whether the pGal MAP is likely to possess biological relevance, we have isolated and sequenced cDNA clones encoding rat galanin (rGal).§ These clones demonstrate that rGal is very similar to pGal and that there is a large region of the putative galanin MAP that is conserved between the two species. The isolated clones have been used to determine the tissue distribution of rGal mRNA synthesis and will be useful for the study of regulation of the rGal gene.

METHODS

Screening of the cDNA Library. A recombinant cDNA library containing rat hypothalamic mRNA sequences in the λgt11 vector was kindly provided by Richard Goodman (Tufts University). Approximately 4×10^5 plaques were screened as described (16) with three pools of mixed oligonucleotides. The oligonucleotides were designed as described below. Plaques that hybridized to at least two of these probes were isolated and characterized.

Nucleic Acid Preparation. Recombinant λ phage DNA, rat liver genomic DNA, and total RNA from rat tissues were prepared by standard methods (17). Radiolabeled cRNA probes were prepared by subcloning the rGal cDNAs from clones rG-2 and rG-5 into pGEM3-blue (Promega Biotec, Madison, WI) and transcribing with T7 RNA polymerase as described (18). Synthetic oligonucleotides were prepared by

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Abbreviations: rGal, rat galanin; pGal, porcine galanin; prepro-Gal, primary translation product of galanin; MAP, mRNA-associated peptide; cRNA, complementary RNA.

§The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03624).

using an automated DNA synthesizer (Applied Biosystems, Foster City, CA) and were 5′-end-labeled using [γ-^{32}P]ATP and T4 DNA kinase (19).

Nucleic Acid Hybridization. RNA or DNA was fractionated on agarose gels and transferred to Nytran membranes (Schleicher & Schuell) according to standard methods (20). Gels containing genomic DNA were exposed to short-wave ultraviolet light for 10 min before transfer. RNA blots were hybridized with complementary RNA (cRNA) probes at 65°C for 18–24 hr in 50% (vol/vol) formamide/5× NaCl/sodium citrate (1× NaCl/sodium citrate = 0.15 M NaCl/0.015 M sodium citrate)/5× Denhardt's solution (1× Denhardt's solution = 0.02% Ficoll-400/0.02% bovine serum albumin/0.02% polyvinylpyrrolidone-40)/0.5% $NaDodSO_4$/500 μg of sonicated denatured salmon sperm DNA per ml/500 μg of yeast tRNA per ml, and washed in 0.2× NaCl/sodium citrate/0.5% $NaDodSO_4$ at 65°C. DNA blots were hybridized for 24–36 hr at 50°C and washed at 65°C with the same hybridization and wash solutions used for RNA blots. Bacterial and phage lifts were hybridized to oligonucleotide probes at 42°C for 48 hr in 6× NaCl/sodium citrate/5× Denhardt's solution/0.1% $NaDodSO_4$, and washed in 6× NaCl/sodium citrate/0.1% $NaDodSO_4$ at the same temperature.

DNA Sequence Analysis. Nucleotide sequences were determined by the dideoxynucleotide method with a single-stranded template prepared after subcloning the DNA into phage M13 (21). Initial sequence information was obtained by using an oligonucleotide primer complementary to M13 sequence adjacent to the rGal insertion site and was extended using synthetic oligonucleotide primers complementary to derived rGal sequence. All reported nucleotide sequences have been confirmed by independently sequencing the complementary DNA strand. Nucleotide sequence analysis was aided by use of MicroGenie software (Beckman) on an IBM PC-XT computer and Intelligenetics (Palo Alto, CA) software on a DEC VAX 785 computer.

RESULTS

To isolate cDNA clones encoding rGal, we used a library prepared from rat hypothalamus, a tissue known to be rich in galanin-like immunoreactive material (3). The library was screened at low stringency with three synthetic oligonucleotide probes whose design was based on the known amino acid sequence of pGal (1). Each probe consisted of a mixture of oligonucleotides to accommodate the redundancy in the genetic code. The number of oligonucleotides in each mixture was minimized by accommodating only the codons most commonly used by mammals (22) and assuming that weak G·T base-pairing would contribute to hybridization. Probe A corresponded to amino acids 1–10 of pGal; probe B, amino

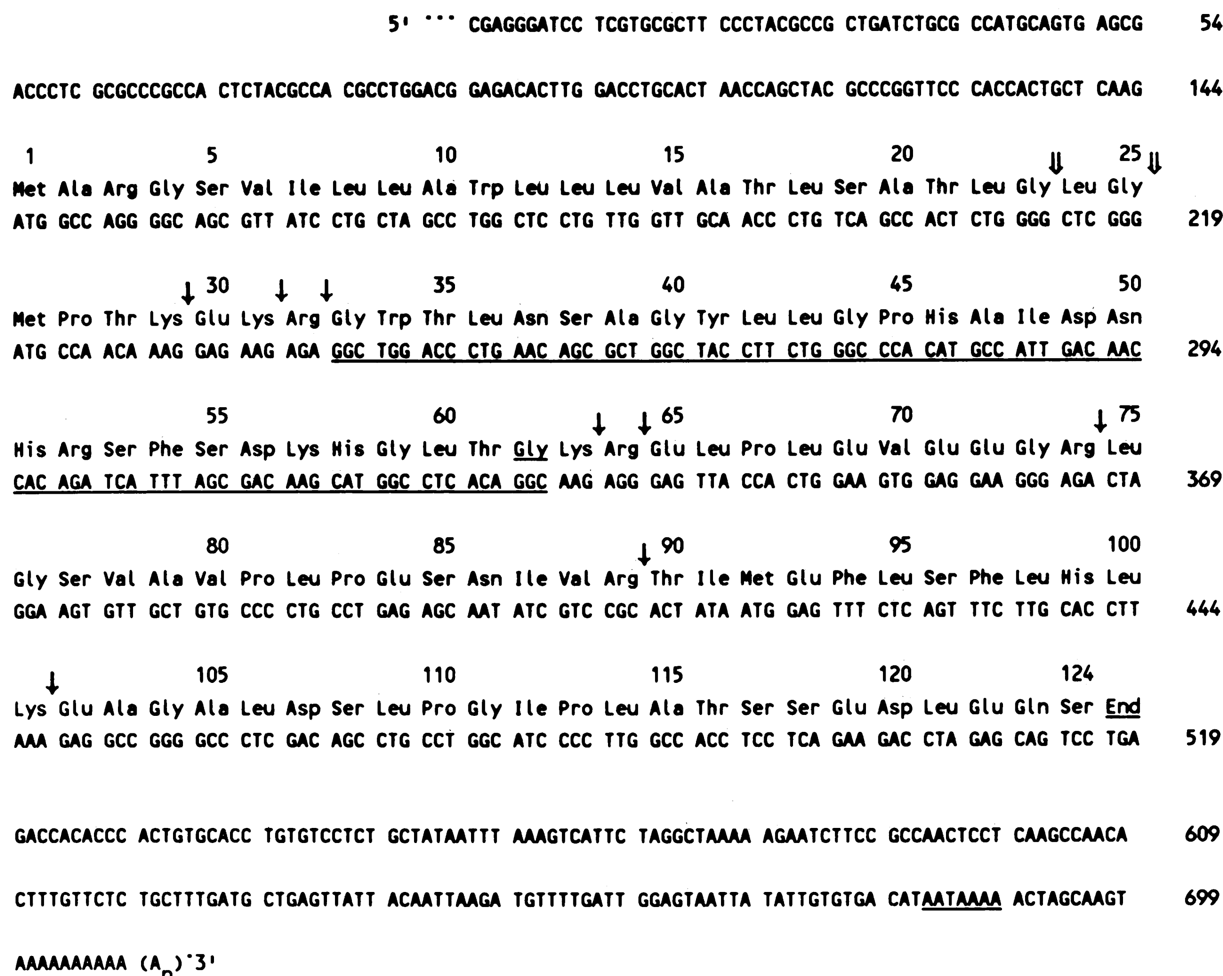

FIG. 1. Nucleotide sequence of the cDNA encoding rGal showing the predicted amino acid sequence of prepro-rGal. Arrows indicate potential tryptic cleavage sites. Large arrows demonstrate potential signal peptide cleavage sites, as predicted from published consensus sequences (23). The sequence encoding galanin is underscored and represents amino acids 33–62 of the predicted primary translation product. The glycine at position 62 likely serves as the substrate for amidation of the N-terminal threonine. Galanin MAP extends from amino acids 65 to 124 and includes the C terminus of the predicted primary translation product. The underscored region from nucleotides 683–689 represents a putative polyadenylylation signal.

acids 11–19; and probe C, amino acids 22–29. The structures of these mixed oligonucleotide probes are as follows:

Probe A: 5′-AGGTAGCCGGCG$^{GA}_{CT}$GTT$^{G}_{C}$AGGGTCCAGCC-3′

Probe B: 5′-GTGGTTGTCGATGGCGTGGGGGCC$^{G}_{C}$AG-3′

Probe C: 5′-CCGGC$^{G}_{C}$AGGCCGTACTTGTCGTGGAA-3′.

Screening of 4 × 10^5 plaques yielded four clones that hybridized to at least two of the probes. In all cases, these clones hybridized to probes A and B and failed to hybridize to probe C, suggesting that the N-terminal and middle portions of galanin would exhibit the greatest conservation among species. Galanin-specific DNA fragments from three of these clones were inserted into a variety of vectors to allow restriction enzyme analysis, cRNA production, and nucleotide sequencing.

Two of the cDNA clones (rG-2, rG-5) were subjected to nucleotide sequence analysis as described in *Methods*. The two derived sequences were identical except that rG-5 was missing three nucleotides present in rG-2 corresponding to the extreme 5′ end of the rGal mRNA. Analysis of the sequence of clone rG-2 (Fig. 1) reveals that the rGal mRNA encodes a 124-amino acid galanin precursor peptide (prepro-rGal). The N terminus of prepro-rGal includes a hydrophobic sequence (23–25 amino acids) that likely serves as the signal for transport into the endoplasmic reticulum. The galanin portion comprises amino acids 33–62 of the precursor peptide and is flanked on both sides by Lys-Arg tryptic cleavage sites. That the glycine at position 62 may serve as an amide donor is suggested by the known C-terminal amidation of pGal (1). The C-terminal region of prepro-rGal is a 60-amino acid sequence (rGal MAP) that contains two internal arginine residues. As shown in Fig. 1, there is relatively little sequence homology between the rGal and pGal mRNAs in the 5′- and 3′-untranslated regions. Notable exceptions include a 7-nucleotide identical sequence immediately upstream of the ATG initiator codon (nucleotides 138–144) and near-identity in the region surrounding the polyadenylylation signal (nucleotides 680–696).

Comparison of the deduced sequence of prepro-rGal with that of prepro-pGal (Fig. 2) reveals striking size and sequence homology of rGal and pGal. All three amino acid variations are located in the C-terminal heptapeptide, including a threonine at the C terminus of rGal in place of the alanine in pGal. Comparison of the sequences of rGal MAP with its porcine analogue reveals 78% identity of the C-terminal portion, corresponding to amino acids 90–124 of prepro-rGal. This sequence is set off in both species by an arginine residue (amino acid 89 of prepro-rGal) and includes a region of 94% identity (amino acids 90–106). In contrast, there is only 29% homology between the two species in the N-terminal portion of galanin MAP. This region of the cDNA differs in length by 3 nucleotides, causing rGal MAP to contain one amino acid more than pGal MAP (shown as prepro-rGal amino acid 70 in Fig. 2).

Hybridization analysis of rat liver genomic DNA using a rGal cRNA probe is consistent with the presence of a single gene encoding prepro-rGal (Fig. 3). Digestion of the genomic DNA with *Eco*RI or *Nco* I generates a single hybridizing band. *Hin*dIII and *Bam*HI digestion generates two hybridizing bands, suggestive of a single cleavage site within the galanin gene (a *Bam*HI site is present at the 5′ end of the isolated cDNA clones).

RNA blot analysis demonstrates a single band of hybridizing RNA ≈900 nucleotides long (Fig. 4). This species is

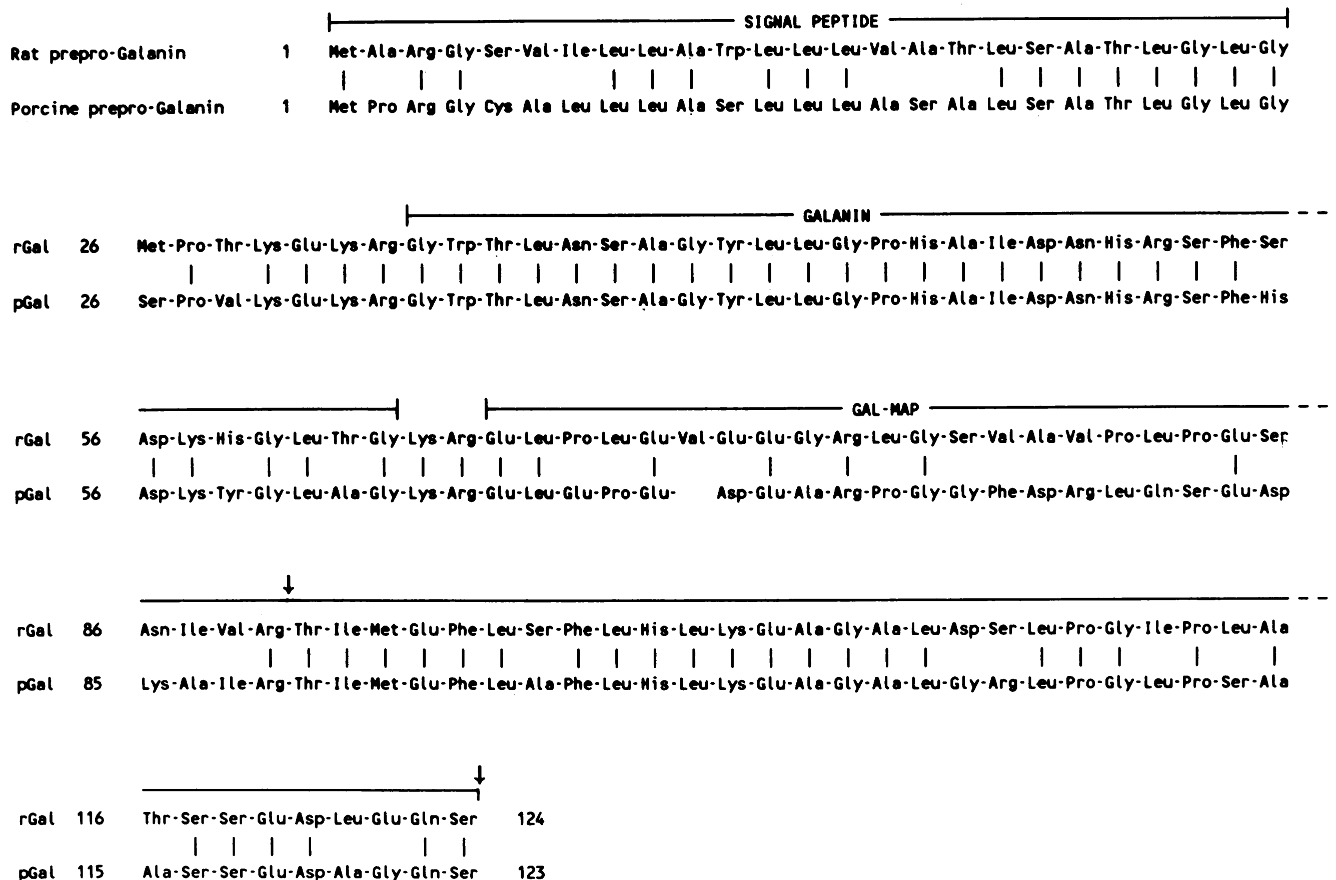

FIG. 2. Comparison of the amino acid sequences of rat and porcine prepro-Gal. Vertical lines denote identical amino acid residues. Regions of the precursor peptides corresponding to the signal peptide, galanin, and galanin MAP are indicated. Arrows define the highly conserved region of galanin MAP.

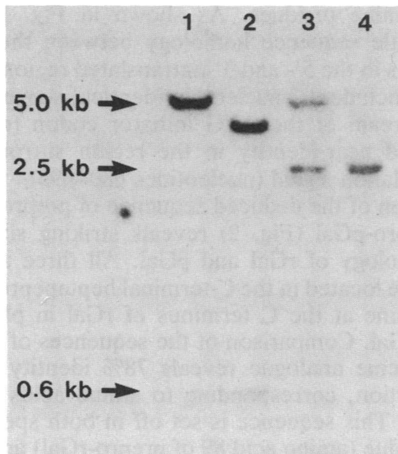

FIG. 3. Southern blot analysis of rat genomic DNA. Purified rat liver DNA (7.5 μg) was digested with various restriction enzymes for 3 hr at 37°C in standard buffers (20), separated by electrophoresis on a 0.8% agarose gel in 1× Tris/borate/EDTA (1× Tris/borate/EDTA = 89 mM Tris·HCl, pH 7.8/89 mM sodium borate/2 mM EDTA), transferred to Nytran membranes, and hybridized to ^{32}P-labeled rGal cRNA as described in *Methods*. Autoradiography was at −70°C with an intensifier screen (E. I. duPont, Wilmington, DE) for 14 days. Lanes: 1, *Eco*RI digestion; 2, *Bam*HI; 3, *Hin*dIII; 4, *Nco* I. Fragment sizes were determined from the migration of *Hin*dIII-cut λ DNA run in parallel lanes. kb, Kilobases.

retained on oligo(dT)-cellulose as would be expected for polyadenylylated mRNA (data not shown). Analysis of the tissue distribution of rGal mRNA reveals that rGal mRNA is abundant in the central nervous system and the gastrointestinal tract. In particular, highest tissue levels are found in the hypothalamus, cerebral cortex, duodenum, and stomach. We detect little galanin-specific mRNA in the adrenals, lungs, pancreas, or esophagus.

DISCUSSION

This report describes the isolation and characterization of cDNAs encoding rGal and its precursor peptide. Blot hybridization analysis reveals that rGal is encoded by a single gene and that rGal mRNA migrates as a single species of ≈900 nucleotides [including the poly(A) tail] in several tissues.

Prepro-rGal shows striking homology with the prepro-pGal sequence reported by Rokaeus and Brownstein (15). Amino acids 1–22 of galanin itself are completely conserved between the two species and there are three amino acid substitutions in the C-terminal heptapeptide. Previous studies have demonstrated heterogeneity of the antibodies that react with pGal. In particular, antibodies that detect galanin-like immunoreactivity in several species appear to be directed at the N-terminal domain of the molecule. Those that react specifically with the C-terminal region do not detect galanin-like immunoreactivity in rat, cow, or humans (15), implying interspecies heterogeneity in this region. The data presented in this report confirm interspecies conservation of the N-terminal and central portions of the galanin molecule. They suggest that biological activity resides in these regions, although species-specific activities of the C terminus cannot be excluded. Further studies comparing the biological activities of rGal and pGal, as well as fragments of these peptides, will be necessary to resolve these questions.

The tight conservation of the 35-amino acid region at the C terminus of rGal and pGal MAP suggests that this peptide possesses a biological function. It is noteworthy that in both species this region is set apart from the less-conserved region of galanin MAP by an arginine residue, which could serve as a tryptic cleavage site. The N-terminal portion of rGal MAP demonstrates far less sequence homology with the porcine sequence. However, in both species this region contains several acidic residues, and Rokaeus and Brownstein (15) have reported that the analogous portion of bovine galanin MAP is similarly acidic. Conservation of ionic properties without sequence specificity suggests that this region could mediate binding to polyvalent cations and participate in subcellular localization or transport processes. We are preparing antibodies to synthetic rGal MAP and galanin MAP fragments to help elucidate their biological functions.

Blot hybridization analysis reveals that the distribution of rGal mRNA is similar to the distribution of the galanin peptide. The highest concentrations of rGal mRNA have been detected in hypothalamus. Microdissection of this tissue has revealed particularly high concentrations in the medial basal hypothalamus and preoptic area (data not shown). The cortex and brainstem also contain high concentrations of rGal mRNA. In contrast, there is little rGal-specific mRNA in the median eminence or neurointermediate lobe of the pituitary, suggesting that galanin-like immunoreactivity detected in these regions is limited to storage in nerve terminals. In the gastrointestinal tract, highest mRNA concentrations are found in the duodenum, stomach, and small intestine. There is relatively less expression in the colon and no detectable mRNA in rat esophagus. This distribution is consistent with the distribution of galanin-like immunoreactivity (2). The rat esophagus, unlike that of higher mammals, contains no smooth muscle and therefore

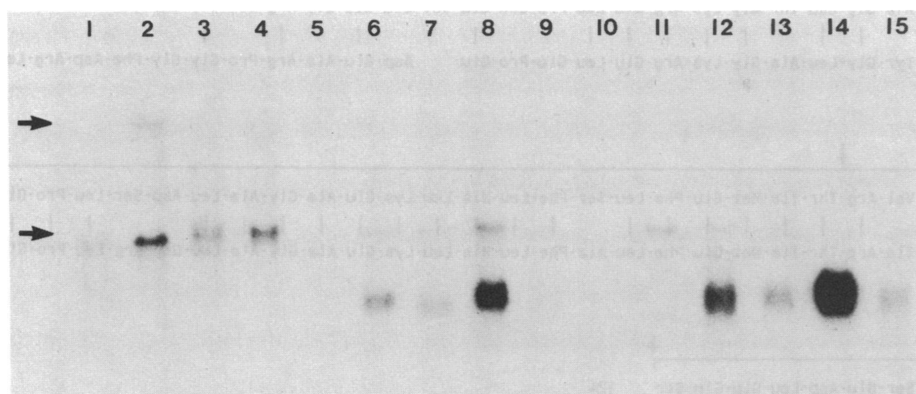

FIG. 4. RNA blot analysis of rat RNA. Total RNA (10 μg) was isolated from various rat tissues, separated by electrophoresis on a 1.5% agarose gel in 2.2 M formaldehyde/20 mM morpholinopropanesulfonic acid/5 mM sodium acetate, pH 7.0/1 mM EDTA, transferred to Nytran membranes, and hybridized to ^{32}P-labeled rGal cRNA as described in *Methods*. Autoradiography was for 3 days at −70°C with an intensifier screen. Arrows indicate the positions of unlabeled 28S and 18S rRNA. Lanes: 1, lung RNA; 2, kidney; 3, spleen; 4, liver; 5, pancreas; 6, stomach; 7, small bowel; 8, duodenum; 9, colon; 10, esophagus; 11, adrenal; 12, brain; 13, cerebral cortex; 14, hypothalamus; 15, brainstem.

lacks the intrinsic nervous plexuses present in other parts of the gastrointestinal tract (24). We were unable to detect rGal mRNA in the pancreas, suggesting that the immunoreactive material observed adjacent to the islets of Langerhans (25) represents neuronal galanin transported from extrapancreatic tissue. The lack of detectable adrenal rGal mRNA or peptide (26) is unexplained, given the high levels seen in porcine adrenal medulla. Understanding of this species specificity will await further delineation of the role of galanin in the porcine adrenal gland.

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1. Tatemoto, K., Rokaeus, Å., Jornvall, H., McDonald, T. J. & Mutt, V. (1983) *FEBS Lett.* **164**, 124–128.
2. Rokaeus, Å. (1987) *Trends NeuroSci.* **10**, 158–164.
3. Skofitsch, G. & Jacobowitz, D. M. (1986) *Peptides* **7**, 609–614.
4. Melander, T., Hokfelt, T. & Rokaeus, Å. (1986) *J. Comp. Neurol.* **248**, 475–517.
5. Ch'ng, J. L. C., Christofides, M. D., Anand, P., Gibson, S. J., Allen, Y. S., Su, H. C., Tatemoto, K., Morrison, J. F. B., Polak, J. M. & Bloom, S. R. (1985) *Neuroscience* **16**, 343–354.
6. Buchanan, J. T., Brodin, L., Hokfelt, T., Van Dongen, P. A. M. & Grillner, S. (1987) *Brain Res.* **408**, 299–302.
7. Servin, A. L., Amiranoff, B., Rouyer-Fessard, C., Tatemoto, K. & Laburthe, M. (1987) *Biochem. Biophys. Res. Commun.* **144**, 298–306.
8. Amiranoff, B., Servin, A. L., Rouyer-Fessard, C., Couvineau, A., Tatemoto, K. & Laburthe, M. (1987) *Endocrinology* **121**, 284–289.
9. Kyrkouli, S. E., Stanley, B. G. & Leibowitz, S. F. (1986) *Eur. J. Pharmacol.* **122**, 159–160.
10. Ottlecz, A., Samson, W. K. & McCann, S. M. (1986) *Peptides* **7**, 51–53.
11. Koshiyama, H., Kato, Y., Inoue, T., Murakami, Y., Ishikawa, Y., Yanaihara, N. & Imura, H. (1987) *Neurosci. Lett.* **75**, 49–54.
12. Nordstrom, O., Melander, T., Hokfelt, T., Bartfai, T. & Goldstein, M. (1987) *Neurosci. Lett.* **73**, 21–26.
13. Silvestre, R. A., Miralles, P., Monge, L., Moreno, P., Villanueva, M. L. & Marco, J. (1987) *Endocrinology* **121**, 378–383.
14. Silvestre, R. A., Miralles, P., Monge, L., Villanueva, M. L. & Marco, J. (1987) *Life Sci.* **40**, 1829–1834.
15. Rokaeus, Å. & Brownstein, M. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6287–6291.
16. Spindel, E. R., Chin, W. W., Price, J., Rees, L. H., Besser, G. M. & Habener, J. F. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5699–5703.
17. Davis, L. G., Dibner, M. D. & Battey, J. F. (1986) *Basic Methods in Molecular Biology* (Elsevier, New York).
18. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1987) *Nucleic Acids Res.* **12**, 7035–7056.
19. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65**, 499–560.
20. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
21. Messing, J. (1983) *Methods Enzymol.* **101**, 20–78.
22. Grantham, R., Gautier, C., Gouy, M., Jacobzone, M. & Mercier, R. (1981) *Nucleic Acids Res.* **9**, R43–R74.
23. Perlman, D. & Halvorson, H. O. (1983) *J. Mol. Biol.* **167**, 391–409.
24. Van Kruiningen, H. J. (1982) *Comparative Gastroenterology* (Thomas, Springfield, IL), pp. 8–16.
25. Dunning, B. E., Ahren, B., Veith, R. C., Bottcher, G., Sundler, F. & Taborsky, G. J. (1986) *Am. J. Physiol.* **251**, E127–E133.
26. Bauer, F. E., Christofides, N. D., Hacker, G. W., Blank, M. A., Polak, J. M. & Bloom, S. R. (1986) *FEBS Lett.* **201**, 327–331.