STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE CHROMOGRANIN A GENE AND STUDY OF THE REGULATION OF EXPRESSION IN THE PARATHYROID

By

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SHORTENED VERSION OF THESIS TITLE

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ABSTRACT

Chromogranin A (CgA) is the major member of a family of acidic secretory glycoproteins (collectively known as granins) that are expressed in all endocrine and neuroendocrine cells. Granins have been proposed to play multiple roles, both intracellular and extracellular, in the secretory process. The regulation of synthesis and secretion of CgA in primary cultures of bovine parathyroid cells was studied and compared to that of parathyroid hormone (PTH). The effects of both extracellular calcium and the active metabolite of vitamin D_3, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] were shown to modulate CgA synthesis and secretion in the parathyroid. Whereas a reduced medium calcium level stimulated both CgA and PTH secretion, it had no effect on CgA mRNA levels. In contrast, $1,25(OH)_2D_3$ stimulated CgA mRNA levels by increasing the CgA gene transcription rate. Thus, $1,25(OH)_2D_3$ had opposite actions on the CgA and PTH genes in the parathyroid cell. The relationship between $1,25(OH)_2D_3$-stimulated CgA mRNA levels and total CgA protein levels (intracellular and extracellular) was not simple. Although $1,25(OH)_2D_3$ increased CgA mRNA levels several-fold, there was only a modest increase in CgA protein. This was the result of a specific effect of $1,25(OH)_2D_3$ on decreasing the translational efficiency of CgA mRNA as demonstrated by an increased ribosome transit time for CgA mRNA. To better understand the functional domains of CgA, and its evolution, and to study the basis of its neuroendocrine cell-specific gene expression, the human CgA gene was cloned and characterized. It spans 15 kb and contains eight exons. There is high conservation between the human, mouse, and bovine CgA genes with respect to exon number and placement of exon/intron borders. Transient transfection studies with constructs containing portions of the 5' gene flanking region upstream of a chloramphenicol acetyl transferase reporter gene showed that sequences between -53 to +32 bp relative to the transcription initiation site were sufficient to confer neuroendocrine cell-specific gene expression. These studies have provided important insights into the regulation of synthesis and release of CgA in the parathyroid, have characterized the human CgA gene, and have begun a functional analysis of the basis of the gene's neuroendocrine cell expression.

RÉSUMÉ

La chromogranine A (CgA) est le membre majeur d'une famille de glycoprotéines acidiques, nommées "granines" exprimées dans toute glande endocrine et neuroendocrine. On pense que les granines ont plusieurs fonctions dans le processus de sécretion, à l'intérieur et à l'extérieur de la cellule. Le contrôle de la synthèse et de la sécrétion de la CgA dans les cultures primaires de cellules parathyroïdiennes a été étudié, et comparé à celles de l'hormone parathyroïdienne (PTH). On a démontré que le calcium extracellulaire et le metabolite actif de la vitamine D_3, la 1,25-dihydroxyvitamine D_3, [$1,25(OH)_2D_3$] modulaient la synthèse et la sécrétion de CgA dans les cellules parathyroïdiennes. Quoiqu'une baisse de la concentration de calcium dans le milieu stimule la sécrétion de CgA et de PTH, cela n'a aucun effet sur le niveau de 'ARNmessager de la CgA (mCgA). Par contre, la $1,25(OH)_2D_3$ stimule le niveau de mCgA en stimulant son niveau de transcription. Donc, la $1,25(OH)_2D_3$ a des effets opposés sur les gènes de la CgA et de la PTH, dans la cellule parathyroïdienne. Le lien entre le niveau mCgA stimulé par la $1,25(OH)_2D_3$ et le niveau de protéine synthesisé, à l'intérieur et à l'extérieur de la cellule est un processus complèxe, car malgré une élévation importante de l'ARNm de la CgA, une hausse modeste de la synthèse de la protéine est observée. Ceci est le résultat d'un effet spécifique de la $1,25(OH)_2D_3$ qui diminue l'éfficacité de la traduction du mCgA, tel que démontré par l'augmentation du temps de transit des ribosomes sur l'ARNm de la CgA. Pour mieux comprendre l'importance des domaines fonctionnels de la CgA, et son évolution, et afin d'étudier les bases de son expression spécifique dans les cellules neuroendocrines, le gène humain de la CgA a été cloné et caractérisé. Le gène mesure 15 kpb, et contient huit exons. Il existe une conservation marquée du nombre et de l'emplacement des exons quand on compare le gène humain, murin, et bovin. Des études de transfections temporaires utilisant des constructions de taille différénte de la région promotrice en amont du gène rapporteur chloramphenicol acetyl transferase, ont démontré qu'une séquence entre -53 et +32pb, en relation du site initiateur de la transcription, est suffisante pour conférer l'expression spécifique du gène dans les cellules neuroendocrines. Ces études ont donc contribué à la compréhension de la régulation de la synthèse et de la sécretion de la CgA dans la glande parathyroïdienne, à la caractérisation du gène humain de la CgA, et à la découverte, avec l'analyse fonctionnelle préliminaire, de la base de l'expression spécifique de la CgA dans les cellules neuroendocrines.

FOREWORD

The Guidelines Concerning Thesis Preparation (Section 2) issued by the Faculty of Graduate Studies and Research at McGill University reads as follows: "Candidates have the option, **subject to approval of the Department,** of including, as part of the thesis, copies of the text of a paper(s), submitted for publication, or the clearly duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis. If this option is chosen, **connecting texts, providing logical bridges between the different papers, are mandatory.** The thesis must still conform to all other requirements explained in the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published.. **The thesis must include, as separate chapters or sections:** (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary.

Additional material (procedural and design data as well as descriptions of equipment used) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement on who contributed to the work and to what extent;** supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear. Candidates following this option must inform the Department before it submits the thesis for review."

I have chosen to write my thesis according to the above format with two papers that have been published and one paper submitted for publication. The thesis is divided in six chapters. Chapter 1 is a general introduction, literature review and historical perspective. Chapters 2 to 4 represent the experimental part of the thesis and are in the form of original papers, each with its own preface, abstract, introduction, materials and methods, results, discussion and references. Chapter 5 discusses my work as it relates to current studies on chromogranin A, and discusses future research directions. Chapter 6 lists the claims to original research.

PUBLICATIONS ARISING FROM THE WORK OF THIS THESIS AND CONTRIBUTIONS MADE BY CO-AUTHORS

1. **Mouland, A.J., Hendy, G.N. 1991. Regulation of synthesis and secretion of chromogranin A by calcium and 1,25-dihydroxy-cholecalciferol in cultured bovine parathyroid cells.** Endocrinology **128**:441-9.

   The candidate was responsible for all aspects of the study. The manuscript was prepared and revised for publication in consultation with Dr. G.N. Hendy.

2. **Mouland, A.J., Hendy, G.N. 1992. 1,25-dihydroxy-cholecalciferol regulates chromogranin A translatability in bovine parathyroid cells *in vitro*.** Molecular Endocrinology **6**:1781-8.

   The candidate was responsible for all aspects of the study. The manuscript was prepared and revised for publication in consultation with Dr. G.N. Hendy.

3. **Mouland, A.J., Bevan, S., White, J.W., Hendy, G.N. 1993. Human chromogranin A gene: molecular cloning, structural analysis and neuroendocrine cell-specific expression.** Journal of Biological Chemistry. Submitted.

   The candidate was responsible for most aspects of the study including the isolation of the chromogranin A clones, restriction enzyme mapping, sequencing and transient transfection studies. Dr. Sarah Bevan helped to completely map and characterize the CgA gene and prepare most of the fusion constructs. Dr. John White (Department of Physiology, McGill University) provided helpful tactical discussions and technical advice. The manuscript was prepared for publication in consultation with Dr. G.N. Hendy.

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DEDICATION

I dedicate this thesis to my wife, Régine, for her love, understanding and encouragement that she gave me without fail

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ABBREVIATIONS

+D	With 1,25-dihydroxycholecalciferol
-D	Without 1,25-dihydroxycholecalciferol
^{3}H	Tritium
5' UTR	5' untranslated region
^{14}C	Carbon 14
$1,25(OH)_2D_3$	1,25-dihydroxycholecalciferol
ACTH	Adrenocorticotrophic hormone
AGII	Angiotensin II
AP-1	Activator protein-1
AP-2	Activator protein-2
b-	Bovine
bp	Basepairs
Ca^{++}	Calcium ion
Ca^{2+}	Ionized calcium
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine-3',5'-monophosphate
cDNA	Complementary DNA
cf.	Compare
CgA	Chromogranin A
CgB	Chromogranin B
CgC	Chromogranin C
CPE	Carboxypeptidase E
cpm	Counts per minute
CRE	cAMP response element
CRH	Corticotrophic releasing hormone
CREB	cAMP response element binding protein
dCTP	Deoxycytidine-5'-triphosphate
DEAE	Diethylamine
DRB	5,6-Dichloro-1-β-d-ribofuranosylbenzimadazole
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(ß-aminoethyl ether)N,N,N',N'-tetraacetic acid
FBS	Fetal bovine serum

FCS	Fetal calf serum
FSH	Follicle stimulating hormone
g	Gram
G-protein	Guanine nucleotide regulatory protein
GRE	Glucocorticoid response element
GTC	Guanidium thiocyanate
h	Hour
H_2O_2	Hydrogen peroxide
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HRE	Hormone response element
K^+	Potassium ion
kb	Kilobase pairs
kDa	Kilodalton
LH	Luteinizing hormone
M	Molar
MBP	Myelin basic protein
ME	Mercaptoethanol
MEM	Modified Earle's medium
MEN	Multiple endocrine neoplasia
MES	2-(N-morpholino)ethanesulfonic acid
$MgCl_2$	Magnesium chloride
min	Minutes
mL	Millilitre
mM	Millimolar
mOhms	Milliohms
$MnCl_2$	Manganese chloride
mRNA	Messenger RNA
MTC	Medullary thyroid carcinoma
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Na_3PO_4	Sodium phosphate
ng	Nanogram (10^{-9} g)
NH_2	Amino
$NH_4(SO_4)_2$	Ammonium sulfate
NH_4COOCH_3	Ammonium acetate
o-	ortho-

OD_{260}	Optical density at wavelength, 260 nm
PBS	Phosphate buffered saline
PC-12	Pheochromocytoma PC-12 cells
PGE2	Prostaglandin E_2
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol-12-myristate-13-acetate
PMSF	Phenylmethylsulfonyl fluoride
POMC	Proopiomelanocortin
proPTH	Propararthyroid hormone
proPTHrP	Proparathyroid hormone related peptide
PSP	Parathyroid secretory protein
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone related peptide
RGD	Arg-Gly-Asp
RIA	Radioimmunoassay
RIN5F	Rat insulinoma 5F
RNA	Ribonucleic acid
RNAse	Ribonuclease
SEM	Standard error of the mean
SgI	Secretogranin I (CgB)
SgII	Secretogranin II (CgC)
SgIII	Secretogranin III (*IB1075*)
SgIV	Secretogranin IV (HISL-19)
SgV	Secretogranin V (protein 7B2)
SP-1	Secretory protein-1
TBP	TATA-binding protein
TCA	Tricarboxylic acid
TPA	12-O-tetradecanoylphorbol 13-acetate
Tris-HCL	Tris(hydroxymethyl)aminomethane-hydrochloride
TSH	Thyroid stimulating hormone
UTP	Uridine-5'-triphosphate
v/v	Volume/volume
VDR	Vitamin D_3 receptor
VDRE	Vitamin D_3 response element

WE-14	Amino-terminal tryptophanyl, carboxy-terminal glutamyl 14 amino acid peptide (CgA 324—337)
x g	Relative centrifugal force
µL	Microlitre

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PART I

INTRODUCTION AND LITERATURE REVIEW

CHAPTER 1

1.1 PREFACE

Several important advances in the understanding of endocrine cell secretory activity regulation have been made since the identification of chromogranin A (CgA), the major constituent of adrenal medulla chromaffin cell granules (Blaschko and Helle, 1963; Banks and Helle, 1965; Smith and Winkler, 1967). Smith and Winkler (1967) coined the term "chromogranin A" for this acidic glycoprotein which is now appreciated to be the principal member of the granin family of proteins. The granins share many biochemical and structural characteristics (Scammell, 1993), and because of this it is believed that they also have similar biological functions. The occurrence of one or more granins together with peptide hormones and neurotransmitters in secretory granules of virtually every endocrine and neuroendocrine cell indicates that these proteins have a critical role in cell secretory activity. This finding provided the rationale to study their biological role in the secretory process.

The recent characterization of the cDNAs encoding the granins of many species has provided clues to their function. It is believed that at least one function of the granins is as a precursor to bioactive peptides that affect endocrine cell secretory activity (e.g., Gerdes *et al.*, 1989; Simon *et al.*, 1988; Fasciotto *et al.*, 1991; Nielsen *et al.*, 1991). It has also been suggested that the granins have intracellular roles in modulating hormone processing, and in hormone sorting and exocytosis by virtue of their physicochemical nature.

There are two major secretory products of the parathyroid, parathyroid hormone (PTH), and CgA. They are costored within secretory granules in the parathyroid and are cosecreted. Recent investigations, including those presented in this thesis, indicate that a complex relationship exists between the regulation of CgA and PTH biosynthesis in the parathyroid.

In order to study the regulation of parathyroid CgA biosynthesis and secretion, and compare the responses to those of PTH, primary cultures of bovine parathyroid cells were used as the model endocrine system. This cell culture system provides a valuable means to study the regulation of these parathyroid secretory proteins. Parathyroid secretory activity is modulated by calcium and 1,25-dihydroxycholecalciferol [1,25$(OH)_2D_3$]. How these factors influence CgA expression at the molecular level in the parathyroid is a major focus of this thesis.

1.2 THE GRANINS

Splanchnic stimulation of the adrenal elicits the secretion of the contents of the secretory granules (Blaschko *et al.*, 1967). Apart from the catecholamines, chromaffin granules contain a large amount of soluble proteins, of which some are granins. Presently, at least six granins have been described (Figure 1.1): CgA (Konecki *et al.*, 1987; Helman *et al.*, 1988a), chromogranin B (CgB; Benedum *et al.*, 1987), secretogranin II (SgII; Gerdes *et al.*, 1989), the *IB1075* gene product (SgIII in Figure 1.1; Ottiger *et al.*, 1990), the HISL-19 antigen (SgIV in Figure 1.1; Krisch *et al.*, 1986), and protein 7B2 (SgV in Figure 1.1; Seidah *et al.*, 1983; Martens, 1988). The granins are exclusively expressed in endocrine and neuroendocrine cells, and by immunohistological studies, are localized within secretory granules. However, their tissue distribution does not always overlap (Rosa *et al.*, 1985; Jin *et al.*, 1993). This characteristic may be important for a granin's specialized function in a particular cell, for example. Whatever the biological significance is, the widespread distribution of the granins indicates that they have important functions in endocrine cells.

The granins share many biochemical properties (Table 1.1). A high percentage of acidic amino acids (glutamic acid, Glu, and aspartic acid, Asp) contributes to their acidic isoelectric points (pI). Heat stability is conferred to the granins by their hydrophilic nature, so they remain soluble even after boiling. At least three of the granins have been shown to have the capacity to bind calcium. Only two of the granins (CgA and CgB) possess a disulphide loop structure. The most striking feature of the granin proteins, however, is the presence of multiple pairs of basic amino acids (Figure 1.1 & Table 1.1).

1.3 FUNCTION

Many functions for the granins have been suggested. These include an extracellular role as a precursor to bioactive peptides. In addition, the granins may have intracellular roles. Examination of their biochemical characteristics suggests that the granins also play a role in the hormone sorting or packaging step in the secretory process. For simplicity, the following section limits the discussion to the many putative functions of CgA (Table 1.2). The other granins will likely have similar functions to those described for CgA.

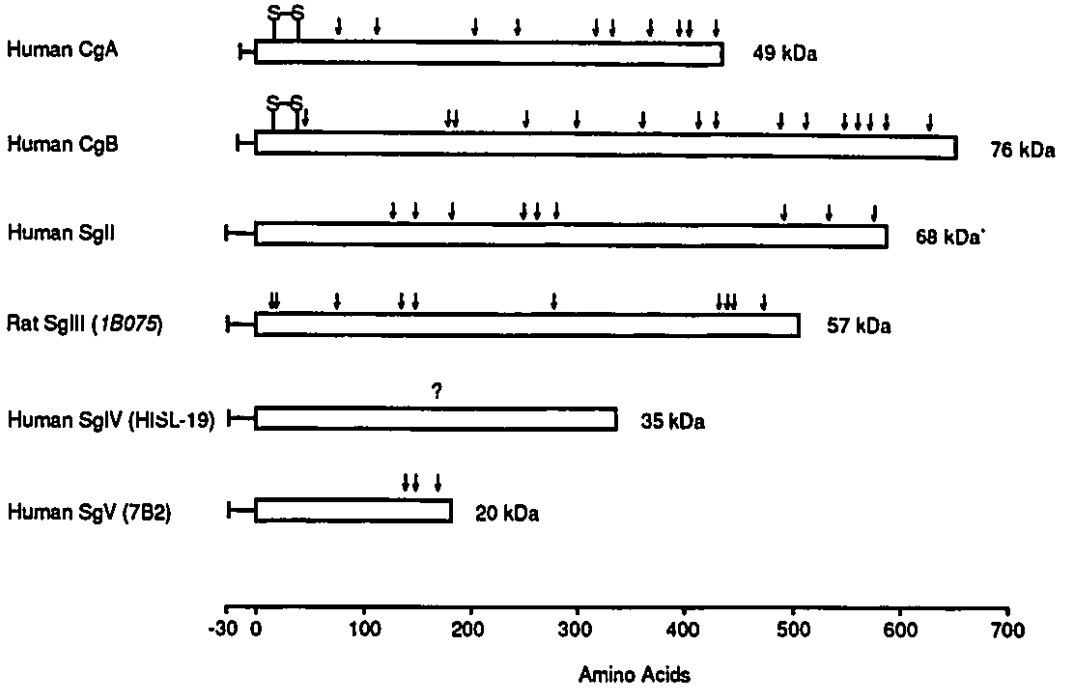

Figure 1.1 The granin family of endocrine secretory proteins. There are at least six members of this family of secretory proteins. The human proteins are shown (except for SgIII where only the rat cDNA has been cloned). They possess multiple pairs of basic amino acid residues (indicated by arrows). In CgA and CgB, a disulphide loop is present in the amino-terminal region. ? denotes that SgIV cDNA has not yet been cloned nor has the protein been sequenced. It is not known whether SgIV contains pairs of basic amino acid residues. See text for a description of the other features of the CgA molecule.

Table 1.1

General Properties of the Granin Family of Proteins[1]

Granin[2]	Mass (kDa)	mRNA (kb)	Chromosome Assignment	pI	% Glu/Asp	Dibasic Sites	Heat Stability	Calcium Binding	Disulphide Loop
CgA	49	2.1	14q32.2-32.3	4.9	25	10	yes	yes	yes
CgB	76	2.5	20pter-p12	5.1	24	15	yes	yes	yes
SgII	68	2.5	ND[3]	5.0	20	9	yes	yes	no
SgIII (*IB1075*)	57	2.2	9*	5.1	19	10	ND	ND	no
SgIV (HISL-19)	ND	ND	ND	5.6	ND	ND	ND	ND	ND
SgV (7B2)	21	1.4	15q11-q15	5.2	16	3	yes	ND	no

[1] Adapted from Huttner *et al.*, (1991).

[2] Data for the human granins are shown, except for SgIII where that for the rat (and mouse*) are shown.

[3] Not determined (ND).

1.3.1 EXTRACELLULAR FUNCTIONS FOR CHROMOGRANIN A

One main goal of the research to date has been to examine the extracellular role of CgA, where CgA-derived peptides are thought to function as modulators of endocrine cell secretory activity (Figure 1.2). Proteolytic processing of CgA can occur both intracellularly and extracellularly, and it has been reported that processing is important for biological activity (Simon *et al.*, 1988; Galindo *et al.*, 1991b; Fasciotto *et al.*, 1992). The resulting peptides may then interact with specific membrane receptors to elicit their biological responses. The experimental data cumulatively suggest that CgA acts as a precursor to bioactive peptides which can regulate endocrine cell secretory function.

The evidence in support of the notion that CgA is cleaved to smaller peptides, at least *in vitro*, arose from the isolation of a 49 amino acid, carboxy-amidated peptide, pancreastatin, that was shown to inhibit glucose-stimulated insulin release from perfused rat pancreas (Tatemoto *et al.*, 1986; Efendic *et al.*, 1987) and from a pancreatic tumour cell line (Lorinet *et al.* 1989). It was demonstrated that pancreastatin shared identity to CgA(250—301), and therefore was thought to be generated from the CgA molecule *in vivo* (Eiden, 1987; Huttner and Benedum, 1987). Pancreastatin is also active in other cell types, for example in inhibiting secretion from the parathyroid (Fasciotto *et al.*, 1989; Drees and Hamilton, 1992), in inhibiting parietal cell secretion (Lewis *et al.*, 1989), and in affecting glucose metabolism in rat hepatocytes (Sanchez *et al.*, 1992; Sanchez-Margalet *et al.*, 1992).

In addition to pancreastatin, a 20 kDa protein (β-granin) derived from the amino-terminus of the CgA protein (amino acids 1—113) was isolated from rat insulinoma cells, RIN5f (Hutton *et al.*, 1987a, 1987b). Although identity to the amino-terminus of rat CgA was established, β-granin's function in the pancreas has yet to be resolved. A biological function of β-granin in the parathyroid has recently become clear where it was shown to inhibit parathyroid cell secretion *in vitro* (Drees *et al.*, 1991). It is likely that β-granin has a similar function in the pancreas and in other endocrine tissues, but this awaits direct study.

Contained within the β-granin sequence is the recently described vasostatin (Aardal and Helle, 1992). Vasostatin is the term which refers to fragments containing the amino-terminal domain of CgA(1—76). It has been shown to inhibit arterial smooth muscle contraction *in vitro* (Aardal and Helle, 1992). CgA immunoreactivity has been localized to nerve terminals in skeletal muscle (Voknaidt *et al.*, 1987) which supports a role of CgA in

Table 1.2

Putative Functions for Chromogranin A

EXTRACELLULAR
• Precursor to biologically active peptides
INTRACELLULAR
• Role as an intracellular calcium-binding protein
• Role in the packaging of peptide hormones and neuropeptides, granule condensation and exocytosis
• Intracellular regulator of prohormone processing

muscle contraction. A truncated version, CgA(1—40), was synthesized and was shown to inhibit parathyroid hormone related protein (PTHrP; Deftos *et al.*, 1989) and calcitonin (Deftos *et al.*, 1990) secretion from BEN cells. This data suggests that the amino-terminal region of CgA may be responsible for eliciting these biological effects, and that the activity is not restricted to one cell type. Vasostatin and β–granin possess pairs of basic amino acid cleavage sites at their carboxy-termini which may serve as targets for processing enzymes and thus may be generated *in vivo*. The authenticity of these cleavage sites, however, remains to be determined.

Endoproteinase Lys-C digestion of bovine CgA generates a peptide that has inhibitory activity on chromaffin cell secretion. Sequence analysis demonstrated that amino acids 124—143 of bovine CgA encode the amino-terminus of this biologically active sequence. The sequenced peptide started at amino acid 124, but extends through 143 to an, as yet undetermined, carboxy-terminus. A synthetic peptide, termed chromostatin, corresponding to CgA(124—143) inhibited L-type voltage-sensitive calcium channels and blocked the transient calcium spike through a serine/threonine acid phosphatase (Galindo *et al.*, 1992b), thereby inhibiting bovine chromaffin cell secretion *in vitro* (Galindo *et al.*, 1991b; Galindo *et al.*, 1992a). In an immunohistological study, an antiserum that was raised to bovine chromostatin revealed the presence of chromostatin immunoreactivity in the secretory granules of normal human pancreatic β-cells, but not in human adrenal medulla cells (Cetin *et al.*, 1993). A species difference in the distribution of chromostatin between the bovine and human adrenal may explain this finding. It is not known how the chromostatin sequence is generated from CgA *in vivo*, nor is it known whether chromostatin has biological activity in other species and tissues. CgA may be cleaved extracellularly to generate chromostatin-like peptides, but this also remains to be established.

Fasciotto *et al.* (1992) have recently identified a peptide, parastatin (amino acids 347—419 of porcine CgA), generated by endoprotease Lys-C digestion of CgA, that inhibits parathyroid cell secretion *in vitro*. Further studies are in progress to determine the significance of the biological activity of this peptide (Fasciotto *et al.*, 1993).

The mechanism by which most of the CgA-derived peptides exert their biological effects is at present unclear. For chromostatin, however, a membrane receptor was demonstrated to exist by cross-linking studies (Galindo *et al.*, 1992a). Because it has been demonstrated that a specific membrane receptor is involved in transmitting chromostatin's effect on secretion, receptor-mediated effects may also be involved in the actions of the other CgA-derived peptides (Figure 1.2).

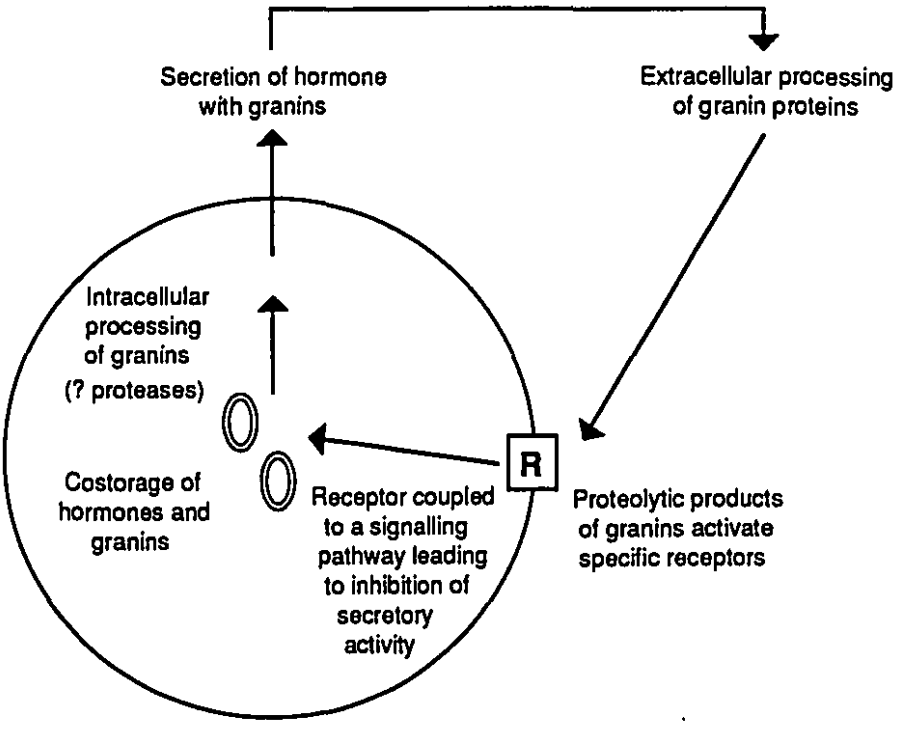

Figure 1.2 Putative mechanism of action of CgA and/or CgA-derived peptides on hormone secretory activity in endocrine cells. CgA is synthesized and shuttled into and stored in secretory granules of endocrine and neuroendocrine cells. CgA is processed within the granule and secreted upon stimulation of exocytosis. CgA is also processed extracellularly, and can then exert its biological actions to modulate secretion. A membrane receptor (R) coupled to an intracellular transduction pathway are believed to transduce the biological actions of the CgA-derived peptides on secretion. The actions of the CgA-derived peptides are believed to act in an autocrine or paracrine manner.

There is no evidence to support the notion that the CgA peptides act in an endocrine manner and it is likely that the CgA-derived peptides described above have effects on cell secretory activity by acting extracellularly in a paracrine or autocrine manner.

Cleavage of CgA has been shown to be necessary for their biological activity, at least *in vitro* (Simon *et al.*, 1988; Galindo *et al.*, 1991b; Fasciotto *et al.*, 1992), and CgA processing may occur during storage in secretory granules, during secretion, or in the extracellular milieu (Figure 1.2). More information about this process, and how these peptides act will be required.

1.3.2 INTRACELLULAR FUNCTIONS FOR CHROMOGRANIN A

The molecular cloning of the bovine (Benedum *et al.*, 1986; Iacangelo *et al.*, 1987; Ahn *et al.*, 1987), human (Konecki *et al.*, 1987; Helman *et al.*, 1988a), rat (Iacangelo *et al.*, 1988a; Parmer *et al.*, 1990), pig (Iacangelo *et al.*, 1988b), and mouse (Wu *et al.*, 1990) CgAs revealed that regions in the CgA molecule share sequence homology to well characterized calcium binding proteins (Benedum *et al.*, 1986; Parmer *et al.*, 1989; Yoo, 1992). The first site constitutes the consensus high affinity calcium-binding sequence in the E-helix region of the calmodulin E-F hand, E-L-Q-D (Watterson *et al.*, 1980; amino acids 55—60 of bovine brain calmodulin), and is conserved in all CgAs. This region is also associated with calmodulin binding *in vitro* (Yoo, 1992). The other calcium-binding domains in CgA share homology to the calcium binding proteins, S100β, oncomodulin and intestinal calcium binding protein (ICBP; Parmer *et al.*, 1989; Table 1.3).

Several lines of evidence demonstrate that CgA binds calcium. Direct evidence to support CgA calcium binding includes studies which use specific dyes, such as Stains-all, and [^{45}Ca] nitrocellulose binding assays (Reiffen and Gratzl, 1986; Leiser and Sherwood, 1989). CgA binds calcium with low affinity and several studies have reported dissociation constants (Kd) ranging from 54 μM (Reiffen and Gratzl, 1986), to 0.13 mM (Videen *et al.*, 1992), to 1 mM (Leiser and Sherwood, 1989), and to 2-4 mM (Yoo and Albanesi, 1991). It has been reported that 7.5 – 35 moles calcium per mole CgA protein can be bound (Reiffen and Gratzl, 1986; Leiser and Sherwood, 1989; Yoo and Albanesi, 1991; Videen *et al.*, 1992), demonstrating that CgA has a high calcium binding capacity. Because CgA calcium binding is influenced by pH, as well as by magnesium and ionic strength (Gratzl, 1987; Gorr *et al.*, 1989; Leiser and Sherwood, 1989; Yoo and Albanesi, 1990), calcium binding properties of CgA can change during the maturation and acidifica-

Table 1.3[1]

Calcium Binding Domain Homologies in Chromogranin A

Homology	Sequence
Calmodulin E-F Hand	
Calm 55-60	AELQDM
rCgA 59-64	K----L
mCgA 59-64	K----L
bCgA 59-64	K----L
hCgA 59-64	K----L
S100β Protein Homology	
S100β 61-65	DSDGD
rCgA 140-144	TTE-P
mCgA 137-142	---KG
bCgA 127-131	-----
hCgA 127-131	AT--A
Intestinal Calcium Binding Protein Homology	
ICBP 16-20	QLSQE
rCgA 328-332	R--R-
mCgA 330-334	R--R-
bCgA 304-308	-----
hCgA 313-317	R----
Oncomodulin	
ONCO 96-102	GKIGADE
rCgA 271-277	--T--S-
mCgA 271-277	--TE-S-
bCgA 258-264	--H--E-
hCgA 263-269	--P--E-

[1] Adapted from Parmer *et al.* (1989).

tion of secretory granules, and these could be important for CgA function. It is likely that CgA binds calcium *in vivo* and this characteristic is related to an important intracellular role for CgA in hormone secretion.

pH and calcium concentration can induce conformational changes in CgA (Yoo and Albanesi, 1990; Yoo and Albanesi, 1991). The intracellular localization of CgA can also be influenced, where calcium can enhance CgA association with membranes (Gorr *et al.*, 1988; Leiser and Sherwood, 1989). These characteristics provide a mechanism by which CgA can act to sort hormones into the regulated secretory pathway and package hormones into secretory granules (Figure 1.3). Although CgA can co-aggregate with a peptide hormone such as PTH, but excludes constitutive secretory proteins such as serum albumin (Gorr *et al.*, 1989), there is no direct evidence to show that CgA functions in cells in this manner. More direct evidence in support of this function, however, is available for the granin, CgB. It has been demonstrated that CgB can divert immunoglobulins into the regulated secretory pathway (Rosa *et al.*, 1989). mRNAs encoding the light and heavy chains of a monoclonal antibody to CgB were microinjected into pheochromocytoma PC-12 cells. The encoded antibody immunocomplexed with CgB and by noncovalently binding to CgB, this otherwise constitutively secreted polypeptide was shown to be diverted into the regulated secretory pathway (Rosa *et al.*, 1989). A similar type of experiment could be used to compare CgA's function in the sorting process.

CgA has been shown to have an intracellular role in regulating prohormone processing. Due to the presence of multiple pairs of basic amino acid sites, CgA may serve as a competitive substrate for serine protease-like activity in cells. Indeed, CgA can serve as a competitive inhibitor for IRCM-serine protease in the processing of pro-opiomelanocortin (POMC; Seidah *et al.*, 1987). This function may have important consequences in the appropriate processing of many prohormones, in the generation of functional hormones such as insulin and POMC-derived peptides, for example. This function could be confirmed by introducing the CgA gene into non-expressing cells and examining the effects on endogenous hormone processing events.

1.4 CHROMOGRANIN A

CgA is the best studied member of the granin family. Since its discovery, several advances have been made to understand CgA biochemistry and function. The following sections of the thesis focus on the biochemistry, structural features, distribution, regulation of biosynthesis, and clinical importance of CgA.

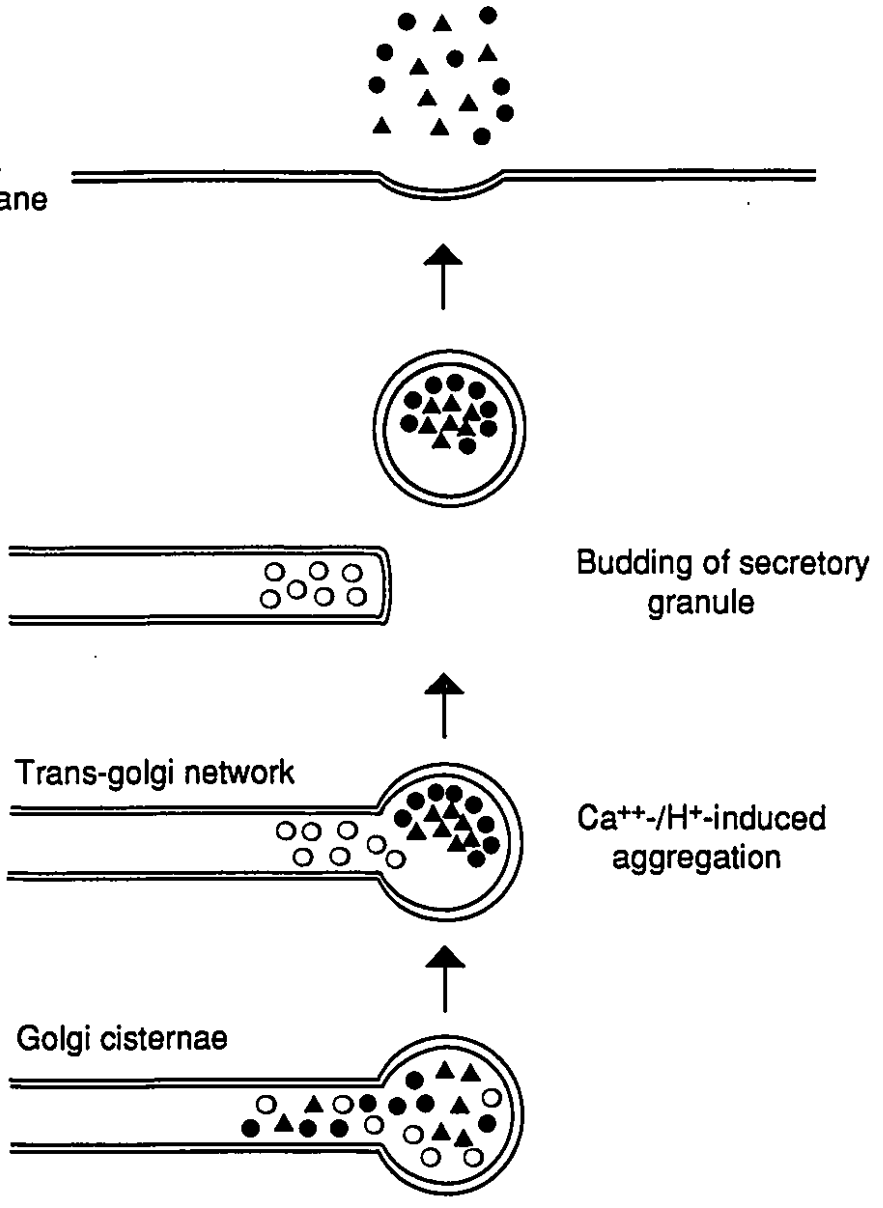

Figure 1.3 Schematic diagram illustrating the sorting of regulated secretory proteins by the granins. The pH of the golgi cisternae is neutral. Acidification and increase in calcium concentration in the trans-golgi promote the selective aggregation of granin proteins (l) and those proteins which can co-aggregate with them (s; e.g., PTH). Budding of an immature secretory granule ensues, containing proteins/hormones which follow the regulated secretory pathway, and excluding constitutive secretory proteins (m).

1.4.1 BIOCHEMISTRY AND STRUCTURAL FEATURES

1.4.1.1 PRIMARY AMINO ACID STRUCTURE

CgA is synthesized with an 18 amino acid signal sequence which targets it to the endoplasmic reticulum for synthesis on membrane-bound polyribosomes. CgA has a calculated molecular weight of approximately 49 kDa, and the mature CgA protein varies in length from 431 amino acids in the cow to 445 in the mouse. There is an unusually high content of glutamic and aspartic acid residues (about 25%) and of proline (10%) which contribute to its acidic pI of 4.5-5, its extreme hydrophilicity and its aberrant mobility on sodium dodecyl sulphate (SDS)-polyacrylamide gels. CgA contains a disulphide bonded loop structure at its amino-terminus (Figure 1.4).

The primary amino acid sequence of CgA contains from eight to ten pairs of basic amino acid residues which may serve as potential targets for endoproteolytic attack by processing enzymes. Several peptides as described above are putatively derived from the CgA molecule: pancreastatin, β-granin, chromostatin, vasostatin, and parastatin. These may be generated, in part, by cleavage at these paired basic amino acid sites. CgA also possesses a cell adhesion sequence, Arg-Gly-Asp (RGD) near its amino-terminus in most species, except in the rat where it is located at the carboxy-terminus. Many proteins such as fibronectin and fibrinogen, that possess the RGD sequence are involved in attachment of anchorage-dependent animal cells to extracellular matrix or in cell-cell adhesion, which involve a class of membrane receptors, the integrins (Hynes, 1987). Despite the presence of an RGD sequence, there is no evidence yet to show that it is functional in the CgA molecule.

The presence of several oligoglutamic stretches of amino acids characterize the CgA molecule. In the human gene, there are four such motifs with four to nine contiguous glutamic acid stretches (Figure 1.4). These motifs are conserved and may have a role in determining the secondary and tertiary structures of the polypeptide. Bundles of negatively charged glutamic acid residues in the CgA molecule, which would oppose the formation of a stable α-helix, indicate that CgA possesses a disorganized structure termed "random coil" in these regions (Simon and Aunis, 1989). Analysis of adrenal medulla CgA by circular dichroism showed a predominance of random coil structure (60-65%), with the α-helical and ß-sheet conformations representing about 25% and 10%, respectively (Yoo and Albanesi, 1990).

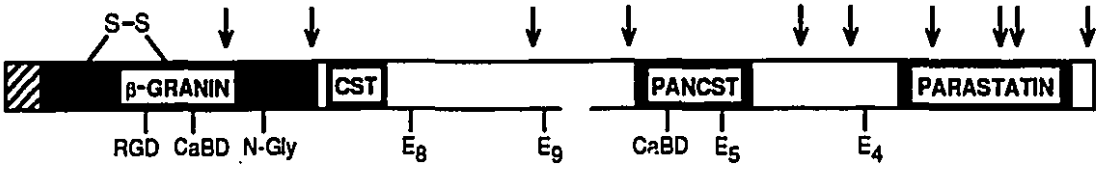

Figure 1.4 Structural and functional domains of human CgA. CgA is synthesized with an 18 amino acid signal sequence (hatched box). A disulphide loop (S-S) is present at the amino-terminus. The primary amino acid sequence contains ten pairs of basic amino acids (indicated by arrows), which may serve as substrates for processing enzymes. Biologically active peptides believed to be derived from CgA include β-granin, chromostatin (CST), pancreastatin (PANCST), and parastatin. A cell adhesion sequence (RGD), two calcium binding domains (CaBD), a putative N-glycosylation site (N-Gly), and four oligoglutamic acid stretches (E_n) are also present within the sequence. Biochemical analysis of CgA showed that CgA in O-glycosylated. O-glycosylation may occur on serine and threonine residues, and these residues are distributed throughout the CgA molecule (not indicated). See text for discussion.

A characteristic which is unique to rat and mouse CgA is the presence of a polyglutamine tract in these proteins. In the mouse CgA molecule, a tract of 11 repeating glutamine residues begins at amino acid 84 (Wu *et al.*, 1991). In the rat CgA molecule, however, a polymorphic polyglutamine tract is found. Depending on the cDNA clone examined, 16 to 20 repeating glutamine residues are found in the amino-terminus of the rat CgA beginning at amino acid 74 (Parmer *et al.*, 1989; Iacangelo *et al.*, 1988b). The functional significance of the polymorphism in the rat CgA molecule is presently unclear.

1.4.1.2 POST-TRANSLATIONAL MODIFICATIONS

Despite a calculated molecular mass of 49 kDa, on SDS-polyacrylamide gels CgA runs with an aberrant low relative mobility (M_r), approximately 1.5 times its true molecular weight. Originally this was thought to be due to post-translational modifications of the CgA molecule. It has been demonstrated, however, that the *in vitro* transcription/translation of bovine and human full-length cDNAs yields a translation product of 68-72 kDa on SDS-PAGE (Benedum *et al.*, 1986; Helman *et al.*, 1988a). Therefore, the aberrant M_r is likely due to the molecule's hydrophilicity and high content of acidic amino acids.

The following discussion presents the many ways in which CgA is post-translationally modified in endocrine cells.

1.4.1.2.1 GLYCOSYLATION

Adrenal medulla CgA is a glycoprotein containing 5.4% carbohydrate whose sugars are mainly present as O-glycosidally linked tri- and tetrasaccharides composed of N-acetylgalactosamine, galactose and sialic acid (Kiang *et al.*, 1982; Apps *et al.*, 1985). Parathyroid CgA was initially reported to contain 18% carbohydrate (Takatsuki *et al.*, 1981), however a later study showed that it was similar in carbohydrate composition (approximately 5%) to adrenal medulla CgA (Cohn *et al.*, 1982). Despite the presence of consensus sites for N-glycosylation in human and rat CgAs (cf. Winkler and Fischer-Colbrie, 1991), experiments using tunicamycin to block N-linked glycosylation in the endoplasmic reticulum failed to show that N-glycosylation of CgA takes place (Rosa *et al.*, 1985). This is also true for the granin, SgIII (*1B1075*), in that there are five consensus

sites for N-linked glycosylation (Asn/X--Ser/Thr), yet SgIII is not an N-glycosylated protein (Ottiger *et al.*, 1990).

1.4.1.2.2 PHOSPHORYLATION

CgA is phosphorylated *in vitro* (Bhargava *et al.*, 1983; Settleman *et al.*, 1985; Rosa *et al.*, 1985; Cote *et al.*, 1986). This occurs primarily on serine and tyrosine residues, with little occurring on threonine residues. The significance of phosphorylation of CgA remains unclear.

1.4.1.2.3 SULPHATION

Sulphation can play a role in rendering a precursor protein amenable to proteolytic processing and may potentiate the biological activity of certain peptides. For example, a positive relationship is suggested to exist between proteolytic processing and sulphation of the gastrin precursor (Andersen and Stadil, 1983). Furthermore, sulphated cholecystokinin and leuenkephalin are more potent in eliciting their biological effects than are the nonsulphated forms of these peptides (Huttner, 1988). In bovine chromaffin cells, CgA is sulphated and the sulphate moiety is mainly bound to carbohydrates, and not to tyrosine residues (Rosa *et al.*, 1985). Originally the sulphate moiety was reported to be linked to the parathyroid CgA molecule on tyrosine residues in the pig (Kumarasamy and Cohn, 1986), but a later study (Gorr *et al.*, 1991) showed that both porcine and bovine parathyroid CgA molecules are sulphated on oligosaccharides. A small portion (1-2%) of bovine CgA can be found in a proteoglycan form (Gowda *et al.*, 1990), and this form can only be found in the bovine parathyroid, and not in that of the pig (Gorr and Cohn, 1991; Barbosa *et al.*, 1991). Proteoglycans have several functions including binding to extracellular proteins such as fibronectin, and serving to bind growth factors such as transforming growth factor-β (TGF-β) and fibroblast growth factor (FGF). It is suggested that the proteoglycan binding serves to mediate the effects of these growth factors on their target cells at a short range (Ruoslahti and Yamaguchi, 1991). It is possible, therefore, that the effects of CgA on cell secretion may be partly mediated by the actions of the proteoglycan form of CgA. However, the precise function for the proteoglycan form of CgA remains to be determined.

1.4.1.2.4 FORMATION OF A DISULPHIDE BOND: THE CHROMOGRANIN A LOOP

CgA contains two amino-terminal cysteine residues (Cys^{17}–Cys^{38}) that participate in the formation of a disulphide bond by disulphide isomerase in the lumen of the endoplasmic reticulum (Benedum *et al.*, 1987). The presence of this structure is conserved in CgA in all species, and is also found in an identical region of the CgB protein, which makes CgA and CgB more closely related to each other than to any other granin (Figure 1.1). Disulphide bond formation may play an important role in determining CgA's function, but this remains to be determined.

1.4.1.2.5 CARBOXYMETHYLATION

Nguyen *et al.* (1987) presented evidence for a cotranslational carboxymethylation of CgA in adrenal medulla chromaffin cells. Carboxymethylation may represent a requirement for a protein destined for endoproteolytic attack (Clarke, 1985; Nguyen *et al.*, 1987). Because CgA may be processed to generate several bioactive peptides, this post-translational modification may be important for this process. However, the known carboxymethylase enzyme is cytosolic, and it is not clear how CgA may come into contact with this enzyme.

1.4.2 THE CHROMOGRANIN A GENE

The CgA gene structure consists of eight exons spanning 11.5 kb, 12.5 kb, and 15 kb in the mouse (Wu *et al.*, 1991) cow (Iacangelo *et al.*, 1991), and human (Chapter 4), respectively. The single copy CgA gene is located on chromosome 14 as determined by the use of flow-sorted chromosomes (Murray *et al.*, 1987) and the use of somatic cell hybrids (Modi *et al.*, 1989). It was more precisely localized to 14q32.2-32.3 by *in situ* hybridization analysis (Modi *et al.*, 1989).

Comparison of the gene structure to those known for the other granins reveals similarities only between the CgA and CgB genes. Homologous domains in the amino- and carboxy-termini of these granins become apparent when the mouse genes are compared. The mouse CgA gene consists of eight exons spanning about 11.5 kb; that for CgB consists of 5 exons and spans 12.1 kb (Pohl *et al.*, 1990). Exon 2 of both the mouse CgA and CgB genes encodes the last three amino acids of the signal peptide and the next thirteen amino

acids of the mature proteins. Exon 3 encodes the next 33 amino acids in both CgA and CgB. Exon 8 of CgA, and exon 5 of CgB encode twenty-five and twenty-seven of the carboxy-terminal amino acids, respectively. The mid-molecule region of both granins is variable, between species and granins, and this is also reflected in the different genomic organization in this region. Similarly, the gene structures of the other granins are distinctly different than those for CgA or CgB (Modi *et al.*, 1989; Murray *et al.*, 1987; Blatt *et al.*, 1985; Jenkins *et al.*, 1991; Schimmel *et al.*, 1992).

Similarites exist in the 200 bp of published promoter sequence between the mouse, bovine and the human CgA genes in the proximal promoter regions (Chapter 4). There is an approximately 80% homology between the human and bovine promoters, with the homology being somewhat less between the human and mouse gene promoters (50%). In each of the promoters, just upstream of a conserved TATA box sequence, a consensus cyclic-adenosine-3'-5'-monophosphate (cAMP) response element (CRE) is found. In addition, consensus sites for the transcription factors, Sp-1 and activator protein-1 (AP-1) are found in all three genes. It is interesting to note that the CgB (Pohl *et al.*, 1990) and the SgII (Schimmel *et al.*, 1992) genes also contain two of these elements in their promoter regions. Table 5.1 identifies many putative response elements within 2.3 kb the human CgA promoter. Chapter 4 describes the characterization of the human CgA gene, and presents the results of the functional analysis of the DNA element in the 5' flanking region of the gene that confers neuroendocrine cell-specific gene expression.

In general, exons can organize the coding region into several distinct structural and functional domains. For example, the third exon of the POMC gene encodes all the functional peptides derived from the mature POMC proprotein (Drouin *et al.*, 1990). Elucidation of the human CgA gene structure and comparison to the other species would be expected to provide insight into the conserved functional domains of the CgA protein. This aspect is discussed in detail in Chapter 4.

1.4.3 PROTEOLYTIC PROCESSING

The presence of multiple pairs of basic amino acid residues within the CgA molecule suggests that CgA functions as a precursor protein where it is specifically processed to generate bioactive peptides. CgA is processed, but CgA processing does not produce a constant pattern of peptides in neuroendocrine cells (Deftos *et al.*, 1990; Barbosa *et al.*, 1991). CgA is also processed to varying degrees in many endocrine tissues. In the parathyroid and adrenal medulla for example, very little processing of CgA occurs (Barbosa

et al., 1991; Simon *et al.*, 1989a). In contrast, CgA is processed extensively in the pancreas (Hutton *et al.*, 1987b), and in gastrointestinal tissues (Watkinson *et al.*, 1989, 1991). The variability in CgA processing from one tissue to another makes the evaluation of the function of the peptide products complicated, and may indicate that the putative CgA-derived peptides have tissue-specific roles.

Direct evidence in support of a precursor-product relationship between CgA and its peptides has been obtained in only a few cases. For instance, by pulse-chase analysis a precursor-product relationship was demonstrated to exist between CgA and ß-granin in rat insulinoma tissue (Hutton *et al.*, 1987a). In addition, a similarly sized fragment, which shares identity to the amino-terminus of CgA, was found to be secreted by bovine parathyroid cells (Drees *et al.*, 1991), suggesting that CgA also serves as a precursor to ß-granin in the parathyroid. However, the demonstration that a true precursor-product relationship exists between CgA and the other putative CgA-derived peptides remains to be established.

Very little information has been reported about the regulation of proteolytic processing of CgA. Two groups have examined the effects of hormones and pharmacologic agents on CgA protein processing (Simon *et al.*, 1989a; Watkinson and Robinson, 1992). Regulators of chromaffin cell function including nicotine, glucocorticoids, and the phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), did not affect the rate of intracellular CgA processing in this cell (Simon *et al.* 1989a). These authors suggested that extracellular processing may be more important in the metabolism of CgA in chromaffin cells since CgA was found to be actively processed only after secretion (Simon *et al.* 1989a). CgA processing can be induced to occur within chromaffin cells, however. The effect of reserpine on CgA processing in chromaffin cells was examined (Watkinson and Robinson, 1992). Reserpine, which induces proenkephalin processing and induces the activity of the peptidyl-glycine amidating enzyme to produce bioactive enkephalins, was also shown to induce CgA processing. A marked increase in the appearance of pancreastatin immunoreactivity occurred in the presence of reserpine (Watkinson and Robinson, 1992). Although extracellular proteolysis of CgA can not be ruled out, at least in part to explain the appearance of pancreastatin-like peptides, this data underscores the fact that CgA processing can be regulated. Furthermore, extracellular processing of CgA can be important, and this has been shown to be a prerequisite for the biological actions of CgA on secretion in parathyroid cells (Fasciotto *et al.*, 1992), and in chromaffin cells (Simon *et al.* 1988; Galindo *et al.*, 1991b).

Several calcium-dependent serine proteases have recently been identified in mammalian neuroendocrine tissues whose recognition sites are pairs of basic amino acid

residues and whose pH optima are acidic (for two recent reviews see Smeekens, 1992; Seidah and Chrétien, 1992). This type of protease activity is active in the proteolysis of POMC, for example. Each cryptic peptide in POMC is flanked by a pair (or a pair doublet) of basic amino acid residues (Thomas *et al.*, 1988). Cleavage of prohormones by proteases may also occur at monobasic, and tribasic sites. CgB, for instance, may be processed at single and triplet basic amino acid residues, as well as at paired basic amino acid residues (Nielsen *et al.*, 1991). The enzymes responsible for these latter cleavages have been much less well characterized, however.

Several CgA-related peptides have been isolated from tumours, suggesting that these peptides are generated from the CgA molecule *in vivo*. The generation of pancreastatin *in vivo* (Schmidt *et al.*, 1988; Watkinson *et al.*, 1989, 1991) is likely to be the result of a concerted action of several consecutive proteolytic activities (Watkinson and Robinson, 1992), including proteases that recognize pairs as well as single basic amino acid sites. β-granin is believed to be formed by cleavage at a paired basic amino acid site in CgA in insulinoma cells by a calcium-dependent serine protease and trimmed by a carboxypeptidase H (Hutton *et al.*, 1987b; Smeekens, 1992). WE-14 [CgA(324—337)] is found in tumour extracts (Curry *et al.*, 1992; Conlon *et al.*, 1992), and may be generated by endoproteolysis at flanking pairs of basic amino acid residues. Multiple endoprotease activities, therefore, appear to be active in the processing of CgA. Indeed, processing of CgA can start at both carboxy- and amino-terminal cleavage sites (Wohlfarter *et al.*, 1988; Schmid *et al.*, 1989; Barbosa *et al.*, 1991). The generation of other putative CgA-derived peptides, such as parastatin for example, will likely require several proteolytic activities.

Because CgA processing is tissue-specific, a differential distribution of proteases may ultimately determine the cell-specific cleavage pattern of CgA. For example, a differential expression of prohormone convertase 2 (PC-2) and PC-1 serine proteases could explain the different pattern of POMC processing between the anterior and intermediate lobes of the pituitary (Seidah *et al.*, 1991). Examination of the differential expression of these prohormone convertase enzymes will be important to compare the distinct pattern of CgA processing in each tissue.

CgA is also a substrate for other proteases including cathepsin D (Laslop *et al.*, 1990), and other tryptic-like activities (Leduc *et al.*, 1991). In addition CgA can be cleaved by acetylcholinesterase (Small *et al.*, 1986). Acetylcholinesterase has recently been localized to adrenal chromaffin cell granules (Bon *et al.*, 1990) and thus may cleave CgA *in vivo* (Simon *et al.* 1989a). As the proteolytic activities in each endocrine tissue becomes known, the importance of these processes for cleavage of CgA will become clearer.

1.4.4 DISTRIBUTION

CgA has a widespread evolutionary distribution in the diffuse endocrine system. For example, a peptide was isolated from the ostrich pituitary that represented the amino-terminal of the ostrich CgA protein. The amino-terminal 76 amino acids were sequenced and were found to share 72-80% homology to the amino-terminal regions of the bovine, human, porcine and rodent CgA molecules (Lazure *et al.*, 1990). This evolutionary conservation suggests a functional importance for CgA.

CgA immunoreactivity has been detected in extracts of *Drosophila* (Winkler and Fischer-Colbrie, 1992), in fish (Deftos *et al.*, 1987), and in single-celled eukaryotic organisms such as the protozoan, *Paramecium tetraurelia* (Peterson *et al.*, 1987). As is the case for neuroendocrine and endocrine cells, CgA immunoreactivity is found in secretory granules or their submammalian counterparts, such as the trichocysts of *Paramecium*. For two comprehensive reviews on the distribution of CgA and the methods used to determine CgA expression, see Winkler and Fischer-Colbrie (1992) and Simon and Aunis (1989).

Many immunohistological and molecular *in situ* techniques have been employed to investigate CgA's expression, each with its own technical advantages. Some conflicting data have been reported regarding the CgA distribution in some tissues (Ravazzola *et al.*, 1988) where the results can depend on the fixation method used in immunohistochemical studies. Furthermore, positive results using immunostaining and molecular *in situ* analysis may corroborate the presence of CgA gene expression, but do not provide information about the molecular nature of the CgA species present in the cells.

1.4.4.1 ENDOCRINE SITES OF SYNTHESIS

Originally CgA was found in chromaffin granules of the adrenal medulla (Blaschko and Helle, 1963; Banks and Helle, 1965; Smith and Winkler, 1967), and in dense-core vesicles of sympathetic nerves (Banks *et al.*, 1968). Later, its expression was determined to be more widespread, in the parathyroid (Cohn *et al.*, 1982b), for example, in most normal neuroendocrine tissues examined (Lloyd and Wilson, 1983; O'Connor, 1983; Siegel *et al.*, 1988; Cohn *et al.*, 1984; Table 1.4), and in many neuroendocrine tumours (Hearn, 1987; Deftos *et al.*, 1989; Levine *et al.*, 1990).

The amount of CgA varies from one neuroendocrine cell to another. For example, the largest amount of CgA is found in the adrenal medulla (O'Connor, 1983; Takiyyuddin *et al.*, 1990). In both bovine and human adrenal medulla, CgA represents

40% of the total soluble content of the secretory granule, and approximately 25% of the chromaffin cell's weight. In bovine and human pituitary, CgA represents 5.5% (O'Connor, 1983), and 24.6% (Takiyyuddin *et al.*, 1990), respectively, of that found in the adrenal medulla. This might be indicative of a species difference in the relative stores of CgA in this tissue, or within a tissue that may contain more than one type of endocrine cell, the distribution of CgA may be cell-specific. This occurs, for example, in the pituitary where CgA immunoreactive staining is restricted to the peptide hormone storage cells which contain LH, FSH (Fischer-Colbrie *et al.*, 1989; Lloyd *et al.*, 1992), and not to those of adrenocorticotrophic hormone (ACTH) or prolactin of the anterior pituitary (Lloyd *et al.*, 1985; Lloyd *et al.*, 1989).

In the human parathyroid, it has been reported that CgA represents 0.41% of that found in the adrenal medulla as determined by radioimmunoassay of tissue homogenates (expressed as microgram per gram wet weight; Takiyyuddin *et al.*, 1990). In earlier studies, however, CgA mRNA content in the parathyroid was shown to be as great or even greater than that in the adrenal (Benedum *et al.*, 1986; Ahn *et al.*, 1987; Helman *et al.*, 1988a). A difference in the synthesis or the metabolism of CgA between these tissues may account for this, or alternatively, the amount of CgA immunoreactivity as determined in the study of Takiyyuddin *et al.* (1990) might have been underestimated due to the presence of contaminating tissues, like fat cells for example.

In the thyroid, CgA represents about 0.1% of that found in the bovine or human adrenal (O'Connor, 1983; Takiyyuddin *et al.*, 1990), and has been identified in thyroid calcitonin-producing C cells (Nolan *et al.*, 1985). CgA is detected in polypeptide-storing cells including the insulin, the glucagon, and exocrine cells of the pancreas (Hearn, 1987; Ravazzola *et al.*, 1988). In bovine exocrine tissue, for example, CgA represents 0.004% of that found in the adrenal medulla (O'Connor, 1983). Scattered neuroendocrine cells in nonendocrine tissues such as the breast (Bussolatti *et al.*, 1985), lung and trachea (Lauweryns *et al.*, 1987), prostate and uterus (Vittoria *et al.*, 1989), and cells in the spleen, lymph node and thymus (Hogue-Angeletti and Hickey, 1985), and several areas in the gastrointestinal tract (Bretherton-Watt *et al.*, 1988; Weidenmann *et al.*, 1988) have been shown to be positive for CgA immunoreactivity.

Clearly, the widespread distribution of CgA is suggestive of an important function in secretory cells, but based on its relative levels in many cells, its relative activity or function may be variable. Also, the expression of the other granins does not necessarily reflect the pattern of CgA expression in endocrine cells, and this could be indicative of different functions for them.

Table 1.4

Tissue Distribution of Chromogranin A

Endocrine Tissues	
Adrenal medulla	
Parathyroid chief cells	
Thyroid	C cells
Pancreas	Insulin cells
	Glucagon cells
	Somatostatin cells
	Pancreatic polypeptide cells
Anterior Pituitary	
	Thyroid stimulating hormone cells
	Luteinizing hormone/Follicle stimulating hormone cells
	Growth hormone cells
Posterior Pituitary	
Neuroendocrine cells of	
	Breast
	Heart
	Lung, trachea
	Prostate
	Uterus
Other tissues	
Immune cells	
	Spleen
	Thymus
	Lymph node
Stomach	
	Enterochromaffin-like cells
Gastrointestinal tract	
	Antrum
	Duodenum
	Jejunum
	Ileum, Colon

Table 1.4 (continued)

Tissue Distribution of Chromogranin A

Nervous tissues
Neurons
Ganglia
Peripheral neurons
Cerebral cortex
Spinal cord
Motor perikarya
Splenic nerve
Hippocampus

1.4.4.2 SITES OF SYNTHESIS IN NERVOUS TISSUES

In the brain, the distribution of CgA is widespread (Somogyi *et al.*, 1984; Nolan *et al.*, 1985; Lloyd *et al.*, 1988; Weiler *et al.*, 1990a; Table 1.4), but only represents as little as 0.1-0.58% of that found in the bovine or human adrenal gland (O'Connor, 1983; Takiyyuddin *et al.*, 1990). CgA immunoreactivity is found in different neuron subpopulations (Siegel *et al.*, 1988; Somogyi *et al.*, 1984), and CgA has been localized in the peripheral sympathetic neurons as well (Nolan *et al.*, 1985; Fischer-Colbrie *et al.*, 1985; Lassman *et al.*, 1986). CgA immunoreactivity is found in the medulla and cerebral cortex of the human brain (Munoz *et al.*, 1990), anterior hypohysis of the rat and ox (Fischer-Colbrie *et al.*, 1985), and its mRNA was found in both anterior and neurointermediate lobes of the pituitary (Siegel *et al.*, 1988; Iacangelo *et al.*, 1986). Secretory vesicles of lumbar motor perikarya (Li and Dahlstrom, 1992; Munoz, 1991) and cholinergic nerve terminals in skeletal muscle contain CgA immunoreactivity (Voknaidt *et al.*, 1987). The CgA-derived peptide vasostatin can affect smooth muscle contraction and the presence of CgA in nerve terminals in muscle may also suggest a role for CgA in local skeletal muscle function. Just as an elevation in circulating plasma CgA levels is considered a marker for some neuroendocrine tumours, the relative plasma CgA level can be considered to be a marker for adrenergic neural activity (Fischer-Colbrie, 1985).

Conflicting results with regard to the distribution of CgA in nervous tissue have been reported, however. With the use of monoclonal antibodies to CgA both Schmid *et al.* (1989) and Wilson and Lloyd (1984) were unable to demonstrate the presence of CgA in nervous tissue. In the sheep (Somogyi *et al.*, 1984) and bovine brain (Nolan *et al.*, 1985) CgA is reported to be abundantly expressed. This suggests, on the one hand, that there may be a difference in the abundance of CgA between species, or there may be a difference in antigen recognition by the various antisera used in these studies.

CgA may have identical intracellular and extracellular functions in nervous tissues as those described above for neuroendocrine cells. In addition to these functions, nervous tissue CgA has been reported to increase the longevity of sensory neurons *in vitro* (Huttner *et al.*, 1991), and may also have a role in systemic metabolism by increasing blood glucose, free fatty acids and corticosterone in rats (Gunion *et al.*, 1989). It will be interesting to further define the function of CgA in nervous tissue and how they relate to the roles of CgA in neuroendocrine tissues.

1.4.4.3 SUBCELLULAR LOCALIZATION

Originally described as the major soluble protein in chromaffin cell secretory granules (Winkler and Smith, 1966), recent studies confirm this initial observation and show that CgA is exclusively localized to secretory granules in chromaffin cells and in other neuroendocrine cells (Hearn, 1987). For example, CgA has been co-localized in secretory granules with parathyroid hormone (Ravazzola *et al.*, 1978; Arps *et al.*, 1987), with insulin in pancreatic islet β-cells (Erhart *et al.*, 1986; Lukinius *et al.*, 1992), with thyrotropin and luteinizing hormone (LH) in the bovine anterior pituitary (Bassetti *et al.*, 1990), and with atrial natriuretic factor in the heart (Steiner *et al.*, 1990). CgA-positive granules were also detected in the stomach and ileum, and in granules associated with some types of endocrine tumours (Hearn, 1987).

CgA has also been shown to be associated with cellular membranes. Morrissey *et al.* (1980) indicated that CgA can not only be found with the soluble portion of the secretory granule, but can also be associated with parathyroid cell membrane fractions. In adrenal chromaffin cells, approximately 15% of the granule protein was reported to be membrane-bound CgA (Settleman *et al.*, 1985b), with the remaining CgA being soluble and virtually identical to the membrane-bound form. Calcium may promote changes in the distribution in the soluble or membrane-bound forms of CgA. Recently, a calcium-dependent membrane association of parathyroid CgA to granule membrane proteins has been described (Gorr *et al.*, 1988; Leiser and Sherwood, 1989). This association could facilitate CgA aggregation in secretory granules, or could place it closer to enzymes or other factors within the membrane fraction (Leiser and Sherwood, 1989). Calcium binding can induce conformational changes in CgA depending on the pH (Yoo and Albanesi, 1991), and may place CgA in a more favourable context for binding or aggregation, for example. It may ultimately be found that both soluble and membrane-associated forms of CgA exist, as is the case for CgB in PC-12 cells (Pumplikar and Huttner, 1992). At least 10% of the cellular CgB was determined to be membrane-bound, and this form could possibly play a key role in the sorting and targeting of CgB and other proteins to the regulated secretory pathway (Pumplikar and Huttner, 1992).

It has recently been demonstrated that subpopulations of secretory granules divided by size can show a differential storage of CgA and the resident hormone. In the parathyroid, for example, PTH and CgA show a differential distribution in granules separated by size (Bajpai and Hamilton, 1991). In this study, parathyroid cell granules were separated into three populations. CgA was concentrated in populations 1 and 2, whose diameters ranged from 70-100 nm, and 200-500 nm, respectively, while population

3 (200-700 nm) contained a very small amount of CgA. Most of the PTH was concentrated in population 3, but it appeared that PTH was also present in the two other granule populations. This phenomenon is also seen in pituitary gonadotrophs, but in these cells CgA was found to be colocalized in larger (500 nm) secretory granules with follicle stimulating hormone (FSH) and LH, while CgA was absent in the smaller (200 nm) granules containing LH and SgII (Watanabe *et al.*, 1991). These data indicate that CgA and resident hormones and other granins can be differentially sorted in endocrine cells.

Granins can also show differential intercellular distribution. For instance, one report demonstrated that immunoreactivity between CgA and CgB, and between CgA and gastrin both correlated negatively in a population of gastrin-secreting cells, while CgB and gastrin positively correlated (Cetin *et al.*, 1992). Thus, this intercellular coordination of granin gene expression in individual gastrin-producing cells might may be related to the specific functions of the granins in individual endocrine cells, for example, in hormone sorting, in hormone processing or in the control of hormone secretion.

1.4.5 REGULATION OF BIOSYNTHESIS AND SECRETION

A mature mRNA of approximately 2.1 kb encodes for a precursor protein which varies in amino acid length from 448 in the pig (Iacangelo *et al.*, 1988), 449 in the cow (Iacangelo *et al.*, 1987; Ahn *et al.*, 1987; Benedum *et al.*, 1986), 457 in man (Konecki *et al.*, 1987; Helman *et al.*, 1988a), 461 in the mouse (Wu *et al.*, 1990) and 462 in the rat (Iacangelo *et al.*, 1988b; Parmer *et al.*, 1989). CgA synthesis follows a pathway characteristic of secretory proteins (Regis and Kelley, 1990; Lingappa, 1991). An eighteen amino acid hydrophobic signal sequence targets CgA to the secretory pathway. This sequence is removed cotranslationally as the emerging polypeptide traverses the endoplasmic reticulum membrane and is shuttled into the lumen of the endoplasmic reticulum. CgA is post-translationally modified and is ultimately packaged into secretory granules. Because CgA secretion is regulated, CgA is stored temporarily in secretory granules until cell secretion is stimulated by a secretagogue.

When neuroendocrine cell secretion is stimulated the content of the secretory granules is released. CgA secretion usually parallels the secretion of the resident hormone or neurotransmitter of these cells. For example, CgA secretion from the adrenal medulla parallels catecholamine secretion after splanchnic nerve stimulation of the adrenal medulla (Blaschko *et al.*, 1967). Similarly, low extracellular calcium stimulates the secretion of both CgA and PTH in the parathyroid (Kemper *et al.*, 1974; MacGregor *et al.*, 1988).

Following the secretory response, there is a compensatory increase in CgA biosynthesis, which may be due to concerted actions at the level of the gene or at other levels within the cell.

The regulation of CgA synthesis by steroid hormones is more complex, however. Expression of CgA and the resident hormone can be oppositely regulated by steroid hormones at the gene level in several cells. This has been described for glucocorticoid regulation of CgA and POMC synthesis in corticotroph cells (Wand *et al.*, 1991; Horiubi *et al.*, 1993), estrogen regulation of CgA and LH/FSH synthesis in the pituitary (Fischer-Colbrie *et al.*, 1992), and $1,25(OH)_2D_3$ regulation of CgA and PTH synthesis in the parathyroid (Russell *et al.*, 1990; Chapter 2). Thus, regulation of CgA gene expression is complex, but its study may provide clues to its function in neuroendocrine cells.

Differential regulation of CgA biosynthesis is indicative of specialized functional roles for CgA in neuroendocrine cells. For example, the opposite gene regulation described above results in an altered cellular CgA content, suggesting that the role of CgA in cell secretory function could change merely by its level within a cell. In dexamethasone-treated corticotrophs and estrogen-treated gonadotrophs, the CgA content changes, and these stoichiometric changes may prove to be important for the relative functions of CgA in neuroendocrine cells.

The following discussion describes the regulation CgA synthesis in various endocrine organs. Bovine adrenal chromaffin cells have been used in large part to study many processes in neurobiology and pharmacology. Because CgA is highly expressed in this tissue, it has prompted several groups to study CgA biology, and examine its role in chromaffin cell secretory activity.

1.4.5.1 CATIONS AND SECOND MESSENGERS

Several intracellular messenger systems, including cAMP, protein kinase C (PKC), and protein kinase A (PKA) are involved in the hormonal regulation of CgA biosynthesis. Activation of these signalling pathways begins a cascade of events which ultimately leads to gene regulation and altered protein synthesis.

Splanchnic nerve stimulation rapidly activates the secretory response in chromaffin cells and evokes a transient rise in intracellular calcium just beneath the plasma membrane (Galindo *et al.*, 1991). Various potent chromaffin cell secretagogues such as carbamyl choline, histamine, and potassium (K^+) can also elicit the secretory response in

this cell by a similar mechanism. It was therefore of interest to determine the effects of these secretagogues on CgA synthesis.

A two-fold increase in CgA protein synthesis by nicotine and K^+ can be induced in chromaffin cells (Simon *et al.*, 1989). Other factors such as histamine, bradykinin, prostaglandin E2 (PGE2) and angiotensin II (AGII) all induce CgA protein synthesis in chromaffin cells (Galindo *et al.*, 1991). These substances cause an increase in intracellular Ca^{++} by two different mechanisms. The cholinergic agonists nicotine, carbamyl choline, and histamine are potent secretagogues and increase intracellular Ca^{++} by activating a voltage-dependent calcium channel that results in a spatial increase in calcium just beneath the plasma membrane. Bradykinin, PGE2, and AGII, on the other hand, increase intracellular calcium by releasing it from internal stores, resulting in a more even spatial general rise in intracellular calcium. These latter factors, however, do not cause an increase in CgA secretion, but cause an accumulation of CgA in chromaffin cells (Galindo *et al.*, 1991). Therefore, in chromaffin cells, intracellular calcium appears to be a primary signal in the induction of CgA biosynthesis.

cAMP regulates the activity of a variety of genes by activating protein kinase A (PKA; Meyer and Habener, 1993). cAMP-dependent protein kinases can phosphorylate protein substrates which may then act to alter cellular protein synthesis at a distal step (Taylor, 1989). In chromaffin cells, cAMP does not mediate any changes in CgA protein synthesis (Galindo *et al.*, 1991; Simon *et al.*, 1989; Eiden *et al.*, 1987), although it can cause an increase in the chronic release of CgA from the cell (Eiden *et al.*, 1987). In a medullary thyroid carcinoma (MTC) cell line, forskolin, which directly activates adenylate cyclase activity (Seamon *et al.*, 1981), causes an almost two-fold increase in CgA mRNA, and a marked increase in CgA secretion, implicating the cAMP-dependent PKA in the regulation of CgA synthesis in this cell. However, in another CgA-producing cell, the lung carcinoma BEN cell line, forskolin does not affect CgA gene expression or secretion (Murray *et al.*, 1988a), suggesting that regulation by cAMP-dependent mechanisms may be cell-specific in nature.

Other protein kinases important for intracellular signalling in neuroendocrine cells are those of the PKC family (Davis *et al.*, 1992). PKC activity is calcium-dependent, is only active when associated with the membrane, and can be activated by diacylglycerol, which is generated by the hydrolyzing actions of phospholipase C on phospholipids. In addition, phorbol esters can mimick the effects of diacylglycerol, and this has facilitated the study of PKC actions in neuroendocrine cells (Davis *et al.*, 1992).

Phosphorylation events mediated by PKC are implicated in the regulation of CgA biosynthesis. For example, the tumour-promoting phorbol ester, phorbol-12-

myristate-13-acetate (PMA) enhances CgA gene expression in human MTC and lung cancer cell lines (Murray *et al.*, 1988a). Another phorbol ester, TPA, enhances CgA protein synthesis in chromaffin cells (Galindo *et al.*, 1991; Simon *et al.*, 1989), but decreases CgA gene expression and protein synthesis in a human neuroblastoma cell line (Weiler *et al.*, 1990b). With the use of PKC inhibitors such as staurospaurin and sphingosine, PKC has been shown to mediate the stimulation of CgA protein synthesis in chromaffin cells by the cholinergic agonists, bradykinin, PGE2, and AGII (Galindo *et al.*, 1991; Simon *et al.*, 1989), but not by histamine (Galindo *et al.*, 1991). In some cases a requirement for micromolar concentrations of intracellular calcium derived from the extracellular milieu is necessary for the enhanced synthesis (Simon *et al.*, 1989). The regulation of CgA biosynthesis by phosphorylation events mediated by PKC is thus a cell-specific phenomenon and intracellular calcium may also play an accessory role in this regulatory event.

1.4.5.2 STEROID HORMONES

CgA gene expression is also regulated by steroid hormones. Glucocorticoids, estrogen, and $1,25(OH)_2D_3$ have all been demonstrated to regulate CgA synthesis. In the parathyroid, CgA synthesis is enhanced by the action of $1,25(OH)_2D_3$ on the CgA gene transcription rate (Russell *et al.*, 1990; Chapter 2). Glucocorticoids and estrogen modulate CgA gene expression in pituitary cells, as demonstrated by measurement of the steady-state mRNA levels and CgA protein synthesis. It remains to be determined, however, how these effects are achieved in pituitary cells. Steroid hormones also act in a cell-specific manner to regulate CgA gene expression in neuroendocrine cells. This suggests that cellular factors, which are unique to each cell, are involved in mediating the steroid hormones' effects on CgA biosynthesis in neuroendocrine cells.

Steroid hormones are lipophilic and thereby readily traverse the plasma membrane and bind to their cognate cytosolic or nuclear receptors. The steroid hormone/receptor complex is then targeted to the nucleus where it can directly associate with cis-acting DNA response elements to exert either positive or negative transcriptional control (Beato, 1991).

1.4.5.2.1 GLUCOCORTICOIDS

The synthetic glucocorticoid agonist, dexamethasone, positively regulates CgA mRNA levels in PC-12 cells *in vitro* (Rausch *et al.*, 1988; Scammell, 1989), and in the AtT-20 corticotrophic cell line (Wand *et al.*, 1991; Horiubi *et al.*, 1993). *In vivo* studies in the rat also revealed that CgA gene expression is regulated by glucocorticoids. Specifically, when rats are hypophysectomized [which prevents the synthesis of glucocorticoids by inhibiting corticotropin releasing hormone (CRH) secretion], a dramatic decline in adrenal medullary CgA mRNA is observed (Fischer-Colbrie *et al.*, 1988). Alternatively, adrenalectomy (i.e., ablation of the glucocorticoid source, the adrenal cortex) caused a greater that 80% decrease in CgA mRNA in the pituitary (Grino *et al.*, 1989). In these two *in vivo* studies, the experimentally induced effects were reversed by dexamethasone infusion. Infusion of dexamethasone into normal male Sprague-Dawley rats caused an increase in CgA mRNA and protein in the pituitary gland, however no effect was observed on CgA levels in the adrenal medulla (Fischer-Colbrie *et al.*, 1989), suggesting that factors derived from the hypothalamus or elsewhere may come into play to coordinate CgA gene expression *in vivo* . Further studies are needed to fully define the role of glucocorticoids in the whole animal.

To directly assess the effects of dexamethasone on CgA gene expression and to rule out any interference that the *in vivo* setting may contribute, the effect of dexamethasone on CgA synthesis in dispersed rat pituitary cell aggregates was tested (Anouar and Duval, 1988). Dexamethasone again caused a dramatic increase in CgA mRNA. This data implicated a direct effect of the steroid on the CgA gene in the pituitary, and is consistent with the above *in vivo* reports.

1.4.5.2.2 ESTROGEN

CgA, and SgII are costored and coreleased with LH and FSH in gonadotroph cells. Because luteinizing hormone releasing hormone release is regulated by estrogen in the female where it in turn regulates LH and FSH release, the regulation of CgA and SgII synthesis and secretion by estrogen was also examined in pituitary gonadotroph cells.

The expression of the granin, SgII, in the female rat pituitary, was determined to be regulated during maturation, where SgII protein and mRNA content increased up to 21 days and plateaued thereafter (Anouar and Duval, 1991). In addition, SgII gene expression was regulated during the estrous cycle in the mature rat where it peaked during mid-cycle

and declined afterwards. In contrast, a marked decrease in the expression of the CgA protein was observed with age, where CgA protein in the pituitary increased to a maximum between 14 and 21 days, and then declined rapidly to barely detectable levels at 60 days (Anouar and Duval, 1991; Fischer-Colbrie *et al.*, 1992). Because of the very low level of CgA gene expression at maturity (60 days), CgA gene expression could not be examined during the estrous cycle. Also, because of the negative effect of estrogen on CgA gene expression in the pituitary, a sex-related difference in the expression of CgA is established (Fischer-Colbrie *et al.*, 1992).

While ovariectomy causes an increase in pituitary CgA immunostaining (Lloyd *et al.*, 1992) and CgA content (Anouar *et al.*, 1991), these effects can be reversed by infusing estrogen into rats after ovariectomy (Lloyd *et al.*, 1992; Anouar *et al.*, 1991). Infusing estrogen into male rats also causes the CgA protein content and mRNA levels to decrease in their pituitary glands (Fischer-Colbrie *et al.*, 1992). In *in vitro* studies using pituitary cell aggregates, physiological concentrations of estrogen (10^{-12}M) cause a general decrease in CgA protein and mRNA levels (Anouar and Duval, 1992). Thus, these effects are identical to those observed *in vivo* and support a physiological role for estrogen in the control of CgA gene expression in the pituitary.

The regulation of CgA gene expression by estrogen appears to be tissue-specific and localized to the pituitary. Experimentally modulating the estrogen levels in rats by ovariectomy, or estrogen infusion, caused no difference in the level of CgA expression in other rat tissues such as the adrenal medulla, hippocampus, and hypothalamus. Furthermore, treatment of the CgA-producing pheochromocytoma PC-12 and pituitary corticotroph AtT-20 cell lines with estrogen had no effect on CgA gene expression (Anouar *et al.*, 1991; Fischer-Colbrie *et al.*, 1992). The differential responses of CgA synthesis to estrogen may be related to the expression of estrogen receptors in these tissues, however. For instance, a lack of a response to estrogen in the adrenal chromaffin cells may be explained by the restricted expression of estrogen receptors in adrenal cortical cells (Cutler *et al.*, 1978).

1.4.5.2.3 1,25-DIHYDROXYCHOLECALCIFEROL

1,25-dihydroxycholecalciferol [$1,25(OH)_2D_3$] is a known regulator of parathyroid gland function and can directly modulate the transcription rate of at least two genes in this gland, including that for CgA. $1,25(OH)_2D_3$ also has nongenomic effects in cells. In the parathyroid for instance, rapid stimulation of phospholipid metabolism

precedes the action of $1,25(OH)_2D_3$ on gene transcription (Bourdeau *et al.*, 1990). In other cells, nongenomic actions of $1,25(OH)_2D_3$ have been reported. $1,25(OH)_2D_3$ stimulates the rapid intestinal transport of calcium in intestinal cells (Boland and Norman, 1990), and activates the calcium signalling system in osteoblast cells (Civitelli *et al.*, 1990). $1,25(OH)_2D_3$ can bind polyribonucleotides *in vitro* suggesting that a direct association between RNA and $1,25(OH)_2D_3$ can be achieved in the absence of a vitamin D_3 receptor (VDR; Franceschi, 1984).

Chapters 2 and 3 present the effects of the $1,25(OH)_2D_3$ on CgA and PTH biosynthesis in the parathyroid.

1.4.5.3 MODEL FOR THE REGULATION OF CHROMOGRANIN A BIOSYNTHESIS BY STEROID HORMONES

The study of the regulation of the biosynthesis of CgA provides insight into the biological role of CgA in neuroendocrine cells. The nature of the CgA gene is characterized by the ability of several factors including three steroid hormones to regulate its expression. This suggests that CgA has specialized functions, other than those which are characteristic of any constitutively expressed housekeeping gene. CgA's putative role as a precursor to bioactive peptides will likely be important, supported by many reports which show that CgA and CgA-derived peptides can have modulatory activity on neuroendocrine cell secretion. The regulation of CgA biosynthesis by three steroid hormones, estrogen, glucocorticoids (dexamethasone), and the active metabolite of vitamin D_3, $1,25(OH)_2D_3$, fits the model where a cell system regulates its own secretory activity in response to an external stimulus, with CgA playing an integral role (Figure 1.5).

By differentially regulating the transcription rate of a resident hormone's gene, a cell system can provide an autonomous means to regulate its secretory activity. For example, in the pituitary, the opposing actions of CRH and glucocorticoids on the regulation of the POMC gene transcription rate provides a physiological transcriptional mechanism to tightly control POMC-peptide secretory rates (Gagner and Drouin, 1987). Similarly, opposite gene regulation of the resident hormone and that of CgA by the same hormone can provide a secretory control mechanism. Specifically, dexamethasone and $1,25(OH)_2D_3$ decrease PTH and POMC synthesis in the parathyroid and pituitary corticotroph, and estrogen increases FSH (and LH) gene expression in the pituitary gonadotroph. The CgA gene is regulated in an opposite direction in each cell by these steroid hormones, where $1,25(OH)_2D_3$ and dexamethasone enhance CgA synthesis and

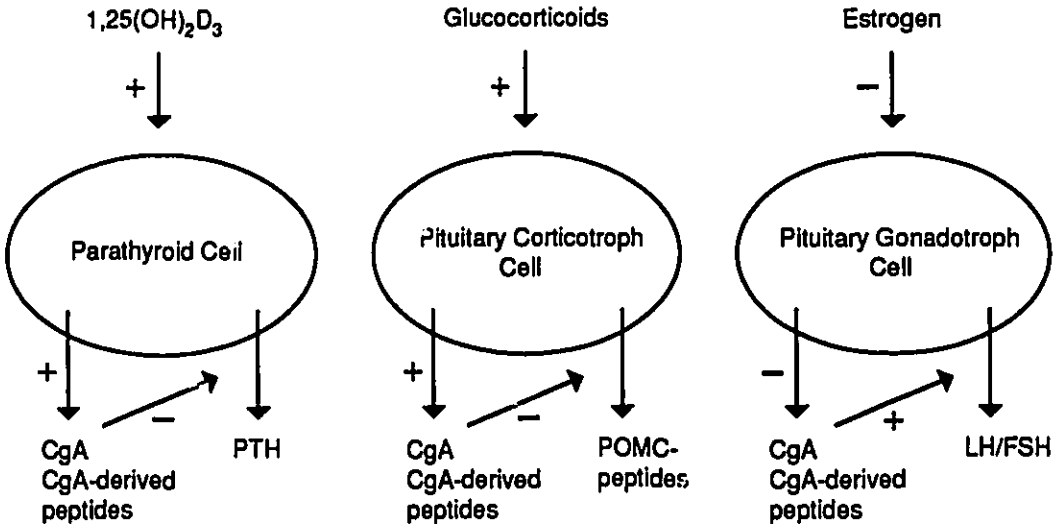

Figure 1.5 Model for endocrine cell gene regulation of CgA by steroid hormones. $1,25(OH)_2D_3$, glucocorticoids and estrogen regulate CgA gene expression in several cell types. Specifically, $1,25(OH)_2D_3$ and dexamethasone upregulate (+) CgA gene expression and secretion in the parathyroid and pituitary corticotroph cells, respectively. PTH synthesis in the parathyroid, and POMC synthesis in pituitary corticotrophs are concomitantly down-regulated. CgA and CgA-derived peptides, therefore, can exert their inhibitory effects (–) on cell secretion, and enhance the suppressed secretion from these cells caused by the effects of the steroids on the resident hormone's gene expression. As shown on the right, in pituitary gonadotrophs, estrogen down-regulates CgA gene expression and secretion *in vitro*, and concomitantly enhances LH and FSH synthesis and secretion. Elevated levels of estrogen in the mature animal may act to constitutively suppress CgA gene expression and thus secretion, thereby allowing for high levels of secretion of LH and FSH from this cell in mature cycling animals.

secretion in the parathyroid cell (Ridgeway and MacGregor, 1988; Russell *et al.*, 1990; Chapter 2), and corticotroph cell (Wand *et al.*, 1990; Horiubi *et al.*, 1993), and estrogen suppresses CgA gene expression in the pituitary gonadotroph (Anouar *et al.*, 1991; Fischer-Colbrie *et al.*, 1992).

By stimulating CgA secretion in the parathyroid and pituitary corticotrophic cells, $1,25(OH)_2D_3$ and dexamethasone can increase the amount of CgA or CgA fragments that can act to further suppress the release of PTH and POMC-peptides from these cells (Figure 1.5). Similarly, estrogen suppresses CgA gene expression in the female pituitary so that gonadotroph cell secretion is not influenced by the negative effects of CgA peptides, and may function to secrete FSH and LH in a pulsatile fashion during the female rat's estrous cycle.

1.4.5.4 DEVELOPMENTAL REGULATION

The developmental expression of CgA, or of particular molecular forms of CgA, has only been examined in rodents and humans in a small number of tissues. Generally, CgA gene expression changes with age. The importance of this, however, is unclear, but may merely reflect the maturation of neuroendocrine tissues that express CgA, and/or the appearance of factors that regulate CgA gene expression. For example, CgA gene expression is influenced by glucocorticoids produced by the adrenal cortex and developmental regulation of CgA gene expression may be a reflection of the maturation of the adrenal gland and its increased secretory function. Similarly, in the pituitary, CgA synthesis is under the influence of estrogen, and this could explain why CgA expression in the female rat pituitary falls to very low levels at maturity (Anouar and Duval, 1991; Fischer-Colbrie *et al.*, 1992).

CgA gene expression during development in some rat and human tissues has been examined. In the rat adrenal medulla, for example, CgA protein content within the developing secretory granule parallels the adrenalin/noradrenalin content and shows a dramatic rise in expression from fetal day 17 onwards (Schober *et al.*, 1989). Similarly, in human chromaffin tissues, CgA mRNA, which can be detected as early as 8 weeks of gestation, progressively increases in abundance during the first year of life (Helman *et al.*, 1988b). By immunohistochemical analysis, CgA in the human striate cortex was shown to undergo developmental changes from birth to 25 years of age (Ang *et al.*, 1992). Generally, CgA immunoreactivity increased with age (1 day to 25 years), indicated by negligible immunostaining at day 1 to very strong staining at 25 years. In this study the

investigators demonstrated that CgA consisted of a major 75 kDa band by Western analysis, but did not demonstrate changes in the number or the size of the CgA bands with age. The changes in CgA immunostaining parallelled the developmental pattern of the post-natal changes observed for other neuropeptides such as vasoactive intestinal peptide, somatostatin, cholecystokinin, avian pancreatic polypeptide and substance P. In contrast to the observed increases in CgA gene expression in the adrenal and striate cortex during development, in motor perikarya in the anterior horn of the developing rat, CgA immunoreactivity was shown to progressively decrease from early embryonal stage to adulthood (Li and Dahlstrom, 1992). This suggests that CgA gene expression may have precise developmentally regulated and tissue-specific functions.

The 70 kDa form of CgA on SDS-polyacrylamide gels represents the intact protein. A higher molecular weight form of CgA can be observed as a smear of approximately 80-100 kDa. This higher molecular weight form represents proteoglycan CgA. In the rat adrenal gland, the relative amount of proteoglycan CgA changes during development in that this form is very prominent in the prenatal stage and at parturition, but declines to barely detectable levels in the adult (Schober *et al.*, 1989). The significance of this finding is unclear, but may suggest that the proteoglycan form of CgA is associated with less well differentiated nervous tissue than with the chromaffin cell endocrine phenotype.

1.4.5.5 STIMULUS-SECRETION-SYNTHESIS COUPLING

Stimulus-secretion-synthesis coupling, or "transynaptic induction of biosynthesis", are terms used to describe a compensatory cellular process to increase synthesis of a neuropeptide in response to a secretory stimulus (Eiden *et al.*, 1984; Thoenen, 1984). CgA synthesis is coupled to its secretion to maintain intracellular levels in chromaffin and parathyroid cells. For example, nicotine and forskolin treatment elicit a secretory response in bovine adrenal chromaffin cells, and these stimuli are followed by a compensatory increase in proenkephalin synthesis (Eiden *et al.*, 1987), and in CgA synthesis (Simon *et al.*, 1989). A similar mechanism exists in $1,25(OH)_2D_3$-treated bovine parathyroid cells, where $1,25(OH)_2D_3$ indirectly stimulates CgA secretion, but the intracellular CgA protein level is maintained at a constant level (Chapter 2). The CgA level within these cells must be maintained, and this may be important for CgA function. It remains to be established whether this stimulus-secretion-synthesis coupling mechanism exists in other CgA expressing cells.

1.4.6 CHROMOGRANIN A AS A NEUROENDOCRINE TUMOUR MARKER

Elevated plasma levels of a polypeptide hormone can represent the earliest clinical indication of a neuroendocrine neoplasm. Screening patients for specific hormones represents a diagnostic tool in patients suspected to have a particular neuroendocrine tumour. For example, examination of the circulating levels of peptide hormones such as pancreatic polypeptide, human choronic gonadotropin-α and -β, calcitonin and somatostatin are used in screening patients with carcinoid tumours who do not present with any clinical syndromes (Eriksson and Öberg, 1991). The distribution of CgA which is more widespread than any other peptide hormone, has made the measurement of CgA a valuable tool in the diagnosis of neuroendocrine neoplasms. Its measurement in plasma is important in the detection of a variety of diseases associated with many neuroendocrine tumours (O'Connor and Bernstein, 1984), and also in patients who present with these tumours as a consequence of multiple endocrine neoplasia syndrome type I or II (O'Connor and Deftos, 1986), in which hyperplasia or tumour formation occurs in endocrine glands containing secretory granules (Landsberg, 1984). Measuring circulating CgA in patients with nonfunctioning, hormone-negative neuroendocrine tumours (i.e., those which do not produce a secretory hormone product) can be helpful in identifying this tumour type (Sobol *et al.*, 1989). In tissue studies, immunohistological determination of CgA can help to identify the neuroendocrine nature of tumours (Lloyd and Wilson, 1983), which may have implications in the method of treatment, for example. Also, the CgA gene has been reported to be preferentially expressed in poorly differentiated and nonfunctioning pituitary tumours relative to any other granin (Jin *et al.*, 1993), and comparison of CgA gene expression with that of the other granins, by Northern blot for example, can provide further clues to the nature of the neoplasm.

1.4.6.1 CIRCULATING LEVELS OF IMMUNOREACTIVE CHROMOGRANIN A IN PATIENTS WITH NEUROENDOCRINE TUMOURS

CgA is produced by endocrine and neuroendocrine tumours including pheochromocytoma, medullary thyroid carcinoma, parathyroid neoplasia, anterior pituitary tumours, pancreaticoduodenal tumours, small-cell lung carcinoma, carcinoid tumours, neuroblastoma, and some hormone-negative tumours (Deftos, 1991). Many neuroendocrine tumours can be marked by abnormal CgA production as determined by radioimmunoassay (RIA) of circulating CgA levels. For example, patients with

neuroendocrine tumours including pheochromocytoma, hyperparathyroidism [primary and secondary (i.e., renal)], parathyroid adenoma, pancreatic insulinomas, C-cell disorders, and oat-cell lung carcinoma, presented with substantially elevated serum CgA levels (O'Connor and Deftos, 1986; Deftos, 1991; columns A & B in Table 1.5).

While many studies have shown that plasma CgA levels are elevated in patients with these conditions, the data is at present incomplete (cf. Table 1.5). Several studies, for example (O'Connor and Bernstein, 1984; O'Connor and Deftos, 1986; O'Connor *et al.*, 1989; Cryer *et al.*, 1991; Table 1.5), demonstrate that most patients with pheochromocytoma present with vastly elevated plasma CgA levels. However, few studies have examined circulating levels of CgA in patients with medullary thyroid carcinoma, neuroblastoma and primary hyperparathyroidism, for example. Further studies will therefore be required to confirm these initial findings.

Plasma CgA was originally reported to be elevated in all patients with primary hyperparathyroidism, although this was more marked in patients with parathyroid hyperplasia (28X) than in those with parathyroid adenoma (3.6X; O'Connor and Deftos, 1986; column B in Table 1.5). The sample size in this original study was small. A later study confirmed these findings, however. With the use of an immunoluminometric assay, 23 of 35 surgically-confirmed primary hyperparathyroidism patients presented with elevated (up to sixteen-fold over the control values) circulating levels of CgA (Bender *et al.*, 1992; column F in Table 1.5). No further clinical information about these patients was presented, however. Nanes *et al.* (1989), using a modification of the RIA of O'Connor *et al.* (1989), measured CgA in patients with primary hyperparathyroidism. They found that elevated levels of plasma CgA were not a general feature of primary hyperparathyroidism, but were only restricted to a subset of patients who also presented with Zollinger-Ellison Syndrome (gastrinoma; column D in Table 1.5). In these patients, the elevated circulating levels of CgA are believed to be the result of the enhancing effects of hypercalcemia or PTH on the synthesis and secretion of gastrin (Nanes *et al.*, 1989), and not due to secretion from the parathyroid. Furthermore, plasma CgA correlated best with indices of pancreatic islet function than with parathyroid function.

In primary hyperparathyroid patients, the circulating levels $1,25(OH)_2D_3$ are elevated. It might be expected, therefore, that the circulating CgA levels would be elevated due to the action of $1,25(OH)_2D_3$ on CgA biosynthesis in the parathyroid (Chapter 2). Immunoreactive CgA may not be elevated, or as elevated as expected, in primary hyperparathyroidism for at least two reasons. Firstly, the contribution of parathyroid CgA production to overall circulating CgA pool would be small even when a hypersecreting

Table 1.5

Circulating Levels of Chromogranin A, B, Pancreastatin, and SgV in Health and Disease

	Circulating Levels[1]									
	A	B	C	D[2]	E	F	G	H[2,3]	I[2,3]	J[2,3]
Normal	117-141 (129)	27-103 (60)	20-50 (28)	25 (±8)	48-200	10-53 (30)	100-330	8.5-18 (±13)	74 (±5)	25 (±6)
Pheochromocytoma	129-4600 (1614)	35-5000 (736)	60-250 (209)	ND[4]	135-2410 (333)	ND	694-1162	ND	114 (±19)	25 (±6)
Carcinoid Tumour	ND	60-101000 (35400)	30-30000 (7758)	ND	ND	ND	1090-19054	10-120 (35)	744 (218)	500 (25-2500)
Medullary Thyroid Carcinoma	ND	100-1200 (564)	ND	ND	ND	ND	ND	ND	100 (±5)	600 (25-2400
Neuroblastoma	ND	ND	180-2000 (744)	ND	ND	ND	3785 n=1	ND	ND	140 (25-700)
Hyperparathyroidism										
Adenoma	ND	200-230 (218)	ND	34 (±10)	ND	Elevated 20-500	ND	ND	83 (±14)	190 (25-1000)
Hyperplasia		220-2000 (1680)	ND	55 (±33)	ND	ND	ND	ND	ND	ND
+ ZES	ND	ND	ND	120 (±127)	ND	ND	ND	ND	ND	ND
Pancreatic Tumours	ND	200-20000 (4960)	ND	ND	ND	ND	765-12510	ND	596 (±181)	ND
Renal Failure	ND	1000-10000 (3820)	391 (±98)[2]	ND	ND	Elevated	ND	110-700 (527)	ND	ND

[1] Range in ng/mL (average), using a chromogranin A RIA reported in: column *A*, O'Connor and Bernstein (1984); *B*, O'Connor and Deftos (1986); *C*, O'Connor *et al.* (1989); *D*, Nanes *et al.* (1989); *E*, Cryer *et al.* (1991); *F*, immunoluminometric assay for CgA of Bender *et al.* (1992); *G*, Chromogranin A and B RIA of Eriksson *et al.* (1990); *H*, Pancreastatin RIA of Tateishi *et al.* (1989); *I*, Chromogranin B [GAWK(20-38)] RIA of Sekiya *et al.* (1989); *J*, SgV RIA of Suzuki *et al.* (1986).

[2] No range reported, ± standard deviation.

[3] Expressed as pmol/L.

[4] Not determined (ND).

parathyroid tumour is present. Secondly, since CgA levels are not abnormal in these patients, resistance to the stimulatory actions of $1,25(OH)_2D_3$ on CgA synthesis might be suspected to exist. This hypothesis would be consistent with the previous observation that PTH mRNA levels and PTH release are unresponsive to $1,25(OH)_2D_3$ in dispersed adenomatous parathyroid tissue in culture (Karmali *et al.*, 1989).

In secondary hyperparathyroidism, elevated blood CgA levels are due to progressive retention of CgA and CgA fragments. CgA immunoreactivity increases progressively with renal failure (O'Connor *et al.*, 1989; columns B, C, and F in Table 1.5), and this increase occurs independently of etiologic diagnosis or parathyroid status (Hsiao *et al.*, 1990a). The circulating forms of CgA consist principally of mid-molecule CgA fragments (Hsiao *et al.*, 1990a), including pancreastatin (Tateishi *et al.*, 1989; column H in Table 1.5), consistent with the bidirectional proteolytic processing of CgA as demonstrated in chromaffin cell granules (Wohlfarter *et al.*, 1988).

Measurement of CgA in nonfunctioning endocrine tumours represents an important tool for diagnosis. While a hormone product could not be identified in some medullary thyroid carcinomas, pituitary adenomas and pancreatic islet cell carcinomas, elevated blood CgA levels could be detected (Sobol *et al.*, 1989; Deftos *et al.*, 1989). In addition to measuring blood CgA levels, the analysis of CgA gene expression can also be useful in identifying this tumour type. In a recent study, CgA mRNA was demonstrated to be preferentially expressed in some nonfunctioning pituitary tumours than any other granin (Jin *et al.*, 1993). Also in the identification of endocrine tumours in nonendocrine tissues, for example in a subgroup of colon adenocarcinomas, molecular analysis of CgA gene expression by Northern blot and *in situ* hybridization analysis, can be very helpful (Helman *et al.*, 1988b).

Circulating CgA can be correlated with the disease stage and tumour burden. In small-cell lung carcinoma for example, elevated serum CgA was more frequently observed in patients with extensive disease than in patients with limited disease (Sobol *et al.*, 1986). Plasma CgA also correlated positively with tumour mass in pheochromocytoma (O'Connor and Bernstein, 1984). In neuroblastoma, serum CgA was shown to be an excellent diagnostic, and prognostic tool for this disease in children (Hsiao *et al.*, 1990b). And in another study, circulating CgA correlated positively with clinical improvement of patients with gastroenteropancreatic endocrine tumours (Moattari *et al.*, 1989).

Eriksson *et al.* (1990) produced a polyclonal antiserum against CgA and CgB with the use of a purified CgA and CgB fraction from chromaffin cell vesicles. In patients with pheochromocytoma, carcinoid, and pancreatic tumours, a combined measurement of CgA and CgB showed greater sensitivity in the detection of these tumours than

measurement of CgA alone. In this study, all patients with these endocrine tumours presented with elevated combined CgA and CgB levels. However, when CgA levels were measured by the RIA of O'Connor *et al.* (1989), only two-thirds of the patients presented with elevated CgA levels (Eriksson *ei al.*, 1990, column G in Table 1.5). Plasma CgA and CgB was also demonstrated to be a sensitive marker for pheochromocytoma, where circulating levels decreased to normal post-operatively after removal of the tumour in all patients examined (Gröndal *et al.*, 1991). Circulating levels of CgA and CgB can also be used to monitor treatment, as demonstrated in the successful treatment of patients with gastrointestinal neuroendocrine tumours (Eriksson and Öberg, 1991). Thus, circulating levels of CgA and CgB may prove to be important in the early detection of endocrine tumours, with potentially even greater sensitivity than measuring circulating CgA levels alone.

The development of RIAs for the other granin proteins may not only be helpful in diagnosis, but it may also prove to be valuable in the diagnosis of particular types of neuroendocrine neoplasms, from which one granin is more greatly secreted than another (Sekiya *et al.*, 1989). For instance, using an RIA for a CgB-derived peptide [GAWK, CgB(420—493)], elevated plasma levels of GAWK in patients with pancreatic islet tumours (especially VIPoma and glucagonoma) were more prevalent than in other tumour types (Sekiya *et al.*, 1989). These tumours are characterized by a low tissue content of GAWK, suggesting that GAWK is secreted very rapidly from them. Measurement of plasma GAWK levels, therefore, could be useful in the early detection of these pancreatic tumour types.

Monitoring plasma GAWK levels after tumour resection has also been shown to be helpful for prognosis. For example, plasma GAWK levels fell to normal levels post-operatively after removal of the tumour in pheochromocytoma patients (Sekiya *et al.*, 1989). Finally, plasma levels of GAWK were found to be elevated in patients with other endocrine tumours, including pheochromocytoma, medullary thyroid carcinoma, and carcinoids (Sekiya *et al.*, 1989; column I in Table 1.5).

SgV (7B2) has also been detected in tumour tissue and in plasma from patients with several neuroendocrine tumours (Suzuki *et al.*, 1986; column J in Table 1.5). Elevated plasma levels of SgV (7B2) were also found to be more prevalent in the pancreatic tumour types (Suzuki *et al.*, 1986), and may represent another diagnostic tool for them.

1.4.6.2 TISSUE SOURCES OF CHROMOGRANIN A IN DISEASE

Increased levels of a particular polypeptide hormone is usually indicative of a tumour in the cognate tissue. For CgA, however, the source of elevated circulating CgA levels in neuroendocrine neoplasia is more difficult to assess because many tissues contribute to the circulating levels of CgA in humans (Takiyyuddin *et al.*, 1991). It would therefore be of interest to determine the principle source of CgA in disease states. Two possibilities exist. Either CgA is overproduced and secreted by the tumour itself, or, secondly, CgA is derived from a source other than that of the primary tumour and its secretion is influenced by factors produced as a consequence of the primary tumour itself.

To examine the first possibility, one study compared CgA gene expression in the normal and hyperplastic parathyroid tissue (Levine *et al.*, 1990). No difference in CgA mRNA content was found between these tissues, as determined by Northern blot analysis (Levine *et al.*, 1990). Patients who presented with other endocrine disease or metabolic disturbances were excluded from this study since these could potentially influence parathyroid cell function. This data is conistent with recent data that indicates that an elevated circulating level of CgA is not a general feature of primary hyperparathyroidism (Nanes *et al.*, 1989).

The second possibility, where the primary source of CgA is not the tumour itself, occurs in Zollinger-Ellison syndrome and diseases associated with it. As described earlier, in patients with primary hyperparathyroidism and Zollinger-Ellison syndrome, CgA levels better correlated with pancreatic function than with parathyroid function. The source of the elevated circulating CgA was not the parathyroid, but was believed to be the pancreas (Nanes *et al.*, 1989). Similarly, in patients with Zollinger-Ellison syndrome only, plasma CgA levels did not correlate with gastrin levels in patients (Stabile *et al.*, 1990), suggesting that in Zollinger-Ellison syndrome, which is characterized by hyperplasia of enterochromaffin-like cells in the gastric fundic mucosa, the tumour was not the source of the elevated CgA levels. Rather, a positive correlation was found between CgA and pepsinogen levels, indicating that the enterochromaffin-like cells of the stomach, which express CgA (Weidenmann *et al.*, 1988), were the primary source of the elevated levels of CgA in Zollinger-Ellison syndrome (Stabile *et al.*, 1990).

1.4.6.3 TUMOURS HAVE SERVED AS A USEFUL SOURCE OF CHROMOGRANIN A-DERIVED PEPTIDES

Several investigations have characterized the presence of CgA-derived peptides in tumour tissues. For example, several pancreastatin-like peptides have been isolated from human tumours, and from cultured cells, ß-granin has been shown to be specifically generated by rat insulinoma cells. It is not known whether the CgA peptides found in these tumours have the same biological activity on secretory activity as their counterparts secreted from normal cells. Furthermore, a systematic evaluation and comparison of the pattern of CgA peptides between normal and tumour tissues have not been done.

Pancreastatin immunoreactivity has been found in normal tissues such as the pancreas, duodenum, and stomach (Ravazzola *et al.*, 1988), and also in some types of tumours (Schmidt *et al.*, 1988; Tamamura *et al.*, 1990). From a liver metastasis of a human carcinoid tumour, for example, Schmidt *et al.* (1988) isolated a precursor to pancreastatin, hCgA 210—301, and an amino-terminal truncated form of the full-length pancreastatin molecule, hCgA 273—301. From a liver metastasis of an insulinoma, a molecule hCgA 116—301, containing the pancreastatin sequence, was isolated (Tamamura *et al.*, 1990). A truncated version of pancreastatin has also been isolated from a pancreatic glucagonoma (Sekiya *et al.*, 1988). In two cases above (Schmidt *et al.*, 1988; Tamamura *et al.*, 1990), tryptic cleavage of the large CgA-derived precursor peptide yielded smaller peptides consistent in size to the full-length and amino-terminal truncated pancreastatin molecules, indicating that pancreastatin could potentially be generated *in vivo* in some tumours.

A fourteen amino acid peptide derived from hCgA 324—337, WE-14, which has been previously named as CAP-14 (Galindo *et al.*, 1991) was isolated from a human midgut carcinoid tumour (Curry *et al.*, 1992) and a human pheochromocytoma (Conlon *et al.*, 1992). This peptide is derived from a highly conserved region of the CgA protein and is flanked by pairs of basic residues which may serve as targets for endoproteolytic attack. Unlike pancreastatin, however, WE-14 has not yet been shown to be biologically active, and requires further characterization.

1.5 PARATHYROID CHROMOGRANIN A

Parathyroid hormone (PTH), of molecular weight 10 kDa, is one of the two major secretory protein of the parathyroid gland. It plays a highly essential role in maintaining calcium homeostasis by its ability to maintain blood calcium within tight

constraints. PTH can act at the level of the kidney to enhance renal Ca^{++} retention and cause excess serum phosphate to be excreted by decreasing renal tubular phosphate reabsorption. On bone cells PTH acts to stimulate osteoclastic bone resorption to increase osteoclastic Ca^{++} release and also acts to stimulate osteoblast activity and recruitment. PTH also acts in an indirect fashion at the level of the intestine. Intestinal absorption of calcium is increased by the action PTH on the kidney where it acts to enhance the 1-α-hydroxylation of 25-dihydroxycholecalciferol to form 1,25-dihydroxycholecalciferol.

In addition to PTH, higher molecular weight proteins were found to be secreted from the parathyroid (Sherwood *et al.*, 1970; Licata *et al.*, 1972). A second higher molecular weight protein, found to be secreted with PTH, was named parathyroid secretory protein (PSP). This represented the second major parathyroid secretory protein, whose apparent molecular weight was approximately 72 kDa (Kemper *et al.*, 1974; Cohn *et al.*, 1982). The secretion of PTH and PSP from the parathyroid was shown to be regulated in the same direction by extracellular calcium, where an inverse sigmoidal relationship exists between extracellular calcium and their secretory rates (Kemper *et al.*, 1974; Morrissey *et al.*, 1978; Cohn *et al.*, 1982a). By immunohistological studies, PSP was localized in parathyroid cells with PTH (Ravazzola *et al.*, 1978), and colocalized to the same secretory granules (Arps *et al.*, 1987).

Cohn *et al.* (1982b) were the first to demonstrate that PSP was similar in immunological cross-reactivity, amino acid and carbohydrate compositions to the well characterized secretory protein of the adrenal medulla, chromogranin A. Kruggel *et al.* (1985) extended these findings by demonstrating that human and bovine CgA, and bovine PSP possess identical amino-terminal sequences. Furthermore, the products of cell-free *in vitro* translation of RNA from the adrenal medulla (Falkensammer *et al.*, 1985) and the parathyroid (Majzoub *et al.*, 1979) closely resemble each other and further support their identity.

1.6 PRIMARY PARATHYROID CELL CULTURES

In earlier studies that examined the regulation of parathyroid cell function, cultures of parathyroid tissue slices (e.g., Sherwood *et al.*, 1971; Habener *et al.*, 1975) and organ cultures of rat parathyroids (e.g., Au, 1976) have been used. Although several advances in our knowledge of parathyroid function came about using such systems, some disadvantages to these two culture systems exist. For example, culture of tissue slices considerably increases the diffusion distances of substances from the cells, and, because the

parathyroid gland consists of a heterogeneous cell population (including relatively large numbers of fat cells), interpretation of studies using parathyroid organ cultures can be complicated.

A major emphasis of this thesis is to describe the mechanisms underlying the regulation of parathyroid CgA synthesis by extracellular calcium and $1,25(OH)_2D_3$. Primary cultures of bovine parathyroid cells were used in these studies (LeBoff *et al.*, 1983), and were prepared by adapting the methods from the earlier work of Brown *et al.* (1976) who used isolated parathyroid cell suspensions. Parathyroid glands are minced and digested with collagenase to generate single cells and small aggregates consisting of approximately 3-10 cells. Despite a rather harsh treatment with enzymes, these cells are viable, are virtually free of fat cells, and display responses to secretory stimuli (Morrissey and Cohn, 1978; Moran *et al.*, 1981). Many important advances in the understanding of parathyroid cell secretory function and gene regulation by calcium and $1,25(OH)_2D_3$ have been made using this cell culture system.

For example, mechanisms underlying calcium-regulated parathyroid cell secretion have partly been elucidated using this culture system. A calcium-sensor or receptor on the cell surface (Brown, 1993), which has recently been cloned from bovine parathyroid tissue (Brown *et al.*, 1993; Rask *et al.*, 1993; Garrett *et al.*, 1993), detects minute changes in calcium levels, and therefore begins the cascade of transduction events which direct parathyroid cell secretion. Subsequent to this, the secretory response has been shown to involve the activation of guanine nucleotide regulatory proteins (G-proteins; Fitzpatrick *et al.*, 1986), the activation of calcium channels (Fitzpatrick *et al.*, 1988), and a cascade of intracellular transduction systems including the PKC signalling pathway (Brown *et al.*, 1984; Kobayashi *et al.*, 1988; Clarke *et al.*, 1993), with inositol phosphates (Brown *et al.*, 1987), diacylglycerol and possibly arachidonic acid (Bourdeau *et al.*, 1992) playing critical roles.

These cells also provide an optimal system to study parathyroid cell gene regulation. In bovine parathyroid cells, calcium was shown to down-regulate PTH mRNA levels (Russell *et al.*, 1983; Brookman *et al.*, 1986) presumably by acting directly to suppress the PTH gene transcription rate. The relationship between circulating $1,25(OH)_2D_3$ levels and PTH gene expression has also been elucidated. Using parathyroid cell cultures, $1,25(OH)_2D_3$ was shown to regulate PTH mRNA levels (Silver *et al.*, 1985), due to a direct effect of $1,25(OH)_2D_3$ on the PTH gene transcription rate (Russell *et al.*, 1986). This cell culture system also provides the best model to study the proliferation of parathyroid cells. For example, the regulation of the genes involved in parathyroid cell proliferation by calcium and $1,25(OH)_2D_3$ has been examined. In primary cultures of

bovine parathyroid cells, $1,25(OH)_2D_3$, but not calcium, was shown to influence both the proliferation of cells as well as the expression of the proto-oncogene c-myc, indicating that $1,25(OH)_2D_3$ influences parathyroid cell growth (Kremer *et al.*, 1989).

The secretory responses in human parathyroid cells have also been examined. Human parathyroid cells are prepared in a similar manner, where cells are dispersed from resected adenomatous parathyroid tissue by collagenase digestion. The effects of calcium and $1,25(OH)_2D_3$ on cell secretory function and gene regulation were examined, and compared to the responses in normal parathyroid cells. Defects in responsiveness to both calcium (Brown et al., 1979; Brookman et al., 1988; Farrow et al., 1988) and $1,25(OH)_2D_3$ (Karmali et al., 1989) have been observed in these cells relative to that observed in normal cells. Experimentation using cultured cells from human adenomatous tissue has revealed some ways in which these cells respond differently to parathyroid cell regulators, and should provide a better understanding of the abnormalities found in parathyroid neoplasias.

Although no *in vitro* model system is better than the *in vivo* setting, dispersed bovine parathyroid cells provide a reproducible, and practical system to study parathyroid cell gene regulation and secretory function.

PART II

EXPERIMENTAL SECTION

CHAPTER 2

REGULATION OF SYNTHESIS AND SECRETION OF CHROMOGRANIN A BY CALCIUM AND 1,25-DIHYDROXYCHOLECALCIFEROL IN CULTURED BOVINE PARATHYROID CELLS

2.1 PREFACE

Primary cultures of bovine parathyroid cells were used to study the effects of calcium and 1,25-dihydroxycholecalciferol on the synthesis of chromogranin A in the parathyroid. Parathyroid cells were treated with varying concentrations of calcium and 1,25-dihydroxycholecalciferol and their effects on chromogranin A gene transcription, synthesis and secretion were measured. This chapter demonstrates that while calcium does not alter the chromogranin A gene transcription rate, 1,25-dihydroxycholecalciferol enhances it resulting in a large increase in steady-state chromogranin A mRNA, and increased synthesis and secretion of chromogranin A. Also, the effects of 1,25-dihydroxycholecalciferol on the chromogranin A gene transcription rate is shown to oppose the effects of 1,25-dihydroxycholecalciferol on the PTH gene transcription rate. The biological significance of this finding is discussed in this chapter.

2.2 ABSTRACT

A radicimmunoassay for bovine parathyroid chromogranin A was developed and used to study the regulation of CgA synthesis and release in cultured bovine parathyroid cells. As previously demonstrated an elevated medium calcium concentration [2.5 mM] led to a reduced release of CgA into the medium. In contrast, the intracellular concentration of CgA was not changed by alterations in the medium calcium concentration. 1,25 dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] stimulated a dose-dependent increase in the release of CgA, whereas intracellular CgA levels were not markedly altered. Alterations in medium calcium concentration did not affect CgA mRNA levels, whereas, 1,25$(OH)_2D_3$ caused a time and dose-dependent increase in CgA mRNA levels. Therefore, changes in CgA release occurred in response to calcium and 1,25$(OH)_2D_3$ with little change in intracellular CgA concentration. This could arise by a change in synthetic rate, to match the alteration in secretion rate, and/or a change in degradation rate. Calcium does not appear to affect CgA synthesis at a pretranslational level, whereas 1,25$(OH)_2D_3$ stimulated a marked increase in CgA mRNA levels via an effect on CgA gene transcription. Furthermore, 1,25$(OH)_2D_3$ had no effect on CgA mRNA stability. The relationship between steady-state levels of CgA mRNA and total CgA levels (intracellular and extracellular) is not simple, and possibly indicates that an important regulatory step for CgA synthesis occurs at the level of mRNA translation. The effect of 1,25$(OH)_2D_3$ on CgA mRNA levels was not modulated by medium calcium concentrations. In addition, the effect of 1,25$(OH)_2D_3$ in reducing parathyroid hormone (PTH) mRNA levels was not affected by medium calcium concentrations, with 1,25$(OH)_2D_3$ and calcium acting independently to reduce PTH mRNA levels.

2.3 INTRODUCTION

The two major secreted products of the parathyroid gland are parathyroid hormone (PTH) and a larger protein, initially known as parathyroid secretory protein (1) but now known to be identical to chromogranin A (CgA) (2). CgA, an acidic protein first identified in chromaffin granules of the adrenal medulla, is a member of the chromogranin family of proteins which are present in virtually all endocrine and neuroendocrine cells (3). The function of the chromogranins in secretory cells remains unclear, but their ubiquitous presence in this cell type suggests an important function in hormone secretion (4).

The major regulators of PTH synthesis and secretion are the extracellular

calcium ion and 1,25 dihydroxyvitamin D concentrations (5-9). Initial studies in cultured parathyroid cells indicated that the secretion of both PTH and CgA were regulated in a similar manner by extracellular calcium concentrations (10). More recent experiments using other modulators of either parathyroid synthetic or secretory activity have indicated that the synthesis and secretion of PTH and CgA may not always be coregulated. For example, $1,25(OH)_2D_3$ inhibits PTH secretion via its effects on transcription (8,9), but it stimulates CgA secretion (11) again apparently by an effect at the transcriptional level (12).

In these studies we have investigated the effect of the extracellular calcium and $1,25(OH)_2D_3$ concentration on CgA synthesis and release in cultured bovine parathyroid cells and compare the responses with those of PTH.

2.4 MATERIALS AND METHODS

2.4.1 ISOLATION OF BOVINE PARATHYROID CHROMOGRANIN A

Bovine parathyroid glands were acetone/hexane dried and defatted and parathyroid hormone (PTH) was extracted with phenol followed by solubilization in acetic acid as described (13,14). The tissue residue was homogenized in acetone four times, to remove the phenol and acetic acid, and lyophilized. The acetone-dried powder was homogenized in 0.1 M Tris-HCl, pH 8.2, 10 mM EDTA, 2.5 mM phenyl methyl sulphonyl fluoride (40 ml/g). Solid material was removed by centrifugation and solid ammonium sulphate was added to the supernatant to a final concentration of 50%. The suspension was left at 4°C overnight and then centrifuged and the supernatant was decanted. The precipitate was stored at -20°C. Aliquots of the precipitate were dissolved in 0.15 M ammonium acetate pH 8.2 and chromatographed on a 100 x 2.6 cm column of BioGel A1.5M. The running buffer was 0.15 M ammonium acetate pH 8.2. The protein peak eluting at a Kd of 0.25 was lyophilized and subjected to ion-exchange chromatography on a 10 x 0.9 cm column of DEAE-cellulose. A linear gradient of increasing conductivity was established using 500 ml buffer A (0.1 M ammonium acetate, pH 8.2) and 500 ml buffer B (0.5 M ammonium acetate, pH 8.2). Following completion of the gradient, 1 M ammonium acetate pH 8.2 was run through the column. The protein peak eluting at a conductivity of 9-11 mMhos was collected and lyophilized. SDS-PAGE under reducing conditions of the purified chromogranin A revealed a single band at 69 kilodaltons. Aliquots of the protein were subjected to amino acid analysis and amino acid sequencing of the NH_2-terminal 40 amino acids, to confirm the homogeneity and structural correctness of the isolated material.

The DEAE-cellulose purified chromogranin A was used for both antibody production and as tracer and standard in the radioimmunoassay.

2.4.2 BOVINE CgA RADIOIMMUNOASSAY

Antibody Production: Rabbits received 150 µg bovine CgA emulsified in Freund's complete adjuvant and injected intradermally at numerous dorsal sites. Animals were reinjected subcutaneously once a month for three months with 150 µg bovine CgA emulsified in Freund's incomplete adjuvant. Animals were bled 10-14 days after injection.

2.4.3 PREPARATION OF IODINATED BOVINE CgA

2.5 µg bCgA were dissolved in 50 µl 0.05 M sodium phosphate buffer, pH 7.0. 10 µl $Na^{125}I$ [1 mCi], 4 µg lactoperoxidase (grade B, 20.3 IU/mg, Calbiochem) in 20 µl phosphate buffer and 20 µl (1:100,000 v/v) H_2O_2 in water were added. After 30s, the reaction was stopped by the addition of 100 mM dithiothreitol. Incorporation of radioiodine into protein was greater than 40% by precipitation of aliquots of labeled bCgA with 20% trichloroacetic acid. Labeled bCgA was purified on a 2.5 x 90 cm column of BioGel A1.5M eluted with 0.15 M NaCl, 0.01 M sodium phosphate pH 7.4, 0.1% ovalbumin, 0.1% sodium azide, 0.005% Triton X-100 at 4°C. The elution profile was defined by counting aliquots in an LKB gamma counter (80% efficiency) (LKB Instruments, Gaithersburg, MD).

2.4.4 RADIOIMMUNOASSAY OF bCgA

The assay buffer was 0.15 M NaCl, 0.01 M sodium phosphate pH 7.4, 1% (w/v) ovalbumin 0.1% (w/v) sodium azide (15). The total volume per assay tube was 800 µl, being comprised of; 100 µl [^{125}I] bCgA (10,000 cpm), 100 µl Rabbit anti-bCgA serum (final dilution 1:8000), 500 µl bCgA (standard or unknown), all made up in assay buffer, and 100 µl 0.1 M EDTA pH 7.4. After incubation at 4°C for 24 h, 100 µl 1:12 (v/v) sheep anti-rabbit gamma-globulin and 100 µl carrier 2% (v/v) normal rabbit serum in assay buffer were added and incubation at 4°C continued for a further 24 h. Bound and free counts were separated by centrifugation at 5000 *xg* for 20 min at 4°C. The supernatants were decanted

and the pellets counted in the gamma spectrophotometer.

Aliquots of medium and cell extracts were assayed in multiple dilutions. Cell extracts were prepared with guanidinium thiocyanate (GTC) buffer as described for RNA analysis below. At the dilutions routinely employed the residual GTC had no influence on antibody-tracer binding.

2.4.5 PREPARATION OF BOVINE PARATHYROID CELLS

Bovine parathyroid glands were obtained from a local slaughterhouse and transported in ice-cold Minimum Essential Medium (MEM) containing antibiotics, penicillin (100 U/ml), streptomycin (100 µg/ml). In the laboratory the glands were rinsed briefly in 70% ethanol and rinsed in fresh medium. Glands were trimmed of excess fat, minced with scissors, separated from the medium by centrifugation and resuspended in MEM, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, antibiotics and 2.5 mg/ml collagenase (Worthington, Freehold, N.J.). The tissue was digested at 37°C for three-five hours with vigorous pipetting every 20 minutes to disperse lumps. Cells were filtered through a nylon mesh of size 150 µm, washed three times with sterile phosphate-buffered saline (PBS) and aliquots taken for cell counting [(model AM, Coulter Electronics, Hialeah, FL) or hemacytometer.

2.4.6 PARATHYROID CELL CULTURES

Cells were suspended in MEM, 10 mM Hepes, antibiotics, 5% FCS, 0.5 mM $MgCl_2$, and various concentrations of $CaCl_2$ (0.5 mM, 1 mM or 2.5 mM) with and without $1,25(OH)_2D_3$ (10^{-9} to 10^{-9} M) and plated at a concentration of approximately 600,000 cells per ml. At the indicated times, medium was aspirated for CgA radioimmunassay, and extracts of the cells made for protein estimation, CgA radioimmunoassay, RNA analysis, and nuclear run-on assay. Cell viability was routinely checked by trypan blue exclusion which was >95%.

2.4.7 ASSESSMENT OF mRNA LEVELS

Dot blot analysis: Cells were washed twice with ice-cold PBS, pH 7.4, and 300 µl of a mixture (GTC) of 4 M guanidinium thiocyanate, 25 mM trisodium citrate, 1 mM

EDTA, and 0.1 M β-mercaptoethanol added to the cell monolayer which was left at room temperature for 10 min with occasional swirling. The GTC extracts could be stored at -80°C before analysis. Aliquots of the extracts were denatured in formaldehyde and standard saline citrate (1 x SSC= 0.15 M NaCl and 0.015 M trisodium citrate) at 65°C for 15 min as described (16). Serial dilutions were made in 20 x SSC and applied to a nylon membrane (Nytran, Schleicher and Schuell, Keene, NH.) with the aid of a dot-blot manifold apparatus (BioRad, Mississauga, ON). Filters were air-dried and baked at 80°C for 2 h.

2.4.8 NORTHERN BLOT ANALYSIS

RNA was isolated from cells using an NP-40 lysis buffer (17). 200 µl of lysis buffer (10 mM Tris-HCl, pH 7.5, 0.15 mM NaCl, 1.5 mM $MgCl_2$, 0.65% NP-40) were added directly onto cell monolayers which were then scraped into an Eppendorf microfuge tube using a cell scraper. The cells and buffer were vigorously vortexed for 30 s and the nuclei removed by centrifugation (500 *xg*, 5 min). The supernatant was removed to a tube containing 200 µl 7M urea, 1% SDS, 0.35 M NaCl, 10 mM EDTA, 10 mM Tris-HCl (pH 7.5) and 400 µl phenol/chloroform/isoamyl alcohol. (50/49/1;v/v/v). The phases were mixed by vortexing vigorously and either stored frozen at -80°C or processed immediately by centrifugation (15,000 *xg*, 1 min) to separate the phases. The aqueous phase was recovered and the RNA precipitated by addition of 1 ml 95% ethanol and freezing. RNA was recovered by centrifugation and quantitated by measurement of the absorbance at 260 nm. One to five µg of RNA were fractionated on a 1% agarose 0.66 M formaldehyde gel, transferred to Nytran by blotting and fixed as above. The integrity of the RNA was assessed by ethidium bromide staining.

Prehybridization and hybridization of dot blots and Northern blots were performed in 50% formamide, 5 x SSC, 25 mM $NaPO_4$, pH 7.4, 1% SDS, 2 mM EDTA, 1 x Denhardt's and 200 µg/ml sheared salmon sperm DNA. DNA probes, which were labeled with [^{32}P]dCTP to specific activities of 10^9 cpm/µg by the random primer method (18), were as follows: 1) bovine CgA, the 528-basepair PstI restriction fragment from plasmid pCHRG4A (19); 2) bovine PTH, the 500-basepair PstI restriction fragment from plasmid pPTHm29(20); 3) rat cyclophilin, the 800-basepair BamHI restriction fragment from plasmid pCD15.8.1 (21). Membranes were washed to high stringency (0.1 x SSC, 0.1% SDS, 60°C) and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY). The relative intensities of the resulting autoradiographic images were assessed using an

LKB Ultroscan XL densitometer (LKB, Baie D'Urfé, QC). The signals for CgA and PTH mRNA were related to that for cyclophilin mRNA which was not influenced by changes in calcium or $1,25(OH)_2D_3$.

2.4.9 IN VITRO NUCLEAR RUN-ON ASSAYS

Relative transcription rates of CgA, PTH and cyclophilin genes were measured using a nuclear run-on assay (22,23). Nuclei were prepared from 10-20 x 10^6 parathyroid cells incubated with either $1,25(OH)_2D_3$ or ethanol carrier alone. Cells were scraped into ice-cold PBS, pH 7.4, pelleted at 4°C, and lysed with NP-40 lysis buffer (0.3 M sucrose; 60 mM KCl; 15 mM NaCl; 15 mM HEPES, pH 7.5; 2 mM EDTA; 0.5 mM EGTA; 0.15 mM spermine; 0.5 mM spermidine; 14 mM β-ME; 0.2% NP-40). After 8 minutes on ice, nuclei were pelleted at 800 x g. They were rinsed once with 1 mL nuclei storage buffer (50% glycerol; 20 mM Tris, pH 7.9; 75 mM NaCl; 0.5 mM EDTA; 0.85 mM DTT; 0.125 mM PMSF), snap frozen in liquid nitrogen and stored at -80°C until assay. Run-on reactions were carried out at 30°C in 300 mM $NH_4(SO_4)_2$; 100 mM Tris-HCl, pH 7.9; 4 mM $MgCl_2$; 4 mM $MnCl_2$; 50 mM NaCl; 0.4 mM EDTA; 1.2 µM DTT; 0.1 mM PMSF; 10 mM creatine phosphate; 29% glycerol; 150 µCi [^{32}P]UTP, 650 Ci/mmol (ICN, Mississauga, ON); 1.5 mM each of CTP, ATP and GTP (Boehringer Mannheim Canada Ltd, Dorval, QC) for 45 minutes. Reactions were quenched with tRNA and treated with (RNAse-free) DNAse and proteinase K, and phenol-chloroform-isoamyl alcohol extracted. [^{32}P]-labelled transcripts were spun column chromatographed through Sephadex G-50, TCA-precipitated, NaOH-treated and ethanol precipitated. 5 µg plasmid DNA containing CgA, PTH, cyclophilin inserts as described above or no insert (pBR322) were NaOH-denatured and slot-blotted (HybriSlot, Gibco/BRL, Burlington, ON) and hybridized with 2 x 10^7 cpm [^{32}P]-labelled transcripts in 50% formamide; 50 mM Hepes, pH 7.3; 0.75 M NaCl; 2 mM EDTA; 0.5% SDS; 10X Denhardt's and 200 µg/ml ssDNA for a minimum of 40 h. In any single experiment, equal numbers of counts were used for all conditions. Nytran filters were exposed to autoradiographic film and quantitation of the relative rates of transcription was achieved by scanning laser densitometry.

2.4.10 mRNA STABILITY ESTIMATIONS

Freshly dispersed parathyroid cells were treated with either 10^{-8}M $1,25(OH)_2D_3$

or ethanol carrier alone for 24 hours. Medium was replaced with identical medium containing the RNA polymerase II inhibitor, 5,6-dichloro-1-β-d-ribofuranosylbenzimadazole (DRB, Sigma Chemical Co., St. Louis, MO; 25 μg/mL) or Dactinomycin (Sigma; 0.5 μg/mL). Total RNA was isolated at 0, 10, 16, 24, 36 and 48 hours and amounts of CgA, PTH, and cyclophilin mRNAs were assessed by Northern blot as described above.

2.4.11 TOTAL RNA SYNTHESIS ESTIMATIONS

In order to quantitate total RNA synthesis parathyroid cells were treated with transcriptional inhibitor or ethanol carrier alone for 24 hours. Cells were then pulsed with 5 μg/mL [^{3}H]Uridine (ICN; 17 Ci/mmol). Cells were washed twice with cold PBS and cold 10% TCA was added. After 3 hours at 4°C, TCA was aspirated and 0.6N NaOH was added for solubilization. An aliquot was added to scintillation cocktail (Cytoscint, ICN) and counted.

2.4.12 STATISTICAL ANALYSIS

Data was analyzed by an unpaired Student's t-test with $p<0.05$ indicating a significant difference between the means.

2.5 RESULTS

2.5.1 RADIOIMMUNOASSAY FOR BOVINE CHROMOGRANIN A

A radioimmunoassay using ^{125}I-labeled bovine CgA and antiserum 4252 at a final dilution of 1:8000 is illustrated in Fig. 2.1. Serial dilutions of conditioned medium from cultured bovine parathyroid cells as well as cell extracts paralleled the dose response curve of bovine CgA. Bovine PTH did not displace the tracer.

The intraassay variation and interassay variation of the assay were 8% and 15%, respectively.

2.5.2 EFFECT OF EXTRACELLULAR CALCIUM CONCENTRATION ON CgA RELEASE AND INTRACELLULAR CgA CONCENTRATION IN CULTURED BOVINE PARATHYROID CELLS

Extracellular calcium concentration had a marked influence on CgA release as shown in Fig. 2.2. Immunoassayable CgA in the high calcium medium was consistently 40% of that in the low calcium medium. In contrast, the intracellular concentration of CgA was not changed by alterations in the medium calcium concentration. For example, at day 2 of culture the intracellular CgA concentration was 306 ± 26 (mean ± SEM) ng/600,000 cells [0.5 mM Ca^{2+}]; 317 ± 15 [1 mM Ca^{2+}] and 300 ± 26 [2.5 mM Ca^{2+}]. The mean concentrations expressed per μg protein were 9.3, 9.9 and 9.52 ng, respectively. The results described in this and subsequent sections are representative of experiments that were repeated at least three or four times.

2.5.3 EFFECT OF $1,25(OH)_2D_3$ ON CgA RELEASE AND INTRACELLULAR CgA CONCENTRATION IN CULTURED PARATHYROID CELLS

As shown in Fig. 2.3, $1,25(OH)_2D_3$ stimulated a dose-dependent increase in the release of CgA from parathyroid cells. Conditioned medium from parathyroid cells cultured for two days in 10^{-7}M $1,25(OH)_2D_3$ contained a 40% greater amount of immunoassayable CgA than conditioned medium from control cells. However, intracellular levels of CgA generally did not change in cells treated with $1,25(OH)_2D_3$ up to 2 days as shown.

2.5.4 EFFECT OF CALCIUM ON CgA mRNA LEVELS IN CULTURED PARATHYROID CELLS

By Northern blot analysis the CgA cDNA probe identified a single mRNA species of 2.1 Kb. In contrast to the marked effect of calcium on CgA secretion, it had no effect on CgA mRNA levels as shown in Fig. 2.4.

2.5.5 EFFECT OF $1,25(OH)_2D_3$ ON CgA mRNA LEVELS IN CULTURED BOVINE PARATHYROID CELLS

In contrast to the modest increase in cga release, $1,25(OH)_2D_3$ had a marked effect on steady-state levels of CgA mRNA. As shown in Figure 2.5, this effect was dose- and time-dependent with CgA mRNA increasing by four-fold with 10^{-8}M $1,25(OH)_2D_3$ and seven-fold with 10^{-7}M $1,25(OH)_2D_3$ at day 3 of culture. These effects of $1,25(OH)_2D_3$ were reversible (data not shown). In addition, modulation of extracellular calcium concentrations had no effect on the $1,25(OH)_2D_3$ stimulation of CgA mRNA (data not shown).

2.5.6 EFFECTS OF CALCIUM AND 1,25(OH)2D3 ON PTH mRNA LEVELS IN CULTURED BOVINE PARATHYROID CELLS

The lack of effect of calcium on CgA mRNA and the stimulatory effect of $1,25(OH)_2D_3$ on CgA mRNA is in contrast to the known effects of these agents to reduce PTH mRNA levels. As shown in Figure 2.6a the reduction in PTH mRNA levels caused by $1,25(OH)_2D_3$ is no different in high calcium (2.5 mM) than in low calcium (0.5 mM) medium suggesting that calcium does not modulate the $1,25(OH)_2D_3$ effect. Likewise, in Fig. 2.6b the reduction in PTH mRNA levels caused by elevated extracellular calcium concentration is no different in the presence or absence of $1,25(OH)_2D_3$ indicating that $1,25(OH)_2D_3$ does not modulate the calcium effect.

2.5.7 EFFECT OF $1,25(OH)_2D_3$ ON PARATHYROID GENE TRANSCRIPTION

The results of nuclear run on assays which were performed on extracts of parathyroid cells cultured with and without $1,25(OH)_2D_3$ for 10 h are shown in Figure 2.7. CgA gene transcription was stimulated 1.6 fold by 10^{-8}M and 2.2 fold by 10^{-7}M $1,25(OH)_2D_3$. In contrast, PTH gene transcription fell to 33% of the control value in 10^{-7}M $1,25(OH)_2D_3$. Cyclophilin gene transcription remained unchanged in the presence of $1,25(OH)_2D_3$

2.5.8 EFFECT OF $1,25(OH)_2D_3$ ON CgA, PTH AND CYCLOPHILIN mRNA STABILITY IN BOVINE PARATHYROID CELLS

Figure 2.8 shows the decay curves for CgA, PTH and cyclophilin mRNAs over a 48 hour time period. $1,25(OH)_2D_3$ had no effect on the rate of disappearance of either CgA, PTH or cyclophilin mRNA. The $t_{1/2}$ of disappearance of CgA mRNA was 30 h, of PTH mRNA was 30 h, and of cyclophilin mRNA was 20 h. Similar results were obtained using both transcription inhibitors, DRB and Dactinomycin, and they inhibited total [^{3}H]Uridine incorporation by 95% and 92%, respectively, at the concentrations used. The transcriptional inhibitors had no apparent cytotoxic effects on the cells throughout the timecourse of the experiments.

2.6 DISCUSSION

Although Ca^{2+} affects CgA secretion it has no apparent effect on its synthesis as evidenced by both measurement of steady-state mRNA levels and intracellular immunoassayable CgA levels. This is in marked contrast to the effect of Ca^{2+} on PTH synthesis as well as on secretion (5,7). This emphasizes the fact that effects of a change in the extracellular calcium concentration can be manifest at multiple levels within the cell. In contrast to the effect of $1,25(OH)_2D_3$ in bringing about a reduction in PTH release into the medium of cultured parathyroid cells (6,8) which is an indirect effect, $1,25(OH)_2D_3$ caused a modest increase in CgA release which appeared to be a consequence of a stimulation in CgA synthesis. However, despite a several-fold stimulation of CgA mRNA levels the resulting increase in intracellular CgA was small implicating an important regulatory step at the level of translation of CgA mRNA. The mechanism of this regulation as well as its significance, remains to be elucidated.

Members of the steroid hormone family have been shown to regulate specific gene expression by both transcriptional and post-transcriptional mechanisms. In the case of $1,25(OH)_2D_3$ it has been shown to modulate mRNA stability as well as affect gene transcription rates. For example, $1,25(OH)_2D_3$ increases calcium-binding protein mRNA stability (24,25), reduces granulocyte-macrophage-colony stimulating factor mRNA stability (26), and has no apparent effect on the half-lives of c-myc mRNA (27) and parathyroid hormone-like peptide mRNA (28). In the present study no effect of $1,25(OH)_2D_3$ was found on the stability of CgA, PTH or cyclophilin mRNAs. Previously the relative stabilities of parathyroid cell mRNAs have been little studied, although Heinrich

et al. (29) showed that PTH mRNA was stable in bovine parathyroid gland slices for up to 7 hours. In the studies described here both CgA mRNA and PTH mRNA were found to be very stable with half-lives of disappearance of approximately 30 hours.

In a study of chromogranin A synthesis and secretion in bovine adrenal chromaffin cells (30) in response to a secretory stimulus by either nicotine or forskolin the release of chromogranin A was not compensated for by an increase in CgA biosynthesis and intracellular levels of CgA were depleted. This is in contrast to our findings in the parathyroid cell in which in response to a secretory stimulus of $1,25(OH)_2D_3$ intracellular CgA levels are either maintained at a constant level or after several days of culture are slightly increased [(11) and our unpublished results]. This compensatory regulation of intracellular CgA levels could occur by a change in synthetic rate and/or a change in degradation rate, and this remains to be determined. More recent studies in cultured bovine chromaffin cells (31) indicates that CgA synthesis is coupled to its secretion, just as it appears to be in parathyroid cells, as shown here.

The effect of $1,25(OH)_2D_3$ on both PTH and CgA synthesis is a direct action on gene transcription. However, whereas PTH gene transcription is downregulated, that of CgA is stimulated. Therefore, although $1,25(OH)_2D_3$ has been shown to regulate the activity of many genes, both positively and negatively, in a variety of different cell types (32), the cultured parathyroid cell may offer a unique model in which to simultaneously study the upregulation and downregulation of gene transcription by $1,25(OH)_2D_3$. Presumably, the sterol receptor complex is able to act as both an effector and inhibitor by either binding to specific positive or negative cis-acting elements as has been proposed for the glucocorticoid receptor and/or by interacting with other trans acting factors (33). Although the 5'-flanking region of the PTH gene has been shown to mediate negative regulation by $1,25(OH)_2D_3$ (34) the precise cis-acting elements have not yet been defined. The 5'-flanking region of the CgA gene has yet to be characterized, but would be expected to contain sequences homologous to the putative *cis*-acting elements which have recently been identified in the osteocalcin gene (35,36,37) which is positively regulated by $1,25(OH)_2D_3$.

Alterations in extracellular calcium concentrations influence some $1,25(OH)_2D_3$-mediated processes but not others. For example, the $1,25(OH)_2D_3$ induction of terminal differentiation of mouse skin epithelial cells (38) and the $1,25(OH)_2D_3$ induction of chick kidney cell calbindin-D_{28k} mRNA levels (39) are modulated by extracellular calcium concentrations, whereas $1,25(OH)_2D_3$-induction of renal 24-hydroxylase activity is not so influenced (40). Our results in the parathyroid cell indicate that neither the $1,25(OH)_2D_3$-stimulated increase in parathyroid CgA mRNA levels nor the $1,25(OH)_2D_3$-inhibition of

PTH mRNA levels are modulated by the extracellular calcium concentration. In addition, there was no evidence of $1,25(OH)_2D_3$ modulating the calcium-mediated reduction in PTH mRNA levels thus confirming that the effects of calcium and $1,25(OH)_2D_3$ on PTH gene expression are purely additive, indicating that they act at different sites (8).

A study of the regulation of CgA synthesis and secretion should help to shed light on its function in the parathyroid cell. CgA may function as a prohormone from which biologically active peptides are released (41). For example, pancreastatin which is a 49-amino acid peptide with a carboxyl-terminal amide, and which was initially isolated from porcine islets, is encoded within the CgA molecule (42). Pancreastatin inhibits glucose-stimulated insulin release from perfused pancreatic islet cells (43) and inhibits low calcium-stimulated PTH and CgA release from cultured parathyroid cells (44). The *in vivo* significance of these observations remains unclear, but one might speculate that the action of elevated $1,25(OH)_2D_3$ levels to inhibit PTH synthesis and thus its secretion might be reinforced by the action of $1,25(OH)_2D_3$ to increase CgA synthesis and release, thereby promoting formation of pancreastatin-like peptides, and thus inhibit PTH secretion further.

CgA has regions of similarity to calcium binding proteins such as intestinal calcium binding protein, calcium binding protein S100β, and oncomodulin (45). These structural observations are consistent with reports that CgA is a functional calcium binding protein (46,47,48). Chromogranins and calcium are co-stored within secretory granules of many endocrine cells, and therefore, chromogranins may be involved in calcium sequestration and mobilization from secretory vesicles during stimulus-secretion coupling. In addition it has been proposed that CgA could be the 'condensation-protein' of secretory granules (48) and also modulate enzymes important for hormone processing (49,50).

Therefore, CgA and/or peptides derived from it may have multiple functions, both extracellular and intracellular. The relative importance of extracellular functions may depend upon the extent of processing of CgA which appears to be tissue-specific (51). For example, small CgA peptides are found in islet cells (52), but in the parathyroid preliminary studies indicate that relatively little processing normally takes place (53). The relationship between the regulation of synthesis of PTH and CgA is complex, but further study in this area will undoubtably provide insight into the multiple functions of CgA in the parathyroid cell.

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2.9 FIGURES

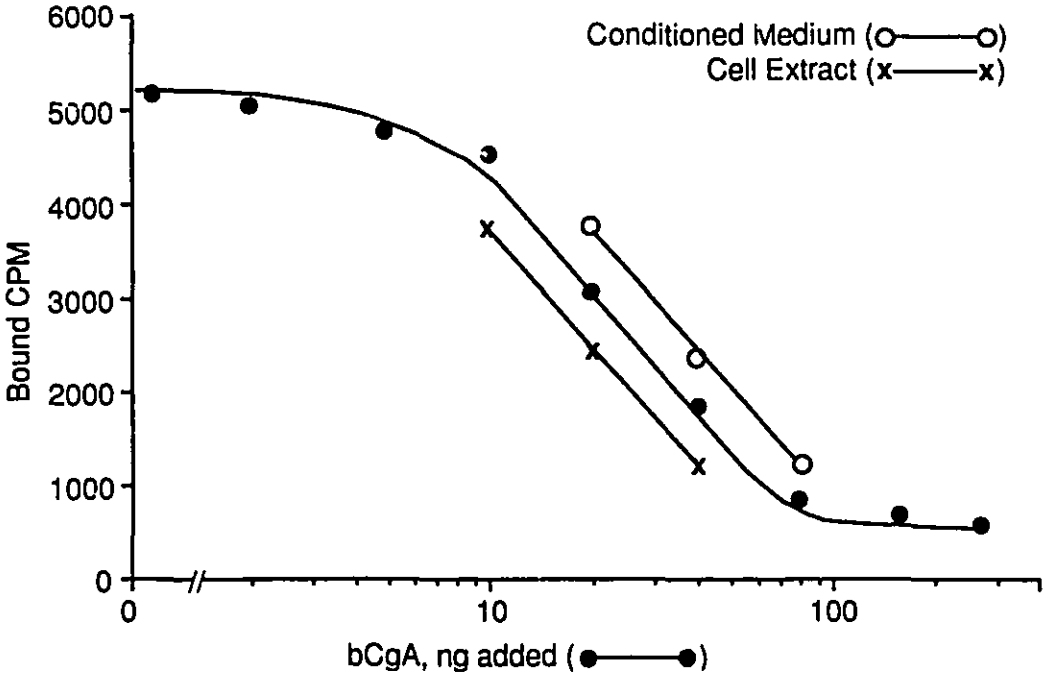

Figure 2.1 Radioimmunoassay for bovine CgA. Antiserum from rabbit 4252 was added at a final dilution of 1:8000 with ^{125}I-labeled bovine CgA as tracer. Standard native bovine CgA (●---●); Serial dilutions of conditioned medium from bovine parathyroid cells (O---O) and cell extracts (X---X).

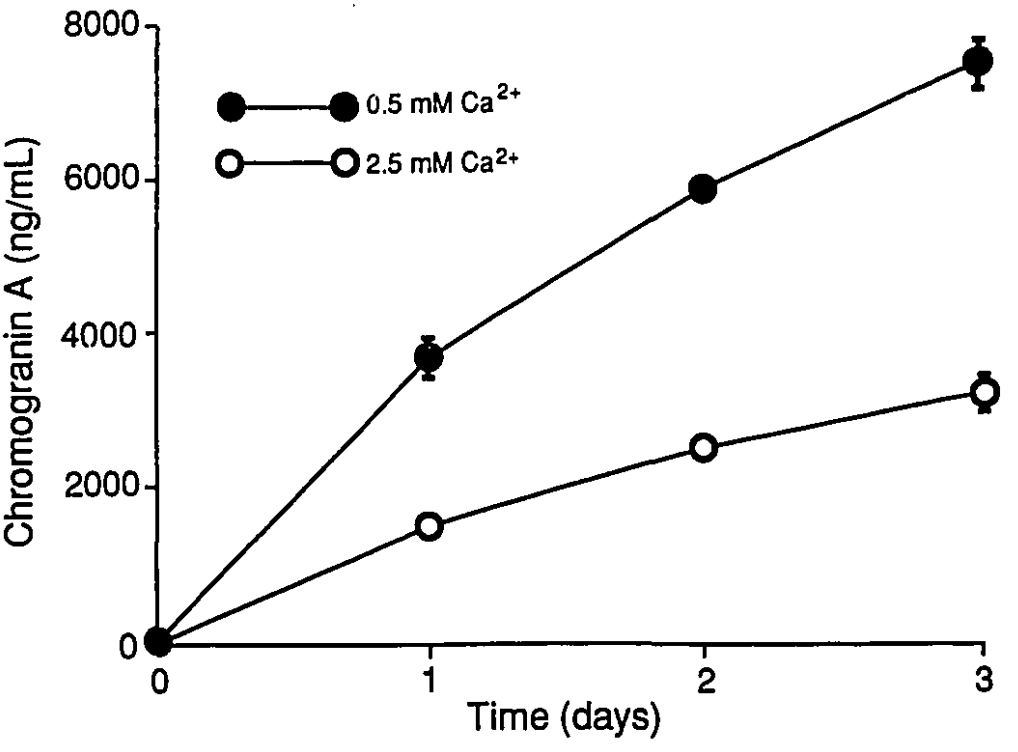

Figure 2.2 Release of CgA into conditioned medium of bovine parathyroid cells cultured in either a low calcium 0.5 mM Ca^{2+} (●---●) or high calcium, 2.5 mM Ca^{2+} (O---O) containing medium. Each point is the mean ± SEM of six estimations.

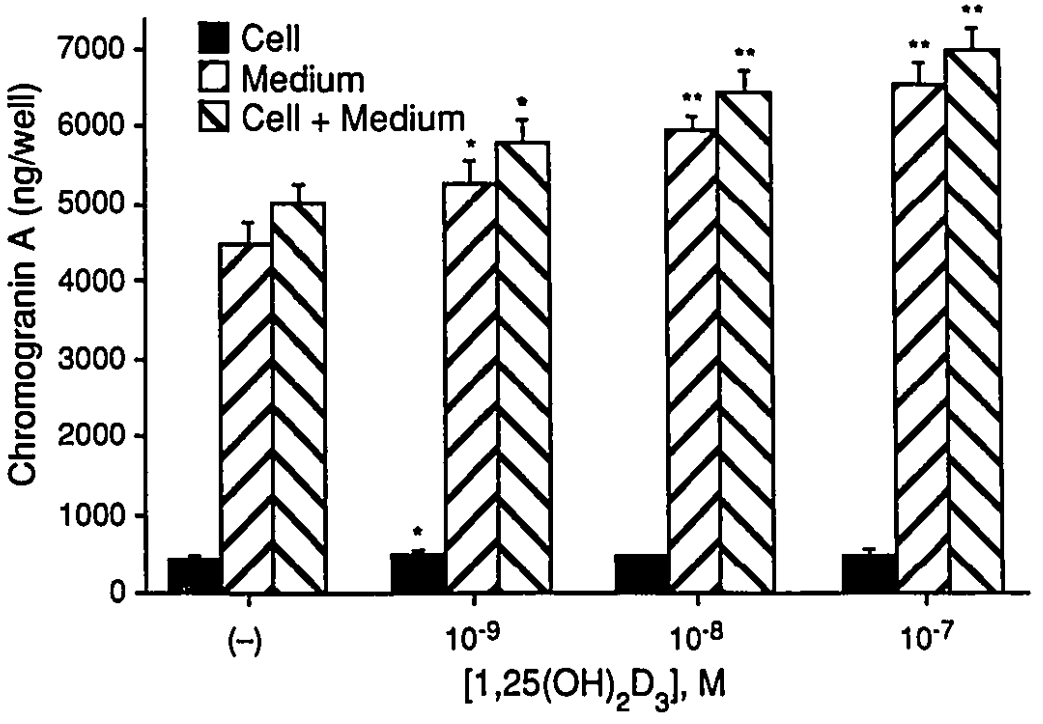

Figure 2.3 Effect of increasing concentrations of $1,25(OH)_2D_3$ on intracellular and extracellular CgA. Bovine parathyroid cells were plated at 600,000 cells per well and cultured for 48 h in the absence or presence of increasing concentrations of $1,25(OH)_2D_3$. Medium and cells were harvested for CgA immunoassay. Each point is the mean ± SE of triplicate determinations (from different wells). Cells, filled bar; medium, left-hatching; total, right-hatching. Asterisks indicate a significant difference from control value *$p<0.05$; **$p<0.005$.

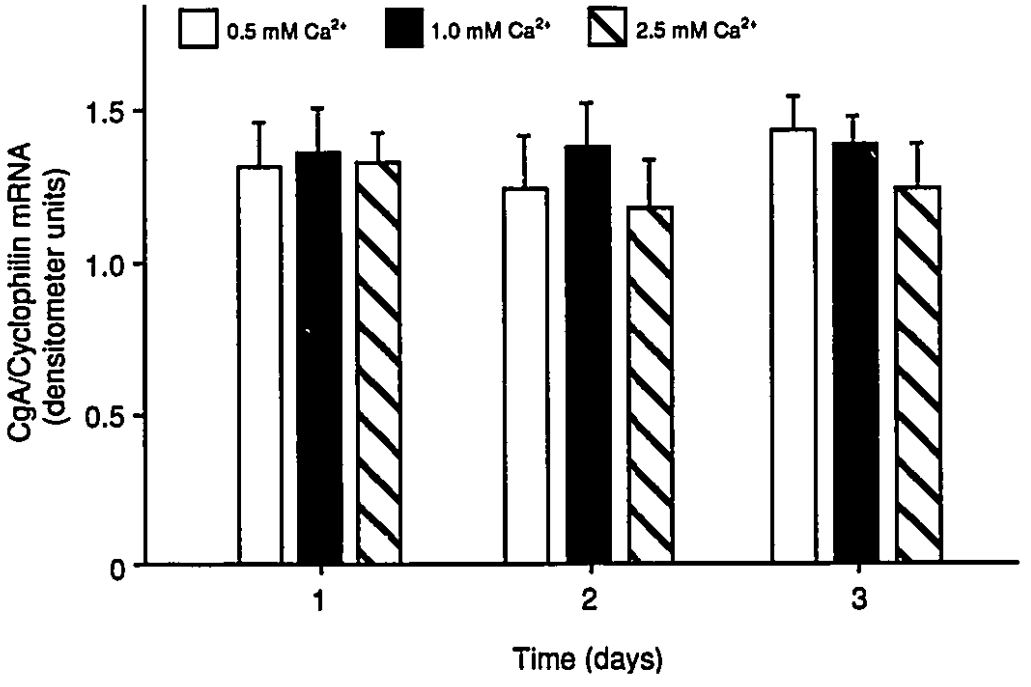

Figure 2.4 Effect of medium calcium concentration on steady-state levels of CgA mRNA. Bovine parathyroid cells were cultured in either 0.5 mM Ca^{2+} (open bar), 1.0 mM Ca^{2+} (filled bar) or 2.5 mM Ca^{2+} (hatched bar) for 1-3 days, harvested and analyzed for CgA mRNA as described in *Materials and Methods*. Each point is the mean ± SE of quadruplicate determinations (from different wells).

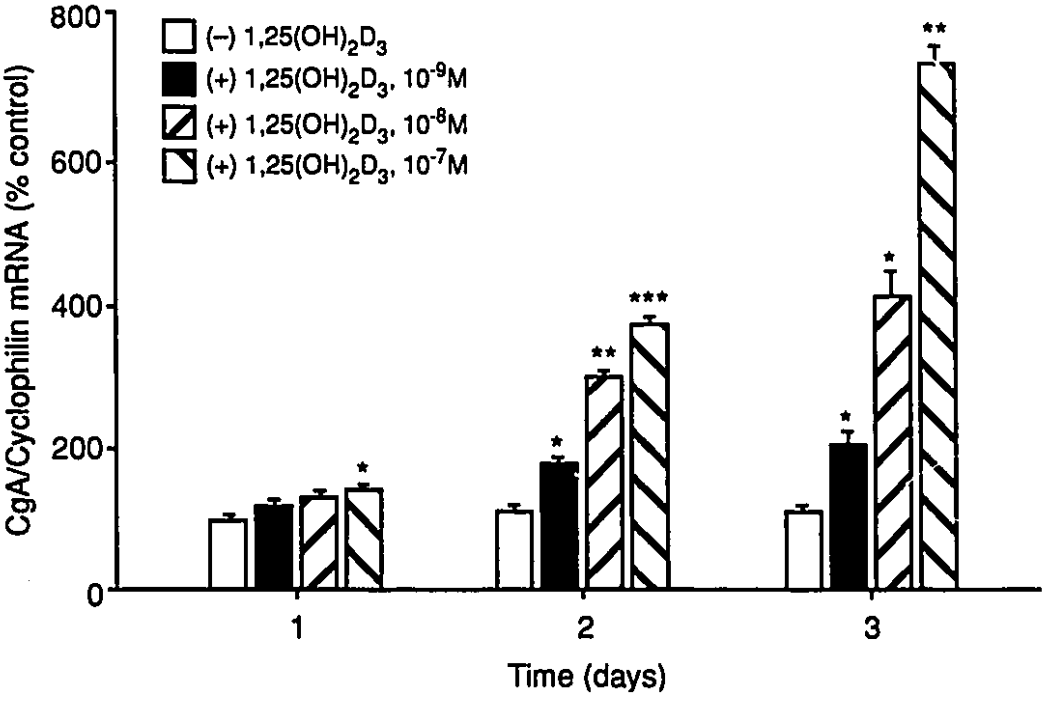

Figure 2.5 Effect of $1,25(OH)_2D_3$ on steady-state CgA mRNA levels. Bovine parathyroid cells were cultured in the absence (open bar) or presence of $1,25(OH)_2D_3$: 10^{-9}M (filled bar), 10^{-8}M (left hatching), 10^{-7}M (right hatching) for 1-3 days, harvested and CgA mRNA levels assessed as described in the text. Each point is the mean ± SE of quadruplicate determinations (from different wells). Asterisks indicate a significant difference from control *p<0.02; **p<0.001; ***p<0.0001.

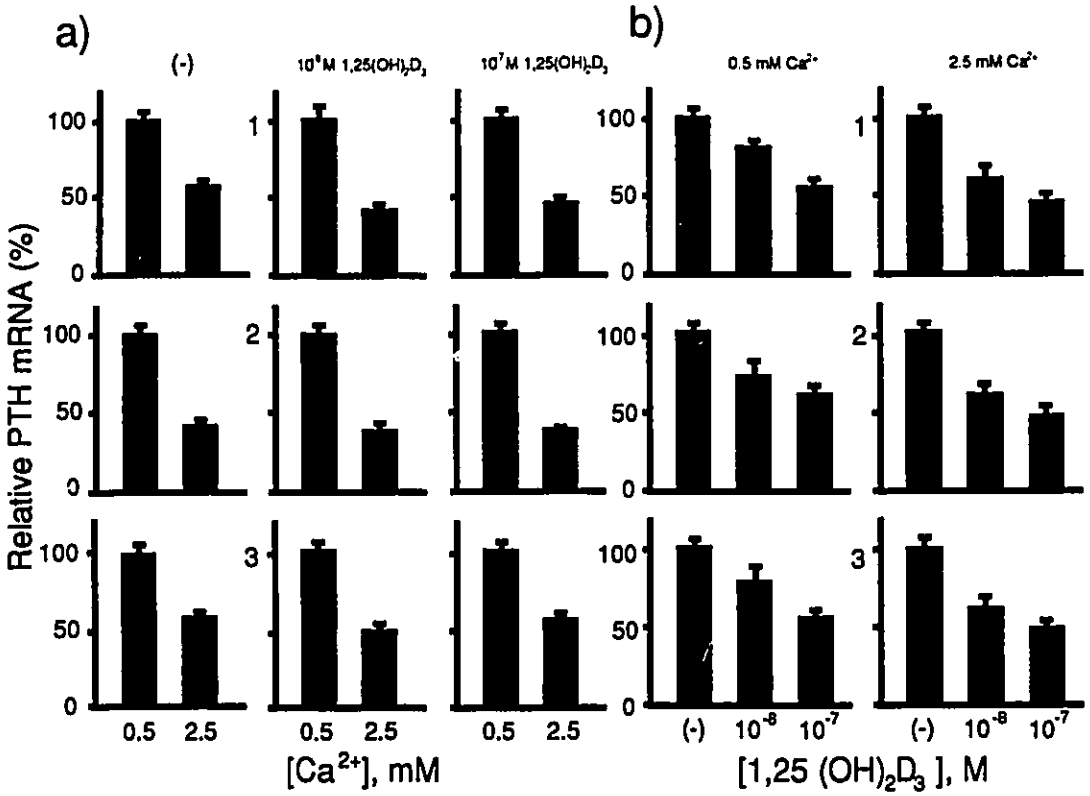

Figure 2.6 Effects of extracellular calcium and $1,25(OH)_2D_3$ on PTH mRNA levels. Bovine parathyroid cells were cultured up to three days in either a low (0.5 mM) or high (2.5 mM) calcium concentration, in the absence or presence of 10^{-8} M or 10^{-7}M $1,25(OH)_2D_3$. Cells were harvested at 1, 2 and 3 days and PTH mRNA measured as described in *Materials and Methods*. Each point is the mean ± SE of quadruplicate determinations. In Fig. 6a all data for cells cultured in 0.5 mM calcium [with and without $1,25(OH)_2D_3$] are expressed as 100% in order to assess the effect of 2.5 mM Ca^{2+} vs 0.5 mM Ca^{2+} on PTH mRNA. In Fig. 6b all data for cells cultured without $1,25(OH)_2D_3$ (in 0.5 mM and 2.5 mM Ca^{2+}) are expressed as 100% in order to assess the effect of $1,25(OH)_2D_3$ on PTH mRNA.

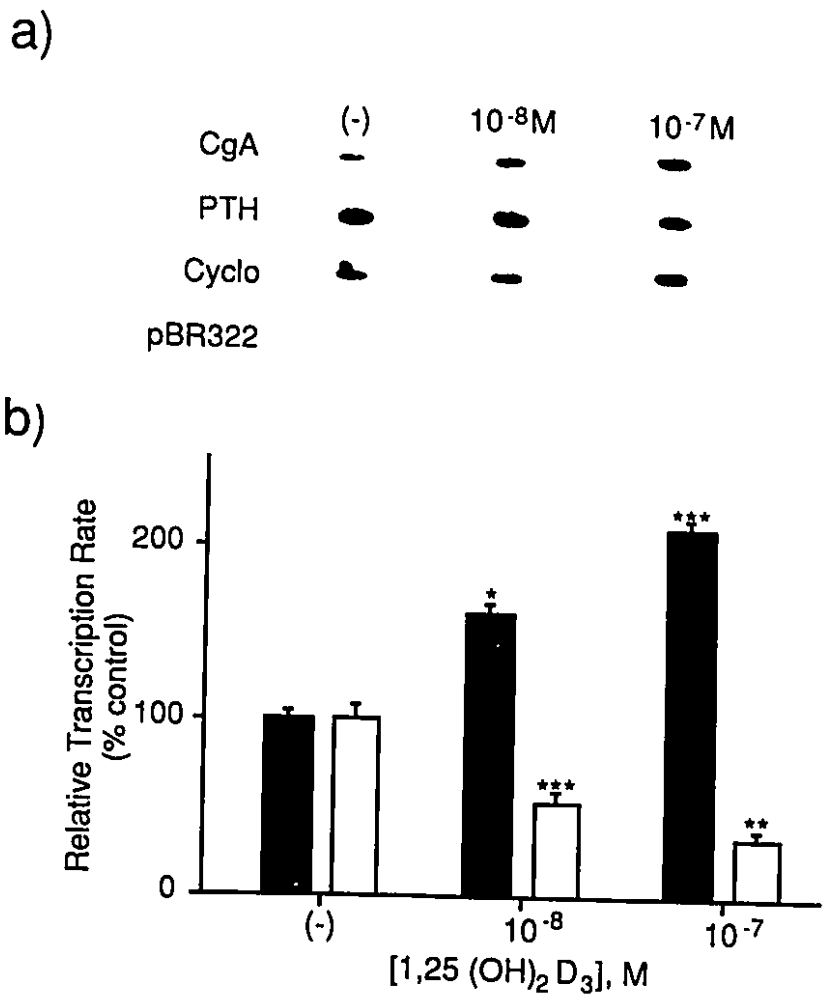

Figure 2.7 Effect of $1,25(OH)_2D_3$ on parathyroid gene transcription. Nuclear run-on assays were performed as described in Materials and Methods on extracts of parathyroid cells cultured for 10 hours in the absence or presence of $1,25(OH)_2D_3$. Autoradiographs of a representative experiment are shown in the top panel: CgA, PTH, cyclophilin (Cyclo) and plasmid pBR322 with no insert to control for nonspecific binding. The mean relative transcription rates ± SE (from three separate experiments) for CgA (filled bar) and PTH (open bar) are shown in the bottom panel. Asterisks indicate a significant difference from control: *$p<0.01$; **$p<0.005$; ***$p<0.001$.

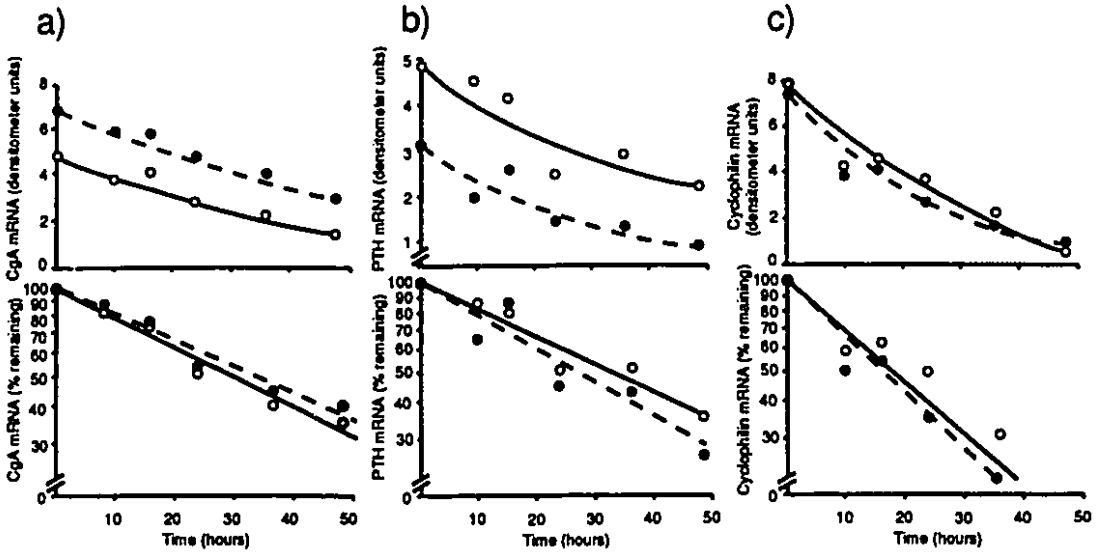

Figure 2.8 Effect of $1,25(OH)_2D_3$ on CgA, PTH and cyclophilin mRNA stability. Bovine parathyroid cells were treated with (●--●) or without (O--O) 10^{-8}M $1,25(OH)_2D_3$ for 24 hours after which medium was replaced with the same medium with the addition of 25 µg/mL DRB (Time 0). Total RNA was isolated at 0, 10, 16, 24, 36 and 48 hours and analyzed by Northern blot. Panels (a), (b) and (c) show the decay curves for CgA, PTH and cyclophilin mRNAs, respectively, with the data as a semi-logarithmic plot in the bottom panels.

CHAPTER 3

1,25-DIHYDROXYCHOLECALCIFEROL REGULATES CHROMOGRANIN A mRNA TRANSLATABILITY IN BOVINE PARATHYROID CELLS

3.1 PREFACE

Chapter 2 described the detailed analysis of the regulation of the synthesis and secretion of chromogranin A by calcium and 1,25-dihydroxycholecalciferol. A poor correspondence between the effects of 1,25-dihydroxycholecalciferol on chromogranin A gene transcription and chromogranin A protein synthesis in parathyroid cells was found. This chapter extends the findings presented in Chapter 2 and examines the regulation of chromogranin A synthesis at the level of mRNA translation. The data presented in this chapter reveals an important effect of 1,25-dihydroxycholecalciferol on parathyroid cell mRNA translation, where 1,25-dihydroxycholecalciferol is shown to have a specific effect on CgA mRNA translatability. Specifically, 1,25-dihydroxycholecalciferol causes a two-fold increase in the CgA mRNA ribosome transit time, and thereby explains the lack of quantitative correspondence between CgA gene transcription and CgA synthesis in parathyroid cells treated with 1,25-dihydroxycholecalciferol.

Figures 3.1, 3.6-3.9 were not included in the published manuscript but have been included in this chapter for completeness.

3.2 ABSTRACT

Our previous studies indicated that regulation of bovine parathyroid chromogranin A (CgA) by 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] may occur at the level of CgA turnover or of mRNA translation. In the present study, immunoprecipitation of extracts of bovine parathyroid cells which had been pulse-chased with [^{35}S]methionine revealed that 1,25$(OH)_2D_3$ had no effect on the disappearance time of intracellular CgA. Therefore, we examined the effect of 1,25$(OH)_2D_3$ on the polyribosome profile of CgA mRNA analyzed by sucrose density gradients. In the presence of 1,25$(OH)_2D_3$, there was a dose-dependent recruitment of CgA mRNA into the denser polyribosomal fractions by 24 hours. In order to determine whether this increased ribosome loading represents increased or decreased efficiency of mRNA translation, ribosome transit time experiments were conducted. The average ribosome transit time and the specific PTH ribosome transit time were not altered by 1,25$(OH)_2D_3$. However, that for CgA was doubled in the presence of 1,25$(OH)_2D_3$. Thus, parathyroid CgA synthesis is regulated by the vitamin D sterol at the level of peptide chain elongation. These studies, therefore, explain the lack of quantitative correspondence between 1,25$(OH)_2D_3$-induced CgA gene transcription and CgA protein levels by revealing a previously unsuspected level of regulation of mRNA translation in the parathyroid cell.

3.3 INTRODUCTION

Chromogranin A (CgA) is an acidic glycoprotein found in many endocrine cells which is co-stored and co-secreted with the resident hormone (1). It is likely that CgA serves as a precursor to bioactive peptides which modulate secretion in an autocrine and/or a paracrine manner (2-7) and that regulation of CgA expression and processing is tissue-specific (8,9). CgA may also have intracellular roles such as sequestration of calcium in processing, sorting, granule condensation or exocytosis (2,10,11). Despite an incomplete picture of CgA's functions, it probably plays a major role in secretory activity because of its widespread distribution in endocrine and neuroendocrine cell types, of both mammalian and fish species (12).

In the parathyroid, CgA is stored and secreted with parathyroid hormone (PTH) (13,14). The secretion of both these polypeptides is regulated in a similar fashion by extracellular calcium concentrations such that low calcium stimulates, and high calcium concentrations inhibit, secretion (15,16). Calcium inhibits PTH gene transcription (17), but

does not affect CgA gene transcription (18,19). Besides calcium, another major regulator of parathyroid cell function is 1,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$] which inhibits PTH synthesis at the level of gene transcription (20,21). On the other hand, 1,25$(OH)_2D_3$, has been shown recently to enhance CgA gene transcription in the parathyroid (18,19). However, while the magnitude of the changes in PTH gene transcription are reflected at the synthetic level (22), there is a lack of correlation between the relative changes in CgA mRNA levels by 1,25$(OH)_2D_3$ and the changes in CgA synthesis. This would suggest an additional level of regulation by 1,25$(OH)_2D_3$ on either CgA protein turnover and/or CgA mRNA translation. This report addresses both issues.

In this study, metabolic labelling and pulse-chase studies showed that 1,25$(OH)_2D_3$ has no effect on cellular CgA protein turnover. However, the vitamin D metabolite negatively influences the translational status of CgA mRNA as assessed by polyribosome profile and ribosome transit time analyses. This explains the lack of correspondence between 1,25$(OH)_2D_3$-induced effects on CgA gene transcription and CgA protein levels and reveals an important effect of the vitamin D sterol on mRNA translatability.

3.4 RESULTS

3.4.1 EFFECTS OF 1,25(OH)2D3 ON CgA mRNA AND CgA PROTEIN LEVELS

Bovine parathyroid cells were cultured in the absence or presence of 10^{-8}M 1,25$(OH)_2D_3$ for 48h. Whereas CgA mRNA was stimulated 3-fold by 1,25$(OH)_2D_3$ there was only a 40% increase in total immunoassayable CgA (cells + medium) in response to the vitamin D metabolite (Figure 3.1). The results described in this and subsequent sections are representative of experiments that were repeated three or four times.

3.4.2 EFFECT OF 1,25$(OH)_2D_3$ ON CgA PROTEIN TURNOVER

As shown in Figure 3.2, 1,25$(OH)_2D_3$ did not affect the clearance rate of metabolically-labelled CgA from parathyroid cells. The half-times of disappearance of CgA in 1,25$(OH)_2D_3$-treated and untreated parathyroid cells were virtually identical. The initial rapid rate of CgA accumulation in the medium was greater in 1,25$(OH)_2D_3$-treated cultures, however at 6h the rate of accumulation decreased in both 1,25$(OH)_2D_3$-treated and

untreated cultures. This later, slower rate of accumulation was unaffected by $1,25(OH)_2D_3$.

3.4.3 POLYRIBOSOME PROFILE ANALYSIS OF PARATHYROID CELL mRNA

Polyribosome sucrose density gradient profiles from parathyroid cells harvested and processed in lysis and gradient buffers containing $MgCl_2$ or without $MgCl_2$, but with EDTA, are shown in Figure 3.3. In the presence of EDTA there was an increase in OD_{260} absorbing material in fractions 18-22 which corresponds to the 80*S* monosomes and free ribosomal subunits, which have been liberated by the action of the chelating agent. This effect of EDTA was reflected in the shift in position of the CgA mRNA in the sucrose gradient. In the presence of $MgCl_2$ the CgA mRNA eluted with the denser polyribosome fractions indicating active translation. In the presence of EDTA the CgA mRNA eluted only in the lighter fractions indicating that polyribosome formation has been disrupted.

The OD_{260} profiles in the presence and absence of $MgCl_2$ were identical for $1,25(OH)_2D_3$-treated and untreated cells (data not shown).

3.4.4 EFFECTS OF $1,25(OH)_2D_3$ ON THE CgA, PTH AND α-ACTIN mRNA POLYRIBOSOME PROFILES

After 2 days of culture with $1,25(OH)_2D_3$, CgA mRNA levels of bovine parathyroid cells were increased, whereas PTH mRNA levels were decreased (Figure 3.4A). Polyribosome profile analysis revealed that CgA mRNA sedimented in both heavy and light polyribosome fractions and there was a marked increase in CgA mRNA sedimenting in the heavier polyribosome fractions after $1,25(OH)_2D_3$ treatment (Figure 3.4B, C and Figure 3.5). This recruitment of CgA mRNA into the more rapidly sedimenting polyribosome fractions was dose-dependent through a tested dose-range of 10^{-9}M to 10^{-7}M (data not shown). PTH mRNA also sedimented in both heavy and light polyribosome fractions; after $1,25(OH)_2D_3$ treatment there was a reduction in the light polyribosome fractions, but the amount of PTH mRNA sedimenting in the heavy polyribosome fractions did not change (Figure 3.4B, and C and Figure 3.5). The polyribosome profile of α-actin mRNA was not affected by $1,25(OH)_2D_3$ (Figure 3.5). The effects of $1,25(OH)_2D_3$ on the respective mRNAs noted at 2 days were also observed at the 24 and 72 hr time points.

3.4.5 EFFECT OF CYCLOHEXIMIDE ON POLYRIBOSOME LOADING

The altered polyribosome profile observed for CgA mRNA could be brought about by a decrease in the rate of peptide elongation. Therefore, low-dose cycloheximide, which itself reduces the rate of peptide chain elongation, was used to determine the rate-limiting step for translation. Cycloheximide treatment alone was effective in increasing the proportion of CgA mRNA in the denser polyribosomes. Cycloheximide treatment of $1,25(OH)_2D_3$-stimulated parathyroid cells caused no further loading of ribosomes onto the CgA mRNA polyribosomes compared to that seen with $1,25(OH)_2D_3$ alone (Figure 3.6). This indicated that the effect of the vitamin D sterol was at the level of elongation.

3.4.6 EFFECT OF $1,25(OH)_2D_3$ ON POLYRIBOSOME DISAGGREGATION

In order to assess whether $1,25(OH)_2D_3$ caused a generalized slowing of the elongation rate of all CgA mRNAs or only blocked elongation of a fraction of the CgA mRNAs, pactamycin (an initiation inhibitor) was used to disaggregate the polyribosomes. The rate or extent of polyribosome disaggregation was not affected by the vitamin D sterol (Figure 3.7). Pactamycin treatment was only partially successful in disrupting polyribosome formation; the denser polyribosomes were affected, but the lighter ones were not.

3.4.7 EFFECTS OF $1,25(OH)_2D_3$ ON PARATHYROID CELL mRNA RIBOSOME TRANSIT TIMES

$1,25(OH)_2D_3$ had no effect on the average ribosome transit time (Figure 3.8) or on the specific PTH mRNA ribosome transit time (Figure 3.9) in parathyroid cells (Table 3.1). However, that for CgA mRNA was increased approximately 2-fold in $1,25(OH)_2D_3$-treated cells (Figure 3.10 and Table 3.1).

3.5 DISCUSSION

We have demonstrated that $1,25(OH)_2D_3$ influences the translational efficiency

of CgA mRNA in parathyroid cells. Administration of the vitamin D metabolite resulted in an approximate doubling of the ribosome transit time of CgA mRNA. The marked recruitment by $1,25(OH)_2D_3$ of CgA mRNA into the dense polyribosome fractions shown by sucrose density gradient analysis could be interpreted in one of two ways. On the one hand, this might have suggested that CgA mRNA was being more actively translated by having a greater number of ribosomes attached to it. On the other hand, the sedimentation profile observed would be consistent with a decrease in the rate of polypeptide elongation, without a concomitant decrease in translation initiation. The use of low-dose cycloheximide as an elongation inhibitor (23) indicated that the effect of $1,25(OH)_2D_3$ was at the level of peptide elongation.

Having established this, the altered polyribosome profile of CgA mRNA in the presence of the vitamin D sterol could then be due to either a generalized slowing of the elongation rate of all CgA mRNAs or blocked elongation of CgA nascent peptide for most CgA mRNAs with a fraction of the mRNAs escaping the block. In order to distinguish between these two possibilities we used the initiation inhibitor, pactamycin (24). There was a release of CgA mRNA from polyribosomes in both $1,25(OH)_2D_3$-treated and untreated cells in the presence of pactamycin. This indicated that the reduced rate of CgA mRNA translation is due to a slowing of the CgA mRNA elongation rate rather than a selective blockade.

The polyribosome profiles of all three mRNAs (CgA, PTH & actin) studied indicated that they were associated with a dense and a light polyribosome pool. In the case of PTH mRNA the light polyribosome pool was reduced by $1,25(OH)_2D_3$ treatment whereas, in contrast to the result with CgA mRNA, the dense polyribosome pool was unaffected. However, this alteration in polyribosome profile did not lead to a change in ribosome transit time. Therefore, there is no evidence that $1,25(OH)_2D_3$ affects PTH mRNA translatability *per se*. Most likely the PTH mRNA is preferentially recruited into the dense polyribosome pool and the alteration in the profile is brought about by the reduction in PTH mRNA levels due to the negative effect of $1,25(OH)_2D_3$ on PTH gene transcription rate (20,21). The elucidation of the precise nature of the different polyribosome pools will require further study.

Members of the steroid hormone family have been shown to act at post-transcriptional sites by, for example, altering mRNA stability (25-27), affecting peptide chain initiation (28,29) and elongation (30, 31; this study), and at post-translational sites by activating protein glycosylation (32) and the pattern of processing of secretory proteins (33,34). With respect to the latter point, we found no evidence in our study for an effect of $1,25(OH)_2D_3$ on the rate of parathyroid CgA degradation. This was also the conclusion

reached previously by Ridgeway and McGregor (35) in their studies of CgA synthesis in cultured parathyroid 'organoids'.

Earlier studies which examined steroid hormone effects on protein biosynthesis in liver explants led to the description of a similar phenomenon to the one reported here. Whereas estradiol stimulated vitellogenin gene transcription, it also resulted in increased polyribosome size and ribosome transit time (30). In most cases, how steroid hormones influence mRNA translatability and specifically, the mechanism whereby $1,25(OH)_2D_3$ modulates CgA mRNA translation, remains to be elucidated. It is likely that $1,25(OH)_2D_3$ acts by affecting the binding of protein factor(s) which influence peptide chain elongation and/or by altering the secondary structure of CgA mRNA. It has been reported in preliminary form (36) that $1,25(OH)_2D_3$ caused a dissociation of a repressor RNA-binding protein from the 3'-untranslated region of creatinine kinase-b mRNA in U937 cells, although the precise mechanism is not known. Recently, a nonanucleotide sequence in the 5'-untranslated region of myelin basic protein mRNA was identified which confers responsiveness to hydrocortisone (37). This effect was demonstrated in an in vitro cell-free translation assay and did not require the presence of the nuclear glucocorticoid receptor. Similar studies to these will be helpful in elucidating the mechanism where by $1,25(OH)_2D_3$ modulates parathyroid CgA mRNA translational efficiency.

In summary, our results explain the lack of quantitative correspondence between $1,25(OH)_2D_3$-induced CgA gene transcription and CgA protein synthesis. Thus, in response to $1,25(OH)_2D_3$ there was an approximate three-fold increase in steady state CgA mRNA level, but a doubling in ribosome transit time which led to the overall slight increase in CgA protein observed. Potentially, therefore, the physiological significance of these findings may be that translational regulation by the vitamin D sterol provides a fine control mechanism to counterbalance the exhuberent positive CgA gene transcriptional effect. In the light of CgA's putative function as a precursor to bioactive peptides which modulate hormone secretion (38), regulation of CgA synthesis at the level of translation may be another way in which protein synthesis is tightly coupled to secretion in endocrine cells.

3.6 MATERIALS AND METHODS

3.6.1 PREPARATION OF BOVINE PARATHYROID CELLS

Bovine parathyroid glands were obtained from a local slaughterhouse and transported in ice-cold Minimum Essential Medium (MEM) containing penicillin (100 U/ml)

and streptomycin (100 µg/ml). Dispersed cells were prepared from the glands as described (19).

3.6.2 PARATHYROID CELL CULTURES

Cells were suspended in MEM, 10 mM HEPES, antibiotics, 5% FCS, 0.5 mM $MgCl_2$, and 1mM $CaCl_2$ with and without added 10^{-9}M, 10^{-8}M or 10^{-7}M $1,25(OH)_2D_3$ (kindly provided by Dr. M. Uskokovic, Hoffman-LaRoche, Nutley, NJ) and plated at a concentration of 600,000 cells/ml in 100 mm NUNC tissue culture dishes (Fisher Scientific, Montreal, Que). For ribosome transit time analyses and pulse-chase studies, cells were plated in 60 mm LUX (Miles Scientific, Naperville, IL) tissue culture dishes. Medium was replaced on a daily basis. Viability was typically over 95% as assessed by trypan blue exclusion. Radioimmunoassay of CgA in cells and medium was carried out as described previously (19).

3.6.3 PROTEIN TURNOVER ESTIMATIONS

To determine the effects of $1,25(OH)_2D_3$ on the rate of CgA turnover, pulse-chase studies were performed. Parathyroid cells were plated in 60 mm LUX tissue culture dishes in methionine-free medium for 24 h, in the absence or presence of 10^{-8}M $1,25(OH)_2D_3$. Cells were then pulsed with 200 µCi/ml [^{35}S]methionine (1000 mCi/mmol; Trans[^{35}S]-label, ICN, Baie d'Urfé, Que) for 24 h. Cells were chased at this time point (time 0) with methionine replete medium. At 1, 3, 6, 12 and 24 h supernatants were collected and cell extracts made by lysing cells in 1 ml immunoprecipitation buffer (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM EDTA, and 1% NP-40) containing 10 µM leupeptin, 1 mM PMSF and 10 µM pepstatin (39). Cells were treated with ethanol carrier or $1,25(OH)_2D_3$ (10^{-8}M) throughout. Cell extracts were spun at 20 000 *x g* for 30 min to remove cell debris and membranes and stored at -80°C until assay.

Immunoprecipitation of secreted CgA was performed by diluting the conditioned medium 1:5 in immunoprecipitation buffer in a total volume of 1 ml. To minimize non-specific binding, cell lysates and diluted supernatants were incubated for 2 h with 30 µl 50:50 (v/v) in water slurry of Protein A-Sepharose (Pharmacia, Mississauga, Ont) and 15 µl non-immune rabbit serum. After removal of the Protein A-Sepharose beads, a saturating amount of a rabbit anti-bovine CgA antiserum 4253 (19) was added and the

reaction was incubated for 1 h at 4°C with constant rotation. To control for non-specific binding, either normal rabbit serum was added as above, or the immune serum was preabsorbed with 50 µg purified CgA for 2 h before it was added to the immunoprecipitation reaction. Twenty µl of 50:50 (v/v) slurry of Protein A-Sepharose in water were added and incubated an additional hour to bind the immune complexes. The Protein A-Sepharose-antibody/antigen complexes were centrifuged 16 000 *x g*, for 20 seconds and the pellets were washed three times with immunoprecipitation buffer. After resuspension of immune complexes with buffer, an aliquot was counted by liquid scintillation counting, and the counts obtained at each time point were related to the number of counts at zero time.

3.6.4 POLYRIBOSOME ANALYSIS AND SUCROSE GRADIENT PREPARATION

At 1, 2 or 3 days of culture post-mitochondrial supernatants were prepared from parathyroid cells and fractionated on 10-50% sucrose gradients (40). Cells were scraped with a rubber policeman into 10 ml ice-cold PBS, pH 7.4 containing 1 mM EDTA. Cells were pelleted and lysed with 1 ml lysis buffer [250 mM KCl, 10 mM $MgCl_2$, 20 mM HEPES, pH 7.3, 0.25 M sucrose, 2 mM DTT, 150 µg/ml cycloheximide, 0.5% NP-40 and 40 U/ml RNAde (Bio/Can Scientific, Mississauga, Ont)]. Cells were allowed to swell for 8 min and homogenized with twenty strokes of a dounce homogenizer. Sodium deoxycholate was added to 0.5% and the homogenate was centrifuged at 16 000 *x g* for 20 min. Equal quantities of the post-mitochondrial supernatants, determined by absorbance at 260 nm, were layered onto 10-50% sucrose density gradients in a Beckman SW40 ultracentrifuge tube and spun for 5 h at 26 000 rpm, (85,330 *x g*) 8°C. From each gradient, thirty 0.4ml fractions were collected and the protein-RNA complex precipitated with 2.5 volumes of ethanol. This was resuspended in 300 µl NETS (100 mM NaCl, 2.5 mM EDTA, 1% SDS, 20 mM Tris-HCl, pH 7.4) and the RNA extracted once with phenol:chloroform:isoamylalcohol (50:48:2) and ethanol precipitated. RNA was taken up in 5 µl DEPC-treated water, fractionated on a 1% agarose-1.6M formaldehyde gel, and transferred to nylon membranes (Nytran, Schleicher & Schuell, Keene, NH). For some experiments, thirty 0.4 ml fractions were collected and the absorbance (260 nm) for each fraction was read.

The gradient buffer was identical to the homogenizing buffer except that the concentration of KCl was 0.5M, and 10U/ml sodium heparin was added instead of RNAde.

In control experiments 20 mM EDTA was used instead of 10 mM $MgCl_2$ in both homogenization and gradient buffers to disrupt Mg^{2+}-dependent association of ribosomes and mRNA.

3.6.5 ASSESSMENT OF mRNA LEVELS

Prehybridization and hybridization of Northern blots were performed in 50% formamide, 5 x SSC, 25 mM $NaPO_4$, pH 7.4, 1% SDS, 2 mM EDTA, 1 X Denhardt's and 200 µg/ml sheared salmon sperm DNA. DNA probes, labeled with [^{32}P]dCTP to specific activities of 109 cpm/µg by the random primer method (41), were as follows: 1) bovine CgA, the 528-basepair PstI restriction fragment from plasmid pCHRG4A (42); 2) bovine PTH, the 500-basepair PstI restriction fragment from plasmid pPTHm29 (43); 3) human α-actin, the 500-basepair EcoRI restriction fragment from plasmid pHMαA-PX (44). Membranes were washed to high stringency (0.1 x SSC, 0.1% SDS, 60°C) and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY). The relative intensities of the resulting autoradiographic images were assessed using an LKB Ultroscan XL densitometer (LKB, Baie D'Urfé, Que).

3.6.6 POLYRIBOSOME LOADING IN THE PRESENCE OF CYCLOHEXIMIDE

These experiments were carried out as described previously (24) using low-dose cycloheximide. Parathyroid cells were cultured in the presence or absence of $1,25(OH)_2D_3$ for 48h. The medium was then replaced with addition of cycloheximide (1 µg/ml) to all samples. This concentration inhibited radiolabelled amino acid incorporation into acid-precipitable radioactivity by 95% (data not shown). Post-mitochondrial supernatants were prepared at 0, 6, and 10 min after cycloheximide addition. Polyribosome and Northern blot analyses were performed as described above.

3.6.7 ESTIMATION OF POLYRIBOSOME DISAGGREGATION RATE

Parathyroid cells were treated with 10^{-8}M $1,25(OH)_2D_3$ or ethanol carrier alone for 48 h. Medium was then replaced with addition of 10^{-8}M pactamycin (The Upjohn Company, Kalamazoo, MI) as described (45). This concentration inhibited [^{3}H]leucine

incorporation into acid-precipitable protein by over 97% (not shown). At 0, 3, 7, and 20 min after addition, post-mitochondrial supernatants were prepared. Polyribosome analysis and assessment of RNA levels were performed as described above.

3.6.8 RIBOSOME TRANSIT TIME ESTIMATIONS

Ribosome transit time analyses were performed essentially by the method of Gehrke et al. (30,31). Parathyroid cells were either treated with 10^{-8}M $1,25(OH)_2D_3$ or ethanol carrier alone for 48 h. Medium was replaced with leucine-deficient MEM containing 500 µCi/ml L-[4,5-^{3}H]leucine (Amersham; 82 Ci/mmol). After two h, cells were pulsed with 25 µCi/ml L-[U-^{14}C]leucine (Amersham; 310 mCi/mmol) and post-mitochondrial and post-ribosomal supernatants were prepared from cells harvested at 10, 15, 20, 25, and 30 min after [^{14}C]leucine addition. Cells were washed twice with ice-cold PBS, pH 7.4, and scraped into 2 ml cold MEM containing 200 µg cycloheximide, 100 mM L-leucine, 10 mM HEPES, pH 7.5, and 1 mM $CaCl_2$. Cells were pelleted, resuspended in 0.5 ml buffer (10 mM MES, pH 5.8, 300 mM NaCl, 20 mM $MgCl_2$, 2 mM DTT, 0.5% NP-40, 0.5% sodium deoxycholate, 150 µg/ml cycloheximide, 10 U/ml sodium heparin and 1.72 mM PMSF) and homogenized with ten strokes of a tight-fitting dounce homogenizer. The homogenate was made to 1 ml with lysis buffer and the nuclei and mitochondria were removed by centrifugation at 500 *x g* and 16 000 *x g*, respectively. Duplicate 50 µl aliquots were precipitated with 10% trichloroacetic acid. A post-ribosomal supernatant was prepared by centrifugation at 140 000 x g for 2 h, 4°C, in a Beckman Ti50 rotor (40 000 rpm). Duplicate aliquots were removed from the supernatant and precipitated. Two aliquots were diluted 1:5 in immunoprecipitation buffer and immunoprecipitation of CgA and PTH was performed as described above. Rabbit anti-bovine PTH(1-84) 7495 (unpublished) was used to immunoprecipitate PTH from the post-ribosomal supernatants. Each time point is the mean of two estimations and each estimation differed from the mean by 7% or less. Ribosome transit time analyses were performed at least four times on four separate occasions.

3.6.9 STATISTICAL ANALYSIS

Student's unpaired t test was used to test for significant differences between means. A p value < 0.05 was judged significant.

3.7 ACKNOWLEDGEMENTS

We thank Johanne Bourdon and Lee Ehler for their expert technical assistance, Drs. Lee Gehrke, David Goltzman and Nahum Sonenberg for their helpful comments, Drs. Lee Eiden and Hank Kronenberg for providing plasmids, The Upjohn Company for the pactamycin, and Shelley Hall and Karuna Patel for preparation of the manuscript.

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3.9 FIGURES

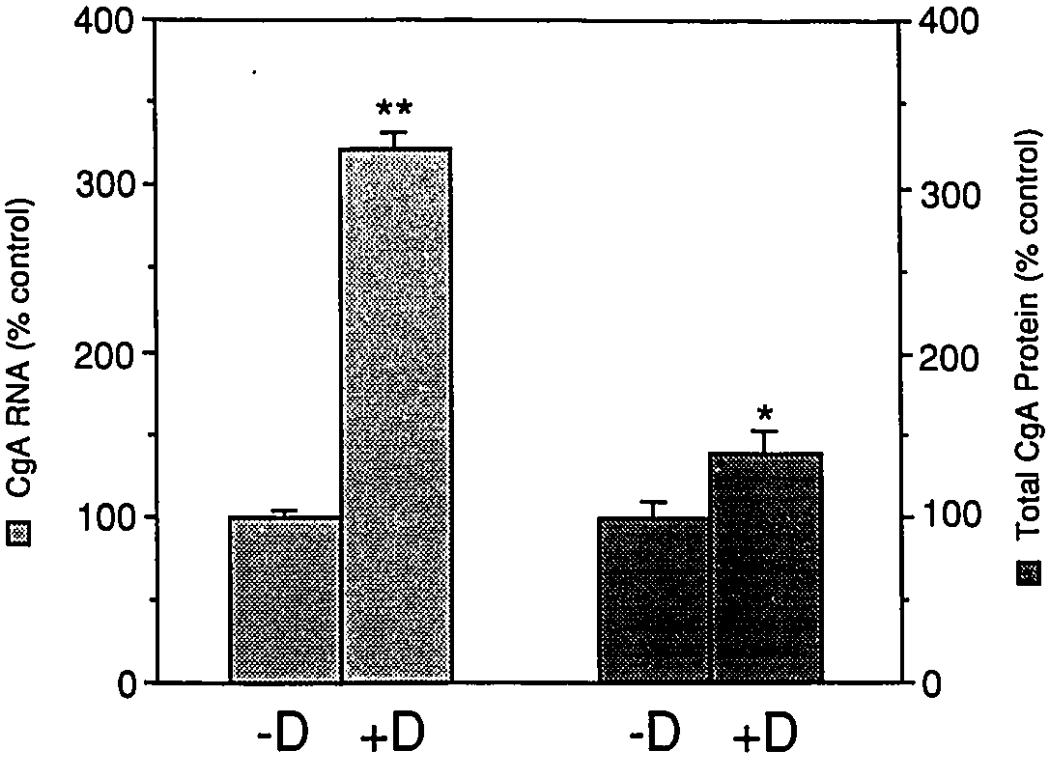

Figure 3.1 Effect of $1,25(OH)_2D_3$ on CgA steady-state mRNA and CgA protein levels in bovine parathyroid cells. Parathyroid cells were cultured for 48 h in the absence (-D) or the presence of 10^{-8}M $1,25(OH)_2D_3$. CgA mRNA (left) was assessed by Northern blot and CgA protein (right) was measured by RIA in cells and medium.

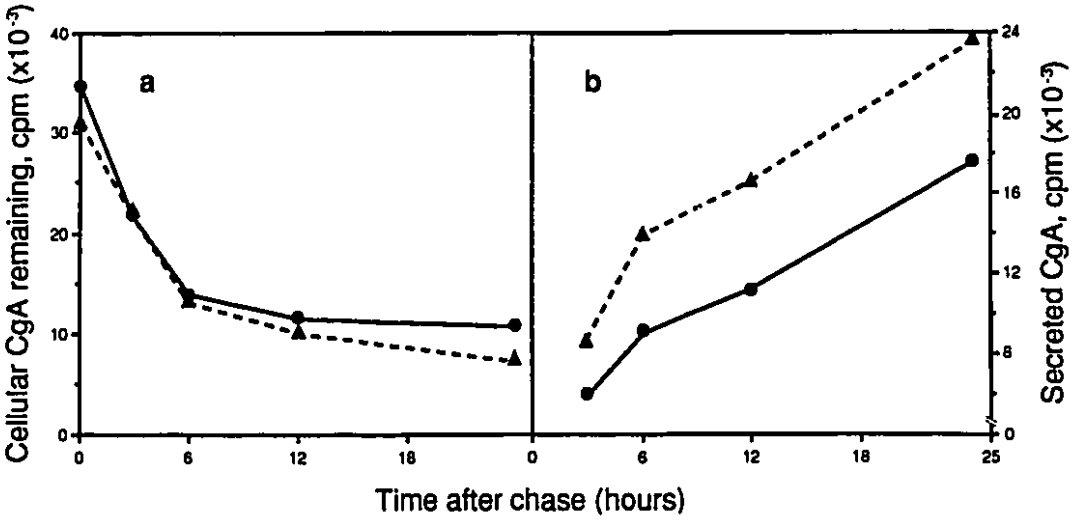

Figure 3.2 Effect of $1,25(OH)_2D_3$ on CgA protein turnover. Bovine parathyroid cells were cultured with (s) or without (l) 10^{-8}M $1,25(OH)_2D_3$ for 24h in methionine-deficient MEM after which cells were pulsed with radiolabelled methionine as described under *Materials and Methods.* The radioactive medium was then removed and replace with unlabelled methionine-replete MEM. Cell extracts were prepared and medium sampled at 0, 3, 6, 12, and 24 h, CgA was immunoprecipitated, and quantitated as described. Labelled CgA is expressed as the cpm that remained in cellular extract (panel a) or cpm that accumulated in medium (panel b).

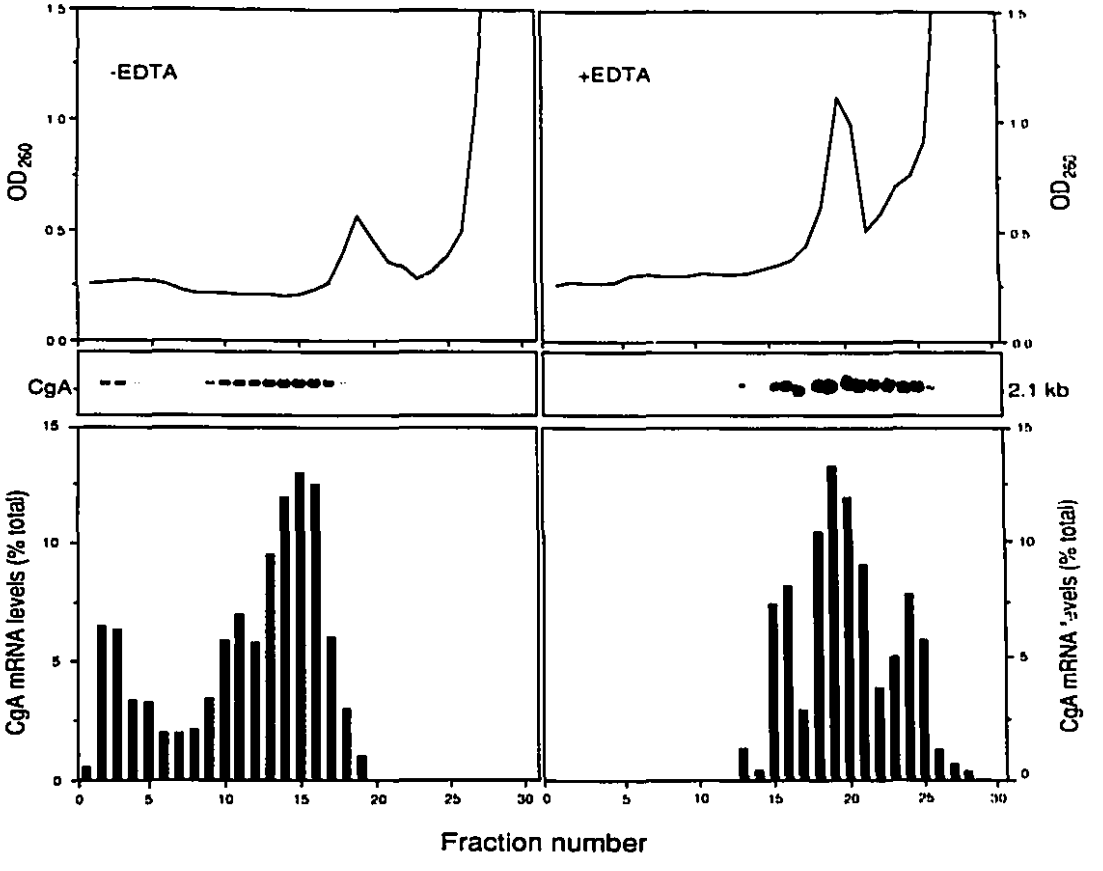

Figure 3.3 Polyribosome profile analysis of parathyroid cell mRNA. Bovine parathyroid cells were cultured for 48h and then lysed in buffers containing either 10 mM $MgCl_2$ (-EDTA, left-hand side), or 20 mM EDTA (+EDTA, right-hand side). Post-mitochondrial supernatants were prepared and analyzed by sucrose gradient and Northern blot analysis as described under *Materials and Methods*. Top panel: Sucrose density gradient OD_{260} absorbance profile. Center panel: Sucrose density gradient Northern blot analysis using a [^{32}P]-labelled CgA cDNA probe. Bottom panel: Results of densitometric scanning of the Northern analysis autoradiograph, expressed as % of the total hybridization signal.

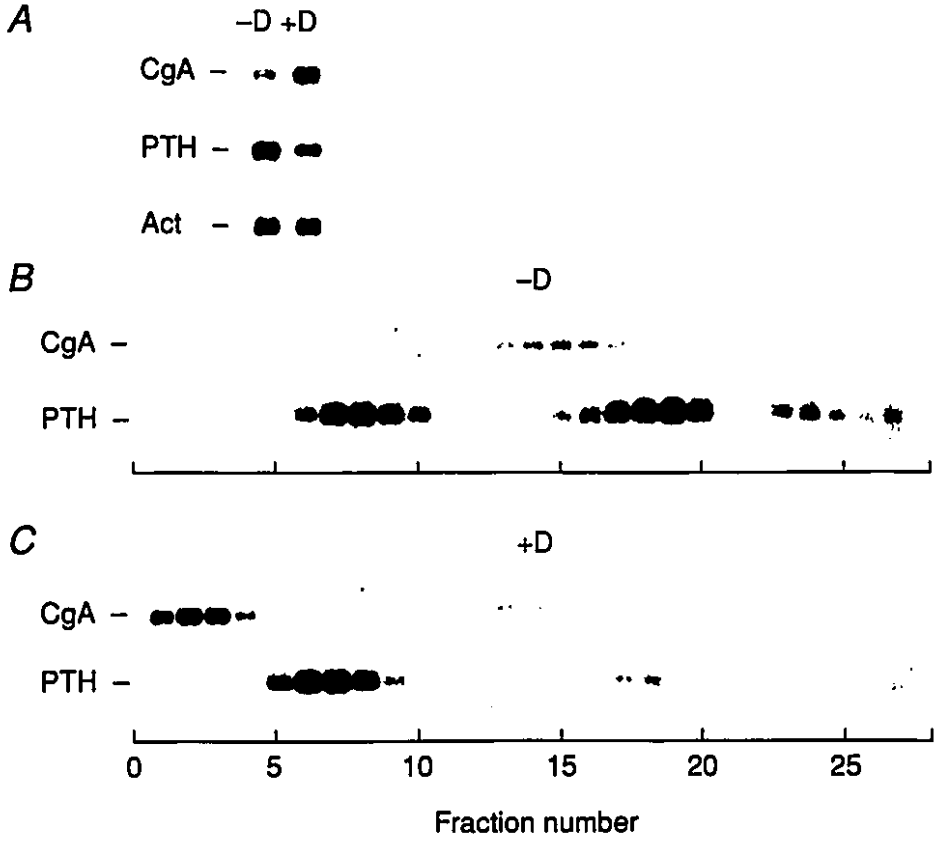

Figure 3.4 Effect of $1,25(OH)_2D_3$ on CgA, PTH and α-actin mRNA levels and CgA and PTH mRNA polyribosome profiles. Parathyroid cells were cultured for 48 h in the absence (-D) or presence (+D) of 10^{-8} M $1,25(OH)_2D_3$. Panel A: Total RNA was prepared and analyzed by Northern blot analysis using probes for CgA, PTH and α-actin mRNA. Panel B (-D) and panel C (+D): Post-mitochondrial supernatants were prepared and analyzed by sucrose-density gradient and Northern blot analysis as described under *Materials and Methods* and in the Figure 3.2 legend.

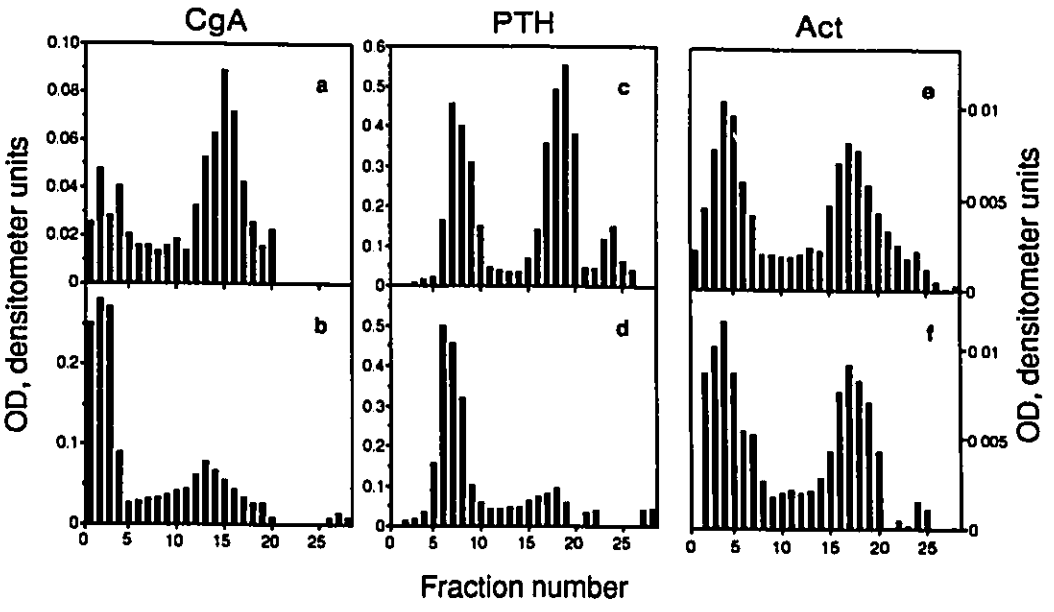

Figure 3.5 Effect of $1,25(OH)_2D_3$ on CgA, PTH and α-actin mRNA polyribosome profiles. Parathyroid cells were cultured for 48 h in the absence (a,c, and e) or presence (b, d, f) of 10^{-8}M $1,25(OH)_2D_3$. Postmitochondrial supernatants were prepared and analyzed by sucrose density gradient and Northern blot analysis, as described in *Materials and Methods* and Fig. 2. a and b, CgA mRNA; c and d, PTH mRNA; e and f, α-actin mRNA. Identical results were obtained at 24 and 72 h.

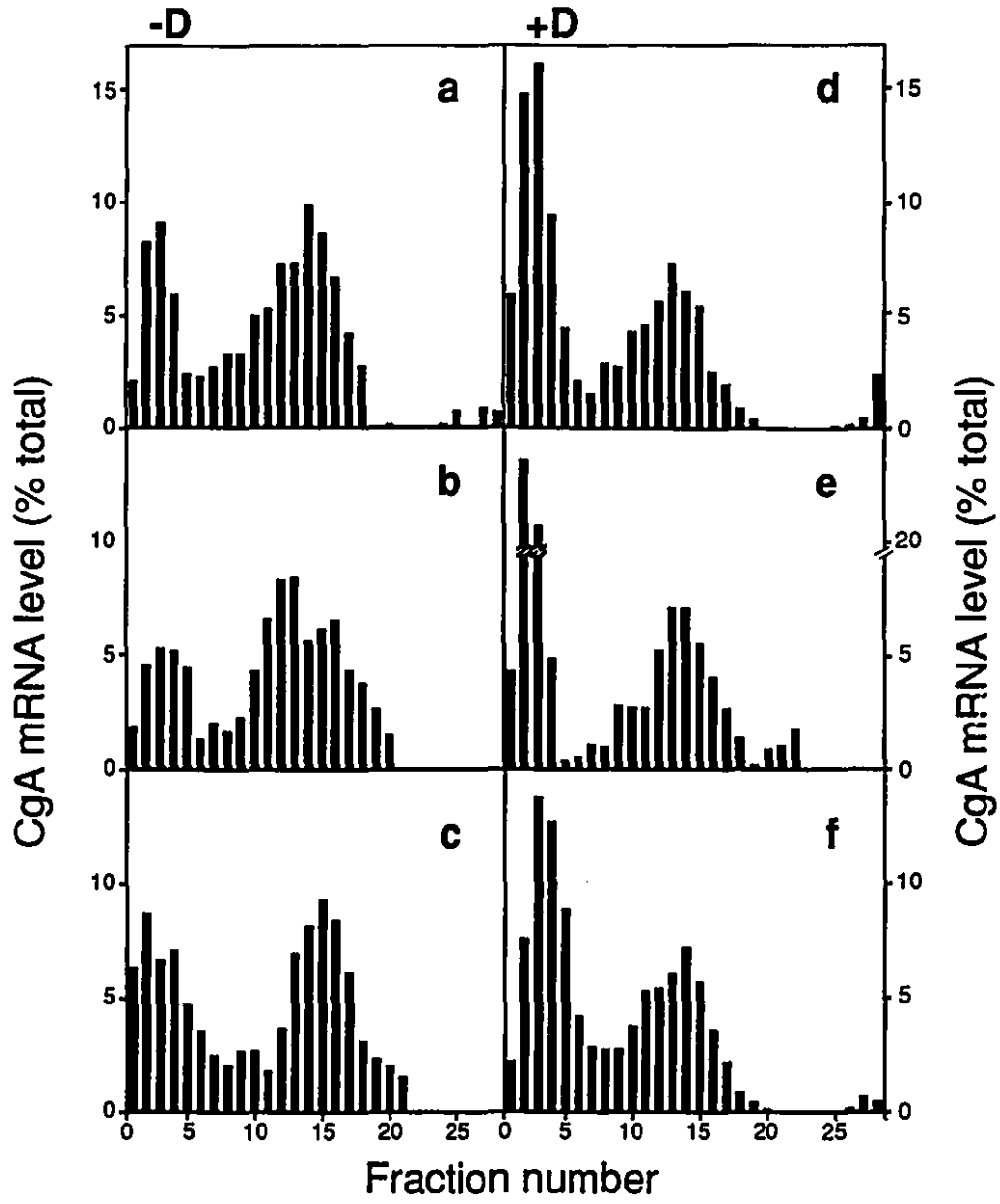

Figure 3.6 Polyribosome profile analysis in the presence of the translation elongation inhibitor, cycloheximide. Parathyroid cells were cultured for 48 h in the absence (-D; a, b, c) or presence (+D; d, e, f) of 10^{-8} M $1,25(OH)_2D_3$ after which cycloheximide was added (1μg/ml). Post-mitochondrial supernatants were prepared at 0 (a, d), 6 (b, e), and 10 (c, f) min and analyzed by sucrose-density gradient and Northern blot analysis as described under *Materials and Methods* and in the Figure 3.3 legend. A longer incubation (20 min) with cycloheximide resulted in a more marked recruitment of CgA mRNA into the denser polyribosomes in the absence of

$1,25(OH)_2D_3$ (not shown). The polyribosome profile of CgA mRNA in $1,25(OH)_2D_3$-treated parathyroid cells remained unchanged at this later time point (not shown). This demonstrated that no further loading of ribosomes occurred on the CgA mRNA in $1,25(OH)_2D_3$-treated parathyroid cells, and indicated that the effects of $1,25(OH)_2D_3$ were at the level of translation elongation .

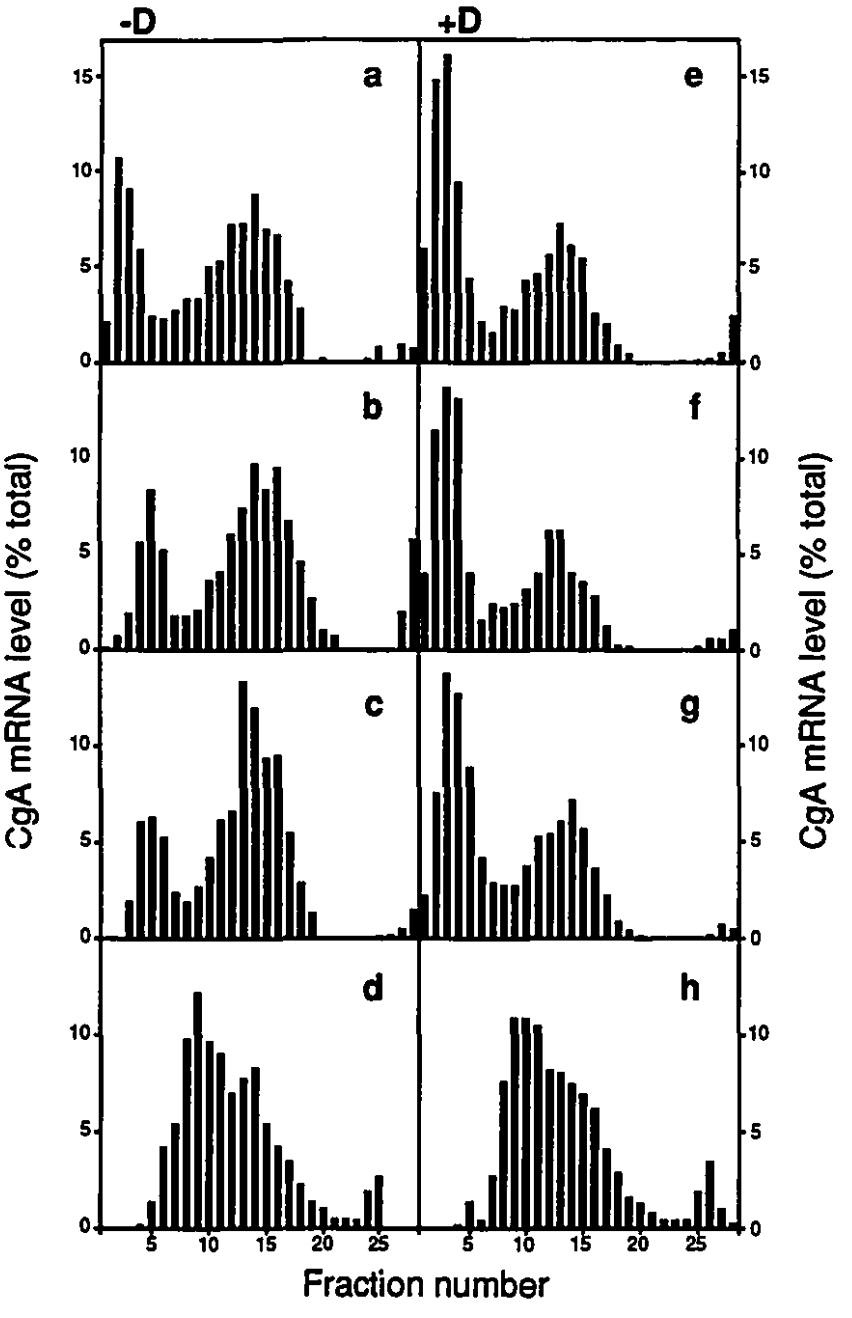

Figure 3.7 Polyribosome profile analysis in the presence of the translation initiation inhibitor, pactamycin. Parathyroid cells were cultured for 48 h in the absence (-D; a, b, c, d) or presence (+D; e, f, g, h) of 10^{-8} M $1,25(OH)_2D_3$ after which pactamycin (10^{-7} M) was added. Post-mitochondrial supernatants were prepared at 0 (a, e), 3 (b, f), 7 (c, g), and 20 (d, h) min and analyzed by sucrose-density gradient and Northern blot analysis as described under *Materials and Methods* and in the Figure 3.3 legend.

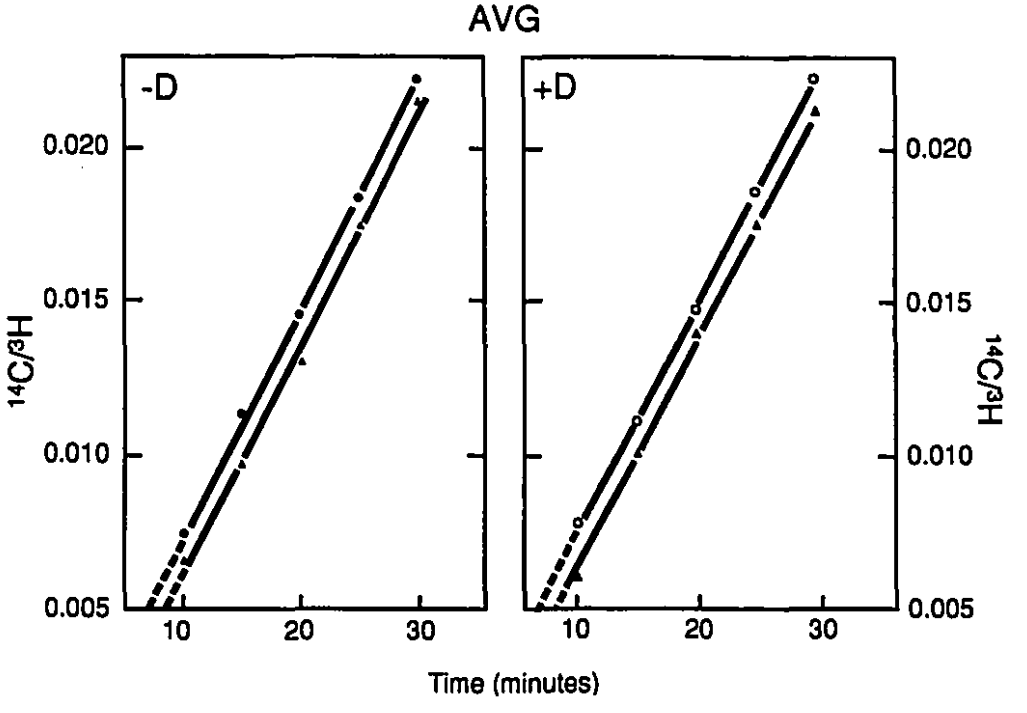

Figure 3.8 Effect of $1,25(OH)_2D_3$ on the average ribosome transit time of parathyroid cell mRNA in cultured parathyroid cells. Parathyroid cells were cultured for 48h in the absence (-D) or in the presence (+D) of 10^{-8}M $1,25(OH)_2D_3$. Cells were then pulsed with [^{3}H]leucine for 120 min, and then pulsed with [^{14}C]leucine. Cells were harvested up to 30 min post-[^{14}C] leucine addition. Acid-precipitable radioactivity was measured in post-mitochondrial (l) and post-ribosomal (s) supernatants as described under *Materials and Methods*. The ratio of [^{14}C]/[^{3}H] radioactivity was plotted versus time and the half-transit times calculated as described (Gehrke et al, 1981a,b). Each point is the mean of two estimations. Each estimation differed from the mean by 7% or less.

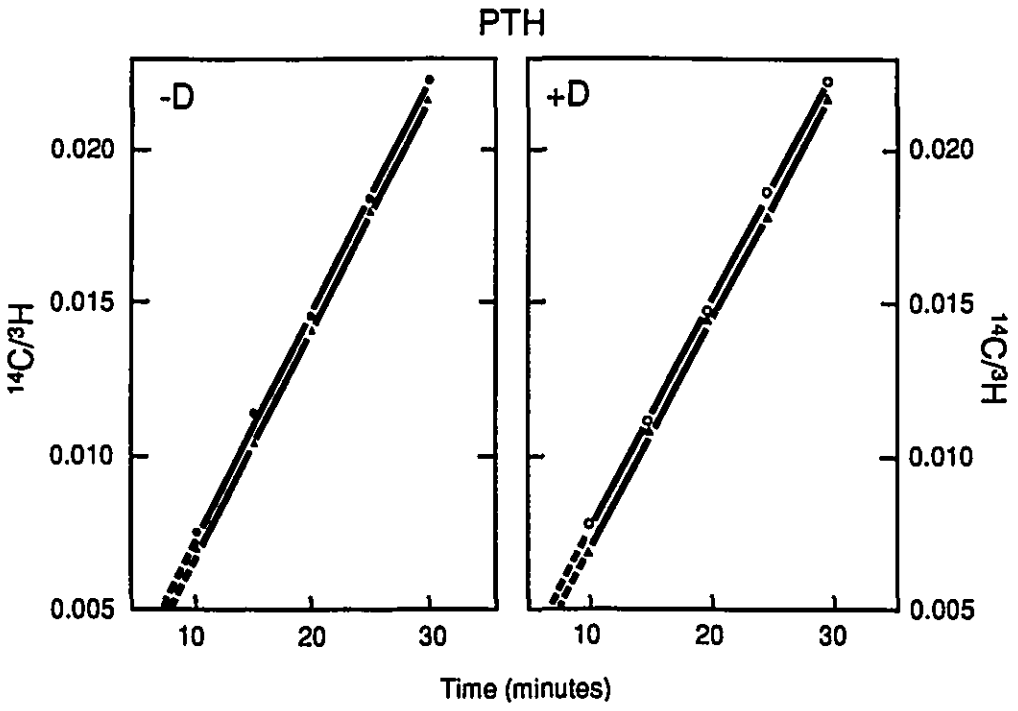

Figure 3.9 Effect of $1,25(OH)_2D_3$ on the ribosome transit time of PTH mRNA in cultured parathyroid cells. Parathyroid cells were cultured for 48h in the absence (-D) or in the presence (+D) of 10^{-8}M $1,25(OH)_2D_3$. Ribosome transit time analysis was described as in Figure 3.8 and as described under *Materials and Methods*. PTH was immunoprecipitated from the post-ribosomal supernatant using a rabbit anti-bovine PTH antiserum.

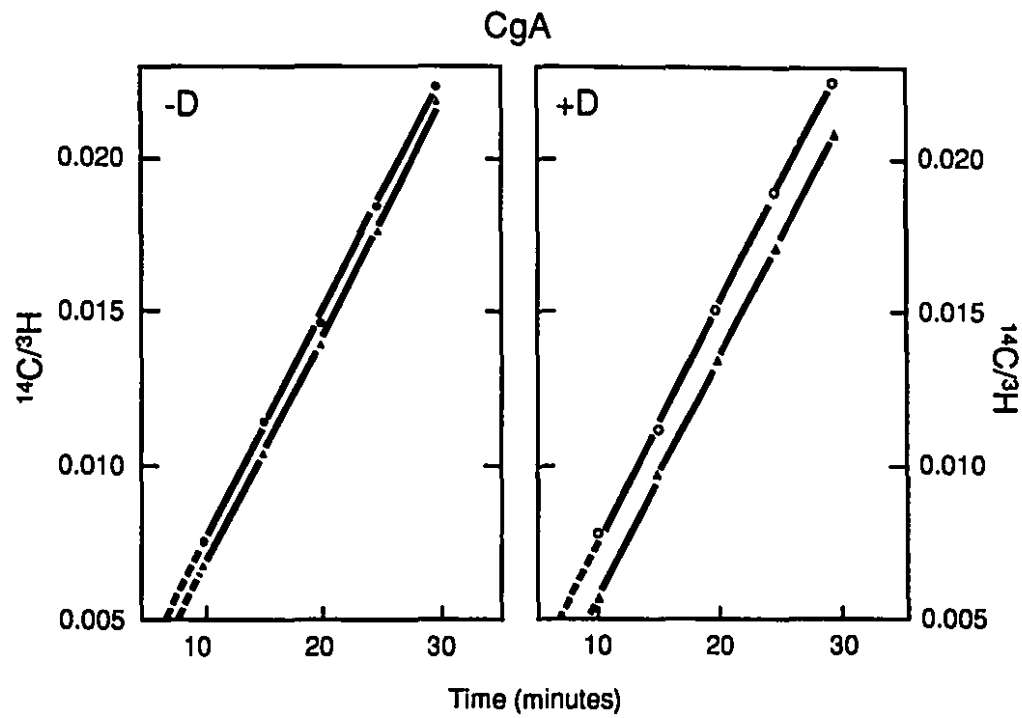

Figure 3.10 Effect of $1,25(OH)_2D_3$ on the ribosome transit time of CgA mRNA in cultured parathyroid cells. Parathyroid cells were cultured for 48h in the absence (-D) or in the presence (+D) of 10^{-8}M $1,25(OH)_2D_3$. Ribosome transit time analysis was described as in Figure 3.8 and as described under *Materials and Methods*. CgA was immunoprecipitated from the post-ribosomal supernatant using a rabbit anti-bovine CgA antiserum.

3.10 TABLES

Table 3.1

Effect of $1,25(OH)_2D_3$ on Average and Specific Ribosome Transit Times (in Minutes) of CgA and PTH mRNAs in Bovine Parathyroid Cells Cultured for 2 Days in the Absence (-D) or Presence (+D) of 10^{-8} M $1,25(OH)_2D_3$

	-D	+D
Average	2.33 ± 0.17[1]	2.5 ± 0.29
CgA	2.08 ± 0.08	4.0 ± 0.29[2]
PTH	1.58 ± 0.22	1.67 ± 0.17

[1] Results are shown as mean ± SEM of three separate experiments.

[2] Significantly different from -D value: $p<0.02$.

CHAPTER 4

HUMAN CHROMOGRANIN A GENE: MOLECULAR CLONING, STRUCTURAL ANALYSIS AND NEUROENDOCRINE CELL-SPECIFIC EXPRESSION

4.1 PREFACE

This chapter describes the complete structural characterization of the human chromogranin A gene. Also, because the chromogranin A gene is exclusively expressed in neuroendocrine cells, we also sought to define the cis-acting DNA element that confer this neuroendocrine cell specificity. By functional assays, the region corresponding to -53 to +32 relative to the transcriptional initiation site is shown to confer gene expression in neuroendocrine cell lines, but not in nonendocrine cells. Defining this region in greater detail and analysis of the protein factors that bind to it will provide important information about the neuroendocrine cell-specific gene expression of the CgA gene.

4.2 ABSTRACT

Chromogranin A (CgA) is an acidic glycoprotein which is widely expressed in endocrine and neuroendocrine cells. It is thought to play important roles in the process of regulated hormone secretion. The single copy human (h) CgA gene was isolated from a human fetal liver gene library. The gene spans 15 kb and contains eight exons. Exon I encodes the 5' noncoding region and the majority of the signal peptide coding region. Exons II-V collectively encode the highly conserved amino-terminal domain (the β-granin sequence). Exon VI encodes a variable domain within which is the chromostatin sequence, and exon VII encodes another variable domain which contains the pancreastatin sequence. Exon VIII encodes the highly conserved carboxy-terminal domain and the 3' noncoding region. A single transcription initiation site was identified for the CgA gene in human medullary thyroid carcinoma TT cells, and human and bovine parathyroid tissue. The human gene promoter has a consensus TATA box, cAMP response element and Sp-I sequence. 2.3 kb of the upstream regulatory region of the hCgA gene directed efficient transcription of a reporter chloramphenicol acetyltransferase gene in several neuroendocrine cell lines, including human medullary thyroid C-cell tumor, mouse pituitary corticotroph, rat pituitary tumor, and rat pheochromocytoma. The promoter was virtually inactive in nonendocrine cell lines including a T-antigen-transformed monkey kidney cell, a mouse fibroblast cell and a rat fibroblast cell. Further transient transfection studies with deleted promoter constructs showed that sequences lying between -53 and +32 bp relative to the transcription initiation site were sufficient for neuroendocrine cell-specific expression. It will now be possible to elucidate the precise cis-acting elements involved both in the general neuroendocrine cell expression of CgA and in response to hormonal stimuli.

4.3 INTRODUCTION

Chromogranin A (CgA), an acidic glycoprotein first identified in chromaffin granules of the adrenal medulla (1), is the major member of the chromogranin/secretogranin (granin) family of proteins which are present in virtually all endocrine and neuroendocrine cells (2, 3). These are costored within secretory granules and cosecreted with the resident hormone. Their ubiquitous presence in this cell type suggests they play important roles in hormone secretion. Extracellularly, peptides formed as a result of proteolytic processing of granins regulate hormone secretion in an autocrine manner. In addition, granins may play a role in secretory granule formation by virtue of their ability to aggregate in the low-pH,

high-calcium environment of the trans-Golgi network and be sorted to the regulated secretory pathway.

The cloning and sequence analysis of CgA cDNA from several species has been reported. The gene encodes a preprotein of molecular weight 50 kDa. The signal sequence is 18 amino acids long and the mature CgA protein is 430 amino acids in the porcine (4), 431 amino acids in the bovine (5, 6, 7), 439 amino acids in the human (8, 9), 444 amino acids in the rat (9, 10, 11) and 445 amino acids in the murine (12) species. Mature CgA contains several conserved pairs of basic residues which raised the possibility that biologically active peptides could be released from the molecule by precursor processing enzymes (13,14). In fact, it has now been reported that several peptides encoded within the CgA molecule, such as vasostatin (15), β-granin (16, 17), chromostatin (18), pancreastatin (19) and parastatin (20) are biologically active, at least *in vitro*, and inhibit hormone or neurotransmitter release probably in an autocrine or paracrine fashion. Pancreastatin, which is a 49-amino acid peptide [encoded by porcine (p)CgA(240-288)] with a carboxy-terminal amide, inhibits glucose-stimulated insulin release from perfused pancreatic islet cells (20). Chromostatin, which corresponds to a sequence beginning at bovine (b)CgA amino acid 124, inhibits secretion from adrenal medullary cells apparently via a mechanism involving closing of a calcium channel (21).

CgA may also be functional intracellularly and play a role in processing (22), sorting (2,3), and granule condensation (23). Some of these putative functions may be related to the molecule's ability to bind calcium which leads to changes in its structure and aggregation and membrane-association properties (24, 25, 26, 27, 28). Therefore, CgA and/or peptides derived from it may have multiple functions, both extracellular and intracellular. The relative importance of extracellular functions may depend upon the extent of processing of CgA which is tissue specific (2, 29).

The biosynthesis of CgA is regulated by many different factors, including steroid hormones and agents that modulate the protein kinase A and protein kinase C signalling pathways (see ref. 2 for review). In theory, its biosynthesis could be regulated in concert with the other components of the secretory granule of the particular cell involved. Alternatively, the biosynthesis of CgA and the resident hormone or neurotransmitter could be regulated differentially. Evidence for the latter type of regulation has been found in several systems. For example, in the parathyroid cell, whereas 1,25-dihydroxyvitamin D inhibits parathyroid hormone (PTH) gene transcription, it stimulates CgA gene transcription (30,31,32).

The CgA gene is a single copy gene. The bovine and mouse CgA genes have been characterized and are comprised of eight exons (12, 33). The exon-intron boundaries

are conserved. There is some limited homology between the amino-terminal and carboxyl-terminal domains of the CgA gene and the related chromogranin B (CgB) gene which has been cloned in the mouse (34). The available sequence of the 5' flanking region of the bovine and mouse CgA genes is limited to about two hundred basepairs (12,33).

In order to further understand the regulation of CgA gene expression we have determined the structure of the entire human CgA gene and delineated a minimal region of its promoter necessary for neuroendocrine cell specific expression. By primer extension analysis of both human parathyroid and medullary thyroid C-cell mRNA a single transcription initiation site downstream of a consensus TATA box sequence was identified. The nucleotide sequence of over 2 kb of the 5' flanking region was determined. We show that an 85 bp fragment of the promoter region containing the transcriptional start site, a TATA box homology and a consensus cyclic AMP response element is sufficient to direct specific expression of a chloramphenicol acetyltransferase reporter gene after transient transfection into a variety of neuroendocrine cell lines of both human and rodent origin.

4.4 EXPERIMENTAL PROCEDURES

4.4.1 MATERIALS

Cell lines and the fetal liver genomic library were obtained from the American Tissue Type Culture (Rockville, Md). Dulbecco's Modified Earle's Medium (DMEM), antibiotics, horse serum and fetal bovine serum (FBS) were obtained from Gibco/BRL (Mississauga, Ont). Kaign's modified Ham's F12 (F12K) medium was obtained from Irvine Scientific (Irvine, CA). [^{14}C] chloramphenicol (45 Ci/mmol) and [^{32}P] adenosine-5'-triphosphate (3000 Ci/mmol) were obtained from ICN Biomedicals (Baie d'Urfé, Que). Oligonucleotides were synthesized by standard phosphoramidite chemistry on a Biosearch oligonucleotide synthesizer. The T7 sequencing kit and acetyl CoA were from Pharmacia (Baie d'Urfé, Que). The lipofection reagent [a 50:50 suspension of 1,2 dioleoyloxy-3-(trimethylammonio) propane and dioleoylphosphatidylethanolamine (DOTAP and DOPE) in sterile, pyrogen-free water] for transfection experiments was kindly provided by Dr. J.R. Silvius (McGill University). Restriction enzymes, polynucleotide kinase and murine mammary leukemia virus (MMLV) reverse transcriptase were from Pharmacia or Gibco/BRL. The subcloning vector pBluescript II KS was from Stratagene (LaJolla, CA), and the β-galactosidase expression vector, pCH110 was from Pharmacia.

4.4.2 SCREENING OF THE GENOMIC DNA LIBRARY

A human fetal liver genomic DNA library (35) was plated and approximately 500,000 plaques were screened by filter hybridization using a polynucleotide kinase [^{32}P] labelled oligonucleotide (45-mer) complementary to amino acids +1 to +15 of bovine CgA. The sequence of the oligomer is described below under oligo **b**. Prehybridization and hybridization were performed in 40% formamide, 5X SSC, 25 mM $NaPO_4$, pH 7.4, 1 % SDS, 2 mM EDTA, 1X Denhardt's, and 200 µg/ml salmon sperm DNA at 42°C. The filters were washed twice with 2X SSC/0.1% SDS at 50°C. Positive plaques, identified after autoradiography, were purified by secondary and tertiary screening. In this way, ten positive plaques for hCgA were obtained which represented two independent clones which were designated λghCgA3 and λghCgA8.

4.4.3 RESTRICTION ENZYME MAPPING ANALYSIS

Recombinant clones λghCgA3 and λghCgA8 were singly digested with the restriction enzymes BamHI, XbaI, SalI, PstI, KpnI, XhoI, HindIII, and EcoRI. In addition, the phage DNAs were digested with these enzymes and with a second enzyme to obtain all possible restriction enzyme combinations. 0.5 µg DNA from each digestion was electrophoresed on a 0.8% agarose gel, Southern blotted and successively hybridized with each of nine [^{32}P]-labelled oligonucleotides. The sequences of the oligomers was as follows: **a**, complementary to nucleotides -31 to -1 upstream of the translation initiation codon, 5' - GGC GGA CCC GGG CGC CGT GCA GCT GGC GGT G: **b**, complementary to the coding region for amino acids +1 to +15, 5' - CAT CTC CTC GGT ATC CCC TTT CTT CAT AGG GCT GTT CAC AGG GTC; **c**, complementary to the coding region for amino acids 14 to 22, 5' - GAT GAC CTC AAC GAT GCA TTT GGC - 3'; **d**, complementary to the coding region for amino acids 45-53, 5' - TCT CAG AAT GGA AAG GAT CCG TTC ATC - 3'; **e**, complementary to the coding region for amino acids 68 to 76, 5' - CTG CTG ATG TGC CCT GTC CTT GGC GCC - 3'; **f**, complementary to the coding region for amino acids 101-109, 5' - CTT GGA TGA TGG CTG TTG GAG CGC CTC -3'; **g**, complementary to the coding region for amino acids 248-256, 5' - AGC CTC CGA CCG ACT CTC GCC TTT CCG - 3'; **h**, complementary to the coding region for amino acids 321-327, 5' - CTG GTC CAT CTT GCT CCA GCG TTT GGA - 3'; **i**, complementary to the coding region for amino acids 413 to 421, 5' - CTC GTC CTC GAC CTC TCG GAC AGC CGG - 3'. The sequences of oligomers a,

and c-i were based upon the human CgA cDNA characterized by Konecki *et al.* (9) and that of oligomer b based upon the bovine CgA cDNA of Iacangelo *et al.* (7).

Analysis of the generated fragments giving positive hybridization signals with the oligomer probes on Southern blot analysis culminated in the construction of a restriction map.

4.4.4 DNA SEQUENCE ANALYSIS

Seven restriction fragments of λghCgA8 which represented the entire human CgA gene were subcloned into pBluescript II KS for further restriction mapping and nucleotide sequence analysis which was carried out by the dideoxynucleotide chain termination method (36) using the Pharmacia T7 sequencing kit. Restriction fragments from selected subclones were [^{32}P]-labelled by the random primer method and used in Southern blot analysis of restriction enzyme-digested human leukocyte DNA.

4.4.5 PRIMER EXTENSION ANALYSIS OF CgA MRNA

Total RNA was isolated from normal bovine parathyroid glands, a human parathyroid adenoma and the human medullary thyroid carcinoma (TT) cell line by the guanidium thiocyanate/cesium chloride method (37). The primer used was oligonucleotide a, described earlier under **Restriction Enzyme Mapping** which is complementary to -31 to -1 upstream of the translation initiation codon of hCgA mRNA. Primer extension analysis was performed as described (38). Ten μg of each total RNA and 25 μg of yeast tRNA (as negative control) were hybridized overnight at 60°C in 10 mM Pipes, pH 6.4, 0.4 M NaCl, 1 mM EDTA. The RNA templates were then reverse transcribed using 200 U MMLV-reverse transcriptase. The extension products were phenol-chloroform extracted, ethanol precipitated and analyzed on a 6% acrylamide/7M urea sequencing gel. As a DNA size marker, an unrelated sequencing reaction using pUCmp18 DNA (Pharmacia) and the universal primer, M13, was run in parallel.

4.4.6 HUMAN CgA GENE PROMOTER ANALYSIS

The hCgA promoter construct (p2300CAT) was prepared by a BamHI-XhoI restriction fragment spanning 2316 bp upstream of the cap site to 32 bp downstream of the cap site into the promotorless CAT expression reporter vector, pBLCAT3 (39) which had been cleaved with BamHI and XhoI. The p750CAT and p450CAT constructs were prepared by ligating the HindIII-XhoI (-716 to +32) and XbaI-XhoI (-447 to +32) restriction fragments, respectively, into the pBLCAT3 vector. Constructs p260CAT and p53CAT were created by ligating PCR-generated fragments into pBLCAT3. The forward primer for the p260CAT fragment was 5'-CCTTGAGGATCCAAGGCAAATCGGTG-3' which consists of six irrelevant nucleotides followed by a BamHI site followed by nucleotides -258 to -245, and the forward primer for the p53CAT fragment was 5'-AAGTCAGGATCCGCTGACGTCATTTC-3' which consists of six irrelevant nucleotides followed by a BamHI site followed by nucleotide -51 to -38. For both fragments the reverse primer was 5'-CACGGGGCTCGAGCAC-3' which is the complement of +25 to +41 which contains the XhoI site. Fragments were PCR-amplified, cleaved with BamHI and XhoI, gel-purified and ligated into BamHI-XhoI-cut pBLCAT3. All plasmid DNAs were prepared by the alkaline lysis/cesium chloride gradient method.

4.4.7 CELL CULTURE

The neuroendocrine cell types were the mouse pituitary corticotroph cell line (AtT-20), the rat pituitary cell line (GH_4C_1), the rat pheochromocytoma cell line (PC-12), and the human medullary thyroid carcinoma (TT) cell line. The nonendocrine cell lines used were the T-antigen-transformed monkey kidney cell line (COS-7), the mouse fibroblast cell line (NIH-3T3), and the rat fibroblast cell line (Rat-2). TT cells were maintained in Ham's F12K medium with 10% FBS. PC-12 cells were maintained in DMEM with the addition of 10% FBS, 5% horse serum. All other cell lines were maintained in DMEM with 10% FBS. All maintenance media contained 100U/ml penicillin and 100 µg/ml streptomycin.

4.4.8 CELL TRANSFECTIONS

The majority of the cell lines were transfected by a lipofection procedure (40). Cells were trypsinized, seeded at $5x10^5$ cells per 35 mm tissue culture dish and incubated

overnight. Medium was aspirated and cells were washed twice with serum-free DMEM. Transfections with the phCgACAT constructs, with the positive control simian virus SV-40 promoter-driven plasmid pSV2CAT, and the negative control promotorless plasmid, pBLCAT3, were done in parallel experiments in all cell lines. Each transfection involved co-transfection with 2 µg of control plasmid pCH110 in which the gene for ß-galactosidase is under the control of the SV-40 early promoter. Eight µg total DNA was mixed with 1.5 ml serum-free DMEM. In a separate tube the lipofection reagent was added to 1.5 ml DMEM and the DNA solution was mixed gently with the lipofection mixture. This was allowed to sit at room temperature for 30 min, and then added drop-wise to cell monolayers. After 6 hr an equal volume of maintenance medium with 20% FBS was added. After 48 hr, cells were washed once with ice-cold PBS and harvested in 1 ml PBS by scraping into 1.5 ml Eppendorf tubes. Cells were spun at 500 *x g* at 4°C for 7 min and resuspended in 250 µl 0.5 M Tris-HCl, pH 7.5. Cells were lysed by three freeze-thaw cycles and the DNA and cell debris were removed by centrifugation at 16 000 *x g* for 10 min at 4°C. Cell lysates were assayed for protein using the Biorad protein assay kit, and assayed for β-galactosidase and CAT activity as described below. CAT activities were normalized to both protein concentration and β-galactosidase activity.

TT cells were transfected by the electroporation method using the BioRad Cell Porator with an extended capacitance unit. Approximately 10 X 10^6 cells were resuspended in 0.4 ml DMEM with 10% FBS. Plasmid DNA was added and the cell suspension was pulsed at 250 V, with a capacitance of 960 µFD. This typically resulted in a time constant of 21-29 msec. The cells were then gently transferred into 10 ml prewarmed Ham's F12K medium. Forty-eight hours after transfection, cells were washed once with ice-cold PBS and harvested in 1 ml PBS by scraping into 1.5 ml Eppendorf tubes. Cells were spun at 500 x *g* at 4°C for 7 min and resuspended in 250 µl 0.5 M Tris-HCl, pH 7.5. Cells were lysed by three freeze-thaw cycles and the DNA and cell debris were removed by centrifugation at 16 000 x *g* for 10 min at 4°C. Cell lysates were assayed for protein using the Biorad protein assay kit, and assayed for β-galactosidase and CAT activity as described below. CAT activities were normalized to β-galactosidase activity.

4.4.9 CAT AND β-GALACTOSIDASE ASSAYS

For CAT assays 5-20 µg protein were made up to 120 µl with 0.5M Tris-HCl, pH 7.5, containing 0.2 µM acetyl CoA and 0.1 µCi [^{14}C]chloramphenicol. Reactions were incubated at 37°C 0.5-4 hr. For the longer incubations, 1 µl 4 mM acetyl CoA was added

after 2 hr incubation. Reactions were stopped by addition of 1 ml ethyl acetate and vigorously vortexed for 20 sec, and spun 5 min at 16 000 *x g*. The top phase containing all chloramphenicol derivatives was dried for 1 hr in a Speedvac (Savant). The reaction products were separated by TLC using 95:5 chloroform:methanol as the running solvent. The TLC plates were exposed to autoradiographic film at -80°C. Percent conversion was calculated by excising the radioactivity from the TLC plates and expressing the percent of acetylated [^{14}C]chloramphenicol counts over the total radioactivity. β-galactosidase assays were determined by measuring an increase in optical density at 420 nm using O-nitrophenyl-β-D-galactopyranoside as substrate.

4.4.10 NORTHERN BLOT ANALYSIS

Total cellular RNA was isolated as described (41) from cells grown to confluency. Gel electrophoresis and Northern blotting was as described (42). Oligonucleotide **b** was [^{32}P] end-labelled as described earlier and used to probe RNA from each of the cell lines tested in this report. Hybridization were carried out overnight in 1% BSA, 7% SDS, 0.5 M Na_2PO_4, pH 6.8, 1 mM EDTA at 65°C. The membranes were washed twice in 0.5% BSA, 5% SDS, 40 mM Na_2PO_4, pH 6.8, 1 mM EDTA, at 65°C, and three times in 1% SDS, 40 mM Na_2PO_4, pH 6.8, 1 mM EDTA at 65°C, and then autoradiographed.

4.5 RESULTS AND DISCUSSION

4.5.1 IDENTIFICATION AND RESTRICTION MAPPING OF GENOMIC CLONES

At the initial screening of 500 000 plaques using an oligomer complementary to the amino-terminal coding region of bovine CgA, ten positive phage plaques were identified. After purification through secondary and tertiary screening procedures, and restriction enzyme digestion of the purified recombinant phage DNA, it was revealed that these phage represented two independent clones designated λghCgA3 and λghCgA8 (see Fig. 4.1) which both contained 15 kb of human genomic DNA. In order to facilitate restriction mapping and DNA sequence analysis, a variety of restriction fragments were

subcloned. A physical map of the human CgA gene and flanking regions is shown in Fig. 4.1.

4.5.2 CHARACTERIZATION OF THE 5' END OF THE GENE BY PRIMER EXTENSION

Primer extension analysis of oligomer a with RNA from bovine parathyroid gland, human parathyroid adenoma and thyroid TT cell line yielded a single major extension product for each RNA (Fig. 4.2). The extension product obtained with bovine parathyroid RNA was 193 bp, while a 213 bp extension product was obtained with both human sources of RNA. The bovine CgA gene transcription initiation site is 30 bp downstream of a typical TATA box sequence, and the human CgA gene transcription initiation site is 27 bp downstream of the TATA box. The data obtained for the transcription initiation site of the bovine gene in parathyroid is identical with that reported by Iacangelo *et al.* (33) using bovine adrenal medulla RNA. The 5' noncoding region of the bovine CgA mRNA is 193 bases and, as shown here, that of the human CgA mRNA is 213 bases.

4.5.3 DNA SEQUENCE ANALYSIS AND STRUCTURAL ORGANIZATION OF THE hCgA GENE

The nucleotide sequence of the human CgA gene is shown in Fig. 4.3. All exons were completely sequenced, as were all the exon/intron junctions. The hCgA gene spans 15 kb and contains eight exons. Exon I encodes the 5' noncoding region and the majority of the signal peptide coding region. Exons II-V collectively encode the highly conserved amino-terminal domain (the β-granin sequence). Exon VI encodes a variable domain within which is the chromostatin sequence, and exon VII encodes another variable domain which contains the pancreastatin sequence. Exon VIII encodes the highly conserved carboxy-terminal domain and the 3' noncoding region.

A comparison of the exon organization of the human, bovine and mouse CgA genes (Fig. 4.4) emphasizes the similarities in their structures. Each has eight exons with their absolute sizes being especially well conserved for their amino-terminal coding region exons. All exon/intron junctions have the consensus GT-AG sequence. The positions and sequences of the splice sites are well conserved between the human and mouse CgA genes

(see Fig. 4.5) and the human and bovine CgA genes (data not shown). Likewise, the relative sizes of the introns are generally well conserved between species (Fig. 4.5).

Southern blot analysis of restriction enzyme digested human leukocyte DNA, using as probes subcloned human DNA from the recombinant phage clones, revealed a simple pattern of restriction fragments which was identical to that seen with the recombinant DNA (data not shown).

4.5.4 COMPARISON OF THE hCgA GENE EXONS AND hCgA cDNAS

Previously, two human CgA cDNAs, both from pheochromocytoma libraries, were independently cloned and characterized (8, 9). These two sequences differed at 40 individual nucleotides throughout the coding region and 3' noncoding region which raised the possibility of allelic variability and/or cloning artifacts or sequencing errors. The nucleotide sequence we obtained for the hCgA gene exons is identical to that of the hCgA cDNA of Konecki *et al.* (9) at 38 of the 40 nucleotides. For the other 2 out of 40 nucleotides, the gene structure is the same as that of the hCgA cDNA of Helman *et al.* (8). These latter two substitutions are for codons 381, TGG (Trp) and 423, GAG (Glu) in the present report and cDNA of Helman *et al.* (8), which are CGG (Arg) and GAA (Glu) respectively, in the cDNA of Konecki *et al.* (9).

4.5.5 COMPARISON OF GENE ORGANIZATION AND DOMAINS ENCODING REGULATORY PEPTIDES

Figure 4.6 emphasizes the relationship between CgA gene exons and regions which encode peptides which have been reported to be biologically active. Vasostatin is the term given to a mixture of NH_2-terminal peptides the shortest of which is bCgA-(1-76), which were purified from bovine adrenomedullary granules (15). These peptides have a vasoinhibitory effect on isolated segments of human blood vessels (15). The CgA-(1-76) peptide could potentially be released from CgA by cleavage at the dibasic Lys^{77}-Lys^{78}. The related peptide CgA(1-113) was first identified in rat insulinomas and pancreatic islets and was given the name β-granin (17). A function of this peptide in pancreas has yet to be described, however, it has been reported that the homologous bovine CgA peptide inhibits PTH and CgA release from bovine parathyroid cells in culture (16). This peptide could be cleaved from CgA by proteolytic attack at the dibasic Lys^{114}-Lys^{115}. Vasostatin and β-

granin are encoded by several gene exons (II to VI in the case of β-granin). This arrangement is absolutely conserved in all CgA genes characterized to date, as are the dibasic cleavage site at Lys^{114}-Lys^{115} and a potential disulphide bridge encoded by exon III. A synthetic CgA-(1-40) peptide has also been reported to be active in modulating hormone secretion in cultured cells (43, 44).

The term chromostatin secretion refers to the sequence CgA (124-143) which was synthesized on the basis of the ability of tryptic and Lys-C generated-peptides of bCgA to inhibit chromaffin cell secretion (18, 45). Chromostatin is encoded within exon VI. The precise amino-terminal and carboxyl-terminal ends of the "natural" chromostatin sequence, or if indeed any cells produce chromostatin from CgA, are as yet unknown. However, binding and cross-linking studies suggest that chromostatin receptors are present in the adrenal medulla (21) and that they mediate the closing of an L-type calcium channel via stimulation of a soluble protein phosphatase (46).

Pancreastatin which is encoded by the 5' end of exon VII was initially identified as a biologically active 49-amino acid amidated peptide from porcine pancreas (19). Cloning of porcine CgA mRNA demonstrated that indeed pancreastatin was encoded within its sequence (4). Pancreastatin's biological activity resides in the carboxy-terminal region where the sequence is most conserved (19). By contrast, the amino-terminal region is very variable (4). Konecki *et al.* (9) speculated on the basis of the hCgA cDNA sequence that cleavage could take place at the non-conserved Arg^{248}-Lys^{249} to yield hCgA(250-301) as the human pancreastatin molecule. However, human pancreastatin peptides isolated and characterized to date do not provide evidence of such a cleavage as they are either amino-terminally extended or truncated relative to this putative cleavage site and to the known amino-terminus of porcine pancreastatin [pCgA-(240-304)]. In contrast to other prohormones, such as proPTH and proPTHrP, in which a prohormone cleavage site is precisely bisected by intron/exon boundaries (47), in the hCgA the splice junction between exon VI and exon VII lies downstream of the Arg^{248}-Lys^{249} sequence and upstream of the Arg^{253} which is another potential cleavage site. The amino acid sequence of this particular region of the CgA molecule, at the carboxy-terminal end of exon VI and the amino-terminal end of exon VII is the most variable between species. Knowledge of the exon/intron structure of this region of the porcine CgA gene may help to shed further light on the relationship between the placement of the exon boundaries and the cleavage site that releases the amino-terminus of pancreastatin.

Several prohormone convertase dibasic cleavage sites occur in the carboxy-terminal half of exon VII and the coding portion of exon VIII. A peptide derived from porcine(p)CgA by Lys-C digestion, termed "parastatin", pCgA(347-419) [which is

homologous to hCgA(356-428)] has been reported to be inhibitory to parathyroid cell secretion (20). Although the potency of this peptide is low in comparison to other CgA-derived peptides such as chromostatin and pancreastatin it may be indicative that there are other biologically active peptides yet to be identified which are derived from this highly conserved portion of the CgA molecule.

4.5.6 COMPARISON OF THE PROMOTER OF THE HUMAN, BOVINE AND MOUSE GENES

Alignment of the first approximately two hundred nucleotides of the human CgA gene 5' flanking region with the equivalent sequences of the bovine and mouse CgA genes is shown in Fig. 4.7. The human and bovine promoter regions are well conserved (76% identity), while the human and mouse promoters are somewhat less similar (49% identity). In all species there is a cyclic AMP response element (CRE) homology just upstream of the TATA box promoter element.

All the promoters contain Sp-1 sites, and possess a high GC content (human, 76%; bovine, 70%: mouse, 65%) which would be consistent with the wide distribution of expression of CgA. A highly-conserved purine-rich region extending from -77 to -58 in the human, -80 to -58 in the bovine, and -101 to -78 in the murine promoter is noteworthy. A similar, but not identical, purine-rich sequence, GGAAGG, is the recognition sequence for PU.1, a trans-acting factor that confers lymphoid cell-specific gene expression (48).

4.5.7 ENDOGENOUS CgA mRNA EXPRESSION IN NEUROENDOCRINE AND NONENDOCRINE CELLS

By Northern blot analysis (Fig. 4.8) CgA mRNA was undetectable in all nonendocrine cells tested (Rat-2, NIH-3T3 and COS-7), whereas a single transcript was detectable in all neuroendocrine cell lines tested (PC-12, AtT-20, TT and GH_4C_1).

4.5.8 NEUROENDOCRINE CELL SPECIFICITY OF HUMAN CgA PROMOTER ACTIVITY

As shown in Fig. 4.9, the 5' flanking region of the human CgA gene exhibited neuroendocrine cell-specific promoter activity. A plasmid (p2300CAT) containing a BamHI/XhoI fragment extending from 2.3kb upstream, to 32bp downstream of the transcription initiation site inserted in the proper orientation upstream of the CAT gene demonstrated strong promoter activity after transfection into various neuroendocrine cell lines of different types and species including rat pituitary (GH_4C_1), mouse pituitary corticotrophs (AtT-20), adrenal chromaffin cells (PC-12) and medullary thyroid C-cells (TT). The hCgA promoter fragment construct was some three- to fifteen-fold more active than the positive control construct in which CAT expression was under the control of the SV-40 early region promoter (Fig.4.9 and Table 4.1). In contrast, in nonneuroendocrine cell lines (COS-7, NIH-3T3, and Rat-2) the hCgA promoter had only very weak activity.

The specificity of the hCgA promoter in directing expression in neuroendocrine cells and not in other types can be compared to data recently reported for the carboxypeptidase E gene promoter (49). CPE is widely, although not exclusively, expressed in cells synthesizing peptide hormones and neurotransmitters. However, in contrast to the CgA gene promoter, the CPE gene promoter was active in both neuroendocrine and nonendocrine cell lines tested.

In order to localize the hCgA gene promoter region that confers specific neuroendocrine cell expression deleted constructs containing approximately 750, 450, 260 and 53 bp of the 5' flanking sequence were transfected into both neuroendocrine and nonendocrine cell lines. All the deletion constructs were just as active as the p2300CAT construct in neuroendocrine cells (Fig. 4.9 and Table 4.1). All constructs demonstrated low activity in the nonendocrine cells.

Therefore, in the present study we have delineated a mininal region of the CgA gene promoter necessary for neuroendocrine cell specific expression. Thus, an 85 bp fragment of the human promoter region containing the transcription start site, a TATA box homology, and a consensus cyclic AMP response element is sufficient to direct expression of a reporter gene in various rodent as well as human neuroendocrine cell lines. This region does not contain the purine-rich GAGA sequence, similar to the PU.1 recognition motif which confers lymphoid cell-specific gene expression (see above), and which, it might have been speculated, played a role in the neuroendocrine cell-specific expression of the CgA gene. The role of this conserved motif in the regulation of CgA gene expression remains to be elucidated.

In addition, it is not known if activation of the CgA gene involves enhancer action via specific positively-acting neuroendocrine cell transacting factors, or release of negative repressor activity in nonendocrine cells. This remains to be determined.

In summary, we have isolated and characterized the human CgA gene, which is similar in both size and exon/intron organization to the mouse and bovine CgA genes. The 5' human CgA gene flanking region within 53 bp of the transcription initiation site confers specific neuroendocrine cell expression. It will now be possible to elucidate the precise cis-acting elements involved in this neuroendocrine cell expression and in those which bind other specific trans-acting factors such as the steroid hormone receptors.

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4.8 FIGURES

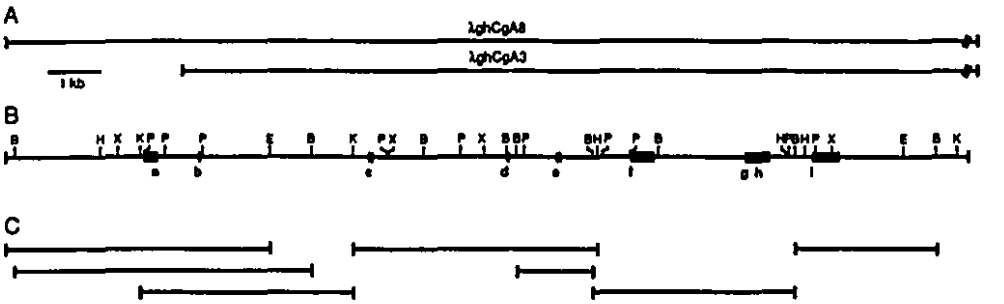


Figure 4.1 Physical map of human DNA encoding the CgA gene - Panel A: Two independent bacteriophage recombinant clones, λghCgA3 and λghCgA8 were isolated after screening a human fetal liver genomic DNA library as described under *Experimental Procedures*. Panel B: Restriction map of the human CgA gene. Solid boxes denote exons. The lower case letters refer to exon-specific oligonucleotide probes (a-i) used to construct the restriction map. See text for detailed description of the oligonucleotides. Panel C: The seven restriction fragments of λghCgA8 which represent the entire human CgA gene. They were subcloned into the pBluescript KS II vector for restriction mapping and nucleotide sequence analysis. B, BamHI; H, HindIII; X, XhoI; K, KpnI; P, PstI; E, EcoRI.

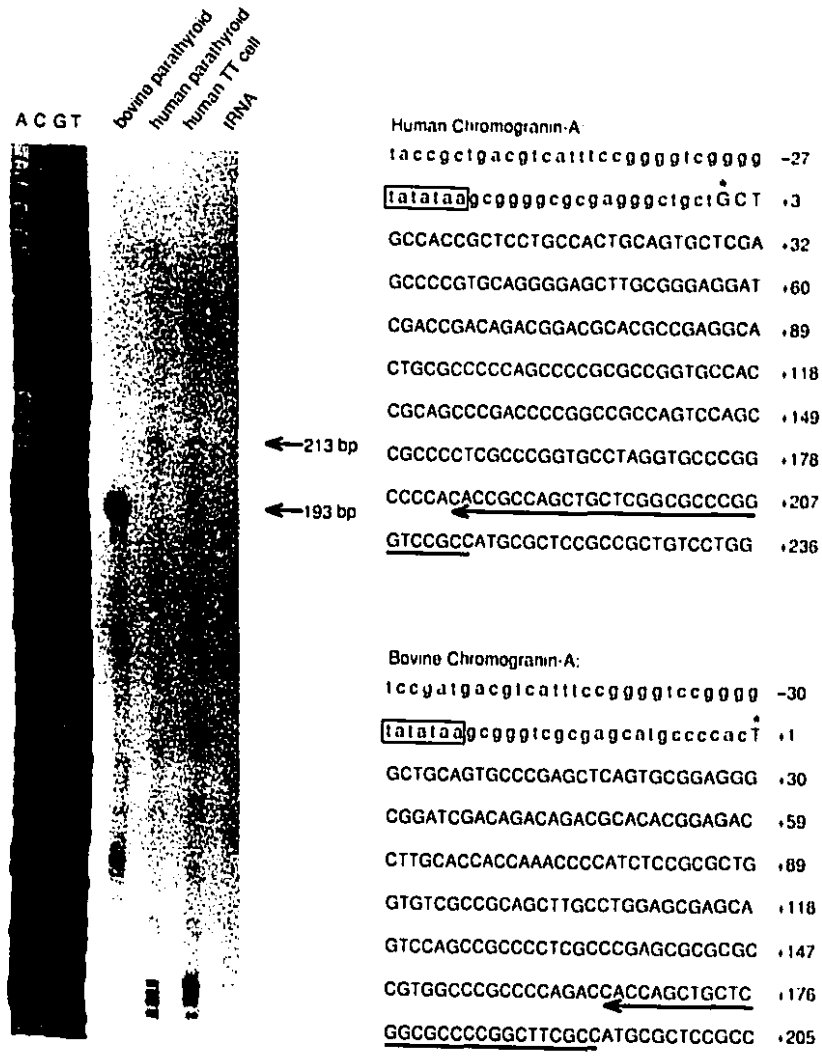


Figure 4.2 Primer extension analysis of CgA mRNA. Ten μg of total RNA from bovine parathyroid gland, human parathyroid adenoma, thyroid TT cell line, and 25 μg tRNA (control) were hybridized with an excess of polynucleotide kinase-labelled oligonucleotide M6 and the extension reaction performed as described under *Experimental Procedures*. The products were analyzed on an 6% acrylamide, 7M urea gel (left). A DNA sequence ladder served as size markers for the extension products. Arrows indicate the extension product of 193 nucleotides obtained with bovine parathyroid mRNA, and the extension product of 213 nucleotides obtained with human parathyroid and thyroid TT cell line mRNA. Nucleotide sequence of the human and bovine CgA genes to indicate the relationship between the antisense oligomer primer, represented by the arrow, and asterisks (*) to show their transcriptional start sites indicated by the primer extension analysis, and TATA box motifs which are boxed (right).

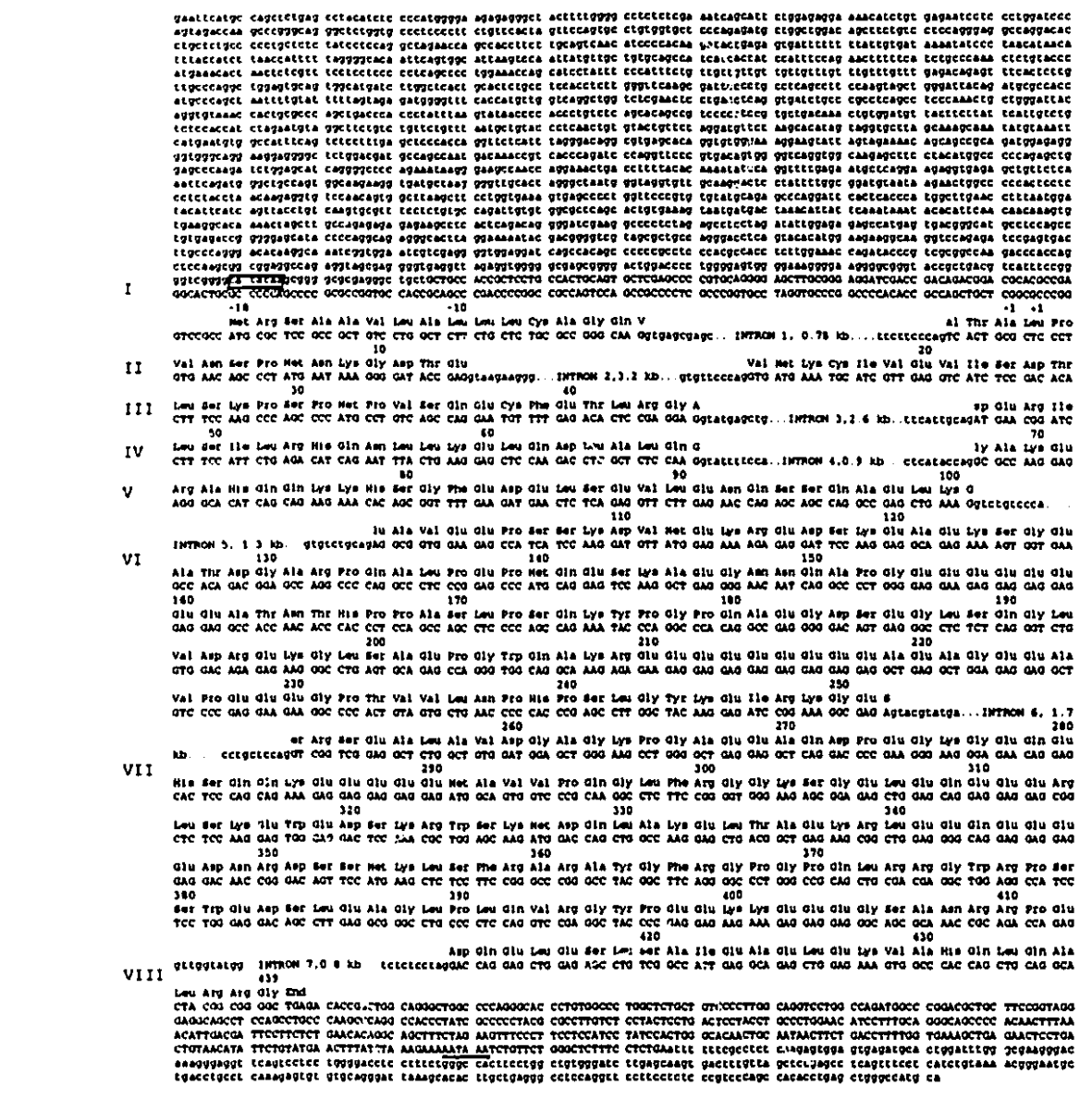


Figure 4.3 Nucleotide sequence of the human CgA gene - The nucleotide sequence was determined as described under *Experimental Procedures* from the subcloned DNAs shown in Fig. 4.1. Exonic sequence is shown in capital letters and intervening sequences and flanking DNA in lower-case letters. Each exon is designated by a roman numeral on left. The TATATA sequence in the promoter is boxed, and the AATAAA polyadenylation signal in exon VIII is underlined. In the protein coding sequence of the gene, positive numbers are placed above assigned amino acids so that +1 (Leu) begins the sequence of CgA; the minus numbers denote the prepeptide.

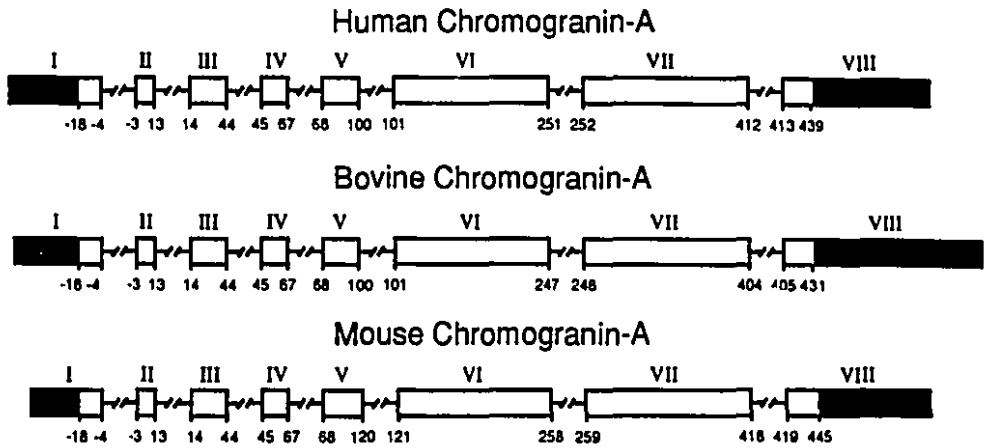


Figure 4.4 Comparison of the exon organization of human, bovine and mouse CgA genes. The relative sizes of the eight CgA exons (Roman numerals) of the human, bovine, and mouse CgA genes are shown. Black boxes denote noncoding regions; open boxes indicate coding regions. The numbers at the intron/exon borders denote the amino acids of the preCgA protein indicated by +1. Note that exon V of the mouse chromogranin A gene demonstrates variability in size because of the insertion of a polymorphic $(CAG)_n$ polyglutamine tract which is not present in either the human or bovine genes.

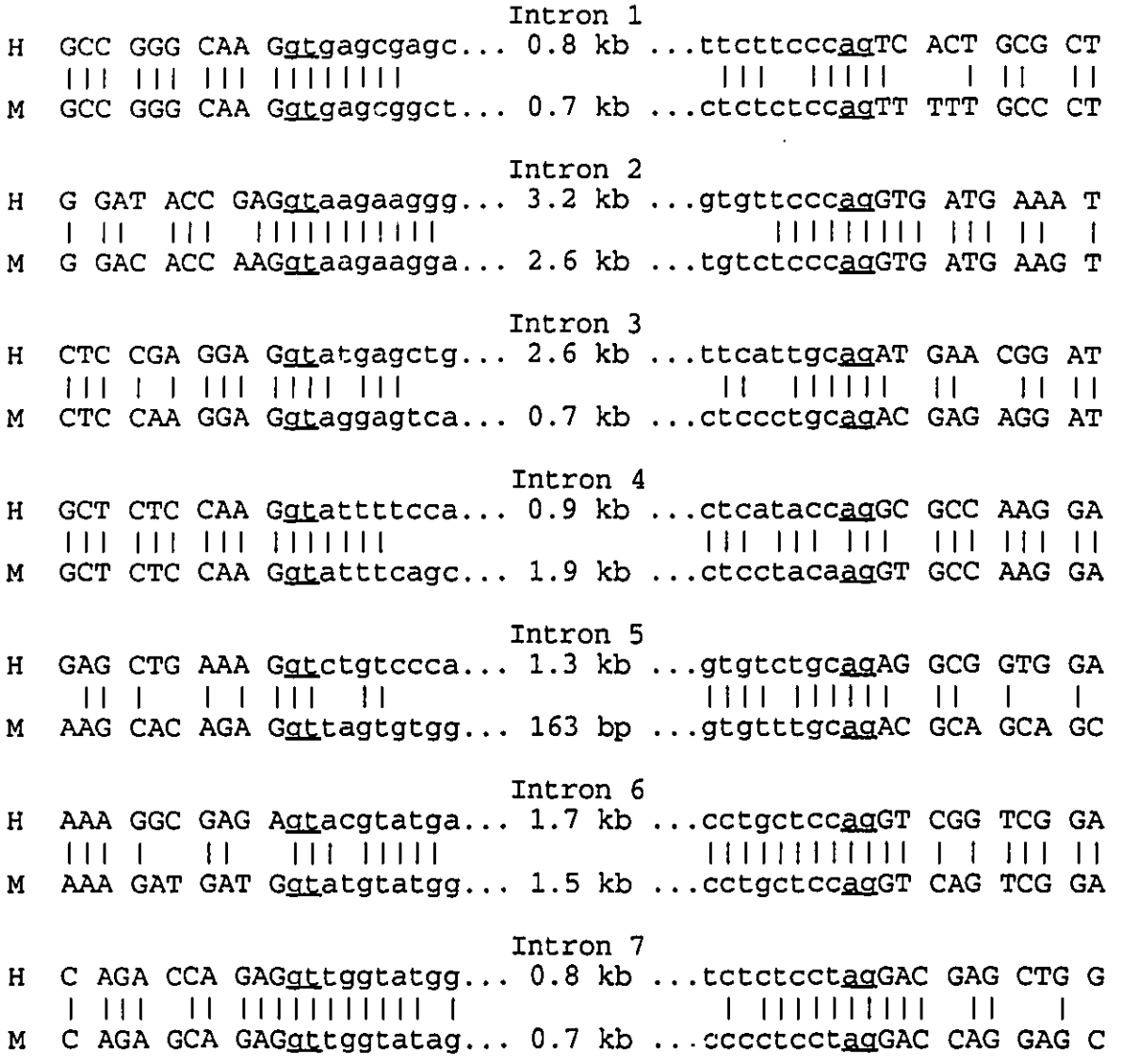


Figure 4.5 Comparison of intron/exon borders in the human and mouse CgA genes. Nucleotides in exons are grouped in triplet codons and are shown in capitals whereas nucleotides in introns are shown in lower case. The splice donor (gt) and acceptor (ag) sites are underlined. Nucleotides which are conserved in the human (H) and the mouse (M) sequences are indicated by vertical lines.

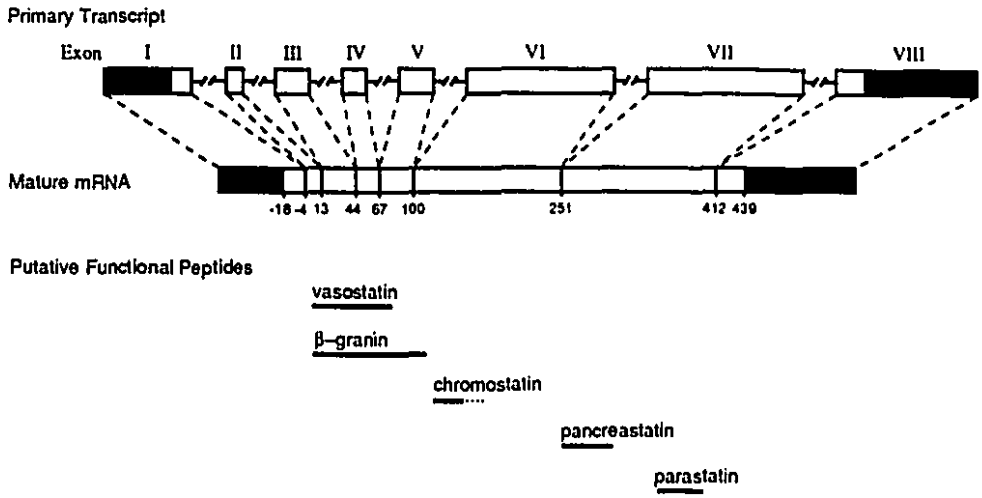
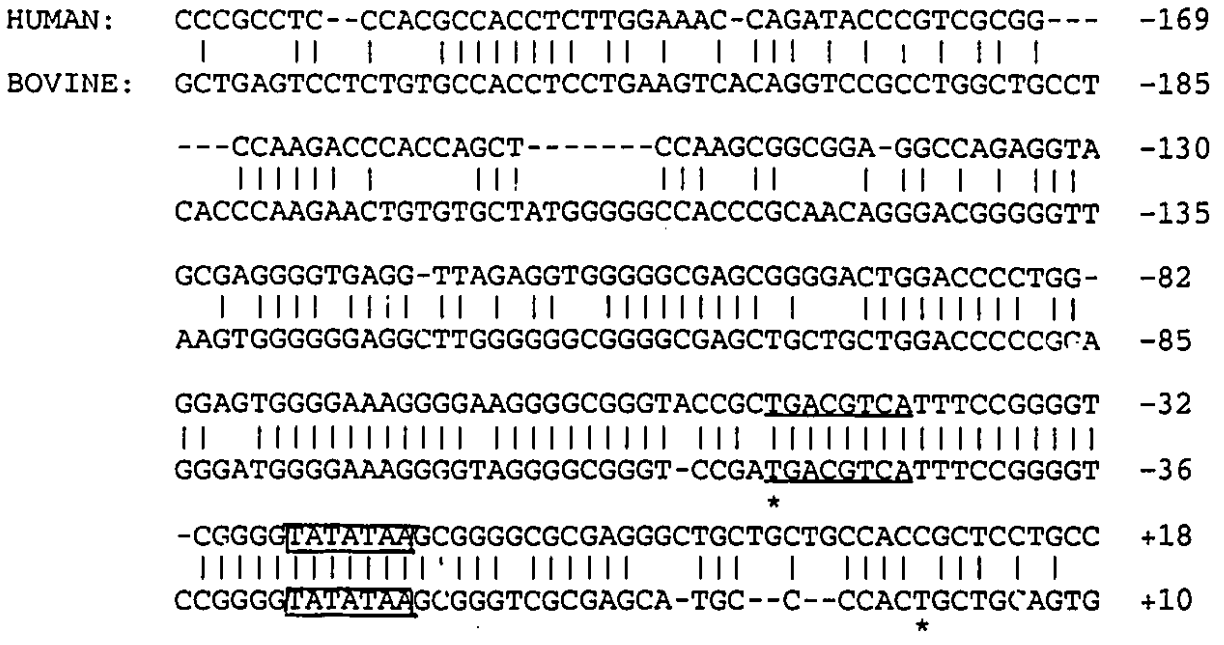


Figure 4.6 Relationship between the human CgA gene structure and functional peptides in the coding region. The exon/intron organization of the hCgA gene is related to the known CgA-derived peptides which have been reported to be biologically active.

A



B

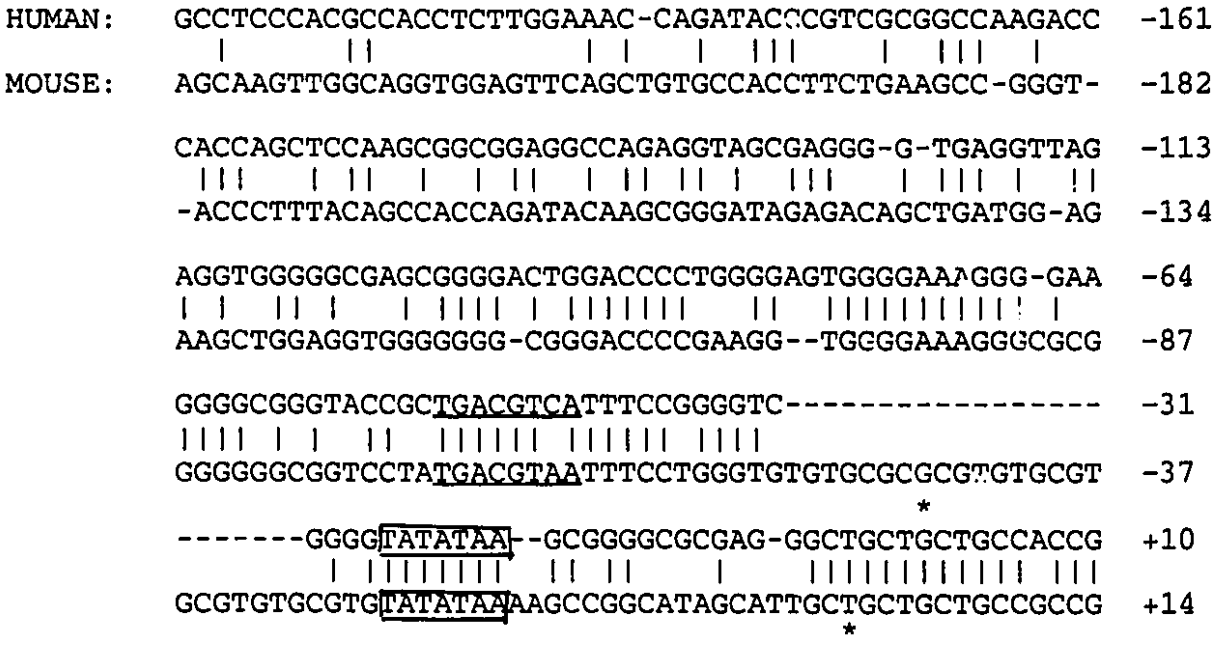


Figure 4.7 Comparison of the promoters of the human, bovine and mouse CgA genes. Panel A shows the alignment of the promoters of the human and bovine CgA genes. Panel B shows the alignment of the promoters of the human and mouse CgA genes. Nucleotides are numbered from the transcription initiation site (+1) which is indicated by an asterisk (*). TATA box motifs are boxed and cyclic AMP-response element homologies (CRE) are underlined.

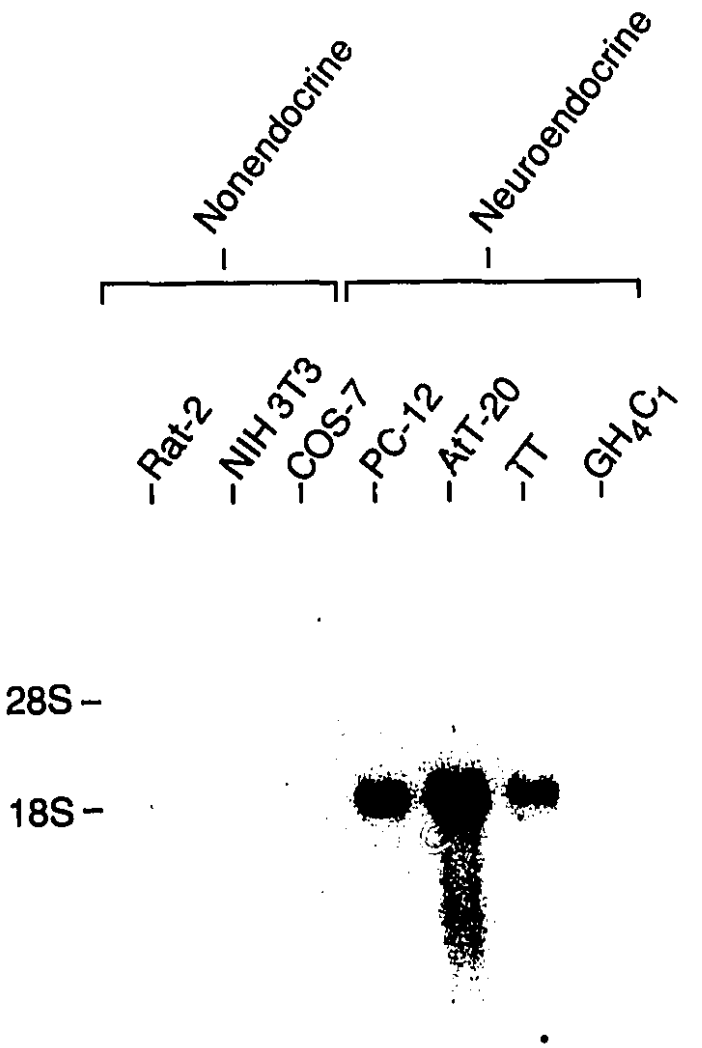
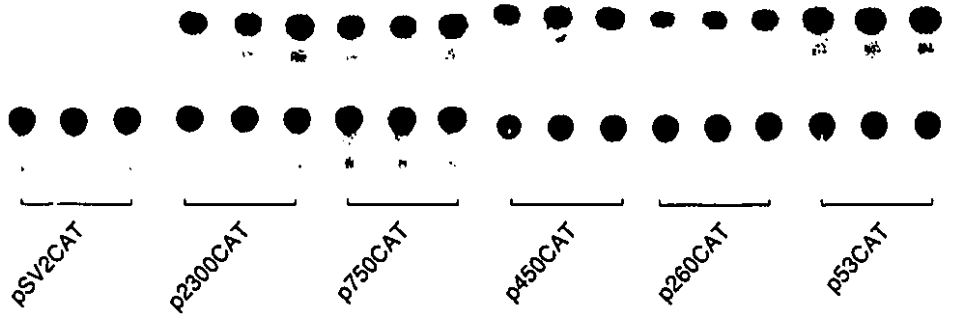


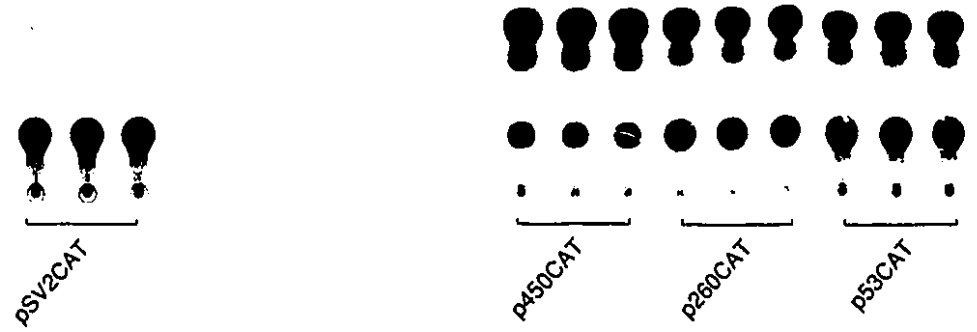
Figure 4.8 Endogenous expression of CgA mRNA in neuroendocrine and nonendocrine cell lines. Cytoplasmic RNA was isolated from the neuroendocrine and nonendocrine cell lines shown and Northern analysis was performed as described under *Experimental Procedures*. CgA mRNA was detected in all neuroendocrine cells, but not in nonendocrine cells.

A: Neuroendocrine

PC-12

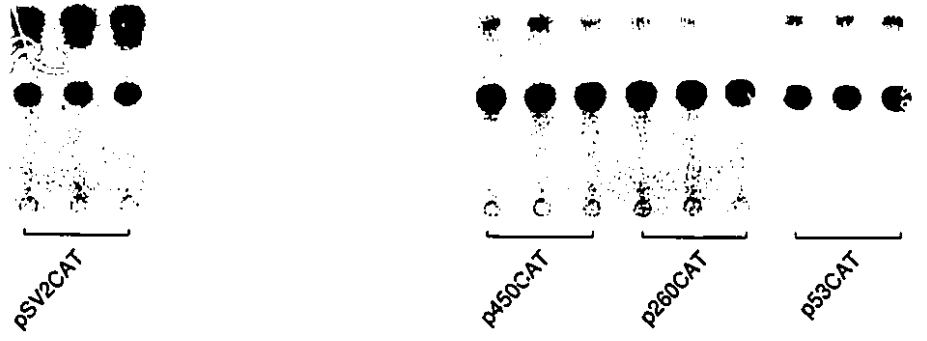


GH_4C_1



B: Nonendocrine

COS-7



RAT-2

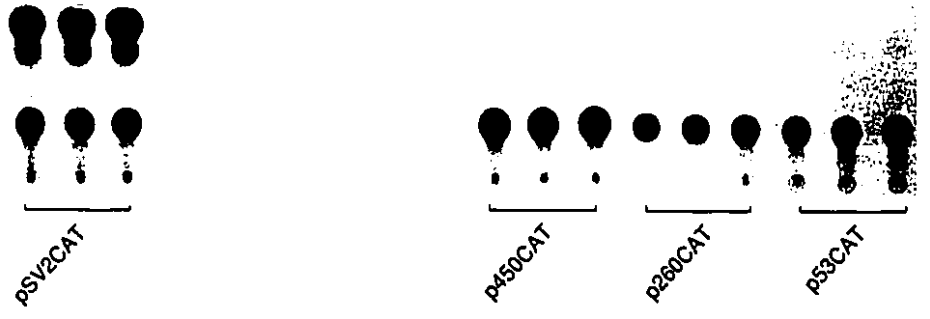


Figure 4.9 Delineation of minimal region necessary for the neuroendocrine cell specific activity of the human CgA gene promoter. The results of the CAT assays are shown for the neuroendocrine cells, PC-12 and GH_4C_1 (Panel A) and for the nonendocrine cells COS-7, and Rat-2 (Panel B). Cells were transfected with plasmid DNAs and CAT activity was determined as described under *Experimental Procedures*. DNAs transfected were: pBLCAT3, a promoterless CAT construct as negative control (not shown); pSV2CAT, in which the CAT gene is driven by the SV40 early region promoter as positive control; p2300CAT, p750CAT, p450CAT, p260CAT and p53CAT, constructs which contain sequences from -2300, -750, -450, -260 and -53 bp to +32 bp relative to the hCgA gene transcription initiation site inserted upstream of the CAT gene in pBLCAT3.

4.9 TABLES

Table 4.1

Transcriptional Activity of the Human CgA Promoter in Neuroendocrine and Nonendocrine Cells

Neuroendocrine and nonendocrine cell lines were transfected with construct p2300CAT or p750CAT or p450CAT, p260CAT, and p53CAT (respectively containing 2300bp, 750bp, 450bp, 260bp, and 53bp of the 5' flanking region of the hCgA gene cloned upstream of the CAT gene in pBLCAT3) and CAT activity was assessed as described under *Experimental Procedures*. For each cell type, the fold-CAT activity is given relative to the activity of pSV2CAT which expresses CAT under the control of the SV40 early region promoter.

Cell Line	Cell Type	p2300CAT	p750CAT	p450CAT	p260CAT	p53CAT
Neuroendocrine						
AtT-20	Pituitary	3.19±0.27	4.97±0.92	6.15±1.0	3.63±0.2	1.58±0.6
TT	C-Cell	3.3±0.33*	3.35±0.36	3.36±0.18	2.67±0.37	5.34±0.79
GH_4C_1	Pituitary	10.07±1.38	9.81±1.06	15.4±1.41	12.98±0.73	11.55±0.92
PC-12	Chromaffin	14.85±0.44	14.41±2.41	13.20±1.29	7.86±1.28	13.54±0.76
Nonendocrine						
COS-7	Kidney	0.02±0.001	0.04±0.003	0.04±0.003	0.03±0.001	0.05±0.003
NIH-3T3	Fibroblast	0.13±0.01	0.15±0.001	0.14±0.007	0.15±0.02	0.12±0.03
Rat-2	Fibroblast	0.06±0.02	0.14±0.007	0.11±0.014	0.09±0.006	0.09±0.01

* Mean ±S.E. of triplicate or quadruplicate determinations.

PART III

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER 5

5.1 PREFACE

The first three chapters of this thesis present a review of the chromogranin A literature to date, its regulation of biosynthesis in several tissues, and the experimental findings on the regulation of the synthesis and secretion of chromogranin A by calcium and 1,25-dihydroxycholecalciferol in the parathyroid. This highlights the nature of the regulation of chromogranin A biosynthesis which is that chromogranin A gene expression can be regulated by a variety of factors, in a cell-specific manner. Chapter 4 presents the complete structural characterization of the human chromogranin A gene, and the functional characterization of the DNA sequence that confers endocrine cell-specific gene expression. Overall, these findings have provided insights into the molecular nature of chromogranin A gene expression and regulation.

This chapter discusses some of the future directions that can be followed in the CgA field based on my own research and that of others. The primary goals of the research should therefore be to define the molecular nature of the regulation of gene expression by hormones and other factors, to define the molecular nature of the cell-specific gene expression and tissue-specific processing, and to elucidate the function(s) of CgA.

5.2 PARATHYROID CELL CULTURES AND GENERATION OF CELL LINES

With the use of primary cultures of bovine parathyroid cells, many advances in the understanding of parathyroid cell gene regulation and secretory function have been made, such as those reported in Chapters 2 and 3. In these studies, experimentation followed a time-course of up to three days following cell dispersal. When these cells are cultured for longer, however, and experimentation is not initiated immediately after cell dispersal, these cells can lose some of their functional properties. For example, in studies with bovine parathyroid cells in their second and fifth passage, the relative responses to both extracellular calcium and $1,25(OH)_2D_3$ at the secretory and gene levels were similar to those observed in primary parathyroid cell cultures, except that the overall secretion of PTH decreased with increasing passage number (Ishimi *et al.*, 1990). In another study, cultures of bovine parathyroid cells were maintained for over 140 population doublings (approximately 5 months), and were able to maintain the secretory rate of PTH throughout this time (Brandi *et al.*, 1986). However, a reduced sensitivity to extracellular calcium, as well as a delay in the secretory response to extracellular calcium were observed. In addition, these cells eventually became senescent (Brandi *et al.*, 1986).

Since the properties of primary cultures of parathyroid cells may vary from one batch to another, it would be advantageous and practical to have a parathyroid cell line. This would provide a source of cells with constant functional properties. From hyperplastic rat parathyroid tissue, a parathyroid cell line was produced (Sakaguchi *et al.*, 1987). Initially, this cell line (named PT-r) was believed to secrete PTH, since conditioned medium from these cells was capable of eliciting a cAMP response in a bioassay (Sakaguchi *et al.*, 1987). However, the PTH bioactivity was later determined to be due to PTH-related protein (PTHrP) in the conditioned medium (Zajac *et al.*, 1989). PTHrP secretion from these cells has been shown to be regulated in an inverse fashion by extracellular calcium (Ikeda *et al.*, 1989), in a similar fashion as that observed for PTH in normal parathyroid cells. More importantly, however, this cell line does not express the PTH gene (Ikeda *et al.*, 1989; Zajac *et al.*, 1989), which makes the usefulness of this parathyroid cell line limited to the study of PTHrP gene regulation.

Stable transformed cell lines have been successfully produced by introducing DNA encoding one or more oncogenes into cells. For example, SV40 DNA and Ha-*ras* oncogene DNA were introduced into cells to generate a granulosa cell line (Suh and Amsterdam, 1990). A strategy such as this could be attempted to produce a functional parathyroid cell line.

5.3 REGULATION OF PARATHYROID CELL SECRETION BY CALCIUM

Calcium is the major regulator of parathyroid secretory function. Parathyroid cells are unusual (LeBoff *et al.*, 1985) in that hypocalcemia stimulates the secretory activity in these cells, whereas in other endocrine cells, such as in chromaffin cells, hypercalcemia elicits the secretory response (Shahid *et al.*, 1989). Central to the understanding of this process is the elucidation of the primary sensor of minute changes in calcium concentrations, the intracellular mechanisms which transduce the calcium signal, and how calcium can modulate PTH secretion (Brown, 1993).

A secretory response in bovine parathyroid cells is transduced through a mechanism that involves a calcium receptor or sensor on the cell surface (Nemeth and Scarpa, 1987). The presence of a membrane receptor represents the primary effector of the calcium-regulated secretion by the parathyroid, since the secretory response to extracellular calcium is abolished, for example, by treating parathyroid cells with trypsin (Muff and Fischer, 1989). Three groups have recently reported in a preliminary form the cloning of a parathyroid calcium receptor (Brown *et al.*, 1993; Rask *et al.*, 1993; Garrett *et al.*, 1993). This appears to be a member of the seven membrane-spanning G-protein coupled receptor family.

G-proteins mediate the signals between membrane-bound receptors and intracellular effectors. For example, they can stimulate phospholipase C activity to generate diacylglycerol, which in turn can activate PKC. It is likely that the extracellular calcium signal is transduced in this way since it has been demonstrated that PKC is involved in the calcium regulated secretion of PTH (Brown *et al.*, 1984; Kobayashi *et al.*, 1988; Clarke *et al.*, 1993). For example, in the presence of high extracellular calcium, PMA, which directly activates PKC, stimulates PTH secretion (Morrissey, 1988). High extracellular calcium also stimulates the accumulation of inositol phosphates in bovine parathyroid cells (Brown, *et al.*, 1987; Kifor *et al.*, 1992), and PKC can prevent this accumulation. Because the regulation of PTH and CgA secretion by calcium is usually coupled in parathyroid cells, PKC may also be involved in the regulation of CgA secretion in this tissue, but this remains to be determined. Indeed, a protein kinase C signalling pathway has been shown to be involved in the regulation of CgA secretion by nicotine in chromaffin cells (Simon *et al.*, 1989).

Calcium-regulated parathyroid cell secretion also involves a cAMP-mediated mechanism. For instance, high extracellular calcium can inhibit agonist-stimulated cAMP accumulation, and this action is blocked by preincubation with pertussis toxin, which

causes ADP-ribosylation and inactivation of the alpha subunit of the inhibitory G-protein, G_i (Fitzpatrick *et al.*, 1986). Therefore, the calcium receptor can also be coupled to the inhibition of adenylate cyclase by the inhibitory G-protein, G_i (Brown, 1993), suggesting that more than one intracellular signalling pathway is involved in the calcium-regulated secretion in the parathyroid cell.

5.4 REGULATION OF CHROMOGRANIN A SYNTHESIS BY CALCIUM

Despite an effect of calcium on the secretory rate of CgA, we (Chapter 2) and others (Russell *et al.*, 1990) have demonstrated that calcium has no effect on steady-state CgA mRNA levels in acutely dispersed bovine parathyroid cells, implying that calcium does not regulate CgA gene transcription. In a recent study, however, using cultured porcine parathyroid cells, calcium was shown to markedly downregulate CgA mRNA levels (Zhang *et al.*, 1993), and these changes were reflected at the secretory level. These differences are likely due to methodological differences between these reports, namely, Zhang *et al.* (1993) initiated experiments after six days, whereas we (Chapter 2) and Russell *et al.* (1990) initiated experiments immediately. The different responses in bovine and porcine parathyroid cells could also possibly reflect a species difference in the response of the CgA gene to calcium. Further studies on the regulation of CgA gene expression in the parathyroid of other species will be required.

Post-transcriptional events are regulated by calcium in the parathyroid. For example, elevated extracellular calcium levels have been shown to directly enhance intracellular PTH degradation (Habener *et al.*, 1985), and this has been shown to be linked to the activation of PKC (Tanguay *et al.*, 1991). Protein synthesis can be modulated independently of transcription, at a translational step, for instance. This provides a mechanism where the synthesis of a protein can be rapidly changed in response to cell stressors. Whereas we have demonstrated that calcium has no effect on the PTH mRNA ribosome transit time in bovine parathyroid cells (Mouland and Hendy, unpublished), extracellular calcium has been reported to regulate the association of PTH mRNA with polyribosomes in these cells (Hawa *et al.*, 1993). They demonstrated that PTH mRNA association with polyribosomes was increased in the presence of low (0.4 mM) extracellular calcium concentration, compared to normal calcium concentration (1.0 mM) in bovine parathyroid cells. These results explained the previous observation of an increase in PTH biosynthesis at a low calcium concentration, without a concomitant change in the PTH

mRNA levels (Heinrich *et al.*, 1983).

We have reported in a preliminary form (Mouland and Hendy, 1991a), that the PTH mRNA that is polyribosome bound, sediments into two distinct polyribosome pools in a polyribosome gradient. Whereas in low and normal (0.4 and 1.0 mM) calcium concentrations PTH mRNA is distributed throughout the two polyribosome pools, treatment of parathyroid cells for 48 hours with a high (2.5mM) calcium concentration caused a loss of PTH mRNA from the less dense polyribosome pool, likely the result of the effect of high calcium on PTH gene transcription. Thus, in the high calcium concentration tested, there was less PTH mRNA, and the remaining PTH mRNA was associated with more dense polyribosome fractions than with the less dense fractions. The nature of the PTH mRNA in these dense polyribosome fractions is unclear. This could, however, represent a fraction of PTH mRNA which is associated with a large number of ribosomes, which is prevalent at all calcium concentrations. The results of the polyribosome profile would be consistent with a population of mRNA whose translation initiation rate is increased, without a change in elongation rate. Since calcium does not affect the PTH mRNA ribosome transit time (Mouland and Hendy, unpublished), the role of calcium on translation intitiation could be addressed. This could be assessed, for example, with the use of the translation initiation inhibitor, pactamycin, which was used in the studies in Chapter 3 to assess the role of translation initiation in the effects produced by $1,25(OH)_2D_3$. We can not directly compare the calcium effects on PTH polyribosome pools between our results and those of Hawa *et al.* (1993) because of the methodological differences that existed between the two studies.

High extracellular calcium also affected the polyribosome profile of CgA mRNA. Similar to the effect observed on the CgA mRNA polyribosome profile by $1,25(OH)_2D_3$ (Chapter 3), calcium caused a shift in the distribution of CgA mRNA into the denser fractions of the gradient, without any effect on the ribosome transit time (Mouland and Hendy, unpublished). Calcium has been shown to be crucial for the integrity of mRNA translation initiation in mammalian cells (Brostrom and Brostrom, 1991), and elevated levels in the parathyroid could be having an effect at this level of regulation. The effects of calcium on translation initiation could be determined as described above.

These studies demonstrate that translational control is undoubtedly an important mechanism in the calcium-mediated effects on parathyroid cell gene expression. Further investigation of the mechanism of the effects on parathyroid cell mRNA translatability is likely to be a fruitful area of study.

The study of the intracellular signalling pathways involved in producing the calcium-mediated changes in polyribosome profiles would contribute to our understanding of the regulation of protein biosynthesis. For example, alteration of intracellular calcium

with the use of the calcium ionophore, A23187, has been shown to affect mRNA translatability by altering the polyribosome profiles of some members of the protein synthesis translational machinery in pituitary cells (Prostko *et al.*, 1992). In the parathyroid, a rise in extracellular calcium results in a transient spike in intracellular calcium derived from intracellular stores by the actions of inositol phosphates on calcium mobilization from the endoplasmic reticulum (Nemeth and Scarpa, 1987; Kifor *et al.*, 1992). A more sustained increase in intracellular calcium levels is achieved by the influx of calcium through plasma membrane calcium channels (Fitzpatrick *et al.*, 1988). Several events including phosphorylation by PKC and calcium/calmodulin-dependent kinases are calcium-dependent (Taylor, 1989) and could be activated by increased calcium concentrations, for example. Analysis of these intracellular signalling pathways and how they affect the polyribosome profiles of both the CgA and PTH mRNAs would contribute to our understanding of the regulation of their biosynthesis.

5.5 REGULATION OF CHROMOGRANIN A SYNTHESIS BY 1,25-DIHYDROXYCHOLECALCIFEROL

The second major regulator of parathyroid cell function is $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ is produced in the proximal tubule of the kidney by the actions of the 1-α-hydroxylase enzyme on 25-hydroxycholecalciferol. Its production can be directly stimulated by circulating PTH levels (Reichel *et al.*, 1989). Conversely, $1,25(OH)_2D_3$ can act back on the parathyroid gland to decrease PTH synthesis and release in one direct and one indirect way. $1,25(OH)_2D_3$ acts to directly suppress the PTH gene transcription rate in the parathyroid gland due to the presence of a VDRE in the PTH gene promoter (Demay *et al.*, 1992). Alternatively, a long feedback loop is established by the actions of $1,25(OH)_2D_3$ to increase the blood Ca^{++} level (by increasing rapid calcium transport in the intestine), which then acts on the parathyroid to suppress PTH synthesis and release.

In addition to these effects, $1,25(OH)_2D_3$ can also regulate mRNA translation at the polypeptide elongation step. In the parathyroid, $1,25(OH)_2D_3$ causes a recruitment of CgA mRNA into dense polyribosomes, and this is reflected in an increase in the CgA mRNA ribosome transit time (Chapter 3). Translational regulation at this post-initiation site can be important for the fine control of gene expression (Tate and Brown, 1992), and in the model presented in this thesis, regulation at the level of CgA polypeptide chain elongation provides a fine control mechanism to counterbalance the enhancing effect of $1,25(OH)_2D_3$ on CgA gene transcription in the parathyroid. The regulation of CgA mRNA translation

may be prevalent in another tissue as well, for example in adrenal chromaffin cells, where translational regulation of CgA mRNA has been suggested to explain a poor correlation between gene transcription and CgA synthesis (Simon *et al.*, 1989).

The intracellular transduction pathways that are involved to produce the effects of $1,25(OH)_2D_3$ on CgA mRNA translatability are unclear. It has been demonstrated, however, that protein kinase C is involved in many actions of $1,25(OH)_2D_3$ in cells. For example, the action of $1,25(OH)_2D_3$ in bone cells involves the PKC signalling pathway (van Leeuwen *et al.*, 1992), and in HL-60 leukemia cells, $1,25(OH)_2D_3$ can stimulate the expression of protein kinase C by stimulating PKC gene transcription (Obeid *et al.*, 1990). Since $1,25(OH)_2D_3$ can act at other levels within cells, in addition to the level of gene transcription, in the transport of calcium in the intestine, for example, it will be of interest to study the PKC signalling pathway, and other transduction pathways that may be involved in the $1,25(OH)_2D_3$-induced changes in CgA mRNA polyribosome profile and ribosome transit time.

The effect of $1,25(OH)_2D_3$ on CgA synthesis is not a direct effect of $1,25(OH)_2D_3$ on CgA mRNA translation but is likely to be the result of *de novo* protein synthesis, since, for example, changes in the polyribosome profile were observed at 24 hours and thereafter (Chapter 3), and not at four and seven hour time points (Mouland and Hendy, unpublished). Furthermore, a direct effect of $1,25(OH)_2D_3$ on CgA mRNA translation was ruled out since *in vitro* translation of mRNA from parathyroid tissue in the presence of $1,25(OH)_2D_3$ did not result in a difference in the synthetic rate of preCgA protein when the translation products were assessed on SDS-polyacrylamide gels (Mouland and Hendy, unpublished).

Direct effects of steroid hormones on mRNA translation have been shown to occur, however. For example, a nonanucleotide sequence (5' AGAAGACCC 3') in the 5' untranslated region (5' UTR) of myelin basic protein mRNA was found to confer responsiveness to hydrocortisone and other steroid hormones including estradiol and progesterone (Campagnoni and Verdi, 1990). The first six basepairs of this sequence may be sufficient to confer responsiveness if they are in a favourable secondary structure in the single-stranded region of a base-paired stem. This steroid modulatory element is found twice in the coding region of bovine CgA mRNA (Ahn *et al.*, 1987), at codons 94 to 96, and 121 to 123, and once in the human CgA mRNA (Chapter 4) at codons 76 to 78. In the bovine molecule, one of the motifs is in a favourable secondary structure context in that the six nucleotides are found in an accessible loop structure (Mouland and Hendy, unpublished). The significance of this sequence motif in the CgA mRNA remains unclear, but could mediate a direct effect of steroid hormones, such as hydrocortisone, on CgA

mRNA translatability. This, however, remains to be established.

One would expect that translational regulation could provide a mechanism to modulate protein synthesis more rapidly to physiological regulators of cell function than transcription. This is true for the translational regulation of ribosomal protein L32 following serum stimulation (Kaspar *et al.*, 1990), and for the translational control of ferritin translation by iron in the liver (Aziz and Munro, 1981; Klausner *et al.*, 1993). However, regulation at the level of translation may occur in a more delayed fashion. For instance, the effect of interleukin-1 (IL-1) on insulin mRNA translation only follows the effect of IL-1 on insulin gene transcription after seventeen hours (Hughes *et al.*, 1990). Similarly, the effects of $1,25(OH)_2D_3$ on CgA mRNA translation in the parathyroid are only observed sometime after seven hours. Thus, regulation at this level may become more important in a delayed fashion in the regulation of parathyroid secretory function, for example. It is expected, therefore, that the action of $1,25(OH)_2D_3$ on CgA mRNA translation requires the presence of newly synthesized factors. These factors may somehow be involved in altering the molecular conformations, or in RNA-binding, in association with other proteins within messenger ribonucleoprotein (mRNP) complexes. CgA mRNA may possess a binding site for a $1,25(OH)_2D_3$-regulated RNA-binding protein, similar to that which was observed to bind to creatine kinase mRNA in U937 cells (Ch'ng *et al.*, 1990), for example.

Differential phosphorylation of protein factors associated with polyribosomes can play an important role in their activity state (Hershey, 1989). Several phosphoproteins are found in both polyribosome-bound and free cytoplasmic poly(A)mRNP fractions (Moore and Sharma, 1980). Proteins within mRNP complexes may be coordinately phosphorylated in response to $1,25(OH)_2D_3$, or calcium, and could determine CgA mRNA translatability in the parathyroid, for example. It will be of great interest to evaluate the regulation of translation factors such as these and how their activity state can be regulated by the major regulators of parathyroid cell function [i.e., extracellular (and intracellular) calcium and $1,25(OH)_2D_3$] and by other hormones that can affect the CgA gene.

5.6 INTERACTIONS BETWEEN CALCIUM AND 1,25-DIHYDROXYCHOLECALCIFEROL IN THE REGULATION OF PARATHYROID HORMONE mRNA

In Chapter 2 we demonstrated that neither the $1,25(OH)_2D_3$-induced increase in CgA mRNA levels nor the $1,25(OH)_2D_3$-induced inhibition of PTH mRNA levels was

modulated by extracellular calcium concentration. In addition, we demonstrated that there was no evidence that $1,25(OH)_2D_3$ modulated the calcium-mediated reduction in PTH mRNA levels, indicating that there was no interaction between these two parathyroid cell regulators, and each acted independently in bovine parathyroid cells in culture. In chicken parathyroid glands, however, Russell *et al.* (1993) have reported that the actions of $1,25(OH)_2D_3$ are modulated by calcium. By increasing calcium concentration in the blood, the actions of $1,25(OH)_2D_3$ on the regulation of the PTH and vitamin D receptor (VDR) mRNAs are enhanced, whereas $1,25(OH)_2D_3$ was not shown to be required for the actions of calcium on these genes (Russell *et al.*, 1993). Similarly, Naveh-Many *et al.* (1989) demonstrated that in *in vivo* studies in the rat, a decreased calcium appeared to slightly modulate the $1,25(OH)_2D_3$-induced decrease in PTH mRNA levels, where the decrease in PTH mRNA levels was not as marked with a lower circulating calcium level. Despite the lowering of serum calcium levels by phosphate infusion, $1,25(OH)_2D_3$ caused a decrease in PTH mRNA levels, indicating that $1,25(OH)_2D_3$ has a dominant role on PTH gene expression, over the enhancing effects of a lowered calcium on PTH gene expression in the rat (Naveh-Many *et al.*, 1989). The difference between our results and those of Russell *et al.* (1993) and Naveh-Many *et al.* (1989) is unclear. However, differences in the responses to calcium and $1,25(OH)_2D_3$ may occur between the *in vitro* and the *in vivo* settings. For example, the substratum to which a cell is attached *in vivo* may determine the final shape, and can in turn influence growth and the cellular responses to hormones and growth factors (Elias *et al.*, 1990).

5.7 DNA RESPONSE ELEMENTS IN THE CHROMOGRANIN A GENE

The presence of consensus hormone response elements (HREs), and other defined binding sites for transacting transcription factors that mediate enhancing or repressing activities in a gene's 5' flanking region, suggests that the activity of the gene may be controlled by these factors. In the CgA gene, there are DNA sequence motifs that are homologous to consensus sequences of some well defined transcription factors (Table 5.1). For example, basal transcription factors such as the GC box, which binds the transcription factor Sp-1, begins at -61 to -56, relative to the transcriptional initiation site. In addition, the presence of a TATA box that begins at -26 to -20 not only serves as a specific binding site for TATA-binding protein (TBP) binding, but also serves to program accurate initiation of transcription (Mitchell and Tijan, 1988).

A cAMP-response element (CRE; Mitchell and Tijan, 1989) begins at -49 to -42, and binds the cAMP-response element binding protein (CREB). Forskolin can upregulate CgA gene expression in chromaffin cells (Iacangelo *et al.*, 1991) and in MTC cells (Murray *et al.*, 1988), and these data are consistent with the presence of a CRE domain in the CgA gene. But this regulation is cell-specific (see earlier), since cAMP and the protein kinase A signalling pathways do not modulate CgA mRNA levels in the rat insulinoma cell line, RIN14B (Iacangelo *et al.*, 1991). Since a CRE can exhibit a multiplicity of actions (de Groot and Sassone-Corsi, 1993), the specific function, as well as the cell specific activity of this response element, can be tested in the human CgA gene. For example, cAMP or forskolin treatment of cells that have been transfected with hCgA gene constructs containing the CRE upstream of a reporter gene would be expected to show a change in reporter gene expression. Similarly, a loss of CRE activity should be conferred by deleting or mutating one or more of the critical basepairs within the CRE. These avenues are currently being explored.

As described earlier, CgA synthesis can be modulated by several steroid hormones whose genomic actions are mediated by cytosolic receptors which bind to specific DNA response elements. Both estrogen- and glucocorticoid-like response elements have been localized in the 5' flanking regions of the bovine and human CgA genes (Iacangelo *et al.*, 1992; Chapter 4; Table 5.1).

The presence of AP-1- and AP-2-like sites (Table 1.5) in the 5' flanking region of the CgA gene also supports the involvement of PKC and PKA in the regulation of CgA gene expression *in vitro* in neuroendocrine cells.

Because CgA gene transcription was shown to be enhanced by $1,25(OH)_2D_3$ in our studies (Chapter 2) and in those of others (Russell *et al.*, 1990), it was of interest to search for the presence of a VDRE within the CgA gene. Recent investigations have demonstrated that $1,25(OH)_2D_3$-regulated transcription is complex, involving two classes of VDREs (Carlberg *et al.*, 1993). The first class of receptor is composed of directly repeated pairs of motifs (GGTCCA, AGGTCA, or GGGTGA) spaced by three nucleotides. The second class of VDRE can be activated by VDR alone, and is composed of the motif A/GGGTGA, arranged as a direct repeat with a spacing of six nucleotides or as a palindrome with a twelve-nucleotide spacing (Carlberg *et al.*, 1993). Two VDRE-like motifs consistent with the structure of the second class of VDRE were localized beginning at -661 to -644, and at -124 to -107 (both are direct repeats with a six basepair spacing; Table 1.5). Two VDRE half-sites were also localized within the 2300 basepair (bp) of the 5' flanking region of the human CgA gene (Table 5.1).

Unusual DNA sequences can determine the conformation of the DNA molecule,

Table 5.1

Putative Cis-Acting Response Elements* in the 5' Flanking Region of the Human Chromogranin A Gene

Element (position[1])		Consensus Sequence[2]	Sequence in hCgA Gene
TATA	(-26)	TATATAA	TATATAA
CRE	(-49)	TGACGTCA	TGACGTCA
Sp-1	(-61)	GGCGGG	GGCGGG
VDRE-like	(-124)	GGGTGAN$_6$GGGTGA	GGGTGAN$_6$AGGTGG
	(-661)	TCACCCN$_6$TCACCC	TCACCCN$_6$TGAACC
VDRE $^1/_2$ sites	(-891)	$^{A}_{G}$GGTCA	AGGTGA
	(-846)	$^{A}_{G}$GGTCA	AGGTGA
ERE	(-1545)	GGTCACAGTGACC	GGTCAGGCTGGTC
GRE	(-891)	GGT$^{T}_{A}$CA$^{C}_{A}$T$_A$NTGTTCT	GGTGAGAGcTGTTCT
AP-1	(-418)	TGA$^{C}_{C}$TCA	TGAGTGA
AP-2	(-374)	G$^{G}_{C}$G$^{T}_{C}$A$^{C}_{G}$CC	CCCCAGGC
IR[3]	(-1736)	—	GTTTGAGAcAGAGTTTC
	(-1917)	—	GCATTAAgtccAATTATG
PR[4]	(-77)	—	[(GGG)AGN]$_3$[5]

* To be confirmed by functional studies.

1 Number indicates beginning of element relative to transcriptional initiation site.

2 When alternate basepairs are indicated (Locker and Buzard, 1990) the best fit to the hCgA sequence is chosen.

3 Inverted repeats.

4 Purine-Rich (PR) sequence.

5 "N" denotes any basepair.

which may be critical for the binding of regulatory protein factors. For example, DNA cruciforms can form at inverted repeat sequences (Wells, 1988). In the CgA 5' flanking region, two inverted repeats begin at -1736 to -1720 and at -1917 to -1900 (Table 1.5). The function of these sequence motifs is presently unclear. In eukaryotic genes, such as those for rat thyroglobulin and chicken ß-globin, sequences that consist of oligopurine and oligopyrimidine sequences on one strand can cause DNA to adopt unusual DNA conformations in that region, or at a distal region (Wells, 1988). Functionally, these sequences can potentially direct the binding of regulatory proteins and direct gene transcription initiation. For example, cell-specific gene expression in lymphoid cells is conferred by the presence of a purine-rich sequence element that binds the PU.1 transcription factor (Goebl, 1990). There is a conserved 20 bp purine-rich sequence [{(GGG)AGn}$_3$] just upstream of the TATA box that begins at -77 to -58 in the human CgA gene (Table 1.5). This region is similar to the lymphoid-specific element (Goebl, 1990). It was originally speculated that this sequence element played a role in conferring neuroendocrine cell-specific gene expression of the CgA gene. However, this sequence is not contained within our smallest construct (p53CAT), which is able to confer neuroendocrine-cell specificity used in our transient transient transfection studies (Chapter 4), suggesting that it serves another function.

Cis-acting DNA elements have not only been found in the 5' flanking regions of eukaryotic genes. They have also been found in intronic sequences, for instance (Wang and Chen, 1992). It is possible that the sequence analysis of the CgA promoter regions further upstream than those reported (Wu *et al.*, 1991; Iacangelo *et al.*, 1991; Chapter 4), or examination of the sequence in introns, for example, will reveal other cis DNA elements that may mediate CgA gene expression in neuroendocrine cells.

Even high sequence homology to well defined functional cis-acting DNA elements is suggestive but not proof of functionality. Without functional studies to examine the activity of putative DNA response elements, one can only infer that the regions are active. For the putative HREs contained within the 5' flanking region of the human CgA gene listed in Table 1.5, including those for glucocorticoids, estrogen, and 1,25(OH)$_2$D$_3$, evaluation of their functionality is clearly of interest. This analysis can be performed with the use of constructs containing these DNA elements upstream of a reporter gene. Testing transfected cells with the respective hormones should provide insight into the HREs' functionality. Furthermore, the examination of binding to specific DNA sequences can be addressed with the use of DNAse I footprinting assays. A band- or mobility-shift assay can provide direct proof that protein factors can bind these elements. In this analysis, radiolabelled oligonucleotides containing defined sequences, usually the areas that are

protected on footprint gels or the sequences that contain a putative HRE, are incubated with nuclear protein extracts in the presence (or absence) of hormone to confirm the presence of DNA binding. This provides direct information about the sequences to which the steroid hormone receptor/ligand bind. Moreover, binding can be competed off with unlabelled DNA to demonstrate the specificity and avidity of binding. Placing the HRE in front of a heterologous promoter and conferring hormone responsiveness is the ultimate test for functionality. This has been done to demonstrate the specific function of the VDRE in conferring $1,25(OH)_2D_3$ responsiveness to the mouse osteopontin gene (Noda *et al.*, 1990). These types of studies will provide information about the molecular mechanisms underlying CgA gene regulation, and provide for an exciting area of study.

5.8 ENDOCRINE CELL-SPECIFIC NATURE OF CHROMOGRANIN A GENE EXPRESSION

Cell-specific expression of a gene can be achieved by a combination of cell-specific transacting factors and specific DNA elements in the gene's promoter region (Maniatis *et al.*, 1987). For example, immunoglobulin genes are expressed in a cell-specific manner due to 1), octamer motifs in their gene promoter, and 2), lymphoid-specific proteins that bind this DNA motif (Staudt *et al.*, 1986). With the characterization of the CgA gene, the question: 'What determines the cell-specific nature of CgA gene expression?' can now be addressed. Specifically, the cis DNA element(s) can be functionally defined in detail. The activity of the putative response element can be assessed in functional assays by preparing deletion constructs of the CgA gene promoter fused to a reporter gene. We have done this in Chapter 4 where we show that -53 to +32 of the 5' flanking region confers neuroendocrine cell specificity. To further assess the functionality, this region can be further mutagenized to evaluate the critical DNA bases for function in neuroendocrine cells.

In addition, cell-specific transacting factors are necessary to program cell-specific gene expression (Maniatis *et al.*, 1987). For example, transcription factors that are specifically expressed in pituitary gonadotrophs are required for growth hormone gene expression in these cells (West *et al.*, 1987), and can confer transcriptional activity from the growth hormone promoter in nonexpressing cells (Bodner and Karin, 1987). Neuroendocrine cells will likely express cell-specific factors that bind to the CgA promoter region, downstream of -53. Elucidation of the cell-specific transacting factors in neuroendocrine cells that are able to promote CgA gene transcription initiation will provide for another area of study. This information should also provide clues to the cell-specific

gene expression of the other granins in neuroendocrine cells. Techniques that would be used to identify specific transacting factors of the CgA gene include DNAse I footprinting assays to identify the binding regions on the promoter; mobility-shift assays using nuclear extracts and DNA oligomers to demonstrate DNA binding; and once having identified DNA-binding proteins that are exclusively expressed in neuroendocrine cells, for example, purification of these could be achieved by biotin/streptavidin column chromatography. In this technique DNA fragments that contain the binding sites for DNA-binding proteins are immobilized and used to purify the specific binding proteins.

Characterization of the CgA gene will also shed light on the cell-specific nature of CgA gene regulation and help to identify transactivating factors responsible for this. CgA gene expression can be regulated by many factors, but as described above, the regulation is cell-specific. For instance, estrogen regulates CgA biosynthesis in the pituitary, but not in other tissues such as the adrenal medulla, hippocampus, or hypothalamus (Anouar *et al.*, 1991; Fischer-Colbrie *et al.*, 1992). At least three possibilities exist here to explain this phenomenon: 1), the hormone receptors may be absent, 2), the hormone receptors may be deficient in some aspect of signal transduction subsequent to hormone binding in selected tissues, or 3), other cell-specific factors may be involved to obtain a hormone response. These ideas have been suggested to explain the lack of responsiveness to thyroid hormone (T_3) of growth hormone in fibroblast and kidney cells, despite the presence of high affinity nuclear T_3 receptors (Larsen *et al.*, 1986). Clearly, a combinatorial effect of both cell-specific positive and negative elements direct the expression of the CgA gene. These factors remain to be elucidated and provide for another exciting and fruitful area of study.

5.9 EVALUATION OF THE FUNCTION OF CHROMOGRANIN A

As described earlier, many functions for CgA have been suggested. One focus in the literature has examined the role of CgA as a precursor to bioactive peptides. Many *in vitro* and *in vivo* studies have examined the role of CgA peptides on secretory activity in a variety of cells. CgA may have other important roles as well, such as that of a condensation protein in endocrine cell secretory granules, or as a modulator of prohormone processing. The studies presented in Chapters 2 and 3 examine the regulation of the biosynthesis of CgA in the parathyroid. With the results of these studies, one can only infer the function of CgA. Similarly, by examining the regulation of CgA biosynthesis in other cells, for example, in pituitary corticotroph and gonadotroph cells, a functional role for CgA can be

suggested. These studies further support the role of CgA as a modulator of secretory activity in these cell types.

Membrane receptors are likely to transduce the biological effects of the CgA-derived peptides. However, only one study to date (Galindo *et al.*, 1992a) has demonstrated the presence of a membrane receptor specific for the CgA-derived peptide, chromostatin, and a protein phosphatase was implicated in the transduction of the biological response (Galindo *et al.*, 1992b). Further work will be required to study the mechanisms by which the CgA-derived peptides exert their biological response, namely the identification of each peptide's receptor (if they are different, of course), and how these signals are transduced intracellularly to affect secretion.

Many sophisticated methods are available to directly assess a protein's function. These include several antisense RNA methodologies, homologous recombination and the generation of transgenic animals, and the generation of mutant animals or lower eukaryotes defective for the gene of interest. Despite the availability of these procedures, no report has yet examined CgA function in any of these ways.

Several antisense methodologies can be used to evaluate protein function in cells. Antisense RNA methodology mimicks a naturally occurring mechanism in prokaryotes (Green *et al.*, 1986) and eukaryotes (Spencer *et al.*, 1986; Williams and Fried, 1986) which renders a gene nonfunctional or incapable of being expressed. This method has been applied successfully *in vitro* in cultured cells as a tool to elucidate the role of many genes in cancer and cell growth, for example (Calabretta, 1991). The first antisense RNA methodology consists of taking a plasmid containing all or part of the gene of interest --in an antisense orientation-- and transiently or stably expressing it in cells. It is believed that an RNA duplex forms with the targetted gene's transcript, and that protein synthesis can be specifically inhibited by this mechanism. A more simple antisense approach consists of adding antisense oligomers to the tissue culture medium where they can diffuse into the cell and inhibit mRNA translation in the cytosol by the formation of a RNA:DNA hybrid. In addition, with the use of short oligodeoxyribonucleotides, RNA polymerase can be blocked in the nucleus by the formation of triple-helices between the oligodeoxynucleotides and the double-stranded genomic DNA (Riordan and Martin, 1991; Young *et al.*, 1991). Furthermore, gene expression can be blocked by hybrid formation and interference with pre-mRNA splicing (Munroe, 1988; Cotten *et al.*, 1989).

We initiated studies to analyze the function of CgA in the parathyroid using the antisense RNA approach where oligodeoxynucleotides are added to medium of cultured cells. Several factors were considered in the oligomer design strategy. In order to achieve duplex formation, single-stranded regions of the mRNA should be chosen as targets (Stein

and Cohen, 1988). A few computer programs are available to predict the secondary structure of RNA molecules (Jacobson *et al.*, 1984; Zuker, 1989). These programs require a large amount of processing power, and provide an estimation of the secondary structure. For example, Heikkila *et al.* (1987) were successful in achieving antisense inhibition of mitogen-induced c-myc protein expression. They predicted that the initiation codon and the four downstream codons lay in a single-stranded RNA loop structure. The antisense oligomers should have therefore formed a stable duplex within this RNA region, and this could explain, at least in part, their success with this strategy. Therefore, with the help of an RNA secondary structure analysis program (Jacobson *et al.*, 1984), a complementary 15 bp oligodeoxynucleotide was designed to a single-stranded region of the CgA mRNA, which potentially forms at codons 96-100 (oligo 1). In addition, because the inhibition of translation initiation in apparently more effective than inhibiting elongation, a 15 bp antisense oligomer (oligo 2) was synthesized to the initiation codon and the four downstream codons of CgA mRNA. This RNA region, however, was predicted to be involved in more secondary structure compared to oligo 1 (Mouland and Hendy, 1989), where twelve ribonucleotides are involved in basepairing and the other three form a bulge loop. We were then able to compare the effectiveness of both antisense oligomers in our experiments.

Initially, in order to judge the feasibility of the approach, we tested these oligomers in a cell-free *in vitro* translation system. Whereas oligo 2 was able to specifically inhibit CgA mRNA translation in a dose-dependent manner, oligo 1, which targets the initiation codon region, was ineffective at all concentrations tested (Mouland and Hendy, 1989). Therefore, the secondary structure of the targeted region of the mRNA is indeed an important consideration in the design of antisense oligomers.

We therefore tested the effectiveness of oligo 2 in the inhibition of CgA protein expression in cultures of bovine parathyroid cells. The oligomer was added to the medium of primary cultures of parathyroid cells. A wide range of oligomer concentration was tested, but neither CgA mRNA nor CgA synthesis was affected. Some limitations to the antisense RNA technique immediately became apparent in a system such as ours, however. Firstly, the targeted mRNA is very stable, with a half-life of over 30 hours (Chapter 2). C-myc as well as other protooncogene mRNAs are very short-lived, and successful antisense inhibition of their synthesis may be a function of their short mRNA half-lives (Calabretta, 1991). Furthermore, examination of the stability of these oligomers revealed that they were metabolized very rapidly in our parathyroid cultures (with a half-life of approximately 3 hours), in a cell-dependent manner. In cultures of HL-60 cells, for example, oligomers have a much longer half-life, of 24-48 hours (Holt *et al.*, 1988).

Several nuclease-resistant oligomer analogues are available (Stein and Cohen, 1988). For example, phosphorothioate and methylphosphonate analogues, where a sulphur atom and a methyl group replaces one of the oxygen atoms on the internucleotide linkage, respectively, are more resistant to nuclease activity in mammalian cells. Furthermore, the methylphosphonate analogue is more lipophilic, and therefore can more readily traverse the plasma membrane. However, even with the use of an antisense CgA methylphosphonate oligomer, metabolism of the oligomer was only attenuated in parathyroid cell cultures, with a half-life of approximately 6 hours. Thus, because primary cultures of parathyroid cells have a high endogenous nuclease activity, the use of another antisense RNA approach would be necessary to address CgA function in a cell system such as this. To pursue this avenue of research, the method can be optimized for this cell system in that more resistant oligomer analogues could be used, or, alternatively, the approach of transfecting parathyroid cells with the CgA gene in an antisense orientation could be used.

Several cellular processes and how CgA is involved in them could be examined. For example, the role of CgA in the endogenous processing of hormones could be evaluated, especially since CgA has been shown to be a competitive substrate in the processing of POMC (Seidah *et al.*, 1987). And, by inhibiting CgA protein expression, this approach could directly verify CgA's putative intracellular and extracellular roles in neuroendocrine cell secretion.

Along the same lines, one major direction in CgA research is the evaluation of the endogenous proteolytic processing of CgA, which is central to the understanding of CgA function. With the use of antisense RNA technology, the role of recently described prohormone convertases [see Smeekens (1993) and Seidah and Chrétien (1992) for reviews] can be evaluated in the prohormone processing in neuroendocrine cells. For instance, by introducing an expression vector containing the cDNA of PC-1 in the antisense orientation into mouse AtT-20 corticotroph cells, the prohormone convertase, PC-1, was demonstrated to be active in the processing of POMC (Bloomquist *et al.*, 1991). By a similar approach, the processing enzymes responsible for the processing of CgA could be evaluated in neuroendocrine cells. The distribution of prohormone convertases is tissue-specific and this characteristic will determine the specific pattern of CgA-derived peptides in each tissue. Thus, an important area of study involves the evaluation of CgA-derived peptides and their function in each tissue.

A "knock-out" experiment takes advantage of homologous recombination to ablate a gene, and the consequences of this can be evaluated *in vivo* (Mansour *et al.*, 1988). This approach has not yet been used to address CgA function. However, Kingsley *et al.*, (1990) generated mutant mice deficient for another member of the granin family, *1B1075* in

the brain (SgIII in Figure 1.1). They did not find any detectable phenotypic, developmental, or other abnormalities in these mice. It is possible that subtle changes were overlooked in this study, or ablation of *1B1075* could be compensated by the presence of another granin in this tissue suggesting functional redundancy for this family of secretory proteins.

The yeast, *Saccharomyces cerevisiae*, provides another genetic tool to evaluate the role of genes in cellular processes *in vivo*. For example, the generation of yeast mutants defective for the phospholipid transfer protein gene, *sec14*, produces a yeast which has a dysfunctional golgi complex, and as a consequence, a defective secretory process (Bankaitis *et al.*, 1990). Thus, a critical role for this gene product was demonstrated. Similarly, since several lower eukaryotes contain CgA immunoreactive proteins (Winkler and Fischer-Colbrie, 1992), a yeast strain defective for the CgA gene, for example, could be generated to evaluate its function in the secretory process.

The above technologies represent invaluable tools, which should provide important clues to the function of CgA and the other granins in the secretory activity of endocrine and neuroendocrine tissues.

CHAPTER 6

CLAIMS TO ORIGINALITY

1. Demonstration of an opposite regulation of PTH and CgA genes by 1,25-dihydroxycholecalciferol in the parathyroid.

2. Demonstration that CgA secretion is coupled to its synthesis in 1,25-dihydroxycholecalciferol-stimulated bovine parathyroid cells.

3. Demonstration that calcium and 1,25-dihydroxycholecalciferol act independently on the CgA and PTH genes.

4. Demonstration that translational regulation is important in the regulation of gene expression in the parathyroid.

5. Demonstration that the PTH and CgA mRNAs are both polyribosome-bound and sediment into two polyribosomal pools, one of intermediate and the other of greater density.

6. Demonstration that 1,25-dihydroxycholecalciferol can specifically increase the CgA mRNA ribosome transit time.

7. Complete structural and functional characterization of the human CgA gene.

8. Demonstration that a high conservation exists in the exon structure and organization between the human, bovine and murine CgA genes.

9. Definition of several putative DNA response elements in the CgA gene promoter.

10. Partial characterization of the endocrine cell-specific DNA element in the human CgA promoter (located between -53 and +32, relative to the transcription initiation site).

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