Calcitonin is expressed in gonadotropes of the anterior pituitary gland: its possible role in paracrine regulation of lactotrope function

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Abstract

Previous studies from this laboratory have shown that salmon (S) calcitonin (CT)-like immunoreactive peptide (CTI) is synthesized and secreted by the anterior pituitary (AP) gland. These studies also co-localized CTI to gonadotropes, and demonstrated that SCT is a potent inhibitor of lactotrope function. However, the molecular structure of putative gonadotrope-derived CTI that inhibits lactotrope function has not been defined.

The present studies cloned CT cDNA (pit-CT cDNA) from a mouse gonadotrope LβT2 cell line using RT-PCR and rapid amplification of cDNA ends (RACE) techniques. Alignment of nucleotide sequences of pit-CT and mouse CT revealed greater than 99% homology between the sequences. The pit-CT cDNA was ligated into a mammalian expression vector, and the construct was transfected into LβT2 cells. Two stable transfectant cell lines (CT.U6/A and B) were obtained by selection in G418. Subsequent S1-nuclease protection assay and immunocytochemistry results have shown that: (1) pit-CT peptide expressed by CT.U6 cell lines immunoreacted with GCT1-anti-SCT serum; (2) secretions of CT.U6 cells inhibited prolactin (PRL) release, PRL mRNA abundance and DNA synthesis of PRL-secreting GGH3 cells; and (3) CT.U6-induced inhibition was abolished by GCT1-anti-SCT serum. The studies also generated a riboprobe from the cloned pit-CT cDNA, and localized CT mRNA expression in gonadotropes of rat AP gland by *in situ* hybridization histochemistry.

These results demonstrate that pit-CT mRNA is closely homologous to mouse CT mRNA; it is expressed by gonadotropes of the rat AP gland, and the peptide may significantly affect lactotrope function by inhibiting PRL release and cell proliferation.

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Introduction

Calcitonins (CTs) are a group of polypeptide hormones containing 32 amino acid residues (Potts 1976, Stevenson 1980, Fischer & Born 1985, Azria 1989). All share an amino terminal ring structure, with cysteines at 1 and 7 linked by a disulfide bridge, as well as a carboxy terminal prolinamide. CT-like immunoreactivity (CTI) is widely distributed in the CNS and the pituitary gland of various mammalian species including rats and humans (Watkins & Moore 1980, Fischer *et al.* 1981, 1983, Flynn *et al.* 1981, Sexton & Hilton 1992). Receptors recognizing salmon (S) CT have been detected in specific regions of rat brain and the anterior pituitary (AP) gland (Fischer *et al.* 1981, Henke *et al.* 1983, Sexton *et al.* 1993, Sheward *et al.* 1994), and complementary (c) DNAs for two such receptors have been cloned from a rat brain cDNA library (Albrandt *et al.* 1993). Supporting the physiological relevance of CT actions in the AP gland are the findings of the presence of SCTI and human (H) CTI in rat and human hypothalami and the pituitary glands by several investigators (Deftos *et al.* 1978, Margules *et al.* 1979, Cooper *et al.* 1980, Fischer *et al.* 1981, 1983, Flynn *et al.* 1981, Balabanova *et al.* 1985, Deftos 1987, Sexton & Hilton 1992, Shah *et al.* 1993, Hilton *et al.* 1998). The evidence for the synthesis and secretion of CT-like immunoreactive peptides by primary cultures of the rat AP gland has also been presented (Deftos 1987, Shah *et al.* 1993). Pituitary derived CT (pit-CT) may share antigenic sites with human (or rat) CT (HCT) and SCT since antisera raised against these peptides immunoprecipitate molecules of similar electrophoretic mobility from AP cell lysates (Shah *et al.* 1993). Using GCT1, the anti-SCT serum raised in this laboratory, we have shown that CTI is selectively localized in gonadotropes, and not in thyrotropes, somatotropes, lactotropes, corticotropes or folliculo-stellate cells of rat AP gland (Shah *et al.* 1993, Chronwall *et al.* 1996).

Previous findings from this laboratory have shown that exogenously added SCT significantly attenuates prolactin

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(PRL) release from perifused rat AP cells without altering the secretion of growth hormone (GH), follicle-stimulating hormone, luteinizing hormone (LH) or thyroid stimulating hormone (Shah *et al.* 1988, 1990). SCT is also a potent inhibitor of PRL gene transcription and lactotrope cell proliferation in rats (Zhang *et al.* 1995, Shah *et al.* 1999). Interestingly, the addition of GCT1-anti-SCT serum immunoneutralizes endogenous CT, stimulates PRL release from cultured rat pituitary cells and raises serum PRL levels in conscious ovariectomized rats (Shah *et al.* 1993, 1996). These results raise the possibility that gonadotrope-derived GCT1-immunoreactive CT is a paracrine inhibitor of lactotrope function. Since the molecular sequence of gonadotrope-derived CT-like peptide that regulates lactotrope function has not been determined, we attempted to determine the identity of this important regulatory peptide. We report that we have cloned and sequenced CT cDNA from a mouse gonadotrope-derived LβT2 cell line (Thomas *et al.* 1996). The study compared the cloned pit-CT cDNA sequence with mouse CT sequence, and also tested the effects of the encoded protein on PRL release, PRL gene expression and proliferation of rat somatomammotrope-derived GGH3 cell line. Additional studies co-localized pit-CT mRNA in rat AP gland by *in situ* hybridization histochemistry.

Materials and Methods

dCTP, [^{32}P]dUTP and ^{125}I were purchased from Dupont-New England Nuclear (Boston, MA, USA). Dulbecco's modified Eagle's medium (DMEM), RPMI-1640, penicillin G-streptomycin mixture, horse sera, fetal calf sera and Superscript II reverse transcriptase were obtained from Gibco Laboratories (Grand Island, NY, USA). PCR amplimers were synthesized by Genemed Synthesis, Inc. (San Francisco, CA, USA). Synthetic salmon (S) CT was obtained from Peninsula Laboratories (Belmont, CA, USA), and the rat PRL RIA kit was provided by the National Hormone and Pituitary Program (Harbor-UCLA Medical Centre, Torrance, CA, USA). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise stated.

Cell lines

LβT2, a gonadotrope-derived mouse cell line that secretes α as well as β subunits of LH was provided by Dr Pam Mellon (University of California San Diego, USA). The cells were maintained in the complete medium as recommended by Dr Mellon's group. The complete growth medium was prepared in DMEM containing 4·5 mg/ml glucose and supplemented with 10% fetal calf serum.

Preparation of RNA and RT-PCR

Total RNA from LβT2 cells was extracted using RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). One microgram total RNA was used for reverse transcription (RT). Oligo dT primer annealing and reverse transcription were performed using Superscript II reverse transcriptase according to the manufacturer's protocol (GIBCO-BRL, Gaithersburg, MD, USA).

Polymerase chain reaction (PCR)

Reverse transcribed cDNA was amplified with a PCR reagent kit purchased from Gibco-BRL. Since HCT-like as well as SCT-like peptides have been detected in the AP gland (Gagel *et al.* 1983, Sexton & Hilton 1992, Shah *et al.* 1993, Hilton *et al.* 1998), we used two sets of primer pairs derived from either mouse (m) or SCT cDNA sequences: mCT-forward: 5′-agagtcaccgcttcgcaa-3′; mCT-reverse: 5′-ccagagaggaactacatgcatc-3′; SCT-forward: 5′-gcaagcaag atccacatg-3′; SCT-reverse: 5′-agagcaaccgctatgcaagcta-3′.

The hot start method was employed to minimize non-specific amplification. The amplified product or products were fractionated on a 1% agarose gel, the bands were cut, and the DNA was extracted and subcloned in pGem-T vector (Promega Laboratories, Milwaukee, WI, USA). The recombinant plasmids were sent for DNA sequencing at the Texas Tech University Biotechnology Facility.

RACE and screening

The first series of PCR reaction yielded two clones that were highly homologous to the mouse CT sequence. Since these sequences were partial, RACE reaction was employed to obtain longer sequences. The gene specific primer (GSP) (5′-caaggatcaagagtcaccgcttcgcaagcactgcct ggc-3′) and universal primer mix (CLONTECH, Palo Alto, CA, USA) were used for 3′-RACE. The reaction was first cycled 5 times (5 s, 94 °C; 3 min, 72 °C), and cycled 5 times again (5 s, 94 °C; 10 s, 70 °C; 3 min, 72 °C) based on the manufacturer's recommendations, and then subjected to 30 cycles of PCR (5 s, 94 °C; 10 s, 60 °C; 2 min, 72 °C). PCR products were purified with a DNA purification kit (Bio-RAD, Hercules, CA, USA) and ligated into the pGEM-T vector. Plasmid DNA from several clones was prepared and identified by Southern blotting using the partial CT cDNA probe. Positive RACE clones were sent for DNA sequencing.

Expression of recombinant pit-CT in LβT2 cells

pit-CT cDNA insert was cloned downstream of the cytomegalovirus promoter in pcDNA3·1/Myc-His(+)B vector (Invitrogen, San Diego, CA, USA). The presence and orientation of the insert in the recombinant plasmid (pcDNA3·1-CT) was verified by digestion with appropriate restriction enzymes as well as by DNA sequencing of the insert. Vector pcDNA3·1 has two distinct C-terminal epitopes to detect the recombinant proteins. The

C-terminal of the expressed protein will carry a c-*myc* epitope that can be identified by anti-*myc* antibody. This epitope is followed by a polyhistidine tag, which can be identified by anti-His (C-term) antibody.

The recombinant plasmid was then used for transfection in LβT2 cells. LβT2 cells in mid-to-late log phase were harvested and resuspended in ice-cold PBS at 10 million cells/ml, and mixed with plasmid DNA (recombinant plasmid pcDNA 3·1-CT or vehicle plasmid pcDNA 3·1, 30 μg/ml) in an electroporation cuvette, and electroporated at 800 μF and 250 V (Gene Pulser II, Bio-RAD). Transfected cells were incubated in a 6-well plate with DMEM for 48 h and then selected with 400 μg/ml G418. Individual colonies were selected after four weeks of culture, dispersed with trypsin/EDTA and propagated further into fresh flasks. Two cell colonies, CT.U6/A and CT.U6/B, displaying the highest CTI secretion were chosen for further investigation.

Detection of CT mRNA in CT-transfectants

The cell lines LβT2 (parental), V (carrying vehicle plasmid), CT.U6/A and CT.U6/B were cultured as described above. Three hundred thousand cells of each of these cell lines were seeded individually into 100 mm dishes, and were grown to exponential phase. The total RNA from these cell lines was extracted as previously described (Xie & Rothblum 1991), and was used to determine CT mRNA abundance by S1-nuclease protection assay.

S1-nuclease protection assay

UTP-labeled antisense riboprobes of pit-CT, PRL and β-actin were generated using T7 RNA polymerase (Promega) and appropriate linearized DNA templates. Total RNA (20 μg) was incubated with the appropriate riboprobe for 18 h at 45 °C. Following this, the samples were treated with S1-nuclease for 30 min at 37 °C. The protected RNA was precipitated and fractioned on 4·5% polyacrylamide gel with 8 M urea. The gel was then dried and autoradiographed. Each experiment was repeated three separate times.

Secretion of CTI in the conditioned media

Levels of CTI and PRL in the conditioned media of LβT2 cells and CT-transfectants were determined by RIA. The PRL RIA used the reagents provided by the NIDDK, and the assay protocol recommended by the NIDDK was followed. CTI was determined as described previously (Shah *et al.* 1989). All samples from this series of experiments were concurrently run in duplicate in the same RIA. Protein A (IgG Sorb) was used for the separation of bound from free hormone antigen. The experiments were repeated at least three separate times, and data from all the experiments were pooled for statistical analysis.

CT immunocytochemistry (ICC)

Approximately 10 000 LβT2 cells or CT-transfectants were plated onto polylysine-coated microscope slides. The cells were incubated for 18 h at 37 °C, washed with PBS and fixed for 1 h in Zamboni's solution. The fixed cells were processed for pit-CT ICC as previously described except that the second antibody was conjugated to horseradish peroxidase (HRP) and therefore the color was developed using diaminobenzidine tetrahydrochloride (DAB) as a substrate (Chronwall *et al.* 1996). The negative controls were treated with primary antiserum that was preincubated with 1 μM SCT at 37 °C for 1 h. The experiments were repeated two more times.

Immunodetection of recombinant pit-CTI by Western blot using GCT1 and anti-His antibodies

Crude cell lysates from the parental LβT2 cells and CT.U6 (A and B) were prepared as previously described (Chien *et al.* 1999). In brief, 10 million cells from each cell line were homogenized in Buffer A (25 mM Tris, pH 7·4 containing 10% glycerol, 1% Nonidet P-40, 50 mM NaF and freshly supplemented with 10 mM sodium pyrophosphate (PPi), 1 mM sodium vanadate, leupeptin (10 μg/ml), aprotinin (5 μg/ml), and phenylmethylsulfonyl fluoride (1 mM)). Nuclear fraction and debris were separated by centrifugation at 2000 **g** for 10 min at 4 °C, and the supernatant was used for Western blot analysis. Protein concentrations of cell lysates were determined using Bio-Rad protein reagent (Hercules, CA, USA). The lysates were then boiled for 5 min in 2 × Laemmli solution containing 20 mM dithiothreitol (DTT), and 50 μg protein per lane were loaded onto 12·5% SDS-polyacrylamide gel. The separated proteins were electrically transferred to nitrocellulose, and the blots were incubated with previously characterized GCT1 rabbit anti-SCT serum (1:500) as well as mouse anti-histamine serum (C-terminal, 1:3000, Invitrogen, San Diego, CA, USA) for 18 h at 4 °C. Following three washes, the membranes were incubated with either anti-rabbit or anti-mouse IgG-HRP (1:1000). Following three successive washes, the immune complexes were visualized using Western blot ECL detection system (Radiochemical Center, NEN Life Science Products, Boston, MA, USA). The same experiment was repeated one more time.

Biological activity of recombinant pit-CT: co-culture of CT-transfectants and GGH3 cells and its effect on PRL secretion, PRL mRNA abundance and DNA synthesis of GGH3 cells

To further examine the role of pituitary-derived CT in lactotrope function, we developed a two-tier co-culture

system where 110^5 LβT2 cells or CT transfectants (CT.U6/A or CT.U6/B) are cultured separately in an upper chamber insert, whereas 2×10^5 GGH3 cells per well (target cells) are cultured in a 12-well plate. During the experimental period, the upper chamber is inserted on top of a well of a 12-well plate containing GGH3 cells so that CT-secretors (in the upper chamber) do not come in direct contact with GGH3 cells (in the lower chamber) but are exposed to their secretions. The GGH3 cells in each set of the experiment were treated as follows: (1) vehicle control where the upper chamber contained GGH3 cells instead of CT-secretors + 10 μl non-immune serum (NIS) in the lower chamber; (2) upper chamber contained either LβT2 or CT.U6 (A or B), and 10 μl NIS were added in the lower chamber; and (3) upper chamber contained either LβT2, CT.U6/A or CT.U6/B cells, and 10 μl anti-SCT serum were added to the medium in the lower chamber. After the incubation period of 24 h, either the conditioned media or GGH3 cells in the lower chamber were harvested. The conditioned media were analyzed for PRL by RIA. The cell lysates were used to analyze PRL mRNA abundance as described in the S1-nuclase protection assay. Each data point was run in triplicate and the data from three independent but similar experiments were obtained.

The results on PRL release are expressed as ng PRL released by 100 000 cells over 24 h. The results on PRL mRNA were digitized, normalized with β-actin mRNA and expressed as normalized densitometric units. The data from all experiments were pooled and expressed as means ± S.E.M. The results were statistically evaluated by one-way ANOVA and the significance was derived by Newman-Keul's test.

[^{3}H]Thymidine incorporation of GGH3 cells

GGH3 cells in log phase were seeded at 1×10^5 cells/well in 1 ml complete medium in 12-well culture plates. The growth rate of cells was slowed down by overnight incubation in low-serum-containing medium (2% FCS) followed by 2-h incubation in serum-free basal medium. The cells were then co-incubated with CT-transfectants as described above for 24 h. Four hours prior to the termination of the assay, the GGH3 cells (in the lower chamber) received [^{3}H]thymidine (0·5 μCi/well). At the end of the incubation, the cells were washed twice with PBS containing 100 μM unlabeled thymidine, and solubilized in Triton X-100 (0·1% vol/vol in distilled water). The incorporated [^{3}H]thymidine was quantified by liquid scintillation counting. Each data point was run in quadruplicate and the data from three independent but similar experiments were obtained.

The results are expressed as d.p.m. of [^{3}H]thymidine incorporated per 1×10^5 cells ± S.E.M. The data from all experiments were pooled and analyzed by one-way ANOVA and the level of significance was derived from Newman-Keul's test.

Localization of pit-CT mRNA in rat AP gland: in situ *hybridization histochemistry (ISH)*

Preparation of sense/anti-sense digoxigenin-labeled pit-CT riboprobes Plasmid containing partial CT.U6 (86–580) was linearized, and antisense riboprobe was transcribed using T7 RNA polymerase. Similarly, a sense riboprobe was generated using SP6 RNA polymerase. Digoxigenin 11-UTP (Boehringer Mannheim, Indianapolis, IN, USA) was used in both transcription reactions, and the manufacturer's instructions were followed. The reaction mixtures were digested with RNAse-free DNAse (Boehringer), the riboprobes were extracted with phenol/chloroform, and purified on TE microselect-D G-50 spin columns (5 Prime-3 Prime, Inc., Boulder, CO, USA).

Preparation of LH-β and PRL cDNA-rhodamine probes cDNA inserts for rat LH-β or rat PRL were labeled with tetramethyl rhodamine-6-dUTP by random primer labeling using klenow fragments of DNA polymerase (Feinberg & Vogelstein 1983), and the probes were purified on TE Microselect-D G-50 spin columns (5 Prime-3 Prime, Inc.).

Double *in situ* hybridization histochemistry (ISH) The rat AP glands were rapidly frozen by submersion in an isopentane–dry CO_2 bath after mounting in the embedding medium (OCT compound, Tissu-Tek, Miles Laboratories, Elkhart, IN, USA). The frozen tissues were sliced to 5–10 μm thick sections and thaw-mounted on Superfrost plus glass slides (Fisher Scientific, Pittsburgh, PA, USA). The sections were stored frozen at −70 °C until ISH analysis.

The frozen tissue sections were rapidly thawed, washed with PBS at 4 °C, and fixed in 4% paraformaldehyde-PBS (pH 7·2) for 10 min. The double ISH procedure was performed using antisense pit-CT RNA and LH-β or PRL cDNA probes as described before (Iczkowski *et al.* 1998). Serial sections of the specimens were concurrently probed with sense probes, which served as negative controls. The hybridization signal of CT mRNA was detected by incubating the hybridized sections with mouse anti-digoxigenin-FITC for 6 h at 4 °C, whereas the cDNA probes for LH-β or PRL contained fluorescent ribonucleotide and did not need additional processing. Three animals per group were used for these experiments. Sections from all animals were processed simultaneously. Two researchers independently evaluated the slides, scoring all slides at the same time to avoid comparing preparations that had been stored or exposed to UV-light for different periods of time. The sections (at least twelve/experiment from three different animals) were observed

under a Nikon Optiphot microscope with epifluorescence attachment. The digital images were captured on a G3 Power PC computer by a Spot camera attached to the microscope and examined for co-localization between pit-CT mRNA and LH-β or PRL mRNA.

Results

Amplification and sequencing of pit-CT mRNA from LβT2 cells

Initial PCR experiments with mCT amplimers yielded a partial cDNA of 564 bp length. The cDNA sequence displayed greater than 90% homology with mouse CT mRNA sequence (emb/X97991·1). Thereafter, the deduced amino acid sequence of the known mouse CT was aligned with partial pit-CT sequence, and the conserved region was used to design a specific primer for 3′-RACE. First-strand cDNA produced by RT with 3′-CDS primer and universal primer mix was used as template for 3′-RACE. 3′-RACE products were ligated into pGEM-T vector. Nineteen clones were identified by Southern blotting and DNA sequencing. The alignment of nucleotide sequences revealed that a clone, pit-CT.U6, had greater than 99% homology with the 23–829 bp segment of mouse CT mRNA (emb/X97991·1) (Fig. 1). Only seven out of 806 bases in the pit-CT mRNA sequence differed from mouse CT mRNA sequence. The mismatches were at positions 383, 418, 463, 469, 580, 831 and 851 of mouse CT sequence. Homology with rat calcitonin gene-related protein (CGRP) sequence was much less and covered only 58–232 bp segment of rat α CGRP mRNA (emb/V01229·1). Moreover, the presence of exon 4 in the pit-CT sequence, which is specific for CT but not CGRP, suggests that the CT gene of LβT2 cells transcribes CT, and not CGRP, mRNA.

Stable LβT2-CT transfectants express pit-CT mRNA and GCT1-immunoreactive CT

Cell clones CT.U6/A and CT.U6/B were obtained by selecting LβT2 transfectants with G418. The results from S1-nuclease protection assay showed that CT.U6 (A and B) cells displayed markedly greater abundance of pit-CT mRNA than the parental LβT2 cells (Fig. 2). Relative densitometric value of pit-CT mRNA in CT.U6 cells increased by 97% over parental LβT2 cells.

Since pcDNA3·1/Myc-His(+)B vector expresses fusion protein, the recombinant pit-CT.U6 protein should also express fusion protein poly Myc-His. The results from Western blot analysis of transfectants reveal that anti-His antibody identified three major immunoreactive bands in CT.U6 lysates (Fig. 3). However, the band with the highest molecular size was also observed in control LβT2 cells which did not express the recombinant protein, and may be a plasmid-related band. Two other bands were specific for CT.U6 transfectants. Interestingly, GCT1 antiserum also identified these two molecular species, suggesting that fusion proteins in these two bands contain the encoded pit-CT peptide that cross-reacts with GCT1-anti-SCT serum.

Consistent with the results from Western blot analysis, ICC results also show that both CT.U6 clonal cell lines (A and B) stained strongly for CTI (GCT1). In contrast, control LβT2 cells were only weakly positive (Fig. 4).

Secretion of CTI by CT.U6 cells

Results from CT RIA of spent media suggest that CT.U6 cell lines released 122·47 pg/100 000 cells of CT-Eq in 24 h. The corresponding release from control LβT2 cells was very close to the detection limit of the assay (30 pg CT-Eq).

CT.U6 cells inhibit PRL mRNA and PRL release from GGH3 cells

To test whether the actions of pit-CT on lactotrope function are consistent with previously demonstrated actions of GCT1-reactive CT, we developed a two-tier co-culture system where GH3 cells received secretions of either LβT2 or CT.U6 cells but did not come in direct contact with them. As presented in Figs 5 and 6, secretions of both CT.U6 cell lines (A and B) caused a dramatic inhibition in PRL secretion as well as PRL mRNA levels. Parental LβT2 cells also caused a decrease in PRL mRNA abundance and PRL release, but the decrease was smaller compared with that caused by CT.U6 cell lines.

Secretions from CT.U6 cells inhibit DNA synthesis of GG3 cells: this action is reversed by GCT1-anti-SCT serum

Since exogenously added CT inhibited proliferation of lactotropes under *in vitro* as well as *in vivo* conditions (Shah *et al.* 1999), we tested the effect of co-culture of CT-transfectants on DNA synthesis of GGH3 cells. The results presented in Fig. 7 show that [^{3}H]thymidine incorporation in GGH3 cells cocultured with CT.U6 cell lines (bars 4 and 6) was dramatically lower when compared with the vehicle controls (bar 1). In parallel experiments, control LβT2 cells were co-cultured with GGH3 cells. Similar to CT.U6 cells, LβT2 cells also decreased DNA synthesis of GGH3 cells (bar 2). However, this inhibition was much smaller as compared with that produced by CT-transfectants (bar 2 vs bars 4 and 6). GCT1-anti-SCT antiserum almost abolished the inhibitory effect of LβT2 and CT.U6 cell lines on DNA synthesis of GGH3 cells (bars 3, 5, and 7).

Localization of pit-CT mRNA in gonadotropes of rat AP gland

Since the pit-CT clone obtained from LβT2 cells expressed 91% homology with rat CT mRNA

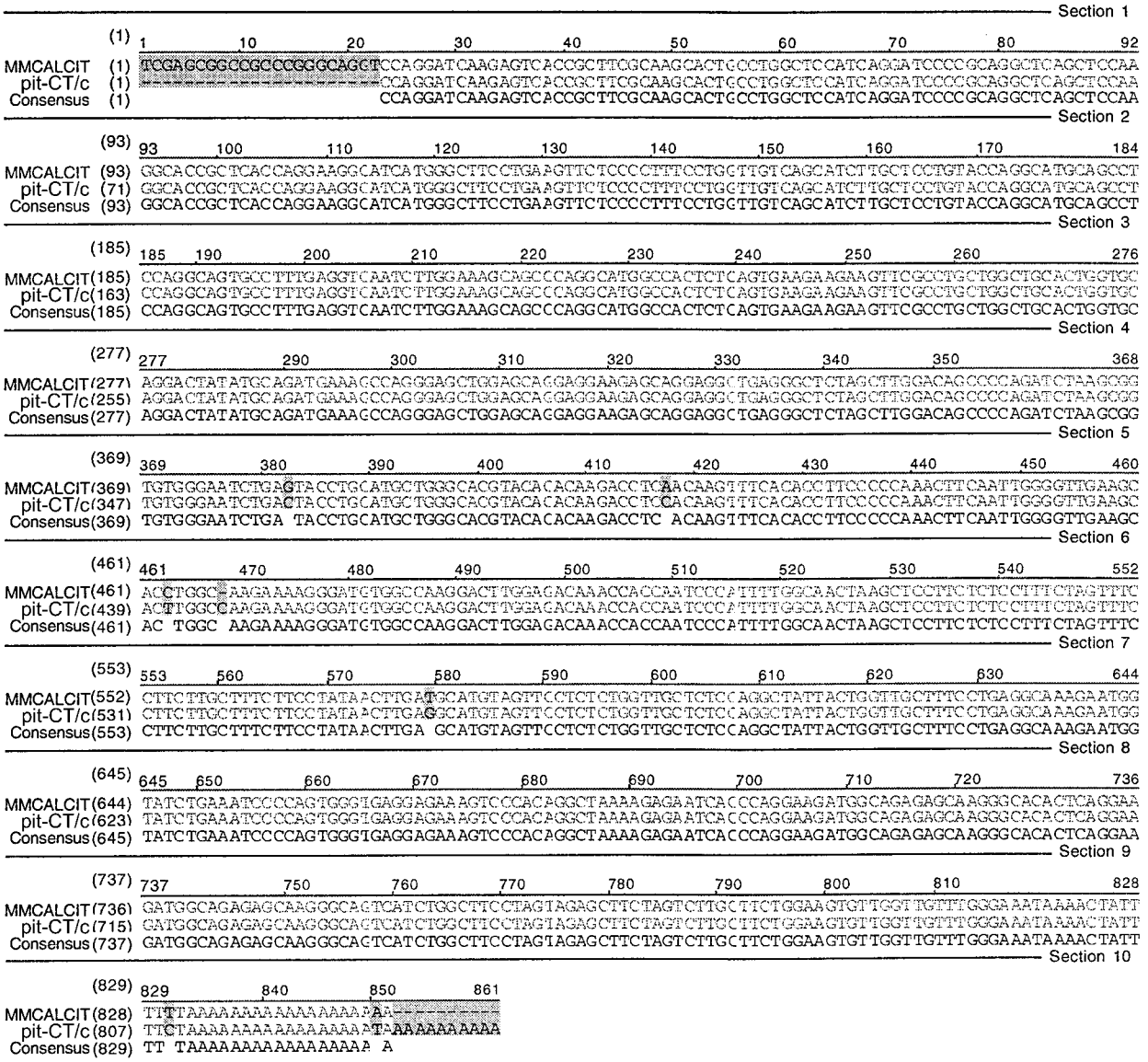

Figure 1 Comparison of sequences of pit-CT mRNA (pit-CT/c) and mouse CT mRNA (MMCALCIT). The sequences from six clones were determined from both ends, assembled and a consensus pit-CT sequence was derived using vector NTI and contig express computer programs. Pit-CT mRNA sequence was then aligned with mouse CT mRNA sequence (emb/X97991·1) by the computer program Align-X (Informax Inc., Bethesda, MD, USA). Mismatches are shaded.

(gb/M26137·1), we constructed a partial pit-CT cDNA vector for riboprobe generation. The sequence of this pit-CT cDNA corresponded with the 4–493 bp segment of rat calcitonin mRNA (RCALC2), and displayed 92% homology. The digoxigenin-UTP-labeled anti-sense probe was used for *in situ* hybridization histochemistry with frozen sections of the AP glands obtained from cyclic female rats in the diestrous phase. Approximately 7–8% of total AP cells displayed pit-CT message (lower panel of Fig. 8). A similar distribution profile was also observed for LH-β mRNA (upper panel of Fig. 8), and co-localization of both these messages could be observed. Sense controls for CT as well as LH probes did not display significant staining (see insets on Fig. 8, lower and upper panels). These results are consistent with our previous findings using GCT1 and rat LH-β antisera (Chronwall *et al.* 1996).

In another experiment, a relationship between CT cells and lactotropes was examined. The results presented in Fig. 9 show that CT mRNA-positive cells (green) were in apposition with PRL mRNA-positive cells (red). However, no co-localization of PRL and CT mRNA was observed. Again, sense controls did not display any

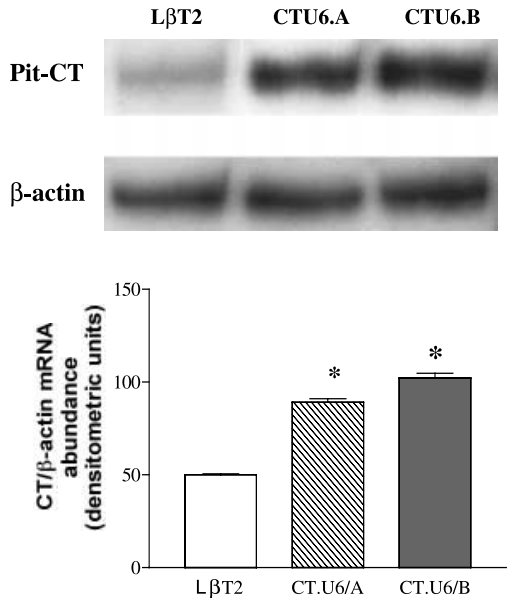

Figure 2 Overexpression of CT mRNA in stable CT.U6 transfectants. S1-nuclease protection assay showed that the CT.U6 cell lines (A and B) expressed higher levels of CT mRNA than parental LβT2 cells. The data from three independent experiments were digitized to obtain relative densitometric units. Pooled data from these experiments showed an almost twofold increase in CT mRNA abundance of the transfectants as compared with parental LβT2 cells. The results are expressed as means ± S.E.M. densitometric units ($n=6$). The data were analyzed by one-way ANOVA and significance was derived by Newman-Keul's test. *$P<0{\cdot}05$.

staining. These findings are also in agreement with our previously published results using GCT1-anti-SCT serum (Chronwall *et al.* 1996).

Discussion

Although there have been reports of the expression of CT-like peptides in the pituitary gland, these results for the first time define the sequence of pituitary-derived CT. cDNA sequence of pit-CT showed greater than 99% homology with mouse CT mRNA sequence (Rehli *et al.* 1996). It is conceivable that a few mismatches found in pit-CT sequence may have occurred due to spontaneous mutations known to occur in immortalized cell lines. It is also possible that Taq polymerase may have introduced mutation(s) during amplification. Additional studies with mouse AP glands will be necessary to explain these differences. However, the present results demonstrate that the mRNA sequence of pit-CT is closely homologous to mouse CT sequence, and the peptide encoded by the pit-CT mRNA immunoreacts with GCT1-anti-SCT serum. These results support our earlier observations that gonadotrope-derived CT immunoreacts with GCT1-anti-SCT serum (Shah *et al.* 1993, 1996, Chronwall *et al.* 1996).

The second objective of the present study was to test whether pit-CT mRNA is localized in gonadotropes of rat AP gland. Using an anti-sense riboprobe derived from cloned pit-CT cDNA, the present results have shown that pit-CT mRNA is co-expressed with β-LH mRNA, and lactotropes (PRL mRNA-positive cells) display close anatomical apposition to pit-CT mRNA-positive cells. Both these results are consistent with our earlier findings (Chronwall *et al.* 1996). Although the presence of SCT-like and HCT-like immunoreactive peptides in the AP gland has been reported by several investigators, there is only one report on the detection of rat CT mRNA in this organ (Jacobs *et al.* 1982). The present results are at variance with the earlier study that reported the lack of detectable CT mRNA in rat AP gland. It is conceivable that low copy numbers of pit-CT mRNA in rat AP gland and utilization of the less sensitive Northern blot technique may have been responsible for this discrepancy. Indeed, we also could not detect pit-CT mRNA in rat AP gland by Northern blot analysis; however, the detection was possible with more advanced techniques such as RT-PCR, S1-nuclease protection assay and *in situ* hybridization, which can detect single copy messages.

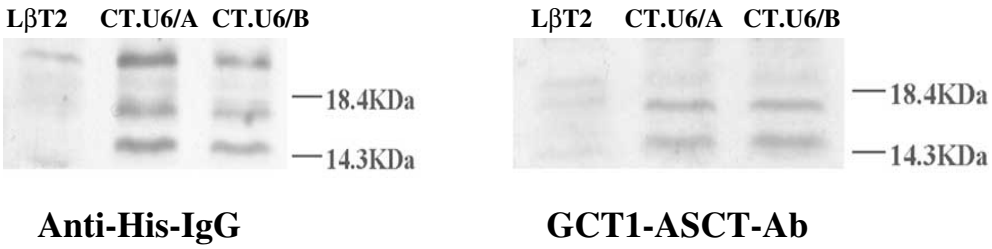

Figure 3 A typical profile of Western blotting of CT.U6 and parental LβT2 cell extracts. Fifty micrograms cell lysate proteins were size-fractioned on 12·5% SDS-polyacrylamide gel. Following electric transfer, the nitrocellulose membranes were immunoblotted with anti-His antibody (Anti-His-IgG; left panel) and GCT1-anti-SCT antibody (GCT1-ASCT-Ab; right panel). Reactivity was demonstrated using ECL Western blotting detection reagents. Positions of protein markers are indicated on the right. Since pcDNA3·1/Myc-His(+)B vector expresses fusion protein, the expressed pit-CT.U6 peptide will be fused with poly Myc-His. Anti-His antibody detected two CT.U6 cell-specific immunoreactive bands. Interestingly, the same bands were also identified by GCT1-anti-SCT antibody. The experiment was repeated one more time, and a similar profile was observed.

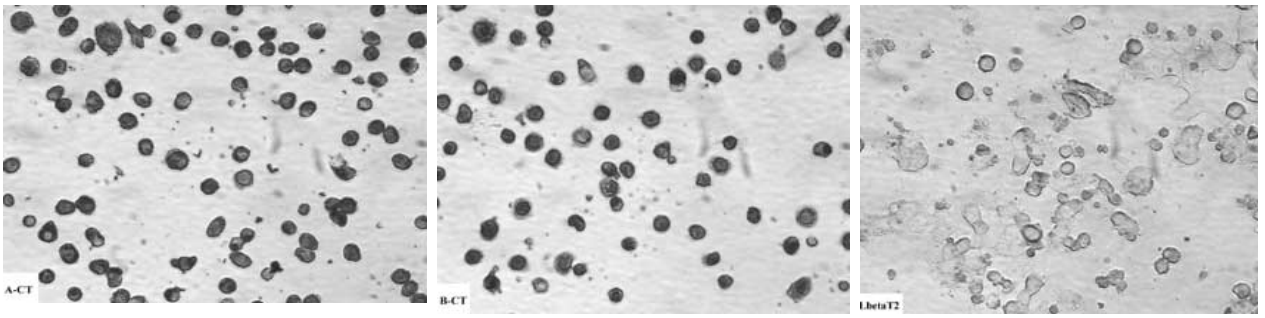

Figure 4 CTI-ICC of LβT2 cells and CT.U6 transfectants: LβT2 cells as well as CT.U6 (A and B) cell lines were processed for CT ICC as described in the Materials and Methods section. GCT1-anti-SCT serum was used as primary antiserum. Both CT.U6 cell lines (A and B; left and middle panels) stained intensely for CTI. In contrast, LβT2 cells (right panel) stained only lightly under the same experimental conditions. Negative controls with preabsorbed antiserum did not display any staining. The experiment was repeated with three different cultures of LβT2 and CT.U6 (A and B).

The third objective of the present study was to test whether the translated product of pit-CT cDNA inhibits lactotrope function. Previous evidence has shown that SCT, when administered either centrally or peripherally, inhibits PRL release in rats (Olgiati *et al.* 1981, 1982, 1983). *In vitro* experiments from this and other laboratories have extended these earlier findings by demonstrating that SCT acts directly at the level of lactotropes to inhibit PRL release, PRL gene transcription and lactotrope cell proliferation (Shah *et al.* 1988, 1990, 1993, 1999, Judd *et al.* 1990, Sortino *et al.* 1991, Zhang *et al.* 1995). A role for the endogenous peptide in these processes is demonstrated by the findings that immunoneutralization of pit-CT with GCT1 anti-SCT serum causes a significant increase in PRL release from cultured AP cells as well as in ovariectomized conscious rats (Shah *et al.* 1993, 1996). Consistent with these findings, the present results show

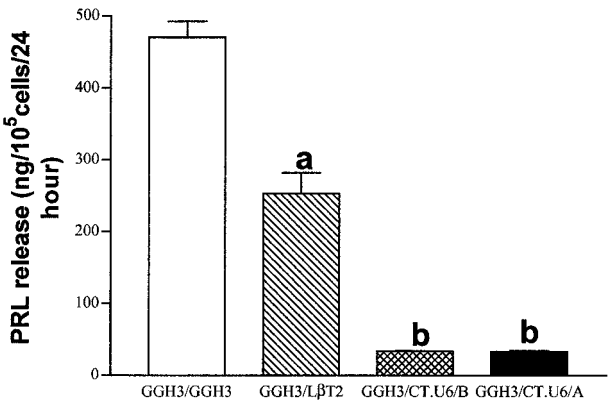

Figure 5 Co-culture with CT.U6 cells dramatically attenuates PRL release from GGH3 cells. GGH3 and LβT2/CT.U6 cells were co-cultured with GGH3 cells in transwell culture plates as described in the Materials and Methods section. Spent media were collected after 24 h of co-cultures and analyzed for PRL by RIA. Controls were GGH3/GGH3 homologous co-cultures. Each data point was run in triplicate, and the experiment was repeated three times. The pooled results are presented as mean ng PRL released ± S.E.M. (n=9). The results were analyzed by one-way ANOVA and significance was derived by Newman-Keul's test. a, P<0·01 (GGH3/GGH3 vs LβT2/GGH3); b, P<0·001 (GGH3/GGH3 vs CT.U6 (A and B)/GGH3).

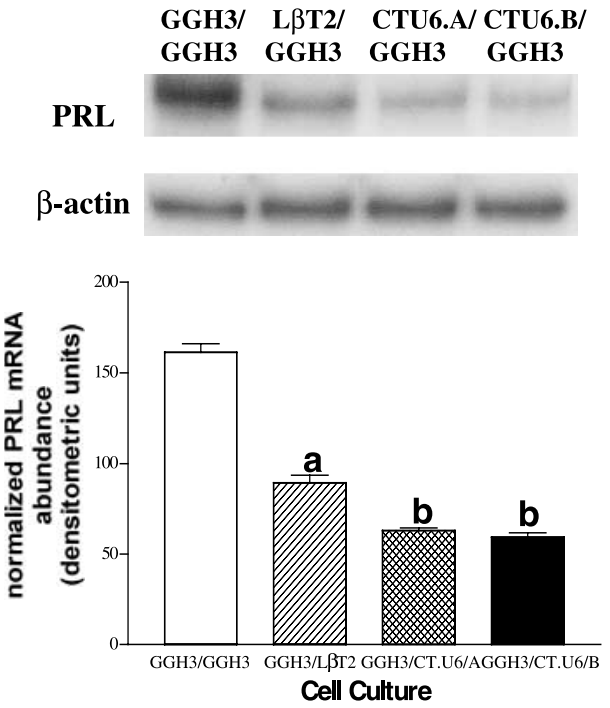

Figure 6 Co-culture with CT.U6 cells causes a marked decrease in PRL mRNA abundance of GGH3 cells. GGH3 and LβT2 or CT.U6 (A and B) cells were co-cultured with GGH3 cells in transwell culture plates as described in the Materials and Methods section. The GGH3 cells were lysed, RNA was extracted and PRL mRNA abundance was determined by S1-nuclease protection assay. β-actin mRNA abundance was also measured. Controls were GGH3/GGH3 homologous co-cultures. The results from three separate experiments were quantified by densitometry, normalized and are expressed as mean ± S.E.M. densitometric units (n=6). The data were statistically analyzed by one-way ANOVA, and significance was derived by Newman-Keul's test. a, P<0·01 (GGH3/GGH3 vs LβT2/GGH3); b, P<0·001 (GGH3/GGH3 vs CT.U6 (A and B)/GGH3).

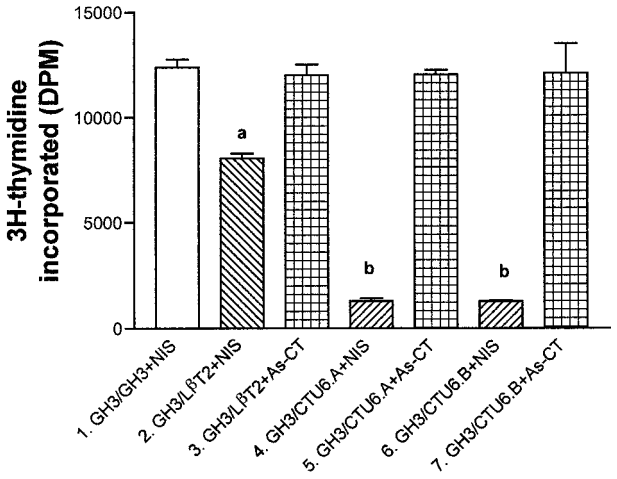

Figure 7 Co-culture with CT.U6 cells causes a dramatic decline in DNA synthesis of GH3 cells. GGH3 and LβT2 or CT.U6 (A and B) cells were co-cultured with GGH3 cells in transwell culture plates as described in the Materials and Methods section. In addition, the cells received either non-immune serum (NIS, 1:50) or GCT1-anti SCT serum (As-CT, 1:50). The GGH3 cells were treated with 0·5 μCi [^{3}H]thymidine during the last four hours. The cells were lysed and the incorporated [^{3}H]thymidine was determined. Each experimental data point was run in quadruplicate, and the experiments were repeated three separate times. The results are expressed as mean [^{3}H]thymidine incorporated ± S.E.M. (n=12). The results were further analyzed by one-way ANOVA and Newman-Keul's test. a, P<0·01 (GGH3/GGH3 vs LβT2/GGH3); b, P<0·001 (GGH3/GGH3 vs CT.U6 (A and B)/GGH3).

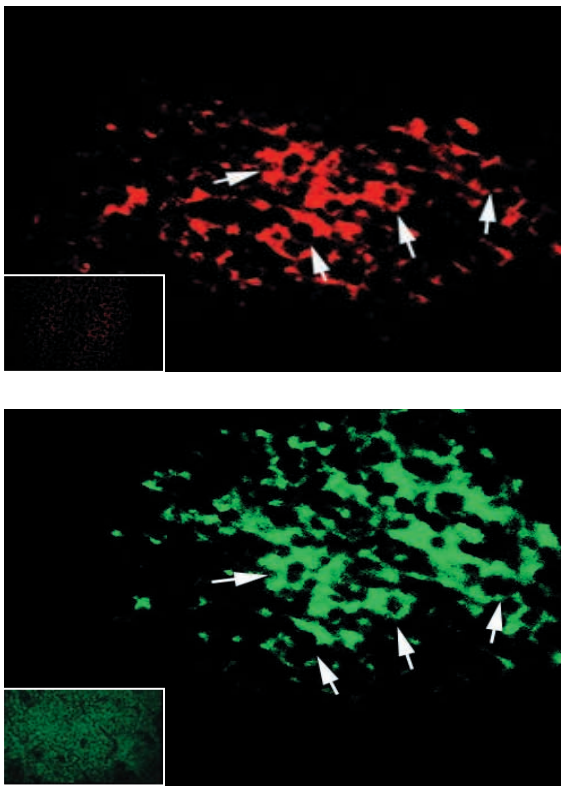

Figure 8 Co-localization of CT mRNA with β-LH mRNA in rat AP gland. Fluorescent probes for β-LH mRNA (TRITC) and CT mRNA (FITC) were prepared as described in the Materials and Methods section. Frozen rat AP sections (5 μm thick) were hybridized with these probes. Arrowheads in a typical micrograph depict colocalization of β-LH mRNA (red, upper panel) and CT mRNA (green, lower panel) in the same cells. Sense controls are presented in the inset. Magnification: × 400.

that stable pit-CT transfectants secrete high concentrations of GCT1-reactive CTI, and they markedly inhibit PRL secretion, PRL mRNA abundance, and also attenuate proliferation of GGH3 cells. A role of pit-CT in these effects is implicated by the findings that GCT1-anti-SCT serum severely attenuates this inhibition.

There is accumulating evidence for the role of paracrine/autocrine peptides in the function of the AP gland. Different cell types display different proportions in the AP gland, and their relative proportions change continuously with changing hormonal environment. For example, there is a dramatic increase in lactotrope cell populations during pregnancy and lactation (Aoki *et al.* 1994). There is also a drastic decrease in these populations with the cessation of lactation (Orgenero de Gaisen *et al.* 1993). Moreover, pituitary cell types respond to hormonal stimuli differently in different physiological conditions or hormonal milieu (Pasolli *et al.* 1992, Aoki *et al.* 1994, De Paul *et al.* 1997). The evidence suggests that signals provided by gonadal or neuroendocrine hormones to their target AP cells may be amplified or modulated by paracrine/autocrine factors. For example, pituitary-derived vasoactive intestinal polypeptide (VIP) and galanin stimulate PRL secretion and also induce lactotrope proliferation (Kaplan *et al.* 1988, Hsu *et al.* 1990, Wynik *et al.* 1993, 1998, Carretero *et al.* 1995, Balsa *et al.* 1996, 1998).

It has been suggested that several effects of estrogens on lactotrope function and proliferation are mediated by lactotrope-derived VIP and galanin (Wynik *et al.* 1993, Cai *et al.* 1998). Similarly, our studies suggest that CT inhibits PRL release and PRL gene transcription, and also attenuates thyrotropin-releasing hormone (TRH)- and suckling-induced PRL release and synthesis (Shah *et al.* 1988, 1990, Judd *et al.* 1990). CT is also a potent inhibitor of lactotrope proliferation (Shah *et al.* 1999). Expression of pit-CT is almost undetectable in early and mid-lactation but displays a dramatic increase in late lactation (Chronwall *et al.* 1996). Moreover, estrogens, which stimulate PRL synthesis and lactotrope proliferation, attenuate CT expression in the AP gland (Li & Shah 1995). Considering the secretion of CT by gonadotropes and the antagonistic functional relationships between gonadotropes and lactotropes, these results raise the strong possibility that gonadotropes may remodel the AP gland by modulating lactotrope cell number and function through the secretion of CT.

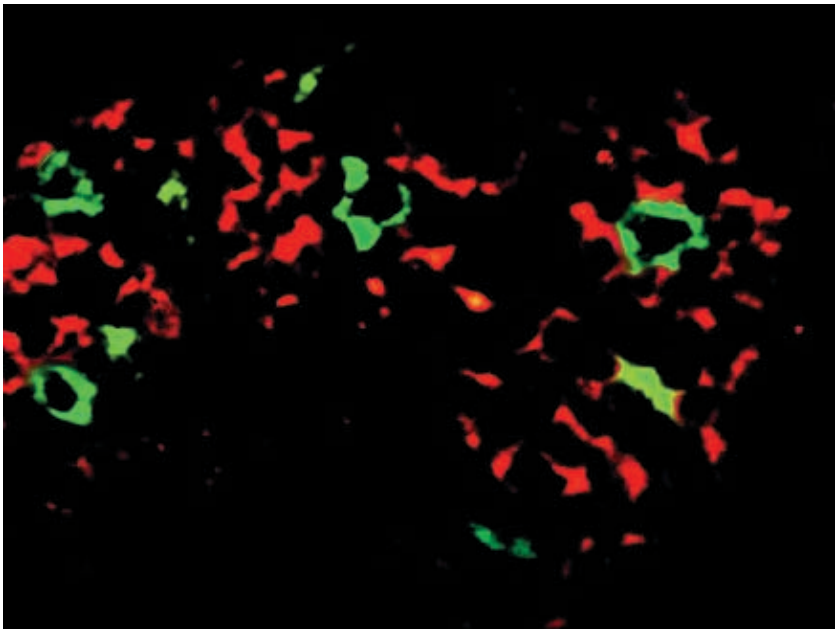

Figure 9 Localization of CT and PRL mRNAs in rat AP gland. The AP sections were hybridized simultaneously with PRL and CT mRNA probes as described in the Materials and Methods section. A typical micrograph shows that the AP cells labeled either for PRL mRNA (red) or for CT mRNA (green), and co-localization of both mRNAs in the same cells was not observed. However, most CT mRNA cells seemed to have been surrounded by PRL mRNA cells. Magnification: × 400.

In conclusion, we have cloned pit-CT cDNA from the gonadotrope-derived LβT2 cell line, and its sequence shows close homology to mouse CT mRNA. Pit-CT mRNA has been localized to gonadotropes, and the encoded peptide may regulate lactotrope function. The availability of the pit-CT cDNA will facilitate further investigations on the role of pit-CT in regulation of the AP gland.

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