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TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL REGULATION OF SOMATOSTATIN GENE EXPRESSION BY GLUCOCORTICOIDS

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February 1995

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of Doctor of Philosophy

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This thesis is dedicated to Beijing and Beijing Medical Universities where I completed the first decade of my scientific career; to my wife, Xiao-Yan Sun, and son, George Liu, in appreciation for their understanding and continuous support; to my parents and formal mentors.

ABSTRACT

Glucocorticoids and somatostatin both influence a broad spectrum of biological activities and their actions are cooperative in growth control, pancreatic islet function, immune suppression, and stress response, e.g. *in vivo* studies indicate that glucocorticoids may act through somatostatin to suppress growth, growth hormone secretion and inflammation. Recent studies have suggested that glucocorticoids influence somatostatin production but the precise nature of this effect has remained unclear. In this thesis, I characterized the actions of glucocorticoids on somatostatin gene expression and their molecular mechanisms of action in three consecutive studies. (1) I started with an investigation of **the *in vivo* and *in vitro* effects** of glucocorticoids and found that dexamethasone exerts significant effects on somatostatin peptide and steady state mRNA levels in normal somatostatin-producing tissues in rats, in cultured cerebral cortex, pancreatic islet and islet somatostatinoma ($1027B_2$) cells. Glucocorticoids stimulate somatostatin production in peripheral tissues (stomach, pancreas, and jejunum) and suppress its biosynthesis in cerebral cortex and hypothalamus. Glucocorticoids induce dose-dependent biphasic effects on steady state somatostatin-mRNA levels in normal rat islet and $1027B_2$ cells, characterized by stimulation at low doses (10^{-10} M) and marked inhibition at high doses ($\geq 10^{-7}$ M). This suggests a complex molecular mechanisms of glucocorticoid action on the somatostatin gene involving multi-level regulation. (2) I further discovered that glucocorticoids stimulate **somatostatin gene transcription** in PC12 (pheochromocytoma) cells transfected with somatostatin promoter-CAT (chloramphenicol acetyl transferase) reporter gene. Dexamethasone induces a dose-dependent 2.2 fold stimulation of somatostatin-CAT expression in PC12 cells and exerts an additive

effect on cAMP-induced gene transcription. The dexamethasone effect is abolished in A126-1B2 (protein kinase A-deficient mutant PC12) cells and with CRE (cAMP response element) mutant construct in PC12 cells, suggesting that glucocorticoid-induced transactivation is dependent on protein kinase A activity, and may be mediated via protein-protein interaction between the glucocorticoid receptor and the CRE binding protein (CREB). Promoter deletions and gel mobility shift assay indicated that DNA sequences upstream from the CRE between -250 and -71 bp in the somatostatin promoter are the target of glucocorticoid action. (3) Finally, I investigated the molecular mechanism underlying glucocorticoid-induced suppression of somatostatin-mRNA levels in thyroid medullary carcinoma (TT) cells. Dexamethasone ($\geq 10^{-8}$ M) produced a dose-dependent reduction of both somatostatin secretion and somatostatin-mRNA levels after a lag period of 12~24 h. To exclude a transcriptional effect, I found that dexamethasone did not inhibit somatostatin-CAT activity and failed to influence the rate of somatostatin gene transcription determined by nuclear run on assay. Both actinomycin D (inhibitor of mRNA synthesis) and cycloheximide (inhibitor of protein synthesis) effectively blocked glucocorticoid effect on somatostatin-mRNA accumulation. The results suggest that glucocorticoid inhibition of somatostatin-mRNA level in TT cells is not mediated through direct transcriptional control of the gene. It requires transcription of another gene(s) whose product(s) accelerates **somatostatin-mRNA degradation**. Therefore, at low doses and in peripheral tissues, glucocorticoids activate somatostatin gene transcription via positive interaction with CREB. At high doses, glucocorticoids decrease somatostatin-mRNA levels through accelerated mRNA degradation, in both brain and peripheral tissues as well as in thyroid carcinoma TT cells.

RÉSUMÉ

Glucocorticoïdes et la somatostatine influencent un large spectre de processus biologiques et ils oopèrent à la croissance, au fonctionnement des îlots de Langerhans, à l'immunosuppression et aux réponses au stress. Par exemple, des études in vitro suggèrent que les glucocorticoïdes puissent agir via la somatostatine en supprimant la croissance et l'inflammation. Les études récentes suggèrent que les glucocorticoïdes induisent la production de la somatostatine, mais la nature de cet effet n'est pas connue. Dans ce mémoire, j'ai étudié l'influence des glucocorticoïdes sur l'expression de gène de la somatostatine et le mécanisme moléculaire de ce processus. Cette étude comporte trois volets consécutifs. J'ai commencé avec l'examen des effets des glucocorticoïdes dans les conditions in vitro et in vivo. J'ai trouvé que le dexamethasone a un effet important sur les niveaux de la somatostatine et de son mRNA dans les tissus qui produisent de la somatostatine dans les conditions normales chez le rat, dans les cultures du cortex cérébral, des îlots de Langerhans et des cellules de somatostatinome des îlots (1027B,). Glucocorticoïdes stimulent la production de la somatostatine dans les tissus du système nerveux périphérique (estomac, pancréas, jéjunum) et ils suppriment sa biosynthèse dans le cortex cérébral et l'hypothalamus. Glucocorticoïdes influencent de deux différentes façons le niveau de mRNA chez les îlots normales de rat et chez des cellules 1027B, qui sont stimulées par une faible dose (10^{-10}M) et inhibées par une dose élevée($>10^{-7}$ M). Ces données suggèrent l'existence d' un complexe mécanisme moléculaire de l'action des glucocorticoïdes sur le gène de la somato-

statine, impliquant la régulation à plusieurs niveaux. En plus, j'ai trouvé que les glucocorticoïdes stimulent la transcription de gène de la somatostatine chez les cellules de PC12 transfectées avec somatostatine à l'aide de gène rapporteur : promoteur-CAT. Dexamethasone provoque une stimulation de 2.2 fois, dépendante du dosage, de l'expression de la somatostatine - CAT chez les cellules de PC12. Elle a aussi un effet additif sur la transcription du gène induite par l'AMP cyclique. L'effet de la dexamethasone est aboli chez les cellules de A126 1B2 (mutant de PC12 déficient en protéinkinase A). Le même effet est obtenu en utilisant un CRE (élément de réponse de AMP cyclique) ce qui suggère que la transactivation induite par les glucocorticoïdes dépend de l'activité du protéine kinase A. Cette transactivation peut être transmise via l'interaction protéine-protéine entre les récepteurs glucocorticoïde et la protéine de liaison de CRE. La suppression du promoteur et un essai par gel de retardement indiquent que la séquence de DNA, en remontant à partir de CRE (entre 250 et -71 bp) sur le promoteur de somatostatine, est la cible de l'action des glucocorticoïdes. Finalement, j'ai étudié le mécanisme moléculaire étant à l'origine de la suppression du niveau de SS-mRNA induite par les glucocorticoïdes chez les cellules médullaires du carcinome de thyroïde (TT). Dexamethasone cause la réduction de la sécrétion de la somatostatine et du niveau de SS-mRNA après un décalage de 12 à 24 h En écartant l'effet de la transcription, j'ai trouvé que la dexamethasone n'inhibe pas l'activité de la somatostatine - CAT et, en plus, elle n'influence pas le taux de la transcription de gène de la somatostatine tel que déterminé par un essai de transcription nucléaire. Actinomycine D (inhibiteur de la synthèse de mRNA) et cycloheximide (inhibiteur de la synthèse des protéique) bloquent efficacement l'effet des glucocorticoïdes sur l'accumulation de SS-

mRNA. Les résultats indiquent que l'inhibition de niveau de SS-mRNA chez les cellules de TT n'est pas transmise via le contrôle direct de la transcription de gène. La transcription d'un autre gène, produit duquel accélère la dégradation de SS-mRNA, est requise. Cependant, à faible dose et dans le tissu périphérique, les glucocorticoïdes activent la transcription du gène de la somatostatine via une interaction positive avec CREB. A forte dose les glucocorticoïdes baissent le niveau de SS-mRNA en accélérant la dégradation de mRNA dans les tissus central et périphérique ainsi que chez les cellules TT du carcinome de thyroïde. (Traduit par Olga Dembinska)

PREFACE

Somatostatin is a secretory peptide important in controlling hormone secretion in pituitary, pancreatic islet and the gut, as well as in neurotransmission, gut function, and immune response. It is produced in a variety of mammalian cells notably those in the brain, pancreas, and gut. Somatostatin secretion and biosynthesis are influenced by agents such as nutrients, ions, peptide hormones, and neurotransmitters, many of which act through intracellular cAMP, which activates somatostatin gene transcription through the cAMP-response element (CRE). Glucocorticoids are also known to regulate secretion and gene expression in many endocrine and nonendocrine target cells. Previous reports have suggested that glucocorticoids are capable of influencing somatostatin function but the precise nature and molecular mechanisms of this effect have remained unclear. In this thesis, I have systematically examined the effects of glucocorticoids on somatostatin peptide and steady state mRNA levels using *in vivo* and *in vitro* models. I have further analyzed the molecular mechanisms of glucocorticoid actions on somatostatin gene transcription and mRNA degradation. Following Chapter I (review and introduction), the main body of the thesis consists of three individual chapters (III to V) based on original publications, each with their own brief introduction, result, and discussion.

Most of the studies described in this thesis have been (are to be) published in the following **ORIGINAL ARTICLES:**

1. DN Papachristou, JL Liu, and YC Patel. Glucocorticoids regulate steady state somatostatin mRNA and peptide levels in normal rat tissues and in a somatostatin producing islet tumor cell line ($1027B_2$). **Endocrinology** 1994; 134: 2259-66.

2. JL Liu, DN Papachristou, and YC Patel. Glucocorticoids activate somatostatin gene transcription via positive interaction with the cAMP signalling pathway. **Biochem J** 1994; 301: 863-9.

3. JL Liu, and YC Patel. Glucocorticoids inhibit somatostatin (SS) gene expression through accelerated degradation of SS-mRNA in human thyroid medullary carcinoma (TT) cells. **Endocrinology** 1995; 136(6) (in press)

Apart from Dr. Yogesh C. Patel, my thesis supervisor, the participation of the coauthor in the above listed publications was as follows:

#1. Dimitrious N. Papachristou initiated the study, performed half of the *in vivo* study, experiments with $1027B_2$ cells and pancreatic islet culture. I contributed the remaining half of the same studies, and additionally performed the experiments with cerebral cortical cultures. I analyzed the data with Dr. Papachristou's help, prepared all the Tables and Figures, and wrote the manuscript with Dr. Patel.

#2. Dimitrious N. Papachristou participated in setting up the transfection condition and CAT assay in preliminary stage.

In the development of the PhD project, I have been involved in other related projects in this laboratory and made significant contributions in the following four manuscripts and various symposia presentations (The manuscripts are not included in the thesis but summarized in the Appendix):

4. DN Papachristou, JL Liu, and YC Patel. Cysteamine-induced reduction in tissue somatostatin immunoreactivity is associated with alterations in somatostatin mRNA. **Regulatory Peptide** 1994; 49: 237-47.

5. MT Greenwood, R Panetta, LA Robertson, JL Liu, and YC Patel. Sequence analysis of the 5'-flanking promoter region of the human somatostatin receptor 5 (SSTR5). **Biochem Biophy Res Comm** 1994; 205: 1883-90.

6. YC Patel, JL Liu, A Warszynska, G Kent, and DN Papachristou. Quinolinic acid differentially stimulates somatostatin but not neuropeptide Y gene expression in cultured cortical neurons. **J Neurochem** 1995 (in press)

7. YC Patel, AS Galanopoulou, SN Rabbani, JL Liu, M Ravazzola, and M Amherdt. Prosomatostatin is efficiently processed at dibasic and monobasic cleavage sites to both SS-14 and SS-28 via the constitutive pathway in islet somatostatin tumor cells ($1027B_2$). **J Biol Chem** 1995 (submitted)

ABSTRACTS:

1. JL Liu, DN Papachristou, and YC Patel. Glucocorticoids regulate somatostatin gene transcription positively in $1027B_2$ islet and PC12 pheochromocytoma tumor cells. **3rd IBRO World Congress of Neuroscience**, August 4-9, 1991, Montreal, Canada. Abstract P17.8

2. JL Liu, and YC Patel. Glucocorticoids activate somatostatin gene transcription through cooperative interaction with the cAMP signalling pathway. **74th Annual Meeting of the Endocrine Society**, June 24-27, 1992, San Antonio, TX. Abstract #573.

3. YC Patel, A Warszynska, JL Liu, G Kent, DN Papachristou, and SC Patel. NMDA differentially stimulates somatostatin (SS) but not neuropeptide Y (NPY) gene expression in cortical SS/NPY producing neurons. **22nd Annual Meeting, Society for Neuroscience**, October 25-30, 1992, Anaheim, CA. Abstract #613.12

4. JL Liu, and YC Patel. Glucocorticoids inhibit somatostatin (SS) gene expression through accelerated degradation of SS-mRNA in human thyroid medullary carcinoma (TT) cells. **76th Annual Meeting of the Endocrine Society,** June 15-18, 1994, Anaheim, CA. Abstract #1210

5. MT Greenwood, R Panetta, JL Liu, O Dembinska, and YC Patel. Cloning and sequence analysis of the human somatostatin receptor 5 (SSTR5) 5'-flanking promoter region. **24th Annual Meeting, Society for Neuroscience**, November 13-18, 1994, Miami Beach, FL. Abstract 220.2

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ABBREVIATIONS

$1,25\text{-}(OH)_2D_3$	1,25-dihydroxyvitamin D_3
aa	amino acid
ACh	acetyl choline
AP-1	activator protein 1
APV	2-amino-5-phosphonovaleric acid
ARE	AU-rich element
ATF	activating transcription factor
ATP	adenosine triphosphate
AU-A(B,C)	AU-rich element binding proteins A (B, C)
AVP	arginine vasopressin
β-Gal	β-galactosidase
bp	base pair(s)
bZIP	basic region-leucine zipper (domain)
cAMP	3',5'-cyclic adenosine monophosphate
CAT	chloramphenicol acetyl transferase
α-CBF	α-CAAT box-binding factor
CBG	corticosteroid-binding globulin
CBP	CREB-binding protein
CCK	cholecystokinin
C/EBP	CCAAT box/enhancer binding protein
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene related peptide
CNS	central nervous system
COUP-TF	chicken ovalbumin upstream promoter transcription factor
CRE	cAMP response element
CREB	cAMP response element binding protein
CREM	cAMP response element modulator protein
CRH	corticotrophin releasing hormone
CTP	cytidine triphosphate
dbcAMP	dibutyryl cyclic AMP
DBD	DNA binding domain
DEPC	diethyl pyrocarbonate
DEX	dexamethasone
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DRB	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
DTT	dithiothreitol
FBS	fetal bovine serum

G protein	GTP-binding protein
GABA	γ-amino butyric acid
GH	growth hormone
GHRH(GHRF)	growth hormone releasing hormone
GI tract	gastrointestinal tract
GM-CSF	granulocyte-macrophage colony stimulating factor
GR-DBD	glucocorticoid receptor-DNA binding domain
GRE	glucocorticoid responsive element
GRU	glucocorticoid responsive unit
GST	glutathione S-transferase
GTC	guanidinium isothiocyanate
GTP	guanosine triphosphate
hCG	human chorionic gonadotropin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPA	hypothalamic-pituitary-adrenal
HRE	hormone response element
HS	horse serum
hsp	heat shock protein
HSV	herpes simplex virus
icv	intracerebroventricular
IDX-1	islet/duodenum homeobox 1
IGF-I	insulin-like growth factor I
IL-1	interleukin 1
IL-3	interleukin 3
IL-6	interleukin 6
IPF1	insulin promoter factor 1
IRE	iron-response element
IRE-BP	iron-response element binding protein
IRS	insulin response sequence
iv	intravenous
KID	kinase inducible domain
MMTV-LTR	mouse mammary tumor virus long terminal repeat
MT	metallothionein
NMDA	N-methyl-D-aspartate
NO	nitric oxide
ONPG	o-nitrophenyl-β-D-galactopyranoside
ORF	open reading frame
PABP	poly(A) tail binding protein
PAN	poly(A) nuclease

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEPCK	phosphonolpyruvate carboxykinase
PKA	protein kinase A
plfG	proliferin gene enhancer element
PMSF	phenylmethylsulfonyl fluoride
Pol I	DNA-dependent RNA polymerase I
Pol II	DNA-dependent RNA polymerase II
Pol III	DNA-dependent RNA polymerase III
POMC	pre-opiomelanocortin
PSS	pro-somatostatin
QUIN	quinolinic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element
PDB	phorbol 12,13-dibutyrate
RIA	radioimmunoassay
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SMS-UE	somatostatin gene upstream enhancer
SP-A	surfactant protein A
SS	somatostatin
SS-14	somatostatin 14
SS-28	somatostatin 28
SS-mRNA	somatostatin-mRNA
SSLI	somatostatin like immunoreactivity
SSPE	$NaCl$-NaH_2PO_4-EDTA (buffer)
SSTR	somatostatin receptor
STF-1	somatostatin transactivating factor 1
SV40	simian virus 40
T_3	3,3',5-triiodo-L-thyronine
TAFs	TATA binding protein (TBP) associated factors
TBP	TATA box-binding protein
TCA	trichloro acetic acid
TFIIA	transcription factor IIA
TFIIB	transcription factor IIB
TFIID	transcription factor IID
TFIIE	transcription factor IIE
TFIIF	transcription factor IIF
TFIIH	transcription factor IIH
TFIIJ	transcription factor IIJ
TK	thymidine kinase
TLC	thin layer chromatography

TNF	tumor necrosis factor
TRE	thyroid hormone response element; phorbol ester response element
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone, thyrotropin
UTP	uridine triphosphate
UTR	untranslated region
VIP	vasoactive intestinal peptide

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CHAPTER I. LITERATURE REVIEW AND INTRODUCTION

This thesis deals with the regulation of somatostatin gene expression by glucocorticoids, at both transcriptional and post-transcriptional levels, in cooperation with the cAMP signalling pathway. Accordingly, this review chapter starts with somatostatin, its gene and regulation of gene expression; followed by a brief discussion of basic transcription mechanism; in-depth discussions of cAMP stimulated and pancreatic islet D-cell specific somatostatin gene transcription; the mechanisms of glucocorticoid regulated gene transcription; and mRNA stability, its regulation. The objectives of the study will be introduced following these discussions.

1. SOMATOSTATIN, ITS GENE AND REGULATION

Somatostatin (somatotropin release inhibitory factor, SS) was discovered in sheep hypothalamic extracts in 1973 in the laboratory of Guillemin at the Salk Institute, in an unsuccessful search for the growth hormone releasing factor (GHRF or GHRH) (1). It proved to be a cyclic peptide of 14 amino acid residues. A second biologically active form somatostatin-28 (SS-28), consisting of an amino terminally extended form of somatostatin-14 (SS-14), was isolated in 1980 (4). Subsequent studies revealed that somatostatin is not only produced in the hypothalamus, but also in other regions of the central nervous system, the gastrointestinal tract and the pancreatic islet of Langerhans. Its wide anatomical distribution is paralleled by an equally broad spectrum of biological effects including endocrine inhibition of growth hormone (GH), thyrotropin (TSH), insulin and glucagon; inhibition of exocrine and endocrine secretions in the gastrointestinal tract; and neuromodulatory activities (2, 3).

1.1 ANATOMICAL DISTRIBUTION OF SOMATOSTATIN CELLS

Somatostatin-producing cells occur at high densities throughout the central and peripheral nervous systems, in the endocrine pancreas, in the gut, and in small numbers in the thyroid, adrenal, submandibular gland, kidney, prostate and placenta (3) (Table 1-1). The typical morphological appearance of a somatostatin cell is that of a neuron with multiple branching processes or of a secretory cell often having short cytoplasmic extensions (D cells) (3). Within the hypothalamus, the most prominent collection of somatostatin neurons lies in the anterior periventricular region (5-7). Outside the hypothalamus, somatostatin-positive neurons and fibres are distributed in the entire central nervous system, with the notable exception of the cerebellum (5, 6). Brain regions rich in somatostatin cells include the deeper

layers of the cortex, all limbic structures, the striatum, the periaqueductal central grey and all levels of the major sensory systems (Table 1-1). The approximate relative amounts of somatostatin in the major regions of the brain are as follows: cerebral cortex 49%, spinal cord 30%, brain stem 12%, hypothalamus 7% (8).

Table 1-1. Major sites of somatostatin localization.

(reproduced from ref. 3)

Body region and cell type		Location
nervous system:	neurons	hypothalamus cerebral cortex limbic system basal ganglia major sensory systems spinal cord dorsal root ganglia autonomic ganglia
pancreas:	D cells	islets
gut:	D cells neurons	mucosal glands submucous and myenteric plexuses

Somatostatin in the pancreas is confined to D cells in the islets of Langerhans (9). In the fetus and neonate, somatostatin cells are the second most abundant islet-cell type after insulin cells, accounting for up to 40% of the total endocrine cell population; in the adult, however, they make up only about 3% of islet cells (3). Islet D cells are characteristically

adjacent to glucagon cells and pancreatic polypeptide cells and are located in the peripheral mantle zone (3). Gastrointestinal somatostatin cells are of two types: D cells and neurons intrinsic to the gut (10). The former are located in mucosal glands from the cardiac portion of the stomach down to the rectum, their highest concentration being in the antrum; the latter populate both the submucous and myenteric plexuses in all segments of the gastrointestinal tract (10). In the thyroid, somatostatin coexists with calcitonin in a subpopulation of C cells (2). Recent studies demonstrate that somatostatin is also widely expressed in cells of the immune system, such as lymphoid organs (spleen, thymus), mononuclear leukocytes, mast cells, polymorphonuclear leukocytes and B lymphocytes (12-14). In the rat, the gut accounts for about 65% of total body somatostatin, the brain for 25%, the pancreas for 5% and the remaining organs for 5% (15).

1.2 ACTIONS OF SOMATOSTATIN

Along with its wide anatomical distribution, somatostatin acts on multiple targets including the brain, gut, pituitary, endocrine and exocrine pancreas, adrenals, thyroid and kidneys. Its actions include inhibition of virtually every known endocrine and exocrine secretion; various behavioral and autonomic effects of centrally administrated somatostatin; and effects on gastrointestinal and biliary motility, vascular smooth muscle tone; and intestinal absorption of nutrients and ions (2, 3). At a cellular level, the broad array of biological actions can be resolved into five processes that are regulated by somatostatin (3, 16):

(1) neurotransmission;

(2) endocrine inhibition: secretion of thyrotropin-releasing hormone (TRH), corticotrophin-releasing hormone (CRH), GH, TSH, insulin, glucagon, gastrin, secretin,

cholecystokinin (CCK), vasoactive intestinal peptide (VIP), motilin, neurotensin;

(3) exocrine inhibition: secretion of pancreatic enzymes and bicarbonate, gastric acid and pepsin, bile;

(4) smooth muscle contractility: both inhibition (late phase of gastric emptying, gastric migrating motor complexes, gallbladder contraction, ileal longitudinal muscle contraction) and stimulation (early phase of gastric emptying, intestinal migrating motor complexes);

(5) inhibition of cell proliferation: gastrointestinal mucosa.

Somatostatin is secreted in different ways to function as a true hormone, a neurotransmitter, or a paracrine regulator. As a classical hormone, somatostatin is secreted into the blood mainly from the gastrointestinal tract and acts on distant targets. Somatostatin released from neurons acts as a neurotransmitter or a neuromodulator (3). In the gastrointestinal tract, somatostatin cells with long cytoplasmic extensions contact directly on parietal (HCl-producing), chief (enzyme-producing) and gastrin-producing cells and thereby regulate these cells locally by paracrine modulation (16). On the target cells, the actions of somatostatin are mediated by high affinity plasma membrane receptors that are coupled via G proteins to adenylyl cyclase, K^+ and Ca^{2+} ion channels, exocytotic vesicles and protein tyrosine phosphatase (3). Pharmacological studies have suggested that somatostatin receptors (SSTRs) are heterogenous and feature subtypes selective for SS-14 and SS-28 (17). The recent cloning of the SSTRs has confirmed the existence of molecular subtypes but additionally revealed a greater genetic diversity than previous suspected. Five distinct SSTR subtype genes have been identified, encoding a family of G-protein coupled receptors - SSTR1, 2, 3, and 4 being relatively selective for SS-14, and SSTR5 having 13-fold greater affinity for SS-28

than SS-14 (18). A comparison of the amino acid sequence of SSTRs shows that these receptors are closely related in size and structure. They diverge most at their amino and carboxyl termini and show the greatest similarity within the putative 7 trans-membrane domains (18).

1.3 SOMATOSTATIN BIOSYNTHESIS

Biosynthesis of somatostatin is directed by a single gene which is localized on the long arm of human chromosome 3 (19). The rat somatostatin gene was cloned and characterized in 1984 in the laboratories of Habener and Dixon (20, 21). The transcriptional unit consists of exons of 238 and 367 bp separated by an intron of 621 bp (877 bp in the human gene) (Figure 1-1). Like other peptide hormones, somatostatin is synthesized as part of a large precursor protein that is cleaved into the prohormone form and processed enzymatically to yield the mature products (3). Somatostatin exons encode a prepro-somatostatin of 116 amino acid residues, which is synthesized on ribosomes and gives rise to pro-somatostatin (PSS, 92 amino acids) by cleaving a signal peptide (24 amino acids) right after translation. PSS is translocated into the lumen of the endoplasmic reticulum, and transported by budding non-clathrin-coated vesicles through the Golgi stacks to the trans Golgi network. Here the protein is sorted via clathrin-coated vesicles into a regulatory compartment consisting of secretory granules or into a constitutive, non-regulated pathway through non-clathrin-coated vesicles which exit from the Golgi and migrate to the plasma membrane (22). The maturation of PSS to active peptides involves endoproteolytic cleavage at di- or monobasic amino acid sites (3). Processing of PSS occurs principally at the C-terminal segment of the molecule and generates the two bioactive forms SS-14 and SS-28. In addition, a monobasic cleavage at the N-terminal

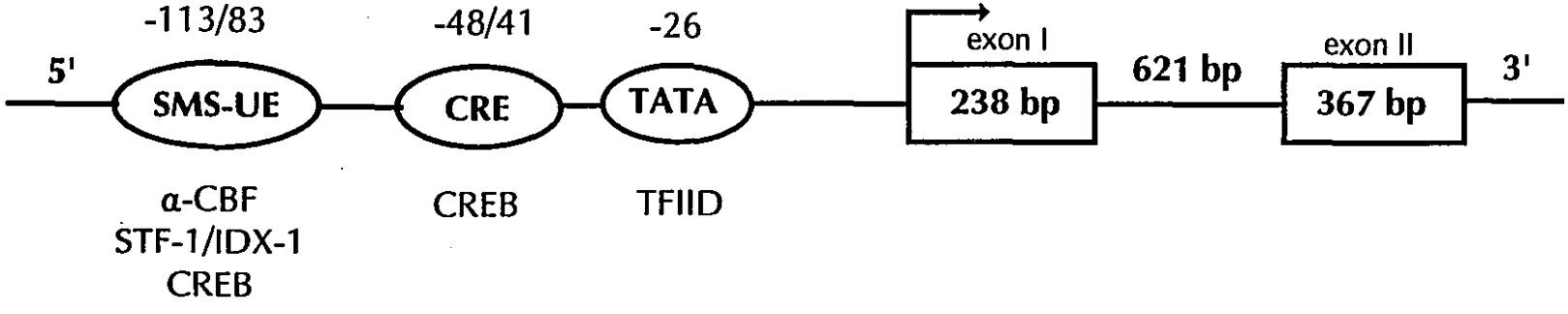


Figure 1-1. Rat somatostatin gene structure. SMS-UE, somatostatin gene upstream enhancer; CRE, cAMP-response element; TATA, TATA box.

segment generates the decapeptide PSS_{1-10} (antrin) without any known biological activity (22).

Analysis of the nucleotide sequence 5' to the start site of transcription has revealed a number of DNA elements that may be involved in initiating somatostatin gene transcription. A variant of the TATA box, TTTAAA, lies 26 bp (23 bp in human) upstream from the start site. A cAMP response element (CRE) is located upstream at -48 to -41 bp (23). An upstream enhancer element (SMS-UE) has been identified at -113 to -83 bp which confers pancreatic islet D-cell specific gene expression, under the influence of transcriptional factors including α-CAAT box binding factor (α-CBF), a homeodomain protein (named STF-1, IDX-1, or IPF1), and CRE binding protein (CREB) (24-26).

1.4 REGULATION OF SOMATOSTATIN SECRETION AND SYNTHESIS

Early studies have revealed various regulators of somatostatin secretion. Neuronal somatostatin secretion is stimulated by depolarization, cAMP, glutamine, aspartic acid, acetyl choline (ACh), VIP, while it is inhibited by γ-aminobutyric acid (GABA). In pancreatic islets, somatostatin secretion is stimulated by glucagon, amino acids, glucose, neurotransmitters including ACh, β-adrenergic agonists, gut peptides including VIP and CCK and suppressed by insulin and catecholamines acting through α-receptors. In the gastrointestinal tract, somatostatin secretion is stimulated by glucagon, secretin, gastrin, CCK and gastric acid; inhibited by substance P, the endorphins, and ACh (2, 13, 16, 27).

Many agents that influence somatostatin secretion are also capable of altering somatostatin gene expression, as assessed by changes in steady-state mRNA levels. **Cyclic AMP** is the main intracellular regulator of somatostatin gene transcription. The effect of cAMP and the tissue-specific expression of somatostatin gene will be discussed in detail in

subsequent sections (3 and 4) in this chapter. Another major regulator of somatostatin synthesis is **glucocorticoids**, the role of which will be discussed in Section 7 of this chapter and in greater detail in Chapter III to V. In addition, somatostatin production and mRNA accumulation are stimulated by GH, GHRH, insulin like growth factor I (IGF-I), testosterone and estradiol in the hypothalamus; by interleukin 1 (IL-1), tumor necrosis factor (TNF), and N-methyl-D-aspartate (NMDA) receptor agonists in the cerebral cortex; inhibited by insulin in the pancreatic islets, and by vitamin D_3 in thyroid tumor cells (3). Both hypoglycaemia (induced by insulin) and hyperglycaemia (induced by glucose infusion) increase hypothalamic SS-mRNA level and inhibit GH secretion (28).

(1) Calcium and depolarization. Tolon *et al.* (29) analyzed the stimulatory effect of potassium depolarization on SS-mRNA levels in primary cultures of fetal cerebrocortical cells. Acute depolarization with 56 mM K^+ increased somatostatin release and decreased SS-mRNA level. Prolonged depolarization (3 h or more) stimulated somatostatin secretion as well as SS-mRNA levels. These changes were inhibited by the Ca^{2+} channel antagonist verapamil. Examination of the rate of disappearance of SS-mRNA levels after inhibition of transcription by actinomycin D revealed that K^+ stimulation stabilized the SS-mRNA. Time-course studies confirmed that the K^+-induced SS-mRNA accumulation is time dependent, and dependent on chronic activation of Ca^{2+} channels (29). This study indicates that prolonged depolarization (K^+) augments SS-mRNA levels in cerebrocortical cells through a Ca^{2+}-dependent pathway by stabilizing the mRNA. In addition, intracellular Ca^{2+} may activate somatostatin gene transcription directly by activating calcium-calmodulin kinase, which has been shown to be able to activate CREB through phosphorylation and glucagon gene transcription (30-32).

(2) Growth hormone and insulin like growth factor I. Physiological evidence

suggests that GH acts through a short-loop feedback mechanism to inhibit its own secretion by stimulating hypothalamic somatostatin release. Patel (33) first reported that chronic administration of GH increases hypothalamic somatostatin concentration in rats. In addition, GH administration restores somatostatin content reduced by hypophysectomy in the median eminence of the hypothalamus. Rogers *et al.* (34) reported that in both hypophysectomized and sham-operated rats, bovine GH significantly increases SS-mRNA levels in the periventricular nucleus assessed by *in situ* hybridization, indicating that GH augments hypothalamic somatostatin biosynthesis. Hurley and Phelps (35) studied the influence of endogenous GH excess or deficiency (induced in transgenic mice expressing excessive GHRH or somatotroph-specific toxin) on hypothalamic SS-mRNA level by *in situ* hybridization. GH overproduction resulted in giant transgenic mice which exhibited a 2.3 fold increase in hypothalamic SS-mRNA (without a change in the density of somatostatin-producing neuron), while transgenic dwarf mice with undetectable GH had a 40% reduction in SS-mRNA signal. This further supports a stimulatory effect of GH on somatostatin biosynthesis. A recent study by Sato *et al.* (36) does not support a stimulatory effect of GH on somatostatin gene expression. They found that in GH-deficient dwarf rats GH has no effect on hypothalamic SS-mRNA level when administrated either systemically or intracerebroventricularly (icv). The discrepancy may be explained in part by the genetic strain of rats used in this study.

In an attempt to elucidate the molecular mechanism of GH action on somatostatin gene, GH was found to stimulate expression of the reporter gene CAT (chloramphenicol acetyl transferase), driven by somatostatin promoter, 4~5-fold when transfected into a rat islet tumor cell line (RIN 5-AH). The promoter element involved in the GH effect was located between -71 to -44 bp of somatostatin gene, which includes the CRE, suggesting a CRE-

dependent mechanism (37). As the GH receptor belongs to the tyrosine kinase receptor family, it will be interesting to determine the nature of the molecular interaction between the receptor and CRE in activating somatostatin gene transcription.

GH could also influence somatostatin secretion and synthesis through a long-loop feedback involving IGF-I produced from the liver. IGF-I has been reported to stimulate somatostatin secretion (38). In the report of Sato *et al.* (36), systemic injection of recombinant human IGF-I for 7 days did not influence hypothalamic SS-mRNA level in dwarf rats. While continuous icv infusion of IGF-I for 7 days increased SS-mRNA 1.5~1.9 fold in rats (36). Although IGF-I is produced peripherally and has no access to the brain, it may act on its receptors at the median eminence which is outside of the blood-brain barrier (36). Furthermore, the existence of IGF-I and IGF-I mRNA has been reported in various brain regions including hypothalamus (39, 40).

(3) Cytokines. Somatostatin is produced in cells of the immune system and has been documented to function as an anti-inflammatory agent. Cytokines stimulate somatostatin production in various culture systems. Scarborough *et al.* (52, 53) have found that IL-1, TNF, and IL-6 stimulate the synthesis and release of somatostatin from cultured fetal rat diencephalic cells. The effect of IL-1β on cellular somatostatin content is obtained with concentrations as low as 10^{-11} M and is dose-dependent up to a maximally effective dose of 10^{-8} M. It is detectable as early as 24 h, continues for up to 6 days, and is accompanied by increases in SS-mRNA levels. The exact mechanisms are unclear and may involve stimulation by cytokines of cAMP formation, CRH secretion, central noradrenergic neurons, or proliferation of somatostatin neurons (52, 53). In addition, Honegger *et al.* (41) reported that IL-1β, but not IL-6 or TNF-α, caused a drastic release of somatostatin from a rat

hypothalamus explant system, which was antagonized by the cyclo-oxygenase inhibitor (indomethacin), indicating the involvement of prostaglandins.

(4) NMDA receptor agonists. Somatostatin is produced in a subpopulation of neurons in the striatum that are selectively resistant to NMDA neurotoxicity and showed a 3~5 fold increase in somatostatin content in Huntington's disease (54-56). Recently, Patel *et al.* (42, 57) reported that quinolinic acid (QUIN), an NMDA receptor agonist, augments SS-mRNA accumulation in cultured fetal rat cortical neurons. QUIN and NMDA induced a dose-dependent, maximal 4-fold increase in SS-mRNA level, which can be blocked by 2-amino-5-phosphonovaleric acid (APV, a NMDA receptor antagonist). Time-course studies showed that the effect of QUIN and NMDA on SS-mRNA occurred after a latency of 8 h. Cortical cells transfected with somatostatin promoter-CAT showed no stimulation of CAT activity with QUIN or NMDA. These data revealed a dose-dependent, NMDA receptor-mediated stimulation of SS-mRNA accumulation. Such stimulation is likely to be post-transcriptional, as suggested by CAT assay and the delay in SS-mRNA induction (42, 57).

(5) GHRH, nitric oxide and cGMP. Aguila (50) found that both GHRH and cGMP increased somatostatin release and SS-mRNA levels in explants of rat periventricular nucleus. The effect of GHRH could be abolished by a specific inhibitor of nitric oxide synthase (L-NMMA) but not by its inactive isomer (D-NMMA). GHRH increased intracellular cGMP (but not cAMP) formation, which was also abolished by L-NMMA but not by D-NMMA. The results suggest that GHRH releases nitric oxide from neighbouring cells, which diffuses into somatostatin neurons, where it activates guanylate cyclase, leading to increased formation of cGMP. cGMP may increase SS-mRNA level and somatostatin release from the periventricular nuclei (50). The mechanism underlying cGMP dependent augmentation of somatostatin gene

expression, however, remains unclear.

(6) Sex steroids. Werner *et al.* (43) reported that gonadectomy decreased SS-mRNA level in hypothalamus by 67% in male and 75% in female rats. SS-mRNA level was similarly reduced in the periventricular and ventromedial regions of the hypothalamus. Estradiol or testosterone treatments reversed the decrease in SS-mRNA level in castrated rats. In contrast, there was no change in cerebral cortical SS-mRNA levels after gonadectomy. These results suggest that sex steroids are involved in tissue-specific regulation of the somatostatin gene in the hypothalamus. Argente *et al.* (44) further demonstrated that testosterone increases SS-mRNA levels in hypothalamus through activation of androgen receptors and not by aromatization to estradiol and activation of the estrogen receptor. They found that dihydrotestosterone, a non-aromatizable androgen could mimic the effect of testosterone in castrated male rats; while 17β-estradiol had no effect in preventing post-castration decline in SS-mRNA content (44). Therefore both androgen and estrogen are capable of restoring the loss of SS-mRNA due to castration.

(7) Inhibitors: insulin and vitamin D_3. Negative regulation of somatostatin gene regulation is relatively rare in the literature. Somatostatin is a well recognized inhibitor of insulin secretion from pancreatic islet. SS-28 released from intestinal D cells following food ingestion attenuates islet B-cell secretion of insulin (45-49). Insulin, in turn, may inhibit somatostatin secretion and biosynthesis. Patel *et al.* (58) examined the direct effect of insulin on somatostatin synthesis and secretion in a somatostatin-producing islet tumor cell line ($1027B_2$). Insulin induced a dose-dependent reduction in somatostatin secretion, a slight decrease in somatostatin content, and a 50% reduction in SS-mRNA accumulation, suggesting that insulin exerts a direct suppressive effect on somatostatin secretion and synthesis.

Rogers *et al.* (51) studied the effect of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] on basal and cAMP-stimulated SS-mRNA accumulation and somatostatin secretion in human thyroid carcinoma TT cells. 1,25-$(OH)_2D_3$ alone decreased SS-mRNA level and peptide content in the cells. Four days pretreatment with 1,25-$(OH)_2D_3$ also inhibited cAMP-mediated increase in SS-mRNA accumulation, but had no effect on stimulated somatostatin secretion (51). GABA receptor agonists have no significant effect on somatostatin gene expression in cultured hypothalamic neurons, although they do inhibit somatostatin release (59). It is reported that dopamine D1 and D2 receptor antagonists decrease SS-mRNA accumulation in rat striatum (60).

In summary, somatostatin is a unique and important regulatory peptide in that it is produced in many parts of the body and influences many biological processes including neurotransmission, glandular secretion and cell proliferation. In turn, somatostatin secretion and biosynthesis are stimulated by peptides and neurotransmitters generally through the cAMP signalling pathway. GH, cytokines, NMDA receptor agonists, sex steroids, GHRH, depolarization and intracellular Ca^{2+} also influence somatostatin gene function but the exact molecular mechanisms remain to be defined. Negative regulators include insulin and vitamin D_3. Glucocorticoids either augment or suppress somatostatin biosynthesis dependent on the target tissue and doses used, as discussed later in this thesis (Table 1-2).

Table 1-2. Major regulators of steady state SS-mRNA accumulation.

Action/Regulator	Target tissue
Stimulation	
cAMP	neuron, D-cell, C-cell (20,51,61,62)
glucocorticoids	pancreas, stomach, jejunum (63)
growth hormone	hypothalamus (34,35,37)
NMDA agonists	cerebral cortical culture (42, 57)
interleukin 1β	diencephalic cells (52, 53)
GHRH, nitric oxide, cGMP	hypothalamus explant (50)
estradiol	hypothalamus (43)
testosterone	hypothalamus (43,44)
hypo- & hyperglycaemia	hypothalamus (28)
Inhibition	
insulin	pancreatic islet (58)
$1,25\text{-}(OH)_2D_3$	thyroid carcinoma (51)
glucocorticoids	cerebral cortex, hypothalamus (63)
	thyroid carcinoma (64)

2. BASIC TRANSCRIPTION

Genes consist of DNA segments which encode information that specifies functional products, either RNA molecules or proteins used for various cellular functions. The base pairs of the encoding element specify the amino acid residues to be linked together into a protein chain. As shown in Figure 1-2, the coding region of a gene contains exons and introns. Exons start 5' from the transcription start site, or the first nucleotide in mRNA sequence, and include the entire sequences corresponding to the mature mRNA (including 5'- and 3'-untranslated regions). Introns are stretches of noncoding DNA interrupting exons (66, 67). For instance, the rat somatostatin gene contains two exons of 238 and 367 bp and an intron of 621 bp (Figure 1-1).

In addition to the coding region, genes also include regulatory elements. Nucleotide sequences that influence the rate of transcription lie in regions of DNA upstream of the transcription start site. They often include an element rich in adenine and thymine, known as the TATA box, and other sequence motifs lying within about 100 bp of the start site (Figure 1-2). Collectively called the promoter of a gene, these sequences comprise binding sites for RNA polymerase and its numerous cofactors. The position of the promoter with regard to the transcription start site is relatively inflexible (65-67).

In contrast to promoters, other DNA regulatory elements, enhancers, occur in unpredictable locations, often at a considerable distance from the start site and augment transcription from the gene promoter (Figure 1-2). Enhancers, like promoters, form binding sites for regulatory proteins, but unlike promoters, the position and orientation of an enhancer are flexible with regard to the gene. Indeed, enhancers can dramatically increase gene transcription from positions within or on either side of the gene, even from thousands of bases

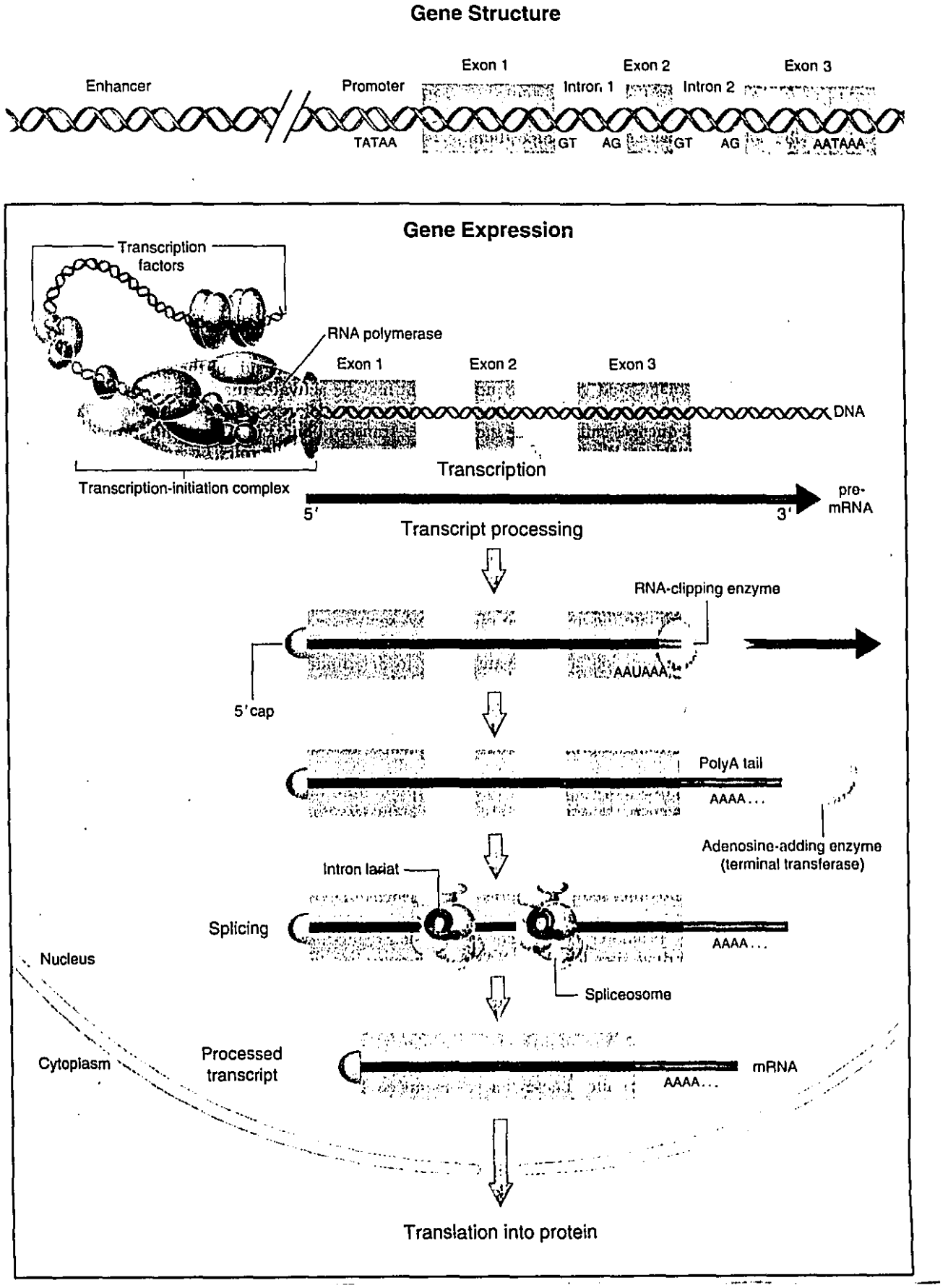


Figure 1-2. Schematic structure of a gene and the process of gene expression. (reproduced from ref. 67)

away (67). Individual elements residing in promoter or enhancer sequences that interact with specific transcription factors consist of small stretchs of DNA (≤ 30 bp) called *cis* elements (such as CRE). The corresponding transcription factors are known as *trans* factors (such as CREB).

Transcription is DNA directed RNA synthesis, the first and usually most important step in the control of gene expression. There are three classes of transcription mediated by different RNA polymerases (Pol I-III). Pol I transcribes ribosomal pre-RNAs; Pol II is the enzyme which facilitates synthesis of the mRNA and its encoding protein; Pol III directs synthesis of tRNA and low-molecular-weight RNAs (68).

The model for the assembly of Pol II-directed transcription initiation complex is based largely on kinetic assays, undenatured gel electrophoresis, and nuclease protection assays (Figure 1-3). An initial committed complex is formed by TATA box-binding protein (TBP) binding to the TATA box of a promoter. It binds the DNA in the minor groove (while virtually all known DNA-binding proteins bind in the wide groove), with its larger outer surface (N-terminal) being available to extend contacts to other proteins. TBP is a small protein (~30 kDa) which is sufficient for recognition of TATA box and subsequent incorporation of other TBP-associated factors (TAFs). But TBP is not enough to mediate transcriptional regulation by upstream regulators, which requires the entire transcription factor IID (TFIID) complex, consisting of TBP and the TAFs. As a central piece of the basal transcription machinery, TBP is highly conserved during eukaryotic evolution (69).

Transcription factor IIA (TFIIA) joins the complex and may activate TBP by relieving a repression caused by the TAFs. TFIIA contains three subunits (two in yeast) and can associate with TBP or TFIID even in the absence of target DNA. TFIIA is not essential for

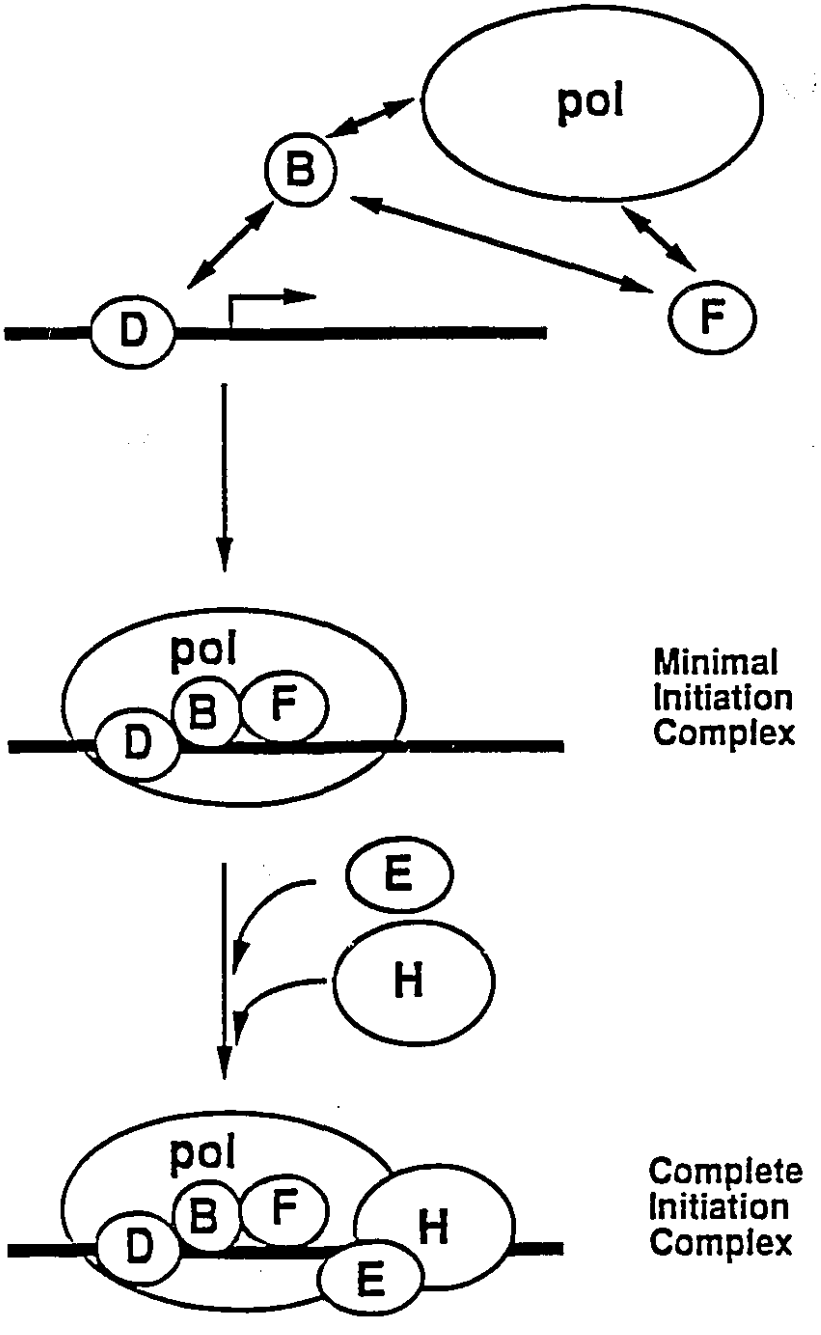


Figure 1-3. Assembly of transcription initiation complex. pol: RNA polymerase II; B, D, E, F, H: TFIIB, D, E, F, H. (adapted from ref. 69)

basal transcription with other purified factors.

Transcription factor IIB (TFIIB) interacts directly with TBP and associates loosely with DNA downstream of the TATA box, which (as a bridging protein) can recruit Pol II and transcription factor IIF (TFIIF) into the complex. This complex is stable in both kinetic assays and undenatured gel electrophoresis.

TFIIF contains two subunits. The larger subunit (RAP74) has an ATP-dependent DNA helicase activity that could be involved in melting the DNA at initiation. The smaller subunit (RAP38), with some homology to the bacterial sigma factor that contact the core polymerase, binds tightly to Pol II. TFIIF may in fact bring Pol II to the assembling transcription complex and provide the means by which it binds. Interactions with TFIIB may be important when TFIIF-polymerase joins the complex. Although it is the catalytic enzyme in the process of gene transcription, RNA polymerase II is not as intensively studied as most of the other basal transcription factors. The yeast enzyme consists of 12 subunits, all of which have been cloned (70). The mammalian counterparts of many of the subunit genes remain to be cloned.

Finally transcription factors TFIIE, and then TFIIH and TFIIJ join the complex. TFIIE is encoded by two genes and is probably a tetramer with two subunits of each type. Its incorporation appears necessary for subsequent recruitment of TFIIH. TFIIH has a kinase activity that can phosphorylate the C-terminal domain of Pol II. It is possible that phosphorylation of the C-tail is needed to release Pol II from the transcription factors so that it can leave the promoter and start elongation. Once the complete complex is assembled, an ATP-dependent activation step is necessary for transcription to occur (Fig 1-3) (69, 71).

There are probably 20 proteins (a total mass of ~500 kDa) involved in the basal

transcriptional apparatus, excluding the Pol II, which alone has 12 subunits with a mass of another 500 kDa (71). An upstream transcription factor (such as CREB) can interact directly with one of the basal transcription factors (such as TFIIB), or indirectly with the TAFs (such as CREB with TAF110), or even more indirectly with a coactivator (such as CREB with CBP, which then interacts with TFIIB), all as discussed later in Section 3.

3. cAMP REGULATION OF SOMATOSTATIN GENE EXPRESSION

The main intracellular regulator of somatostatin gene transcription is cAMP. It is converted from the precursor ATP by the enzyme adenylyl cyclase, located on the inner surface of the plasma membrane and coupled via G proteins to membrane receptors for various hormones and neurotransmitters. Therefore, upon stimulation of the receptor by its ligand, adenylyl cyclase is activated and intracellular cAMP is generated (Figure 1-4). cAMP binds to the regulatory (R) subunit of cAMP-dependent protein kinase (protein kinase A, PKA), leading to the dissociation of the catalytic (C) subunit. PKA catalyses the transfer of the γ-phosphate of ATP to serine or threonine residues in a variety of proteins, including CREB (68).

3.1 cAMP RESPONSE ELEMENT (CRE)

It has been shown that somatostatin secretion is increased by agents that stimulate adenylyl cyclase activity, including VIP, glucagon and epinephrine. In primary diencephalic cultures, Montminy *et al.* (61) reported that steady state SS-mRNA level, as well as somatostatin secretion, were increased by exposure to forskolin, a post-receptor adenylyl cyclase activator. It indicated that cAMP activates somatostatin gene expression.

The CRE was first identified in the somatostatin gene. Montminy *et al.* (23) prepared chimeric genes containing the somatostatin gene promoter fused to the bacterial reporter gene CAT and transfected them into PC12 pheochromocytoma cells. The authors found that forskolin increased CAT activity 7.5-fold. Promoter deletion studies defined a region between -60 and -29 bp that conferred cAMP responsiveness when placed adjacent to the simian virus 40 (SV40) promoter. The CRE consensus was identified as an 8-base palindrome (5'-

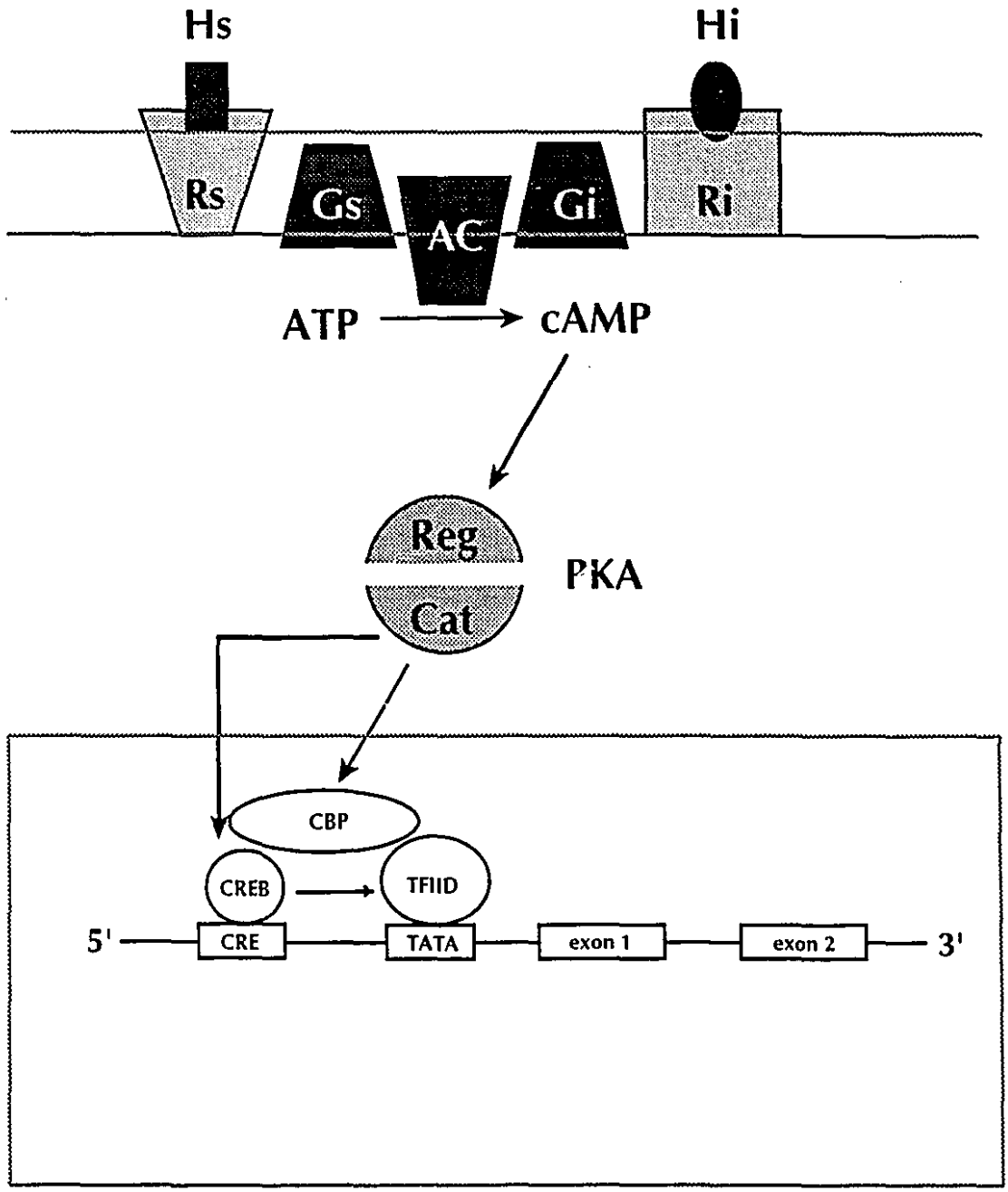


Figure 1-4. Schematic model of cAMP-mediated target gene regulation. cAMP binds to the regulatory subunit (Reg) of PKA, leading to the dissociation and activation of the catalytic (Cat) subunit. It then translocates to the nucleus where it phosphorylates CREB and CBP. CREB activates target gene either through direct interaction with TAFs-TFIID, or indirectly through CBP to activate TFIIB and TFIID.

TGACGTCA-3') which was also found to be present in many other cAMP responsive genes. cAMP responsiveness was greatly reduced when the fusion gene was transfected into the mutant PC12 line A126-1B2, which is deficient in PKA (23). This was the first demonstration that the somatostatin gene is transcriptionally regulated by cAMP, requiring a highly conserved promoter element and PKA activity (23).

3.2 CRE BINDING PROTEIN (CREB)

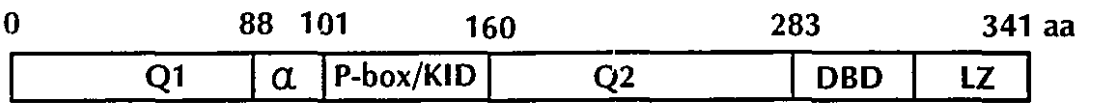
Identification of CRE prompted Montminy *et al.* (72) to further identify a specific nuclear protein which interacts with this conserved region of DNA. By DNase I footprint assay, they characterized a protein in PC12 nuclear extracts which binds selectively to the CRE of the somatostatin gene. The purified 43 kDa CREB turns out to be the first member of a family of transcriptional factors capable of binding to CRE. Forskolin caused 3~4-fold increase in the phosphorylation of CREB, indicating that the cAMP-dependent pathway may regulate transcription by phosphorylating CREB (72). Following hormone stimulation and subsequent activation of adenylyl cyclase, the C subunit of PKA appears to be translocated to the nucleus where it phosphorylates CREB at a single residue, Ser-133 (73). This phosphorylation is very important for somatostatin gene regulation since PKA stimulates somatostatin gene transcription 20-fold *in vitro* (73).

Hoeffler *et al.* (74) isolated the first CREB cDNA by screening an expression library of human placenta. The cDNA clone encodes a CREB which exhibited binding specificity to DNA probes containing CRE. Comparison of the cDNAs from human, rat and mouse revealed the presence of two primary CREB protein isoforms, CREB327 and CREB341, encoded from the same gene by alternative splicing of the mRNA (74). The cloning of CCAAT

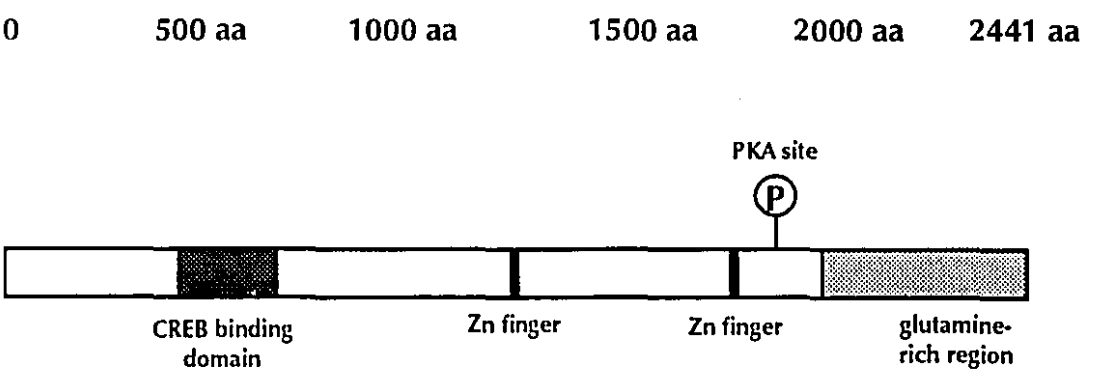
box/enhancer binding protein (C/EBP), AP-1, CREB, and the subsequent cloning of cDNAs for other CREB/ATF proteins, and analysis of their predicted protein sequences has revealed a common structural motif, the basic region-leucine zipper (bZIP) domain. The bZIP domain is essential for binding to specific enhancer sequences. The bZIP family of proteins bind as dimers to their target DNA enhancer sequences. The amino terminal three-fourths of CREB comprises the transactivation domain, and the carboxyl-terminal one-fourth of the sequence contains the DNA-binding and dimerization domains (75) (Figure 1-5).

Within the bZIP family of transcription factors, CREB, CREM and ATF-1 form a distinct subfamily of proteins characterized by highly conserved DNA binding domains and phosphorylated regions. Other possible members include CRE-BP1/ATF-2 and ATF-a (75). Together they represent the final communicative link in the regulation of gene expression in response to the activation of the cAMP-dependent signalling pathway.

Concerning the role of phosphorylation, only CREB, CREM and ATF-1 are activated upon phosphorylation by PKA, and also in some circumstances CREB is activated by calcium-calmodulin kinase (30-32). The serine-133 (or Ser-119 in CREB327) is the consensus site for both PKA and calcium-calmodulin kinase in CREB341 (30, 31). Phosphorylation of the PKA site is essential but not sufficient to activate CREB. An additional region located carboxyl-proximal to CREB (α peptide, amino acid residues 88~101) is required to confer transactivation functions (76). In some circumstances, CREB appears to activate gene transcription independent of its phosphorylation by PKA (e.g. when activating SMS-UE in cooperation with homeodomain proteins) (77). In addition to amplifying the effects of cAMP in a diverse group of biological functions (neuronal excitation, circadian rhythm, pituitary proliferation, gluconeogenesis), CREB is targeted by other signalling molecules such as



CRE binding protein (CREB)



CREB binding protein (CBP)

Figure 1-5. Schematic diagrams of the domain structures of CREB and CBP.

calcium influx (induced by action potential) and TGF-β and may be able to integrate distinct cellular pathways into the nucleus (30-32, 81). Recently, CREB has been found to be a mediator of long-term memory (82).

3.3 MECHANISMS OF CREB-INDUCED TRANSACTIVATION

How does phosphorylated CREB activate gene transcription after binding to its target DNA ? There is evidence for both direct and indirect interaction of CREB with the basal transcription machinery. **(1) Direct CREB-TAF110 interaction.** Mutagenesis studies have revealed a 60-amino acid modulatory domain of CREB, termed the kinase-inducible domain (KID), or P-box because of the presence of a phosphorylation site (Ser-133), which is critical for phosphorylation stimulated CREB activity (76, 83) (Figure 1-5). KID functions synergistically with an adjacent glutamine-rich region Q2, which extends from amino acids 160 to 283 of the CREB protein (84, 85). Q2 sequences resemble the hydrophobic and glutamine-rich Sp1 activation domain B, which is important for interaction with $dTAF_{II}110$ (a TBP associated factor). Ferreri *et al.* (86) tested for similar interactions between Q2 and $dTAF_{II}110$. They prepared CRE oligonucleotide affinity resins bound by purified CREB or Q2 mutant protein. The $dTAF_{II}110$ protein could indeed bind to CRE resin containing wild-type CREB, but not Q2-mutant CREB, indicating direct interaction of CREB (through Q2 domain) with the basal transcriptional complex (through $dTAF_{II}110$). Such an interaction was further confirmed in the yeast two-hybrid system. A yeast expression plasmid encoding the Q2 domain fused to the acidic activation domain of Gal4 was cotransformed into a yeast strain with a second vector expressing a $dTAF_{II}110$-Gal4 DNA binding domain fusion protein. Interaction of the Q2 domain with $dTAF_{II}110$, monitored by the recruitment of the acidic

activation domain onto the promoter of a Gal1-lacZ reporter gene, was readily observed (86). In similar assays, CREB-Q2 did not appear to interact with other components of the TFIID complex, such as TBP, TAF40, and TAF80. The results suggested that CREB-Q2 recruits TFIID to cAMP-responsive promoter, probably by interacting with TAF110. This finding also explains why CREM proteins α, β, and ε, which lack Q2, may function as repressers (86).

(2) CREB acting through a coactivator (CBP) and TFIIB. Activated CREB may also function through coactivator molecules, since Chrivia *et al.* (87) have identified a CREB binding protein (CBP) which binds specifically to phosphorylated CREB (Figure 1-5). CBP is a large nuclear protein of 2441 amino acid residues (265 kDa) and contains a so-called bromodomain, a conserved structural unit thought to be important for protein-protein interactions. This domain is found in *Drosophila* and yeast coactivator proteins involved in signal-dependent (not basal) transcription. It also contains several calmodulin kinase II phorsphorylation sites, a single PKA phosphorylation site, two zinc finger regions, and a carboxyl-terminal glutamine-rich domain (Figure 1-5).

How does CBP contact the transcriptional apparatus upon recruitment by phosphorylated CREB? Kwok *et al.* (88) provided evidence that the activation domain of CBP interacts with the basal transcriptional factor TFIIB through a domain that is conserved in the yeast coactivator ADA-1. In CREB deficient F9 teratocarcinoma cells, in the absence of endogenous CBP, the combination of CREB and PKA could only activate expression of a reporter gene 15-fold; Addition of CBP increased transcription in a dose-dependent manner up to 90-fold. CBP did not activate gene expression in the absence of CREB, even in the presence of PKA. Furthermore, CREB mutated at the PKA phosphorylation site did not allow CBP to activate expression. The finding that CBP induces gene expression only if CREB and

PKA are both present implicates that CBP is the mediator responsible for cAMP-activated gene transcription (88).

Binding of CBP to TFIIB was tested by using glutathione-S-transferase (GST)-TFIIB fusion proteins linked to glutathione agarose beads. Specific binding was observed with minimum CBP fragment containing residues 1680~1812, which contains a zinc finger structure homologous with ADA-1. Binding between CBP and deletion mutants of TFIIB suggested that CBP may require an amino-terminal region of TFIIB and the two repeats required for VP16 binding. These data support the model that CBP serves as a coactivator for phosphorylated CREB. The interaction of the zinc-finger region of CBP with TFIIB is intriguing but it can not exclude interactions with other basal transcriptional factors or even direct interactions between the basal factors and portions of CREB (as discussed earlier) (89).

Since TFIIB interacts with TBP and helps to recruit Pol II to the promoter, a continuous chain of physical contacts is now established, linking the stimulus-activated and phosphorylated CREB, bound distal to the promoter, with the Pol II complex that initiates transcription (90).

Arias *et al.* (89) developed an antiserum against amino acids 634~648 of the CREB binding domain of CBP in order to characterize its functional properties. By Western blot, they detected a 265 kDa protein which co-migrated with the predominant CREB binding activity in nuclear extracts. Microinjection of the anti-CBP serum into fibroblasts completely inhibited transcription from a cAMP responsive promoter. CBP antibody absorbed with synthetic CBP peptide failed to inhibit cAMP-induced gene transcription. Furthermore, CBP also showed cooperation with upstream activators such as c-Jun which are involved in mitogen responsive transcription. These authors proposed that CBP is recruited to the

promoter through interaction with certain phosphorylated factors, and that CBP may thus play a critical role in the transmission of inductive signals from cell surface receptor to the transcriptional apparatus (88-90).

To summarize, in response to cAMP elevation, the catalytic subunit of PKA dissociates from the regulatory subunit and migrates into the nucleus where it phosphorylates CREB at Ser-133 (or Ser-119). Phosphorylation may change the protein conformation of CREB thereby exposing domains necessary for interactions either with (1) the basal transcriptional factors, e.g. TAF110 interacting with Q2 domain of CREB; or (2) CBP as a coactivator, which in turn recruits TFIIB by interaction of a zing-finger region of CBP with regions of TFIIB, leading to activation of target gene transcription (Figure 1-5).

4. PANCREATIC ISLET-SPECIFIC EXPRESSION OF SOMATOSTATIN GENE

The somatostatin gene is widely expressed but in restricted cell types, e.g. neurons, D-cells in pancreatic islets and gastrointestinal tract, and C-cells of the thyroid gland. Little is known about the *cis*-regulatory sequences that determine the restricted cellular specificity of somatostatin gene expression. The insulin-, glucagon-, and somatostatin-producing cells in the pancreatic islets derive from a common precursor stem cell and differentiate sequentially during embryonic development, thereby providing an informative model for the study of the transcriptional mechanisms involved in the control of cell-specific gene expression.

Morphogenesis of the pancreas begins by evagination of the duodenum at the 26 somites stage (day 9.5 of gestation) in the mouse. By embryo microdissection coupled with reverse transcriptase-polymerase chain reaction (PCR), the onset of cell-specific gene expression was recently redefined during pancreatic organogenesis (91). SS-mRNA is the first to be detected, at the 10-somite stage throughout the foregut, long before the onset of insulin and glucagon expression (20-somite) and pancreas evagination. In the fetus and neonate, somatostatin cells make up 40% of the total endocrine pancreas, only next to insulin producing cells (9). However, in adult pancreas and gut, somatostatin expression is restricted to D cells, which make up only 3% of the pancreatic islet cell population (3, 91). In the last several years, we have begun to understand the involvement of both positive and negative transcriptional control mechanisms, which determine the early onset of somatostatin gene expression in pancreas, and its restricted expression in islet D cells in the adult.

4.1 GLUCAGON AND INSULIN GENE EXPRESSION

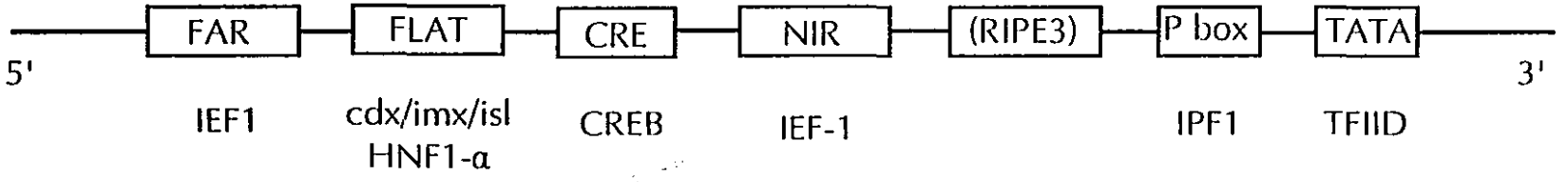
Binding assays and transient transfection analyses of reporter plasmids bearing regulatory sequences of the glucagon or insulin genes have lead to the identification of transcriptional control elements that interact with regulatory proteins to direct pancreatic islet A- or B-cell-specific transcription, respectively.

Glucagon gene. Expression of the glucagon gene is restricted to the A-cells of the pancreatic islet, the L cells of the intestine, and a few distinct neurons in the brain (153). Three hundred bp of the 5'-flanking region of the rat glucagon gene has been found to be sufficient to confer A-cell specificity. There are three transcriptional control elements within this part of the gene: G1 (-97/-65 bp) is A-cell specific enhancer, G2 (-192/-174 bp) and G3 (-268/-238 bp) are islet cell but not A-cell specific. Thus, in pancreatic islets, cell specificity of glucagon gene expression is confered by the glucagon promoter, including the G1 element, which acts together with islet cell-specific enhancer elements (G2 and G3) (275, 276, 278). Further upstream is the CRE (TGACGTCA) at -298/-291 bp (Figure 1-6).

Insulin gene. In adult mammals, insulin is synthesized exclusively in the B-cells of the pancreatic islets. Efficient transcription of the insulin genes require 300~400 bp of the 5'-flanking sequence. In most species, including humans, the insulin gene exists as a single copy. In rodents there are two non-allelic insulin genes, designated insulin I and insulin II, which are transcribed equally. There are considerable similaritiy in length and organization between the human and the murine insulin II genes (277-279). The terminology concerning *cis*-acting DNA elements and *trans*-acting factors for the insulin gene is very confusing. The important ones based on studies of rat insulin I and II, and human insulin genes are summaried here and shown in Figure 1-6:

(1) NIR, an 8 bp enhancer (GCCATCG) at -112/-105 bp of the rat insulin I gene,

insulin gene



glucagon gene

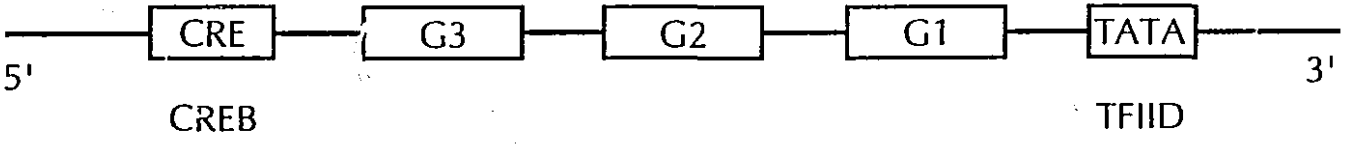


Figure 1-6. Schematic structure of the 5'-flanking DNA regions of insulin and glucagon genes.

named after Uri Nir, essential for basal and B-cell specific expression. It corresponds to the insulin control element (ICE) or GC-I (sequence: GCCATCTG) in rat insulin II or human insulin genes, respectively. The *trans*-acting factor is insulin enhancer factor 1 (IEF-1).

(2) FAR, another identical 8 bp enhancer upstream of the NIR (-238/-231 bp), equally important in function, also bound to IEF-1. In the human gene this element is called GC-II (sequence: GCCACC), but binds to factors other than IEF-1. The rat insulin II gene does not have such an element. In other studies, NIR and FAR have been termed E box elements, being recognized by the ubiquitous helix-loop-helix protein Pan-1 and Pan-2 in rodents (or termed E47 and E12 in human) (294).

(3) CRE, TGACGTCC, at -185/-178 bp, present in all insulin genes, binds to CREB family of proteins.

(4) FLAT (or E2), a mini-enhancer region located just downstream of the FAR box (-223/-208 bp), not present in rat insulin II gene. It binds to cdx-3, lmx-1, Isl-1 (homeodomain proteins) and hepatic nuclear factor 1-α (HNF1-α). In human insulin gene a similar region is named CT-II (sequence: TCTAATG).

(5) P box (-80/-74 bp, TAATGGG), an enhancer element conserved in rat and human insulin genes. P box and FLAT share a common TAAT consensus motif, which are also found in somatostatin gene. The protein factor, insulin promoter factor 1 (IPF1), has been recently cloned and found to be important in pancreas formation during embryonic development (279, 295, 297). IPF1 is identical protein to IDX-1 and STF-1 as discussed later.

4.2 SOMATOSTATIN GENE EXPRESSION

To delineate the location of the positive and negative *cis*-regulatory elements in the

somatostatin gene, Vallejo *et al.* (24) performed transient transfection assays in somatostatin-producing pancreatic islet cell line (RIN-1027-B2) using CAT reporter plasmids bearing somatostatin promoter sequences. Sequential 5' end deletions of the somatostatin promoter (-900 to +55 bp) indicated the presence of both positive (-120 to -65 bp, termed SMS-UE) and negative (-425 to -345 bp, and -250 to -120 bp) regulatory elements. Deletion of CRE located downstream from SMS-UE reduced SMS120-CAT expression 3~5-fold in RIN-1027-B2 cells, indicating that both the SMS-UE and CRE enhance transcription in a synergistic manner.

To test the functional interaction of CRE and SMS-UE, the authors used another cell line, because RIN-1027-B2 cells are known to be defective in cAMP signalling. In hamster insulin-producing HIT-T15 cells, the basal level of SMS120-CAT expression was 3-fold higher than that of SMS65-CAT (without SMS-UE). Incubation with cAMP resulted in a 6.5- and 5.2-fold increase in SMS65-CAT and SMS120-CAT expression, respectively. These results indicate the existence of a functional synergism between the SMS-UE and CRE under basal conditions. Under cAMP-induction SMS-UE and the CRE also act synergistically (24).

When transfected into different types of islet cell lines, SMS120-CAT has the highest expression in RIN-1027-B2 (D-cell, 100%), only 40% activity in InR1-G9 (A-cell), and only 20% activity in RIN-1046-38 (B-cell). This indicates that a functional unit including the SMS-UE, CRE, and TATA box is sufficient for the preferential expression of somatostatin gene in islet D-cells. In addition, when 3 copies of SMS-UE were inserted in front of the minimal promoter of the herpes simplex virus (HSV) thymidine kinase (TK) gene [$(SMS\text{-}UE)_3TK$], the activity was about 20-fold higher than TK promoter alone in RIN-1027-B2 cells, compared to only 2~3-fold in other cell types (including other islet cells) (24). These results indicate that

the SMS-UE is part of a functional unit which includes other elements of the somatostatin gene proximal promoter, and that they act together to regulate D-cell-specific transcription of the somatostatin gene in islet cells (24).

4.3 CHARACTERIZATION OF THE TRANSCRIPTION FACTORS INVOLVED IN PANCREATIC ISLET-SPECIFIC GENE EXPRESSION

In a follow up study, Vallejo *et al.* (25) characterized the nuclear proteins that interact with the SMS-UE. Detailed mutation combined with CAT reporter transfection studies revealed the presence of two domains (A and B) within the SMS-UE. Domain A of the SMS-UE is a DNA enhancer sequence that is identical to that bound by the ubiquitously distributed α-CBF, a transcription factor that also regulates the expression of genes such as human chorionic gonadotropin (hCG) α-subunit gene. The B domain, on the other hand, binds an islet cell-specific protein with characteristics similar to that of Isl-1, a transcriptional activator protein that binds to the FLAT (E2) enhancer of the rat insulin I gene. In addition, the SMS-UE binds transcription factor CREB but not CREM, on a site adjacent to, or overlapping with, the 3' end of domain B. The carboxyl-terminal bZIP domain of CREB alone binds to the CRE of the somatostatin gene but is not sufficient for binding to the SMS-UE, suggesting that CREB/SMS-UE binding requires stabilization by a region of the CREB located within the transactivation domain (25).

Leonard *et al.* (77) identified three consensus recognition sites (CTAATG) for Isl-1 at -96, -295, and -452 bp on somatostatin promoter. Mutation of the proximal Isl-1 site (-96 bp, SMS-UE B domain) caused a 70% reduction in cell-specific activity in MSL-G2-Tu6 (pancreatic tumor) cells. Insertion of a single Isl-1 motif into a minimal reporter enhanced its

activity 5~10-fold in MSL-G2-Tu6 cells, supporting the notion that Isl-1 may be a cell specific transcription factor. However, in the same study, expression of an Isl-1-like protein was also demonstrated in PC12 pheochromocytoma cells by Western blot, indicating that Isl-1 is not restricted to pancreatic islet, and is thus unlikely to be the islet specific transcription factor itself.

Recently, homeodomain-containing transcription factors named STF-1, IPF-1, and IDX-1 were cloned independently from three laboratories, and turned out to be an identical protein. Homeodomain is a sequence first characterized in several proteins coded by genes involved in development regulation in *Drosophila* (homeotic loci) (71). Miller *et al.* (26) cloned islet/duodenum homeobox-1 (IDX-1), a protein of 31 kDa, 283 amino acid residue. IDX-1 mRNA and protein are detected in rat pancreatic islet, duodenum, RIN-1027B2 cells and in rat fetal intestine. In gel shift assay, IDX-1 is able to bind to TAAT1 (-462/-438 bp) and TAAT2 (-303/-280 bp), B domain of SMS-UE, and to the FLAT element in rat insulin I promoter. IDX-1 activates somatostatin gene transcription mainly through interaction with TAAT1 element. Somatostation gene enhancer elements TAAT1 and TAAT2 bear strong similarity to insulin promoter FLAT element (26).

Leonard *et al.* (154) cloned a somatostatin transactivating factor-1 (STF-1) that stimulates somatostatin expression in pancreatic islet cells. STF-1 is expressed in cells of the endocrine pancreas and small intestine. The 284-amino acid protein binds to tissue-specific elements within the somatostatin promoter and stimulates somatostatin gene expression both *in vivo* and *in vitro*. As IDX-1/STF-1 expression is not confined to D-cells, this factor may be more generally involved in promoting peptide hormone expression in the pancreas and small intestine. Further study revealed that immunoreactive STF-1 is located in nuclei of 90%

of B cells, in contrast to 2% of A cells and 19% of D cells, indicating that STF-1 protein is largely confined to a subset of insulin-containing cell types within the pancreatic islets (294). As only 19% of somatostatin-producing cells in adult pancreatic islets contain immunoreactive STF-1, it is probably not required for maintenance of somatostatin expression in most islet cells.

Ohlsson *et al.* (279) described the cloning of insulin promoter factor 1 (IPF1), whose expression is restricted to pancreatic islet B-cells, βTC1 cells (an insulin-producing B-cell line), pancreatic islet, and duodenum. IPF1 binds to the enhancer P box, which is conserved in the rat and human insulin genes, and transactivates insulin gene. IPF1 expression is initiated prior to insulin gene expression at the primitive foregut where pancreas will later form. In fact, IPF1 is required for the formation of the pancreas as Jonsson *et al.* (297) reported. Mice homozygous for a targeted mutation in the *Ipf1* gene can not develop a pancreas and die soon after birth (297).

In summary, current studies mark only the beginning in the understanding of *cis* and *trans* elements involved in pancreatic islet specific somatostatin gene expression. SMS-UE and its binding proteins, in collaboration with CRE-CREB, could well be the key elements in inducing somatostatin gene expression in very early stage of development. But current evidence is not enough yet to identify a D-cell specific *trans* factor which interacts with SMS-UE. In addition, there have to be negative elements/factors which suppress somatostatin gene expression in other cells.

5. THE MOLECULAR MECHANISMS OF GLUCOCORTICOID-REGULATED GENE EXPRESSION

Glucocorticoids (cortisol and corticosterone) are synthesized in the adrenal cortex (*zona fasciculata*) and secreted as hormones in response to ACTH released from the pituitary. In the circulation, they are bound to an α globulin called transcortin (or corticosteroid-binding globulin, CBG) and to a lesser extent to albumin. Their half life is 1~1.5 h. They regulate fundamental functions such as blood pressure, carbohydrate and lipid metabolism, mineral metabolism, immune system, and body response to stress (11). Like other steroid as well as thyroid hormones, glucocorticoids travel via the blood stream to their target cells, enter these cells by simple or facilitated diffusion, and then bind to specific receptors which activate or silence target genes (Figure 1-7).

The steroid hormone receptor superfamily consists of a large number of genes, and represents the largest known family of transcription factors in eukaryotes. It includes receptors for the steroid hormones (estrogen, progesterone, glucocorticoids, mineralocorticoids, and androgen); thyroid hormone, vitamin D, retinoic acid, 9-*cis* retinoic acid; and orphan receptors without known ligand which have sequence homology to the receptor superfamily. Steroid receptors exist in inactive forms either in the cytoplasm or nucleus. Although glucocorticoid receptor (GR) is present in almost all cell types, its concentration depends on the cell type, the state of differentiation, the phase of the cell cycle, and the endocrine status of the cell (174). Unliganded steroid receptors are maintained as part of hetero-oligomeric complexes with the heat shock proteins (hsp90 and hsp70) as well as other less well characterized proteins (96). Upon binding their respective ligands, the receptors undergo an activation or "transformation" step. The activated receptor binds to a DNA response element e.g.

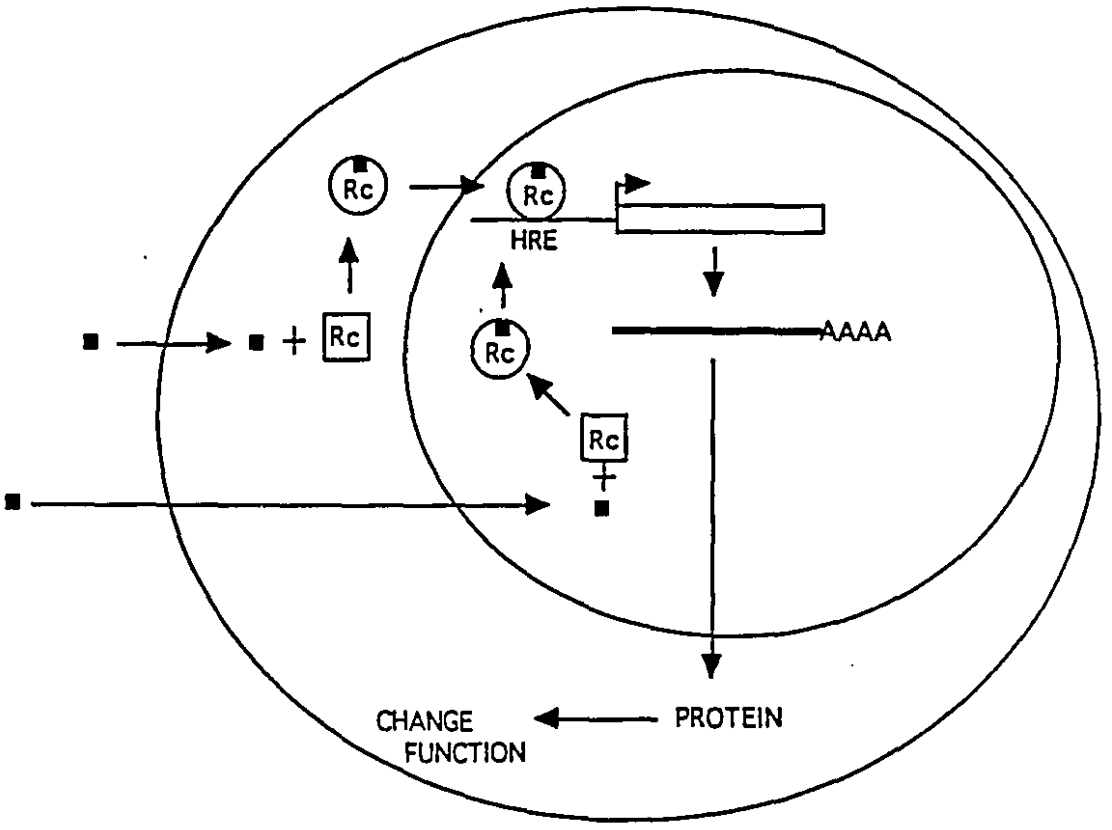


Figure 1-7. A simplified model of steroid action on target gene expression.
■ steroid hormone; Rc: receptor; HRE: hormone response element.
(adapted from ref. 176)

glucocorticoid response element (GRE) and activates transcription of a target gene. In addition to regulating transcription, steroid hormones are also known to regulate gene expression by affecting mRNA stability and translational efficiency (176), as discussed in Section 6 of the chapter.

5.1 RECEPTOR STRUCTURE. Amino acid sequence analysis and mutational dissection of intracellular steroid receptors indicate that they can be subdivided into several domains as indicated in Figure 1-8 (176). The N-terminal A/B domain is highly variable in sequence and in length (GR 1~420). This transactivation domain activates target genes presumably by interacting with components of the basal transcriptional machinery, coactivators, or other transactivators. This region of the receptor may also be important for determining target gene specificity of different receptors, which recognize the same response element, e.g. both glucocorticoid and mineralocorticoid receptors bind to GRE on target DNA. The C region, DNA binding domain (DBD), contains two zinc finger structures, which are responsible for DNA recognition and dimerization (GR 421~496). It is the most conserved region among all steroid receptors cloned so far (174).

Immediately adjacent to the carboxyl terminal end of the C region there is a variable hinge region, the D region (GR 496~528), which may allow the protein to bend or alter conformation, and often contains a nuclear localization domain and/or transactivation domain. The ligand-binding domain, or E region, is located carboxyl-terminal to the D region (GR 528~777). It is relatively large (~250 aa) and functionally complex. It usually contains regions important for HSP association, dimerization, nuclear localization, transactivation, intermolecular silencing, intermolecular repression and, most importantly, ligand binding.

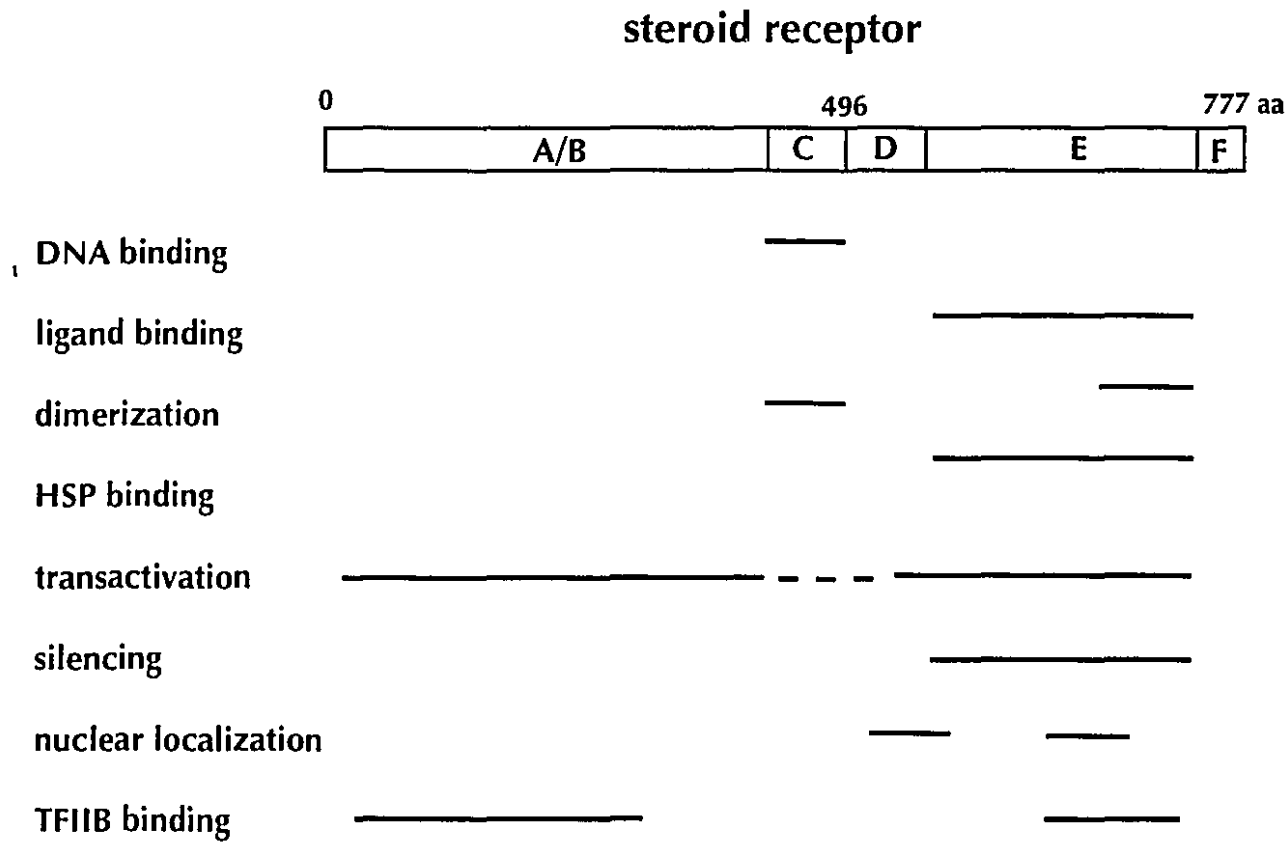


Figure 1-8. Functional domains of steroid (glucocorticoid) receptors.
(adapted from ref. 176)

Although most of these functions require only small stretches of amino acid sequence, the ligand binding domain appears to form a pocket involving a majority of the E region since most of the mutations identified in this region compromise the ability of the altered receptor to bind hormones (176). The major dimerization domain of receptors has been localized in the C-terminal half of the ligand binding domain. This region contains leucine-rich sequences that may form coil-coil interactions as the receptor dimerizes. Finally, located at the C-terminal end of certain receptors is the variable F region, for which no specific function has been identified. For example, deletion of the F region in estrogen receptor does not affect any known receptor function (177).

Although steroid receptors are highly phosphorylated proteins and several phosphorylation sites have been identified on various receptors, their functional significance is unclear (179). It may be more accurate to consider these receptors as inherently active transcription factors whose potential is enhanced by phosphorylation in a cell and promoter specific manner. In addition, phosphorylation of steroid receptors is a way of cross-talk with other signalling pathways. For example, PKA can enhance the DNA binding activity of glucocorticoid receptor thereby activating gene transcription in F9 embryonal carcinoma cells, without the need of endogenous CREB (92). Phosphorylation of progesterone receptor by PKA can occur independent of ligand binding and can be controlled by other signals, such as dopamine (97).

5.2 CONSENSUS GRE AND DIRECT GENE ACTIVATION BY STEROID RECEPTORS. DNA sequences responsive to glucocorticoids was first identified by mutational analysis of the long terminal repeat (LTR) of the mouse mammary tumor virus

(184). A deletion or mutation of that sequence eliminated hormone responsiveness. A short oligonucleotide corresponding to the identified sequence conferred glucocorticoid binding and responsiveness to a heterologous promoter (190). The GRE consists of two short, imperfect inverted repeats separated by three nucleotides ($G_{/T}G_{/T}TACA_{/C}nnnTGTT_{/C}CT$). Later, response elements for progesterone, mineralocorticoid, and androgen receptors were shown to be identical to that of glucocorticoid receptor (176). The conservation among hormone response elements (HREs) is in fact due to conservation of their amino acid sequences in the DNA binding domain of the receptors.

Upon binding hormone, steroid receptors bind to DNA and activate target gene expression. The receptor stimulates the formation of a pre-initiation complex by increasing its rate of formation and/or by stabilizing a preformed complex, through direct interaction with components of the basal transcriptional machinery or an intermediate factor (coactivator). Formation of the pre-initiation complex at a core promoter is a sequential process, as described in section 2 of this chapter. It is possible that a receptor can act on multiple sites to enhance formation of the pre-initiation complex (176).

Early studies indicated that chicken ovalbumin upstream promoter transcription factor (COUP-TF), a member of the steroid/thyroid hormone receptor superfamily, interacted directly with TFIIB without the need for a cofactor, since re-natured COUP-TF from a single gel band was able to bind to TFIIB (192, 198). Similar observations were also made for receptors for progesterone, estrogen, thyroid hormone, and retinoic acid (RAR) (198, 208). Baniahmad *et al.* (208) have found that the N-terminal end of the thyroid hormone receptor interacts specifically with TFIIB, at its C-terminal half which contains a direct repeat. Since binding of TFIIB to the TFIID-DNA complex is one of the rate-limiting steps in pre-initiation

complex formation, it is possible that thyroid hormone receptor enhances the formation of the complex through such an interaction. In addition to TFIIB, TBP has been shown to interact with thyroid hormone receptor and other transactivators (242, 260).

5.3 SYNERGISM OF MULTIPLE cis ELEMENTS. Many eukaryotic genes are under the control of multiple hormones and environmental cues. Consequently, HREs are usually found in multiple copies or clustered with other *cis*-acting elements. For example, the tryptophan oxygenase gene, the MMTV LTR, and the vitellogenin genes contain multiple HREs (261-3). In addition, the MMTV LTR, rat tryptophan oxygenase, and phosphoenolpyruvate carboxykinase (PEPCK) gene contain other *cis*-acting elements in close proximity to HREs (262, 300, 302). In these cases, when either one of the HREs or the adjacent *cis*-acting elements is mutated, promoter activity is drastically decreased. Thus, HREs often interact synergistically with other HREs or with different class of *cis*-acting elements.

Ptashne (303) has proposed two models to explain synergism that are relevant to the steroid/thyroid hormone receptor superfamily members. **(1) Cooperative binding:** Binding of one receptor complex facilitates the binding of a second. This synergistic interaction allows both complexes to bind with greater affinity and consequently in greater occupancy of the *cis*-acting elements, thus promoting greater transcriptional activity. Indeed, cooperative binding of receptors to multiple response elements has been observed with receptors for progesterone, glucocorticoids, and estrogen (304-306). Protein-protein interactions between the two dimers probably facilitate cooperativity. Tsai *et al.* (307) found that two molecules of the *E. coli*-expressed DBD of glucocorticoid receptor were able to bind to a single GRE in a cooperative manner. However, two dimers of the DBD were unable to bind cooperatively to two adjacent

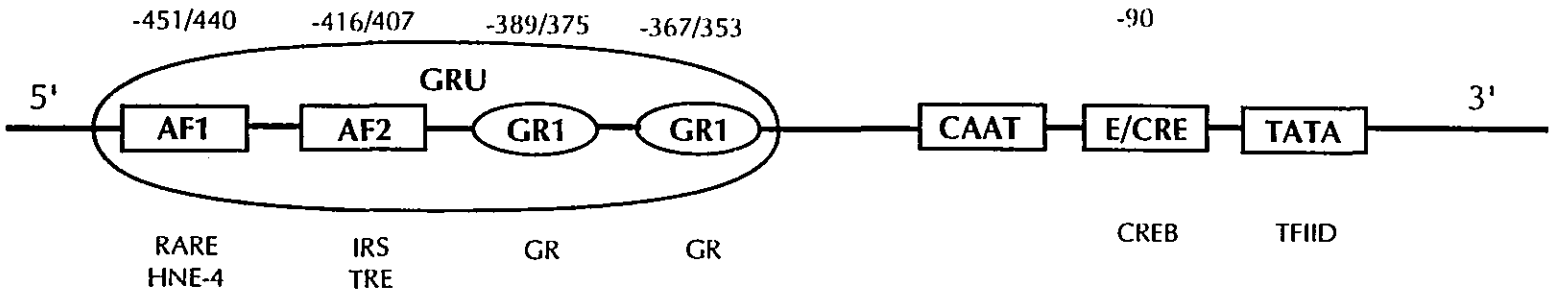
GREs, although the full-length receptor could do so (44). Since both the DBD and the full-length receptor can bind DNA, cooperative binding between two receptors appears to require protein-protein interactions involving regions outside the DBD domains. By deletion analysis, the region required for cooperative binding was localized to amino acid residues 250~417 of progestrone receptor, which includes the DBD (amino acids 280~369) (308).

(2) Cooperative protein-protein interaction. Although cooperative binding is an attractive model, it is unlikely to account for the total level of transcriptional synergism observed at target genes, especially when different HREs are present. For example, a GRE and an ERE can activate transcription of a linked target gene synergistically, but very little cooperative binding between progestrone and estrogen receptors can be observed (308). The second mechanism is proposed that cooperative interactions of receptors or transcription factors with multiple target sites (presumably components of the aggregate transcriptional machinery) may play a role in synergism. Attempts to define the regions important for this type of cooperativity have not been very successful. Mutation of a variety of regions in progestrone receptor decreases its ability to synergize with estrogen receptor (308). Similarly, synergistic interaction has been observed between glucocorticoid receptor and other transcription factors, and the structure important for this interaction has been shown to be complex (313).

There is a good example of functional cooperativity between the glucocorticoid receptor and the cAMP signalling pathway, illustrated by the gene for PEPCK (GTP; EC4.1.1.32). As the rate-limiting gluconeogenetic enzyme, PEPCK catalyzes the conversion of oxaloacetate to phosphoenolpyruvate. The hormones that regulate gluconeogenesis appear to do so by altering the rate of synthesis of PEPCK. Thus, cAMP and glucocorticoids increase

the transcription rate of the gene, and insulin inhibits (309, 144). The cAMP and glucocorticoid effects are synergistic, and the insulin effect is dominant both in rat liver and in H4IIE hepatoma cells (309).

The minimal DNA sequence required for glucocorticoid induction of the PEPCK gene is defined as the glucocorticoid response unit (GRU), which spans about 110 bp and includes two receptor-binding elements plus two accessory factor-binding elements as determined by DNase I protection assay (98, 175, 180) (Figure 1-9). Purified glucocorticoid receptor bound two regions: GR1 (-389/-375 bp) and GR2 (-367/-353 bp). Factors in crude rat liver nuclear extract bound to two additional regions on the promoter, designated accessory factor 1 (AF1, -455/-431 bp) and accessory factor 2 (AF2, -420/-403 bp) elements, respectively. Gel retardation analysis revealed that at least two proteins bound to AF1 and that they were distinct from the protein(s) that bound to AF2. Various combinations of GR1, GR2, AF1, and AF2 were fused to the CAT reporter gene and cotransfected with a glucocorticoid receptor expression plasmid into H4IIE cells to characterize the functional GRU. Neither the glucocorticoid receptor binding regions nor the accessory factor binding regions alone were sufficient to confer glucocorticoid responsiveness. GR1 and GR2 functioned independently, and each accounted for half of the maximal response, provided the accessory factor elements were present. In the absence of the accessory factors, the glucocorticoid receptor binding sites alone are functionally inert, and bind glucocorticoid receptor at a 5~10-fold lower affinity than does the consensus GRE (150). Similarly, deletion of either AF1 and AF2 diminished glucocorticoid induction of the PEPCK gene to approximately half of the maximum. The role of AF1 and AF2 seems to be to enhance or stabilize binding of the glucocorticoid receptors to GR1 and GR2.



AF1: TGACCTTTGGCC

AF2: TGGTGTTTTG

GRE consensus G/TG/TTACA/cNNNTGTT/cCT

GR1 cacACANNNTGTgCa

GR2 aGcAtANNNaGTCCa

Figure 1-9. PEPCK gene promoter elements.

Further studies indicated that AF1 includes a retinoic acid response element (RARE) and an HNE-4 element that may be involved in hepatic-specific expression of genes (98, 145, 146); AF2 functions as both an insulin responsive sequence (IRS) and phorbol ester response element (TRE) (147, 148). Retinoic acid acts on the PEPCK gene synergistically with glucocorticoid, while insulin and phorbol esters inhibit cAMP or glucocorticoid induced responses, both individually as well as in combination. The two glucocorticoid receptor binding sites are distinct from the consensus GRE sequence in which they match in only 7/12 and 6/12 positions, respectively (175) (Figure 1-9).

The response of the PEPCK gene to cAMP is mediated primarily through an E/CRE at -90 bp that is both a part of the basal promoter and a CRE (149). The E/CRE binds the transcription factor CREB and is required for the full response of the PEPCK gene to glucocorticoid. Granner *et al.* (98) found that internal deletions of either the CAAT box and the E/CRE together or of the E/CRE alone produced a marked reduction in the glucocorticoid response. In contrast, glucocorticoid induction was unaffected by deletion of the CAAT box alone. In addition, they found that GRU from the PEPCK gene functions poorly when subcloned into the TK promoter (without a CRE) (98).

Faber *et al.* (151) extended the *in vitro* observations of PEPCK promoter activity to "*in vivo*" genomic footprinting, *e.g.* DNase I footprinting in permeabilized H4IIE cells. Nearly all of the sites of protein interaction observed *in vitro* are protected *in vivo*. The DNase I protection pattern is the same in cells without or with any of the hormone treatments (glucocorticoid, insulin, and cAMP), suggesting that hormonal modulation of transcription does not involve addition or removal of factors from the *cis*-elements of the promoter (151).

Protein-protein interaction was observed *in vitro* between glucocorticoid receptor and

CREB that might account for the role of the E/CRE in the glucocorticoid response of the PEPCK gene (98). [^{35}S]-methionine-labelled CREB was mixed with extracts of HeLa cells infected with a glucocorticoid receptor-expressing recombinant vaccinia virus, or with wild type vaccinia virus. After incubation, the receptor and associated proteins were immunoprecipitated with a monoclonal antibody against glucocorticoid receptor. Following electrophoresis, labelled CREB was found to co-precipitate with glucocorticoid receptor. As a positive control, wild type c-Jun but not C-terminal truncated Δc-Jun complexed with glucocorticoid receptor as well. Therefore, the functional interaction between GRU and E/CRE is most probably through a physical association of the glucocorticoid receptor with CREB (98).

cAMP and glucocorticoids influence many of the same physiologic processes. In some cases one or another of these effectors plays a permissive role, and numerous additive and synergistic responses have been described. A functional relationship between the glucocorticoid and cAMP response elements, through interaction of the proteins that bind to them, may be one way that these physiologic effects are achieved (180, 302).

5.4 MECHANISMS OF GLUCOCORTICOID-INDUCED GENE SILENCING.

Over the years, there have been several models to explain negative regulation of glucocorticoids on target gene transcription, e.g. negative, competitive or composite GREs and even without direct binding to DNA (no GRE). They are briefly discussed below.

(1) Negative GRE: An initial study by Sakai *et al.* (310) implicated the specific DNA sequence recognized by the receptor as the determinant of positive or negative regulation. A 34-bp sequence of the bovine prolactin gene which binds purified glucocorticoid receptor *in*

vitro represses heterologous promoters to which it is fused. This so-called "negative glucocorticoid response element" (nGRE: CAGATCTCAGCATCAT) differs substantially from the consensus sequence identified for the positive GRE (311). It was suggested that the interaction of receptor with nGRE sequences might alter receptor conformation, thereby inhibiting its positive activity (310).

A second example of the negative GRE has been reported in the case of the pro-opiomelanocortin (POMC) gene in the anterior pituitary, the transcription of which is totally inhibited by glucocorticoids (178). An element within the rat POMC gene 5'-flanking region is required for glucocorticoid inhibition of the gene transcription in AtT-20 cells, which contains an *in vitro* binding site at -63 bp for purified glucocorticoid receptor. Site-directed mutagenesis revealed that binding of the receptor to this site is essential for glucocorticoid repression of transcription. The DNA sequence of the POMC negative GRE differs significantly from the GRE consensus, which may result in different receptor-DNA interactions and may account at least in part for the opposite transcriptional properties of these elements. An alternative explanation is based on the fact that glucocorticoid receptor binds to a region (nGRE) on the POMC promoter adjacent or overlapping the enhancer CAAT box. Mutually exclusive binding of two proteins may result in suppression on the gene (178, 248). In the case of both prolactin and POMC genes, the responsible DNA elements have been referred to as nGRE due to their variance from the nucleotide sequence of the consensus GRE. However, their mechanism of action may be better understood as competitive GREs.

(2) Competitive GRE: Repression of the human glycoprotein α-subunit gene by glucocorticoids has been extensively characterized. It was shown that glucocorticoid inhibition was dependent on the presence of a functional CRE closely adjacent to or overlapping the

GRE (93). Thus, negative regulation could be due to the glucocorticoid receptor causing steric hindrance to the binding of a positive factor to the CRE. Similar results were obtained for several other genes. In the case of the bovine prolactin gene, the rat α1-fetoprotein and the rat POMC gene, it was shown that the GREs are always located close to or overlap with binding sites for other transcription factors, which mediate glucocorticoid-induced gene suppression (312).

(3) Composite GRE: is proposed by Diamond *et al.* (311) based on studies of rat proliferin gene promoter. Both glucocorticoid receptor and AP-1 are able to bind to a 25-bp plfG promoter region; and glucocorticoid receptor interacts directly with c-Jun in a solution (311). Glucocorticoids exert three different effects on the proliferin gene due to cell specific differences in AP-1 proteins: (a) In the absence of AP-1 activity, e.g. in F9 cells, glucocorticoids are without effect on the proliferin promoter because of failure of the hormone receptor complex to interact functionally with the target DNA region (plfG element). (b) The c-Jun homodimer alone binds weakly to plfG and activates the promoter, e.g. in HeLa cells. The glucocorticoid receptor interacts both with the plfG sequence and with c-Jun, producing a stable complex that strongly enhances promoter function. (c) In CV-1 cells, the Jun-Fos heterodimer strongly enhances promoter function. The hormone-receptor complex interacts both with plfG and with Jun-Fos producing a complex with altered conformation that is not functional for enhancement, or alternatively, structural alteration may lead to release of all components from the DNA thereby causing gene silencing (311).

In this model, limiting amounts of the receptor can repress efficiently and specifically despite its inability to occupy fully even cognate GRE sequences. The key feature is that the receptor interacts at the composite GRE not merely with a DNA sequence but also with the

bound Jun-Fos complex. Thus, the receptor associates only with activated promoters. The finding that the receptor interacts both with DNA and with Jun-Fos implies that the composite GRE may facilitate or stabilize the protein-protein interaction, and provides a mechanism for selectivity that explains why glucocorticoids do not regulate all promoters that utilize AP-1 (311, 314).

(4) No DNA binding: A different type of negative regulation has been described which does not involve DNA binding of the receptor at all. Treatment of mouse fibroblasts with phorbol esters, ultraviolet light, or serum growth factors induces dramatic changes in their gene expression pattern. One example is the induction of the collagenase gene mediated by binding of AP-1 protein to the promoter, which can be counteracted by addition of glucocorticoids, without the presence of a GRE on the target DNA (94). Glucocorticoid receptor lacking the DBD is also functional in such a repression. *In vitro* experiments using purified glucocorticoid receptor and c-Jun proteins suggest that mutual repression is due to direct interaction between them. Direct interaction between AP-1 and glucocorticoid receptor in the presence of hormone could be demonstrated by immuno-coprecipitation (94, 99). Thus, it appears that AP-1 and glucocorticoid receptor form a complex which is unable to transactivate. Conversely, glucocorticoid can be repressed by overexpression of AP-1. As well, the estrogen receptor was shown to repress the prolactin gene in the absence of DNA binding, by interacting with the pituitary-specific transcription factor Pit-1 (95).

In summary, glucocorticoids influence target gene transcription through activating their receptors. Binding of the glucocorticoid receptor to positive GRE on the target promoter may stabilize the basal transcriptional machinery and active gene transcription. Such activation

requires the direct or indirect interactions of glucocorticoid receptor with TFIIB or other basal transcriptional factors. Synergistic transactivation and/or DNA binding exist with multiple GREs, or GRE in conjunction with other enhancer elements. Negative regulation in most cases is due to either protein-protein interaction with other transcriptional enhancers, or competitive DNA binding with other adjacent enhancers. Negative GRE can also mediate glucocorticoid-suppression of gene transcription probably through destabilizing the basal transcriptional machinery. Finally, glucocorticoid receptor can also inhibit gene transcription through interaction with other transcription factors without direct binding to target promoters (Figure 1-10 and Table 1-3).

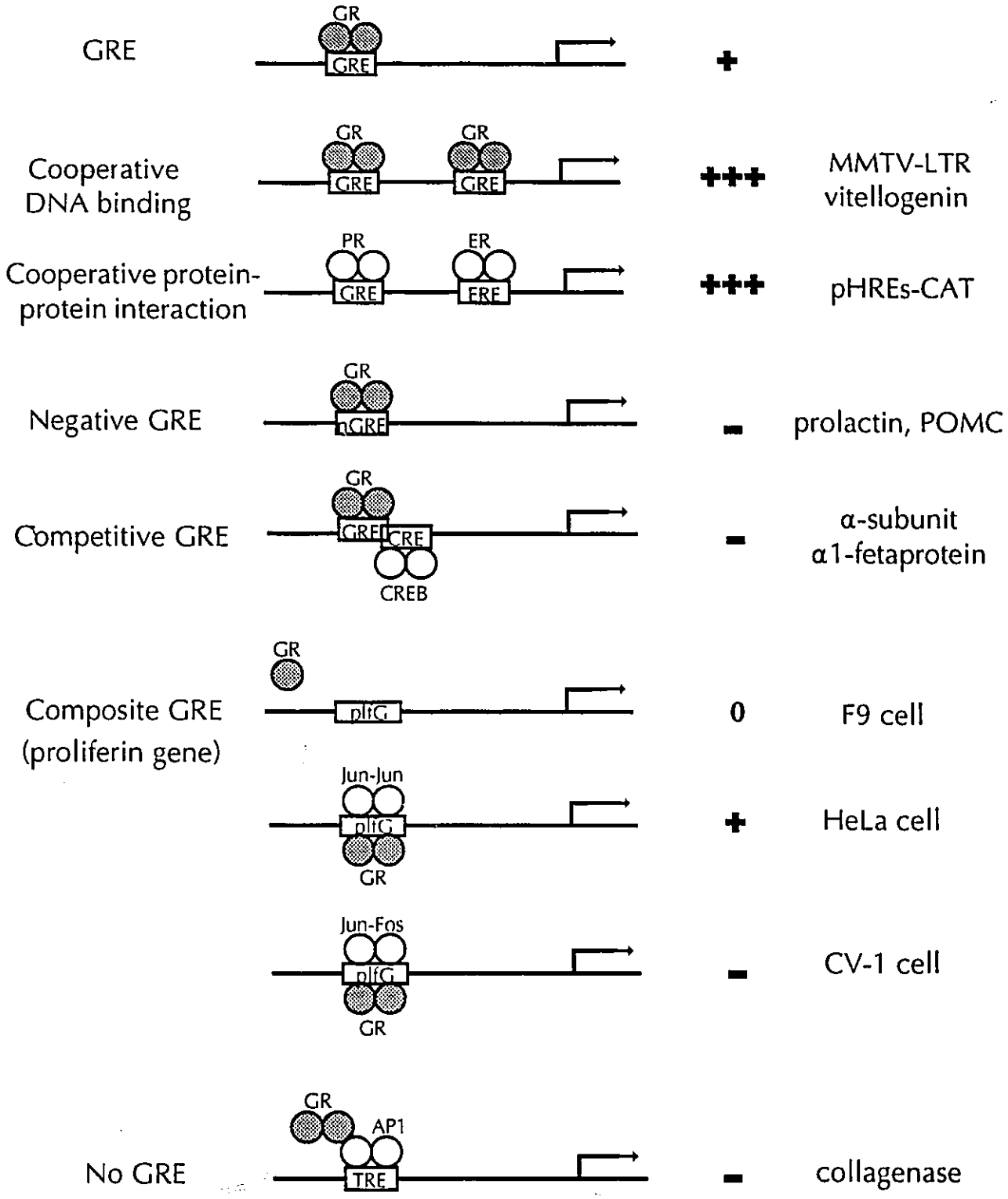


Figure 1-10. Schematic models of glucocorticoid regulated gene transcription. GR, PR, ER: glucocorticoid, progestrone, and estrogen receptrors; GRE, CRE, ERE, TRE: glucocorticoid-, cAMP-, estrogen-, and phorbol esters- response elements; plfG: proliferin gene enhancer element; CREB: CRE binding protein; AP1: activator protein 1.

Table 1-3. Mechanisms of glucocorticoids action on gene transcription.

ROLE OF GRE	TARGET GENE	MECHANISM
positive		COUP-TF, PR, ER, TR, RAR ~ TFIIB TR ~ TBP
synergistic	MMTV LTR PEPCK	cooperative DNA binding GR ~ CREB
negative	bovine prolactin, POMC	
competitive	glycoprotein α-subunit, prolactin, POMC, α1-fetoprotein	GRE ~ CRE
composite	proliferin	GR ~ c-Jun/c-Fos
none	collagenase prolactin	GR ~ AP-1 ER ~ Pit1

6. mRNA STABILITY AND ITS REGULATION

Until recently, most studies of the regulation of eukaryotic mRNA levels have focused on the control of gene transcription. However, the level of a cellular mRNA actually represents a balance between its rates of synthesis and degradation. It is known that eukaryotic mRNAs display diverse half-lives ranging from a few minutes to many hours or even days, and the degradation rate of a given mRNA can be altered in response to external stimuli. The list of examples of genes whose regulation involves mRNA stability has continued to expand. The regulation of cytoplasmic mRNA stability has emerged as an important control point in a variety of biological systems, including oncoproteins, peptide hormones, and cytokines (100, 121, 122, 155, 158).

Following gene transcription, the steps leading to protein synthesis can be summarized as follows (67, 68, 100, 101): (1) Processing of the primary transcript (heterogenous RNA): As shown in Figure 1-2, the primary transcript includes both exon and intron sequences. Post-transcriptional processing begins with changes at both ends of the RNA transcript. At the 5' end, enzymes add a special nucleotide cap: 7-methyl guanine (m^7G); at the 3' end, an enzyme clips the pre-mRNA about 30 bp after the AAUAAA sequence in the last exon. Another enzyme adds a poly(A) tail, which consists of up to 200 adenine nucleotides. Next, spliceosomes remove the introns by cutting the RNA at the boundaries between exons and introns. (2) mRNA transport: Mature mRNA is transported through nuclear pores into the cytoplasm, at which point it may be translated and/or degraded. (3) Translation is initiated by interaction of the 40S ribosomal complex with the 5'-end of mRNA followed by binding of the 60S ribosomal unit and subsequent synthesis, elongation, and termination of polypeptide chains. (4) Transfer of secreted proteins into the lumen of the endoplasmic reticulum is co-

translational after which modifications such as glycosylation and sulphation of the newly synthesized protein may occur. The regulation of synthesis of many polypeptide hormones occurs at one or more steps along these pathways (101). For the purpose of this thesis, mRNA stability and its regulation will be discussed in this section.

6.1 SEQUENCE ELEMENTS IN THE mRNA MOLECULE DETERMINE ITS STABILITY

It is believed that mRNA is inherently stable in cells. Unstable mRNA is due to the presence of various destabilizing sequences, linked to mRNA degradation mechanisms. mRNA exists in the cytoplasm as ribonucleoprotein particles with some proteins protecting the mRNA from digestion by nucleases and others promoting nuclease attack. The proteins function through interaction with mRNA structure or sequences, and their amount is regulated by hormones and other signals (68, 100). Recent work on mRNA decay indicates that sequence elements regulating mRNA stability are found throughout the message, as illustrated by the following examples (summarized in Table 1-4).

(1) The 5' cap structure: The unique 5'-5' phosphodiester bond of the cap makes it intrinsically resistant to general ribonucleases. A specific decapping enzyme has been purified from yeast, and it is possible that activation of this enzyme by mRNA sequences would create an unstable mRNA. Removal of the cap structure would lead to digestion of the mRNA by either a 5'-3' exoribonuclease or an endoribonulcease whose site of action would be masked by the cap (155).

(2) The 5'-UTR and the translational requirement of mRNA degradation: The 5'-UTR of mRNA has not yet been definitively shown to be a destabilizing sequence in any

mRNA. However, the 5'-UTR has been well documented to control the translatability of a mRNA molecule, and it is through negative regulation of translation that this region of mRNA has been shown to influence the degradation process.

There are a number of examples of rapidly degraded mRNAs whose levels are markedly increased in cells treated with inhibitors of protein synthesis, such as mRNAs for c-myc, c-fos, and c-jun (264-267). The increase in mRNA levels appear to be the result of substantial stabilization of the mRNAs by the protein synthesis inhibitors and are seen with various types of inhibitors (i.e., inhibitors of translation initiation and of elongation that act by distinct mechanisms). The mechanism by which protein synthesis inhibitors stabilize certain mRNAs is thought to require a highly unstable protein that is involved in the degradation of these mRNAs (a so-called *trans* effect), or translation *per se* is required for the decay of these mRNAs (a *cis* effect). These two possibilities are not mutually exclusive and each may be applicable in certain instances (158).

The *cis* effect: Consistent with a *cis* effect, a mutation resulting in a decreased rate of translation due to limited functional tRNA also stabilized mRNA (157). There is evidence that ribosome-bound β-tubulin mRNA is degraded after cotranslational recognition of the nascent amino-terminal peptide (Met-Arg-Glu-Ile) by unassembled dimers of α- and β-tubulin (268). This recognition is presumed to activate an RNase that may be ribosome-associated. In fact, such a nuclease has also been invoked in models for regulated turnover of histone mRNAs (268-270). In the case of c-fos mRNA stabilization by cycloheximide, it has been suggested that the rapidity of the effect on mRNA stability is more consistent with a *cis* effect (271). But the major difficulty in distinguishing between a *cis* effect and a *trans* effect of protein synthesis inhibitors is that inhibition of global protein synthesis will, by its nature,

inhibit the translation of the mRNA in question and the synthesis of the putative short-lived, trans-acting protein.

The *trans* effect: To overcome this problem, Koeller *et al* (158) performed a unique experiment with mRNAs for human transferrin receptor and c-fos. They introduced a ferritin iron response element (IRE) into the 5'-UTR of each of these mRNAs. The presence of the IRE allowed the authors to use iron availability to alter the translation of the mRNA in question, without global effects on cellular protein synthesis. The translation of a mRNA is up-regulated by iron in the presence of an IRE in its 5'-UTR (272-274). In this study, although specific translation of these mRNAs could be inhibited by iron chelation to a degree comparable to that seen with cycloheximide (~95% inhibition), no effect on mRNA turnover was observed (158). These data support the model of *trans* effect in which a *trans*-acting labile protein is necessary for the turnover of these mRNAs (158).

(3) Premature termination and mRNA destabilization: Investigations on the mechanism by which nonsense mutations within mRNA lead to instability have provided significant insight into the general pathways of mRNA degradation. It has been appreciated for some time that nonsense mutations can destabilize mRNA. Furthermore, the closer the mutation is to the initiator methionine codon, the less stable the transcript is (160).

(4) Open reading frame (ORF) destabilizing sequences: In what remains a unique example, Yen *et al.* (161) discovered that the N-terminal tetrapeptide encoded by β-tubulin mRNA provided a signal to target rapid degradation of that mRNA under conditions of tubulin monomer excess. Destabilizing sequence is also identified in the ORF of c-fos mRNA (162). The sequence was only utilized when the message was transiently expressed following growth factor stimulation. A closer examination of this element has shown that it is the RNA

sequence, but not the protein product or a biased codon usage, that is required for destabilization (162). Recently, several proteins that recognize the ORF destabilizing region of c-fos mRNA have been identified by ultraviolet cross-linking experiments (165). In a related series of experiments examining an analogous destabilizing sequence in the ORF of c-myc mRNA, Bernstein *et al.* (163) found that the *in vitro* decay rate of the polyribosome-bound c-myc mRNA was dramatically increased in the presence of a synthetic RNA containing the destabilizing region.

(5) 3'-UTR destabilizing sequences: have received the greatest attention in studies on mRNA degradation. Transferrin mRNA degradation is regulated by changes in cellular stores of iron. Located within the 3'-UTR of this mRNA is an IRE that contains five distinct stem-loop structures, capable of binding the IRE-binding protein (IRE-BP). The binding of the IRE-BP to the transferrin mRNA stabilizes the mRNA. Cellular iron regulates the affinity of binding by dissociation and reassociation of an iron-sulphur cluster within the IRE-BP (164).

A well-characterized 3'-UTR destabilizing sequence is the AU-rich element (ARE). Shaw and Kamen (130) made the initial observation that an ARE in the 3'-UTR of the granulocyte-macrophage colony stimulating factor (GM-CSF) mRNA could stimulate the degradation of the β-globin mRNA. The consensus sequence for the AREs is loosely defined as AUUUA repeats, often found within a U-rich region in the 3'-UTR, of the mRNAs including the lymphokine and immediate early genes.

(6) 3'-Poly(A) tail: Most eukaryotic mRNAs are protected by a poly(A) tail from degradation caused by ribonucleases. Its removal may be a pre-requisite for mRNA degradation as discussed later.

Table 1-4. Defined elements in mRNA which determine its stability.

Location	Host mRNA	Element/Function
5'-cap	all mRNAs	removal of the cap destabilizes mRNA (159)
5'-UTR	---	translatability --> mRNA stability (155)
premature termination	---	upstream nonsense mutation --> mRNA instability (160)
open reading frame	β-tubulin mRNA;	encodes a signal for degradation (161);
	c-fos & c-myc mRNA	destabilizing sequences (162, 163)
3'-UTR	transferrin mRNA;	iron-response element stabilizes the mRNA (164);
	lymphokine, immediate early gene mRNAs	AU-rich element destabilizes mRNA (130)
poly(A) tail	many mRNAs	pre-requirement for mRNA degradation

6.2 PROPOSED MECHANISMS OF mRNA DEGRADATION

Three models have been proposed to characterize the molecular mechanisms of mRNA degradation: deadenylation, sequence-specific cleavage of the mRNA independent of poly(A) shortening, and antisense RNA. They are discussed briefly below.

(1) Deadenylation. Several observations have implicated the removal of the poly(A) tail as an early step in the decay of many mRNAs (121). mRNA degradation is initiated by shortening of the 3' poly(A) tail, which leads to decapping (removal of the 5' cap), followed by 5' to 3' degradation of the transcripts. For example, mRNAs lacking poly(A) tails degrade more rapidly than adenylated control mRNAs. In addition, changes in the size distribution of the poly(A) tails of some mRNAs correlate with alterations in their decay rate. Moreover, some sequences that promote rapid mRNA decay cause rapid deadenylation, e.g. sequences in the 3'-UTR of the unstable yeast MFA2 mRNA, sequences within the coding region and an ARE found in the 3'-UTR of c-fos mRNA (121).

More direct evidence that shortening of the poly(A) tail is required for mRNA decay comes from studies following the degradation of "pulses" of newly synthesized transcripts produced by rapid induction and repression of regulatable promoters. These "transcriptional pulse-chase" experiments demonstrate that the unstable mammalian c-fos mRNA and several yeast mRNAs, including both stable and unstable transcripts, do not begin to decay until their poly(A) tails are shortened (121, 131). In addition, chimeric transcripts of stable mRNAs containing either the ARE or the coding region of c-fos mRNA, or containing the 3'-UTR of the MFA2 mRNA, begin to decay earlier than their wild-type counterparts, corresponding to their more rapid deadenylation (121). These observations argue strongly that deadenylation is required for the decay of at least some mRNAs.

Current knowledge of how deadenylation leads to the decay of mRNAs is limited. One possibility is that the poly(A) tail associated with poly(A)-binding proteins (PABPs) protects mRNAs from nonspecific exonucleases. Several observations suggest that deadenylation triggers a nucleolytic event near the 5'-end (121). The ribonuclease involved in PABP-dependent shortening of poly(A) tails from yeast has been purified and cloned (172). This poly(A) ribonuclease is unique among identified eukaryotic RNases in that it requires a protein-RNA complex as a substrate. Furthermore, a mammalian poly(A)-specific nuclease has been partially purified, although its importance in mRNA decay remains to be determined (173).

(2) Sequence-specific cleavage of the mRNA independent of poly(A) shortening. A second mechanism of mRNA turnover is initiated by sequence-specific cleavage within the 3'-UTR, independent of poly(A) tail removal. Evidence for this mechanism comes from analysis of transcripts, such as mammalian 9E3 and IGF-II mRNAs, where mRNA fragments are detected *in vivo* that correspond to the 5'- and 3'-portions of the transcript and that are consistent with internal cleavage within the 3'-UTR (132, 133). After blocking mRNA synthesis, the level of these fragments increases as the level of the full-length transcript decreases, suggesting that these fragments are decay intermediates. In both cases, the 3'-fragments stayed adenylated, indicating that poly(A) tail removal is not required for the endonucleolytic cleavage. Such cleavage sites that are recognized independently of the poly(A) tail have also been identified in the 3'-UTR of *Xenopus Xlhbox2b* mRNA (121, 134).

Sequence-specific cleavage is also critical in the decay of mammalian histone mRNAs. Although not polyadenylated, they possess a characteristic stem-loop structure at their 3'-end. The initial event in histone mRNA decay is removal of a few nucleotides at the 3'-terminus,

which disrupts this stem-loop structure (121, 135). Therefore, all of pathways described above are initiated by a modification of the 3'-end of the transcript (deadenylation, endonucleolytic cleavage within the 3'-UTR, or removal of the histone 3' stem-loop structure), which then triggers later nucleolytic events.

In the deadenylation-dependent pathway, differences in decay rate result from both the rate of poly(A) shortening and the rates of decay of the oligoadenylated transcript. Thus, the decay rate of a mRNA is controlled by the presence of features within the mRNA that affect these two rates. The elements within mRNAs that promote poly(A) shortening (such as the ARE in the 3'-UTR of c-fos mRNA) may recruit different poly(A) nucleases, or modify the rate at which a single poly(A) nuclease functions (121, 131, 136). Differences in decay rates between mRNAs are also due to the presence of sequence-specific endonuclease target sites within some mRNAs and the secondary structures of mRNA (101, 130, 137, 138).

(3) Degradation of mRNA involving antisense RNA. Antisense transcripts introduced into eukaryotic cells have been shown to inhibit gene expression by rapid destabilization of the corresponding endogenous mRNAs (291). However, there is only limited evidence for the participation of endogenous antisense RNAs in eukaryotic gene regulation.

After induction of murine erythroleukemia (MEL) cells to differentiation in response to chemical inducers, oncoprotein p53 is found to undergo down-regulation at a post-transcriptional level involving synthesis of an RNA molecule (127). By probing with various fragments of the p53 gene, Knochbin and Lawrence (127) identified a nuclear RNA molecule of ~1.3 kb, which accumulates when cells are treated by the chemical inducers, while p53-mRNA undergos degradation. It has ~1.2 kb sequence complementary to p53-mRNA and is not a spliced product of p53 pre-mRNA because it can only be detected by a sense probe.

This RNA was found at a low level even in uninduced cells and is accumulated upon induction of cell differentiation. Sequence analysis of the genomic DNA fragment revealed the presence of Pol III consensus boxes (A and B) at 3' end and the antisense orientation. These boxes assemble a potential promoter which may direct the synthesis of the RNA upon cell induction (127).

Down regulation of p53 mRNA under post-transcriptional control has also been described in mouse teratocarcinoma F9 cells upon induction (292) and chick embryonic development (293). In both cases the authors reported a constant rate of p53 gene transcription and the stabilization of p53-mRNA when treated with actinomycin D. Furthermore, the degradation of estrogen receptor mRNA by phorbol esters in human mammary adenocarcinoma (MCF-7) cells is found to be dependent on ongoing RNA synthesis but not on protein synthesis, indicating an RNA molecule being involved in the mRNA degradation (187). Another example of mRNA degradation by antisense RNA comes from the *Dictyostelium discoideum* prespore gene EB4-PSV. In early development and after disaggregation, the 2.2 kb mRNA becomes unstable. In the meantime, a 1.8 kb antisense transcript originating from the same gene locus is detected by a sense strand probe. The inhibition of RNA synthesis during disaggregation prevents the synthesis of the antisense RNA and degradation of the mRNA (290).

An advantage of antisense mediated mRNA degradation is the ability to respond rapidly to environmental changes. A single set of enzymes, including double strand-specific RNase, would provide the means to destabilize all antisense-controlled mRNAs. In contrast, regulation of mRNA stability by specific *trans*-acting factors would be slower in action, and function only on a family of coordinately regulated transcripts. Thus, endogenous antisense

RNA synthesis represents a general, rapid and efficient type of gene regulation.

6.3 CHARACTERIZATION OF RNA BINDING PROTEINS

In order for eukaryotic mRNAs to be regulated at the level of degradation or translational control, the cell must possess a mechanism for their selective recognition. So far, proteins specific for ARE, IRE, and poly(A) tail have been identified. As discussed, ARE in the 3'-UTR represents a common feature of unstable mRNAs. To identify cellular factors that interact with ARE, RNA gel shift and RNA-protein ultraviolet-cross linking assays have been developed (167, 168). A 32 kDa protein was identified predominately in the nuclei of HeLa cells which interacts with AREs from a number of transcripts including GM-CSF, c-myc, and c-fos (168). Another similar factor (36 kDa) which is present in cytoplasmic extracts from Jurkat cells or from human peripheral blood mononuclear cells, interacts with AREs from IL-3, GM-CSF, interferon-γ, and c-fos mRNAs, and forms a major ultraviolet cross-linked complex with RNA that migrates at 44 kDa (167).

Bohjanen *et al.* (169) have identified three RNA-binding proteins from human T lymphocytes that interact with AREs. AU-A is an abundant, constitutively expressed 34-kDa factor that localizes primarily to the nucleus. It binds to AUUUA multimers with low affinity and also binds to other U-rich sequences. AU-B and AU-C are 30- and 43-kDa cytoplasmic factors that are induced following T cell receptor-mediated stimulation and bind to AUUUA multimers with high affinity. Protease cleavage of the RNA-protein complexes indicate that AU-B and AU-C are structurally related to each other but distinct from AU-A (169). These data suggest that AU-B and AU-C RNA-binding factors could be cytoplasmic regulators of lymphokine mRNA metabolism.

More recently, two proteins of 37 and 40 kDa were purified from K562 cells that selectively accelerate degradation of c-myc mRNA in a cell-free system (170). These proteins bind specifically to the c-myc and MG-CSF mRNAs at the 3'-UTR, suggesting that they are in part responsible for selective mRNA degradation. The two proteins are found to be immunologically cross-reactive and both are phosphorylated and complexed with other proteins. Immunologically related proteins are found in both the nucleus and the cytoplasm. The 37-kDa protein was further cloned using antibodies. The cDNA (AUF1) contains an ORF predicted to produce a protein with several features, including two RNA recognition motifs and domains that potentially mediate protein-protein interactions (170). These results provide further support for a role of these proteins in mediating ARE-directed mRNA degradation.

Yu *et al.* (181) purified the IRE-BP and subsequently isolated its cDNA from the rat liver. The protein was predicted to have 889 amino acids and a MW of 98kDa. IRE-BPs from human and rodent share 95% identity. A mRNA of 3.6 kb was expressed in rat liver, spleen, and gut. Injection of iron decreased the RNA binding activity of IRE-BP by 50% but did not change its mRNA level, indicating that IRE-BP functionally responds to iron concentration, through binding to target RNA.

The poly(A)-binding protein (PABP) is 70-kDa, localized exclusively to the cytoplasm (182). PABP occurs at a high concentration in HeLa cells where it displays a low turnover rate. It binds oligo$(rA)_{25}$ in high affinity (Kd of 7 nM) and exists in an approximately 3-fold molar excess of the PABP in relation to available binding sites on cytoplasmic poly(A) (182).

6.4 REGULATION OF mRNA DEGRADATION BY GLUCOCORTICOIDS

Glucocorticoids have been reported to exert both positive and negative effects on

mRNA stability. For example, glucocorticoids enhance the stability of GH (109, 110), fibronectin (111), and PEPCK mRNAs (112) and decrease the stability of mRNAs for insulin (102), surfactant protein-A (SP-A) (117-120), interleukin-1β (113), 3-hydroxy-3-methylglutaryl-coenzyme-A reductase (114), type I procollagen (115), GM-CSF (116), and interferon-β (171). DEX has been reported to induce a dose-dependent decrease in steady state insulin-mRNA levels without changing the rate of insulin gene transcription (102). Inhibition of RNA and protein synthesis by actinomycin D and cycloheximide, respectively, completely abolished the DEX effect, whereas actinomycin D added 9 h after DEX had no effect on DEX-induced insulin-mRNA degradation, indicating that DEX effect on insulin biosynthesis requires transcriptional activation of a gene encoding a protein responsible for the accelerated disappearance of insulin-mRNA (102).

SP-A, the major pulmonary surfactant-associated protein, is a developmentally and hormonally regulated sialoglycoprotein. Glucocorticoids have dose-dependent biphasic effects on the levels of SP-A and its mRNA in human fetal lung *in vitro* (117-120). At concentrations of 10^{-10}~10^{-9} M, DEX increases the levels of SP-A and its mRNA level over those of control tissues, whereas at concentrations $\geq 10^{-8}$ M, the effect is markedly inhibitory. Furthermore, DEX stimulates SP-A gene transcription in human fetal lung and acts synergistically with cAMP to increase the transcriptional activity. On the other hand, DEX ($> 10^{-9}$ M) has a dominant negative effect on SP-A mRNA accumulation and antagonizes the stimulatory effect of cAMP by promoting SP-A mRNA degradation (117-120).

The effects of steroid hormones on mRNA stability are likely to be mediated by their receptor proteins. The best evidence that classical receptors are required for altered mRNA stability comes from a comparison of cells with and without the receptor. RU486, which

antagonize the progesterone receptor, alters the stability of mRNA for fatty acid synthase in MCF-7 cells, which contain progesterone receptor, but not in MDA-MB231 cells, which lack this receptor (139). In addition, the ARE in the 3'-UTR has been reported to mediate the increased turnover of interferon-β mRNA induced by glucocorticoids (171). Human interferon-β mRNA mutants with deletions of ARE in the 3'-UTR displayed no effect on the stability of the mRNA and little effect on its efficiency of translation. However glucocorticoids destabilized mRNA only in cells expressing interferon-β mRNA with ARE in the 3'-UTR, suggesting that glucocorticoids may act by stimulating a ribonuclease which degrades mRNAs containing ARE in the 3'-UTR (171).

At present, there is no evidence that steroid receptors act directly in the cytoplasm to alter mRNA stability. Estrogen, progesterone, and androgen receptors are predominantly nuclear proteins (140). Unliganded glucocorticoid receptor may be localized in the cytoplasm, but translocates to the nucleus upon hormone binding. Most likely receptors participate indirectly in regulated mRNA turnover because they are required for the transcriptional regulation of a turnover factor (122). Consistent with this point, stabilization of PEPCK-CAT chimeric mRNA in rat hepatoma cells occurs only after 8 h of glucocorticoid treatment, and the stabilization of GH mRNA by glucocorticoid in fibroblast cell lines requires 12~24 h (141, 142). On the other hand, more rapid and direct effects of glucocorticoids on mRNA turnover also exist. For example, destabilization of interferon-β mRNA by glucocorticoid occurs within 2 h, perhaps through activation of a pre-existing signalling pathway since it is not blocked by inhibitors of protein synthesis (143).

In summary, in the last several years, mRNA stability has become an important

control point for the expression of many oncoproteins, peptide hormones, and cytokines. Destabilizing sequences located throughout the transcript determine mRNA half-life. mRNA degradation is usually initiated by removal of the 3'-poly(A) track, which then triggers 5'-decapping and 5' to 3' digestion of the transcript. Less commonly, mRNA degradation starts from within the sequence without poly(A) shortening; or is caused by interaction with induced endogenous antisense RNA. mRNA stability is regulated by factors such as glucocorticoids, cAMP, iron, cell cycle- and cell-specific factors, although the precise molecular mechanisms are not well understood.

7. FUNCTIONAL INTERACTIONS OF SOMATOSTATIN AND GLUCOCORTICOIDS

Although glucocorticoids are only produced in adrenal cortex, their receptors are present in almost every cell and they affect fundamental processes throughout the body. Somatostatin is produced in many body tissues and acts on multiple tissue targets. Thus both glucocorticoids and somatostatin influence a broad spectrum of biological activities. In addition, there is evidence that glucocorticoids regulate somatostatin function, and somatostatin, in turn, is able to influence the functions of CRH and ACTH and therefore of glucocorticoid secretion.

7.1 IN VIVO INTERACTIONS OF SOMATOSTATIN AND GLUCOCORTICOIDS

The most important interactions of somatostatin and glucocorticoids are observed in growth control, pancreatic islet function, immune suppression, and stress responses.

(1) Growth and Growth Hormone Secretion. Postnatal growth is controlled by GH and IGFs, thyroid hormone, sex steroids and glucocorticoids, in addition to genetic, socioeconomic and nutritional factors (19). Glucocorticoids in excess are well known to retard growth. Only 2~3 times the normal daily secretion rate of cortisol can attenuate growth (13), e.g. in Cushing syndrome or treatment of children with exogenous glucocorticoids for diseases such as asthma and glomerulonephritis. The effect of glucocorticoids on GH function is rather complicated in that glucocorticoids inhibit GH secretion *in vivo* but stimulate GH biosynthesis and secretion when directly applied to GH producing cells (80, 296). The *in vivo* inhibition is most probably through release of inhibitors of GH secretion, such as somatostatin (19).

The role of hypothalamic somatostatin release in glucocorticoid inhibition of growth and GH secretion was investigated by using passive immunization techniques (105, 106). It was reported that 4-day pretreatment with DEX (40 μg/day) significantly reduced basal and GHRH-stimulated GH secretion in rats. To determine the role of somatostatin in the impaired GH response, another set of animals was injected with somatostatin antiserum 30 min before GHRH. Somatostatin antiserum reversed the responses to GHRH, suggesting that the *in vivo* inhibition of GH secretion by glucocorticoids is at least partially mediated via augmented hypothalamic somatostatin release (105). In a followup study, Wehrenberg *et al.* (106) found that young rats (50 g) treated with daily DEX injection (40 μg/kg) for 33 days grew much slower than control animals. Glucocorticoid-induced growth retardation was partially reversed by concomitant treatment of rats with somatostatin antiserum. Most recently, corticosterone has been reported to directly increase somatostatin content 2~3 fold in fetal rat hypothalamic culture (299). Therefore, the inhibitory effect of glucocorticoids on growth and GH may be madiated, in part, by increased somatostatin secretion (Figure 1-11).

(2) Pancreatic islet function. Glucocorticoids exert profound effects on intermediary metabolism, most of which are counteractive to those of insulin. They enhance hepatic production of glucose, decrease glucose uptake and increase amino acid release from muscle, and increase lipolysis and release of glycerol and free fatty acids from adipose tissue. During fasting, glucocorticoids contribute to the maintenance of plasma glucose levels by increasing hepatic gluconeogenesis, glycogen deposition, and peripheral release of substrates (amino acids, free fatty acids, etc.) (19). Furthermore, glucocorticoids inhibit insulin secretion and biosynthesis from both normal rat islets and HIT cells (315). Philippe *et al.* (102) reported

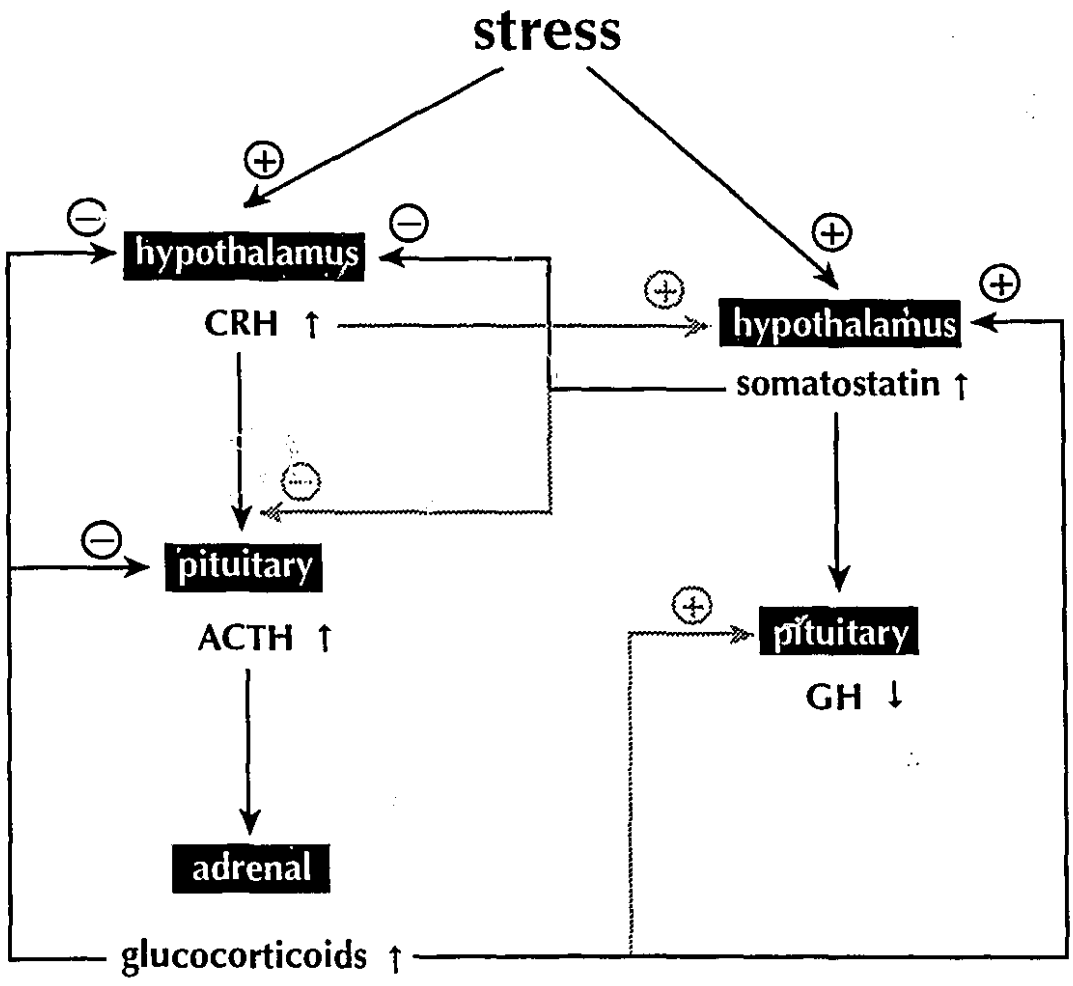


Figure 1-11. Proposed interactions of somatostatin and the hypothalamic-pituitary-adrenal axis in response to stress and in control of growth and GH secretion.

a dose-dependent decrease in steady state insulin-mRNA levels in HIT cells treated with DEX. This effect is due to accelerated mRNA degradation rather than suppression of insulin gene transcription. Somatostatin biosynthesis and secretion are augmented in the gut during fasting (316), and somatostatin inhibits insulin biosynthesis and secretion, thereby, functionally collaborating with glucocorticoids in maintaining a normal metabolic status. In addition, corticosterone has been reported to augment somatostatin content and secretion as well as D-cell number in cultured rat islet cells (107). Therefore, both glucocorticoids and somatostatin inhibit insulin secretion. The glucocorticoid-induced somatostatin production from the islets may constitute an extension of their anti-insulin actions.

(3) Immune Suppression. A prominent effect of glucocorticoids is suppression of the immune response. Recent studies indicate that somatostatin is produced in lymphoid organs (spleen and thymus) and mononuclear leukocytes, mast cells, and polymorphonuclear leukocytes and inhibits the immue response (12-14).

Somatostatin peptide and mRNA are found in the spleen and thymus of rats, and in the spleen, thymus, and bursa of Fabricius in the chicken. Its localization in the bursa indicates that the peptide is synthesized in B lymphocytes since this is the site of origin of B lymphocytes in birds (14). The presence of somatostatin in rat spleen is confined in some B cells and other cell types, but not in T cells. In the rat thymus, somatostatin is present in a small population of T lymphocytes in the medulla, as revealed by the Thy-1.1 marker (14). Fuller and Verity (12) also demonstrated the expression of SS-mRNA in rat thymus. Specific receptors for somatostatin are expressed on quiescent as well as activated monocytes, lymphocytes, lymphocytic leukaemia cells and lymphoid cell lines (209, 210, 215).

Somatostatin produced in immue cells may exert paracrine or autocrine functions. In addition, somatostatin is found in C-fibres and the small myelinated A-δ fibres of peripheral nervous system (215), which may release somatostatin into the immune system. This might represent a direct regulatory interaction between the nervous and the immune systems.

Somatostatin may modulate the immune response by a variety of mechanisms. The peptide exerts both stimulatory and inhibitory effects on the proliferation of lymphocytes (type T and B) and leukocytes (basophils) (185, 210-213); suppresses immunoglobulin synthesis by plasma cells (210); inhibits the release of colony-stimulating factor from activated lymphocytes (214); suppresses tumoricidal activity of macrophages (215); and enhances leukocyte-migration-inhibiting factor formation in activated lymphocytes (210).

Glucocorticoids suppress multiple aspects of immunologic and inflammatory responsiveness. They cause a species- and cell type-specific lysis of lymphocytes and impair release of effector substances such as the lymphokine IL-1, antigen processing, antibody production and clearance, and other lymphocyte functions (19, 68). They suppress the inflammatory response by 1) decreasing the number of circulating leukocytes and the migration of tissue leukocytes; 2) inhibiting fibroblast proliferation; and 3) inducing lipocortins which, by inhibiting phospholipase A_2, blunt the production of the potent anti-inflammatory molecules, prostaglandins and leukotrienes (68).

The immune system, in turn, affects the hypothalamic-pituitary-adrenal (HPA) axis. The inflammatory cytokines TNF-α, IL-1β, and IL-6 activate the axis primarily by causing hypothalamic CRH secretion, which leads to glucocorticoid secretion mediated by ACTH release (201-205). Glucocorticoid secretion then limits the inflammatory reaction by preventing leukocytes from reaching the inflammatory site and by decreasing the secretion and

action of various cytokines and lipid mediators of inflammation (205, 206). Although IL-1β, and probably TNF and IL-6 as well, stimulate somatostatin production from cultured neurons (41, 52, 53), it is not known whether somatostatin participates in such a physiologic process as an anti-inflammatory peptide. It would be of interest to examine the local secretion of somatostatin during glucocorticoid-induced suppression of the inflammatory response.

Recently, the anti-inflammatory effect of somatostatin has been documented (205). In an experimental model of inflammation induced by injection of carrageenin into subcutaneous air pouches in rats, the volume, the leukocyte concentration and the level of local pro-inflammatory mediators (TNF-α, substance P and CRH) in the inflammatory exudate were significantly reduced by systematic or local injection of somatostatin analogues (BIM 23014 and sandostatin) in a time- and dose-dependent manner (205). Furthermore, endogenous somatostatin was shown to participate in DEX-induced anti-inflammation effect. DEX inhibited the production of all pro-inflammatory substances, by stimulating the endogenous production of somatostatin locally (207). These are exciting observations which provide a physiologic basis for a functional interaction between glucocorticoids and somatostatin whereby glucocorticoids inhibit the inflammatory reaction secondarily through activation of somatostatin which in turn inhibits the production of pro-inflammatory peptides.

(4) Stress. Acute physical or psychological stress stimulates several adaptive hormonal responses, including the secretion of glucocorticoids from the adrenal cortex and catecholamines from the adrenal medulla (285). Stress, acting via poorly defined central pathways, activates the HPA axis by stimulating the release of hypothalamic CRH and AVP which in turn enhance pituitary ACTH secretion. ACTH stimulates glucocorticoids which

exert negative feedback control on the pituitary and hypothalamus. In the anterior pituitary, glucocorticoids inhibit both ACTH secretion and POMC gene transcription (178). To a lesser extent, glucocorticoids also decrease CRH and AVP mRNA and peptide levels in the hypothalamic paraventricular nuclei. In addition, glucocorticoids block the stimulatory effects of CRH on POMC gene transcription and acute ACTH release (178, 328) (Figure 1-11).

Stress also activates somatostatin function. Aguila *et al* (280) detected a 2-fold increase in somatostatin release from hypothalamus, sustained for 20 min, following a 2 min ether stress in rats. In the anestrous ewe, prolonged electric foot shock augmented hypothalamic somatostatin release (281). In addition, Engelhardt and Schwille (286) reported that plasma somatostatin concentration is increased 50~90% in response to stress in rats.

Stress-induced somatostatin release suppresses ACTH secretion, possibly by inhibiting CRH. Thus, SS-28 (but not SS-14) given icv inhibits stress-induced pituitary ACTH release. ICV infusion of the somatostatin analogue (octreotide) partially inhibits hemorrhage-induced ACTH release without affecting ACTH secretion in control euvolemic animals (282). The elevation of ACTH after tail-suspension stress is totally prevented by iv administration of antisera against CRH. Since SS-28 does not inhibit CRH-induced ACTH secretion from the pituitary *in vivo* or *in vitro*, these results are consistent with the hypothesis that SS-28 prevents stress-induced ACTH secretion, not by a direct action on pituitary corticotrophs but by inhibition of hypothalamic CRH release (287).

Stress-induced somatostatin release also suppresses GH secretion, in collaboration with CRH or glucocorticoids. The first report of a role of somatostatin on stress-induced GH secretion came from Terry *et al.* (301), who found that antiserum to somatostatin administrated iv to rats subjected to swim stress restored GH secretory pulses which were

suppressed in non immune serum controls, indicating that circulating somatostatin plays a prominent role in stress-induced inhibition of GH secretion. It was reported that after ether exposure, mean plasma GH level was significantly reduced (280). While exposure to electric-shocks markedly lowered plasma GH levels, central administration of the CRH antagonist alpha-Hel CRH-(9-41) totally abolished this effect of stress (288). Furthermore, centrally administrated CRH can mimic stress in reducing GH secretion. The effect of CRH can be blocked by somatostatin antiserum, indicating that the CRH effect is mediated through somatostatin release (288, 289). Such observations support the hypothesis that in the rat, endogenous CRH mediates the inhibitory action of noxious stimuli on GH secretion and further suggest that this effect may involve an increased release of endogenous somatostatin (288). In addition, CRH may inhibit GH secretion indirectly at another level through activation of ACTH and glucocorticoids, since glucocorticoid is a well known inhibitor of *in vivo* GH secretion.

As illustrated in Figure 1-11, stress-induced CRH release (1) activates the HPA axis leading to increased secretion of glucocorticoids, which in turn enhance hypothalamic somatostatin secretion; (2) augments somatostatin release directly from hypothalamus. The somatostatin released in response to CRH and glucocorticoids inhibits GH secretion. Furthermore, somatostatin seems to be an inhibitory factor on stress-activated HPA axis in that it acts at the levels of hypothalamus (CRH) and pituitary (ACTH) to confine the extent of stress reactions.

7.2 REGULATION OF GLUCOCORTICOID SECRETION BY SOMATOSTATIN

As discussed earlier, somatostatin inhibits glucocoticoid production through actions at the levels of CRH and possibly ACTH secretion. Somatostatin suppresses CRH secretion and CRH-stimulated ACTH secretion in pituitary tumor cells and may mediate glucocorticoid-induced ACTH inhibition (216-219). Ferrara *et al.* (152) reported that somatostatin (in the hippocampus) mediates DEX-induced feedback suppression of HPA system in the rat. Systemic administration of cysteamine (a depletor of somatostatin storage), or icv administration of somatostatin antibody, or microinjection into two discrete areas of the hippocampus under sterotatic control, completely blocked DEX-induced inhibition of corticosterone secretion. Although high affinity binding sites for somatostatin have been detected in the rat adrenal cortex (220) and somatostatin inhibits the growth of *zona glomerulosa* of the rat adrenals and angiotensin II-stimulated aldosterone production *in vitro* (13, 221), there has been no report on direct somatostatin action on glucocorticoid production from the adrenal cortex.

7.3 REGULATION OF SOMATOSTATIN FUNCTION BY GLUCOCORTICOIDS

Over the years, sporadic reports have suggested that glucocorticoids are capable of influencing somatostatin function, although there are conflicting reports. Rodriguez *et al.* (38) reported that adrenolectomy in rats led to a significant decrease in the number of somatostatin receptors in hippocampus, striatum and hypothalamus, but not in cerebral cortex, without changing the somatostatin content in these regions. Such a decrease was completely reversed by glucocorticoid replacement. Schonbrunn *et al.* (124) found that excessive glucocorticoid reduced the number of somatostatin receptors in pituitary cells in culture. Adrenalectomy has

been reported to lead to increased pancreatic and circulating somatostatin concentrations in dogs (125).

Direct effects of glucocorticoids on somatostatin peptide synthesis, secretion, and mRNA accumulation have been reported in several systems. In cultures of embryonic quail sympathetic ganglia, corticosterone increased somatostatin 3 fold after 4 day treatment (317). Corticosterone augmented somatostatin content and secretion as well as D-cell number in cultured rat islet cells (107). Corticosterone has been reported to directly increase somatostatin content 2~3 fold in fetal rat hypothalamic culture (299). In thyroid C-cell line 44-2C, which produces calcitonin, calcitonin gene-related peptide (CGRP), neurotensin and somatostatin, Zeytin and Delellis (79) found that DEX increased both somatostatin secretion and cellular somatostatin content. Pharmacological doses of DEX (165 μg/day, 3 days) also increased hypothalamic somatostatin content in the rat (80). It has been reported that 10-day DEX administration reduced rat hypothalamic SS-mRNA 50% (235) while 3-day treatment increased hypothalamic SS-mRNA level in female rats (318). Fuller and Verity (12) demonstrated that levels of thymic SS-mRNA exhibited a bell-shaped stimulatory response to DEX administrating to drinking water in adrenalectomized rats. After 6 days of DEX treatment SS-mRNA level increased 5-fold. But higher doses of DEX decreased somatostatin gene expression. Cote *et al.* (64) have studied glucocorticoid effect on somatostatin production in the human thyroid carcinoma TT cell line. DEX inhibited both somatostatin production and SS-mRNA accumulation in a dose-dependent manner. TT cells treated with DEX showed an almost complete inhibition of peptide production by 48 h. Molecular sizing chromatography displayed a decrease in both the probable somatostatin precursor (13 kDa) and the fully processed peptide. The hybridizable SS-mRNA decreased to ~50% of basal levels within 12

h of treatment.

Therefore, glucocorticoid effects on somatostatin secretion or biosynthesis involves both inhibition (hypothalamus, TT cells) and stimulation (hypothalamus, pancreatic islets, thymus, thyroid tumor cells). While the differences in the reported effects of glucocorticoids can be explained by the use of widely different experimental paradigms involving different normal tissues as well as tumor cells, *in vivo* and *in vitro* models, and different concentrations of glucocorticoids, the precise nature of the effect of glucocorticoids on somatostatin function remains unclear. Accordingly, I have directed my efforts in this thesis towards a systematic investigation of the effects of glucocorticoids on somatostatin gene expression in normal tissues and a characterization of the molecular mechanism of glucocorticoid action at transcriptional and post-transcriptional levels using *in vitro* cell models.

8. AIMS OF STUDY

Glucocorticoids have been reported to influence somatostatin peptide and mRNA levels, involving both stimulation and inhibition with sometimes conflicting results, but the precise nature of this effect and its molecular mechanism are unclear. For the PhD project, I have proposed to systemically examine the effects of glucocorticoids on somatostatin gene expression in various systems (Part A), to determine direct transcriptional effect of glucocorticoids on somatostatin promoter (Part B), and to characterize the role of mRNA stability in glucocorticoid-regulated SS-mRNA levels (Part C).

Part A. To start my PhD project, I have used as models DEX treated rats *in vivo*, primary cultures of rat islet and cerebrocortical cells, and a somatostatin-producing rat islet tumor cell line (1027 B_2), in order to answer the following questions:

Do glucocorticoids influence somatostatin gene expression ?

Is the glucocorticoid effect tissue specific ?

Is the glucocorticoid effect dose dependent ?

Does it differ between normal and neoplastic cells ?

Does it result from a direct action of glucocorticoids on somatostatin cells ?

Part B. In order to determine possible direct action of glucocorticoids on somatostatin gene transcription, I proposed

(1) to examine direct transcriptional control of the somatostatin gene by glucocorticoid using promoter-CAT reporter gene transcription assay;

(2) to compare the effect of glucocorticoids with that of other steroid and thyroid hormones;

(3) to map the 5' upstream regulatory region of the somatostatin gene involved in glucocorticoid transactivation;

(4) to investigate transcriptional interaction between glucocorticoids and the cAMP signalling pathway;

(5) to determine the roles of CRE/CREB and PKA in glucocorticoid transactivation;

(6) to characterize interaction of glucocorticoid receptor with somatostatin promoter by gel shift assay.

Part C. In order to investigate the molecular mechanisms underlying DEX inhibition of SS-mRNA level, I used human thyroid carcinoma TT cells in which DEX has been reported to produce solely an inhibition on somatostatin secretion and SS-mRNA accumulation and proposed

(1) to characterize DEX inhibition on SS-mRNA level and somatostatin secretion by dose-dependence and time course studies;

(2) to determine direct transcriptional effect of DEX on somatostatin gene by nuclear run on transcription assay and reporter gene (CAT) transfection; and

(3) to determine the effects of transcription blockade (by actinomycin D) and translational blockade (by cycloheximide) on DEX effect on SS-mRNA level.

CHAPTER II. MATERIALS AND METHODS

MATERIALS

Dexamethasone sodium phosphate solution (Decadron, Sabex, Quebec) was diluted in water and used for *in vivo* administration. For *in vitro* experiments, dexamethasone was dissolved initially in ethanol and subsequently diluted in water. Dexamethasone, 8-(4-chlorophenylthio)-cAMP, dibutyryl cAMP, actinomycin D, cycloheximide, phorbol 12, 13 dibutyrate (PDB), forskolin, β-estradiol, testosterone, 3,3',5-triiodo-L-thyronine (T_3), and retinoic acid were from Sigma Chemical Co. (St. Louis, MO). 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was from Calbiochem (La Jolla, CA). The stock solutions of actinomycin D and DRB were made in methanol, and cycloheximide in water. Synthetic SS-14 was a gift from Ayerst Laboratories (Montreal, Quebec, Canada). Tyr-SS-14 was purchased from Bachem (Torrance, CA).

Medium 199, Dulbecco's modified Eagle medium (DMEM), phenol red-free DMEM, RPMI medium 1640 (with glutamine, without phenol red), heat inactivated fetal bovine serum (FBS) and horse serum (HS), L-glutamine, and other tissue culture reagents were purchased from GIBCO. Hormone-free serum was prepared by incubating 100 ml serum with 5 ml 0.5% charcoal (Norit A) and 0.05% dextran-T70 in 0.14 M NaCl for 30 min under constant stirring; removing the charcoal by centrifugation; and heating the serum at 56 C for 30 min; followed by filtering sterilization, as reported (183).

[^{32}P]-UTP, [α-^{32}P]-deoxy-CTP, [γ-^{32}P]-deoxy-ATP, D-threo-[dichloroacetyl-1-^{14}C] chloramphenicol, [1,2,4,6,7-^{3}H]-dexamethasone, and [Na^{125}I] were purchased from Amersham Corporation, Arlington Heights, IL.

Restriction endonucleases and other nucleic acid modifying enzymes were from BRL, Bethesda, MD. Oligonucleotides of CRE and GRE consensus were from Promega. Mermaid and GeneClean oligo DNA purification kits were from BIO101. PrimeErase kit was from Stratagene, La Jolla, CA. Oligonucleotide primers for PCR reaction were prepared by Shelton Biotechnology Center, McGill University, Montreal.

Purified bacterially expressed glucocorticoid receptor-DNA binding domain (GR-$DBD_{440-525}$) was from Dr. L.P. Freedman (New York, USA) (189). Prepro-somatostatin cDNA was provided by Dr. R. Goodman, cyclophilin cDNA by Dr. S. Rabbani, and MT-CREB expression vector by Dr. D. Drucker. Plasmids pCAT-Basic was from Promega, pBluescript from Stratagene, and pCH110 (SV40-lacZ) from Pharmacia. All other reagents were of analytical grade and obtained from various commercial sources.

IN VIVO EXPERIMENTS

Groups of 10 male Sprague-Dawley rats (Charles River) weighing ~180 g were injected with saline or DEX (0.5 mg/kg i.p.) for 1, 3 or 8 days and studied immediately thereafter. Another group of 10 rats was injected similarly for 8 days, allowed to recover for 14 days and studied on day 22. Rats were caged in a light controlled room (lights 6 a.m. to 6 p.m.) at 22-25 C with *ad libitum* access to food (pure Purina Chow) and water. Injections were administered at 9:00 a.m. daily. On the day of study, fed rats were weighed and killed by decapitation. The hypothalamus, cerebral cortex, stomach, pancreas, and a 12 cm segment of jejunum were removed, frozen in liquid nitrogen and stored at -80 C pending extraction for RNA and immunoreactive somatostatin (SSLI) measurements.

CELL CULTURE AND IN VITRO EXPERIMENTS

Islet Cell Cultures: Cultures of dissociated pancreatic islet cells from splenic segments of pancreata of 3-4 day old Wistar rats (Charles River) were established in 35 mm diameter plastic Petri dishes as previously described (62, 194), and used from day 5~7 after plating. These cultures contain predominantly B- and D-cells, less numbers of A-cells, and virtually no pancreatic polypeptide cells. Regulation of somatostatin secretion from neonatal islet cells has been previously shown to be comparable to that in adult islets (193, 194). The culture medium consisted of medium 199, supplemented with 10% heat inactivated FBS, 16.7 mM glucose, penicillin (100 U/ml), streptomycin (100 µg/ml) and fungizone (0.25 µg/ml). Medium glucose and serum concentration were reduced to 5.5 mM and 1% respectively, 24 h before the start of experiments. Test incubations were carried out by exposing groups of 5-10 culture dishes for 18 h with control medium or medium containing DEX (10^{-10}-10^{-5} M).

1027 B_2 Islet Tumor Cells: The somatostatin-producing rat islet cell line $1027B_2$, derived from a radiation-induced transplantable islet tumor RIN-r, was obtained from Dr. W. Chick (Worcester, MA) and cultured as previously described (62). 0.5 x 10^6 Cells were plated in 35 mm diameter plastic Petri dishes in medium 199 - 5% FBS - 16.7 mM glucose and grew as a monolayer. They were used on day 4 after plating when they had grown to ~1.5 x 10^6 cells/dish. For test experiments, groups of 8-10 culture dishes were exposed for 18 h to control medium (medium 199 - 5.5 mM glucose - 1% FBS) or medium containing DEX (10^{-10}-10^{-5} M).

At the end of incubations of normal islet cells or $1027B_2$ tumor cells, media were decanted, centrifuged (1,000 rpm x 2 min) to remove floating cells, and the supernatant acidified to pH 4.8 with 1 M acetic acid and assayed for SSLI. Released SSLI was stable in

the incubation medium for up to 48 h. Cells were extracted in guanidine thiocyanate (GTC) solution, 100 µl aliquot removed for SSLI measurements and the remaining extracts pooled for RNA analysis.

Thyroid Carcinoma (TT) Cells: The somatostatin-producing TT cell, a line of transformed parafollicular (C) cell, derived from a human thyroid carcinoma, was obtained from American Type Culture Collection (Rockville, MD). It produces calcitonin and CGRP as well. For Northern blots and radioimmunoassay (RIA) analysis, cells (1×10^6) were plated in 35-mm diameter plastic Petri dishes in RPMI medium 1640 supplemented with 15% FBS and grew as a monolayer. Triplicate dishes of TT cells were used on day 4 after plating. 24 H prior to DEX incubation, fresh medium containing only 5% FBS rendered hormone free by treatment with dextran-coated charcoal was used. For CAT transfection experiments, the cells (2×10^6) were plated in 100-mm diameter Petri dishes and used at day 3. For nuclear run-on assay, confluent TT cells from multiple flasks were pooled and used for nuclei preparation.

Cerebrocortical Cell Cultures: Primary cultures of dispersed fetal rat cerebrocortical neurons were prepared as previously described (194). Embryos were obtained from pregnant SD rats (Charles River) 17-18 days post conception. The cerebral hemispheres were removed and slivers of cortical tissue (~1 mm deep) were obtained by dissection under an operating microscope. Tissue fragments were dissociated by trituration into a homogeneous cell suspension which was plated at a density of ~5×10^5 cells/plate in 35 mm diameter Petri dishes. The culture medium for the first 6 days consisted of DMEM supplemented with 10% heat inactivated HS, 10% heat inactivated FBS, glucose (21 mM), bicarbonate (38 mM), and insulin (8 units/100 ml). Cultures were left undisturbed for 6 days following which the

medium was changed at 48 h intervals. The feeding medium consisted of DMEM supplemented with 15% HS, 5% FBS, 21 mM glucose, 38 mM bicarbonate, and insulin (8 units/100 ml). Cultures were viewed through a Zeiss 1 M inverted microscope. Under these conditions cells grew as a mixed population of neurons and astrocytic glia. Experiments were conducted on day 15 of culture. Groups of 5 culture dishes were incubated for 18 h with control medium (DMEM, 1% FBS, 21 mM glucose, 38 mM bicarbonate, insulin 8 units/100 ml) with or without DEX (10^{-11}-10^{-6} M) and dbcAMP (5 mM). In a time-course study, cortical cultures were incubated with DEX and cAMP for 1, 2, 4, 8, or 18 h.

DETERMINATION OF CYTOSOLIC DEX BINDING SITES

In order to confirm the presence of glucocorticoid receptor in the tumor cells used in these studies, cytosolic DEX binding was determined as reported with minor modifications (195, 196). Briefly, cells were homogenized in ice-cold 10 mM Tris-HCl, 300 mM potassium chloride, 1.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 20 mM sodium molybdate, pH 7.4 (at 23 C). Following centrifugation for 1 h at 105,000 X g at 4 C, 200 µl of the supernatant was incubated on ice with 4 nM [1,2,4,6,7-^{3}H]-DEX for 18 h in presence or absence of 800 nM cold DEX (total volume: 240 µl). Free DEX was precipitated by incubating for 15 min with 40 µl of 2.5% charcoal and 0.25% dextran T-70 followed by 5 min centrifugation at 10,000 rpm. The radioactivity in supernatant was counted as bound DEX. The protein assay was described later in the section of CAT assay. Results were shown in the table (n = 3).

Cells	Specific binding capacity for [^{3}H]-DEX (fmol/mg protein)
1027B$_2$	46 ± 12
PC12	24 ± 7
A126-1B2	37 ± 10

MEASUREMENT OF SSLI, INSULIN, AND GLUCAGON BY RADIOIMMUNOASSAY

SSLI was measured in medium and cell extracts by RIA using antibody R149 (directed against the central segment of SS-14), [^{125}I] Tyr-SS-14 radioligand, and SS-14 standards as previously described (8). GTC extracts of tissues or cultured cells were diluted prior to analysis to eliminate nonspecific interference of GTC in the RIA. Insulin concentrations in the culture media were determined by RIA using the Wright guinea pig antibody, [^{125}I] pork insulin, and rat insulin standards (62, 194). Glucagon was measured in the secretion media by RIA using a sheep antiglucagon antibody (Donald), [^{125}I] pork glucagon, and pork glucagon standards (62, 194).

RNA EXTRACTION AND NORTHERN BLOT ANALYSIS

RNA extraction method A: used for Chapter III of the study. Rat tissues or cell cultures were homogenized in 4 M GTC, 25 mM sodium citrate (pH 7.0), 100 mM β-mercaptoethanol, 1 mM EDTA, 0.5% sodium laurosyl sarcosine with a polytron homogenizer (Brinkman, Switzerland) or a hand held homogenizer (199). Following centrifugation at 9000 X g at 4 C for 10 min, a 100 µl aliquot of the supernatant was kept for measurement of SSLI. Total RNA was isolated from the remaining supernatant by ultracentrifugation over a 2 ml cushion of 5.7 M CsCl, 25 mM NaAc (pH 5.0) at 42,000 rpm for 18 h in a fixed-angle rotor

(70.1 Ti, Beckman, Fullerton, CA). The pellet was collected in 1 ml mixture of 0.25 M NaAc (pH 6.5)-ethanol (1:2.5 v/v), and the RNA was allowed to precipitate at -80 C for 1 h. After removing the ethanol, the precipitate was dissolved in diethyl pyrocarbonate (DEPC)-treated water, and RNA concentration was determined by absorbance at 260 nm (accepted ratio of absorbance at 260/280 nm 1.8-2.2).

RNA extraction method B: used in Chapter V of the study. RNA extraction was simplified with major modifications from method A. Total RNA was isolated from cultured cells by acid GTC-phenol-chloroform extraction (197). Cell cultures in 30 mm-diameter dishes were scraped into 1 ml solution D [4 M GTC, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and added in fresh 0.1 M β-mercaptoethanol (0.7%, v/v)]. Total RNA was extracted in 15-ml conical tubes by adding 100 µl 2 M NaAc (pH 4.0), 1 ml treated phenol followed by shaking, and 200 µl Chloroform-iso-amyl alcohol (24:1, v/v) followed by vigorously mixing for 10 seconds. The mixture was placed on ice for 15 min and then centrifuged in 15-ml Corex tubes at 10,000 X g for 20 min, at 8 C. The aqueous phase was mixed with equal volume of isopropanol and precipitated at -20 C, for ≥ 1 h. The first RNA pellet was obtained by centrifuging at 20,000 X g for 20 min at 8 C, and was resuspended in 0.7 ml solution D, and pelleted again with 0.7 ml isopropanol in 1.5-ml Eppendorf tubes. The second RNA pellet was achieved by centrifuging at 13,000 rpm for 10 min in a microcentrifuge. The pellet was washed once with 70% ethanol, dried briefly under vacuum, and resuspended into DEPC-treated water. The RNA concentration was determined by absorbance at 260 mm.

Northern blot analysis: Equal amount of total RNA (5-20 µg) was electrophoresed on 1.5% agarose formaldehyde gel, transferred to Nytran membranes (Schleicher-Schuell, Keene, NH) by a VaccumeGene system (Pharmacia). The hybridization probe consisted of [^{32}P]-

cRNA transcribed by T7 RNA polymerase from a 0.45 kb prepro-SS-cDNA in pGEM (190, 199). The RNA blots were hybridized for 18 h at 60 C in 5 X SSPE, 1% SDS, 2.5 X Denharts', 0.1 mg/ml ssDNA, and 50% formamide. The blots were washed at 60 C for three times of 20 min in 1 X SSPE and 0.1% SDS, once of 30 min in 0.1 X SSPE and 0.1% SDS. Autoradiograms were prepared by exposing the membranes to Kodak XAR-5 film at -70 C for 1-4 days with an intensifying screen. Autoradiographic signals were analyzed with a Quick Scan R&D densitometer (Helena Laboratories, Beaumont, TX) using the Hoefer graphics computer program (Hoefer Scientific, USA). SS-mRNA levels were expressed in arbitrary (densitometric) units. To minimize variations between blots, analysis was carried out on multiple autoradiograms obtained from multiple electrophoretical gel runs of every sample. As an internal control to confirm equal recovery of mRNA in gel lanes and to validate the specificity of SS-mRNA changes, membranes were stripped (by boiling in 0.02 X SSPE, 0.02% SDS for 15 min) and rehybridized with an [α-^{32}P]deoxy-CTP-labeled 0.8-kb rat cyclophilin cDNA probe (238). The blot was hybridized at 42 C for 18 h and washed in 1 X SSPE and 0.1% SDS for 10 min at room temperature, twice of 20 min at 55 C, and another 10 min at room temperature. Changes in the levels of SS-mRNA was compared with mRNA for cyclophilin (a ubiquitous and abundant eukaryotic protein) used as a negative control.

CELL CULTURE AND GENE TRANSFECTION

PC12 rat pheochromocytoma (200) and **A126-1B2** mutant PC12 cells deficient in PKA activity (222) were obtained from Dr. J.A. Wagner, Dana Farber Cancer Institute, Boston, MA. Both cell lines were cultured in DMEM with 10% FBS, 5% HS, and 27.8 mM glucose. In all experiments involving culture of cells in steroid hormones, transfected cells

were incubated in medium that was phenol red-free, supplemented with FBS and HS rendered hormone-free by treatment with dextran-coated charcoal.

For transient transfection experiments, 2.5 x 10^6 cells were plated in 10 cm diameter plastic Petri dishes. After 48 h of culture at ~70% confluency, they were transfected by calcium phosphate precipitation followed by glycerol shock (223). 20-40 µg of plasmid DNA was mixed in 125 mM $CaCl_2$, 25 mM HEPES-buffered saline, pH 7.05 at room temperature for 30 min and subsequently incubated with the cells for 4-6 h. The medium was then discarded and cells were exposed to 15% glycerol for 3 min, washed with phosphate buffered saline (PBS), and reincubated with 10 ml fresh culture medium. Test agents were added 24 h after transfection and the cells harvested by scraping 48 h later. $1027B_2$ rat islet tumor and TT human thyroid carcinoma cells were transfected under the same conditions.

CHLORAMPHENICOL ACETYL TRANSFERASE (CAT) ASSAY

Cells were washed twice with PBS, harvested in 1 ml of 40 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA (NET solution), and collected by low speed centrifugation. Cell pellet was resuspended in 150 µl of 250 mM Tris-HCl buffer (pH 7.8), and cell extract was prepared by 3 cycles of freezing and thawing in a dry ice-ethanol bath and were cleared by centrifugation at 10,000 x g at 4° C for 10 min. Aliquot of the supernatant were assayed for protein concentration by the method of Bradford with bovine serum albumin (BSA) as standard (224). For standard curve, 0, 1, 2, 3, 4, 5, 7, 10, and 15 µg of BSA in 100 µl water was mixed with 1:5 diluted Bio-Rad protein dye (Bio-Rad Laboratories, Richmond, CA). After 5 min (but no more than 1 h) incubation at room temperature, the optical density was read at 595 nm. The relationship of OD_{595} value and protein concentration

(μg/tube) was analyzed by linear regression with the following equation:

protein concentration (μg/tube) = a + b x [OD_{595nm}]

Cell extract samples (2~5 μl) were read in duplicates under the same condition and their protein concentration was calculated using the above equation. CAT assays were performed at 37° C in a total volume of 150 μl with 0.25 M Tris-HCl (pH 7.8), 0.53 mM acetyl coenzyme-A (Pharmacia), and 0.83 μCi/ml [^{14}C] chloramphenicol (223), prepared as follows:

cell extract	100 μg protein
0.25 M Tris-HCl buffer (pH 7.8)	volume to 125 μl
4 mM acetyl coenzyme A	20 μl
[^{14}C]-chloramphenicol	5 μl
Incubate at 37 C for 2 h,	
4 mM acetyl coenzyme A	10 μl
Incubate at 37 C for 1.5 h.	

At the end of the incubation, chloramphenicol is extracted by 1 ml ethyl acetate, which is evaporated under vacuum. The final samples are resuspended in 30 μl of ethyl acetate and loaded on a thin layer chromatography (TLC) plate. Run the TLC plate above a mixture of 190 ml chloroform and 10 ml methanol for 6 min (~6 cm) in a TLC tank, dry the plate in the air, and expose it to a X-ray film for 1~2 days at -70 C.

Under these conditions, the CAT activity from the assay was linear up to 8 h and 300 μg of protein. CAT activity was quantified by liquid scintillation counting of excised bands containing either the acetylated or nonacetylated forms of [^{14}C] chloramphenicol

and expressed as a percent of [^{14}C] chloramphenicol converted to the acetylated forms. All experiments were repeated at least three times.

β-*GALACTOSIDASE ASSAY*

As an internal control for monitoring transfection efficiency, the plasmid pCH110 (10~20 μg, Pharmacia) was routinely cotransfected with various CAT constructs (225). It expresses constitutively lacZ (β-galactosidase, β-Gal) gene driven by SV40 early promoter. Aliquots of cell extract prepared for CAT assay are also used for β-Gal assay. The following reactions were set up:

(μl of solutions)	Negrative	Positive	Sample
100 X Mg solution	3	3	3
1 X ONPG	66	66	66
extract from mock-transfected cells	30	30	0
cell extract	0	0	30
β-Gal (50 U/ml)	0	1	0
0.1 M sodium phosphate (pH 7.5)	201	200	201

Incubate the mixture at 37 C for 1 h (or until a faint yellow color has developed), stop the reactions by adding 0.5 ml 1 M sodium carbonate, and read the optical density of the reactions at a wavelength of 420 nm. 100 X Mg solution: 0.1 M $MgCl_2$, 4.5 M β-mercaptoethanol. 1 X ONPG: 4 mg/ml of o-nitrophenyl-β-D-galactopyranoside in 0.1 M sodium phosphate (pH 7.5).

The result (mean, n = 3) of a test assay was as follows. From that, 10~20 μg of

pCH110 was chosen as the usual amount of cotransfection with somatostatin-CAT constructs.

(μg) pCH110 transfected	$OD_{420\ nm}$	OD/mg protein
0	0.010	0.14
5	0.021	0.29
10	0.066	1.00
20	0.141	1.86
40	0.037	0.52
negative	0	--
positive	1.941	--

Treatment with test agents did not produce any significant variation in β-Gal activity in any experiment. The result of a representative experiment was shown as follows (n = 3). PC12 cells were cotransfected with 30 μg of pSS-750CAT and 10 μg of pCH110, incubated with test agents for 48 h. Changes in CAT activity were presented in Chapter IV (Table 4-1), while no significant change was observed in β-Gal activity due to the treatments.

	β-Gal (OD/mg protein)	Relative CAT activity
control	0.72 ± 0.04	1.00 ± 0.13
DEX (1 μM)	0.74 ± 0.07	2.12 ± 0.21
forskolin (10 μM)	0.79 ± 0.06	4.89 ± 0.51
forskolin + DEX	0.67 ± 0.05	7.48 ± 0.70

PLASMID CONSTRUCTIONS

The metallothionein-CREB (MT-CREB) expression vector was obtained from Dr. D. Drucker (Toronto, Canada) and has been previously described (226). CREB expression was induced by exposure of MT-CREB transfected cells to $ZnSO_4$ (90 μM). Chimeric gene constructs pSS-750 CAT and pSS-71 CAT (containing somatostatin gene sequences from positions -750 to +55 bp and -71 to +55 bp respectively ligated to the bacterial reporter gene encoding CAT) were obtained from Dr. M.R. Montminy (Salk Institute, La Jolla, CA) (23). pSS-250 CAT was generated by inserting a Sal I fragment (-250 to +55 bp) of the -750 somatostatin promoter into a pUC19-derived pCAT-Basic vector. A CRE substitution mutation in the -250 bp promoter (Δ-CRE pSS-250 CAT) was generated by replacing the octameric CRE (TGACGTCA) with an equal sized nonrelated sequence (GCGTAGTC) by a 2-step PCR procedure for gene splicing by overlap extension as described below (227).

Somatostatin promoter fragment (-250 to +55 bp) was subcloned into Sal I site of pBluescript II KS+ (Stratagene) and used as template for first PCR. Two complementary 38-mer oligonucleotides were synthesized as middle primers, which contain the mutant sequences flanked by 15 nucleotides of wild-type sequences at either side (CREMT1: 5'-TTTAAACTCTCTCTCgcgtagtcGCCAAGGAGGCGCCC-3', and CREMT2: 3'-AAATTTGAGAGAGAGcgcatcagCGGTTCCTCCGCGGG-5'). For the first two sets of PCRs, the middle primers (carrying mutants) were used with either KPNC or SAC+ as end primers and run 25 cycles of: 94 C, 1 min; 50 C, 2 min; 72 C, 1 min. DNA fragments K2 (KPNC-CREMT2, equivalent to -250 to -27 bp) and S1 (SAC+-CREMT1, -

63 to +55 bp) generated from these reactions were purified with a PrimeErase kit. The second PCR was run with K2 and S1 fragments as templates and end primers KPNC and SAC+, which generated a fragment (KS) containing sequence from somatostatin DNA -250 to +55 bp with CRE mutation. KS fragment was purified and subcloned into Sal I site of pCAT-Basic. All constructs and insert orientation were confirmed by restriction mapping and/or sequence analysis using the method of Sanger (228).

GEL SHIFT ASSAY

(1) Nuclear extracts were prepared from PC12 cells as described (229). Briefly, Cells from culture flasks were washed with and scraped into ice-cold PBS, pooled in a graduated conical centrifuge tube, and pelleted by centrifugation for 10 min in a JS-4.2 rotor (Bechman) at 3000 rpm. The cell pellet was rapidly resuspend in 5 pcv (packed cell volume, measured by the graduation on the tubes) of hypotonic buffer (10 mM HEPES, pH 7.9 at 4 C, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF*, 0.5 mM DTT*), and centrifuged for 5 min in the JS-4.2 rotor at 3000 rpm and the supernatant was discarded. [*Both PMSF and DTT are added fresh from stock solutions. 200 mM PMSF (Phenylmethylsulfonyl fluoride, MW 174.2) is dissolved in anhydrous isopropanol.]

Resuspend the packed cells in hypotonic buffer to a final volume of 3 pcv (packed cell volume) and allow to swell for 10 min on ice. For example, if an original pcv of 1 ml has swelled to 2 ml, only 1 ml of additional buffer is required here. The cells should swell at least 2-fold.

The cell suspension was homogenized with a glass Dounce homogenizer with ten

up-and-down strokes using a type B pestle. The nuclei were collected by centrifuging for 15 min in the JS-4.2 rotor at 4000 rpm (3300 X g). The supernatant was removed and could be used for preparation of S-100 cytoplasmic extract.

Resuspend the nuclei with half pnv (packed nuclear volume) of low-salt buffer (20 mM HEPES, pH 7.9 at 4 C, 25% glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT). In a dropwise fashion, while stirring gently, add 0.5 pnv of high-salt buffer (20 mM HEPES, pH 7.9 at 4 C, 25% glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT). Allow the nuclei to be extracted for 30 min at 4 C with continuous gentle mixing on a tiltboard.

Pellet the extracted nuclei by centrifuging 30 min in a Beckman JA-20 rotor at 25,000 X g. Draw off the resulting supernatant. Dialyze the extract against 50 volumes of dialysis buffer (20 mM HEPES, pH 7.9 at 4 C, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT) for 24 h with 3 changes. Centrifuge in 1.5 ml-Eppendorf tubes at 13,000 rpm for 15 min and discard the precipitation. Determine the protein concentration by the method of Bradford as described in the CAT Assay and quickly freeze aliquot in liquid nitrogen and keep at -80 C until use.

(2) Somatostatin promoter fragments -250 to -71 bp (Sal I/Bgl II), -71 to +55 (Bgl II/Sal I), and -250 to +55 (Sal I fragment) were digested from pSS-250CAT, isolated by either Mermaid or GeneClean and end-labelled with [α-^{32}P]-dCTP through Klenow fill-in reactions. Synthetic double-stranded CRE or GRE consensus oligonucleotides were end-labelled with [γ-^{32}P]-dATP by T4 polynucleotide kinase reaction.

(3) The 6% nondenaturing polyacrylamide gel was prepared with two 16-cm glass

plates, cleaned and siliconized. Assemble the plats vertically with 1.5 mm-thick spacers in between for casting the gel. The bottom was sealed with heated 0.5% agarose solution.

Prepare 35 ml high-ionic-strength gel mix with 7.0 ml 5 X Tris-glycine stock solution (0.25 M Tris base, 1.9 M glycine, 12 mM EDTA, pH 8.5), 7.0 ml 30% acrylamide, 1.31 ml 2% bisacrylamide, 1.5 ml 50% glycerol, and 17.9 ml H_2O. Add 88 µl of 30% ammonium persulfate and 30 µl TEMED immediately before use and swirl gently to mix.

Pour the gel mix between the plates and top it with a comb with teeth of 7 mm (or wider). Allow the gel to completely polymerize for 20 min. Remove the comb and attach the plates to the electrophoresis tank after filling the lower reservoir with 1 X high-ionic-strength electrophoresis buffer. Fill the upper reservoir with buffer and pre-run the gel at 100 V (current ~22 mA) for at least 90 min.

For best resolution and confirmation of the results, experiments were performed with 4% and 5% gels and under low-ionic-strength condition as well. For 35 ml of 4% low-ionic strength gel mix, mix 236 µl 1 M Tris-HCl (pH 7.9), 70 µl 0.5 M EDTA, 116 µl 1 M sodium acetate (pH 7.9), 4.7 ml 30% acrylamide, 0.88 ml 2% bisacylamide, and 1.75 ml 50% glycerol. Low-ionic strength electrophoresis buffer contains 6.7 mM Tris-HCl, pH 7.9, 3.3 mM sodium acetate, 1 mM EDTA. In order to counteract buffer polarization during electrophoresis, a pump, with two heads and a flow rate of 10 to 30 ml per min, was set up to exchange buffer between the upper and lower reservoirs.

(4) DNA binding reactions were carried out in the presence of 5 µg sonicated fish sperm DNA, 0.5 mg/ml BSA and specific competitors as indicated (229, 230). Nuclear

extracts (5-10 µg protein) were incubated for 30 min at room temperature with 50,000 cpm (10-20 fmol) radiolabelled probe in a total volume of 20 µl containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 6 mM DTT, 40 mM NaCl, 0.5 mg/ml BSA, 10% glycerol and 0.002% Triton X-100. Competition experiments were carried out by incubation first with 9 pmol cold CRE or GRE oligonucleotides for 20 min at room temperature without probe, followed by 20 min with probe, at room temperature. The reaction mixtures were loaded onto 6% nondenaturing polyacrylamide gels and resolved by electrophoresis at 120 V for 3 h, under high ionic strength running conditions. The gels were subsequently dried and autoradiographed at -70°C with an intensifying screen.

IN VITRO NUCLEAR RUN ON TRANSCRIPTION ASSAY

Relative transcription rates of somatostatin and cyclophilin genes were measured by nuclear run-on assay (231). Nuclei were prepared from 10~20 X 10^6 cells incubated with or without DEX for 18 h. Cells were scraped into ice-cold PBS, pH 7.4, pelleted at 4 C, and lysed with Nonidet P-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-mercaptoethanol, and 0.2% Nonidet P-40]. After 8 min on ice, nuclei were pelleted at 800 X g and resuspended in 75 µl of nuclei storage buffer [50% glycerol, 20 mM Tris (pH 7.9), 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, and 0.125 mM PMSF], and snap-frozen in liquid nitrogen, and stored at -80 C until assay.

Run-on (*in vitro* elongation) reactions were carried out at 30 C in 300 mM ammonium sulfate, 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, and 1.5 mM each of CTP, ATP, and GTP for 45 min. Reactions were quenched with 100 µg tRNA and treated with RQ1 DNase (37 C, 15 min) and proteinase K (60 µg, 42 C, 30 min), and phenol/chloroform extracted. Precipitate [^{32}P]-labelled transcripts with an equal volume of TCA solution (60 mM sodium pyrophosphate in 10% trichloro-acetic acid), by incubating on ice for 10 min, followed by centrifugation at 13,000 rpm for 15 min. Carefully remove the last drop of the supernatant and resuspend the pellet in 250 µl ice-cold 20 mM HEPES, 5 mM EDTA, pH 7.5. Then add 63 µl of 1 N NaOH to denature the RNA and leave on ice for 13 min exactly. Stop the reaction by adding 175 µl of 3 M NaAc, 1 M HEPES, pH 5.0. The reaction mixture (488 µl) was mixed with 1 ml ethanol and kept at -20 C (≥ 1 h) until ready for hybridization. Prior to hybridization, precipitate the RNA by centrifuging 15 min at 13,000 rpm and wash the pellet once with 70% ethanol and dry briefly.

Plasmid DNA (5 µg) containing somatostatin and cyclophilin cDNA inserts, or pBleuscript vector, were linearized and NaOH denatured, slot blotted (BioDot, BioRad), and hybridized with 20 X 10^6 cpm [^{32}P]-labeled transcripts in 50% formamide, 5 X SSPE, 1% SDS, 2.5 X Denhart's, 100 µg/ml ssDNA at 60 C for 48 h. In any single experiment, equal amount of radioactivity were used for all conditions. Nytran filters were washed in 1 X SSPE, 0.1% SDS at 60 C, for 20 min twice and exposed to autoradiographic film. Quantitation of the relative rates of somatostatin gene transcription was achieved by counting the radioactivities of excised strips of DNA blots, corrected with that of cyclophilin signals.

STATISTICAL ANALYSIS

Data are expressed as mean ± SE. Statistical comparisons were made using unpaired Student's t-test and one-way analysis of variance of Anova using InStat computer program (Graph Pad Software, San Diego, CA).

CHAPTER III

GLUCOCORTICOIDS REGULATE STEADY STATE SOMATOSTATIN mRNA AND PEPTIDE LEVELS IN NORMAL RAT TISSUES AND IN A SOMATOSTATIN PRODUCING ISLET TUMOR CELL LINE ($1027B_2$)

Somatostatin secretion is influenced by agents such as nutrients, ions, peptide hormones, and neurotransmitters, many of which act through stimulation of intracellular cAMP (2, 3). cAMP is an important regulator not only for secretion but also for somatostatin gene transcription (23, 61, 62, 72, 74, 232). Glucocorticoids are also known to regulate gene expression in many endocrine and nonendocrine target cells. They stimulate expression of several genes such as PEPCK and suppress others, e.g. POMC, glycoprotein hormone α subunit (174, 302). After diffusing into target cells, glucocorticoids bind to intracellular receptors; the activated receptor then interacts with target DNA to cause an increase or decrease in gene transcription (174). Over the years, sporadic reports have suggested that glucocorticoids are capable of influencing somatostatin function but the precise nature of this effect has remained unclear. Thus, the administration of DEX to rats *in vivo* inhibits growth in part by increased somatostatin secretion (105, 106). Corticosterone has been reported to augment somatostatin content and secretion as well as D cell number in cultured rat islet cells (107). Adrenalectomy in dogs, however, also leads to increased pancreatic and circulating somatostatin concentrations (125). DEX stimulates somatostatin content and release in thyroid 44-2C cells and SS-mRNA accumulation in rat thymus (12, 79). By contrast, DEX reduces somatostatin secretion and mRNA accumulation in TT human thyroid carcinoma cells (64). These conflicting reports can be explained by the use of widely different experimental

paradigms involving different normal tissues as well as tumor cells, *in vivo* and *in vitro* models, and different concentrations of glucocorticoids. In the present study, the effects of glucocorticoids on somatostatin peptide and steady state mRNA levels have been systematically examined. Using as models DEX treated rats *in vivo*, primary cultures of rat islet and cerebrocortical cells, and a somatostatin-producing rat islet tumor cell line (1027 B_2), I have sought to answer the following questions: (1) Do glucocorticoids influence normal somatostatin gene expression? (2) Is the glucocorticoid effect tissue-specific? (3) Is it dose-dependent? (4) Does it differ between normal and neoplastic cells? and (5) does it result from a direct action of glucocorticoids on somatostatin cells?

RESULTS

1. Glucocorticoids influence somatostatin peptide and SS-mRNA levels in normal somatostatin-producing tissues *in vivo*

DEX administration was associated with a significant loss of body weight (6% at 3 d, 28% at 8 d) compared to saline treated controls (Table 3-1). Discontinuation of DEX after 8 days partially restored body weight but the DEX treated animals did not reach the body weights of their saline injected counterparts by the end of study (day 22). Despite these growth differences, organ weights and total RNA concentrations of the removed somatostatin-producing tissues (hypothalamus, cerebral cortex, pancreas, stomach, jejunum) were not significantly different between saline and DEX injected rats at all time points of the study (Table 3-1).

Figure 3-1 depicts the effect of *in vivo* DEX administration on steady state SS-mRNA

Table 3-1. Body weight (A), organ weight and total RNA content (B) in control and DEX-treated rats. In B, data from the 8 day group alone are depicted. The 3 d and 22 d groups (like the 8 d group) showed no change in organ weight or RNA contents. (Mean ± SE, n = 8, *p < 0.05)

A.

		3 d	8 d	22 d
Body Weight (g)	Control	196 ± 3	242 ± 5	359 ± 9
	DEX	184 ± 4*	175 ± 5*	286 ± 12*

B.

Tissue		Weight (g)	RNA Content (mg/g tissue)
Hypothalamus	Control	0.046 ± 0.006	1.77 ± 0.14
	DEX	0.042 ± 0.004	1.87 ± 0.10
Cortex	Control	0.83 ± 0.06	1.46 ± 0.24
	DEX	0.96 ± 0.07	1.48 ± 0.20
Pancreas	Control	0.92 ± 0.03	11.5 ± 0.3
	DEX	0.67 ± 0.04	11.8 ± 0.4
Stomach	Control	1.38 ± 0.06	4.23 ± 0.34
	DEX	1.09 ± 0.02	4.12 ± 0.31
Jejunum	Control	1.72 ± 0.06	2.46 ± 0.14
	DEX	1.34 ± 0.06	2.47 ± 0.20

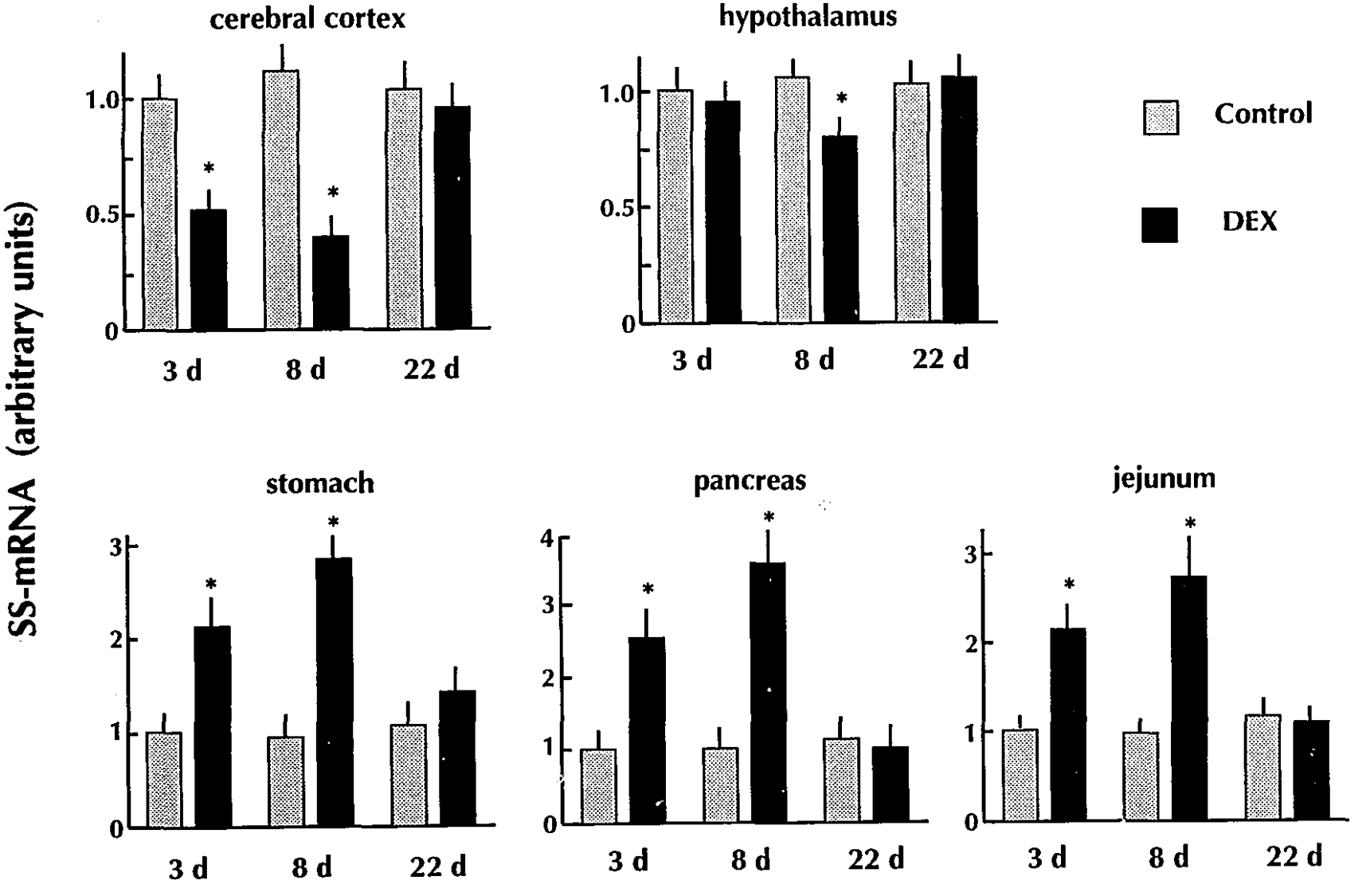

cerebral cortex
hypothalamus
stomach
pancreas
jejunum
SS-mRNA (arbitrary units)
Control
DEX
3 d
8 d
22 d
0
0.5
1.0
1
2
3
4
*

Fig 3-1

FIGURE 3-1. Effect of *in vivo* DEX administration on steady state SS-mRNA accumulation in cerebral cortex, hypothalamus, stomach, pancreas, and jejunum. Rats were injected with saline or DEX (0.5 mg/kg i.p.) for 3 or 8 days and studied immediately thereafter (3 d, 8 d groups). A third group of rats was injected for 8 d, allowed to recover for 14 d and studied on day 22 (22 d group). Cyclophilin-mRNA levels did not change under the same experimental conditions (data not shown). ($n = 8$; $*p < 0.05$)

accumulation in cerebral cortex, hypothalamus, stomach, pancreas, and jejunum. DEX induced significant alterations in SS-mRNA (detected as a 0.75 kb transcript) in all of these tissues. Cerebrocortical SS-mRNA level was reduced by 50% and 60% after 3 and 8 days of treatment respectively. A smaller but significant reduction in SS-mRNA was also observed in the hypothalamus after 8 days of DEX treatment. Alterations in SS-mRNA induced by DEX in peripheral tissues were opposite to those seen in brain. Thus, stomach, pancreas, and jejunum all exhibited 2-3 fold increases in SS-mRNA. As in the case of brain, the DEX effect was time-dependent being more pronounced after 8 days than after 3 days of treatment. In all tissues, SS-mRNA levels returned to control values at day 22, 2 weeks after cessation of DEX.

Changes in tissue SSLI content parallelled those of SS-mRNA (Figure 3-2). Thus, in cerebral cortex there was a 35% and 60% reduction in SSLI after 3 and 8 days of DEX treatment respectively. A reduction in hypothalamic SSLI also occurred with DEX but was less marked than in cerebral cortex and significant only at 8 days when levels fell by 20% from 7.5 to 6 μg/g tissue. The content of SSLI in stomach, pancreas, and jejunum was increased by DEX. There was a significant 60% increase in gastric SSLI at day 8, 33% and 98% increases in pancreatic SSLI at day 3 and day 8 respectively, and 65% and 80% increases in jejunum at day 3 and day 8 of DEX administration respectively. In all tissues, SSLI content was restored to control levels by day 22, two weeks after discontinuation of DEX. At an early time point, 1 d DEX treatment did not induce any significant change in SS-mRNA or SSLI levels in all tissues examined (data not shown).

2. Glucocorticoids influence SS-mRNA level, cellular somatostatin content and

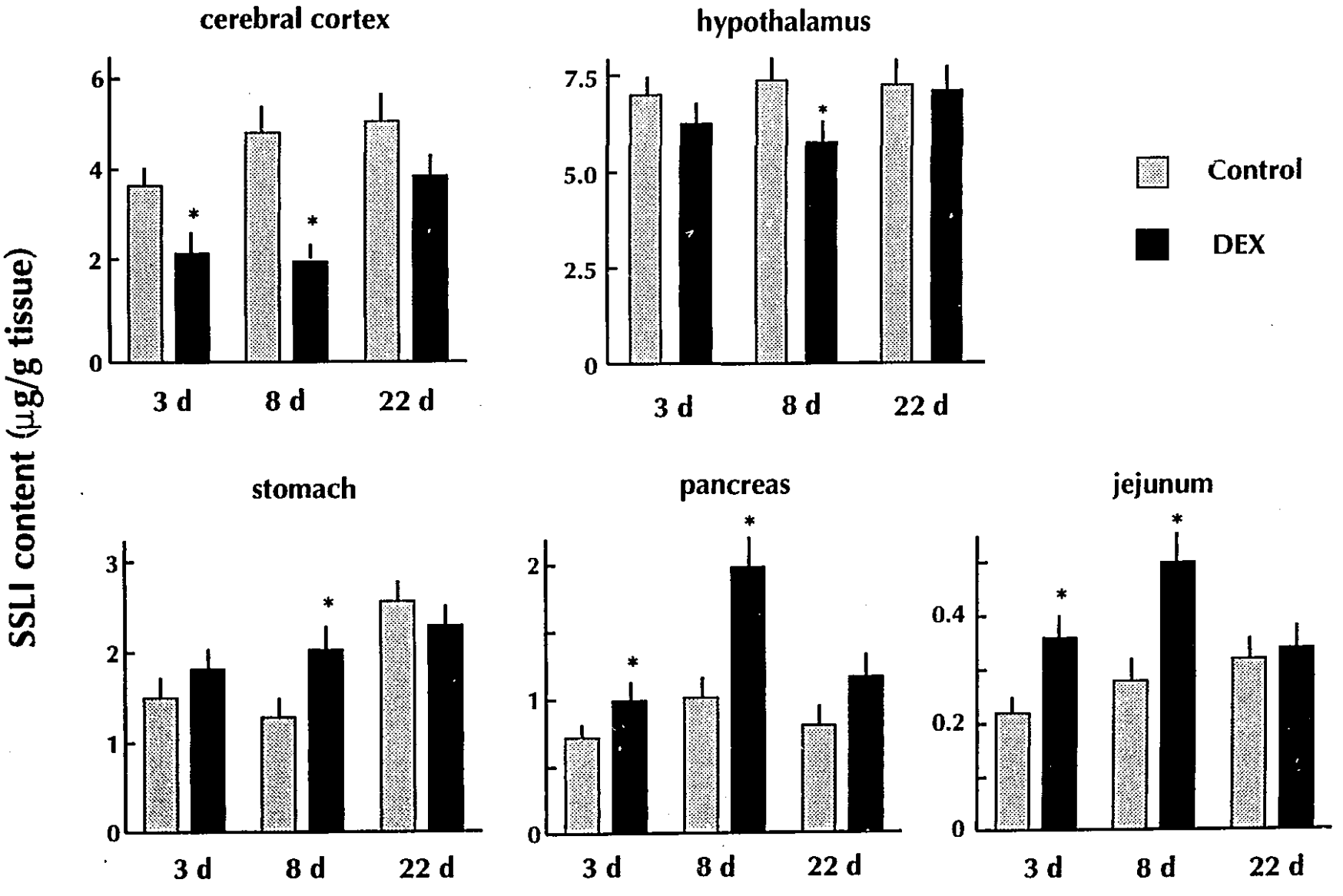

cerebral cortex
hypothalamus
stomach
pancreas
jejunum
SSLI content (μg/g tissue)
Control
DEX
3 d
8 d
22 d
0
2
4
6
2.5
5.0
7.5
1
3
0.2
0.4
*

Fig 3-2

FIGURE 3-2. Changes in SSLI content in brain and peripheral tissues following 3 or 8 days of DEX treatment or on day 22 following termination of 8 days treatment as described in Figure 3-1 ($n = 8$, $*p<0.05$).

somatostatin secretion in cultured normal islet cells

Figure 3-3 illustrates changes in SS-mRNA, cellular SSLI content, and SSLI release from cultured islet cells incubated with DEX (10^{-10}-10^{-5} M) for 18 h. DEX produced significant alterations in all 3 parameters. In the case of SS-mRNA accumulation, there was a dose-dependent biphasic pattern: low dose of DEX (10^{-10} M) stimulated SS-mRNA 40% whereas higher doses produced progressive inhibition of SS-mRNA with a maximum 55% decrease at 10^{-6} M. Changes in cellular SSLI content followed a comparable pattern with stimulation at low doses of DEX and inhibition at higher doses. In the case of SSLI secretion, there was no change with low doses of DEX but higher concentrations (10^{-9}-10^{-5} M) produced up to 45% inhibition. Cell numbers and total RNA concentration in the cultures did not change significantly during the time course of the experiment.

3. Glucocorticoids stimulate insulin secretion and inhibit glucagon release from cultured normal islet cells

Since islet cell cultures represent a mixed population of cells which also secrete insulin and glucagon, both of which could regulate somatostatin gene expression independent of DEX (58, 62), the secretion of these 2 hormones was monitored by RIA (Figure 3-4). Insulin secretion was stimulated by DEX in a dose-dependent manner with maximum stimulation of ~ 40% at 10^{-6} M DEX. In contrast, the secretion of glucagon was inhibited up to 47% with low doses of DEX (10^{-10} M and 10^{-9} M) but higher concentrations of DEX failed to maintain this inhibition resulting in steadily increasing glucagon secretion towards normal control. Therefore, an indirect effect of DEX on somatostatin cells through secondary changes in insulin and glucagon secretion can not be ruled out.

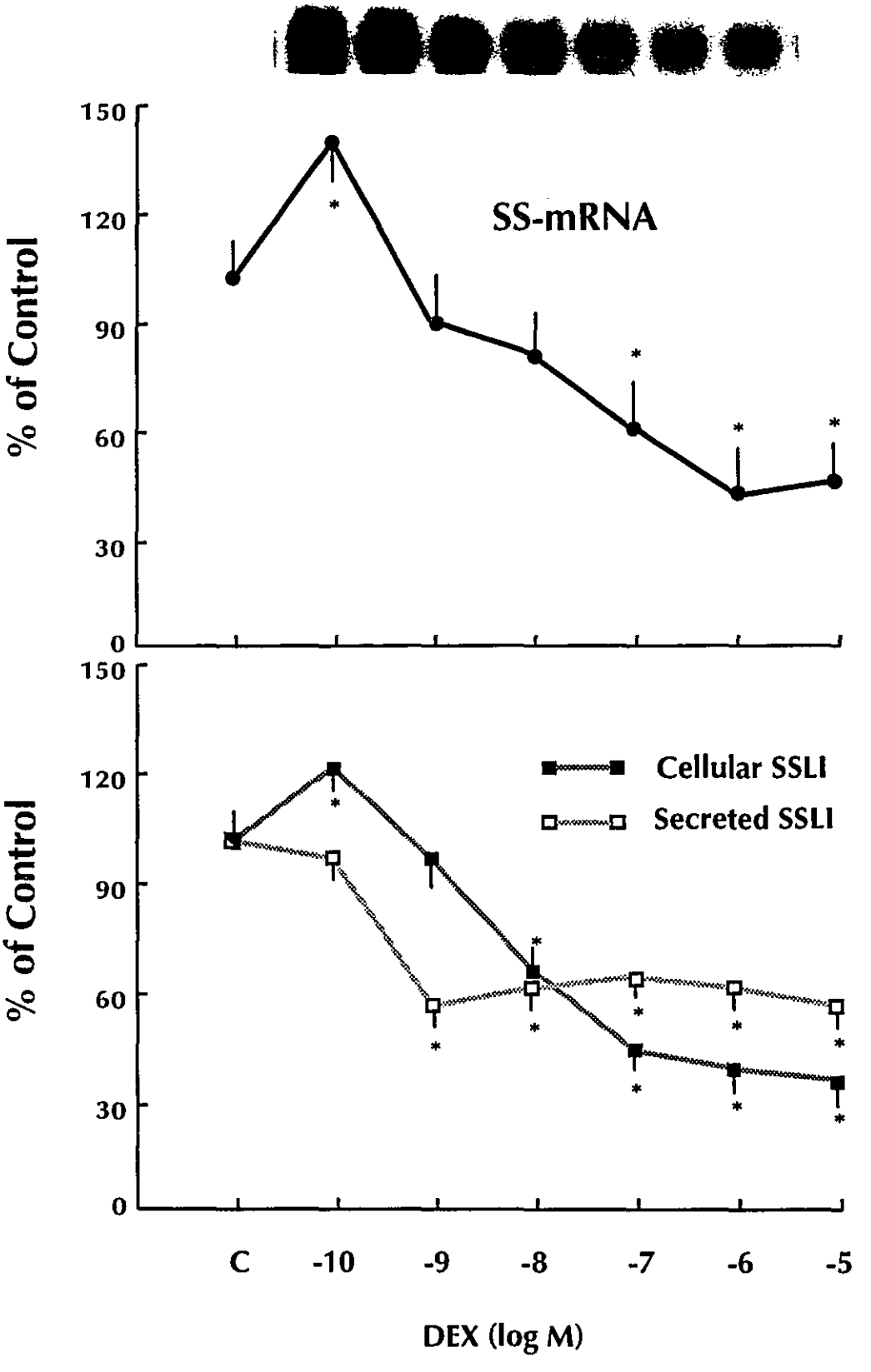

SS-mRNA
% of Control
150
120
90
60
30
0
Cellular SSLI
Secreted SSLI
% of Control
150
120
90
60
30
0
C
-10
-9
-8
-7
-6
-5
DEX (log M)

Fig 3-3

FIGURE 3-3. Steady state SS-mRNA levels (●---● upper panel), cellular SSLI content (■---■) and SSLI release (□---□ lower panel) from cultured rat pancreatic islet cells exposed for 18 h with DEX (10^{-10} ~ 10^{-5} M). Groups of 5 culture dishes were incubated with control medium or medium containing DEX. Cyclophilin-mRNA levels did not change under the same experimental conditions (data not shown). Results of SSLI are representative of 3 complete experiments. Data are expressed as percent of control (n = 5, SSLI; n = 3, SS-mRNA; *$p<0.05$).

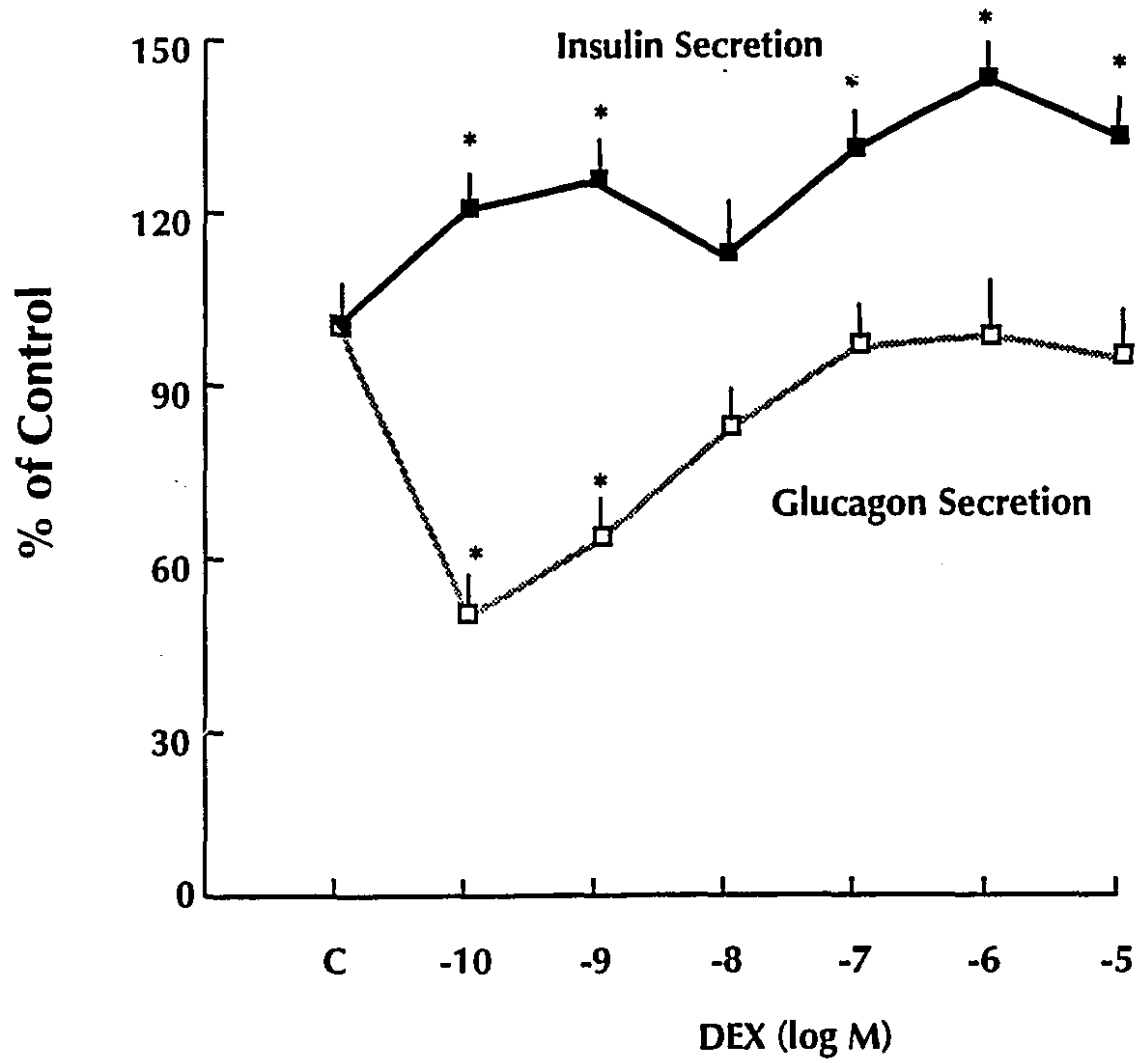


FIGURE 3-4. Effects of an 18 h incubation with DEX (10^{-10} ~ 10^{-5} M) on insulin (■---■) and glucagon (□---□) secretion from cultured rat pancreatic islet cells. Groups of 5 culture dishes were incubated with control medium or medium containing DEX. Data are representative of 3 complete experiments (*$p<0.05$).

4. Glucocorticoids suppress SS-mRNA accumulation in cortical cell cultures

Figure 3-5 illustrates the effect of DEX on SS-mRNA accumulation in primary cultures of cerebrocortical neurons. SS-mRNA levels were suppressed by DEX in a dose-dependent manner with a maximum 48% reduction at 10^{-8}~10^{-6} M DEX. The direction of this change was comparable to that induced by DEX on cortical SS-mRNA *in vivo* (Figure 3-1). In a time course study, the effect of DEX on steady state SS-mRNA level became evident only after 8 h of incubation (Figure 3-6), in comparison to cAMP, whose stimulation on SS-mRNA level became evident within 2 h of incubation and reached 15-fold by 18 h. DEX did not significantly reduce the cAMP-stimulated SS-mRNA accumulation in cortical cell cultures (Figure 3-6).

5. Glucocorticoids influence SS-mRNA level, cellular somatostatin content and somatostatin secretion in $1027B_2$ cells

DEX treatment of the somatostatin-producing islet tumor cell line $1027B_2$ produced dose-dependent changes in the levels of SS-mRNA and SSLI (Figure 3-7). The pattern of these changes was comparable to that observed in cultured normal islet cells. Thus, there was an initial 50% increase in SS-mRNA at low concentration of DEX (10^{-10} M) followed by a dose-dependent inhibition with increasing concentrations of DEX, with a maximum 65% reduction in SS-mRNA at DEX 10^{-6} M compared to control. SSLI content in these cells showed a comparable pattern with initial stimulation followed by suppression. However, only the stimulatory effect was statistically significant. SSLI release from these cells was comparable to that observed in cultured normal islets with no stimulation of release but a dose-dependent inhibition of secretion at DEX concentrations from 10^{-8}~10^{-5} M with a

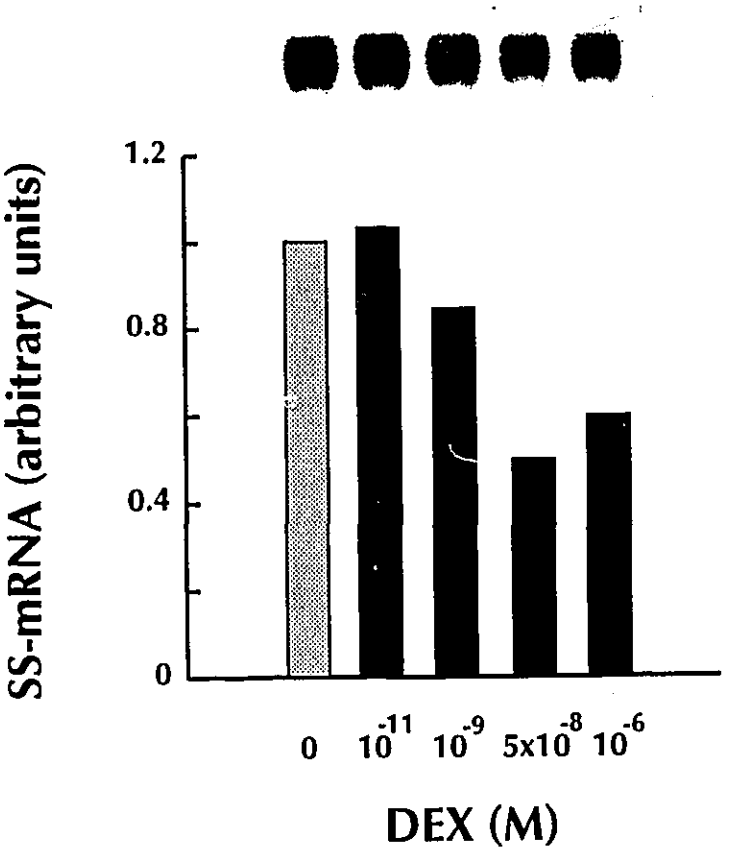


FIGURE 3-5. Effect of an 18 h incubation with DEX (10^{-11} - 10^{-6} M) on steady state SS-mRNA levels in cultured fetal rat cerebrocortical cells. 15 µg total RNA (pooled from 5 culture dishes) was probed with a [^{32}P] labeled SS-cRNA probe. SS-mRNA is expressed in arbitrary (densitometric) units. The blot depicted is representative of 2 complete experiments. Cyclophilin-mRNA levels did not change under the same experimental conditions.

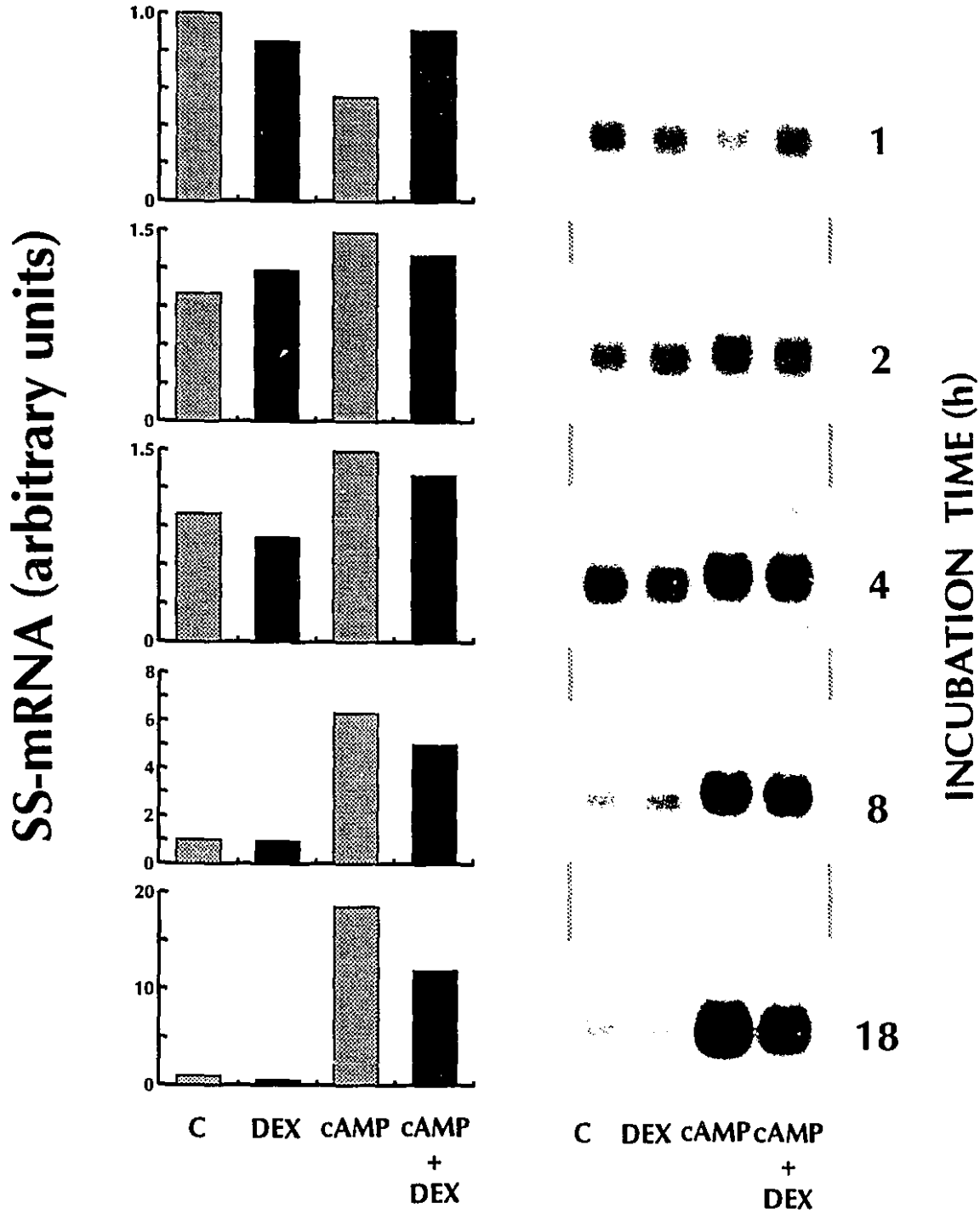


FIGURE 3-6. Time course study of the effects of DEX and cAMP on SS-mRNA accumulation in cultured fetal rat cerebrocortical cells. Cells were incubated with DEX (1 μM) or db-cAMP (5 mM) for the hours indicated and total RNA was extracted for Northern blot analysis of SS-mRNA levels. 15 μg of total RNA was probed with a [^{32}P]-labelled SS-cRNA probe. As not shown, cyclophilin-mRNA levels did not show significant change under the same conditions. Representative blots are depicted from two experiments.

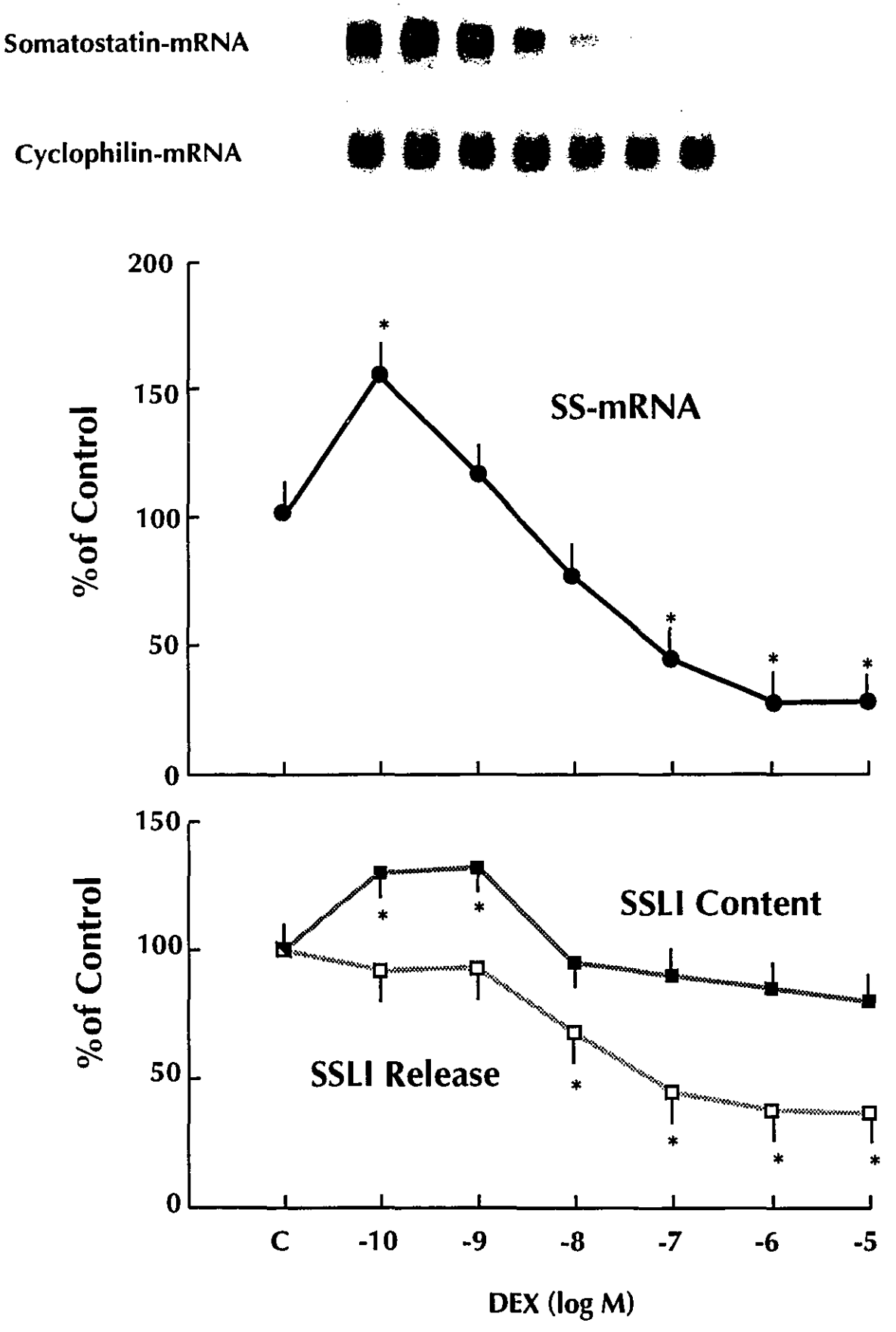

Somatostatin-mRNA
Cyclophilin-mRNA
200
150
100
50
0
%of Control
SS-mRNA
150
100
50
0
%of Control
SSLI Content
SSLI Release
C
-10
-9
-8
-7
-6
-5
DEX (log M)

Fig 3-7

FIGURE 3-7. Effects of an 18 h incubation with DEX (10^{-10} ~ 10^{-5} M) on steady state SS-mRNA levels (●---● top panel), cellular SSLI content (■---■), and SSLI release (□---□ bottom panel) from $1027B_2$ rat islet SS-producing tumor cells. Pools of 5 culture dishes were incubated with control medium or medium containing DEX. 20 μg total RNA was hybridized to a [^{32}P] labeled SS-cRNA probe. Cyclophilin-mRNA was analyzed as an internal control, and unlike SS-mRNA, was unaffected by DEX. Data are expressed as percent of control (n = 3, SS-mRNA; n = 5, SSLI; *$p<0.05$).

maximum 60% inhibition.

DISCUSSION

This study demonstrates that glucocorticoids exert significant effects on somatostatin peptide production and steady state mRNA accumulation in all of the major somatostatin producing tissues such as the brain, gastrointestinal tract, and pancreatic islets. The glucocorticoid effects are tissue specific, dose- and time-dependent, and occurred in opposite directions in brain compared to peripheral organs.

In view of the wide distribution of somatostatin cells and their capacity for interaction with many different body systems, it is not surprising to find that somatostatin function can be influenced by a broad array of secretagogues ranging from ions, nutrients, and cyclic nucleotides to neuropeptides, neurotransmitters, classical hormones, and growth factors (2, 3). Many of the agents that influence somatostatin secretion are also capable of altering somatostatin gene expression as assessed by changes in steady state mRNA levels. For instance, somatostatin secretion and mRNA accumulation are stimulated by cAMP in brain, pancreas and gut (3, 61, 62), by GH in the hypothalamus (34, 35, 233), by IL-1, TNF in diencephalic cells (41, 52, 53) and NMDA receptor agonists in the cultured cortical neurons (42), whereas both are inhibited by insulin in pancreatic islets (58). In the case of steroid hormones, *in vivo* administered testosterone and estradiol have both been reported to augment hypothalamic SS-mRNA levels (43, 234). By contrast, there has been no systematic investigation of the effects of glucocorticoids on somatostatin gene expression in normal tissues despite scattered evidence that this hormone influences somatostatin function at the peptide level in some tissues (107, 125) and on SS-mRNA in thyroid tumor cells and rat

thymus (12, 64).

In the present study, it is shown that *in vivo* administration of DEX produced significant decreases in SSLI and SS-mRNA levels in cerebral cortex and hypothalamus associated with increases in stomach, pancreas, and jejunum. Although DEX treatment led to a loss of body weight, total RNA was unaffected, indicating that the observed changes in cellular SSLI and mRNA levels were specifically induced by the glucocorticoid. Additional evidence for glucocorticoid specificity came from the finding that DEX produced a tissue-specific pattern of both increased and decreased SS-mRNA level together with a dose-dependent effect, the changes being more pronounced at day 8 compared to day 3, and reversing towards normal upon discontinuation of the glucocorticoid treatment. There have been no previous studies of the effects of glucocorticoids on somatostatin function in cerebral cortex or the gastrointestinal tract. In the case of the hypothalamus, our data are in agreement with a preliminary study describing a 50% reduction in SS-mRNA following 10 days of DEX administration in rats (235) but at variance with the reported increase in hypothalamic SS-mRNA following 3 days of DEX administration to female rats (318). Since changes in somatostatin secretion and mRNA accumulation generally occur in parallel, the finding of a reduction in hypothalamic SS-mRNA levels after 8-day DEX treatment does not support the proposal that glucocorticoid-mediated growth inhibition in rats is due to suppressed GH secretion secondary to increased hypothalamic somatostatin release (105, 106). Nevertheless, without direct evidence on somatostatin release from the hypothalamus, one can not rule out that possibility. Furthermore, the observed reversal of glucocorticoid induced GH inhibition by somatostatin antibody could be due to immunoneutralization of circulating somatostatin produced from other sources.

The *in vivo* actions of glucocorticoids on somatostatin function could be a direct one on somatostatin-producing cells, or secondary to the numerous endocrine and metabolic effects that glucocorticoids are known to exert on non somatostatin-producing cells. Changes in the levels of circulating hormones induced by DEX could be one explanation for the differential effect of the glucocorticoid in brain compared to peripheral tissues, since unlike steroid hormones, circulating peptides and many other hormones and transmitter substances do not readily traverse the blood brain barrier. This is also suggested by the *in vitro* experiments comparing the effects of DEX on somatostatin function in primary cultures of normal islet and cerebrocortical neurons. Islet cell cultures exposed to DEX for 18 h displayed a dose-dependent biphasic change in SSLI and SS-mRNA, low dose glucocorticoids being stimulatory and high doses inhibitory. The pattern of SS-mRNA and SSLI in DEX treated brain cultures was different to that in islets with a predominant inhibitory effect at higher concentrations of DEX. To obtain further insights into a potential direct effect of glucocorticoids on normal somatostatin-producing cells, I correlated DEX-induced alterations in SS-mRNA with changes in insulin and glucagon secretion. Neonatal islet cell cultures represent a mixed population of cells containing predominantly B and D cells, lesser numbers of A cells, and virtually no pancreatic polypeptide cells (62, 191). Since both insulin and glucagon can influence somatostatin gene expression, the levels of the two hormones were measured in the culture media. The increase in insulin secretion which occurred with DEX could secondarily inhibit D cell function since insulin is capable of suppressing islet D cell secretion and SS-mRNA expression (58). Likewise, the suppression of glucagon induced by DEX could also contribute to a reduction in somatostatin secretion and mRNA level because glucagon acting on D cells is capable of augmenting both secretion and gene expression of

somatostatin through a cAMP-dependent mechanism (62). Whilst these data support an indirect effect of DEX on somatostatin cells through secondary changes in insulin and glucagon secretion, the absence of a tight relationship between the patterns of insulin and glucagon secretion and somatostatin function leave open the possibility of an additional direct glucocorticoid effect on D cells.

To confirm a direct glucocorticoid effect on D cells, the rat islet somatostatinoma cell line $1027B_2$ was examined. These cells possess DEX binding sites (see Determination of Cytosolic DEX Binding Sites in Chapter II) and responded to DEX with stimulation of SS-mRNA and somatostatin secretion at low doses and inhibition at higher doses comparable to that observed in normal islet cell cultures, providing definitive evidence for a direct action of glucocorticoids on somatostatin gene expression. In the only other study of the direct effects of glucocorticoids on SS-mRNA, Cote *et al.* reported uniphasic inhibition of somatostatin secretion and mRNA levels in medullary thyroid carcinoma (TT) cells by DEX 10^{-8} ~ 10^{-6} M (64). These data are in partial agreement with our findings in $1027B_2$ cells, differing only with respect to the stimulatory component of somatostatin secretion and mRNA levels elicited in $1027B_2$ cells. Present results in $1027B_2$ cells clearly indicate that the pattern of DEX induced alterations in SS-mRNA is complex with dose-dependent stimulatory and inhibitory components suggesting multilevel regulation. Since glucocorticoids not only alter transcriptional rates in target genes (174, 176) but additionally induce effects on mRNA stability and translational efficiency (102, 109, 112, 236, 237), further studies are required to determine whether the alterations in steady state SS-mRNA level produced by DEX are regulated at a transcriptional and/or post-transcriptional level.

In conclusion, this study provides the first evidence that glucocorticoids influence somatostatin peptide and steady state mRNA levels in normal somatostatin-producing tissues *in vivo* and *in vitro*. The glucocorticoid effect is time- and dose-dependent, tissue-specific, and at least in part due to a direct action of the steroid hormone on somatostatin-producing cells. The mechanisms underlying the direct actions of glucocorticoids on somatostatin gene expression are complex and may involve multiple intracellular levels of glucocorticoid action.

CHAPTER IV. GLUCOCORTICOIDS ACTIVATE SOMATOSTATIN GENE TRANSCRIPTION VIA POSITIVE INTERACTION WITH THE cAMP SIGNALLING PATHWAY

As described in Chapter III, DEX induces tissue-specific, time- and dose-dependent alterations in somatostatin peptide production and mRNA accumulation in normal somatostatin producing tissues *in vivo* and *in vitro* (63). Furthermore, DEX produces a biphasic patterns of change in somatostatin secretion and steady state mRNA levels in rat islet culture and somatostatin producing islet tumor cells ($1027B_2$) with dose-dependent stimulatory and inhibitory components (63). This suggests a complex molecular mechanism of glucocorticoid action on the somatostatin gene involving multi-level regulations. In the present study, I have examined transcriptional control of the somatostatin gene by glucocorticoid as one putative level of control. Using the CAT transcription assay, I have compared the effect of glucocorticoids with that of other steroid and thyroid hormones, and investigated transcriptional interaction between glucocorticoids and the cAMP signalling pathway. I demonstrate that glucocorticoids regulate somatostatin gene transcription positively through cooperative interaction with the cAMP signalling pathway and that DNA sequences upstream from CRE in the somatostatin gene are necessary for this effect.

RESULTS

1. Glucocorticoids activate somatostatin gene promoter

To determine whether glucocorticoids can influence somatostatin gene transcription,

the effect of DEX on pSS-750 CAT expression was investigated initially in PC12 cells. DEX induced a significant dose-dependent increase in CAT activity from 10^{-8} to 10^{-6} M, with a maximal 2.2 fold induction at 1 μM (Figure 4-1). To test the specificity of DEX action, the ability of other steroid and thyroid hormones to alter somatostatin gene transcription was determined (Table 4-1). In contrast to DEX, β-estradiol, testosterone, 3,3',5-triiodo-L-thyronine (T3) and retinoic acid in doses between 10^{-9} to 10^{-6} M had no effect on pSS-750 CAT expression in PC12 cells. Likewise, phorbol esters were without effect whereas cAMP and forskolin induced a 4-5 fold stimulation of somatostatin-CAT expression (Table 4-1).

2. The -250 to -71 bp of the somatostatin promoter region is required for DEX-induced transactivation

In order to map the promoter region responsible for glucocorticoid activation, progressive deletions of the 5' flanking somatostatin DNA were performed. Both the -750 and -250 bp promoter constructs exhibited full responsiveness to DEX stimulation (Figure 4-2). Deletion of the 5' somatostatin DNA to -71 bp which retains the CRE and TATA elements but removes the SMS-UE, abrogated DEX-induced stimulation of somatostatin gene transcription. These results further validate the specificity of the DEX effect and suggest that CRE alone is insufficient for DEX-induced transactivation, which requires additional elements located further upstream from CRE between -250 and -71 bp in the promoter region.

3. DEX potentiates cAMP stimulated somatostatin-CAT gene expression

In view of the finding of DNA elements responsive to glucocorticoid stimulation upstream from CRE in the somatostatin gene, the potential interaction between DEX and

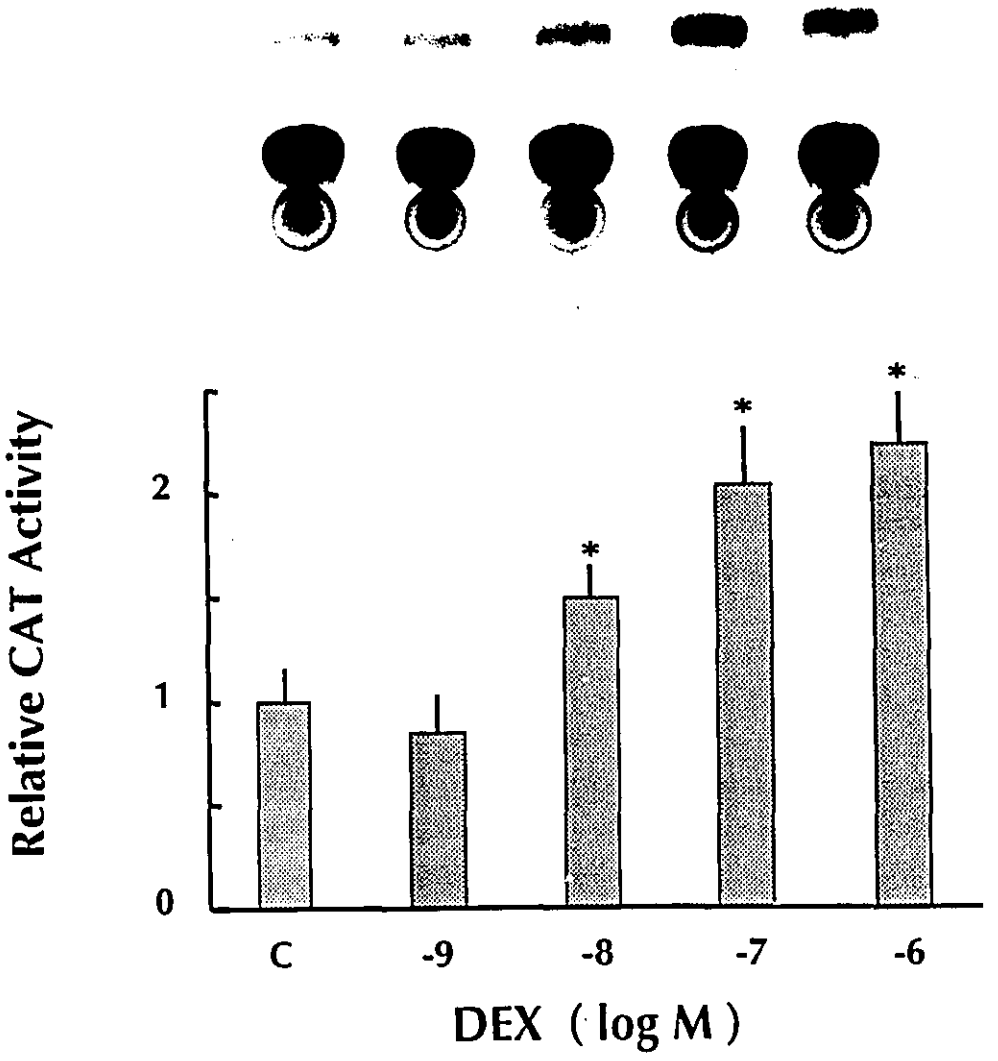


Figure 4-1. Effect of DEX on pSS -750 CAT expression in PC12 cells. Following transient transfection, PC12 cells were incubated with DEX for 48 h and assayed for CAT. CAT activity is expressed relative to untreated controls. DEX induced dose-dependent stimulation of SS-CAT expression with a maximum 2.2 fold effect at 10^{-6} M. The panel depicts an autoradiogram of a representative assay used to determine CAT activity in transfected PC12 cell extracts.

Table 4-1. Effects of DEX, cAMP, forskolin, and other agents on pSS-750CAT expression in transiently transfected target cells. (n = 5, *p < 0.05 vs control, **p<0.05 vs cAMP or forskolin alone)

Agents	Cell Type	CAT Activity (% of control) (mean ± SE)
DEX (1 μM)	PC12	212 ± 21*
cAMP (1.5 mM)	PC12	457 ± 48*
forskolin (10 μM)	PC12	489 ± 51*
β-estradiol (1 μM)	PC12	108 ± 11
testosterone (1 μM)	PC12	106 ± 13
triiodothyronine (1 μM)	PC12	116 ± 16
retinoic acid (1 μM)	PC12	101 ± 8
phorbol 12,13 dibutyrate (1 μM)	PC12	95 ± 15
DEX + cAMP	PC12	776 ± 81**
DEX + forskolin	PC12	748 ± 70**
DEX (1 μM)	A126-1B2	110 ± 12
cAMP (1.5 mM)	A126-1B2	120 ± 21
forskolin (10 μM)	A126-1B2	105 ± 18

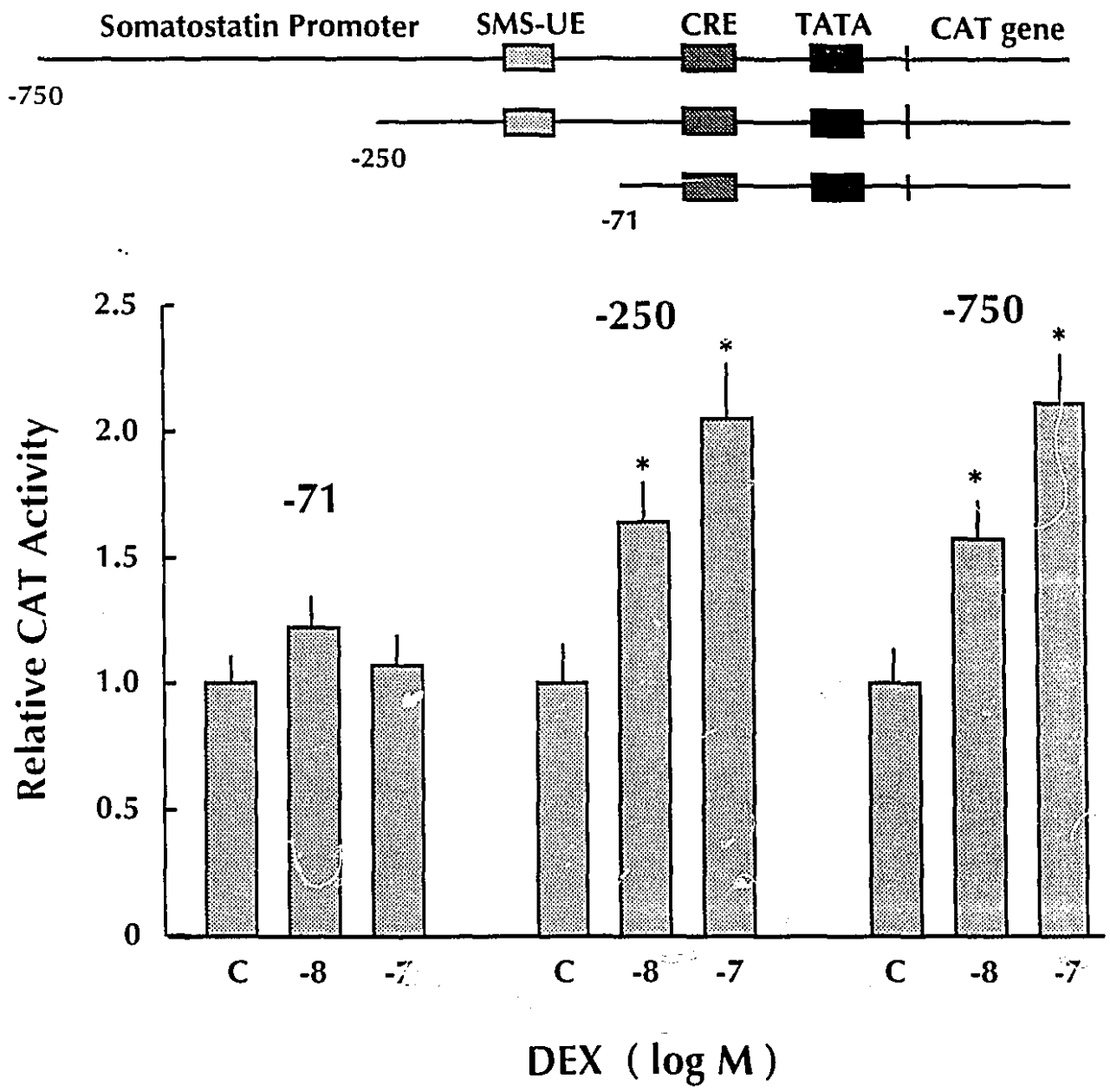


Figure 4-2. Effect of progressive 5' deletion of the somatostatin promoter fragment on DEX-induced transactivation in PC12 cells. Three promoter fragments of -750, -250, and -71 bp were used and are schematically depicted in the upper panel. Both the -750 and -250 nucleotide promoter constructs exhibited full responsiveness to DEX stimulation. Deletion to -71 bp abrogated DEX-induced stimulation of somatostatin gene transcription.

cAMP induced gene transcription was explored in PC12 cells transfected with pSS-750 CAT. 8-(4-Chlorophenylthio)-cAMP (1.5 mM) or forskolin (10 μM) both induced a 4-5 fold stimulation of somatostatin promoter activity (Figure 4-3 and Table 4-1). The combination of DEX with cAMP or forskolin potentiated CAT activity 7-8 fold, suggesting an additive effect between DEX and cAMP-induced transcription. The additive effect of DEX was also observed at low concentration of cAMP (0.1 mM), which stimulated somatostatin gene transcription from 3 to 5 fold (data not shown). The pSS-250 CAT construct exhibited cAMP and forskolin responsiveness as well as the additive effect with DEX, identical to the longer -750 bp promoter. On the other hand, the pSS-71 CAT construct which was insensitive to DEX (Figure 4-2), maintained full cAMP responsiveness (data not shown).

4. DEX transactivation shows dependency on protein kinase A activity

To further characterize the components of the cAMP signalling pathway involved in DEX-induced transactivation of the somatostatin gene, A126-1B2 mutant PC12 cells lacking PKA activity were used (Table 4-1). As expected, A126-1B2 cells transfected with pSS -750 CAT did not respond to either cAMP or forskolin. These cells also proved to be unresponsive to DEX-induced somatostatin gene transcription, confirming a requirement for PKA activity for the DEX effect.

5. Mutation of CRE abolishes DEX effect on somatostatin promoter activity

To test the effect of mutation of the CRE element on DEX-induced somatostatin gene transcription, PC12 cells were transfected with Δ-CRE pSS-250 CAT. Replacement of CRE by a nonrelated sequence reduced basal CAT activity (% conversion of chloramphenicol into

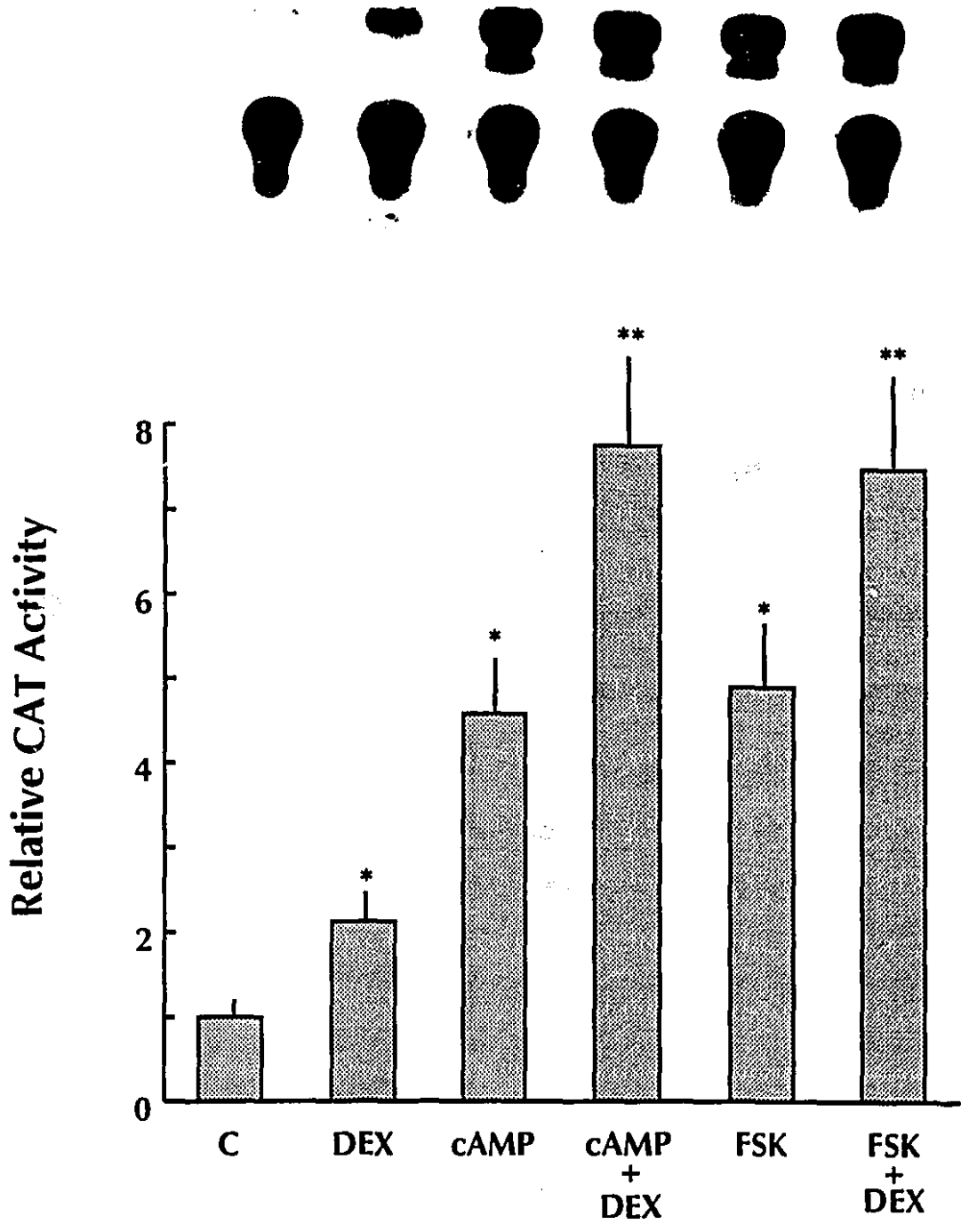


Figure 4-3. Additive effect of DEX and cAMP stimulation of somatostatin gene transcription. cAMP (1.5 mM) or forskolin (FSK, 10 μM) both induced 4-5 fold stimulation of pSS -750 CAT expression in PC12 cells. The combination of DEX (10^{-6} M) with cAMP or FSK potentiated CAT activity 7-8 fold.

acetylated forms) from 1.42 ± 0.28 to 0.52 ± 0.03 ($p<0.01$). As expected, the mutation abolished the stimulatory effect of forskolin but additionally blocked DEX-induced stimulation, as well as the combined forskolin and DEX potentiation of CAT activity (Figure 4-4). Mutation of CRE was further validated by gel electrophoretic mobility shift assay. somatostatin promoter fragments -250 to +55 bp from cognate (containing the CRE) or CRE mutant (Δ-CRE) pSS-250CAT were radiolabelled and incubated with PC12 nuclear extract. Three retarded DNA-protein complexes were observed with the natural promoter, labelling of one of which was significantly inhibited in the presence of 500 fold molar excess of unlabelled CRE consensus oligonucleotide (Figure 4-5). This band was not observed with the CRE mutant promoter.

In a reciprocal experiment, the effect of overexpressed CREB on DEX-induced somatostatin gene transcription was investigated. Cells were transfected with pSS-750 CAT (28 µg) with or without MT-CREB (12 µg) whose expression was induced by the addition of zinc sulphate (90 µM) to the culture medium. Cotransfection of PC12 cells with MT-CREB and pSS-750 CAT had no effect on basal CAT expression and did not enhance DEX, forskolin, or combined forskolin and DEX induced stimulation of CAT activity in these cells (data not shown).

6. Gel shift assay indicates that the -250 to -71 bp somatostatin promoter is glucocorticoid receptor binding region

In order to obtain evidence for a potential DNA-protein interaction between the somatostatin promoter and the glucocorticoid receptor, gel shift assays were performed. Incubation of labelled -250 to -71 bp somatostatin-DNA with nuclear extract from PC12 cells

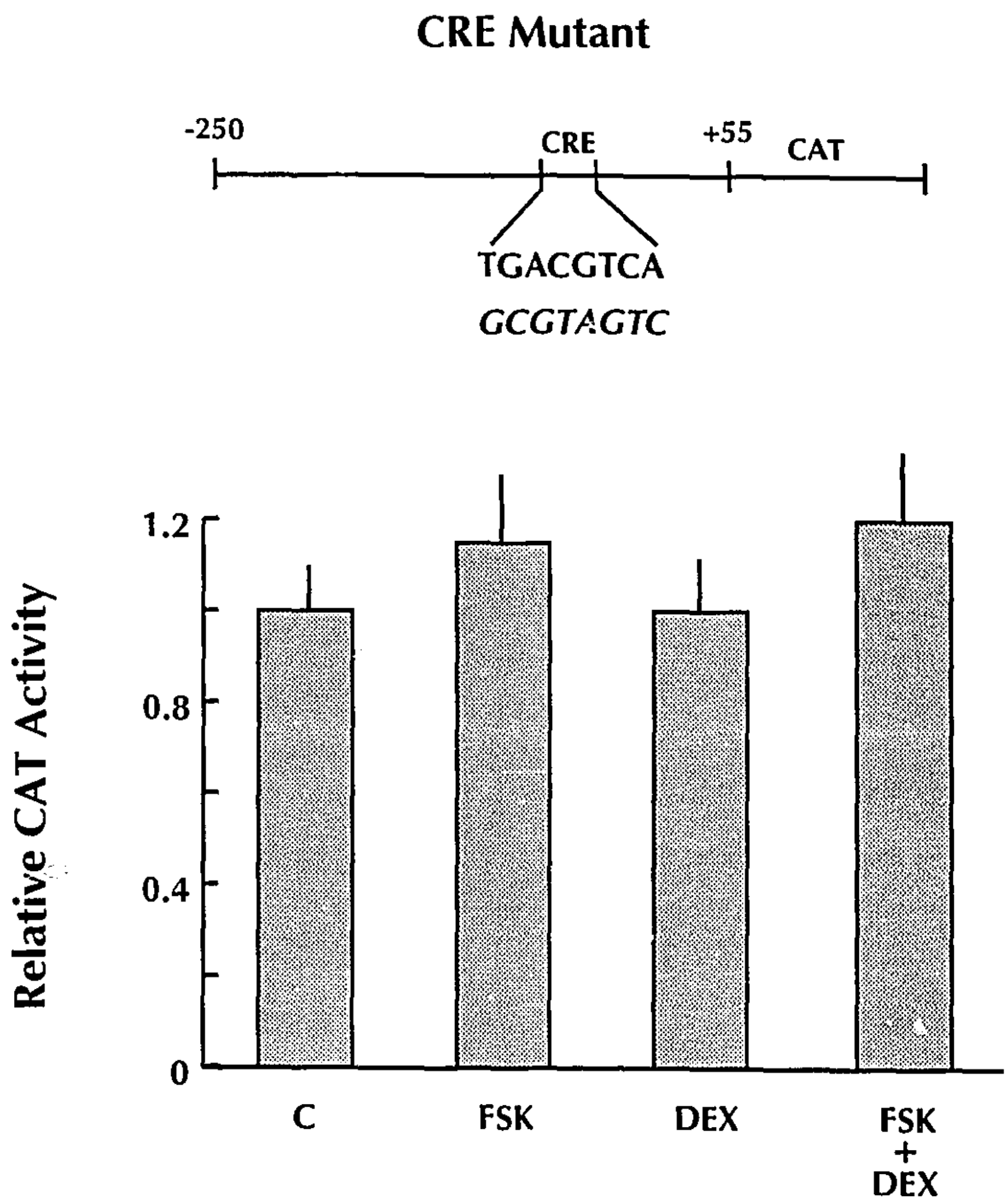


Figure 4-4. Effect of mutation of the CRE element on somatostatin-CAT expression in PC12 cells. Mutation of the CRE element in the -250 promoter abolished all the effects of forskolin (FSK, 10 μM), DEX (1 μM), or combined forskolin and DEX. (n=3)

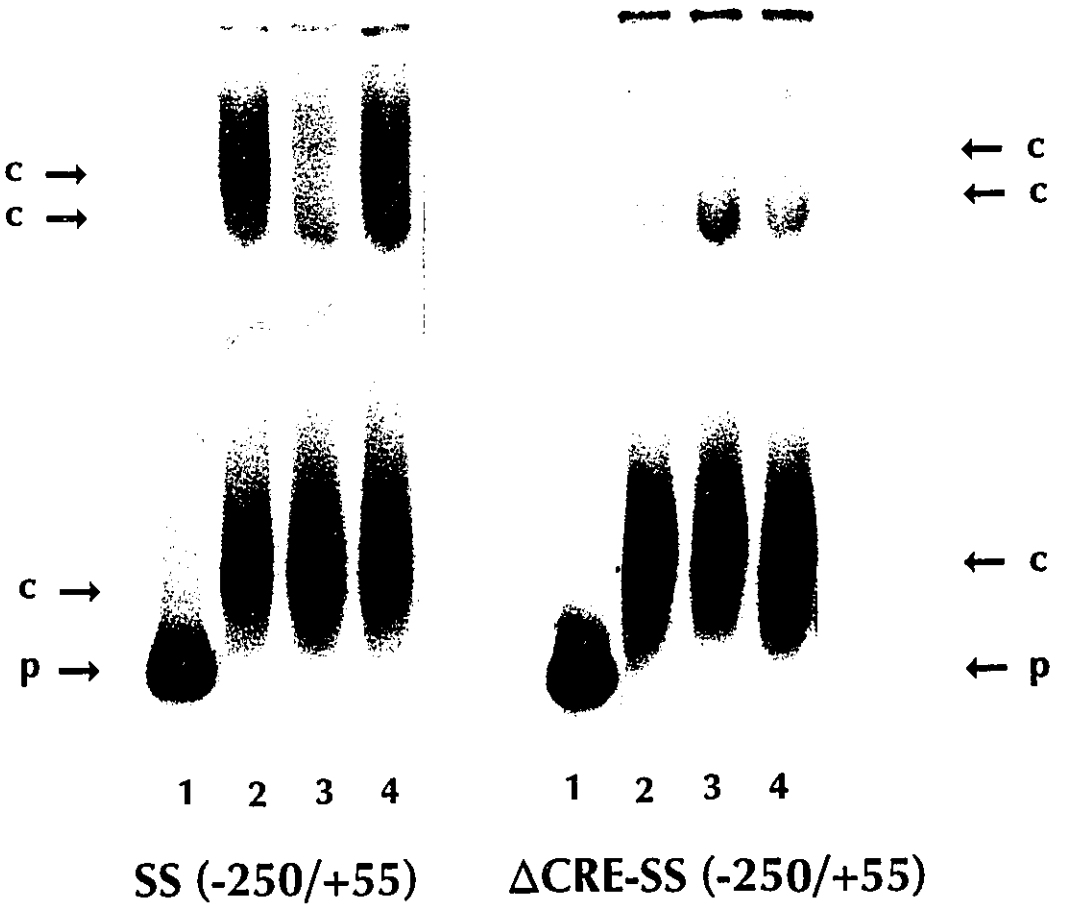


Figure 4-5. Gel mobility shift assay confirmed CRE mutation in SS gene promoter. [^{32}P]-labelled cognate (Panel A) or CRE mutant (Panel B) SS promoter fragments (-250 to +55 bp) were incubated with nuclear extract from PC12 cells. In both panels: lane 1, probe (50,000 cpm) alone; lanes 2 and 4, probe incubated with PC12 nuclear extract (10 μg protein); lane 3, with PC12 nuclear extract and 9 pmol of CRE consensus oligonucleotide (500-fold molar excess). Note that there are three retarded DNA-protein complexes (c, arrows) formed in lane 2 and 4 with cognate promoter (panel A); labelling of the second one is competitively inhibited by cold CRE oligo (lane 3); the same band is not formed in lane 2 and 4 of Δ-CRE SS promoter. (p, free probe; c, DNA-protein complex)

revealed a retarded band (Figure 4-6, panel A, lane 3). The specificity of the labelled complex was tested by competition experiments with synthetic oligonucleotides. Addition of 500 fold molar excess of unlabelled GRE consensus oligonucleotide to the binding reaction specifically inhibited complex formation (lane 5), whereas the same concentration of unlabelled CRE consensus oligonucleotide was without effect (lane 4). To further validate the specificity of somatostatin DNA-protein interaction, parallel gel electrophoretic mobility shift assay were conducted with the -71 to +55 bp somatostatin promoter DNA (Figure 4-6, panel B). A promoter DNA complex between -71 to +55 bp somatostatin DNA and PC12 nuclear extract was detected as a high molecular weight band (lane 3) whose labelling was inhibited by excess unlabelled CRE consensus oligonucleotide (lane 4) but not by GRE oligonucleotide (lane 5). In contrast to the binding observed between somatostatin promoter DNA and PC12 nuclear extract, purified glucocorticoid receptor DNA binding domain protein ($GR\text{-}DBD_{440\text{-}525}$) failed to bind to either somatostatin promoter fragments (Figure 4-6, lane 2 in panel A and B). The receptor protein was capable of retarding the mobility of labelled synthetic consensus GRE but not CRE (data not shown).

7. DEX promotes somatostatin gene transcription independently of tissue specific somatostatin enhancer elements

In view of recent evidence for a pancreatic islet cell-specific transcriptional enhancer SMS-UE, which cooperates with CRE and falls within the somatostatin promoter segment which also confers glucocorticoid responsiveness (-250 to -71 bp), I compared DEX-induced activation of somatostatin gene transcription in PC12 cells with that in 1027 B_2 cells. 1027B_2 cells exhibit high level constitutive expression of the somatostatin gene under the control of

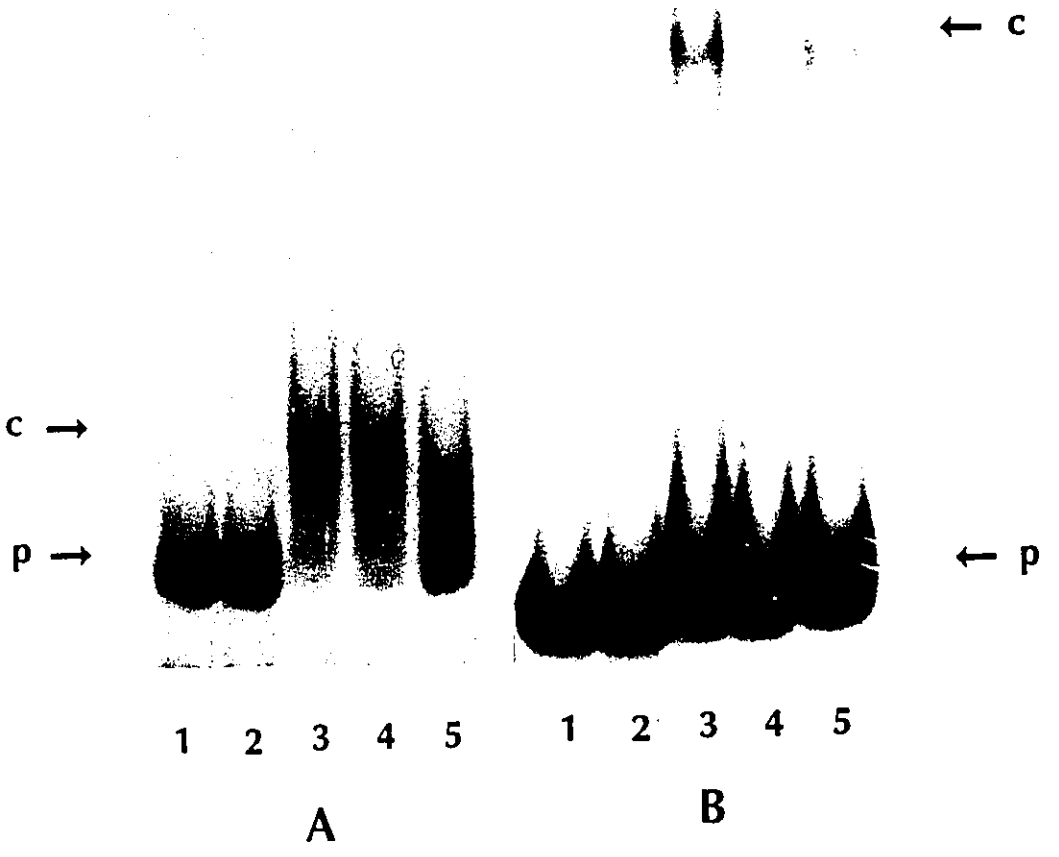


Figure 4-6. Gel electrophoretic mobility shift assays with [^{32}P]-labeled SS promoter fragments and nuclear extract from PC12 cells. **Panel A**, Sal I/Bgl II fragment (-250 to -71 bp) of SS DNA as probe. Lane 1, probe alone (50,000 cpm); 2, with glucocorticoid receptor DNA binding domain protein (GR-DBD$_{440\text{-}525}$) 1 ng; 3, with PC12 nuclear extract (7 µg protein); 4, with PC12 extract and 9 pmol CRE oligonucleotide; 5, with PC12 extract and 9 pmol GRE oligonucleotide. Note the retarded DNA-protein complex (c, arrow) in lane 3, labelling of which is competitively inhibited by preincubation with GRE (lane 5) but not CRE (lane 4). **Panel B**, Identical conditions to those described in Panel A, except for the radiolabelled probe (-71 to +55 bp SS DNA). A higher molecular weight retarded DNA-protein complex (c, arrow) is observed (lane 3), labelling of which is specifically inhibited by unlabelled CRE (lane 4) but not GRE (lane 5). (p, free probe; c, DNA-Protein complex)

islet cell-specific nuclear proteins which bind to and activate the SMS-UE. As in PC12 cells, DEX induced dose-dependent stimulation of CAT activity in $1027B_2$ cells transfected with pSS-750 CAT (Figure 4-7) with a maximum 2.1 fold response virtually identical to that found in PC12 cells. In keeping with their known unresponsiveness to cAMP, $1027B_2$ cells showed no stimulation of pSS-750 CAT expression with forskolin, and no synergism between DEX and forskolin. As in PC12 cells, DEX is inactive to pSS-71CAT construct in $1027B_2$ cells.

DISCUSSION

The present studies provide the first evidence of direct transcriptional enhancement of the somatostatin gene by glucocorticoids. The effect of glucocorticoids was hormone-specific and could not be demonstrated with other steroid and thyroid hormones, or with retinoic acid. The action of glucocorticoids was dependent on a functional interaction with CRE/CREB and related binding proteins (75) and led to an additive response between the two regulatory elements.

Transcriptional control by steroid and thyroid hormones is mediated by a family of nuclear receptors that are activated by ligand and bind to *cis* regulatory elements in specific target genes (140, 176, 243). Recent reports have provided increasing evidence for functional interactions between the nuclear receptors and other transcription factors not involving a natural promoter (244-248, 300). This is particularly the case with glucocorticoid-inducible genes which frequently show GREs contiguous with DNA regulatory sequences for other transcription factors in the promoter region. These include binding sites for SP1, NF1, CACCC and CCAAT box binding proteins (244, 300), CREB (245, 248) and AP-1 factors (246, 247), or a second GRE (244). Interaction between the glucocorticoid receptor and these

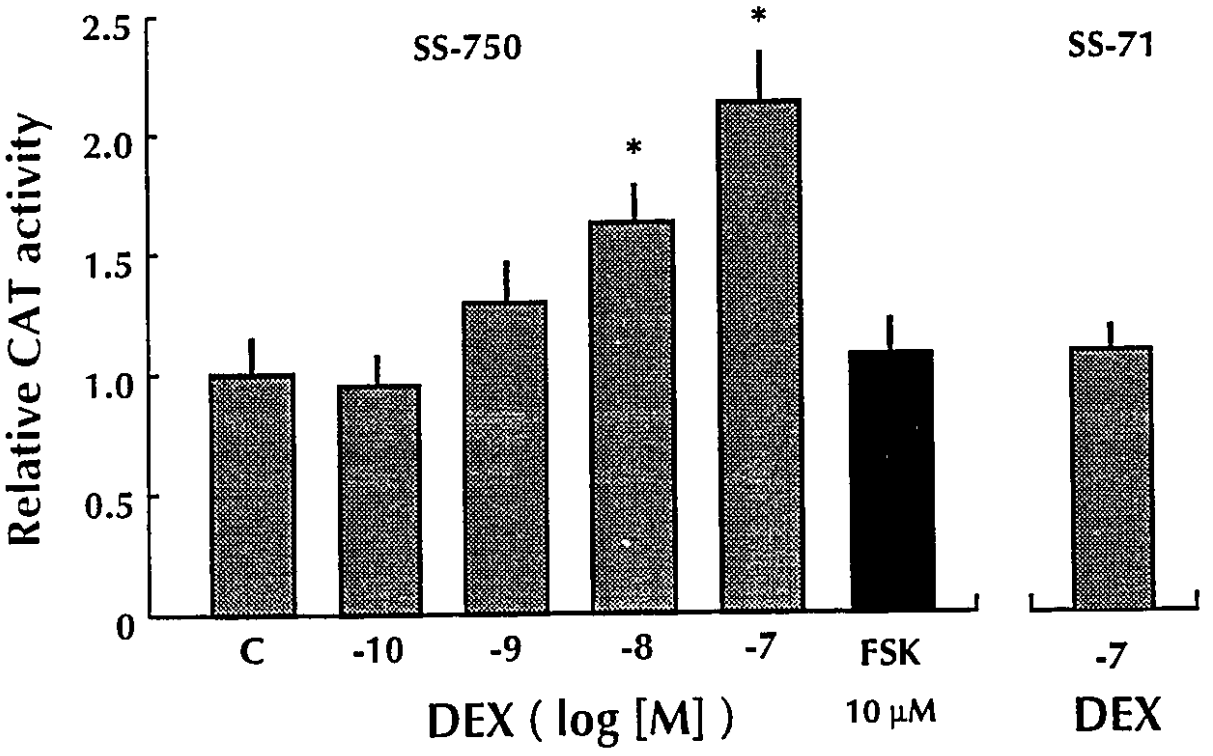


Figure 4-7. Effect of DEX on pSS -750 CAT expression in $1027B_2$ cells. Following transient transfection, the cells were incubated with DEX for 48 h and assayed for CAT. CAT activity is expressed relative to untreated controls. DEX induced dose-dependent stimulation of SS-CAT expression with a maximum 2.1 fold effect at 10^{-6} M. In contrast, $1027B_2$ cells are not responsive to forskolin (10 μM) treatment; DEX is inactive to pSS-71CAT construct. (n = 3, *$p<0.05$)

accessory factors can lead to either enhancement or repression of gene transcription. Synergism between the glucocorticoid receptor and other transcription factors was first reported for NF1, SP1, and CACCC binding proteins in genes encoding tyrosine aminotransferase and tryptophan oxygenase (244, 300). Functional cooperativity in these instances was shown to be critically dependent on the spacing between the regulatory elements but not their orientation, and mediated by protein-protein interaction rather than cooperative DNA binding (244, 300). These studies additionally revealed that a single copy consensus GRE sequence isolated at a distance from the transcription start site was insufficient for gene induction but became active when a second regulatory element including a second GRE was positioned nearby (244). This suggests a basic model of glucocorticoid-induced transcriptional activation requiring multiple GREs or a combination of a GRE with other *cis* elements for constitution of a hormone-inducible enhancer. More recently, the glucocorticoid receptor has been shown to repress gene expression by functional interference with several different transcriptional activators (245-248). For instance, repression of the hCG α-subunit and POMC genes appears to be mediated by competitive binding of the glucocorticoid receptor to DNA regulatory sequences termed negative GRE, thereby displacing transcriptional activators such as CREB in the case of the α-subunit gene (245), and the CAAT box binding factor in the case of the POMC gene (248). For other genes such as those for osteocalcin, proliferin, and collagenase, glucocorticoids repress an AP-1 site by a different mechanism involving direct protein-protein interaction between c-Jun and the glucocorticoid receptor leading to a mutual inhibition of their DNA binding activities (246, 247).

Dependence of glucocorticoid receptor-mediated enhancement of somatostatin gene transcription on CRE/CREB activation was shown here by two lines of evidence. First, the

PKA deficient mutant cell line A126-1B2, which is incapable of inducing cAMP-mediated gene transcription due to an inability to phosphorylate and thereby transactivate CREB, was also unresponsive to glucocorticoid induction. Secondly, replacement of the canonical CRE in the somatostatin gene upstream region with an inert sequence abolished both cAMP and glucocorticoid responsiveness. Failure of DEX to induce the -71 bp somatostatin promoter suggests that the glucocorticoid receptor needs elements further upstream to interact with the CRE/CREB complex. Since the -250 bp promoter showed full glucocorticoid responsiveness, our findings imply a glucocorticoid sensitive region in the -250 to -71 sequence of the somatostatin promoter. Gel electrophoretic mobility shift assay confirmed that the -250 to -71 bp region of the somatostatin promoter (but not the -71 to +55 bp domain) binds specifically to a GRE sensitive nuclear protein suggesting a putative glucocorticoid receptor interaction with somatostatin promoter DNA. Failure of the purified glucocorticoid receptor DNA binding domain protein to bind to somatostatin DNA suggests the requirement of other components of the receptor protein and/or associated binding proteins for responsiveness. For example, retinoid X receptor β is required for both DNA binding and transactivation of retinoic acid receptor to its cognate response element (249); Interaction of glucocorticoid receptor with the promoter of glycoprotein hormone α gene is dependent on cell-specific cofactors (250). Analysis of the somatostatin promoter failed to reveal a classical GRE but did disclose sequences of two possible variants (aGGCTTnnnTtTTCT at -167 bp and aaGATTnnnTGgTCT at -219 bp) which ressemble non consensus GREs. Further studies with promoter deletion mutants combined with DNase I footprint are necessary to map precisely the regulatory elements of the somatostatin gene which interact directly with the glucocorticoid receptor.

There is only one other example of functional cooperativity between the glucocorticoid

receptor and the cAMP signalling pathway, illustrated by the gene for PEPCK (98, 180, 302). The proximal region of the PEPCK gene promoter (-100 to +1 bp) contains the TATA and E/CRE elements in a virtually identical configuration to that found in the somatostatin gene (Figure 1-9). The glucocorticoid response "element" in this gene is a complex unit (hence termed glucocorticoid response unit or GRU) consisting of a tandem array (5' to 3') of a RARE, an IRS, and 2 glucocorticoid receptor binding sites, the entire complex spanning about 110 base pairs (from -451 to -353). The 2 glucocorticoid receptor binding sites are not homologous with the consensus GRE sequence which they match in only 7/12 and 6/12 positions, respectively, and which function independently, each accounting for half of the full response (302). Glucocorticoids and cAMP individually induce PEPCK gene transcription and in combination produce enhancement. This is achieved through a protein-protein interaction between the glucocorticoid receptor and CREB occuring over a distance of 300 bp. Retinoic acid acts on the PEPCK gene synergistically with DEX, and insulin inhibits the DEX and cAMP responses, both individually as well as in combination. In the absence of the accessory factors, the glucocorticoid receptor binding sites alone are inert. In the case of the somatostatin gene promoter, we found evidence of functional cooperativity between glucocorticoids and the CRE/CREB locus which however resulted in an additive rather than a synergistic response. Interestingly, in addition to transcriptional cooperativity between the glucocorticoid receptor and the CRE/CREB unit, somatostatin gene expression is also capable of inhibition by insulin (58). A survey of the somatostatin gene promoter for these accessory factor binding sites shows 2 contiguous putative RARE (-373 to -366 bp) and IRS (-330 to -321 bp) motifs considerably further upstream from the potential glucocorticoid receptor responsive site. Despite these differences, there are sufficient similarities to suggest that the

PEPCK gene could serve as a useful model for further studies to determine the precise nature of the transcriptional interaction between the glucocorticoid receptor, CRE, and perhaps the retinoic acid receptor and insulin sensitive transcription factors in the somatostatin gene.

Since glucocorticoids act on numerous target genes, an interaction between glucocorticoids and the cAMP pathway could also occur at points more proximal to the gene through induction of one or more components of the cAMP signalling pathway. For instance, glucocorticoids have been reported to activate adenylyl cyclase in GH_3 cells (251) and could conceivably enhance cAMP dependent gene transcription via this mechanism. Such an action, however, cannot explain the ability of glucocorticoids to potentiate the effect of high concentrations of exogenous cAMP or forskolin found in the present study. Moreover, the results with the -71 bp construct argue against this possibility. It is not known whether glucocorticoids regulate the expression of PKA, although glucocorticoids stimulate CREB expression in rat C6 glioma cells (252). Induction of either of these 2 molecules could represent an indirect mechanism for glucocorticoid stimulation of somatostatin gene transcription but cannot account for the additive effect between the activated glucocorticoid receptor and the cAMP pathway. Our results with CREB cotransfection of PC12 cells suggest that overexpression of this molecule alone is insufficient for activating somatostatin gene transcription. This is probably due to an abundance of endogenous CREB and related factors in these cells (75, 77, 253), as well as the requirement for not just CREB molecules, but for phosphorylated CREB for transcriptional activation (253, 319).

Recent studies have identified a number of *cis* regulatory elements upstream from CRE in the somatostatin promoter which exercise both positive and negative transcriptional control (24, 25, 77). The positive control element has been characterized as the SMS-UE,

which consists of 3 functionally interdependent *cis* domains arranged in tandem and which bind α-CBF protein (nucleotide positions -113 to -107), a homeodomain protein IDX-1/STF-1 (positions -96 to -88), and CREB (which binds to a half CRE-like palindrome at positions -88 to -83). IDX-1/STF-1 is an pancreatic islet specific transcription factor belonging to the LIM family of homeotic genes which acts in concert with the adjacent accessory transcription factors assembled on SMS-UE to drive high level expression of the somatostatin gene in islet somatostatin producing cells (75, 77, 254). Interestingly, SMS-UE interacts synergistically with consensus CRE located in the proximal promoter region both basally and in response to cAMP induction (24, 25, 77). Since the putative glucocorticoid receptor binding domain in the somatostatin promoter maps to a region which overlaps SMS-UE, we compared glucocorticoid responsiveness in PC12 cells with 1027 B_2 cells which contain islet cell specific proteins and exhibit high level expression of the somatostatin gene. Our finding of approximately equal stimulation of somatostatin-CAT activity by DEX in the two cell lines suggests that the DEX effect does not require SMS-UE binding proteins and is not restricted to PC12 cells. Although 1027B_2 cells show high basal somatostatin gene expression, they lack cAMP responsiveness due to a deficiency of PKA activity (unpublished observations; 298). How then can DEX induction of transcriptional activity in these cells be reconciled with a complete absence of DEX effect in A126-1B2 cells or in PC12 cells transfected with the CRE-substituted promoter construct? One explanation is that unlike A126-1B2 cells, 1027 B_2 cells have a relative rather than an absolute deficiency of PKA and can generate low level CREB phosphorylation sufficient to support DEX-induced gene transcription. A second possibility is that of cooperation between the glucocorticoid receptor and one or more components of the SMS-UE regulatory domain in 1027 B_2 cells, as an alternative to a

glucocorticoid receptor-CREB interaction in these cells.

The concentration of DEX at which significant induction of somatostatin-CAT activity occurred (10^{-8} M) falls within the physiological range especially if allowance is made for differences in the responses between transfected and endogenous somatostatin gene. Since cAMP is the predominant regulator of somatostatin gene transcription and since glucocorticoids and cAMP influence many of the same physiological processes, a cooperative relationship between the two pathways is clearly functionally relevant. Transcriptional activity of the somatostatin gene however can explain only part of glucocorticoid action on steady state SS-mRNA levels that we found in rat tissues, viz. the stimulatory component (63). The additional more potent effect of glucocorticoids on inhibition of SS-mRNA accumulation must therefore be mediated through a post-transcriptional mechanism (63, 64). Studies of the effects of glucocorticoids on SS-mRNA stability are necessary to investigate this question further.

In conclusion, I have demonstrated positive transcriptional control of the somatostatin gene by glucocorticoids. Glucocorticoid-induced transactivation shows dependence on PKA activity, and may be mediated via interaction between the glucocorticoid receptor and CREB. DNA sequences upstream from CRE between -250 and -71 base pairs in the somatostatin promoter appear necessary for the glucocorticoid effect.

CHAPTER V. GLUCOCORTICOIDS INHIBIT SOMATOSTATIN GENE EXPRESSION THROUGH ACCELERATED DEGRADATION OF SS-mRNA IN HUMAN THYROID MEDULLARY CARCINOMA (TT) CELLS

As described in Chapter III, glucocorticoids exert significant effects on somatostatin peptide production and steady state mRNA levels in normal somatostatin producing tissues *in vivo*, *in vitro*, and in cultured rat islet somatostatin producing tumor cells (63). The glucocorticoid effect is tissue-specific, time- and dose-dependent (low doses being stimulatory and high doses inhibitory), and at least in part due to a direct action of the steroid hormone on somatostatin producing cells. At a molecular level, glucocorticoids regulate target gene expression both transcriptionally as well as post-transcriptionally through alterations in mRNA stability. Transcriptional control by glucocorticoids occurs through a DNA protein interaction between the activated glucocorticoid receptor and the GRE (176, 311). GREs subserve complex transcriptional roles as either positive, negative, synergistic or composite regulatory domains in different target genes (311). In an attempt to elucidate the nature of the molecular interactions between glucocorticoids and the somatostatin gene, I have reported that DEX activates somatostatin promoter-CAT activity in transfected PC12 cells through cooperative interaction with CREB suggesting that stimulation of SS-mRNA by glucocorticoids is transcriptionally mediated (Chapter IV, 191).

Although glucocorticoids are well known to regulate mRNA degradation, the molecular mechanisms are less well defined. Mendelson *et al.* have reported that glucocorticoids regulate SP-A and its mRNA accumulation in cultured human fetal lung in a dose-dependent biphasic manner, low doses (10^{-10} - 10^{-9} M) being stimulatory and high

doses ($\geq 10^{-8}$ M) being inhibitory. The stimulatory effect was shown to be mediated by transcriptional induction of the SP-A gene whereas inhibition was found to be due to accelerated SP-A mRNA degradation (117, 117a). This model is virtually identical to the scheme that we have developed for explaining somatostatin glucocorticoid interaction with the exception of glucocorticoid-induced alterations in SS-mRNA stability which remain to be determined. Accordingly the present study was set up to investigate the molecular mechanisms underlying DEX inhibition of SS-mRNA using as a model human thyroid carcinoma TT cells in which DEX has been previously reported to produce solely an inhibition of somatostatin secretion and mRNA accumulation (64). I excluded a direct transcriptional effect of DEX on somatostatin gene by somatostatin promoter - CAT transfection and nuclear run-on assays and analyzed the effects of transcriptional and translational blockade on the DEX effect on SS-mRNA levels. It is shown that as in the case of the SP-A gene, glucocorticoids inhibit SS-mRNA by accelerating its degradation.

RESULTS

1. Glucocorticoids induce a late onset reduction in somatostatin secretion and SS-mRNA in TT cells

Cultured TT cells synthesized and secreted large quantities of somatostatin amounting to 90 ng SSLI content in 10^6 cells and 18 ng SSLI secreted/10^6 cells/24 h. Figure 5-1 shows the time course of somatostatin secretion (bottom panel) and SS-mRNA (top panel) in TT cells incubated with or without DEX (1 μM) for 6, 12, 24, 48, and 72 h. Control cells displayed an exponential increase in SSLI in the medium over the 72 h period of incubation. In the presence of DEX, somatostatin secretion was significantly reduced by 25% at 24 h,

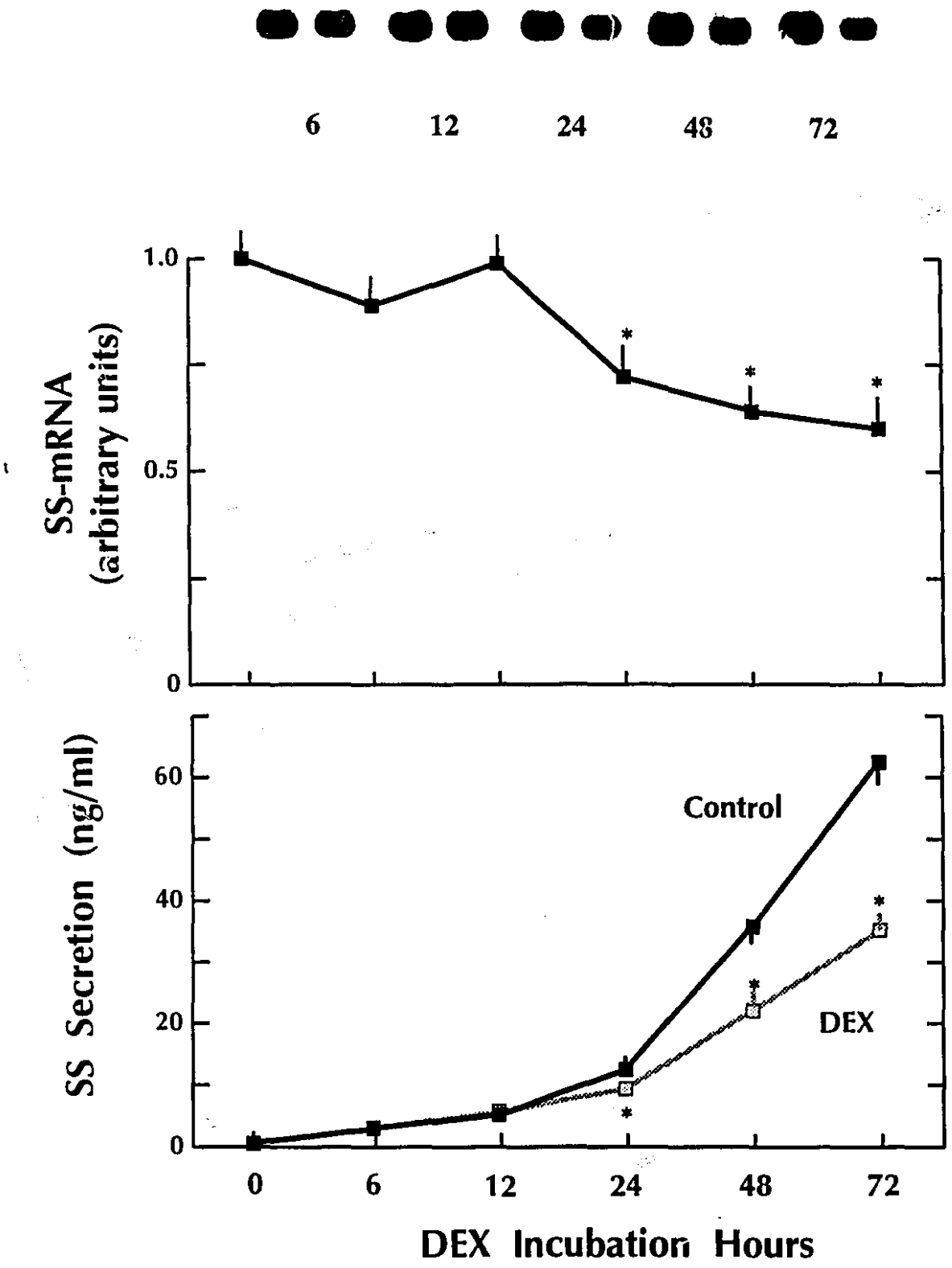

6
12
24
48
72
1.0
0.5
0
SS-mRNA
(arbitrary units)
*
*
*
60
40
20
0
SS Secretion (ng/ml)
Control
DEX
*
*
*
0
6
12
24
48
72
DEX Incubation Hours

Fig 5-1

Figure 5-1. Time course of DEX inhibition of SS-mRNA (top panel) and SS secretion (bottom panel). Cells were incubated with DEX (1 μM) or vehicle for the time periods indicated before being subjected to Northern blot analysis and radioimmunoassay. The inset depicts a representative Northern blot of SS-mRNA in 7 μg total RNA from paired control and DEX-treated dishes at various time points. DEX (10^{-6} M) induced a reduction in both SS-mRNA and SS secretion after a lag period of 12 h. (n=5, *$p<0.05$).

39% at 48 h, and 44% at 72 h. DEX-induced inhibition of somatostatin secretion was parallelled by a comparable suppression of SS-mRNA beginning at 24 h (71% of control), and continuing at 48 h (65% of control), and 72 h (60% of control). Incubation of TT cells with dbcAMP (5 mM) used as a positive control, led to a 3.5 fold increase in secretion of SSLI as well as a 2 fold induction in SS-mRNA levels at 48 h (data not shown).

2. Glucocorticoids induce a dose-dependent reduction in somatostatin secretion and SS-mRNA in TT cells

Figure 5-2 depicts the dose response effect of DEX incubated for 48 h on somatostatin secretion and SS-mRNA levels in TT cells. DEX inhibited somatostatin secretion from 10^{-8} M to 10^{-5} M with a maximum 60% effect. There was a parallel dose-dependent inhibition of SS-mRNA level from 10^{-8} M to 10^{-5} M with a maximum 60% inhibition. Cyclophilin-mRNA used as a control was unaffected by DEX (Figure 5-2, top panel).

3. Glucocorticoids do not affect somatostatin-CAT reporter gene expression in TT cells

Figure 5-3 is an autoradiogram of a representative CAT assay of TT cells transfected with 750 bp length of 5' flanking somatostatin promoter ligated to the reporter CAT gene. This segment of the somatostatin promoter contains a number of well-defined regulatory elements including a TATA box, a CRE, a SMS-UE and an atypical GRE previously shown to be responsible for glucocorticoid dependent activation of the somatostatin promoter (191). Incubation with DEX 1 μM for 48 h had no effect on the basal level of somatostatin-CAT expression in TT cells. This indicates that DEX does not affect somatostatin gene promoter

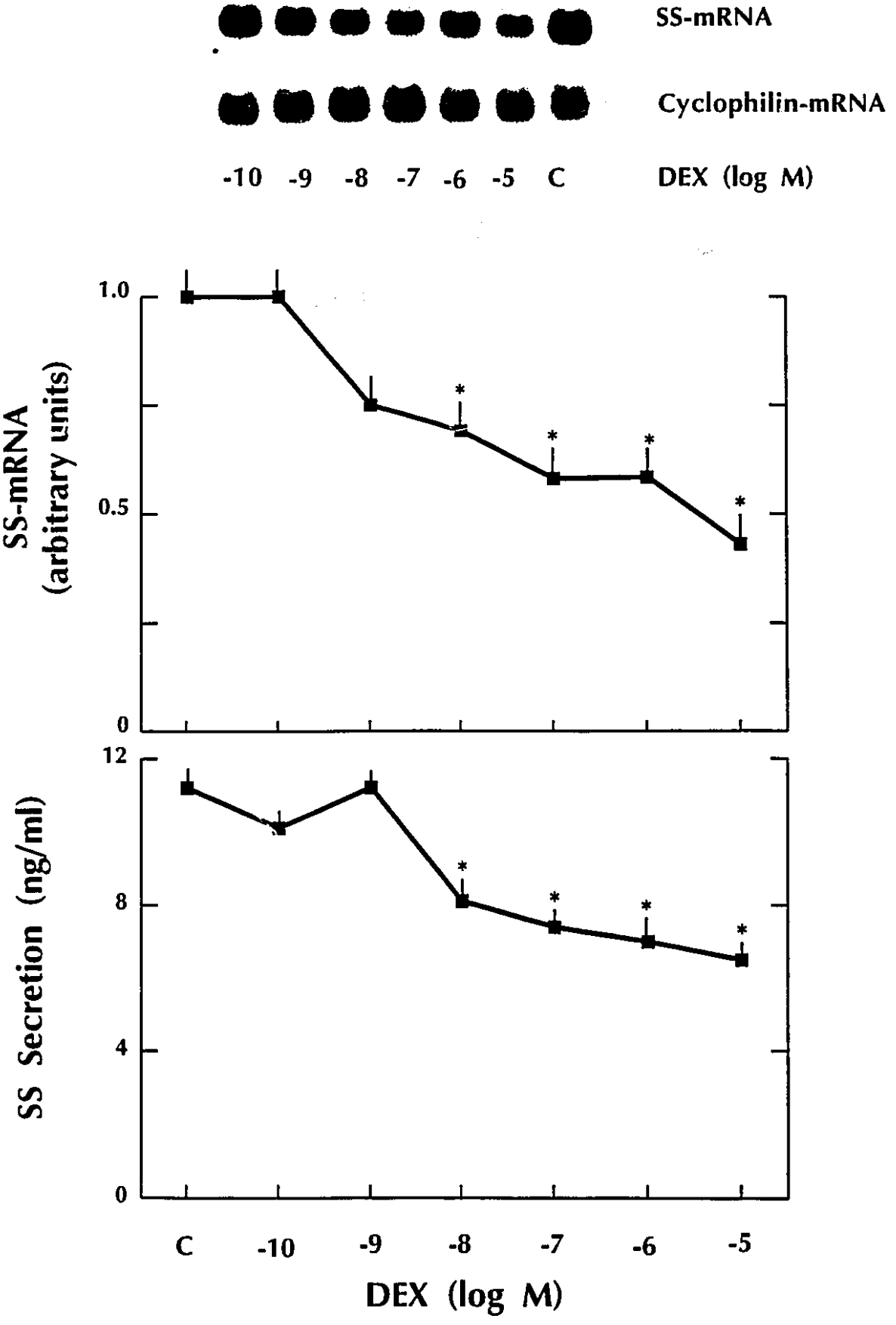
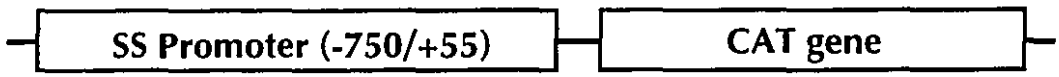

SS-mRNA
Cyclophilin-mRNA
-10 -9 -8 -7 -6 -5 C
DEX (log M)
SS-mRNA (arbitrary units)
1.0
0.5
0
SS Secretion (ng/ml)
12
8
4
0
C -10 -9 -8 -7 -6 -5
DEX (log M)

Fig 5-2

Figure 5-2. Dose-dependent inhibition of SS-mRNA (top panel) and SS secretion (bottom panel) by DEX following a 48-h incubation. DEX produced a maximum 60% inhibition of both SS-mRNA and SS secretion. The inset depict representative Northern blots of SS-mRNA and cyclophilin-mRNA used as control. (n=5, *$p<0.05$)



Reporter Gene Construct

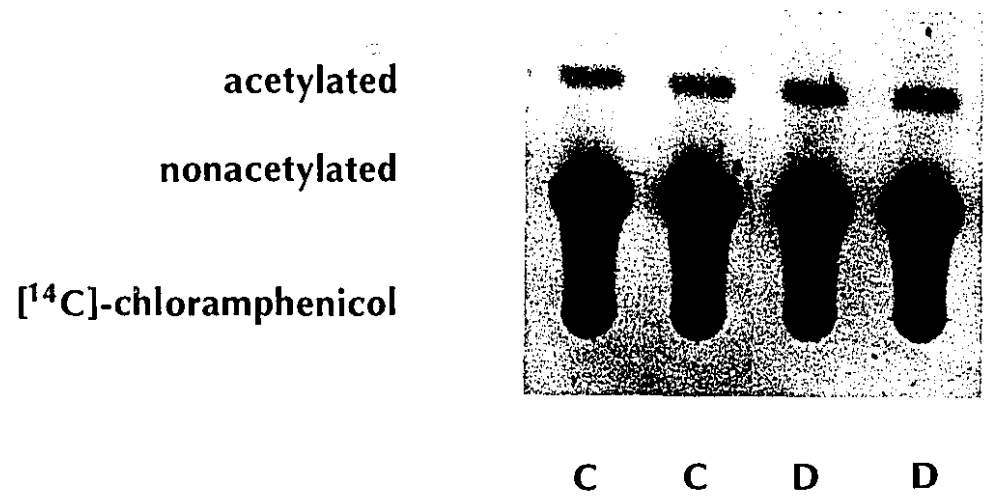


Figure 5-3. DEX does not affect pSS-750CAT expression in TT cells. Cells were transfected with the chimeric reporter gene construct (illustrated on top of the autoradiogram) and incubated with DEX (1 μM) for 48 h and assayed for CAT activity. The figure depicts an autoradiogram of a representative assay from 2 seperate experiments. (C, control; D, DEX-treated)

activity in these cells at least for the part of the promoter studied, and that transcriptional inhibition is not the mechanism through which DEX suppresses steady state SS-mRNA accumulation.

4. Glucocorticoids do not reduce the rate of somatostatin gene transcription in nuclear run-on assays

To further assess transcriptional effects of DEX on the endogenous somatostatin gene, nuclear run-on assays were carried out. The rate of nascent somatostatin transcript formation in isolated nuclei from control TT cells or TT cells exposed to DEX (1 μM) or cAMP (5 mM) for 18 h was measured (Figure 5-4). DEX produced a small but not significant increase in somatostatin gene transcription. By contrast, cAMP used as a positive control, produced a 2 fold enhancement of somatostatin gene transcription. Cyclophilin gene used as a negative control showed no alteration in the level of transcription when treated with DEX or cAMP. The pBluescript vector included as an additional negative control for hybridization specificity in these experiments elicited no signal.

5. Glucocorticoid inhibition of SS-mRNA is blocked by actinomycin D

In order to determine the effect of DEX on SS-mRNA stability, SS-mRNA decay curves were obtained in TT cells exposed for 6, 12, 24, 48, and 72 h with actinomycin D with or without DEX. The results from control, 24 h and 48 h incubations are shown in detail in Figure 5-5. There was no change in SS-mRNA accumulation in control cells at any time point up to 72 h. Treatment of TT cells with actinomycin D to block transcription produced no change in SS-mRNA levels compared to control. Likewise, DRB (an RNA polymerase

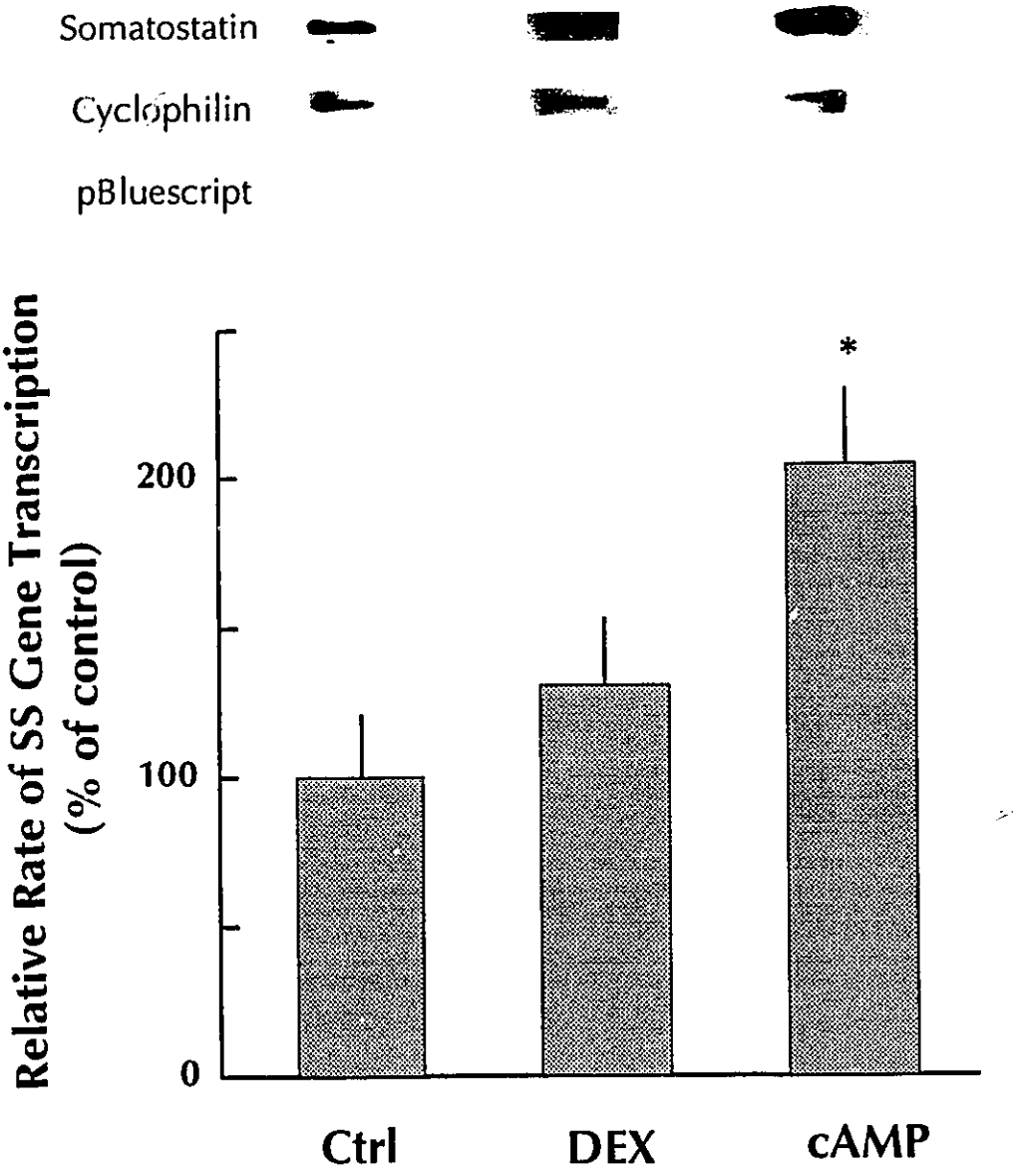


Figure 5-4. Effect of DEX and cAMP on SS gene transcription. Nuclear run-on assays were performed, as described in Materials and Methods, on nuclei of TT cells cultured for 18 h in the presence or absence of DEX (1 μM) or dbcAMP (5 mM). Autoradiographs of a representative experiment illustrating SS and cyclophilin cDNAs, and plasmid pBluescript with no insert to control for nonspecific hybridization are shown in the top panel. The mean relative transcription rates ± SE (from three separate experiments) for SS gene is shown in the bottom panel. (n=3, *$p<0.05$)

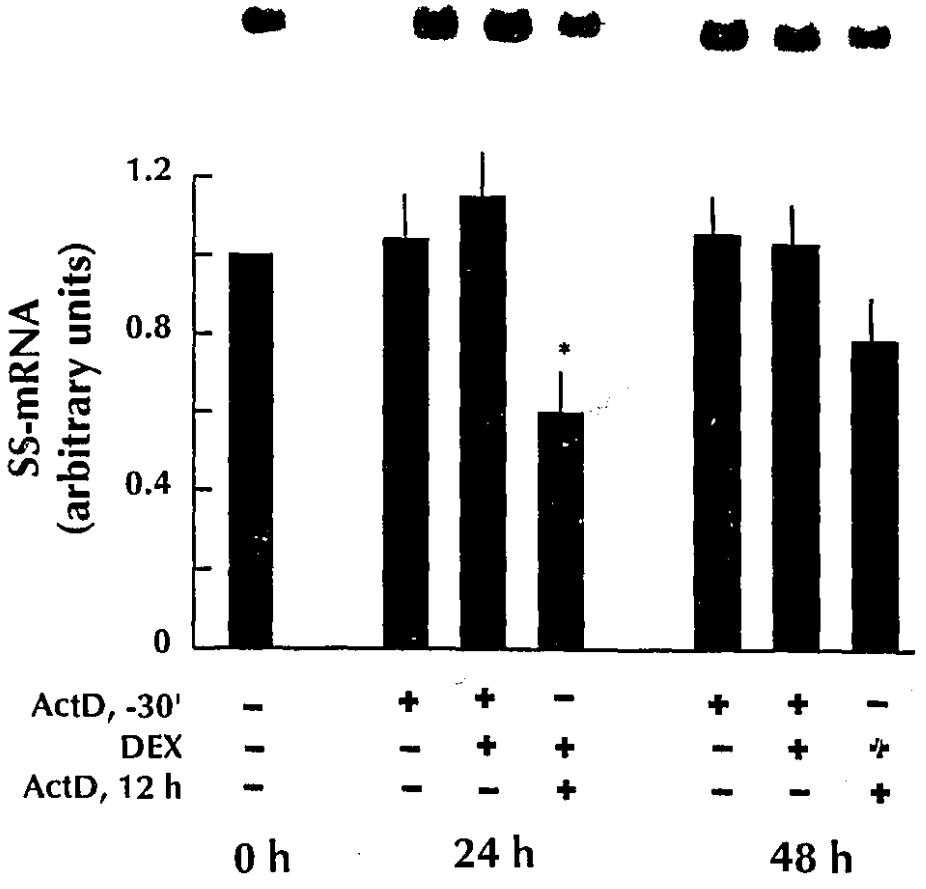


Figure 5-5. DEX effect on SS-mRNA level is blocked by actinomycin D (ActD, 5 μg/ml) when added 30 min in advance, but not when added 12 h after DEX incubation. TT cells were incubated with DEX for either 24 or 48 h as indicated. Actinomycin D alone caused no change in SS-mRNA level. The inset depicts a representative Northern blot. (n=3, *$p<0.05$ vs untreated control)

inhibitor, 20 µg/ml) also failed to affect steady state SS-mRNA levels (data not shown), indicating that SS-mRNA is either very stable or that its degradation is dependent on ongoing transcription. Coincubation with actinomycin D blocked the DEX-induced inhibition of SS-mRNA. However, when actinomycin D was added 12 hours after DEX, the inhibitory effect of DEX on SS-mRNA was partially restored (Figure 5-5).

Although actinomycin D did not change the level of SS-mRNA, with or without DEX treatment, it shortened the size of the SS-mRNA (Figure 5-6, lanes 1-3, lower panel). Cyclophilin-mRNA included as a control in these experiments, showed both a reduction in the level as well as the size of its mRNA (Figure 5-6, lanes 1-3, upper panel). Treatment of TT cells with cAMP increased SS-mRNA level 2.2 fold without affecting cyclophilin mRNA. Actinomycin D reduced cAMP stimulated SS-mRNA levels from 2.2 to 1.6 fold and simultaneously reduced the size of the mRNA transcript. These results demonstrate that actinomycin D reduces steady state mRNA levels for cyclophilin through transcriptional blockade of its gene. In addition, actinomycin D produced a physical alteration in both somatostatin and cyclophilin mRNA transcripts which rendered the SS-mRNA but not the cyclophilin mRNA species resistant to DEX-induced degradation. This suggests that DEX-mediated turnover of SS-mRNA is an active process which is dependent on transcription of other factors.

6. Glucocorticoid inhibition of SS-mRNA is blocked by cycloheximide

To investigate the effect of blockade of new protein synthesis on DEX-induced inhibition of SS-mRNA, control and DEX-treated TT cells were incubated with cycloheximide 10 µg/ml using a similar experimental protocol to that described for the actinomycin D studies

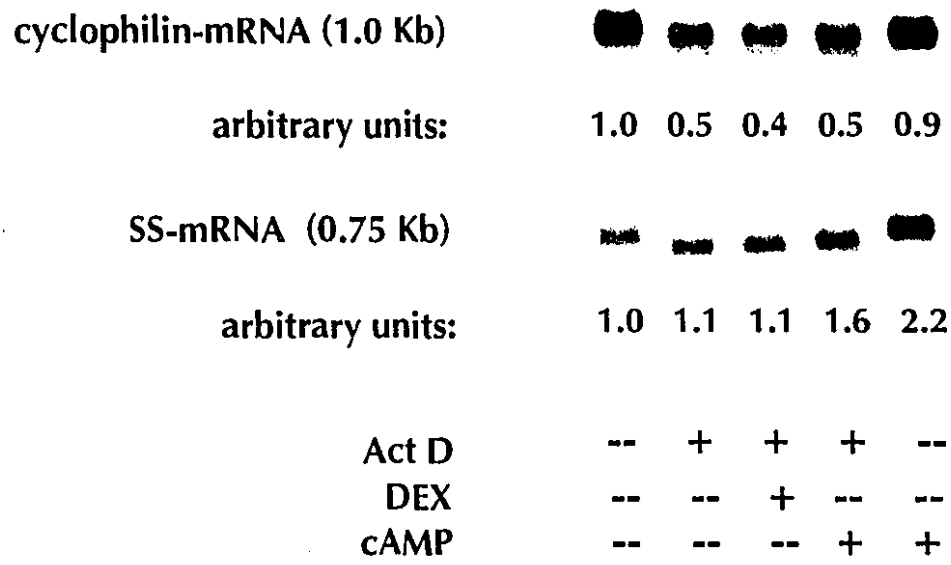


Figure 5-6. Northern blots showing effect of DEX with or without actinomycin D (ActD) on SS-mRNA transcripts. TT cells were incubated with or without test agents for 24 h. DEX alone reduced the level of SS-mRNA 29% without changing its size as shown in Figure 1. Actinomycin D shortened the size of SS-mRNA but failed to induce its mRNA degradation (lanes 1-3, lower panel). Cyclophilin-mRNA, included as control, showed both a reduction in the level as well as the size of its mRNA (lanes 1-3, upper panel). Treatment of TT cells with dbcAMP increased SS-mRNA level 2.2 fold without affecting cyclophilin-mRNA (lane 5). Actinomycin D reduced cAMP stimulated SS-mRNA levels from 2.2 to 1.6 fold and simultaneously reduced the size of the transcript (lanes 4 vs 5, lower panel). Actinomycin D produced a physical alteration in both SS and cyclophilin transcripts which rendered the SS-mRNA but not the cyclophilin mRNA species resistant to DEX-induced degradation. Data representative of 3 experiments.

(Figure 5-7). In contrast to the effect of actinomycin D, treatment with cycloheximide alone produced a significant reduction in SS-mRNA levels comparable to that induced by DEX alone. There was no additive effect of cycloheximide when coincubated with DEX. These data indicate that the DEX effect on SS-mRNA is blocked by cycloheximide.

DISCUSSION

TT cells consist of transformed calcitonin- and CGRP-producing parafollicular (C) cells, derived from a human medullary thyroid carcinoma. Like normal C cells, TT cells have also been found to coexpress the somatostatin gene and have provided a useful model for studying the regulation of somatostatin and SS-mRNA (255). Previous studies with these cells have shown that somatostatin secretion and mRNA levels are stimulated by cAMP treatment and inhibited by glucocorticoids and 1,25-dihydroxy vitamin D_3 (51, 64, 255). Here I have confirmed the stimulatory and inhibitory effects respectively of cAMP and glucocorticoids on steady state SS-mRNA levels and extended these observations to an analysis of the molecular mechanisms underlying the glucocorticoid effect. I demonstrate that DEX inhibition of SS-mRNA in TT cells (i) occurs at high doses of the steroid ($\geq 10^{-8}$ M) and following a lag period of 12-24 h; (ii) is not due to transcriptional inhibition of the somatostatin gene as determined by nuclear run-on assays and by the inactivity of DEX on somatostatin promoter-CAT expression; and (iii) is post-transcriptionally mediated and is dependent on ongoing gene transcription.

Until recently, studies of the regulation of eukaryotic mRNA levels have focused on control of gene transcription. However, the level of a cellular mRNA represents a balance between the rates of its synthesis and degradation. In the last few years, the regulation of

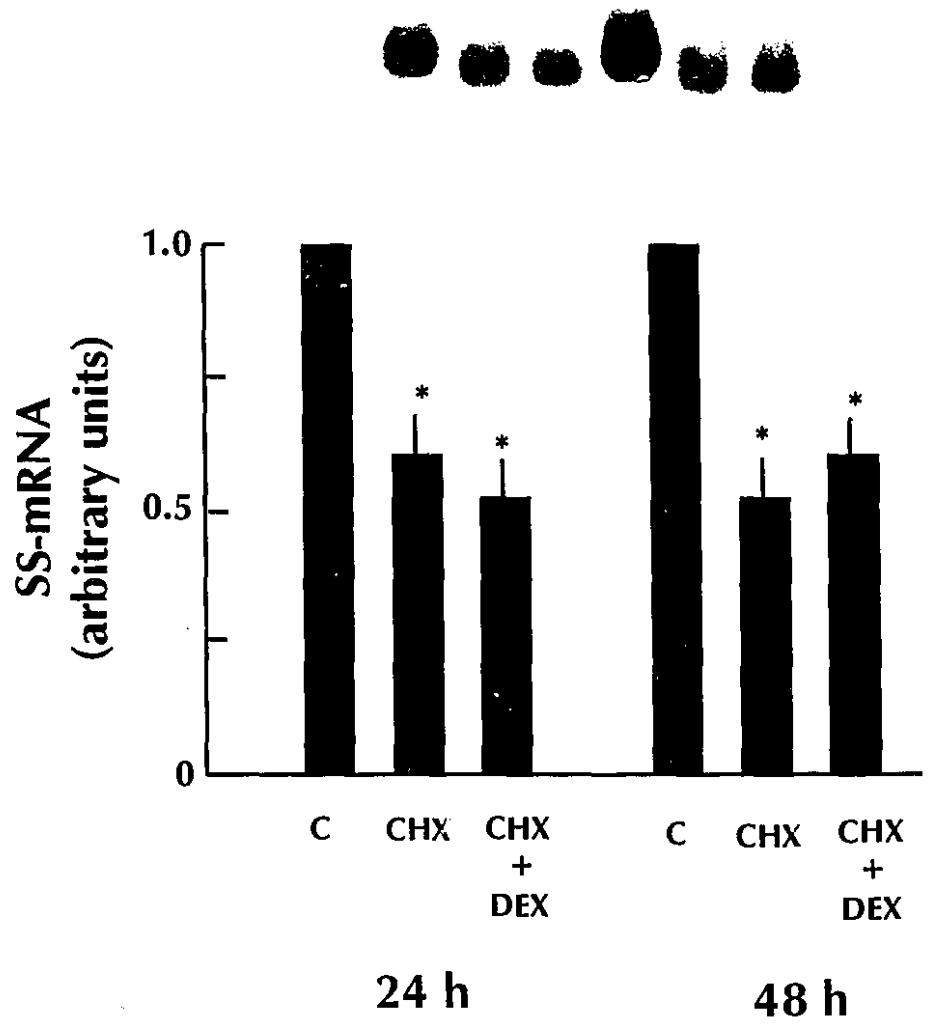


Figure 5-7. DEX effect on SS-mRNAlevel in TT cells is blocked by cycloheximide (CHX). CHX (10 μg/ml) was added 30 min in advance to DEX (1 μM). The cells were then incubated for either 24 or 48 h as indicated. CHX alone dramatically reduced SS-mRNA level. The inset depicts a representative Northern blot. (n = 3, *$p<0.05$ vs untreated control)

cytoplasmic mRNA stability has emerged as an important control point for a variety of genes including those regulated by glucocorticoids (100, 121, 122, 155). Most mRNAs are protected by a 5' cap structure and 3' poly(A) tract complexed to poly(A) binding proteins which render them stable until they are subjected to a nucleolytic attack (100, 182). Nucleases digesting mRNA and other proteins promoting nuclease attack function through interaction with mRNA structures or sequences under regulation of hormones and other signals. Current understanding of the mechanism of mRNA degradation is limited and may involve one of three pathways: (i) destabilizing elements in the transcript such as the ARE in the 3'-UTR of e.g. c-fos mRNA capable of recruiting poly(A) nucleases which remove the poly(A) tract thereby triggering decapping and degradation of the mRNA (121); (ii) sequence-specific cleavage usually within the 3'-UTR at a stem loop structure independent of poly(A) shortening as in the case of histone mRNAs which are not polyadenylated (121, 135); (iii) degradation involving the synthesis of antisense RNA as demonstrated for prolactin transcripts in pituitary cells and for p53 mRNA in murine erythroleukemia cells (126, 127).

At present there is no evidence that steroid hormone receptors act directly in the cytoplasm to alter mRNA stability. Estrogen, progesterone and androgen receptors are predominantly nuclear proteins. The unliganded glucocorticoid receptor can occur in the cytoplasm but localizes to the nucleus upon hormone binding (176, 311). In keeping with the predominant role of steroid hormone receptors as transcription factors, the glucocorticoid receptor most likely regulates mRNA turnover indirectly through transcriptional control of a mRNA regulating protein. Consistent with this notion is the finding that glucocorticoid-regulated destabilization/stabilization of mRNA for somatostatin found in the present study and for several other target genes that have been previously studied, e.g. GH, insulin, and

PEPCK occurs after a lag period of 6-24 h (102, 109, 112).

Human and rat SS-mRNA transcripts are ~750 bp long and consist of a 5'-UTR (100 bp), coding segment (348 bp), a 3'-UTR (150~200 bp), and a poly(A) tail (~ 150 bp) (21, 256). Within the 3'-UTR there are 2 AU rich regions which could serve as potential motifs for promoting mRNA degradation similar to the AUUUA sites in other mRNAs (130). There have been no previous reports of the half-life of SS-mRNA. In TT cells in the present study, SS-mRNA showed no apparent degradation in time-course studies up to 72 h. Likewise in $1027B_2$ islet somatostatin producing tumor cells, SS-mRNA was found to be stable for 48-72 h (unpublished observations). Actinomycin D is widely used in mRNA half-life studies because of its ability to bind double-stranded DNA and to block transcription (257). It does not appear to have any direct effect on mRNA stability or degradation. Blockade of transcription by actinomycin D failed to affect steady state SS-mRNA levels for at least 48 h in TT cells in the present study as well as in $1027B_2$ cells (unpublished observations) further suggesting that SS-mRNA is a stable transcript with a relatively long half-life. In contrast to actinomycin D, DEX produced a 60% decrease in SS-mRNA levels. Since DEX did not alter somatostatin gene transcription, this effect appeared to be post-transcriptionally mediated through glucocorticoid activation of a SS-mRNA degrading system. Actinomycin D reduced the size of the SS-mRNA transcript and rendered it resistant to DEX-induced degradation when coincubated with DEX, but not when it was added after a delay of 12 h. These data provide strong evidence that DEX destabilizes SS-mRNA by an active process requiring ongoing gene transcription. Unlike actinomycin D, cycloheximide reduced steady state SS-mRNA probably by blocking the translation of proteins required for somatostatin gene transcription. Furthermore, the extent of SS-mRNA disappearance by cycloheximide was

the same as that for DEX suggesting that the two agents promote SS-mRNA degradation through a common pathway. Since only actinomycin D blocked DEX-induced decay of SS-mRNA, transcription clearly plays an important role in this mechanism. One possible explanation is that cycloheximide (which superinduces some gene expression in addition to blocking protein synthesis, 258, 259) and DEX both induce the synthesis of an antisense SS-mRNA which promotes SS-mRNA degradation. Further studies of the mechanism of action of cycloheximide on SS-mRNA, including the question of whether actinomycin D abolishes the cycloheximide as well as the DEX effect on SS-mRNA levels will be necessary to resolve this point.

Glucocorticoids exert both positive and negative effects on mRNA stability. For instance, they enhance the stability of mRNAs for GH (109), fibronectin (111), and PEPCK (112), and decrease mRNA stability for insulin (102), SP-A (117), IL-1β (113), 3-hydroxy-3-methylglutarylcoenzyme A reductase (114), type I procollagen (115), and GM-CSF (116). Glucocorticoid regulation of insulin and SP-A gene mRNAs is very similar to that of the somatostatin gene. Thus, glucocorticoids inhibit insulin biosynthesis by destabilizing mRNA rather than decreasing insulin gene transcription in hamster insulinoma cells (102). The steroid hormone induces a dose-dependent decrease in steady state insulin mRNA levels which also occurs after a lag period of 6 h following DEX treatment, is maximal at 24 h, and which is abolished by coincubation of DEX with actinomycin D and cycloheximide but not when the addition of actinomycin D is delayed for 9 h after DEX exposure (102). These are virtually identical findings to those obtained for SS-mRNA in the present study and further affirms that DEX mediated inhibition of insulin- and SS-mRNA requires the transcriptional activation of genes whose product accelerate the disappearance of the two mRNAs. Glucocorticoid

regulation of the SP-A gene involves both transcriptional and post-transcriptional control via mechanisms indistinguishable from those elucidated for the somatostatin gene. Mendelson *et al.* observed that DEX produced a dose-dependent biphasic effect on the levels of SP-A protein and mRNA in human fetal lung *in vitro*, low doses (10^{-10} - 10^{-9} M) being stimulatory whereas high doses ($\geq 10^{-8}$ M) were markedly inhibitory (117, 117a). This situation is virtually identical to our previous finding of DEX regulation of the somatostatin gene in cultured normal rat islet cells or $1027B_2$ islet somatostatin tumor cells (63). Mendelson *et al.* further discovered that the stimulatory effect of DEX on SP-A mRNA was transcriptionally mediated by the glucocorticoid receptor acting synergistically with the cAMP signalling pathway exactly as found by us for the somatostatin gene (117, 117a, 191). The predominant effect of DEX on SP-A mRNA, however, was an inhibitory one occurring at high levels of DEX due to a dose-dependent inhibition of SP-A mRNA stability identical to that found for DEX inhibition of SS-mRNA in the present study. The model of glucocorticoid action on the somatostatin gene that emerges from these studies indicates a complex molecular mechanism involving multilevel regulation. At low doses, glucocorticoids activate somatostatin gene transcription via positive interaction with CREB (191). These effects are predominantly manifested in peripheral tissues. At high doses, glucocorticoids decrease SS-mRNA levels through accelerated mRNA degradation (63). These actions occur in both brain and peripheral tissues as well as in thyroid carcinoma TT cells.

In conclusion, I have shown that glucocorticoids inhibit steady state SS-mRNA levels and the rate of somatostatin secretion in TT cells. This effect does not appear to be mediated through direct transcriptional inhibition of the somatostatin gene. It requires transcription of a gene whose product accelerates SS-mRNA degradation.

CONCLUSIONS

1. Glucocorticoids exert significant effects on somatostatin peptide production and steady state mRNA levels in normal somatostatin-producing tissues *in vivo*, *in vitro*, and in cultured $1027B_2$ cells, as summarized in Table 6-1. The glucocorticoid effect is time- and dose-dependent, tissue specific, and at least in part due to a direct action on somatostatin-producing cells. In normal rat islet and islet somatostatin producing tumor cells ($1027B_2$), glucocorticoid effect on somatostatin biosynthesis is characterized by stimulation at low doses (10^{-10} M) and marked inhibition at high doses ($\geq 10^{-7}$ M).

Table 6-1. Effects of glucocorticoids on somatostatin and somatostatin-mRNA levels in various conditions.

	in vivo	*in vitro*
cerebral cortex	–	
cortical neurons		–
hypothalamus	–	
thyroid TT cells		–
pancreas	+	
pancreatic islet		+/-
islet $1027B_2$ cells		+/-
stomach	+	
jejunum	+	

2. Glucocorticoids stimulate somatostatin gene transcription. Glucocorticoid-induced transactivation shows dependence on PKA activity, and may be mediated via protein-protein interaction between the glucocorticoid receptor and the CREB. DNA sequences upstream from the CRE between -250 and -71 bp in the somatostatin promoter appear to be the target of glucocorticoid action. The model is proposed in Figure 6-1.

3. Glucocorticoids inhibit steady state somatostatin-mRNA level and somatostatin secretion in TT cells. This effect is not mediated through direct transcriptional inhibition of the somatostatin gene. It requires transcription of another gene(s) whose product(s) accelerates somatostatin-mRNA degradation. In Figure 6-2, I proposed two possible mechanisms for this effect.

4. Therefore, at low doses, glucocorticoids activate somatostatin gene transcription via positive interaction with CREB. These effects are predominantly manifested in peripheral tissues. At high doses, glucocorticoids decrease somatostatin-mRNA levels through accelerated mRNA degradation. These actions occur in both brain and peripheral tissues as well as in thyroid carcinoma TT cells.

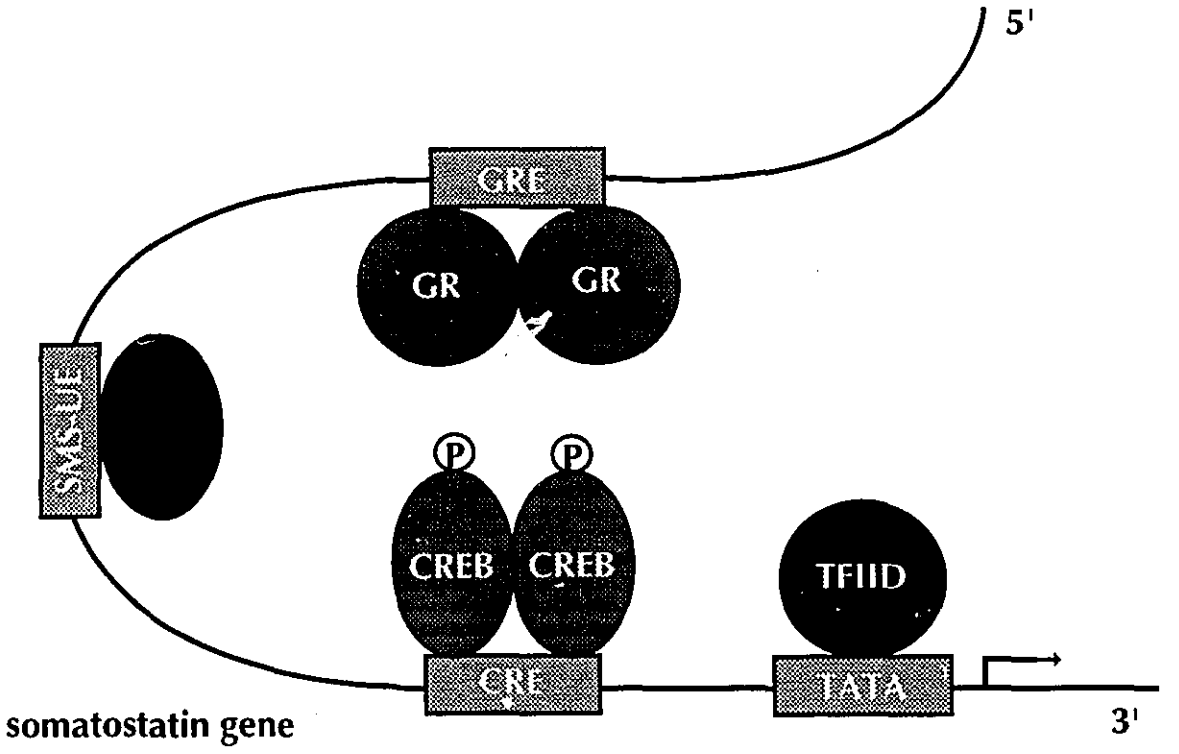


Figure 6-1. Proposed mechanism of glucocorticoid action on somatostatin gene transcription. Glucocorticoid receptor (GR) binds to an atypical GRE at -250/-71 bp region and interacts with phosphorylated CREB. Interaction of GR-CREB then activate gene transcription.

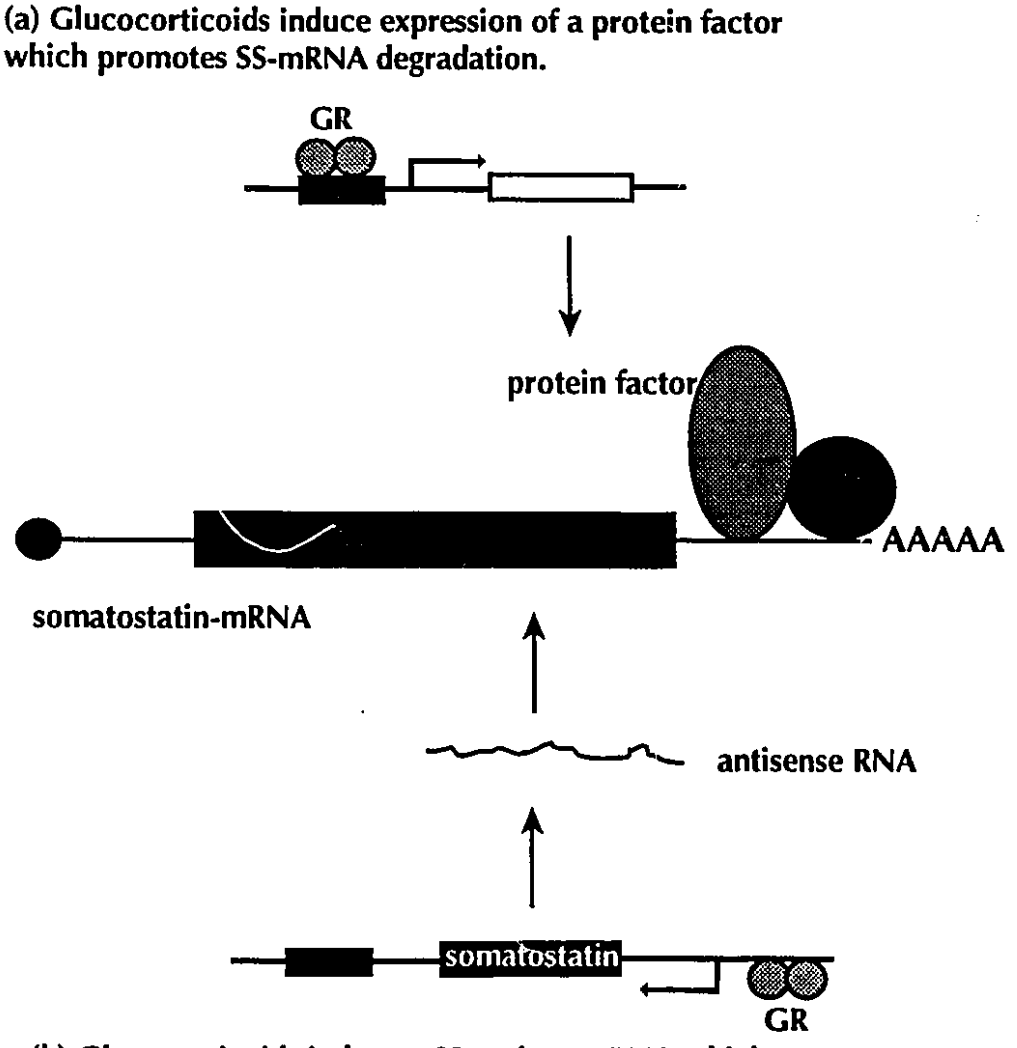


Figure 6-2. Possible mechanisms of glucocorticoid-regulated SS-mRNA degradation in thyroid carcinoma TT cells. (a) Glucocorticoids induce a protein which interacts with SS-mRNA or RNA-binding proteins and promotes SS-mRNA degradation. (b) Glucocorticoids induce an antisense RNA synthesis which is complementary to SS-mRNA and renders its degradation.

CLAIMS FOR ORIGINAL RESEARCH

The studies presented in this thesis constitute the first systematic evaluation of glucocorticoid-regulated somatostatin gene expression. I characterized the molecular mechanisms at both transcriptional and post-transcriptional levels. A number of observations attest to the originality of this research. These include demonstration that

1. Glucocorticoids regulate somatostatin gene expression in a tissue-specific fashion: stimulation in peripheral tissues and inhibition in the central nervous system;

2. Glucocorticoid effect on somatostatin gene expression is dose-dependent and biphasic in pancreatic islet and islet tumor cells: low dose (10^{-10} M) being stimulatory and higher doses (10^{-7} to 10^{-5} M) being inhibitory;

3. Glucocorticoid effect on steady state SS-mRNA and somatostatin production is, at least in part, mediated through direct action on somatostatin-producing cells;

4. Glucocorticoids stimulate somatostatin promoter activity;

5. The glucocorticoid sensitive region in the somatostatin gene has been mapped to -250 to -71 bp through promoter deletions and competitive gel shift assay;

6. There is cooperative interaction of glucocorticoids with the cAMP signalling pathway

in stimulating somatostatin gene transcription;

7. Glucocorticoid receptor interacts with CREB. Based on the present findings and those of others, I have proposed this as a novel mechanism of positive interaction between glucocorticoids and cAMP pathway.

8. Glucocorticoid inhibition on somatostatin gene expression in TT cells is not through direct gene transcription based on nuclear run-on and promoter-CAT transfection studies;

9. Glucocorticoid inhibition on SS-mRNA level is dependent on transcription of another gene(s) whose product(s) accelerates SS-mRNA degradation.

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APPENDIX

Summary of related publications

1. CYSTEAMINE-INDUCED REDUCTION IN TISSUE SOMATOSTATIN IMMUNOREACTIVITY IS ASSOCIATED WITH ALTERATIONS IN SOMATOSTATIN mRNA

D.N. Papachristou, J.-L. Liu, and Y.C. Patel

Regulatory Peptides 1994; 49: 237-47

[SUMMARY] The drug cysteamine induces a profound loss of somatostatin-14 biological and immunological (SS-14 LI) activity from SS cells *in vivo* and *in vitro*. The present study was designed to determine (i) whether cysteamine induced loss of SS is accompanied by secondary increases in SS-mRNA perhaps through loss of autoinhibition of SS cells; (ii) whether cysteamine exerts additional direct effects on SS gene regulation. Cysteamine was administered to rats *in vivo* or applied *in vitro* to primary cultures of rat islet cells, rat islet SS-producing tumor cells ($1027B_2$), and endogenous or *in vitro* synthesized SS-mRNA. *In vivo* administration of cysteamine led to 80% reduction in tissue SSLI by 4 h. These changes were accompanied by significant alterations in SS-mRNA that were both tissue-specific and time-dependent. The pattern in brain and intestine was typified by a significant 60% increase in SS-mRNA at 2 h followed by a gradual reduction to ~55% of control at 8 h. Stomach showed a significant 95% increase in SS-mRNA at 4 h followed by a 37% decrease by 8 h. Pancreatic SS-mRNA displayed a sustained 25-65% reduction for 8 h. Pretreatment of islet cell cultures with cysteamine reproduced the *in vivo* findings with pancreas viz. decreased SSLI (80-90% of control) accompanied by a parallel reduction in SS-mRNA (40-50% of control) sustained from 2-72 h. Cysteamine also induced a reduction in immunoreactive insulin and insulin mRNA in cultured islet cells. As with normal islet cells, cysteamine treatment of $1027B_2$ islet tumor cells led to a profound and sustained decrease in SSLI and SS-mRNA. These changes occured in the absence of any alteration in intracellular cAMP levels. Cysteamine was without effect when incubated directly with SS-mRNA isolated from $1027B_2$ cells or with *in vitro* synthesized SS-mRNA. We conclude that, in addition to its effect on SSLI, cysteamine also induces time- and tissue-dependent alterations in SS-mRNA. The mechanism of cysteamine action on SS-mRNA is complex and may involve both an indirect effect secondary to loss of SS autoinhibition (to account for SS-mRNA increases) and/or a direct inhibition of SS gene expression (to explain SS-mRNA reduction). The precise site of direct cysteamine action on SS gene regulation remains to be defined. Since cysteamine inactivates cellular SS and produces long term reductions in SS-mRNA in all tissues, it represents a useful tool for inducing functional lesions of SS cells at both gene and peptide levels.

2. SEQUENCE ANALYSIS OF THE 5'-FLANKING PROMOTER REGION OF THE HUMAN SOMATOSTATIN RECEPTOR 5

M.T. Greenwood, R. Panetta, L.A. Robertson, J.-L. Liu, and Y.C. Patel
Biochem Biophy Res Comm 1994; 205: 1883-90

[SUMMARY] We have determined the sequence of 2.2 kb of 5' flanking promoter region of the human somatostatin receptor 5 (hsstr5) gene. A number of widely distributed promoter elements were identified including AP1, AP2, AP3, E2A, GCF, and SP1 consensus sequences. hsstr5/CAT gene fusions showed that the 0.9 kb of DNA immediately upstream of the ATG, functions as a promoter in rat pituitary GH_3 but not in CHO ovary cells. Insertion of this hsstr5 fragment in the anti-sense orientation led to a four fold reduction in CAT activity. Dibutyryl cAMP produced a three fold induction of CAT activity whereas estradiol and retinoic acid had no significant effect. These results indicate that we have identified a DNA fragment at the 5' end of the hsstr5 gene which contains both tissue-specific and regulated elements. The absence of CRE consensus sequence suggests that the cAMP effect is mediated by the multiple AP1 and AP2 sites.

3. DIFFERENTIAL STIMULATION OF SOMATOSTATIN BUT NOT NEUROPEPTIDE Y GENE EXPRESSION BY QUINOLINIC ACID IN CULTURED CORTICAL NEURONS

Y.C. Patel, J.-L. Liu, A. Warszynska, G. Kent, D.N. Papachristou, and S.C. Patel
J. Neurochem. 1995, in press

[ABSTRACT] SS and neuropeptide Y (NPY) are coproduced in a subpopulation of neurons that are selectively resistant to N-methyl-D-aspartate (NMDA) neurotoxicity. We have previously reported that quinolinic acid (QUIN), and NMDA receptor agonist, augments SS-mRNA in cultured fetal rat cortical neurons. This study examines coregulation of SS and NPY by QUIN and NMDA in cultured cortical neurons and compares the effects of these agents with those of forskolin and phorbol-12-myristate-13-acetate (PMA), which are known to activate SS and NPY gene transcription by protein kinase A- and protein kinase C-dependent mechanisms. In addition, transcriptional regulation of the SS gene was investigated by acute transfection of cortical cultures and PC12 cells with an SS promoter-CAT construct. QUIN and NMDA displayed dose-dependent four-fold augmentation of mRNA for SS but not for NPY. In contrast, forskolin and PMA increased both SS and NPY mRNA levels. QUIN- and NMDA-mediated induction of SS-mRNA was blocked by the NMDA receptor antagonist (-)-2 amino-5 phosphonovaleric acid (APV), and displayed regional brain specificity since it was not observed in fetal hypothalamic cell cultures. Time-course studies showed that the effects of QUIN/NMDA on SS-mRNA occurred after a latency of 8 h. Cortical cells or PC12 cells transfected with pSS-750CAT showed 3-4 fold stimulation of CAT activity with forskolin but not by QUIN or NMDA. These data reveal a dose-dependent, NMDA receptor-mediated stimulation of SS but not NPY mRNA. Such stimulation of SS gene expression does not require activation of the PKA or PKC signalling pathways, is not transcriptionally mediated, and is likely post-transcriptional, as also suggested by the delay in SS-mRNA induction.

4. PROSOMATOSTATIN IS EFFICIENTLY PROCESSED AT DIBASIC AND MONOBASIC CLEAVAGE SITES TO BOTH SS-14 and SS-28 VIA THE CONSTITUTIVE PATHWAY IN ISLET SOMATOSTATIN TUMOR CELLS ($1027B_2$)

Y.C. Patel, A.S. Galanopoulou, S.N. Rabbani, J.-L. Liu, M. Ravazzola, and M. Amherdt
J Biol Chem 1995 (submitted)

[ABSTRACT] We have characterized the biosynthesis and secretion of the principal mature products of prosomatostatin (PSS) processing in $1027B_2$ rat islet tumor cells previously reported to lack regulated secretion and cAMP responsiveness of the SS gene. Analysis of cell extracts and secretion media by high performance liquid chromatography and specific C- and N-terminal radioimmunoassays showed that the cells contained large quantities of fully processed SS-14 and SS-28 with very little unprocessed PSS (ratio SS-14:SS-28:PSS = 39%:51%:10%). Pulse-chase studies demonstrated that PSS is efficiently processed to SS-14, SS-28, and $PSS_{[1-10]}$ via separate biosynthetic pathways: PSS ▸ SS-14 + 8 kDa; PSS ▸ SS-28 + 7 kDa; PSS ▸ $PSS_{[1-10]}$. Synthesis of SS-14, SS-28, and $PSS_{[1-10]}$ occurred independently from PSS and was demonstrable within 15 min. By light and electron microscopic immunocytochemistry, tumor cells were devoid of secretory granules. There was no specific immunogold labeling of any intracellular compartment. The cells expressed the endoproteases furin, PC1, and PC2. They secreted large amounts of fully processed SS-14, SS-28, and $PSS_{[1-10]}$ constitutively along with PC1 and PC2. Monensin reduced intracellular SS-like immunoreactivity without altering processing efficiency. Transfection with the catalytic subunit of protein kinase A (PKA-C) activated SS promoter-CAT activity indicating that the defect in cAMP-dependent signaling occurs at the level of PKA-C. PKA-C overexpression failed to alter the ratio of processed SS-14 and SS-28. We conclude that efficient processing of PSS can occur constitutively in the absence of secretory granules probably within the Golgi compartment.