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Research Article

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Glucocorticoid Activation of Chromogranin A Gene Expression

Identification and Characterization of a Novel Glucocorticoid Response Element

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Abstract

Glucocorticoids regulate catecholamine biosynthesis and storage at several sites. Chromogranin A, an abundant protein complexed with catecholamines in secretory vesicles of chromaffin cells and sympathetic axons, is also augmented by glucocorticoids. This study reports isolation of the rat chromogranin A promoter to elucidate transcriptional regulation of chromogranin A biosynthesis by glucocorticoids in neuroendocrine cells. Endogenous chromogranin A gene expression was activated up to 3.5-fold in chromaffin cells by glucocorticoid, in time-dependent fashion. Inhibition of new protein synthesis by cycloheximide did not alter the rise in chromogranin A mRNA, suggesting that glucocorticoids directly activate the chromogranin A promoter; nuclear runoff assays confirmed a 3.3-fold increased rate of initiation of new chromogranin A transcripts after glucocorticoid. Transfected rat chromogranin A promoter/luciferase reporter constructs were activated 2.6–3.1-fold by glucocorticoid, and selective agonist/antagonist studies determined that dexamethasone effects were mediated by glucocorticoid receptors. Both rat and mouse chromogranin A promoter/luciferase reporter constructs were activated by glucocorticoid. A series of promoter deletions narrowed the region of glucocorticoid action to a 93-bp section of the promoter, from position -526 to -619 bp upstream of the cap site. A 15-bp sequence ([-583 bp] 5'-ACATGAGTGTGTCCT-3' [-597 bp]) within this region showed partial homology to a glucocorticoid response element (GRE; half-site in *italics*) consensus sequence, and several lines of experimental evidence confirmed its function as a GRE: (a) site-directed mutation of this GRE prevented glucocorticoid activation of a chromogranin A promoter/reporter; (b) transfer of this GRE to a heterologous (thymidine kinase) promoter/reporter conferred activation by glucocorticoid, in copy number-dependent and orientation-independent fashion; and (c) electrophoretic gel mobility shifts demonstrated binding of this GRE by ligand-activated glucocorticoid receptor, though at 2.75-fold lower affinity than the glucocorticoid receptor interaction with a consensus GRE. The rat chromogranin A GRE showed functional and structural similarities to GREs in other genes proportionally regulated by glucocorticoids. We conclude that a discrete domain of

the chromogranin A promoter is both necessary and sufficient to confer glucocorticoid regulation onto the gene, and that the activity of this region also explains the degree of activation of the endogenous gene by glucocorticoid. (*J. Clin. Invest.* 1994, 94:2357–2368.) Key words: chromogranin A • adrenal medulla • catecholamine • glucocorticoid • steroid • promoter • enhancer • pheochromocytoma • PC-12 • chromaffin.

Introduction

After release from the adrenal cortex, glucocorticoids first enter sinusoids that traverse the adrenal medulla before entering the systemic circulation. Exposure to high local glucocorticoid concentration plays a crucial developmental role in tissue-specific activation of genes that characterize the chromaffin cell phenotype (1). In the adult (2), two genes of the catecholamine biosynthetic pathway are directly activated by glucocorticoids: phenylethanolamine-*N*-methyltransferase (3) and tyrosine hydroxylase (4, 5 and references therein). The expression of chromogranin A, the major soluble protein in chromaffin vesicles, is also augmented by glucocorticoids, but the mechanism of activation has not been elucidated (6).

Chromogranin A is the index member of a family of acidic, soluble proteins found in neuroendocrine secretory granules (7). Within granules, chromogranin A binds catecholamines and calcium (8, 9), and may inhibit prohormone processing enzymes (10). After release into the extracellular space, chromogranin A is processed into several biologically active peptides (11, 12). Even though chromogranin A is already abundant, representing 46% of soluble protein in chromaffin vesicles (13), it remains sensitive to glucocorticoids (14–17). In vivo, hypophysectomy decreases adrenal chromogranin A (14), with restoration after glucocorticoid replacement (15). In vitro, chromogranin A protein is consistently up-regulated by glucocorticoid in bovine chromaffin (16) and rat pheochromocytoma (PC-12) cells (17), with proportional induction of its mRNA (16, 17).

Since glucocorticoids and catecholamines play important regulatory roles in metabolic and cardiovascular responses, and chromogranin A influences catecholamine storage and release (8), it is crucial to understand chromogranin A gene regulation by glucocorticoids. Indeed, a thorough understanding of this regulation may assist in elucidating the protein's many intracellular and extracellular functions.

This investigation presents evidence that glucocorticoids directly activate chromogranin A gene expression. We isolated a region of the rat chromogranin A promoter with resemblance to a consensus glucocorticoid response element (GRE),¹ and

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1. Abbreviations used in this paper: GR, Glucocorticoid receptor; GRE, glucocorticoid response element; hGR, human glucocorticoid receptor; RSV, Rous sarcoma virus; TK, thymidine kinase.

Table 1. Plasmids and Oligonucleotides Used to Investigate Glucocorticoid Regulation of Rat Chromogranin A

Experiment type	Plasmid or oligonucleotide	Description
Gene isolation	sCos-1	Supercos-1 cosmid (Stratagene); carries 32–42 kb genomic DNA inserts (18, 19)
	sCos-1rCgA-1	Rat genomic DNA cosmid that spans entire rat CgA gene
	sCos-1rCgA-2	Cosmid containing rat CgA gene with ~ 20-kb overlap with sCos-1rCgA-1
Sequencing/subcloning	pBSrCgAP/P1594	pBluescriptKS– with a 1,594-bp PstI/PstI fragment of rCgA gene subcloned into the multiple cloning site (MCS) at PstI
	pBSrCgAB/S489	pBluescriptKS– with 489 bp BamHI/SstI fragment of rCgA gene subcloned into the MCS
	pSV2ALΔ5	Luciferase reporter gene vector under control of the SV40 early promoter (41)
	pXp2	Promoterless luciferase reporter gene vector with MCS immediately upstream of luciferase open reading frame (35)
	pXp2RCgA	Rat CgA promoter fragment inserted into MCS of pXp2
	pXp2rCgAΔ-523(+)	pXp2rCgA restriction digest-derived [‡] insert: SstI/SstI [5' –523 to +75 [§] –3'] positive orientation
	pXp2rCgAΔ-523(–)	pXp2rCgA restriction digest-derived insert: SstI/SstI [5' +75 to –523 –3']
Transcriptional nuclear runoff assay	pBSm-gDNA5.1	5.1-kb EcoRI/EcoRI fragment of mouse CgA gene (includes exons 1–3) subcloned into MCS of pBluescriptKS– (34)
General transient transfection	pRSVCAT	Chloramphenicol acetyltransferase (CAT) expression driven by RSV promoter (39)
	pRSVhGR	Human glucocorticoid receptor expression driven by RSV promoter (38)
	pXp2	Promoterless luciferase reporter-gene plasmid with MCS immediately upstream of luciferase open reading frame (35)
	pXp2rCgA	Rat CgA promoter fragment inserted into MCS of pXp2 (specifics of a fragment are indicated by number following the deletion symbol “Δ”)
Promoter deletion and expression	pXp2rCgAΔ-523	pXp2rCgA RE-derived [‡] insert: SstI/SstI [5' –523 to +75 [§] –3]
	pXp2rCgAΔ-619	pXp2rCgA PCR-derived insert: HindIII/XhoI [5' –619 to +112 –3' [¶]]
	pXp2rCgAΔ-756	pXp2rCgA PCR-derived insert: HindIII/XhoI [5' –756 to +112 –3']
	pXp2rCgAΔ-1053	pXp2rCgA PCR-derived insert: HindIII/XhoI [5' –1053 to +112 –3']
	pXp2rCgAΔ-1281	pXp2rCgA RE-derived [‡] insert: SmaI/SmaI [5' –1281 to +75 –3']
Promoter GRE mutation	pXp2rCgAΔ-756m	pXp2rCgAΔ-756 mutated from position –597 to –590 by changing [–597:5'-AGGACACA-3':–590] to [–597:5'-gcGgtACc-3':–590] where bold letters indicate the rGRE motif, lower case letters indicate mutated residues, and newly introduced KpnI site (GGTACC) is <u>underlined</u> . Vector constructed by ligating PCR-derived fragments HindIII/KpnI [–756: 5' to 3':–591] and KpnI/XhoI [–595:5' to 3':+112] into MCS upstream of luciferase reporter in pXp2. Mutated plasmid sequence confirmed by dideoxy chain termination sequencing.
Response elements retardation studies	cGRE	Consensus GRE ^{**} 5'-AGAACAagagTGTCT-3' (54, 55), with capital letters indicating consensus motifs
	*cGRE	γ-[³² P]-end-labeled consensus cGRE ^{**}
	rGRE	GRE ^{**} from the rat CgA promoter: [–583 bp] 5'-ACATGAGTGTGTCCT-3' [–597 bp]
	*rGRE	γ-[³² P]-end-labeled GRE ^{**} from the rat CgA promoter
Rat CgA GRE transfer study	pTKluc	Thymidine kinase promoter/luciferase reporter plasmid with MCS upstream of TK promoter (36)
	pTKlucC17	One rGRE inserted ^{§§} (in reverse orientation) into BamHI site of MCS of pTKluc
	pTKlucC19	One rGRE inserted ^{§§} (in forward orientation) into BamHI site of MCS of pTKluc
	pTKlucE9	Two rGREs inserted into BamHI site of MCS of pTKluc
	pcGRETKluc-1	One cGRE inserted into BamHI site of MCS of pTKluc
	pcGRETKluc-2	Two cGREs inserted into BamHI site of MCS of pTKluc

This table serves as a reference guide for plasmids and oligonucleotides used in this investigation. It is organized by experiment type in the order presented in Results. Plasmids and oligonucleotides are described alphabetically in their respective experimental type subsection. Promoter components are in the “positive” or endogenous orientation, unless indicated otherwise. [‡] Derived from a restriction enzyme digest of pBSrCgAP/P1594. [§] Sequence from +1 to +75 bp is within the untranslated region in exon 1 of the rat chromogranin A gene. ^{||} Derived by PCR with primers flanking the reported sequence. HindIII site placed near 5' end of forward primer; XhoI site placed near 5' end of reverse primer. [¶] Sequence from +1 to +112 bp is within the untranslated region in exon 1 of the rat chromogranin A gene. ^{**} In gel retardation studies, cGRE and rGRE are the central sequences in a 22 bp double-stranded oligonucleotide organized as follows: [5'-GATC-(cGRE or rGRE)-CTA-3'] (sense strand) and [5'-TAG-(complementary sequence of cGRE or rGRE)-GATC-3'] (antisense strand). In the rat chromogranin A rGRE transfer studies, cGRE and rGRE are inserted into a BamHI site immediately upstream of the thymidine kinase promoter on pTKluc using double-stranded oligonucleotides with 5' BamHI overhangs: [5'-GATC-(cGRE or rGRE)-3'] (sense strand) and [5'-GATC-(complementary sequence of cGRE or rGRE)-3'] (antisense strand). ^{§§} Refer to Table III for more specific information regarding orientation of the insert. MCS, multiple cloning site; RE, restriction enzyme; CgA, chromogranin A.

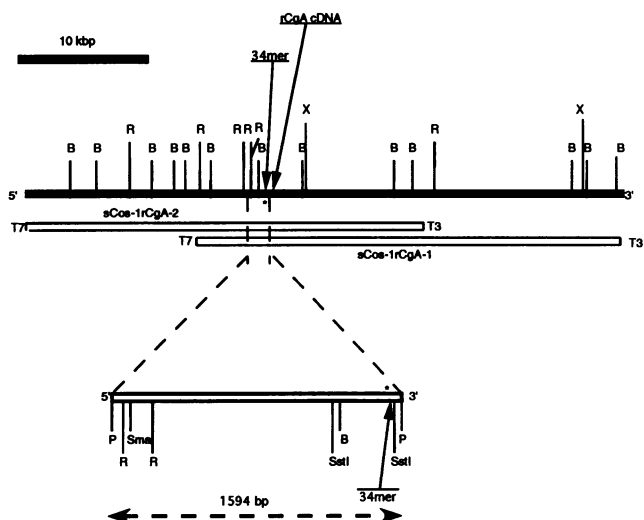


Figure 1. Restriction map of two overlapping rat genomic clones (sCos-1rCgA-1 and sCos-1rCgA-2) derived from the Stratagene SuperCos-1 cosmid vector (18) established the ~ 50-kbp local chromosomal region of the chromogranin A (CgA) gene. This region contains the gene's promoter, which 5' flanks exon 1. Cosmid screening and isolation was by colony hybridization using as probes a 288-bp *AvaI/ApaI* 5' fragment of the rat CgA cDNA (20) and a synthetic 34-bp oligonucleotide (34-mer) complementary to the 5' most known sequence of rat CgA cDNA [5'-AGCGGTGGTGTGGCAGTGG CCGTGATGGT GGTG-3'] (34). Southern hybridization using the 34-bp oligonucleotide yielded a 1,594 bp *PstI/PstI* fragment which, upon subcloning, resulted in a partial restriction map of the 5' regulatory region. The figure depicts on a 10-kbp scale the restriction map from two isolated cosmids (sCos-1rCgA-2 above sCos-1rCgA-1), using three restriction enzymes: *BamHI* (B), *EcoRI* (R), and *XhoI* (X). T3 and T7 indicate orientation of bacteriophage promoters flanking rat genomic DNA inserts. Hybridization regions for the 34 bp oligonucleotide and the 288-bp *AvaI/ApaI* fragment of rat CgA cDNA (*rCgA cDNA*) are indicated by arrows; *denotes the location of the transcription initiation or "cap" site. Within the dashed lines, a more detailed and enlarged restriction map of the 1,594 bp *PstI/PstI* fragment is given. This region contains the first 1,482 bp of the 5' regulatory region upstream of the cap site. The locations of the 34-mer and cap site are indicated as before: *cap site; P, *PstI*; B, *BamHI*; R, *EcoRI*; and Sma, *SmaI*.

demonstrated its functional activation of transcription in response to glucocorticoid, as well as its binding to ligand-activated glucocorticoid receptor.

Methods

Cosmids, plasmids, and many of the oligonucleotides used in this investigation are listed and briefly described in Table I. They are categorized by experiment type as presented in Results, and listed alphabetically within each subsection, with additional details on each.

Isolation of the rat chromogranin A genomic DNA clones. From a rat genomic DNA cosmid (sCos-1 vector) library (obtained from Dr. Glen Evans, Salk Institute, San Diego, CA) (18, 19), 5×10^5 colonies were screened with a random primer-labeled 288-bp 5' fragment (*AvaI/ApaI*) of rat chromogranin A cDNA (20). After initial hybridization yielded three positive colonies, secondary and tertiary screenings were done with a γ -[³²P]-end-labeled 34-bp synthetic oligonucleotide (5'-AGCGGTGGTGGTGGCAGTGGCGGTGATGGTGGTG-3'), corresponding to the complementary (antisense) strand of the most upstream available (5' untranslated) sequence of rat chromogranin A cDNA (21).

```

CTG CAGCTAGITT TTACGAGATG GTATTTTGGG GACAGCATGC CGGGAGCTTG -1430
CGTGTGATCA CAGTGACTTC ATGTCCTAGG ACCTGGAACA CTCTGGAATT CTCCCAGTGC -1370
hGRE-MTIIA
TGAGCTGGAG TCCTTTTCTA GGAACATAA TATATGAATG GAGACGCCCT AGTCAGAAAT -1310
hGRE.7
AAATGCTTTA AATCTGCTGG CCCTTGTCCT CCGGTAGAGC CAGCCTCTCC CATACTTCC -1250
TGTCCTCAC TAGAATCTG CCGTCTTCTC CCCTTTATGC CTGTAGCACG GCCATGACCC -1190
AGCATAGTGT ACACCTTGCT TCTTCTCTG GAAAGGGAAT TCTATAAGGG TTGGGTTTGC -1130
TGTTTGTTTA CTGCCGTGTC TTTGGGCATC TGGCACAGTC AAGTGTGTTT CTGAGGTGTT -1070
hGRE.3 hGRE.3
CTAAGCCAC GTTGATGCTT AACACATGAT TGTTGAATGA ATGCATGCAA AGCAGTTTCT -1010
CATTTAGGGG CATGAGTGGG CAAGAGGTGT GGGCAGGAAG CAGGAAGAG CAGAAGCAGG -950
TGGGGACCGA AGGCGCGCGG GCTCTGAAGG ATGCCAGTCA GTGCCAAACT GTCATCCAGA -890
TACCAGGCTC ATTATGGCAC TGGGTGCAGG CTTCACAGGG CTTCCTCATG GTTCCACAGG -830
Sp1
GTGAGAGCAG AGCTGGGGAT GGAGCGGGGC AGAAGGAAAC CAACCAGGAA GCAAGCTCAC -770
ACCCAAAATA TCCAGCTTTT AAGAGCATT AAAAAAAAAA AAAAGACAAG GCGTGGCTCT -710
TCAAGACAGA GGTGTTCTCG GAGTGTGGA CTAGGACTGA CTACTTTTGT TTTAGCTTAA -650
hGRE.7
TGTTGAGAAC TGCCCTCCAC TGCTACCTGC CTTACTTGCC ACTTGAATA CTAGGACACA -590
hGRE-MTIIA
CTCATGTGTG GGCTGGATCT TCAATGCACA CATTGAACCT GTGTGAAGCC ATTGGTTGTC -530
AGTGAAGGAG TCTCAGCACT GAGAAAGCAG TGACCACACT CCCTATCAA TAACTATTAA -470
ATACACACAG AACAGGGCAC GGGGCTGAGT TTCAGGAGAC GCCTCACTCA GGTAGGGATC -410
CAAGAGCCTT CTCTGGGACC CGTGTAATC TTCCAGGGAG TTCTGAAAGA CACAGCGTGC -350
CTCCAACCGA CTGAAATCAA GAGAAAAGTA CGCTAAGTAT AGGAAAATTC AGCACCCCTGG -290
AGAGGAACCC TAAACACCGA AGGATGTGTA GGCTCAGAGA CAGGAGGACT TGCCCAAGGA -230
hGRE.7 hGRE-MTIIA
CACACAGCAA ATTGACAGGT GGAAGTTCAG CTGTGCCACC TTCTGAAGCC GTGTATCTCT -170
CACAGCCACC AAATAGAAGC AGGATGGAGG CAGCTCACCG AGAAGCTGGA GGTAGCGGGC -110
Sp1
GGACCCCGA AGGTGGGGAA AGGGCGCAGG GGGCGTCTA TGACGTAATT GCCTGGGTGT -50
GTGCGTGTGC GTGCGTGTGT ATAAAAATAG GCATAGCATT GCTTGGGGGC TGCTGTACCG +11
TATA
CCACCACCAT CACCGCCACT GCCACCACCA CCGCT
Complementary to 34 bp screening oligonucleotide

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Figure 2. This figure records the 5' regulatory region sequence of the rat chromogranin A gene (plus strand). Nucleotide numbering indicated in the right column is based on the particular nucleotide's position 5' (upstream) of the transcription initiation or "cap" site. Sequence of each strand was determined by the dideoxynucleotide chain termination method (23). The cap site (+) is assigned by homology to the mouse chromogranin A gene sequence. Within the proximal promoter region of the gene (from -100 to +1 bp), rat and mouse chromogranin A genes share > 85% homology. Consensus response elements are underlined and include: TATA—TATA box (5'-TATAAA-3') (46); Sp1—Sp1 (stimulation protein) promoter element (5'-CCGCCC-3') (48); and CREB—cAMP response element (7/8 bp match; 5'-TGACGTAA-3') (47). In addition, several consensus glucocorticoid response element half-sites (hGRE) are underlined: hGRE.3 (5'-TGTTCT-3') (50); hGRE.7 (5'-AGTCTT-3') (51); and hGRE-MTIIA (5'-TGTCTT-3') (49). Some of these consensus matches are on the minus strand (see Results). The position of the 34-bp oligonucleotide used in isolating the gene is indicated (21).

Two colonies remained positive (sCos-1rCgA-1 and sCos-1rCgA-2). Restriction mapping (Stratagene, La Jolla, CA) by *BamHI*, *EcoRI*, and *XhoI* showed that the genomic DNA inserts overlapped by ~ 20 kbp, and spanned ~ 50 kbp of the rat genome (see Fig. 1).

Southern hybridization (22) of the 34-bp probe (see above) to restriction-digested cosmids yielded several positive fragments, notably 1,594 bp *PstI/PstI* and 489-bp *SstI/BamHI* bands. Since *SstI* and *PstI* sites occurred near the 5' end of rat chromogranin A cDNA (but downstream [3'] of the 34 bp sequence), these fragments were subcloned into pBluescript-KS⁻ (Stratagene), resulting in pBSrCgAP/P1594 and pBSrCgAB/S489. Insert sequencing was done by the dideoxy chain termination method (Sequenase; United States Biochemical Co., Cleveland, OH), initially with "universal" 17-bp primers to phage T3 (5'-ATTAACCCTCACTAAAG-3') and T7 (5'-AATACGACTCAC-

Table II. Activity of Rat Chromogranin A 5' Regulatory Region in Adrenal Chromaffin and Fibroblast Cells

Cell line	Cell type	Promoter/enhancer (plasmid)			
		Rat CgA 0.6 kbp, forward orientation (pXp2rCgAΔ-523[+])	Rat CgA 0.6 kbp, reverse orientation (pXp2rCgAΔ-523[-])	SV-40 early promoter (pSV2ALΔ5)	None (pXp2)
PC-12	Adrenal chromaffin	8.2	1.3	1.0	0.13
NIH 3T3	Fibroblast	0.28	0.025	1.0	0.007

Activity of rat chromogranin A promoter/enhancer is assessed in PC-12 (neuroendocrine) and NIH 3T3 (nonneuroendocrine) cells by a luciferase reporter transient transfection assay. A ~ 0.6 kbp fragment of the rat CgA gene (containing 523 bp of sequence 5' of the "cap" site) was inserted in either orientation into the multiple cloning site of the promoterless luciferase reporter vector pXp2, yielding plasmids pXp2rCgAΔ-523(+) (forward or endogenous orientation) and pXp2rCgAΔ-523(-) (reverse orientation). PC-12 and NIH3T3 cells were compared for luciferase reporter activity after lipofection-mediated transfection with pXp2rCgAΔ-523(+), pXp2rCgAΔ-523(-), pXp2 (negative control), or pSV2ALΔ5 (positive control, in which luciferase activity is expressed under control of the SV-40 early promoter). Cells were cotransfected with pRSCAT to correct for differences in transfection efficiency. Values in the table represent mean luciferase activity of a given plasmid, corrected for transfection efficiency and normalized to the activity of the SV-40 early promoter (= 1.0). $n \geq 3$ transfections.

TATAG-3') promoters flanking subcloned fragments, and later with sequence-derived primers (23, 24).

Cell culture. Rat pheochromocytoma (PC-12) (25), mouse anterior pituitary corticotrope (AtT-20) (26), mouse fibroblast (NIH-3T3) (27), and transformed monkey kidney (Cos) (28) cells were grown in monolayer under 6.2% CO₂ in DME-high glucose media supplemented with serum depleted of steroids by charcoal/dextran adsorption, as previously described (29). Serum supplements included 5% fetal bovine serum and 10% horse serum for PC-12 cells, 10% fetal bovine serum for AtT-20 and NIH-3T3 cells, and 5% fetal bovine serum for Cos cells. Charcoal/dextran-adsorbed serum had cortisol < 1 nM. Cells were split once weekly, and growth medium was replaced every three to four days.

mRNA isolation and quantitation. Total RNA was isolated from PC-12 monolayers in 10-cm tissue culture dishes (~ 5 × 10⁶ cells) by the guanidinium thiocyanate extraction method (RNAzol B; Tel-Test, Friendswood, TX) (30), quantified by UV absorption (A₂₆₀), and its quality verified by A₂₆₀/A₂₈₀ absorbance ratio (= 1.7–2.0) and by appearance on ethidium bromide-stained agarose gel. A typical RNA yield per 10 cm plate was 75 μg. Relative amounts of chromogranin A mRNA were determined by either slot blotting or northern analysis (22) using a random primer radiolabeled rat chromogranin A cDNA fragment (288-bp Aval/ApaI) probe (20, 31). Slot blot lanes received 5, 10, and 15 μg of total RNA, while agarose gels for Northern blots were loaded with 10 μg total RNA per lane. Chromogranin A mRNA was normalized in slot blot studies to the mRNA of a constitutively expressed ("housekeeping") gene, cyclophilin, using a PCR-derived random primer-labeled rat cyclophilin cDNA probe (32). Northern blot autoradiographic bands were quantified by densitometry (StratoScan 7000 densitometer; Stratagene) after equivalent 18 S and 28 S ribosomal RNA bands were verified on ethidium bromide-stained gel lanes. In some studies, cells were pretreated with cycloheximide (5 μg/ml, 8 h) to block protein synthesis at the level of translation; such treatment decreased [³⁵S]methionine incorporation into newly biosynthesized, trichloroacetic acid-precipitable protein by > 95% (33).

Nuclear runoff assay. To measure directly transcriptional events (rate of initiation of new chromogranin A transcripts) associated with dexamethasone induction of chromogranin A biosynthesis, a nuclear runoff transcription assay was performed. In this study, heterogeneous nuclear RNA (hnRNA) was isolated (33) from AtT-20 corticotrope cell nuclei, after 8–24 h of dexamethasone (100 nM) or vehicle. In brief, 3 × 10⁷ nuclei were isolated from two confluent 15-cm cell culture plates by treatment with 0.5% hypotonic buffer (10 mM Tris pH 7.4, 3 mM CaCl₂, 2 mM MgCl₂, 0.5% NP-40) on ice, and stored frozen at -70°C, before biosynthetic labeling of hnRNA with α-[³²P]-UTP.

Labeled hnRNA was hybridized to filters, on which 5 μg of the desired DNA target had been previously affixed by slot-blotting. The chromogranin A genomic DNA probe was a ~ 5.1-kbp mouse chro-

mogranin A EcoRI/EcoRI genomic DNA fragment which spanned exons one through three and introns A through C (34). The negative control probe was the plasmid pBluescriptKS- (Stratagene). Newly labeled transcripts were hybridized for 36 h at 65°C, at two levels of radioactivity (1.0 × 10⁶ cpm/ml and 4.0 × 10⁵ cpm/ml) in 10 mM TES, pH 7.4, 10 mM EDTA, 0.2% SDS, and 0.3 M NaCl. Each blot was washed twice in 2X SSC at 65°C for 1 h. Newly initiated and labeled hnRNA was quantified by transmission densitometry of autoradiographs (StratoScan 7000 densitometer).

Promoter/reporter plasmids. Plasmids were constructed to provide templates for the nuclear runoff assay and to test glucocorticoid responses of the rat chromogranin A promoter and a consensus glucocorticoid response element. Plasmids used are recorded in Table I. Vectors and inserts are grouped alphabetically according to type of experiment, as categorized in Results.

Several restriction fragments of the 5' regulatory region flanking and extending into the 5' untranslated (leader, exon 1) region were inserted into pXp2, a promoterless luciferase reporter plasmid whose polylinker is just 5' of the luciferase open reading frame (35). A 1,389-bp SmaI/SmaI fragment or a 598-bp SstI/SstI fragment from pBSrCgAP/P1594 were inserted in the sense (correct) orientation into the multiple cloning site of the promoterless luciferase reporter pXp2 (35). The resulting expression plasmids were pXp2rCgAΔ-1281 and pXp2rCgAΔ-523 (where Δ = deletion). The nomenclature (Table I) for these and other chromogranin A promoter/luciferase reporter plasmids derives from the number of base pairs of promoter sequence upstream (5') of the transcriptional initiation or "cap" site. Using pXp2rCgAΔ-1281 as a template, and HindIII (upstream) or XhoI (downstream) restriction sites engineered into polymerase chain reaction (PCR) primer ends, PCR created additional promoter deletions which were subcloned into the pXp2 reporter: pXp2rCgAΔ-1053, pXp2rCgAΔ-756, and pXp2rCgAΔ-619.

To create a disrupting mutation within the putative GRE, pXp2rCgAΔ-756 was mutated to pXp2rCgAΔ-756m, by substituting a KpnI (GGTACC) site, as well as two bases (GC) just upstream of the KpnI site, into the candidate GRE sequence. The 15 bp wild-type rat chromogranin A GRE motif, [-597 bp] 5'-AGGACACACTCATGT-3' (-583 bp) was mutated to (-597 bp) 5'-gcGgtACcTCATGT-3' (-583 bp); newly substituted (mutated) bases are shown in lower case, while the newly created KpnI site is underlined. The mutation was by base substitution, rather than insertion or deletion. Two pairs of PCR primers (Table I) creating and bridging the desired mutation were designed to amplify the upstream and downstream portions of the entire promoter region from pXp2rCgAΔ-756. The two PCR products ([-756 bp] 5' to 3' [-591 bp], and [-595 bp] 5' to 3' [+112 bp]) were digested with KpnI, ligated, and reinserted into pXp2, yielding pXp2rCgAΔ-756m.

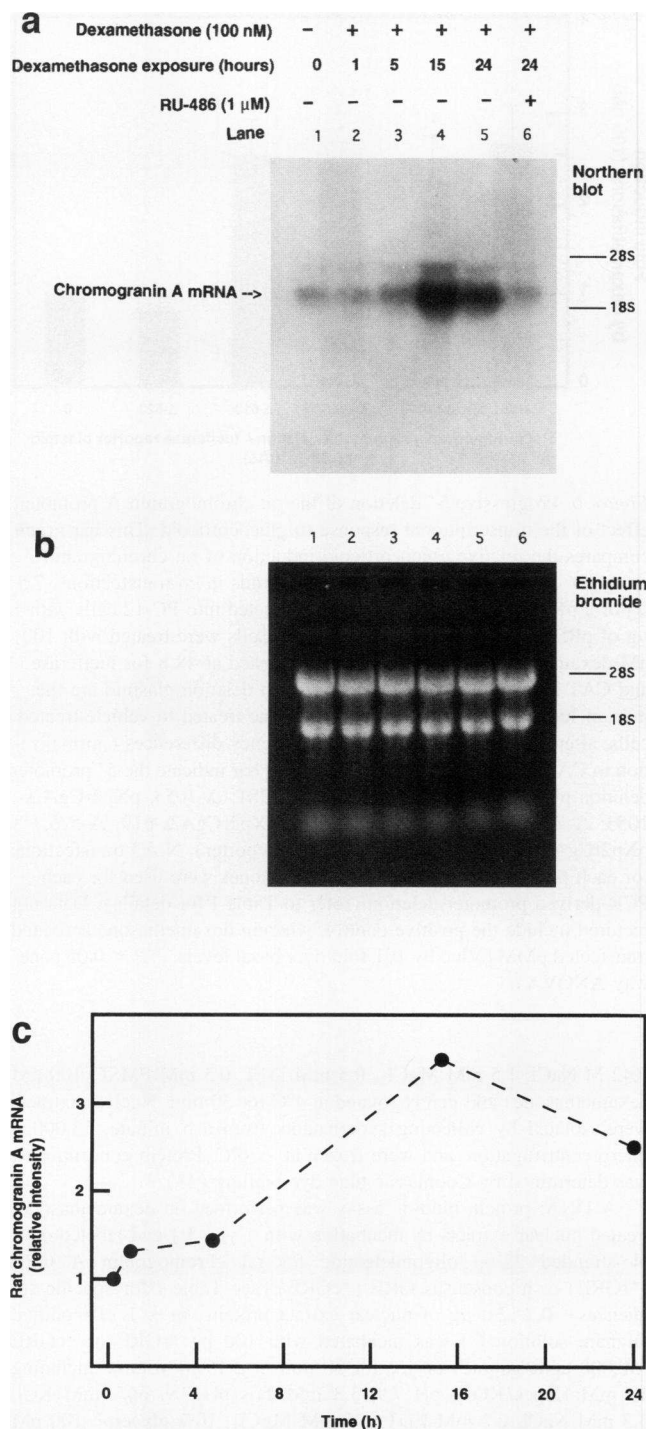


Figure 3. Time course of induction of chromogranin A mRNA by dexamethasone. 24-h time course of rat chromogranin A mRNA response to 100 nM dexamethasone in PC-12 cells. (a) Northern hybridization of rat chromogranin A probe to total RNA (10 μ g/lane). Lanes 1–5 show RNA from cells which received dexamethasone for 0, 1, 5, 15, or 24 h. Lane 6 shows RNA from cells pretreated with the glucocorticoid antagonist RU-486 (1 μ M) for 30 min before and during 24 h exposure to 100 nM dexamethasone. The rat chromogranin A probe was derived by [32 P]-random primer-labeling of a 288-bp Aval/ApaI fragment of the rat chromogranin A cDNA (20). (b) Consistent levels of 18 S and 28 S rRNA loading for all exposures on the ethidium bromide-stained gel. (c) Graphs the time course of rat chromogranin A (densitometric intensity) response to dexamethasone.

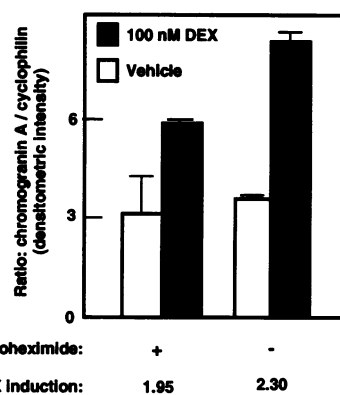


Figure 4. Glucocorticoid regulation of rat chromogranin A mRNA during inhibition of protein synthesis (translation). This graph quantifies the response of rat chromogranin A mRNA to dexamethasone in PC-12 cells in the presence or absence of cycloheximide. PC-12 cells were pretreated for 6 h with 5 μ g/ml cycloheximide (or vehicle), exposed to 100 nM dexamethasone, harvested for total RNA after 18 h, and hybridized to a 288-bp Aval/ApaI fragment of rat chromogranin A cDNA (20). Slot blots were washed and re-hybridized to a labeled cDNA probe for rat cyclophilin (34), a constitutively expressed (“housekeeping”) gene. Hybridization of rat chromogranin A cDNA to mRNA isolated from NIH 3T3 fibroblasts served as a negative control for probe specificity (not pictured). Band intensity was quantitated by densitometry as described in Methods. Data are reported as the ratio of densitometric intensity, rat chromogranin A to cyclophilin bands ($n = 2$). DEX, dexamethasone. Bars represent mean \pm 1 SEM.

To confirm that promoter region –583 to –597 bp was sufficient to confer response to glucocorticoid, a double-stranded oligonucleotide encoding this region (rat chromogranin A GRE, or “rGRE”), flanked by BamHI ends, was inserted into the BamHI site of pTKluc (36), just 5’ of the heterologous herpes simplex virus thymidine kinase (TK) promoter regulating luciferase reporter expression. The rGRE was inserted in both orientations and in multiple copy number, yielding vectors pTKlucC17, pTKlucC19, and pTKlucE9 (see Tables I and III for details of the sequence, BamHI ends, vector copy number and orientation). As a positive control, a double-stranded oligonucleotide encoding a consensus GRE (“cGRE”; 5’-AGAACAGAGTGTTCT-3’), also flanked by BamHI ends, was inserted into this position in single and tandem arrays resulting in vectors pcGRETKluc-1 and pcGRETKluc-2, respectively (refer to Tables I and III) (36). All response element insertions into these vectors were confirmed by sequence analysis.

A 1,133-bp mouse chromogranin A promoter/luciferase reporter vector (34; GenBank accession number L31361) was also used in some interspecies glucocorticoid activation experiments.

Transient cotransfection (trans-activation) studies. To investigate glucocorticoid effects on the isolated rat chromogranin A promoter, 11 μ g of total plasmid DNA were transfected into 6-cm PC-12 cell culture plates by lipofection (37). 7.5 μ g of rat chromogranin A promoter-luciferase reporter constructs were cotransfected with 1 μ g of pRSVhGR (expressing human glucocorticoid receptor [hGR] under control of the strong Rous sarcoma virus [RSV] long terminal repeat) (38) and 2.5 μ g pRSV-CAT (as a transfection efficiency control), expressing the reporter chloramphenicol acetyltransferase (CAT) under control of the RSV promoter (39). pRSVhGR was co-transfected to provide PC-12 cells with ample glucocorticoid receptor, since preliminary experiments ($n = 4$) indicated that dexamethasone maximally (101-fold) activated transfected pMMTVluc (expressing luciferase under control of mouse mammary tumor virus long terminal repeat, containing three functional GREs; 40) only when pRSVhGR was co-transfected. Dexamethasone actions on pMMTVluc (with co-transfected pRSVhGR) served as a positive control, while its effects on promoterless pXp2 served as a

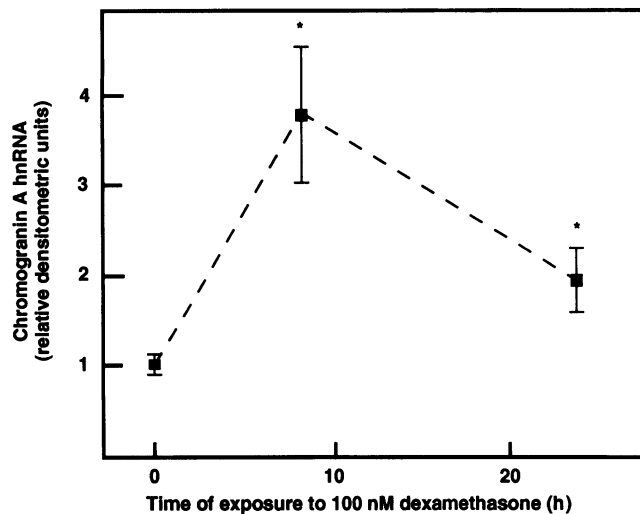


Figure 5. Transcriptional activation of chromogranin A by glucocorticoid. The graph quantitates the transcriptional response from $n = 3$ nuclear runoff assays, with measurement of autoradiographic band intensity by densitometry. Nuclei from AtT-20 mouse anterior pituitary corticotrope cells were isolated after exposure to 100 nM dexamethasone for 0, 8, or 24 h. Equivalent amounts of α -[32 P]-UTP-labeled heterogeneous nuclear RNA (hnRNA) were hybridized to slot blots containing 5 μ g of mouse chromogranin A genomic DNA (5.1 kb EcoRI/EcoRI fragment containing exons 1–3 and introns A–C) or pBluescriptKS⁻ DNA (negative control). hnRNA hybridized only to chromogranin A genomic DNA in each experiment. Values given are relative intensity, normalized to basal rate of CgA transcription (that is, 0 h of exposure to dexamethasone). * $P < 0.01$ (one-way ANOVA). Filled squares with bars, mean \pm 1 SEM.

negative control. Each chromogranin A promoter/luciferase reporter vector was evaluated in at least 2–3 separate transfections. For PCR-derived promoter fragments, two independently isolated amplification products were tested. Luciferase activity was measured by luminometry (41), CAT activity was determined by 14 C-acetylation of chloramphenicol, with organic phase extraction (42), and cell protein was measured by Coomassie blue dye-binding (43).

In some experiments, transfection results were normalized to those of pSV2AL Δ 5 (wherein luciferase expression is driven by the SV40 early promoter) (41).

Reagents. PC-12 (rat adrenal chromaffin) cells, Cos (T antigen-transformed monkey kidney) cells, and AtT-20 (mouse anterior pituitary corticotrope) cells were treated with the following reagents: the glucocorticoid receptor agonist dexamethasone (100 nM; Sigma Chemical Co., St. Louis, MO), the glucocorticoid receptor antagonist RU-486 (1 μ M; Roussel Uclaf, Paris, France), the mineralocorticoid receptor antagonist spironolactone (10 μ M; Sigma), or the protein synthesis (translation) inhibitor cycloheximide (5 μ g/ml; Sigma).

Electrophoretic gel mobility shift studies. Nuclear extracts were prepared from PC-12 or Cos cells according to Dignam and Roeder (44). 10-cm plates were transfected (37) with 10 μ g pRSVhGR or control DNA at 30% (Cos) or 50% (PC-12) confluence, then cultured in the presence or absence of 100 nM dexamethasone, and harvested after 48 hours. After phosphate buffered saline (PBS) washes at 0°C, cells were pelleted for 5 min at 750 g at 4°C. Cell pellets were resuspended in three volumes of modified Dignam solution A (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, with or without 100 nM dexamethasone) and incubated for 15 min on ice. Cells were lysed by passing the suspension five times through a 25 gauge needle. Nuclei were isolated by a 30 second, 13,000 g micro-centrifugation at 4°C. The pellet was resuspended on ice in one original volume of modified Dignam C solution (10 mM HEPES, pH 7.9, 25% glycerol,

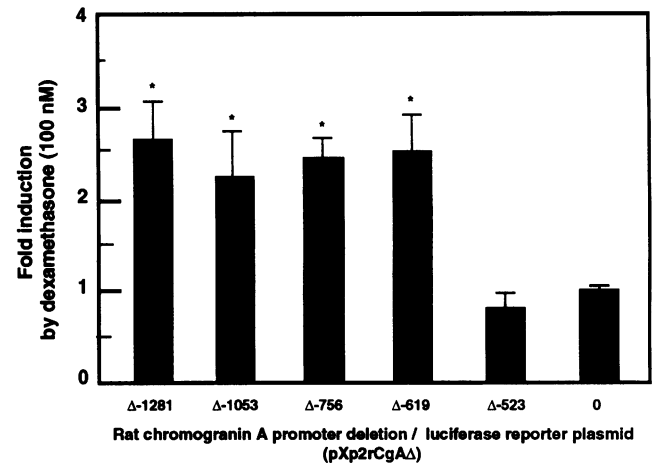


Figure 6. Progressive 5' deletion of the rat chromogranin A promoter: effect of the transcriptional response to glucocorticoid. This bar graph compares the relative glucocorticoid induction of rat chromogranin A promoter deletion/luciferase reporter plasmids in co-transfections. 7.5 μ g of a pXp2rCgA deletion were cotransfected into PC-12 cells with 1 μ g of pRSVhGR and 2.5 μ g pRSVCAT. Cells were treated with 100 nM dexamethasone (or vehicle) and harvested at 48 h for luciferase and CAT assays. Induction values for each deletion plasmid are the ratio of luciferase activity in dexamethasone-treated to vehicle-treated cells, after correcting for transfection efficiency differences (normalization to CAT activity). Numbers under each bar indicate the 5' promoter deletion plasmid: Δ -1281, pXp2rCgA Δ -1281; Δ -1053, pXp2rCgA Δ -1053; Δ -756, pXp2rCgA Δ -756; Δ -619, pXp2rCgA Δ -619; Δ -526, pXp2rCgA Δ -526; 0, pXp2 (promoterless reporter). $N \geq 3$ transfections for each plasmid. Two different plasmid clones were used for each PCR-derived promoter deletion (refer to Table I for details). Data not pictured include the positive control, wherein dexamethasone activated transfected pMMTVluc by 101-fold over basal levels. * $P < 0.05$ (one-way ANOVA).

0.42 M NaCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.5 mM PMSF, 100 nM dexamethasone) and gently rotated at 4°C for 30 min. Nuclear extracts were isolated by collecting supernatants from a 5 minute, 13,000 g micro-centrifugation, and were frozen at -70°C. Protein concentration was determined by Coomassie blue dye-binding (43).

A DNA: protein binding assay was performed on dexamethasone-treated nuclear extracts by incubation with a γ -[32 P]-end-labeled double-stranded 22-bp oligonucleotide for rat chromogranin A GRE (*rGRE) or a consensus GRE (*cGRE) (see Table I for specific sequences). 0.7–2.0 μ g of nuclear extract protein (in \sim 1 μ l modified Dignam solution C) was incubated with 100 pg *rGRE or *cGRE (50,000 cpm/sample) on ice for 30 min in a 15 μ l volume including 10 mM HEPES/KOH, pH 7.9, 3.3 mM Tris pH 7.9, 66.7 mM KCl, 3.3 mM NaCl, 0.2 mM EDTA, 5 mM MgCl₂, 10% glycerol, 100 nM dexamethasone, 0.33 mM DTT, 10 μ g BSA, and 1 μ g poly-dI-dC. Binding samples were applied to a 6% nondenaturing polyacrylamide (37.5:1 acrylamide: bisacrylamide) 0.4X tris-borate-EDTA (TBE) gel and electrophoresed for 2 h at 200 V (< 35 mA). Gels were dried, exposed to x-ray film, and the autoradiographic bands were quantified densitometrically (StratoScan densitometer).

Statistics. Hormone responses were determined to be significant by one-tailed *t* test or one-way ANOVA, as appropriate.

Results

Isolation of the rat chromogranin A gene and its functional promoter. Fig. 1 details a restriction map of rat chromogranin A genomic clones. A 1,594-bp PstI/PstI fragment hybridized

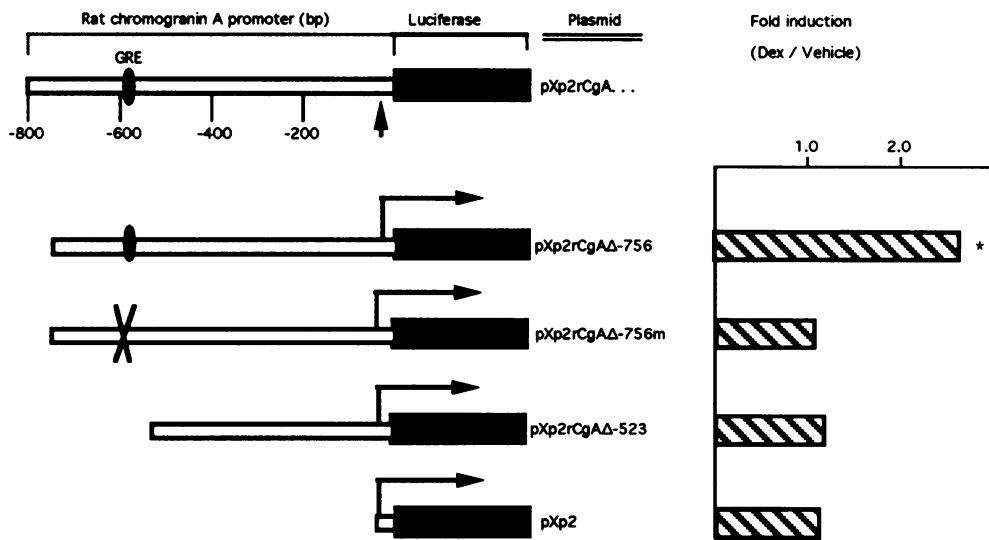


Figure 7. This figure illustrates how mutagenesis of the consensus GRE half-site sequence in the rat CgA promoter ([−592 bp] 5′-TGTCT-3′ [−597 bp]) abolishes response of promoter/luciferase constructs to 100 nM dexamethasone. Three rat chromogranin A promoter deletion/luciferase reporter plasmids (pXp2rCgAΔ-756, pXp2rCgAΔ-756m, pXp2rCgAΔ-523) and one promoterless luciferase expression plasmid (pXp2) were transfected into PC-12 cells. Luciferase reporter activity (normalized to cell protein content) after 100 nM dexamethasone is compared with activity after vehicle. Plasmid structure is diagrammed on the left: (blackened oval)

GRE; (X) GRE site-directed mutation by multiple base substitutions at GRE half-site (see Methods and Table I); (open box) rat chromogranin A promoter; (solid box) luciferase reporter gene; (vertical arrow) CAP site; (horizontal arrow) direction of transcription. In the middle section, the transfected promoter deletion/luciferase reporter plasmid is listed by name. On the right, the mean fold-induction by dexamethasone (versus vehicle) is graphed for each plasmid. $N \geq 2$ transfections for each plasmid. For PCR-derived mutations, two independent clones were evaluated. Not shown is the positive control in which dexamethasone activated transfected pMMTVluc.

to the 34-bp oligonucleotide probe; sequence analysis positioned the 5′ end of the probe recognition site within 75 bp of the 3′ end of the 1,594-bp fragment.

The 1,594-bp fragment was sequenced (see Fig. 2), with the following results: (a) the 34-bp oligonucleotide sequence was within 45 bp of a TATA box homology; (b) the entire sequence showed substantial homology (see below) with the mouse chromogranin A promoter (34) and its adjacent first exon, demarcated by the transcription initiation or “cap” site (nucleotide +1, Fig. 2). Therefore this 1,594-bp fragment of rat genomic DNA contained the 5′ flanking region as well as

the 5′ untranslated exon 1 (leader) region of the rat chromogranin A gene.

In transfections testing whether this 5′ flanking region constituted a functional, cell type-specific promoter (Table II), pXp2rCgAΔ-523(+) showed 29-fold greater expression in PC-12 chromaffin cells than in control (NIH-3T3 fibroblast) cells. In PC-12 cells, pXp2rCgAΔ-523(+) expression (with the promoter in the sense [correct] orientation) exceeded by 6.3-fold that of the corresponding plasmid with the promoter in the opposite (inverted, incorrect) orientation [pXp2rCgAΔ-523(−)], and by 63-fold that of the promoterless reporter vec-

Table III. Effect of Glucocorticoid on Thymidine Kinase Promoter/Luciferase Reporter Constructs with Glucocorticoid Response Element Insertions

Plasmid	Inserted element	No.	Orientation	Dex induction (Dex/no dex)	±SEM	P (versus pTKluc)
pTKluc	None	—	—	1.22	±0.29	—
pTKlucC17	rGRE	1	<	2.46	±0.63	0.043
pTKlucC19	rGRE	1	>	2.06	±0.22	0.019
pTKlucE9	rGRE	2	⊕	3.38	±1.31	0.023
pcGRETkluc-1	cGRE	1	>	8.57	±3.21	0.027
pcGRETkluc-2	cGRE	2	⊕	12.11	±0.96	0.025

Potency of rat chromogranin A GRE (rGRE) induction by 100 nM dexamethasone is compared with that of a consensus GRE (cGRE). Using pTKluc (a thymidine kinase promoter/luciferase reporter) as the insertion vector, plasmids were constructed with single or double insertions of rGRE or cGRE into the BamHI site located adjacent (5′) to the TK promoter. Number and direction (either forward or reverse) of inserted GREs was confirmed by sequence analysis. Experimental protocol included transient co-transfection of each analysis. Experimental protocol included transient co-transfection of each construct with pRSVCAT into PC-12 cells, and treatment with 100 nM dexamethasone (or vehicle). After 48 h cells were harvested and assayed for luciferase and chloramphenicol acetyltransferase (CAT) activity. The fold-induction represents luciferase activity (corrected for transfection efficiency by CAT assay) for each construct in glucocorticoid-treated versus vehicle-treated cells. rGRE, the rat chromogranin A GRE with BamHI ends (Table I); cGRE, consensus GRE with BamHI ends (Table I); No., number of GREs inserted into the BamHI site; >, single insert in forward (endogenous) orientation; <, single insert in reverse orientation; ⊕ or ⊗, double insertion, orientation as described; P, values for one-way T-test relative to pTKluc. $n = 3$ transfections.

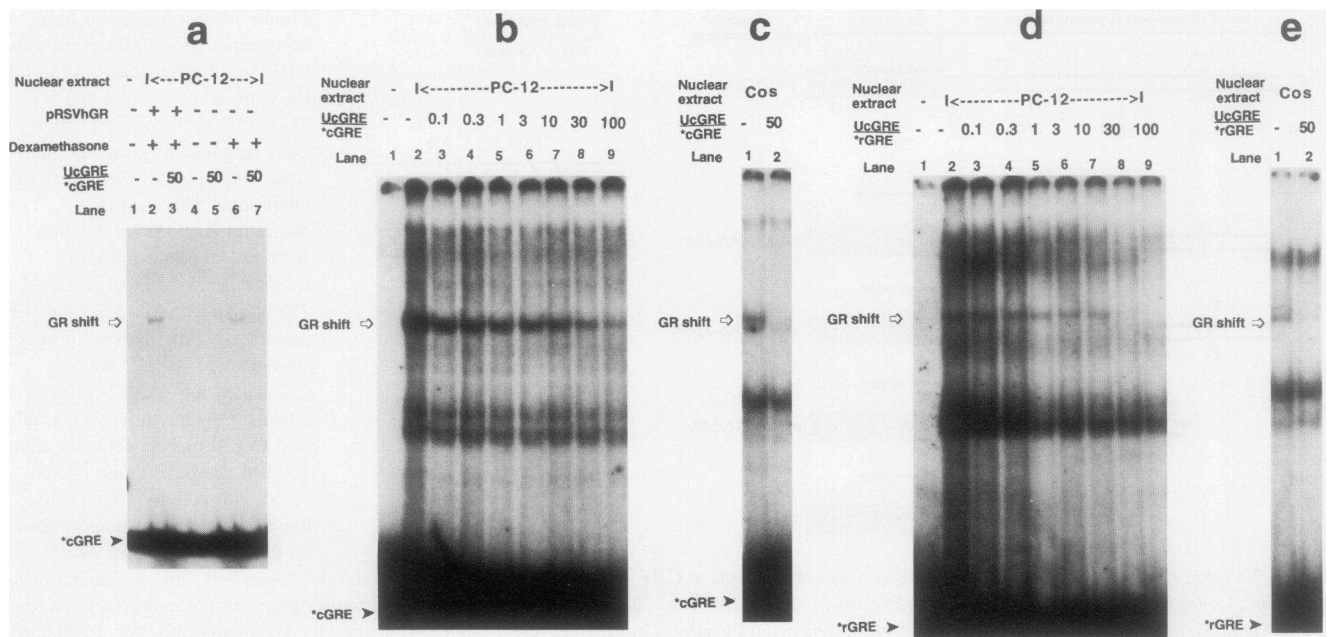


Figure 8. Specific binding of rat chromogranin A GRE (*rGRE*) or a consensus GRE (*cGRE*) to glucocorticoid receptor (*GR*) is investigated in a series of five (*a–e*) *in vitro* electrophoretic mobility shift assays. Nuclear extracts were prepared according to a modification of the protocol outlined by Dignam and Roeder (44; see Methods). (*a*) The band shift of a γ -[³²P]-end-labeled double-stranded consensus GRE probe (**cGRE*), incubated with 1 μ g of nuclear extract protein from PC-12 cells, in the presence or absence of 50X molar excess of double-stranded, cold (unlabeled) consensus GRE (“UcGRE”). PC-12 cells were grown in the presence or absence of 100 nM dexamethasone prior to extraction of nuclei. 50,000 cpm of **cGRE* were electrophoresed in each lane. Lane 1, no nuclear extract; lanes 2 and 3, pRSVhGR-transfected PC-12 cells, with dexamethasone exposure; lanes 4 and 5, untransfected PC-12 cells, no dexamethasone exposure; lanes 6 and 7, untransfected PC-12 cells with dexamethasone exposure. Lanes 3, 5, and 7 were treated with 50X molar excess of UcGRE, while lanes 2, 4, and 6 received an equivalent amount (mass excess) of unlabeled salmon sperm (non-specific control) DNA. (*b*) A competitive gel shift study between γ -[³²P]-end-labeled *cGRE* (**cGRE*) and unlabeled *cGRE* (UcGRE). Nuclear extracts from PC-12 cells were isolated in the presence of 100 nM dexamethasone. All lanes received 50,000 cpm of **cGRE*. Lane 1 contains **cGRE* only; lanes 2–9 received 2 μ g PC-12 nuclear extract protein. The molar ratio of unlabeled to labeled probe (UcGRE:**cGRE*) is listed above each lane. The molar ratios ranged from 0.1:1 to 100:1. Unshifted **cGRE* is indicated by the arrowhead. The open arrow indicates position of the **cGRE* shift by glucocorticoid receptor (*GR*). (*c*) Gel shift similar to *b*, but nuclear extract protein (2 μ g/lane) is from Cos cells, transfected with pRSVhGR and treated with 100 nM dexamethasone. (*d*) A competitive gel shift study between γ -[³²P]-end-labeled *rGRE* (**rGRE*) and unlabeled *cGRE* (UcGRE). Nuclear extracts from PC-12 cells were isolated in the presence of 100 nM dexamethasone. All lanes received 50,000 cpm of **rGRE*. Lane 1 contains **rGRE* only; lanes 2–9 received 2 μ g PC-12 nuclear extract protein. The molar ratio of unlabeled to labeled probe (UcGRE:**rGRE*) is listed above each lane. The molar ratios ranged from 0.1:1 to 100:1. Unshifted **rGRE* is indicated by the arrowhead. The open arrow indicates position of the **rGRE* shift by glucocorticoid receptor (*GR*). (*e*) Gel shift similar to *d*, but nuclear extract protein (2 μ g/lane) is from Cos cells, transfected with pRSVhGR and treated with 100 nM dexamethasone.

tor (pXp2). Thus, the promoter exhibited a typical directional preference, as well as cell type specificity.

Sequence analysis of the chromogranin A promoter. 1,482 bp were sequenced upstream (5′) of the rat chromogranin A promoter cap site, established by comparison with corresponding cap sites in the mouse (34), bovine (16), and human (45) chromogranin A promoters. Promoter sequence consensus homologies (Fig. 2) included a TATA box (TATAAAA; −30 bp; plus strand) (46), a partial (7/8 bp) CREB site match (TGACGTA; −71 bp; plus strand) (47), and three Sp1 sites (GGGCGG; −79, −113, and −936 bp; all on the plus strand) (48). There were eight potential GRE half-sites: TCTCCT (hGRE-MTIIA; 49) at −1,408 bp (plus strand), −592 bp (minus strand), and −228 bp (minus strand); TGTCT (hGRE.3; 50) at −1,083 bp (plus strand), and −1,073 bp (plus strand); and AGTCCT (hGRE.7; 51) at −1,361 bp (plus strand), −672 bp (minus strand), and −240 bp (minus strand).

Promoter interspecies sequence homologies were established with a Pustell DNA database matrix analysis (MacVector; IBI/Kodak, New Haven, CT), and GenBank (Entrez ver-

sion, release 10.0, April 15, 1994; National Center for Biotechnology Information, Bethesda, MD) files for the promoter (5′ flank, up to the cap site) regions of mouse chromogranin A (1,135 bp; reference 34; GenBank accession number L31361), bovine chromogranin A (255 bp; reference 16; GenBank accession number S79277), and human chromogranin A (771 bp; reference 45; GenBank accession number X60682). The rat chromogranin A promoter sequence was 71% homologous with the mouse promoter, 25% homologous with the bovine promoter, and 17% homologous with the human promoter.

Time course of chromogranin A mRNA response to glucocorticoid. After dexamethasone, chromogranin A mRNA increased 1.3-, 3.5-, and 2.5-fold over basal at 5, 15, and 24 h (Fig. 3). Pretreatment with the glucocorticoid antagonist RU-486 (52) blocked the induction.

Regulation of chromogranin A mRNA by glucocorticoid during translational inhibition of protein synthesis. To determine whether chromogranin A mRNA induction by dexamethasone is direct, or requires activation of an intermediary gene, the response to glucocorticoid was compared in the presence

and absence of translation inhibition by cycloheximide (Fig. 4). Dexamethasone augmented chromogranin A gene expression by 1.95-fold in the presence and 2.3-fold in the absence of cycloheximide; therefore, the response to glucocorticoid did not require new protein synthesis.

Transcriptional (nuclear runoff) studies. To test whether the chromogranin A mRNA increase (Fig. 5) is a transcriptional response to glucocorticoid, three independent nuclear runoff studies showed a significant increase in new chromogranin A hnRNA transcripts after 100 nM dexamethasone—by 3.3-fold at 8 h and 1.8-fold at 24 h.

Glucocorticoid effects on chromogranin A promoter-luciferase reporter constructs. To define the glucocorticoid-responsive region of the rat chromogranin A promoter, promoter deletion/luciferase reporter plasmids were transfected (Fig. 6). Reporter expression was increased 2.6–3.1-fold after glucocorticoid. There was a significant dropoff in glucocorticoid response between positions –619 bp (plasmid pXp2rCgAΔ-619) and –523 bp (plasmid pXp2rCgAΔ-523).

A transfected mouse chromogranin A 1,133-bp promoter/luciferase reporter construct (34) (GenBank accession number L31361) was also activated 2.52-fold (light units/mg protein; $n = 4$ replicates; $P < 0.05$) by 10^{-6} M dexamethasone in PC12 cells.

In a dose-response study from 10^{-12} to 10^{-5} M dexamethasone, transfected promoters of both chromogranin A and mouse mammary tumor virus (pMMTVluc [40]) were each maximally activated after 10^{-7} M dexamethasone.

Specificity of glucocorticoid action on the chromogranin A promoter. Since dexamethasone is also a weak agonist at mineralocorticoid receptors (53), the response of the transfected chromogranin A promoter (pXp2rCgAΔ-1281) to dexamethasone (100 nM) was studied in the presence of glucocorticoid receptor- and mineralocorticoid receptor-specific antagonists (added 30 min before agonist). The response to dexamethasone was completely blocked by the glucocorticoid antagonist RU-486 (1 μ M), but was not affected by the mineralocorticoid antagonist spironolactone (10 μ M); in the absence of agonist, neither of these antagonists affected promoter/reporter expression (data not shown).

Isolation of a glucocorticoid response element from the rat chromogranin A promoter. Sequence analysis of the rat promoter between –523 and –619 bp revealed a consensus match for a glucocorticoid receptor-binding half-site at (–597 bp) 5'-AGGACA-3' (–592 bp) (on the opposite strand: [–592 bp] 5'-TGTCCT-3' [–597 bp]). To test the function of this rGRE motif, PC-12 cells were transfected with pXp2rCgAΔ-756 (which contains the wild-type rGRE motif) versus pXp2rCgAΔ-756m (with a substitution mutation at the rGRE motif, [–597 bp] 5'-gcGgtA-3' [–592 bp]; see Methods and Table I), and treated with dexamethasone or vehicle (Fig. 7). Only wild-type pXp2rCgAΔ-756 responded significantly (2.44-fold, $P = 0.005$) to dexamethasone. No dexamethasone response was found for pXp2rCgAΔ-756m, or for the negative controls pXp2rCgAΔ-523 (which lacks sequence upstream of –523 bp) or the promoterless reporter pXp2.

A 15-bp sequence ([–597 bp] 5'-AGGACACACTCATGT-3' [–583 bp] or "rGRE"; consensus half-site in bold; on the opposite strand: [–583 bp] 5'-ACATGAGTTGTCCT-3' [–597 bp]), corresponding in length to the consensus 15-bp glucocorticoid receptor homodimer-binding GRE motif (49, 54, 55), was inserted into the heterologous promoter/luciferase reporter

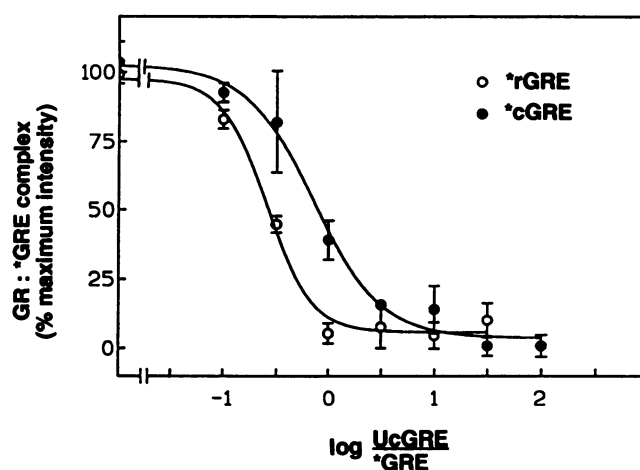


Figure 9. Relative affinities of rat chromogranin A GRE (rGRE) and consensus GRE (cGRE) for glucocorticoid receptor. This figure plots competitive displacement curves generated from the gel retardation studies of Fig. 8. The complexes of GR with labeled ligand (*rGRE or *cGRE), as they are progressively displaced by unlabeled cGRE (UcGRE), are normalized to their respective minimum (0%) and maximum (100%) autoradiographic, densitometric band intensities. The recorded band intensity for a given molar ratio (in \log_{10} units) of UcGRE to labeled probe is graphed as mean percentage of maximum intensity (\pm one SEM) from three separate densitometric measurements. Competition curves and relative EC_{50} values were estimated by non-linear regression, using the InPlot GraphPad statistical package (GraphPad Software, San Diego, CA). Note: *cGRE— γ -[32 P]-labeled double-stranded consensus GRE; *rGRE, γ -[32 P]-labeled double-stranded rat chromogranin A GRE; UcGRE, unlabeled double-stranded consensus GRE; *GRE, labeled probe (*cGRE or *rGRE); (filled circles) displacement of *cGRE by UcGRE; (open circles) displacement of *rGRE by UcGRE; bars, \pm one standard error of mean (SEM). $n = 3$ replicate determinations.

plasmid pTKluc. Single rGRE inserts (pTKlucC17 or pTKlucC19) increased luciferase reporter activity by 2.06 to 2.46-fold after glucocorticoid (Table III); tandem (double) rGRE inserts (pTKlucE9) induced 3.38-fold activation by glucocorticoid. Thus, glucocorticoid response of the rGRE element was copy number-dependent and orientation independent. Glucocorticoid induction of these plasmids' rGREs yielded only 24–28% of the 8.6–12.1-fold induction achieved by similar plasmids with cGREs (pcGRETKluc-1, pcGRETKluc-2; Table III).

Electrophoretic gel mobility shift assays. To determine whether rGRE and cGRE bind glucocorticoid receptor similarly, gel retardation studies were performed using nuclei from PC-12 chromaffin or control Cos cells (Fig. 8).

PC-12 nuclear extracts (Fig. 8a), when prepared from cells treated with dexamethasone (100 nM) alone (lane 6), or dexamethasone plus co-transfected pRSV-hGR (lane 2), shifted the mobility of *cGRE. Without glucocorticoid (lanes 4 and 5), a shift was not seen. Co-transfected pRSVhGR increased the amount of *cGRE shifted (lane 2 versus lane 6), but only when glucocorticoid was also given (lane 2 versus lane 4). Specificity of band shifts was confirmed by abolition after competition by 50-fold molar excess of unlabeled cGRE (lanes 3 and 7). Similar results were obtained with 3 independent preparations of PC-12 nuclei.

Radiolabeled cGRE (*cGRE; Fig. 8b) or rGRE (*rGRE;

Table IV. Comparison of Rat Chromogranin A Glucocorticoid Response Element (GRE) to other GREs

GRE type	5' bp	Sequence					3' bp	Homology	Reference									
		1	6	7	9	10				15								
Consensus		A	G	A	A	C	A	n	n	n	T	G	T	T	C	T	—	(54)
hGRE-MTIIA	-263	G	G	T	A	C	A	c	t	g	T	G	T	C	C	T	—	(49)
Rat CgA GRE	-583	A	c	A	t	g	A	g	t	g	T	G	T	C	C	T	9/12	Present work
ANF GRE I	-963	G	c	c	t	g	t	t	t	g	T	G	T	T	C	T	7/12	(60)
ANF GRE II	-884	t	c	T	c	t	g	t	a	a	T	G	T	C	C	T	7/12	(60)
PEPCK GRE I	-378	c	a	c	a	C	A	a	a	a	T	G	T	g	C	a	6/12	(61)
PEPCK GRE II	-367	A	G	c	A	t	A	t	g	a	a	G	T	C	C	a	8/12	(61)

This table compares homology of GREs that show strong response (consensus, and human metallothionein-IIA [hGRE-MTIIA]) to glucocorticoid, versus GREs that show a more moderate (2–4-fold) response. Strong response GREs are shown in **bold** and aligned according to GRE half-site motifs. They serve as indexes to which homology for less responsive GREs can be compared. Moderately responsive GREs from rat chromogranin A (CgA), atrial natriuretic factor (ANF), and phosphoenolpyruvate carboxykinase (PEPCK) genes are aligned below the index GREs by placing that gene's GRE half-site motif with stronger homology to the sequences 5'-TGTTCT-3' or 5'-TGTCCT-3' at positions 10 through 15. Fraction of homology for moderate GREs is based on exact conservation (upper case lettering) with the base from either index (strongly responsive) GRE for positions 1 through 6 and 10 through 15. Negative numbers flanking GREs indicate position within each GRE's promoter. Among moderately responsive GREs, rat CgA GRE shows the highest homology (9/12) to the index GREs, while PEPCK and ANF require cooperativity between two separate 15-bp motifs (for example, ANF GRE I plus ANF GRE II) to achieve their moderate responses to glucocorticoid.

Fig. 8 *d*) each bound and was shifted by glucocorticoid receptor from PC-12 nuclei. The fractional band shift (shifted band mobility/free band mobility) in the same gel was identical for both *cGRE and *rGRE. Unlabeled cGRE (UcGRE) displaced both *cGRE (Fig. 8, *b* and *c*) and *rGRE (Fig. 8, *d* and *e*) from glucocorticoid receptor, whether the receptor was from PC-12 nuclei (Fig. 8, *b* and *d*) or (pRSVhGR-transfected-) Cos nuclei (Fig. 8, *c* and *e*). Unlabeled nonspecific (salmon sperm) DNA, at 50-fold mass excess, had no effect on the shifted *rGRE band mobility.

As the molar ratio of UcGRE/*cGRE (Fig. 8 *b*) or UcGRE/*rGRE (Fig. 8 *d*) was progressively increased from 0.1 to 100, *cGRE and *rGRE were competitively displaced from glucocorticoid receptor in PC-12 nuclei. The relative affinities (EC₅₀s) of *rGRE and *cGRE for glucocorticoid receptor were estimated by nonlinear regression analysis (GraphPad InPlot competition curve; GraphPad Software, Inc., San Diego, CA) of the log₁₀ molar displacement curves (Fig. 9). *rGRE had 2.75-fold (= antilog₁₀ 0.44) lower affinity for glucocorticoid receptor than *cGRE.

Discussion

Isolation of a functional GRE in the rat CgA gene. To determine the mechanism by which glucocorticoids augment chromogranin A expression, we focused on transcriptional regulation of the gene, since glucocorticoid induction of chromogranin A protein in vitro and in vivo parallels induction of chromogranin A mRNA (14–17). The investigation initially concentrated its efforts on isolating the 5' regulatory region (promoter) of the rat chromogranin A gene (Figs. 1 and 2, and Table II), and determining the extent to which glucocorticoids activated chromogranin A expression at a transcriptional (pre-translational) level. We found that glucocorticoid activated chromogranin A gene expression up to 3.5-fold (Fig. 3), that the response did not require new protein synthesis (Fig. 4), and involved a 3.3-fold increase in rate of initiation of new chromogranin A transcripts (Fig. 5).

A novel glucocorticoid response element ([–583 bp] 5'-ACATGAGTGTGCCT-3' [–597 bp]) bound glucocorticoid receptors (Fig. 8). Functional properties of this novel rGRE included: (a) a 2.6- to 3.1-fold increment in promoter activity in response to glucocorticoid was lost after deletion of this region (Fig. 6); (b) transfer of the motif to a heterologous promoter yielded 2.06–3.38-fold glucocorticoid induction (Table III); and (c) site-directed mutation of the motif abolished the glucocorticoid response (Fig. 7).

Evidence that this rGRE exerts its effects through a selective interaction with ligand-activated glucocorticoid receptor emanated from two studies: dexamethasone induction was blocked by a glucocorticoid antagonist but not a mineralocorticoid antagonist, and the rGRE specifically bound ligand-activated glucocorticoid receptor in vitro (Fig. 8).

Glucocorticoid activation of the rat CgA gene. Glucocorticoids *trans*-activate many genes at the level of transcription (56 and references therein). Ligand-activated glucocorticoid receptor homodimers bind characteristic DNA response elements (GREs). Mutagenesis of the glucocorticoid receptor (36) DNA binding domain indicates that zinc finger motifs (composed of two sets of four cysteine residues per monomer) contain specific amino acids critical for DNA binding specificity to a GRE. X-ray crystallography (57) further established that each monomer in the homodimer interfaces with specific bases in the major groove of the double-stranded DNA, for high affinity binding.

Glucocorticoid receptor homodimers cooperatively bind full length, 15-bp GREs with at least 10-fold greater affinity than their attraction for GRE half-sites (58, 59). Since the 15-bp rGRE motif bound glucocorticoid receptor at only 2.75-fold lower affinity than the 15 bp cGRE (Fig. 9), even the degenerate half-site within the rGRE ([–583 bp] 5'-ACATGA-3' [–588 bp]) may provide sufficient affinity to participate in cooperative binding of the glucocorticoid receptor homodimer.

Functionally, rGRE mediated 2–3.5-fold increments in gene expression after glucocorticoid, in several contexts (Figs. 3–6). In a direct comparison (Table III) of isolated cGRE versus

rGRE effects, cGRE caused 3.6–4.2-fold greater glucocorticoid induction than rGRE, a value consistent with the 2.75-fold affinity differences of these motifs for glucocorticoid receptor (Fig. 9).

Inspection of the rGRE sequence ([-583 bp] 5'-ACATGAGTGTGTCCT-3' [-597 bp]; Table IV) reveals a consensus (49) GRE half-site ([-592 bp] 5'-TGTCCT-3' [-597 bp]) and a degenerate half-site ([-588 bp] 5'-TCATGT-3' [-583 bp]). There are ample precedents for such imperfect (degenerate from consensus) GREs with preserved (though attenuated) response to glucocorticoid. Atrial natriuretic factor (ANF) and phosphoenolpyruvate carboxypeptidase (PEPCK) genes, each of which display 2–4-fold stimulation responses to glucocorticoid, have GREs which are even more degenerate from consensus than the rGRE (60, 61; Table IV).

Functional GREs have not been isolated from other species' chromogranin A genes, although the transfected mouse chromogranin A 1133 bp promoter/luciferase reporter responded 2.52-fold to glucocorticoid (see Results). In the region of the mouse (34; GenBank accession number L31361) chromogranin A promoter ([-583 bp]-5'-ACATGGGTGGTCT-3' [-597 bp]) corresponding to the rGRE, 13/15 bp are identical (in **bold**) to those in the rGRE. The first 1,135 reported bp of the mouse chromogranin A promoter (34; GenBank accession number L31361) also have another GRE half-site (AGTCCT; hGRE.7; 51) match at position -679 bp (minus strand). The first 255 reported bp of the bovine chromogranin A promoter (16; GenBank accession number S79277) contain one GRE half-site match (AGTCCT; hGRE.7; 51) at position -230 bp (plus strand). The first 771 reported bp of the human chromogranin A promoter (45; GenBank accession number X60682) contain no GRE half-site matches.

Biological significance of the chromogranin A response to glucocorticoids. A similar degree of glucocorticoid activation of transfected rat and mouse chromogranin A promoters (2.6- to 3.1-fold, versus 2.52-fold), coupled with GRE sequence homologies in mouse, rat, and bovine chromogranin A promoters, suggest that chromogranin A GREs may be of general functional importance in mammalian species.

One function of chromogranin A is its action to complex or osmotically inactivate cations such as calcium and catecholamines within the catecholamine storage vesicle core (8). Since glucocorticoid exposure also augments catecholamine storage in chromaffin cells by 2- to 4-fold (62–64), a parallel rise in co-stored chromogranin A may provide additional binding capacity for glucocorticoid-stimulated increases in vesicular catecholamine stores (8). Zhang et al. (65) have also shown that the effects of glucocorticoid on the chromogranin A mRNA depend on the prevailing extracellular calcium concentration.

Once secreted, chromogranin A proteolytic fragments are active in the extracellular space, modulating further catecholamine release from chromaffin cells (12). Thus, an increment in chromogranin A may also provide a homeostatic or negative-feedback “brake” on release of steroid-augmented catecholamine stores.

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