

Neuron-Specific Alternative RNA Processing in Transgenic Mice Expressing a Metallothionein–Calcitonin Fusion Gene

E. Bryan Crenshaw III,*† Andrew F. Russo,*
Larry W. Swanson,‡ and Michael G. Rosenfeld*

* Howard Hughes Medical Institute
Eukaryotic Regulatory Biology Program
School of Medicine, M-013
University of California, San Diego
La Jolla, California 92093
† Department of Biology
University of California, San Diego
La Jolla, California 92093
‡ Howard Hughes Medical Institute
Neural Systems Laboratory
The Salk Institute
La Jolla, California 92037

Summary

Alternative RNA processing of the calcitonin/CGRP gene generates transcripts encoding predominantly calcitonin in thyroid C cells or CGRP in the nervous system. To examine the RNA processing choice of this gene in a wide variety of tissues, we created transgenic mice expressing the rat calcitonin/CGRP transcript from the mouse metallothionein-I promoter. Most cells that do not express the endogenous calcitonin/CGRP gene have the capability to make a clear splicing choice for calcitonin or CGRP transcript. In the majority of tissues studied, 90%–97% of the transgene mRNA encodes calcitonin. In contrast, both calcitonin and CGRP mRNAs were detected in the transgenic mice brains. Immunohistochemical and *in situ* RNA hybridization analyses show that CGRP transcripts are selectively expressed in a wide variety of neurons, while calcitonin is expressed predominantly in nonneuronal structures. Splicing choice operates independently of calcitonin/CGRP gene transcription. The data suggest that a specific regulatory machinery is required for the processing of CGRP transcripts and is restricted primarily to neurons.

Introduction

Alternative RNA processing is a developmental strategy that determines the phenotype of several cell types (for reviews, see Rosenfeld et al., 1984; Padgett et al., 1986; Leff et al., 1986). A clear example of regulated alternative RNA processing is the switch of immunoglobulin M or D from the membrane form to the secreted form during B cell development (Alt et al., 1980; Early et al., 1980; Maki et al., 1981). Like immunoglobulins, many proteins have developmentally regulated isoforms that differ by a single functional domain. These structural changes are often made by the choice of exons included in the RNA transcript. For example, splicing choice is used to produce fetal or adult forms of myosin (Nabeshima et al., 1984; Rzek and Davidson, 1986), cellular or plasma forms of

fibronectin (Schwarzauer et al., 1983; Tamkun et al., 1984), and several isoforms of troponin T (Breitbart et al., 1985). In the nervous and endocrine system, the functional domain that is included in or excluded from a prohormone can encompass a complete neuropeptide. Examples of neuropeptide splicing choices include calcitonin/CGRP (Amara et al., 1982), pre-kininogen (Kitamura et al., 1983), and substance P/substance K (Nawa et al., 1984). These choices, which increase the diversity of peptides expressed in the neurons and endocrine cells, are undoubtedly crucial events in the development and function of complex systems like the brain.

The peptides calcitonin and calcitonin gene-related peptide (CGRP) are encoded by alternatively spliced transcripts from the same gene (Amara et al., 1982). The calcitonin/CGRP gene contains six exons. There are two polyadenylation sites one following the fourth and one following the sixth exon, used in generating calcitonin and CGRP mRNA, respectively. CGRP mRNA is formed by splicing sequences representing the first three exons (common to calcitonin and CGRP mRNAs) with the fifth and sixth exons (see Figure 1). The calcitonin/CGRP transcription unit uses an identical cap site and termination site approximately 1 kb downstream from the second (exon 6) polyadenylation site, irrespective of the type of mature transcript generated (Amara et al., 1984). This excludes alternative transcription initiation or termination as the regulated event. Therefore, the choice of poly(A) site and the pattern of splicing of exon 3 to either exon 4 (calcitonin-coding) or exon 5 (CGRP-coding) must be regulated. This RNA processing choice is stringently regulated since greater than 95% of the calcitonin/CGRP gene transcripts are processed to mature calcitonin or CGRP mRNAs in their endogenous sites of expression (Amara et al., 1982; Sabate et al., 1985). Calcitonin transcripts are the primary mRNA observed in the thyroid C cells (Jacobs et al., 1981; Sabate et al., 1985). CGRP transcripts, on the other hand, are the primary mRNA observed in specific neurons distributed throughout the central and peripheral nervous systems (Rosenfeld et al., 1983; Kawai et al., 1985).

To determine whether the machinery for alternative splicing of the calcitonin/CGRP transcript can be found in tissues in which the gene is not normally expressed, we have created transgenic mice that widely express this transcript from a heterologous promoter. We chose the metallothionein I (MT-I) promoter because it is expressed in many different tissues, can be regulated by heavy metals, and has previously been shown to direct expression of fusion genes to a number of tissues in transgenic mice (Palmeter et al., 1983), including discrete subsets of neurons (Swanson et al., 1985). Based on the expression pattern of the calcitonin/CGRP fusion gene in transgenic mice, we propose that the ability to splice the calcitonin/CGRP primary transcript to the mature CGRP transcript requires a specific splicing machinery that is expressed in many neurons and that the majority of tissues

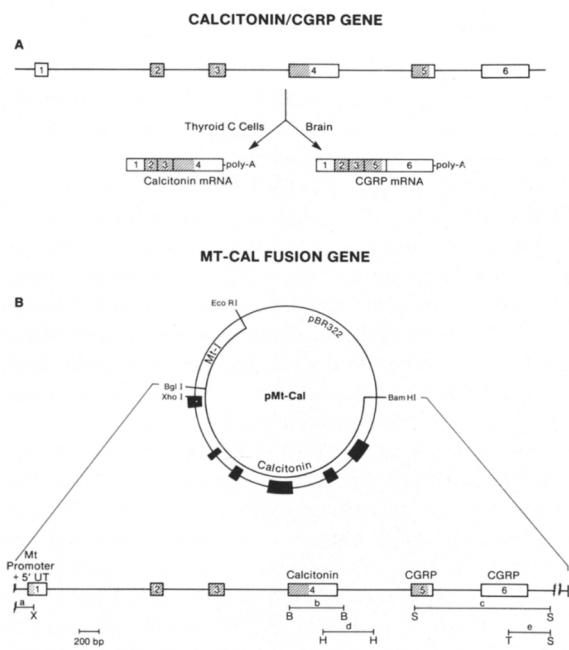


Figure 1. Structure and Expression of the Calcitonin/CGRP and MT-Cal Genes

(A) The endogenous calcitonin/CGRP gene contains six exons that are alternatively processed to produce the calcitonin transcript in thyroid C cells or the CGRP transcript in the nervous system. Coding regions are shown as shaded boxes, and noncoding regions are shown as open boxes.

(B) The MT-Cal fusion gene contains the metallothionein promoter fused to the calcitonin/CGRP gene. The 5'-flanking and a small portion of 5'-untranslated region (BglII at position -185 to XbaI at +62 bases from the MT cap site) of the mouse MT-I gene were fused to the genomic rat calcitonin/CGRP gene at a PstI site, 11 bases upstream of the calcitonin/CGRP CAP site. The coding regions are shown as shaded boxes, the noncoding regions as open boxes, and the MT 5'-untranslated region as a stippled box. The gene and its expected mRNA products are shown to scale. The regions used for hybridization probes (labeled a-e), with the relevant restriction enzyme sites are shown below the gene. Restriction endonucleases are designated by B (BglII), H (HaeIII), S (Sau3A), T (TaqI), and X (XbaI).

express the calcitonin transcript because they lack the requisite CGRP splicing machinery.

Results

Construction and Microinjection of the Metallothionein–Calcitonin Fusion Gene

We constructed a hybrid fusion gene, pMT-Cal, that places the calcitonin/CGRP gene under the transcriptional regulation of the mouse MT-I promoter (Figure 1). The transcript from this fusion gene consists of 62 bp of 5'-untranslated region of MT-I, 11 bp of 5'-flanking sequences from the calcitonin/CGRP gene, and the 6 exons of the calcitonin/CGRP gene. Approximately 200 copies of the BglII–BamHI fragment of MT-Cal were microinjected into the male pronuclei of 210 fertilized eggs, and 115 eggs were selected for transfer into pseudopregnant females. Of 19 mice that developed to term, 7 mice had integrated 1 to 70 copies of the transgene (data not shown). Four

lines were established from the progenitor animals (MC3, MC4, MC5, and MC6). Three of these lines expressed the transgene in the liver, while the MC3 line did not express the transgene in any tissues examined. This result is consistent with the expression pattern seen with other MT fusion genes (Palmiter et al., 1983). The tissue distribution of splicing was examined in greater detail in the three lines that express the transgene.

Tissue-Specific Expression of the MT-Cal Fusion Gene

By using probes specific for calcitonin or CGRP mRNA (see Figure 1, probes b and c), we were able to distinguish the two alternative RNA products, following gel fractionation and RNA blotting analysis. As shown in Figure 2, calcitonin mRNA is the predominant processing choice in the liver and kidney. CGRP probes hybridized to large molecular weight nuclear RNAs (3.6 and 4.2 kb), which contain both the calcitonin and CGRP exons and may represent nonproductive transcripts or precursor RNAs. Only 3%–8% of the mature transcripts of the transgene RNA were expressed as CGRP mRNA (1.2 kb). In contrast, the transgene RNA in the brain was processed to both CGRP and calcitonin RNA (Figure 2). In addition to the liver and kidney, most tissues we tested expressed primarily calcitonin RNA (Table 1). Based on hybridization analysis of size-fractionated total RNA, we found that in skeletal muscle, spleen, gut, and lung greater than 90% of mature transcript was calcitonin RNA. The only non-neuronal location that expressed greater than 10% CGRP mRNA was the heart (Table 1). The relative levels of expression of the transgene (Table 1) parallel that of the endogenous MT-I, as is usually seen with MT fusion genes (Palmiter et al., 1983). The splicing choice is independent of the expression level, because tissues with comparable levels of expression can make very different splicing decisions (Table 1). Furthermore, the MC5 pedigree expressed the transgene at levels 4- to 5-fold higher than the MC4 pedigree in all tissues tested, yet exhibited a virtually identical pattern of relative mRNA processing. The expression level and integration site were not critical determinants for the RNA processing, because the processing choices were qualitatively the same in tissues from all three pedigrees examined, even after induction of liver and kidney MT-Cal RNA levels by zinc treatment. There was no detectable calcitonin or CGRP RNA in these tissues from nontransgenic control mice (with or without zinc), except for relatively low amounts of the endogenous mouse CGRP mRNA in the brain (expressed at roughly 5% the MC5 transgenic CGRP mRNA levels).

Because the nucleic acid probes used for this analysis do not distinguish between RNA from the rat transgene and endogenous mouse calcitonin/CGRP gene, it was necessary to show that the CGRP expression in the transgenic mouse brain was not due to an unexpected induction of the endogenous gene. To differentiate expression of the transgene from the expression of the endogenous gene, we took advantage of the fact that the MT-Cal gene has 62 bases of the MT-I 5'-untranslated region fused to the calcitonin/CGRP gene. Using this region of the trans-

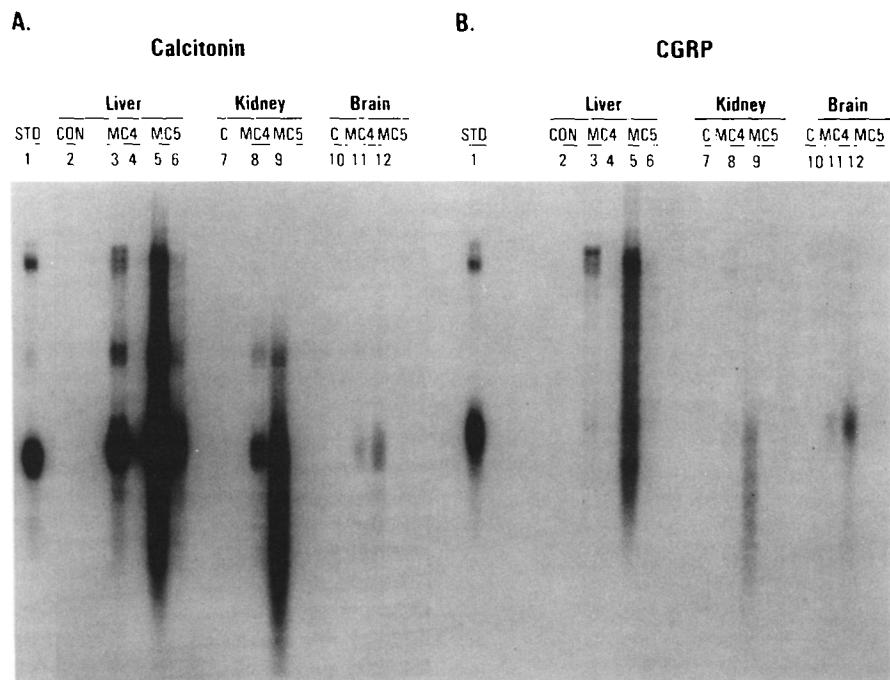


Figure 2. Northern Analysis of Calcitonin and CGRP RNA in the MT-Cal Transgenic Mice Tissues

The RNA processing choice was clearly biased toward calcitonin in the liver and kidney, while the brain produced both calcitonin and CGRP mRNAs. There was no detectable calcitonin or CGRP in the tissues from control mice, except for relatively low amounts of CGRP in the brain (roughly 5% of the transgenic levels). The standard (lane 1) is 50 ng of poly(A)-enriched WA medullary thyroid carcinoma (MTC) RNA, which contains equal amounts of calcitonin and CGRP RNA. Liver poly(A)-enriched RNA samples are lanes 2, 3, 5 (10 µg), 4, and 6 (1 µg). Kidney poly(A)-enriched samples are lanes 7, 8, and 9 (3 µg). Brain poly(A)-enriched RNA samples are lanes 10, 11, and 12 (1 µg). The 4.2 and 3.6 kb RNA species are nuclear species that contain both the calcitonin and the CGRP exons, and the 2.2 kb nuclear species contains only calcitonin, and not CGRP, exons.

Table 1. Tissue Distribution of Calcitonin and CGRP RNAs in MT-Cal Mice

Tissue	Relative RNA Levels	% Calcitonin RNA
Liver	100	97
Kidney	30	93
Skeletal muscle	0.8	98
Lung	1	>90
Spleen	<0.5	>90
Stomach	3	>90
Submandibular	4	>90
Heart	1	58
Brain	4	42

The RNA processing choice for calcitonin versus CGRP mRNA was determined by scanning densitometry of autoradiographs of size-fractionated RNA from zinc-treated MC5 mice using calcitonin- and CGRP-specific probes, as described in Experimental Procedures. For some tissues there was no detectable CGRP. Based on our limits of detection, we estimate that calcitonin RNA in these tissues represents greater than 90% of the mature transgene mRNA.

gene as a probe (see Figure 1, probe a), there was hybridization to two RNA species, which corresponded in size to CGRP and calcitonin RNA, from the transgenic mice brains, but not from control mice brains (Figure 3). For comparison, transgenic liver contained mostly RNA the size of calcitonin, as well as the endogenous MT-I RNA (Figure 3).

Immunohistochemical Analysis of MT-Cal Expression in the Brain

Because both calcitonin and CGRP RNAs were expressed in the transgenic brain, it was necessary to establish whether discrete neuronal regions selectively express only one mature transcript or whether no clear splicing choice is made. Immunohistochemical analysis showed selective CGRP immunofluorescence in the pyramidal cell layer of hippocampal field CA3 and in the mossy fiber axonal projection to CA3 cells from the granule cells in the dentate gyrus (Figure 4). Colchicine pretreatment, which blocks axonal transport of peptides, diminished CGRP immunoreactivity in the mossy fiber afferents and increased the signal in granule and CA3 pyramidal cell bodies. These cell types were not stained by antisera against calcitonin, and were not stained in normal mice by antisera against either calcitonin or CGRP. Calcitonin, but not CGRP, immunostaining was detected in the livers of these animals, confirming that the antibodies can recognize the calcitonin product of the transgene. Calcitonin and CGRP were also detected in the expected endogenous locations in the thyroid and brain, respectively. These results demonstrate that neurons which do not express the endogenous gene can make an unambiguous choice for CGRP.

Although clear staining for CGRP was observed in hippocampal neurons, we were unable to detect clear immunostaining for calcitonin or CGRP in other regions of

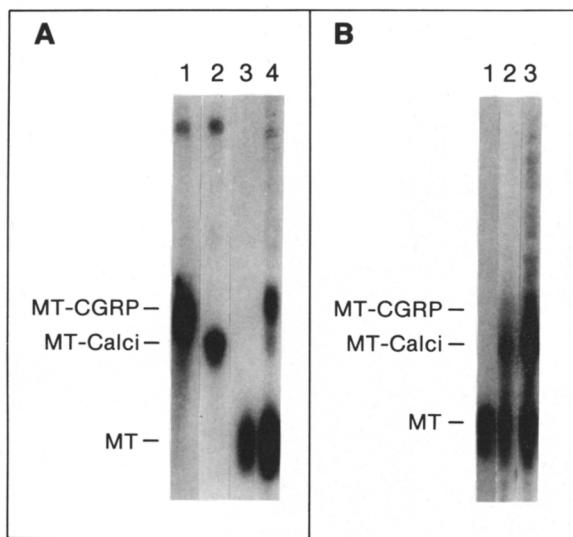


Figure 3. Identification of Calcitonin and CGRP RNAs as Products of the MT-Cal Fusion Gene

Transgene expression in the MT-Cal mice was confirmed by Northern analysis of total RNA hybridized with a probe consisting of metallothionein 5' region (Figure 1, probe a), except as noted in lanes 1 and 2 of (A). (A) Lanes 1 and 2, WA MTC standard RNA (50 ng poly(A)-enriched) hybridized with either calcitonin (lane 1) or CGRP (lane 2) probes to provide size standards; lane 3, control mouse brain RNA (20 µg); lane 4, MT-Cal brain RNA (20 µg, MC5). (B) Lane 1, control mouse liver RNA (10 µg); lanes 2 and 3, MT-Cal liver RNA (10 µg and 20 µg, MC5). The right panel was exposed for about a 10-fold shorter period to obtain comparable intensities for the endogenous metallothionein.

the brain where CGRP is not normally expressed. The discrepancy between the levels of fusion gene transcripts by RNA blot analysis and the immunohistochemical analysis suggested the necessity of an *in situ* hybridization histochemical analysis to examine the splicing choices made within the brain.

Analysis of MT-Cal Expression in the Brain by In Situ Hybridization Histochemistry

To localize the MT-Cal RNA products, *in situ* hybridization of mice brain sections was performed using specific RNA

probes (see Figure 1, probes d and e). We found that the calcitonin RNA was primarily expressed in nonneuronal structures, such as the choroid plexus, ependyma, and pia mater (Figure 5). CGRP RNA was expressed at high levels in the hippocampus (CA3 pyramidal cells and dentate gyrus). It was also the major transcript in several discrete regions, including the reticular nucleus and ventrobasal complex of the thalamus, layer V of the neocortex (and to a lesser extent, layer II), the retrosplenial cortex, and the taenia tecta (Figure 5). A few regions of the brain, such as the granular layer and lateral nuclei of the cerebellum, gave equivalent hybridization signals with both probes. Calcitonin RNA appeared to be the major transcript in only a very few regions that contain neurons, such as the Purkinje layer of the cerebellum and the inferior colliculus (Figure 5).

Because the *in situ* hybridization technique does not distinguish hybridization to mature mRNA from hybridization to precursor RNAs, it was possible that a portion of calcitonin hybridization seen in the brain was actually due to large molecular weight transcripts containing both the CGRP and calcitonin exons (the 3.6 and 4.2 kb species). To address this possibility, we compared the relative amounts of calcitonin and CGRP RNA in the relatively homogeneous liver and kidney tissues by RNA blotting and *in situ* hybridization. Typically, the 3.6 and 4.2 kb nuclear RNAs in liver, kidney, and brain represent 10%–20% of the total calcitonin hybridization detected by RNA blot analysis, while mature CGRP mRNA represents 3%–7% of the mature transcripts in liver and kidney (Figure 2). Densitometric scans of the *in situ* hybridization autoradiographs indicated that liver and kidney contained 80% calcitonin-reactive and 20% CGRP-reactive species. These data are consistent with the interpretation that the *in situ* technique detects the 10%–20% of nuclear transcripts present in liver, kidney, and brain and underestimates the relative expression of mature calcitonin transcripts in the liver or CGRP transcripts in regions of the brain. Consequently, the results of hybridization histochemical analysis, which show that CGRP mRNA represents approximately 80% of the total hybridization in sites such as the hippocampus and neocortex, are likely to reflect a >90%

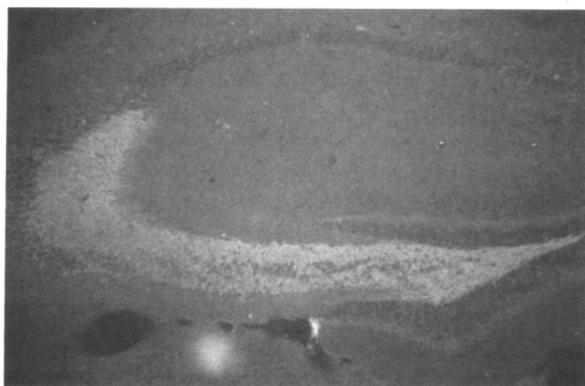


Figure 4. Immunohistochemical Identification of CGRP in the MT-Cal Mouse Hippocampus

The left panel is a bright-field image, and the right panel shows the immunofluorescence seen with anti-CGRP sera (35×). The immunofluorescence is limited to the CA3 pyramidal neurons and mossy fiber projections from the granule cells of the dentate gyrus. No calcitonin immunoreactivity was detected in the hippocampus.

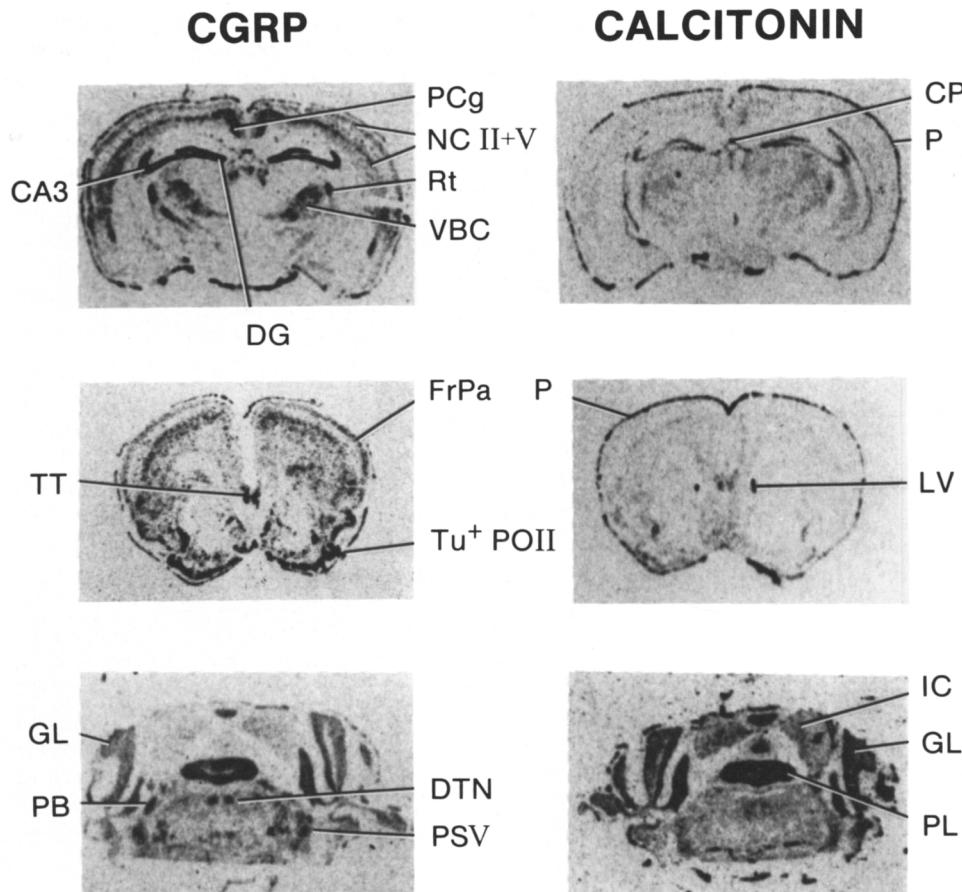


Figure 5. Localization of Calcitonin and CGRP Hybridization in Discrete Neuronal Structures of MT-Cal Mice Brains

In situ hybridization analysis was performed using ^{35}S -labeled RNA probes to identify the regions expressing the MT-Cal transgene. The results show calcitonin primarily in the pia (P), choroid plexus (CP), cerebellum Purkinje layer (PL), ependyma of the ventricles, e.g., lateral ventricle (LV), and inferior colliculus (IC). CGRP is primarily expressed in the hippocampus (CA3 pyramidal neurons and granule cells of the dentate gyrus [DG]), the retrosplenial (post-cingulate) cortex (PCg), the reticular nucleus (Rt) and ventrobasal complex (VBC) of the thalamus, the neocortex layers II and V (NCII+V), the taenia tecta (TT), the frontoparietal cortex (FrPa), the pyriform cortex layer II (POII), the olfactory tubercle (Tu), and the principal sensory nucleus of the trigeminal (PSV). CGRP RNA was also specifically detected in the parabrachial nucleus (PB) and the dorsal tegmental nucleus (DTN), where CGRP is expressed in control mice. Comparable levels of calcitonin and CGRP RNAs were seen in the cerebellum granular layer (GL). Exposure time was 6 days at 4°C.

processing choice for CGRP mRNA. Conversely, the slight amount of CGRP hybridization in nonneuronal structures, such as the pia mater, is likely to reflect the presence of unprocessed nuclear species.

Within the brain, we find that splicing choice is not correlated with the level of mRNA expression. For example, comparable high levels of expression of mature transcripts are observed in the ependyma of the lateral ventricles, which produces predominantly calcitonin mRNA, and in the taenia tecta, which produces predominantly CGRP mRNA (Figure 5). We also observe differential splicing decisions being made in neurons that express significantly lower mRNA levels (e.g., calcitonin mRNA expression in inferior colliculus is comparable to CGRP mRNA expression in the frontoparietal cortex). Although mRNA level is not solely dependent on the transcription rate, the data suggest that the splicing choice is independent of transcription rate in transgenic animals. Because tissues in the transgenic animals represent a heteroge-

neous population of cells, transcription rates in tissues would not accurately reflect the transcription rate in the cells expressing the transgene. To test directly whether transcription rate is independent of splicing choice, calcitonin/CGRP transcription was determined in a series of medullary thyroid carcinomas expressing either predominantly calcitonin or CGRP transcripts. As shown in Table 2, splicing choice is entirely independent of transcription rate. Therefore, these data show that the splicing decision is independent of mRNA levels and transcription rate in animals and in cell culture, respectively.

Analyses of nontransgenic animals with antisense probes and of transgenic animals with sense strand probes confirmed that the hybridization seen with the transgenic mice was specific (Figure 6). In the control animals, CGRP hybridization was detected only at the expected endogenous locations after long exposures of the autoradiographs. No hybridization was detected either to control or transgenic mice brain sections using sense

Table 2. Calcitonin Gene Transcription in a Series of Rat Medullary Thyroid Carcinoma Tumors

Tumor (Line)	Calcitonin Gene Transcription Rate (ppm/kb Probe)	Type of RNA Produced
WG-1	26	>98% calcitonin mRNA
VE	43	>95% calcitonin mRNA
WG-2	150	>98% calcitonin mRNA
CA-1	98	>95% CGRP mRNA
VF	21	>85% CGRP mRNA
WF	145	>95% CGRP mRNA

A series of rat medullary thyroid carcinoma tumors was analyzed for their content of calcitonin and CGRP mRNAs. Several tumors that contained predominantly calcitonin mRNA or CGRP mRNA were analyzed for the calcitonin gene transcription rate, as described in Experimental Procedures. Simultaneous quantitation of mature calcitonin and CGRP mRNAs was accomplished by S1 nuclease assay. Results are the average of quadruplicate determinations using probes specific for the calcitonin coding region; virtually identical results were obtained when probes specific for the 5' terminal or CGRP-specific exons were used.

strand RNA probes (Figure 6). The lack of hybridization in control animals or with sense strand probes indicates that the antisense probes are not hybridizing either to DNA or nonspecifically to RNA in regions of dense cell bodies. Thyroid glands from control and transgenic animals gave strong hybridization signals with calcitonin probe and

weak signals to CGRP probe, as expected by the relative RNA levels in thyroid (Figure 6).

Cellular Resolution of Hybridization Histochemistry Confirms Neuronal Expression of CGRP

Because of the cellular heterogeneity of the brain, it was important to determine whether neuronal or nonneuronal cell types were expressing the fusion gene. In situ hybridization sections were analyzed by exposure to a thin layer of photographic emulsion and counter-stained with thionin for a Nissl stain. Because most cellular RNA has been removed by RNase treatment to reduce background hybridization from the RNA probes, only the nuclei (DNA) were Nissl-stained. CGRP mRNA hybridization was detected in the hippocampal field CA3 over cells containing large, pale nuclei, which are characteristic of the pyramidal neurons in this cortical layer (Figures 7A–7C). Cells containing the smaller, darker staining nuclei, characteristic of glial cells, generally had very few or no grains over them. Although the morphology of the stained nuclei is not an absolute criteria of cell type, the distribution of these nuclei in this cortical layer allows unambiguous assignment of the cells as neurons. Calcitonin probes also show the same grain pattern distribution, but at a much lower density, probably reflecting the contribution of precursor RNA to the autoradiographic grain density.

In most regions of the brain, CGRP hybridization occurs in cells that contain neuronal-like nuclei. The ventrobasal

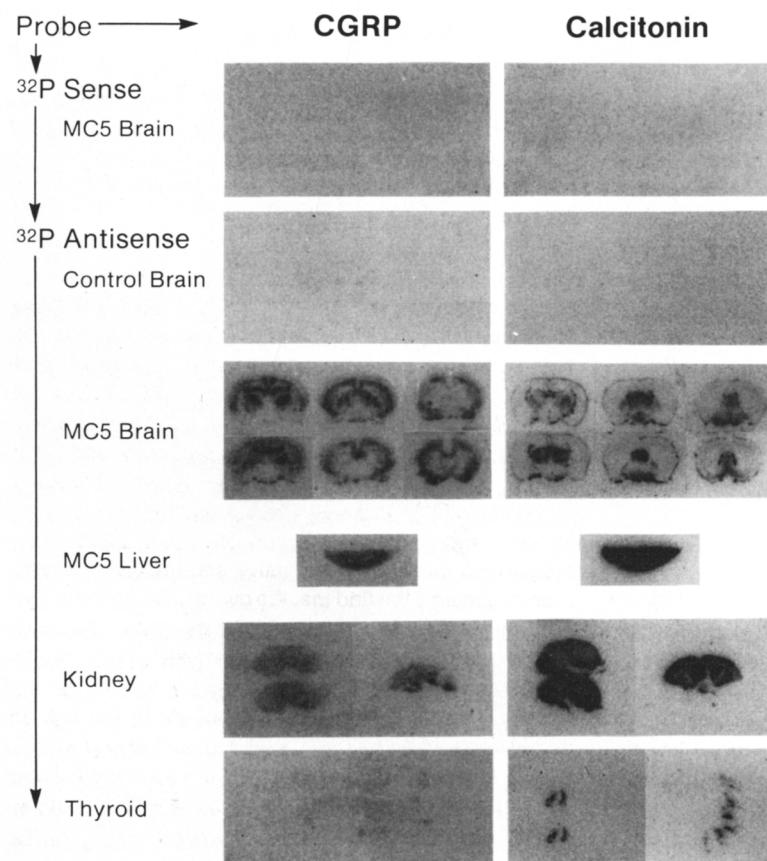


Figure 6. In Situ Hybridization Histochemistry of Calcitonin and CGRP in the Brains of MT-Cal and Control Mice

The specificity and degree of calcitonin and CGRP RNA in situ hybridization analysis were tested by using control mice and both sense and antisense strand ^{32}P -labeled probes. Brain serial sections, mounted caudal to rostral from brainstem to olfactory bulbs (only six sections shown), were hybridized with antisense and sense CGRP and calcitonin probes, as described in Experimental Procedures. From top to bottom: MT-Cal brain sections (MC5 strain) hybridized using sense strand probes; sections from a control mouse hybridized with antisense strand probes; serial sections from a MT-Cal mouse (MC5) hybridized with antisense strand probes; MC5 liver, kidney, and thyroid hybridized with antisense strand probes. Exposure time was 1 week at 4°C.

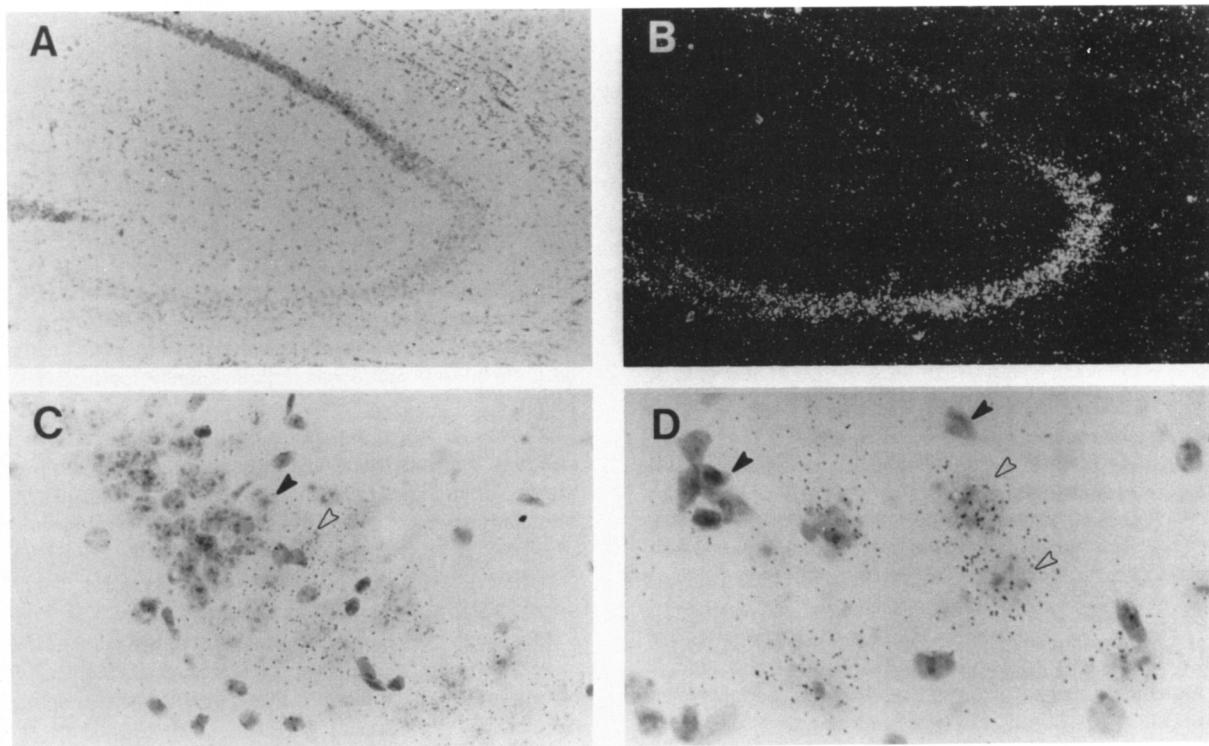


Figure 7. Hybridization Histochemistry of Neurons Expressing CGRP

Sections (20 μ m) were hybridized with 35 S-labeled CGRP RNA probe (Figure 1, probe e) and stained with thionin (see Experimental Procedures). (A) Bright-field and (B) Dark-field view of MC5 hippocampus section shows autoradiographic silver grains over field CA3 (50 \times). (C) Border of fields CA3 and CA1 in hippocampus (190 \times). Open arrow indicates a CA3 neuron (lighter nucleus) that expresses CGRP from MT-Cal transgene, and solid arrow indicates a CA1 neuron (darker nucleus) that does not express the transgene. (D) Higher magnification view of the ventrobasal complex of thalamus (300 \times). Open arrows indicate neurons (lighter nuclei) that express CGRP from the MT-Cal transgene, and solid arrow indicates glial cells (darker nuclei) that do not express the transgene.

complex of the thalamus shows hybridization in cells that contain large, pale nuclei similar to those seen in the hippocampus (Figure 7D). This pattern of hybridization is seen throughout the brain in neuronal structures that express predominantly CGRP transcripts.

While most brain regions that express primarily calcitonin are strictly nonneuronal, the Purkinje layer of the cerebellum is heavily labeled after hybridization with the calcitonin probe, while CGRP hybridization gives a weak signal (Figure 5). The Purkinje layer consists of the Purkinje neurons and specialized types of nonneuronal cells, such as Bergmann glia. Analysis of silver grains over the cerebellum sections shows that calcitonin hybridization is usually excluded from the area around the Purkinje neurons and falls instead over cells containing nuclei with a nonneuronal morphology. This suggests that the calcitonin transcript is being expressed in a cell type other than the Purkinje neurons. Although we cannot unambiguously identify a cell type in this case, it seems likely that the calcitonin transcripts are expressed in specialized glia rather than in neurons.

Although the majority of brain neurons express predominantly CGRP mRNA, the inferior colliculus expresses predominantly calcitonin RNA in cells with neuronal morphology, and the calcitonin transcript is apparently expressed in neurons in the lateral cerebellar nuclei. CGRP

mRNA is also neuronally expressed in this region, but whether coexpression of the transcripts occurs at the cellular level is not established.

Discussion

Because transgenic mouse technology allows the expression of alternatively spliced transcript in many cell types, a comprehensive analysis of splicing decisions *in vivo* provides insights into the organization and relationships of splicing machinery throughout the mouse. Such an analysis has not been previously reported, despite increasing evidence of the widespread use of alternative splicing in a number of genes and cell types. Although alternative RNA processing can be examined in cultured cells, the transgenic mouse model has provided a unique insight into the use of alternative splicing as a developmental strategy. We find that the overwhelming majority of cells throughout the body make unambiguous decisions. Most visceral and muscle tissues express primarily the calcitonin transcript. Tissues, such as liver, lung, and skeletal muscle, are capable of splicing choices that are as definitive as those made in the normal thyroid C cells (Table 1). Predominant calcitonin mRNA expression is found in tissues with ontogenies from endodermal, mesodermal, and ectodermal origin.

In contrast, CGRP mRNA expression is essentially restricted to neurons in MT-Cal mice. Although other tissues express small amounts of the mature CGRP message, we have not detected a subset of cells within these tissues that selectively express CGRP mRNA. These data suggest that cells in which calcitonin mRNA is the predominant product of the transgene may also express CGRP mRNA at low levels. This is analogous to the situation in normal rat thyroid C cells, in which calcitonin mRNA represents >95% of mature transcripts and CGRP has been colocalized to the cells producing calcitonin (Sabate et al., 1985). Within the brain, hybridization histochemistry demonstrates that CGRP expression is localized to neurons. Although most neurons that express MT-Cal generate predominantly CGRP mRNA, there appears to be a very limited set of neurons in which calcitonin mRNA is the predominant transcript.

Immunohistochemical analysis confirms the expression of CGRP in neurons of the hippocampus and dentate gyrus. The reason for the limited detection of CGRP and lack of calcitonin by the immunohistochemical analysis is not known, but could reflect either inadequate sensitivity of the detection method or the inability of these cells to process and store stable peptides that are recognized by our antisera. Because the hippocampus showed the highest level of CGRP expression, it is possible that the other regions of the brain express the transgene at a level below the limits of detection with immunohistochemistry. Alternatively the translation product of these transcripts may not be accurately processed in all neurons. When calcitonin is ectopically expressed in various tumors, the protein precursor is often aberrantly processed (for example, Riley et al., 1986). Low et al. (1985) have demonstrated that the translation product of a metallothionein-somatostatin fusion gene was processed in heterologous pituitary cell types, but not in the liver of transgenic mice. Also, in normal expression of peptide hormones, cell type-specific proteolytic processing of hormone precursors is well documented (for reviews, Liotta and Krieger, 1983; Lynch and Snyder, 1986).

A Model for Calcitonin/CGRP Splice Regulation

We propose that the highly restricted expression of CGRP mRNA is dictated by splice machinery that is restricted to neurons. This would suggest that the calcitonin splice choice is the default or "null" choice when this putative machinery is absent. The model predicts a mechanism that allows for the wide distribution of calcitonin RNA production without invoking the wide distribution of specific factors throughout the animal. Although it is possible that the calcitonin factor is widely distributed and neurons lack this factor, mutational analyses of the calcitonin/CGRP gene argue against this possibility. These analyses show that cells which normally produce calcitonin can express little or no CGRP RNA when the splice site or polyadenylation site of the calcitonin exon is damaged. These mutants are only able to produce mature CGRP transcripts in cells that produce predominantly CGRP (Leff et al., 1987). Furthermore, our results in transgenic animals indicate that the critically regulated step is independent of

the level of gene expression. Several additional observations are consistent with this hypothesis. First, in F9 teratocarcinoma cell lines that are transfected with the calcitonin/CGRP gene, the splice choice is independent of gene expression over a 50- to 100-fold range (Leff et al., 1987). Second, the splice choice in medullary thyroid carcinomas is independent of transcription rate. Third, the transcription rate across the calcitonin and CGRP exons is the same in cells making either splicing choice (Amara et al., 1984). Therefore, it seems likely that critical regulation is independent of the extent or the level of gene transcription and that the neuronal pattern of expression is dependent on RNA processing factors specific for the CGRP transcript.

Our results show that neurons, other than those that normally express the CGRP transcript, are capable of making unambiguous splicing choices for CGRP mRNA. These results predict that the CGRP splice machinery is widely expressed in neurons. This raises the possibility that there are a limited number of neuronal factors which dictate alternative splicing and that two or more alternatively spliced transcription units could be regulated by the same machinery. For instance, it is interesting that CGRP is expressed with the alternatively spliced neuropeptide substance P in sensory ganglion cells (Gibson et al., 1984; Weisenfeld-Hallin et al., 1984; Lee et al., 1985). The parallel between these two genes is even more striking when one considers that the second alternatively spliced transcript, encoding substance K, is found in thyroid C cells with calcitonin (Nawa et al., 1984). The colocalization of the transcripts from these genes is consistent with the possibility that they are coregulated by the same splice regulatory machinery. This neuronal splicing phenotype may represent a developmental strategy common to several alternatively spliced gene transcripts that may be regulated by common splice-regulating factors in neurons. Shared splicing factors would allow many transcription units to be alternatively spliced in a wide range of tissues without requiring a large array of splicing factors. Consequently, transcription factors in conjunction with a limited number of splicing factors would increase the potential complexity of developmental strategies.

Experimental Procedures

Plasmid Construction

The calcitonin/CGRP fragment extended from the PstI site, 11 nucleotides 5' of the calcitonin/CGRP cap site, to the EcoRI site, 1.1 kb 3' of the poly(A) site in the sixth exon. This fragment was linked with Xhol at the 5' PstI site and with BamHI at the 3' EcoRI site, then inserted into a vector containing the MT-I promoter.

Microinjection of Fertilized Eggs and Tail Dot Blot Analysis

Fertilized eggs (C57BL/6J × DBA/2J)F1 or (C57BL/6J × SJL/J)F1 were collected on the morning that a copulation plug was found. Microinjection and transfer to pseudopregnant females was done as described by Costantini and Lacy (1982). Briefly, the BglI-BamHI fragment of MT-Cal was microinjected at approximately 200 copies per cell in a volume of 2 pl. Eggs that survived microinjection or incubated overnight to the two-cell stage and then transferred into (C57BL/6J × DBA/2J)F1 females that had mated with vasectomized males.

Transgenic mice were identified by dot blot analysis of DNA extracted from a segment of the tail as described by Palmiter et al. (1982).

A specific RNA probe was generated by cloning the PstI fragment of the rat calcitonin/CGRP gene that encompasses the first three exons of the gene into pSP65. Run-off transcripts were produced by *in vitro* transcription using SP6 polymerase as suggested by the manufacturer (BRL). Quantitation of copy number was determined by densitometric scanning of autoradiograms from 3-fold dilutions of tail DNA dot blots. Normal rat DNA was used as a standard.

Northern RNA Analysis

Tissues, which were frozen in liquid nitrogen, were pulverized to a coarse powder, and total nucleic acids were isolated by the method of Shields and Blobel (1977). Total RNA was separated from DNA by precipitation using LiCl. Briefly, total nucleic acids were incubated with 2.5 M LiCl containing 20 mM sodium acetate (pH 5.0) for 3 hr on ice. Aliquots were denatured and subjected to electrophoresis on 1.2% agarose-formaldehyde gels (Maniatis et al., 1982). RNA was transferred to nitrocellulose, washed in prehybridization buffer containing 50% formamide, and hybridized to probes nick-translated with [α -³²P]-dCTP as the labeled nucleotide (1×10^8 to 3×10^8 cpm/ μ g). A Sau3A fragment encompassing the fifth and sixth exons was specific for CGRP transcripts (Figure 1, probe c); a BglII fragment from the fourth exon of the genomic clone was specific for calcitonin containing transcripts (Figure 1, probe b). Size standards were provided by migration of calcitonin and CGRP mRNA species from a rat medullary thyroid carcinoma (MTC) cell line designated WA, which contains equal amounts of the two RNA species (Amara et al., 1982).

Quantitation of relative amounts of calcitonin and CGRP transcripts was obtained by laser densitometric scanning of autoradiographs using Quick Scan R & D densitometer (Helena Laboratories). Scans were standardized using medullary thyroid carcinoma RNA in which calcitonin and CGRP mRNAs are present at equivalent levels (WA MTC).

Immunohistochemistry

Tissue was fixed by vascular perfusion, and frozen sections 20 μ m thick were cut on a sliding microtome (Swanson et al., 1983). Immunofluorescence localization of CGRP and calcitonin was carried out as described in detail in Swanson et al. (1983). CGRP serum was used at a dilution of 1:2,000 and calcitonin serum at a dilution of 1:1500.

Hybridization Histochemistry

Tissue was fixed as described above for immunohistochemistry. Sections were mounted on poly-L-lysine-coated slides and air-dried. Pre-treatment, hybridizations, and washing conditions have been described for RNA probes (Cox et al., 1984). Briefly, sections were digested with proteinase K (10 μ g/ml, 37°C, 30 min), acetylated, and dehydrated. After thorough drying, 50 μ l of hybridization mix containing ³²P-labeled probe (10⁷ cpm/ml) was spotted on each slide. Slides were incubated at 50°C for 16 hr. Slides were rinsed, digested with ribonuclease (RNAse A, at 20 μ g/ml, 37°C, 30 min), and washed in 0.1× SSC for 30 min at 55°C. After dehydration, the sections were exposed to Cronex 4 film (DuPont) at 4°C. Hybridizations with ³⁵S-labeled probes were performed in the presence of 10 mM DTT, and 1–10 mM DTT was included in the following washes. Specific RNA probes were generated with *in vitro* transcription vectors. A CGRP vector was produced by cloning the 450 bp TaqI-Sau3A CGRP genomic fragment (Figure 1, fragment e) into pSP64. This fragment contains 170 bp of single copy 3'-noncoding sequence and 280 bp of single copy 3'-flanking sequence. The calcitonin vector was produced by cloning the 530 bp HaeIII genomic fragment (Figure 1, fragment d) into pSP65. This fragment contains 130 bp of 3'-noncoding sequence and 400 bp of 3'-flanking sequence. After the vectors were linearized, run-off transcripts were produced with SP6 polymerase using carrier-free [³²P]UTP (800 Ci/mmol, NEN) or [³⁵S]UTP (1200 Ci/mmol, NEN).

Nuclear Run-On Gene Transcription Analysis

A series of independent medullary thyroid carcinoma (MTC) tumor lines, producing either calcitonin or CGRP as the predominant peptide product (referred to as WG-1, WG-2, VE, CA-1, VF, and WF), were the generous gifts of Dr. B. Roos (Veterans Administration Medical Center, Tacoma, WA). Cells were washed with ice-cold phosphate-buffered saline and lysed by gentle homogenization in 5 ml of ice-cold buffer containing 150 mM KCl, 5 mM MgCl₂, 5 mM DTT, 10 mM Tris (pH 7.8), 0.25

M sucrose, and 0.1% NP40, layered onto a 4 ml cushion of the identical buffer containing 0.5 M sucrose, and centrifuged at 1000 × g for 10 min. The nuclear pellets were washed in the nascent chain labeling buffer (150 mM KCl, 5 mM MgCl₂, 10 mM Tris [pH 7.8], 10% glycerol). Nuclei were then incubated (25°C, 10 min) in 100 μ l of the same buffer supplemented with 1 mM ATP and GTP, 100 μ Ci of [³²P]UTP and [³²P]-CTP (200–400 Ci/mM), and 2 μ l of RNAase inhibitor, allowing nascent transcripts to elongate. Labeled RNA was purified and subjected to hybridization analysis, as previously described (Murdoch et al., 1982; McKnight and Palmer, 1979). Briefly, 8 × 10⁶ to 15 × 10⁶ cpm of ³²P-labeled RNA was hybridized to 2 μ g of linearized cloned calcitonin cDNA (pCal) or CGRP cDNA (pCGRP₂; Amara et al., 1982) bound to a 3 mm disk of nitrocellulose. Immobilized pBR322 was used to determine assay background. Filters were washed with 10 mM Tris-HCl (pH 7.5), 0.1% SDS, 0.2× SSC at 65°C for 30 min with pancreatic RNAase (1.3 μ g/ml) and T1 nuclease (10 U/ml) in 10 mM Tris-HCl (pH 7.4), 0.3 M NaCl at 37°C for 30 min, followed by extensive washing with 10 mM Tris-HCl (pH 7.4), 0.3 M NaCl, 0.1% SDS at 42°C. Each point is the mean +/- SE of the transcription rate (specific cpm bound per 10⁶ TCA precipitable cpm added), based on quadruplicate hybridizations. Calcitonin gene transcription is undetectable when α -amanitin (0.8 μ g/ml) is included in the incubation. The hybridization is linear with respect to added labeled RNA and constant with hybridization to variable cloned DNA/filter (0.5–5.0 μ g), confirming that all hybridizations are in DNA excess and that unlabeled RNA does not compete for elongated nascent transcripts.

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