30 m) requires a minimum sediment accumulation rate of 75 cm kyr^{-1} for N3, a factor of more than 2 higher than the preceding part of the Vrica section. However, a larger terrigenous input was noted in the uppermost part of the section and based on the lithology this option cannot be excluded.

A final decision on the correlation of the Stuni and Vrica magnetostratigraphy to the GRTS must weigh the likelihood that the selected palaeontological events occurred penecontemporaneously in the open ocean and the Mediterranean versus the likelihood of changes in sediment accumulation rate occurring with minor lithological effects. The arguments of Backman *et al.*[15] for the worldwide penecontemporaneity of the LADs of *D. brouweri* and *D. brouweri* var. *triradiatus* appear to be on firm ground, as opposed to the argument for a more constant sediment accumulation rate which has more aesthetic than scientific merit. The preferred correlation is, therefore, option (2), which correlates N1–N2 to the Olduvai and assumes N3 to be a short subchron between the Olduvai and the Jaramillo. A consequence of the correlation adopted here is that the proposed Plio-Pleistocene boundary stratotype defined in the Vrica section by the physical horizon immediately below the first appearance of *C. testudo* is located above the Olduvai subchron at ~1.6 Myr.

Samples were taken during a field workshop organized by the International Geological Correlation Program's project 128 (Late Cenozoic Magnetostratigraphy) and presided over by Professor Raimondo Selli. Additional support was provided by NSF grants EAR 82 12763 and OCE81-19695. The critical comments of G. Kukla, B. M. Clement, M. L. Colalongo, D. Rio, N. J. Shackleton, J. Backman, J. Van Couvering, J. D. Obradovich and E. Hailwood, are greatly appreciated. The palaeomagnetic laboratory was maintained by D. Lafferty and the support of D. Kent is acknowledged. Lamont-Doherty contribution no. 3482.

Received 25 February; accepted 10 May 1983.

1. Lyell, C. *Principles of Geology* 1st edn, Vol 3 (John Murray, London, 1833).
2. Lyell, C. *Pitois-Levranet* (Paris, 1839).
3. Berggren, W. A. & Van Couvering, J. A. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **16**, (1974).
4. Selli, R. *et al. Giorn. Geol.* **42**, 181–204 (1977).
5. Colalongo, M. L., Pasini, G. & Sartoni, S. *Boll. Soc. Paleont. Ital.* **20**, 99–120 (1981).
6. Obradovich, J. D., Naeser, C. W., Izett, G. A., Pasini, G. & Bigazzi, G. *Nature* **298**, 55–59 (1982).
7. Johnson, N. M., Opdyke, N. D. & Lindsay, E. H. *Bull. geol. Soc. Am.* **86**, 5–12 (1975).
8. Dunlop, D. J. & Stirling, J. M. *Geophys. Res. Lett.* **4**, 163–166 (1977).
9. Channell, J. E. T., Freeman, R., Heller, F. & Lowrie, W. *Earth planet. Sci. Lett.* **58**, 189–201 (1982).
10. Fisher, R. A. *Proc. R. Soc.* **217**, 295–305 (1953).
11. Watson, G. S. *Mon. Not. R. astr. Soc., Geophys. Suppl.* **7**, 160–161 (1956).
12. Nakagawa, H. & Niitsuma, N. *EOS* **13**, 517 (1981).
13. Mankinen, E. A. & Dalrymple, G. B. *J. geophys. Res.* **84**, 615–626 (1979).
14. Cati, F. & Borsetti, A. M. *Giorn. Geol.* **43**, 365–384 (1981).
15. Backman, J., Shackleton, N. J. & Tauxe, L. *Nature* **304**, 156–158 (1983).
16. Pasini, G. & Colalongo, M. L. *Pap. at the XI INQUA Congr.* (1982).
17. LaBrecque, J. L., Kent, D. V. & Cande, S. C. *Geology* **5**, 330–335 (1977).

Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing

Michael G. Rosenfeld*, Jean-Jacques Mermod*, Susan G. Amara*, Larry W. Swanson†, Paul E. Sawchenko†, Jean Rivier‡, Wylie W. Vale‡ & Ronald M. Evans§

* Eukaryotic Regulatory Biology Program, School of Medicine, University of California, San Diego, La Jolla, California 92093, USA
† Developmental Neurobiology Laboratory, ‡ Peptide Biology Laboratory and § Molecular Biology and Virology Laboratory, The Salk Institute, San Diego, California 92138, USA

Alternative processing of the RNA transcribed from the calcitonin gene appears to result in the production of a messenger RNA in neural tissue distinct from that in thyroidal 'C' cells. The thyroid mRNA encodes a precursor to the hormone calcitonin whereas that in neural tissues generates a novel neuropeptide, referred to as calcitonin gene-related peptide (CGRP). The distribution of CGRP-producing cells and pathways in the brain and other tissues suggests functions for the peptide in nociception, ingestive behaviour and modulation of the autonomic and endocrine systems. The approach described here permits the application of recombinant DNA technology to analyses of complex neurobiological systems in the absence of prior structural or biological information.

THE nervous system is unique among organ systems in the complexity and specificity of its intercellular communications, which consist primarily of short-range interactions at chemical synapses between the chains of neurones that mediate sensory, integrative and motor functions. It seems likely that the unique phenotypic properties of many of the cells in the nervous system are due in part to their ability to synthesize and release, as well as respond to, a wide variety of polypeptide regulators. The peptide neurotransmitters that have been characterized so far are likely to represent only a small percentage of the total number utilized by the nervous system, and have classically been obtained by purification from large quantities of tissue on the basis of a specific bioassay. Once purified, sequenced and synthesized, study of these known polypeptide regulators using histochemical, physiological and behavioural assays has demonstrated that each polypeptide is localized to a discrete subset of neurones and pathways in the central nervous system. Each polypeptide subserves a wide variety of functions that are, in part, dependent on the site of synthesis. Furthermore, these neuropeptides are also located in tissues outside the brain, such as the gastrointestinal tract or lung, and are known to exert regulatory functions on extraneuronal target tissues.

In this article we describe an approach by which recombinant DNA and molecular biological techniques can be applied to the identification of previously unknown neuropeptides. This approach was applied in the identification of a putative novel

0028-0836/83/280129—07$01.00

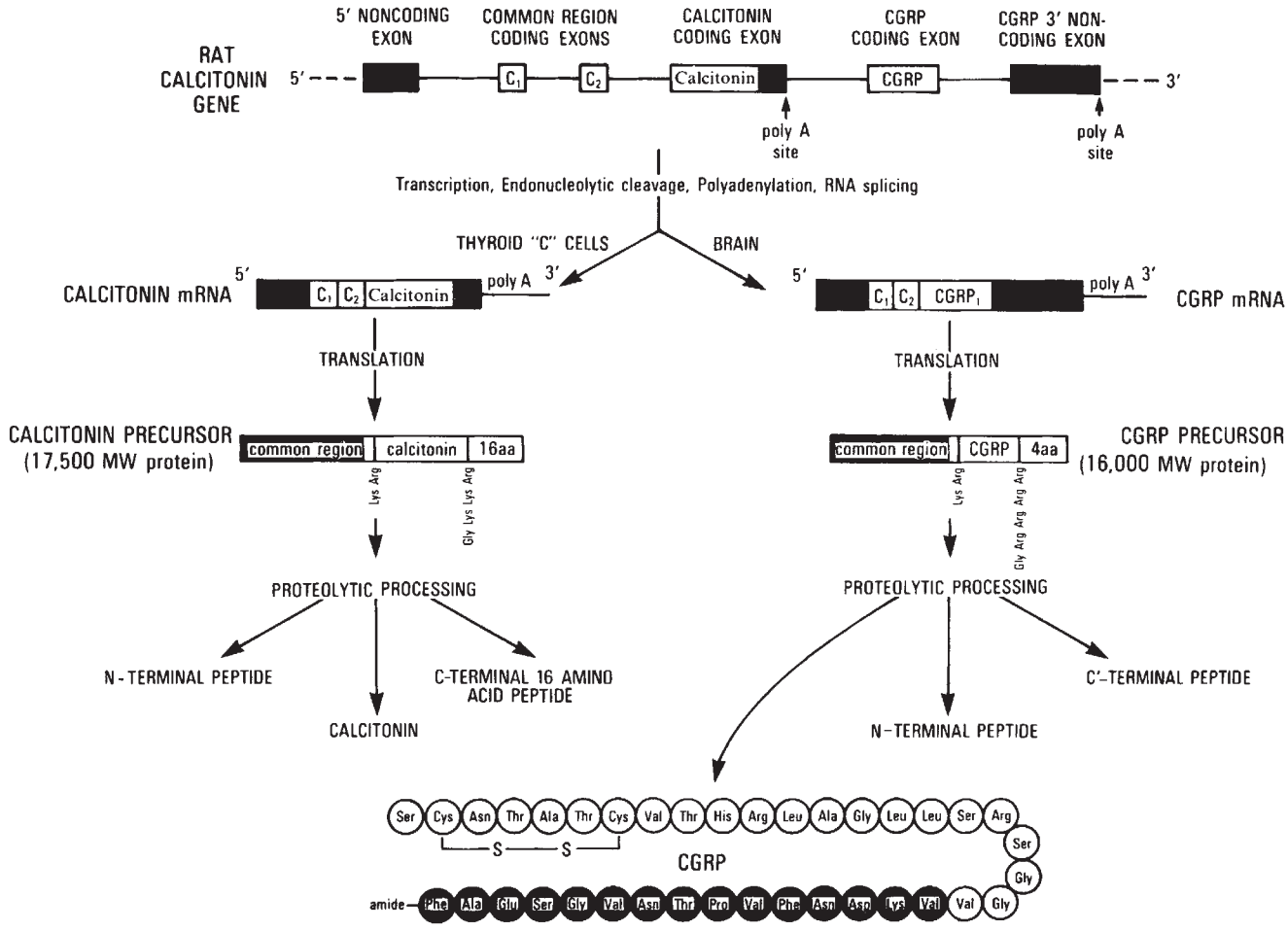

Fig. 1 Alternative RNA processing pathways in expression of the calcitonin gene, which predict the synthesis of a novel neuropeptide (CGRP) in the brain. The structural organization of the rat calcitonin gene is based on DNA sequence and mapping data[1]. The selection of alternative polyadenylation sites is suggested to account for the synthesis of calcitonin in mRNA in thyroidal 'C' cells, and the larger CGRP mRNA in brain. CGRP mRNA from rat medullary thyroid carcinomas encodes a 16,000 MW primary translation product, which we predict would be processed to generate three polypeptides, including the putative 37 amino acid polypeptide referred to as CGRP.
Methods: The immunological strategy was analogous to one successfully used to generate site-directed somatostatin antisera[11]. A synthetic oligopeptide consisting of the predicted ultimate 14 C-terminal amino acids of CGRP (shaded), with an additional N-terminal tyrosine residue to permit linkage and iodination for radioimmunoassay, was assembled automatically using a 990 B Beckman synthesizer, with a previously reported program[12]. Hydrogen fluoride cleavage was performed using the standard technique. The crude peptide was purified on an ion-exchange column (CM52) using a gradient of ammonium acetate from 0.01 M (*p*H 4.5) to 0.3 M (*p*H 6.5). The major component was subjected to countercurrent distribution after it had been desalted by multiple lyophilizations from distilled water. The solvent system used was 0.1% 1-butanol, and 200 transfers were performed with the main fraction being collected in tubes 19–25. The overall yield was 10% based on the substitution per g of starting resin. Reverse-phase HPLC in several systems indicated that the product had a purity of >95%. Amino acid analysis after 4 M methane sulphonic acid (containing 0.2% tryptamine) hydrolysis gave the predicted ratios. The synthetic peptide was linked by bisdiazotization through the tyrosine to human α-globulins (Sigma). Antisera were raised in New Zealand white rabbits by serial multiple intradermal injections of the conjugate emulsified in Freund's adjuvant. Rabbits were injected every 2 weeks, and sera obtained 10 days after the third and sixth bleedings were adsorbed with excess human α-globulins and used for studies reported here.

neuropeptide, predicted on the basis of structural analysis of calcitonin gene expression[1]. Based on the evidence presented below we suggest that, in the brain, the RNA transcribed from the calcitonin gene is processed to a discrete mRNA encoding the precursor to a predicted 37 amino acid peptide, referred to as CGRP (calcitonin gene related peptide).

Alternative RNA processing

We have recently reported how alternative RNA and protein products of the calcitonin gene can be generated in specific tissues by the selection of alternative exons for incorporation into their respective mRNAs[1–5] (see Fig. 1), rather similar to the generation of multiple mRNAs from a single transcription unit in viral and immunoglobulin gene expression[7–10]. Thus, the calcitonin gene encodes two different mRNAs that share an identical 5' sequence, but have entirely different 3' sequences[1]. The resultant mRNAs encode either the 17,500 molecular weight (MW) calcitonin precursor protein, which is proteolytically processed to yield the calcium-regulating hormone, calcitonin, and two other peptides[2–4]; or a 16,000 MW protein, the predicted translation product of CGRP mRNA (see Fig. 1). From the nucleotide sequence of the cloned CGRP cDNA it can be predicted that the encoded protein is proteolytically processed to generate three peptides, including the 37 amino acid CGRP (excised from the precursor by N-terminal cleavage at the dipeptide Lys-Arg, and C-terminal cleavage at the sequence Gly-Arg-Arg-Arg-Arg).

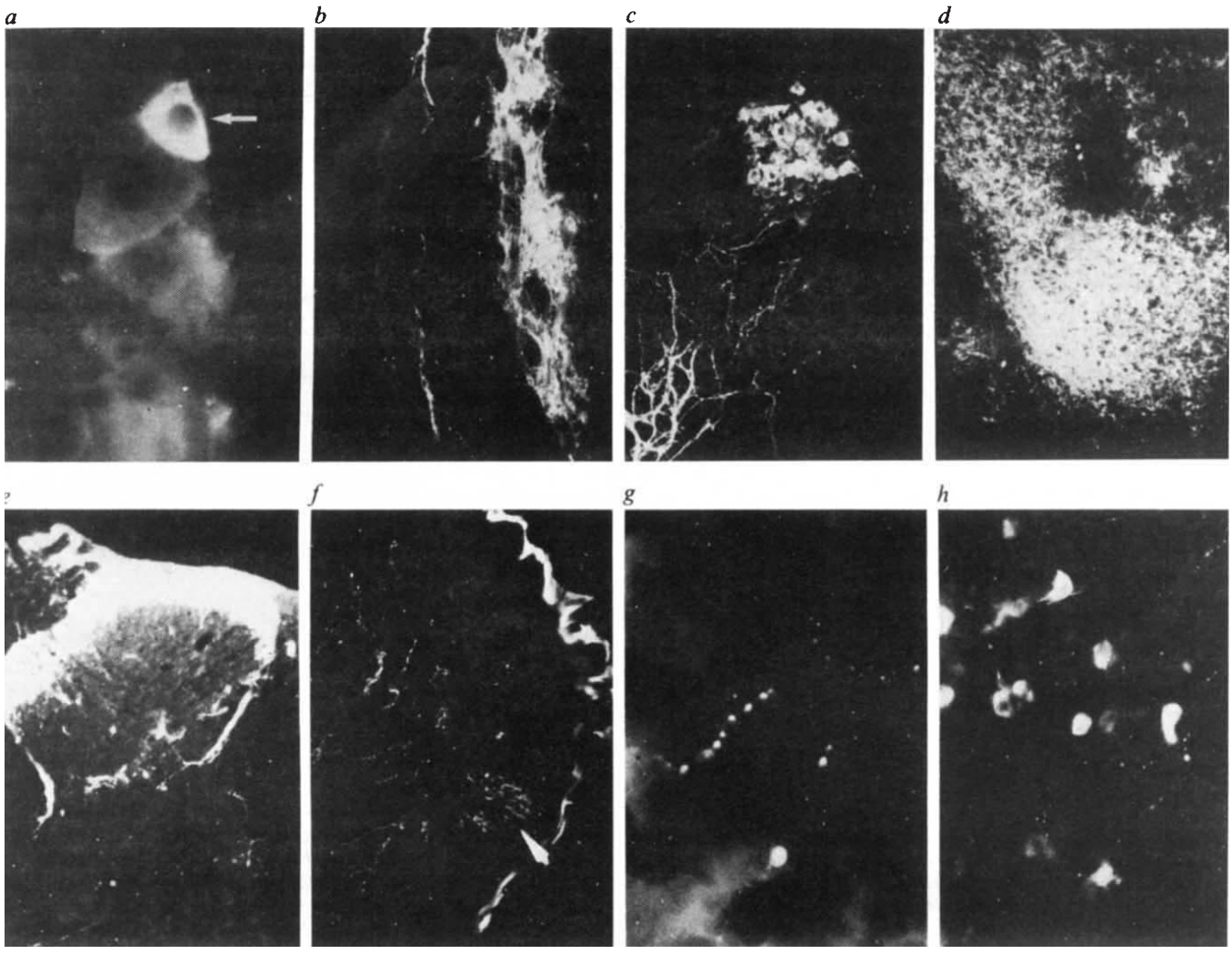

Fig. 2 Immunofluorescence localization of CGRP in the nervous system. *a*, High-power view of a row of four neurones in the trigeminal ganglion. Three large unstained neurones can be seen below one small, intensely stained neurone, the nucleus of which is unstained (arrow)(×500). Trigeminal ganglia contained 0.25–0.7 ng immunoreactive CGRP per mg tissue. ***b***, A dense band of CGRP-stained fibres in the superficial part of the spinal trigeminal nucleus, pars caudalis. The lateral surface of the medulla is to the left. A few stained fibres, which arise in the trigeminal ganglion (*a*), can also be seen in the spinal tract of the trigeminal nerve, which lies just medial to the edge of the brain stem (×75). ***c***, CGRP-stained motorneurones in the rostral part of the nucleus ambiguus (top). Stained fibres associated with the trigeminal imput to the lateral medulla can be seen in the lower lateral field to the left (×75). ***d***, A dense circumscribed CGRP-stained terminal field in the lateral part of the central nucleus of the amygdala. Fibres associated with the terminal field also formed patchy terminal fields in caudal parts of the caudoputamen and globus pallidus (×75). ***e***, CGRP-immunoreactive fibres in the dorsal horn (substantia gelatinosa) of the spinal cord at segment T_{10}. Because many stained fibres were seen in dorsal roots, it seems clear that the fibres shown here arise from dorsal root ganglion cells, as in the trigeminal nucleus. In each section of the spinal cord, several large motor neurones in the ventral horn were labelled, although stained preganglionic sympathetic neurones in the intermediolateral column were not observed (×75). ***f***, A cross-section through the tongue, the lateral edge of which is outlined by scalloped nonspecific staining on the right. Many CGRP-stained fibres were seen among muscle fibres and within the walls of blood vessels, as well as within some, but not all, taste buds (arrow)(×75). ***g***, CGRP-stained fibres in the anterior lobe of the pituitary gland (×500). ***h***, CGRP-stained cells and fibres in the adrenal medulla (×200).
Methods: Immunofluorescence localization of CGRP was carried out as described in detail in ref. 13. Serum was treated by addition of human α-globulin (7.5 μg ml^{-1}). Staining was completely blocked by the addition of synthetic CGRP (23–37) or CGRP (1 mg); addition of calcitonin, vasopressin or somatostatin (1–5 mg) did not influence staining. 24 Hours before perfusion, animals were treated either with 75 μg of colchicine subcutaneously (*a*, *f–h*) or 150 μg of colchicine in the lateral ventricle (*b–d*, *e*). Radioimmunoassay was used to quantitate CGRP content in trigeminal ganglia. Serum was used at a dilution of 1:85,000, with assay sensitivity to 0.5 pg. Competition by intact CGRP was about 30% as effective as CGRP (23–37); CRF, GRF, calcitonin and somatostatin failed to compete with immunoprecipitation of iodinated CGRP (23–37).

The specific hybridization of CGRP exon-specific probes to poly(A)-selected RNA from rat trigeminal ganglia and hypothalamic RNA preparations, but not from thyroidal 'C' cells (data not shown), suggested that the critical physiological consequence of these alternative RNA processing pathways might be the tissue-specific synthesis of CGRP in the brain (see Fig. 1). The results of immunohistochemical studies, peptide isoiation and characterization, and RNA structural analyses, sapport the prediction that CGRP is produced in the central and peripheral nervous systems.

Localization of CGRP immunoreactivity

Antisera raised against a synthetic polypeptide corresponding to a portion of the predicted C-terminal sequence of CGRP (Fig. 1) were used for immunohistochemical studies of the rat. Photomicrographs of representative areas are shown in Fig. 2. Specific immunostaining was observed in restricted components of neuronal systems that are known to subserve sensory, integrative and motor functions (see Fig. 3). Staining in sensory systems includes a subpopulation of small neurones in the

trigeminal ganglion (Fig. 2*a*) and in the spinal sensory ganglia, as well as their terminals in the brain stem and spinal cord, which are believed to relay somatic and cutaneous pain and/or temperature information. The overall distribution of the CGRP-stained neurones and pathways does not correspond to that of any other known neuropeptide. The CGRP content of trigeminal ganglia, as quantitated by radioimmunoassay, is 0.3–0.7 ng per mg tissue (Fig. 2 legend). There are also many CGRP-stained fibres in the tongue, some of which innervate a subset of taste buds (Fig. 2*f*), and a subset of primary olfactory fibres are CGRP immunoreactive. Most of the motor neurones in the facial nucleus, the hypoglossal nucleus (tongue movement) and in rostral parts of the nucleus ambiguus (innervation of the heart and the branchial muscles of the pharynx and larynx that are involved, for example, in swallowing) (Fig. 2*c*) contain immunoreactive CGRP, which suggests that this neuropeptide is produced in neurones that use acetylcholine as a neurotransmitter.

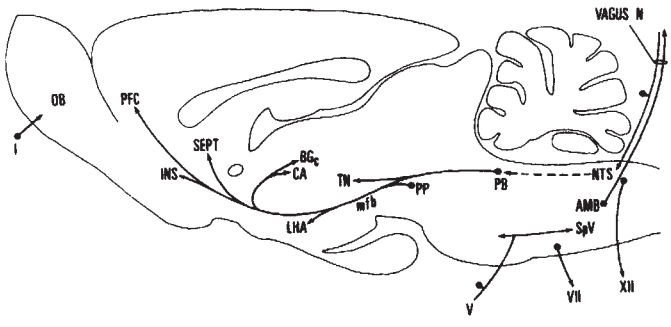

Fig. 3 A summary of the major CGRP-stained cell groups (black dots) and pathways (arrows) projected on a sagittal view of the rat brain. This staining was localized in discrete parts of several functional systems. First, dense terminal fields were stained throughout the substantia gelatinosa of the spinal cord and caudal part of the spinal trigeminal nucleus. These fibres arise in dorsal root and trigeminal ganglion cells (Fig. 2*a*) and probably relay nociceptive or thermal information. Second, CGRP is found in most parts of the taste pathways, including sensory endings in taste buds and the central endings of these fibres in the rostral part of the nucleus of the solitary tract (NTS), and in the relay system from the parabrachial nucleus (PB) to the thalamic taste nucleus (TN) and the taste area of the cerebral cortex (posterior agranular insular area, INS). In addition, most motor neurones in the hypoglossal nucleus (XII), which move the tongue, were stained. Third, a small group of primary olfactory fibres (I) that end in the glomerular layer of the olfactory bulb (OB) were stained, suggesting that CGRP has a role in olfaction as well as taste. Fourth, a small number of fibres, apparently from the 8th nerve, end in the cochlear and vestibular nuclei (not shown). Fifth, CGRP is found throughout the caudal part of the NTS, and throughout the PB, suggesting that it plays a part in the relay of visceral sensory information from the vagus (and glossopharyngeal) nerve, by way of an ascending pathway through the medial forebrain bundle (MFB). This pathway appears to arise in the PB and peripeduncular nucleus (PP), and projects to the lateral hypothalamic area (LHA), the central nucleus of the amygdala (CA), to patches in caudal parts of the caudoputamen and globus pallidus (BGc), to the lateral septal nucleus and bed nucleus of the stria terminalis (SEPT) and to layer III of three cortical areas: the infralimbic prefrontal area (PFC), the INS and the perirhinal area. The ascending projections in the MFB are probably modulated by a massive, non-CGRP containing pathway from the NTS to the PB (dashed line). Sixth, stained motor neurones in the rostral part of the nucleus ambiguus (AMB) project through the vagus nerve and may innervate the heart and/or branchial muscles in the pharynx that are involved, for example, in the control of swallowing. The overall distribution of CGRP-stained pathways does not correspond with any other known peptide, and suggests that it has a particularly important role in the sensory, integrative (by way of the MFB) and motor components of ingestive behaviour, and also in the processing of painful stimuli.

CGRP-staining was also observed in thin, beaded fibres throughout many parts of the body, including the heart, lung and gastrointestinal tract, and these fibres were often associated with the smooth muscle of blood vessels. The thyroid gland, despite the fact that it does receive sensory, sympathetic and parasympathetic innervation, failed to demonstrate CGRP-staining in either cells or fibres. Conversely, antisera against calcitonin failed to stain any region of the brain or sensory ganglia, although bright 'C' cells and beaded fibres were observed in the thyroid.

The possibility that CGRP has a significant role in the endocrine system is suggested by the presence of a subset of cells that are stained by the CGRP antisera in the adrenal medulla (Fig. 2*h*), and by CGRP-stained fibres that project through the adrenal cortex to a subcapsular zone, where they are particularly numerous. A small number of CGRP-stained cells and fibres were also observed at the periphery of pancreatic islets. While no CGRP-stained cells were observed in the small bowel of the rat, occasional cells did stain with the calcitonin antisera as did a few cells in the centre of some pancreatic islets. Scattered CGRP-stained beaded fibres were observed in the anterior lobe of the pituitary gland (Fig.2*g*). These fibres could not be traced from the brain, and may represent 'sympathetic' fibres arising in the superior cervical ganglion.

CGRP mRNA in brain

It is important to demonstrate that it really is CGRP that is being recognized by the antisera in the immunohistochemical experiments, and not just cross-reacting, structurally related antigens. The recent identification of a second gene encoding a CGRP-related peptide (our unpublished data) makes this question particularly pertinent. Evidence that the CGRP staining does represent calcitonin gene expression has been obtained by using S_1 nuclease mapping experiments[14] to detect the presence and distribution of authentic CGRP mRNA in the brain.

The experimental approach used is shown schematically in Fig. 4*a*. The cDNA insert of plasmid $pCGRP_2$ is complementary to the 5′ sequence shared by both calcitonin and CGRP mRNAs, and also to the 3′ sequence unique to CGRP mRNA, so S_1 nuclease protection assays simultaneously quantitate the amount of both calcitonin and CGRP mRNAs. CGRP mRNA is distinguished in this analysis from the product of the second gene encoding a CGRP-related peptide, the mRNA of which has a primary structure that differs considerably from that of CGRP mRNA. The results of such an analysis are shown in Fig. 4*b*. CGRP mRNA was identified in RNA prepared from the hypothalamus (medial preoptic area cells), midbrain (peripeduncular nucleus cells), and trigeminal ganglion. RNA prepared from a region containing primarily terminal fields (amygdala) contained only very low levels of CGRP mRNA. Calcitonin RNA sequences were not detected in any of the brain regions analysed, but were readily detected in a rat medullary thyroid cell line[15] that simultaneously produces calcitonin and CGRP mRNAs. This analysis confirms that authentic CGRP

Table 1 Quantitative analysis of CGRP mRNA in the brain

Region assayed	CGRP mRNA	Calcitonin mRNA
Trigeminal ganglion	100	ND
Lateral medulla	4.8	ND
Midbrain	0.4	ND
Hypothalamus	0.4	ND
Amygdala	<0.1	ND

Quantitative densitometric scan analysis of autoradiograms similar to those shown in Fig. 4*b*, with adjustments for the time of exposure such that each band analysed was within the linear portion of the exposure. Results are presented as per cent of the maximal protection (observed using trigeminal nuclear poly(A)-rich RNA). The medullary thyroid cell line RNA standard is from a clonal line of rat medullary thyroid cells which produce extremely low levels of both calcitonin and CGRP mRNAs. ND, None detectable.

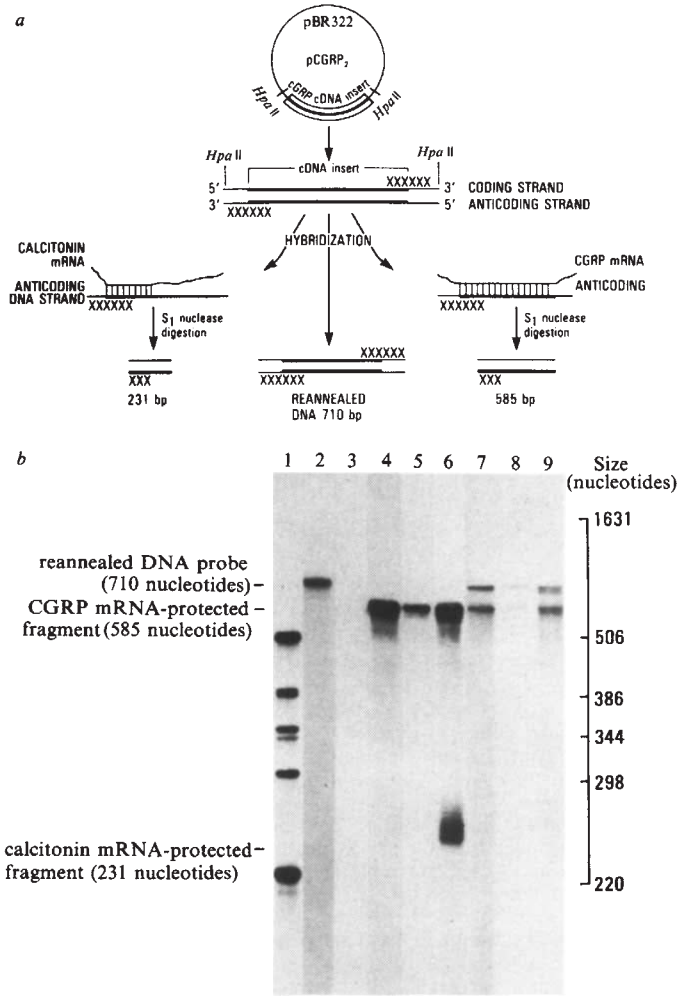

Fig. 4 S_1 nuclease mapping of CGRP mRNA sequences in the brain. *a*, The strategy used for S_1 nuclease analysis. The plasmid $pCGRP_2$, which contains a 585 bp sequence of CGRP cDNA inserted into the *Pst*I site of pBR322, was restricted with *Hpa*II such that an excised 710 bp fragment contains the 585 bp CGRP cDNA insert flanked by short sequences contributed by pBR322. This fragment provided a convenient probe for analysis of CGRP mRNA. The fragment was labelled by first incubating in 33 mM Tris-Ac (*p*H 7.9), 66 mM KAc, 10 mM MgAc, 100 μg ml^{-1} bovine serum albumin, 5.4 mM dithiothreitol for 20 min at 37 °C with 0.4 U ml^{-1} T4 DNA polymerase to permit 3′ exonuclease digestion, and then adding deoxyribonucleotides, including 100 μCi of [α^{32}P]dCTP (300 Ci $mmol^{-1}$), and continuing to incubate for 10 min such that the digested DNA is quantitatively replaced (2 mM unlabelled dCTP was added to 0.1 mM final concentration during the final minute of the reaction). Following phenol/chloroform extraction and ethanol precipitation, the labelled *Hpa*II fragment was subjected to electrophoresis through a 5% polyacrylamide gel. The DNA was eluted, melted and aliquots (50,000 c.p.m., 5×10^{7}–5×10^{8} c.p.m. per μg DNA) were mixed with buffer containing 20 μg of poly(A)-selected RNA from the tissue being analysed in the presence of 10 μg *Escherichia coli* tRNA and ethanol precipitated. The nucleic acids were dissolved in 40 μl of hybridization buffer containing 50 mM PIPES (*p*H 6.4), 500 mM NaCl, 1 mM EDTA, 80% formamide, heated to 85 °C for 10 min, then placed at 55 °C for 3 h. The reaction was diluted 10-fold with buffer containing 280 mM NaCl, 30 mM NaAc (*p*H 4.5), 4.5 mM $ZnSO_4$ and 20 μg ml^{-1} single-stranded DNA and incubated for 30 min at 37 °C with 500 U of S_1 nuclease. Following the nuclease digestion, the sample was subjected to phenol extraction, ethanol precipitated, resuspended in 5 μl of formamide and subjected to electrophoresis using a 4% acrylamide, urea gel. Any reannealed DNA will be 710 bases in length; 585 nucleotides of radiolabelled fragment will be protected from S_1 nuclease-digestion if hybridized to CGRP mRNA, while 231 nucleotides of radiolabelled fragment will be protected from S_1 nuclease digestion if hybridized to calcitonin mRNA. In the absence of added RNA, only the trace amount of reannealed DNA would remain undigested. *b*, Analysis of S_1 nuclease protection of CGRP cDNA using brain poly(A)-rich RNA. Autoradiographs of: lane 1, *Hin*f-digested pBR322 standards (3 h exposure); lane 2, aliquots of undigested probe (21 h exposure); lane 3, probe sham hybridized with carrier RNA only (72 h exposure); lane 4, hybridization to 20 μg poly(A)-rich RNA from trigeminal ganglia (3 h exposure); lane 5, hybridization to 20 μg poly(A)-rich RNA lateral medulla (11 h exposure); lane 6, hybridization to 17 μg poly(A)-rich RNA from the clonal rat medullary thyroid cell line (ref. 19, a gift of F. Zeytinoglu) (11 h exposure); lane 7, hybridization to 20 μg poly(A)-rich RNA from midbrain (72 h exposure); lane 8, hybridization to 20 μg poly(A)-rich RNA from temporal tissue (amygdala) (72 h exposure); lane 9, hybridization to 20 μg poly(A)-rich RNA from hypothalamus (72 h exposure). The migration of reannealed, CGRP mRNA-protected and calcitonin mRNA-protected probe are indicated. Similar results were obtained in three independent analyses.

mRNA is produced in regions of the central nervous system that contain CGRP-immunoreactive cell bodies.

CGRP mRNA levels were quantitated by a densitometric scan analysis of autoradiograms similar to that shown in Fig. 4*b*, and the results of such an analysis are shown in Table 1. The levels of CGRP mRNA are considerably higher in the trigeminal ganglion than in specific regions of the brain. CGRP RNA sequences are approximately 0.5–1% as frequent in the trigeminal ganglion as in rat medullary thyroid tumours, which contain ~10,000 copies per cell. On the basis of immunohistochemical reactivity, it was estimated that about 1% of trigeminal ganglia neurones[16] are CGRP-producing, and that they contain ~4,000–20,000 copies of CGRP mRNA per cell (similar to the levels of prolactin and growth hormone mRNAs in their respective cells of origin in the anterior pituitary[17]).

The rate of transcription of the calcitonin gene in the trigeminal ganglion was estimated by DNA-excess hybridization experiments, after labelling the nascent RNA transcripts in nuclei isolated from trigeminal ganglia by incubation with [α-^{32}P]UTP as described previously[17,18], as 200–300 p.p.m. per kilobase (kb) of DNA probe in each CGRP-synthesizing neurone (which compares well with a rate of 100–300 p.p.m. per kb probe estimated for medullary thyroid calcitonin-producing tumours).

The relative level of CGRP mRNA in each brain region parallels the percentage of CGRP-reactive neurones present in each region assayed (see Table 1). In an area rich in CGRP-stained terminal fields (central nucleus of the amygdala), very little CGRP mRNA was detected. No calcitonin mRNA sequences were detected in any brain region, even though the S_1 nuclease assay used would easily detect transcripts present at concentrations of less than 0.2% of that of CGRP mRNA. Thus, if any calcitonin-synthesizing neurones are present in the brain, they would have to represent a very small number of cells producing extremely small quantities of calcitonin mRNA and immunoreactive calcitonin to escape detection.

Protein encoded by CGRP mRNA

From the sequence of CGRP mRNA it is predicted that it encodes of 16,000 MW protein, the precursor of CGRP. As shown in Fig. 5, hybridization of rat trigeminal ganglia poly(A)$^+$ RNA to an immobilized plasmid containing a CGRP-specific insert specifically selects a mRNA that directs the synthesis of a 16,000 MW protein in a cell-free translation system. This product co-migrates with the protein encoded by the authentic CGRP mRNA isolated from rat medullary tumour cells. mRNA from the lateral medulla (presumably including the nucleus ambiguus and facial nucleus) also directed the synthesis of the 16,000 MW protein. Thus, brain CGRP mRNA sequences appear to direct the synthesis of the protein precursor predicted by the CGRP cDNA sequence.

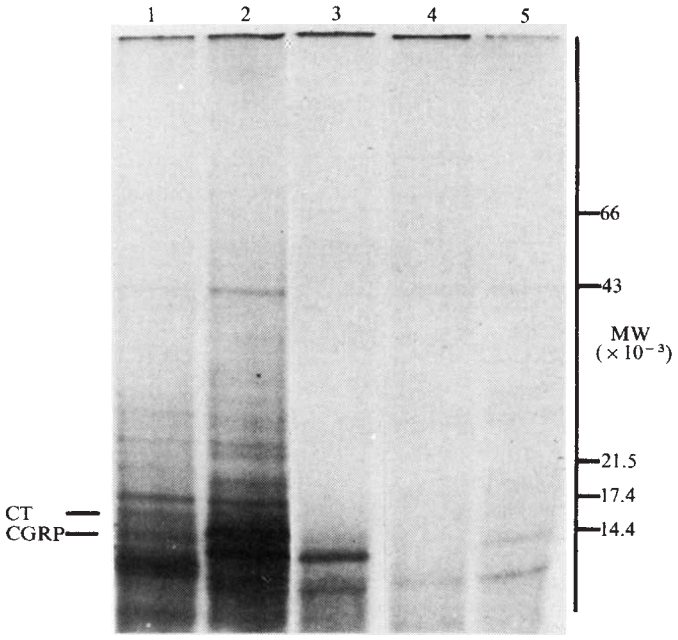

Fig. 5 Cell-free translation of CGRP mRNA from the brain. Autoradiograms demonstrate the translation products directed by: lane 1, poly(A)-rich RNA from trigeminal ganglia; lane 2, poly(A)-rich RNA from a medullary tumour; lane 3, medullary tumour RNA selected by the CGRP-specific plasmid; lane 4, trigeminal ganglion RNA selected by pBR322 as a control; lane 5, trigeminal ganglion RNA selected by the CGRP-specific plasmid. The migration of calcitonin and CGRP precursors is indicated. The ^{35}S-methionine-labelled bands observed in the pBR322 control lane are present in the absence of any added RNA and are thought to represent the translation products of endogenous wheat germ mRNAs.
Methods: Poly(A)-rich RNA prepared from trigeminal ganglia (5 μg) or from a rat medullary tumour producing both calcitonin and CGRP mRNAs (1.6 μg) was allowed to hybridize to plasmids immobilized on nitrocellulose. The hybridization selection and elution were performed as described previously[19] using nitrocellulose filters to which 2 μg of denatured, linearized plasmid containing a CGRP mRNA-specific cDNA insert (lanes 3, 5) or pBR322 (lane 4) had been bound. The RNAs eluted from both control (pBR322) and CGRP-specific filters were translated in a nuclease-treated wheat germ lysate, and the ^{35}S-methionine-labelled products were subjected to SDS-polyacrylamide gel electrophoresis, as described previously[2].

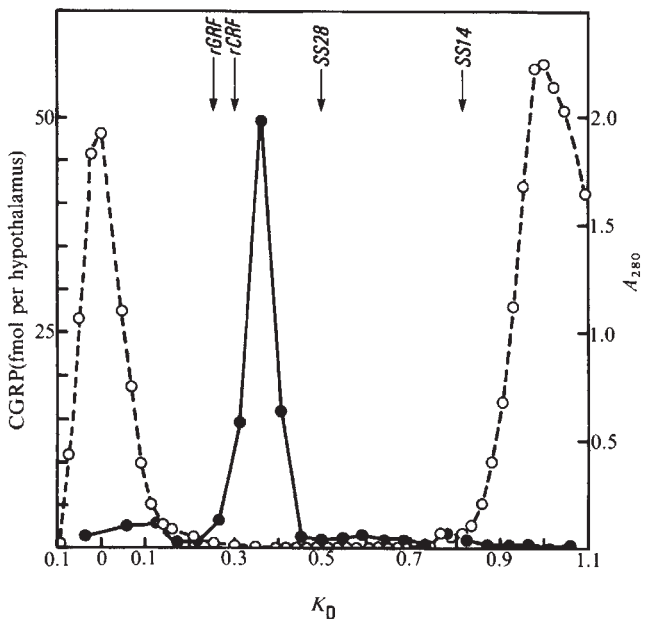

Fig. 6 Gel filtration of a defatted acetic acid extract of 20,000 rat hypothalamic fragments. Extraction and gel filtration (G50 fine) were performed as described previously[20] using a Pharmacia K 15/100 column (21.5×90 cm, $V_t = 32.6$ L). The column was eluted with 3 M acetic acid, 0.1% β-mercaptoethanol at a flow rate of 750 ml h^{-1}. Each fraction was quantitated for material immunoreactive to CGRP antiserum using radioimmunoassay. Elution of rat growth hormone releasing factor (rGRF), rat corticotropin-releasing factor (rCRF), somatostatin (●—●). 14 (SS14), and somatostatin-28 (SS28) are indicated by arrows. $K_D = V_e - V_0 / V_t - V_0$; where V_e = elution volume; V_0 = void volume; V_t = total volume. A semi-logarithmatic plot of molecular weight standards suggests a theoretical molecular weight of the CGRP-immunoreactive material of 4225.

However, the presence of CGRP mRNA and CGRP antisera immunoreactivity in brain cells does not conclusively establish the protein-processing scheme shown in Fig. 1. Evidence that CGRP itself really is the product of calcitonin gene expression in the brain was obtained by characterization of CGRP-immunoreactive material derived from rat hypothalami. The predominant component of the immunoreactive material is a small peptide, estimated by gel filtration to have a MW of 4,200 (Fig. 6), and which co-migrates with synthetic CGRP standard on HPLC (unpublished data). These data strongly support the scheme shown in Fig. 1 for the generation of CGRP in the brain.

Concluding remarks

An analysis of calcitonin gene expression has provided the first example of tissue-specific regulation of RNA processing pathways in the nervous and endocrine systems, which may be one of the ways that diversity in the neuroendocrine system is increased and which may have important physiological consequences. Calcitonin gene expression, therefore, provides a useful model system for exploring the mechanisms responsible for developmentally determined RNA processing, and for defining an approach to investigate the production, distribution and function of neuropeptides predicted to be made by nucleotide sequencing. This approach is based on the use of antisera against a synthetic polypeptide (representing part of the encoded protein) and S_1 nuclease mapping, which permit simultaneous analysis of both the synthetic pathways involving the peptide and the neuronal sites of peptide synthesis. The modified S_1 nuclease protection assay used here confirms that CGRP mRNA is made in the brain and identifies the sites of its biosynthesis. CGRP mRNA in the brain is regionally distributed in a way that corresponds to the immunohistochemical distribution of the reactive peptide, and brain CGRP mRNA directs the cell-free synthesis of the predicted 16,000 MW CGRP precursor protein. That this precursor really is processed by proteolysis in the brain is demonstrated by the size of the brain immunoreactive CGRP; unequivocal evidence that no other post-translational modifications occur can be obtained only by sequencing the purified peptide.

Immunoreactive calcitonin and calcitonin mRNA were not detected in rat brain or pituitary. Earlier reports of immunoreactive calcitonin in the pituitary gland and/or brain of the rat were based on immunohistochemical or radioimmunoassay evidence[21–25]. On the basis of the data presented here, and of RNA blot analyses of the pituitary[2,25], the immunological data in rats probably reflect either the detection of a substance cross-reacting with calcitonin antisera, or the binding of calcitonin from the blood that has entered circumventricular regions of the brain that lack a blood–brain barrier to peptides. Calcitonin membrane receptor sites in the hypothalamus and pituitary of rat and human brain have been reported[26,27], although it is possible that these receptors bind a calcitonin-related peptide rather than calcitonin itself. Reports of immunoreactive calcitonin in the central nervous system of birds[28], reptiles[29], protochordates[30] and sea squirts[31] could reflect either a different organization of the calcitonin gene, altered developmental switching events, or binding of calcitonin from the blood. With regard to these possibilities, we find that the chicken hypothalamus contains large numbers of CGRP-

stained cells and fibres, consistent with the presence and expression of CGRP exons in the chicken calcitonin gene.

The distribution of CGRP in specific sensory, integrative and motor systems suggests several possible functions for the peptide. The differential distribution of CGRP immunoreactivity in the olfactory and gustatory systems, in the hypoglossal, facial and vagal nuclei, and in the hypothalamus and limbic regions, strongly suggests that it may have a functional role in ingestive behaviour. CGRP is present in small trigeminal and spinal sensory ganglion cells, which are known to relay thermal and nociceptive information from the head and body to the brain stem and spinal cord. Applying a method combining immunohistochemical and fluorescence retrograde transport techniques[32], CGRP was also shown to be present in many of the motor neurones in rostral, but not caudal, parts of the nucleus ambiguus (Fig. 2). This part of the nucleus ambiguus is believed to be particularly concerned with certain visceral motor functions mediated by the vagus nerve. The production of a polypeptide hormone by cholinergic cells has already been suggested for vasoactive intestinal peptide (VIP)[33]. CGRP is also present and widely distributed in fibres in blood vessels and the visceral organs, and in the adrenal medulla where, as in the sensory ganglia, CGRP is one of several neuropeptides (for example VIP, substance P, enkephalins), each present in a subpopulation of cells[34,35]. This distribution suggests CGRP may be involved in cardiovascular regulation. In fact, administration of synthetic CGRP exerts profound and unique effects on blood pressure and blood catecholamine levels in the rat (L. Fisher *et al.*, manuscript submitted). Finally, the production of CGRP in several glandular tissues suggests that it may also have endocrine functions.

We thank Dr B. A. Roos and D. Chin, J. Vaughan, A. Corrigan, Robert Galyean, Rick McClintock, Ron Kaiser and M. Richards for their help. This work was supported by grants from the American Cancer Society, the NIH and the American Heart Association–California Affiliate.

Received 30 December 1982; accepted 22 April 1983.

1. Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S. & Evans, R. M. *Nature* **298,** 240–244 (1982).
2. Amara, S. G., David, D. N., Rosenfeld, M. G., Roos, B. A. & Evans, R. M. *Proc. natn. Acad. Sci. U.S.A* **77,** 4444–4448 (1980).
3. Amara, S. G. *et al. J. biol. Chem.* **257,** 2129–2132 (1982).
4. Jacobs, J. W. *et al. Science* **213,** 457–459 (1981).
5. Rosenfeld, M. G., Amara, S. G., Roos, B. A., Ong, E. S. & Evans, R. M. *Nature* **290,** 63–65 (1980).
6. Rosenfeld, M. G. *et al. Proc. natn. Acad. Sci. U.S.A.* **79,** 1717–1721 (1982).
7. Ziff, E. B. *Nature* **287,** 491–499 (1980).
8. Alt, F. W. *et al. Cell* **20,** 293–301 (1980).
9. Early, P., Rogers, J., Davis, M., Calame, K., Bond, M., Wall, R. & Hood, L. *Cell* **20,** 313–319 (1980).
10. Maki, R. *et al. Cell* **24,** 353–365 (1981).
11. Vale, W., Ling, N., Rivier, J., Douglas, C. & Brown, M. *Metabolism* **25,** 1491–1494 (1976).
12. Rivier, J. *J. liquid Chromatogr.* **1,** 343–367 (1978).
13. Swanson, L. W., Sawchenko, P. E., Rivier, J. & Vale, W. W. *Neuroendocrinology* **36,** 165–186 (1983).
14. Weaver, R. F. & Weissman, C. *Nucleic. Acids Res.* **7,** 1175–1193 (1979).
15. Zeytinoglu, F. N., DeLellis, A., Gagel, R. F., Wolfe, H. J. & Tashjian, A. H. *Endocrinology* **107,** 516–521 (1980).
16. Forbes, D. J. & Welt, C. *J. comp. Neurol.* **199,** 133–147 (1981).
17. Evans, R. M., Birnberg, N. & Rosenfeld, M. G. *Proc. natn. Acad. Sci. U.S.A.* **79,** 7659–7663 (1982).
18. Murdoch, G. H., Potter, E., Nicolaisen, A. K., Evans, R. M. & Rosenfeld, M. G. *Nature* **300,** 192–194 (1982).
19. Parnes, J. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **78,** 2253–2257 (1981).
20. Vale, W., Vaughan, J., Yamamoto, G., Spiess, J. & Rivier, J. *Endocrinology* **112,** 1553–1555 (1983).
21. Deftos, L. J. *et al. Life Sci.* **23,** 743–748 (1978).
22. Deftos, L. J. *et al. J. clin. Endocr. Metab.* **47,** 452–459 (1978).
23. Cooper, C. W., Peng, T.-C., Obie, J. F. & Garner, S. C. *Endocrinology* **107,** 98–107 (1980).
24. Mendelsohn, G., D'Agostino, R., Eggleston, J. C. & Baylin, S. B. *J. clin. Invest.* **63,** 1297–1306 (1979).
25. Jacobs, J. W., Goltzman, D. & Habener, J. F. *Endocrinology* **11,** 2014–2019 (1982).
26. Sagar, S. M. & Martin, J. B. *Proc. natn. Acad. Sci. U.S.A.* **78,** 7801–7805 (1981).
27. Nakamuta, H. *et al. Jap. J. Pharmac.* **31,** 53–60 (1981).
28. Galan Galan, F., Rogers, R. M., Girgis, S. I. & MacIntyre, I. *Brain Res.* **212,** 59–66 (1981).
29. MacInnes, D. G., Laszlo, I., MacIntyre, I. & Fink, G. *Brain Res.* **251,** 371–373 (1982).
30. Girgis, S. I. *et al. J. Endocr.* **87,** 375–382 (1980).
31. Fritsch, H. A. R., Van Noorden, S. & Pearse, A. G. E. *Cell Tissue Res.* **202,** 263–274 (1979).
32. Sawchenko, P. E. & Swanson, L. W. *Brain Res.* **210,** 31–51 (1981).
33. Lundberg, J. M., Änggård, A., Fabrenkrug, J., Hökfelt, T. & Mutt, V. *Proc. natn. Acad. Sci. U.S.A.* **77,** 1651–1655 (1981).
34. Hökfelt, T., Lundberg, J. M., Schultzberg, M. & Fahrenkrug, J. *Acta physiol. scand.* **113,** 575–576 (1981).
35. Schultzberg, M. *et al. Neuroscience* **3,** 1169–1186 (1978).

Translocation joins c-*myc* and immunoglobulin γ1 genes in a Burkitt lymphoma revealing a third exon in the c-*myc* oncogene

P. H. Hamlyn* & T. H. Rabbitts†

* Ludwig Institute for Cancer Research, MRC Centre, and † MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

*Recombinant DNA clones have been used to directly demonstrate that the Burkitt lymphoma cell line Raji, t(8;14)(q24;q32), has a rearranged copy of the c-*myc *gene adjacent to the γ1 constant region gene of the human immunoglobulin heavy-chain locus; the genes are arranged in the opposite direction for transcription. At least one further 5′ exon has been detected in the normal c-*myc *gene by analysis of RNA transcripts, and the occurrence of high levels of two c-*myc *mRNA size classes, both apparently initiating in this exon, is described.*

BURKITT'S lymphoma (BL) is a form of B-cell leukaemia that occurs in man. An interesting feature of these tumours is that the cells almost invariably carry specific chromosomal translocations involving a segment of chromosome 8(8q24 → qter)[1]. This translocation frequently involves reciprocal exchange with the long arm of chromosome 14 (ref. 1) at q32. Variant translocations of chromosome 8 with chromosome 2(2p12) or 22(22q11) are also known[2]. Recent genetic mapping studies have shown that the human heavy(H)-chain genes are located on chromosome 14 (refs 3, 4), the κ light(L) chains on chromosome 2 (ref. 5) and the λ light chains on chromosome 22 (ref. 6): furthermore, the localizations have been refined to 14q32, 2p12 and 22q11 for H, κ and λ chains respectively[5,7,8]. Thus, it was strongly suggested that the immunoglobulin genes are directly involved in these specific translocations in BL.

A gene sequence from chromosome 8 which is implicated in the BL translocations is the human homologue of the avian myelocytomatosis virus oncogene, designated c-*myc*. This gene has been mapped to the position on human chromosome 8 which is involved in the BL translocation (8q24)[9,10], and indeed Southern filter hybridization indicates that the c-*myc* and the μ H-chain constant(C) region gene are present, in a small proportion of BL cells, on the same restriction fragment[11,12].

We now present direct proof that the c-*myc* gene is translocated into the H-chain locus in a BL cell line (Raji) by cloning, in λ phage, the rearranged and non-rearranged c-*myc* genes

0028-0836/83/280135—05$01.00