Cell Type-specific Gene Expression in the Neuroendocrine System

A Neuroendocrine-specific Regulatory Element in the Promoter of Chromogranin A, a Ubiquitous Secretory Granule Core Protein

Hongjiang Wu, David J. Rozansky, Nicholas J. G. Webster, and Daniel T. O'Connor

Department of Medicine and Center for Molecular Genetics, University of California, San Diego, California 92161; and Department of Veterans Affairs Medical Center, San Diego, California 92161

Abstract

The acidic secretory protein chromogranin A universally occurs in amine and peptide hormone and neurotransmitter storage granules throughout the neuroendocrine system. What factors govern the activity of the chromogranin A gene, to yield such a widespread yet neuroendocrine-selective pattern of expression? To address this question, we isolated the mouse chromogranin A gene promoter. The promoter conferred cell type-specific expression in several neuroendocrine cell types (adrenal medullary chromaffin cells, anterior pituitary corticotropes, and anterior pituitary somatolactotropes) but not in control (fibroblast or kidney) cells. In neuroendocrine cells, analysis of promoter deletions established both positive and negative transcriptional regulatory domains. A distal positive domain (−4.8/−2.2 kbp) was discovered, as well as negative (−258/−181 bp) and positive (−147/−61 bp) domains in the proximate promoter. The proximate promoter contained a minimal neuroendocrine-specific element between −77 and −61 bp. Sequence alignment of the mouse promoter with corresponding regions in rat and bovine clones indicated that the mouse sequence shares over 85% homology with rat and 52% with bovine promoters. DNaseI footprinting and electrophoretic gel mobility shift assays demonstrated the presence of nuclear factors in neuroendocrine cells that recognized the proximate promoter. We conclude that the chromogranin A promoter contains both positive and negative domains governing its cell type-specific pattern of transcription, and that a small proximate region of the promoter, containing novel as well as previously described elements, interacts specifically with neuroendocrine nuclear proteins, and is thereby sufficient to ensure widespread neuroendocrine expression of the gene. (*J. Clin. Invest.* 1994. 94:118–129.) Key words: PC-12 • AtT-20 • GH3 • chromaffin • catecholamine • enhancer

Introduction

Amine- and peptide-producing neuroendocrine cells possess a highly specialized secretory phenotype; some features of this phenotype, such as dense core granules of the regulated secretory pathway (1, 2), are universal characteristics of such cells.

The chromogranin/secretogranin protein family has a ubiquitous distribution in secretory granules throughout the neuroendocrine system (3–5). Members of this protein group share ancestral exons, and may thus constitute an ancient gene family (6, 7). These acidic, soluble proteins are co-stored and co-released, by exocytosis, with virtually all amine and peptide hormones and neurotransmitters (3–5). Within hormone storage granules, these proteins may osmotically stabilize granule cores by complexing other secretory components (8), and may modulate prohormone processing (9); before and upon release, chromogranins/secretogranins are proteolytically cleaved to a series of active peptides which may act in the extracellular space to control further hormone release from the secretory cell (10–14).

Among the chromogranins/secretogranins, perhaps the most widespread in distribution, especially in neuroendocrine neoplasia (15), is chromogranin A, a 48-kD protein encoded by a recently isolated (6, 7) eight-exon gene whose chromosomal position has been established in homologous (conserved) regions in man (16, 17), mouse (18), rat (18), and cattle (19).

Because the chromogranin A gene has such a widespread neuroendocrine pattern of expression (3–5, 11, 15), yet its expression is confined to the neuroendocrine system, excluding expression in purely exocrine tissues or nonpeptide-producing endocrine tissues (3–5, 11, 15), it occurred to us that its transcriptional control region might provide unique insights into acquisition of the neuroendocrine secretory phenotype.

We therefore isolated a functional transcriptional control region from the chromogranin A gene, characterized its expression in neuroendocrine (versus control) cells, defined functional domains within this promoter, and analyzed interactions between the promoter and neuroendocrine nuclear proteins. Our results suggest that the chromogranin A 5′ flanking region contains multiple positive and negative functional transcriptional control regions, most of which behave as promoter rather than enhancer domains. In the minimal neuroendocrine cell type-specific control region, neuroendocrine nuclear proteins recognized well-known as well as novel DNA sequences.

These results suggest the potential for novel cis- and trans-acting elements giving rise to the neuroendocrine secretory phenotype.

Methods

Mouse and rat chromogranin A genomic clone isolation and characterization. Genomic clones were isolated from mouse (strain AJ) and rat genomic DNA libraries in the BamHI site of the cosmid vector sCos-1 (20, 21), as previously described (6). Clones were restriction mapped as previously described (6).

Address correspondence to D. T. O'Connor, M.D., Division of Nephrology-Hypertension (9111H), University of California, San Diego, 3350 La Jolla Village Drive, San Diego, CA 92161.

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Promoter terminology. Promoter fragments are oriented and numbered with reference to the transcription initiation (cap) site as +1. For example, a fragment from −1135 to +52 spans a region from 1135 bp upstream (5′) of the cap site to 52 bp downstream (3′) of the cap site.

Cap site and sequencing. The transcription initiation (cap) site was established both by primer extension, as previously described (6), and by RNAse protection analysis (22). An EcoRI–PstI genomic fragment, spanning positions −1135 to +52 bp, was subcloned into pBluescript (Stratagene, La Jolla, CA), and linearized with KpnI at position −181 bp. This template was used to synthesize antisense RNA, priming at the T3 phage promoter and using T3 RNA polymerase. The source of sense neuroendocrine mRNA was the mouse pituitary corticotrope cell line AtT-20/D16v (23), while sense control (non-endocrine) mRNAs came from mouse liver and a murine erythroleukemia cell line MEL.

The same EcoRI–PstI fragment (−1135 to +52 bp), was sequenced on both strands by the dideoxy chain termination method (24).

Sequence homology searches. The neuroendocrine cell type-specific region of the chromogranin A promoter sequence (−260/−40 bp) was aligned to known transcription factor recognition sites ("Transcription factors" database), as well as to all sequences recorded in GenBank (Entrez version, release 77, 1993; National Center for Biotechnology Information) using the software package Macvector (IBI/Kodak).

Promoter/reporter expression constructs, and promoter deletions. Promoter fragments were subcloned into the polylinker region of the promoterless luciferase reporter vector pXP1 (25), between the upstream XhoI site and the downstream HindIII site. Correct orientation of all inserts was verified by either asymmetric double restriction digest, or sequencing.

A 4.8-kbp promoter (−4.8 to +42 bp) was subcloned directionally into pXP1, and named pXP-4.8k.

A series of progressive 5′ deletions was prepared either by restriction digestion of this plasmid, or by polymerase chain reaction amplification, using oligonucleotide primers containing 5′ (upstream) XhoI and 3′ (downstream) HindIII adaptors. The regions amplified had various 5′ end lengths, and all terminated on the 3′ end +42 bp downstream of the cap site. The authenticity of polymerase chain reaction fragments was confirmed both by restriction digestion, and by DNA sequencing (24) across their borders as well as functionally important regions. These constructs were named by their upstream (that is, minus) bp number.

One putative positive promoter/enhancer upstream (distant) domain tested in conjunction with the homologous chromogranin A promoter was a 2.6-kbp fragment (from −4.8 to −2.2 kbp upstream of the cap site), inserted in the usual (correct) orientation just 5′ (upstream) of the chromogranin A promoter fragment −426/+42 bp, in the pXP-426 luciferase reporter construct.

Promoter/enhancer domains tested with a heterologous promoter. To test putative positive or negative regulatory domains in the promoter, the following constructs were made, wherein promoter/enhancer fragments were ligated as a single copy into the polylinker immediately upstream (5′) of (adjacent to) the heterologous herpes simplex virus thymidine kinase (TK) promoter in the luciferase reporter vector pTK-LUC (S. Hollenberg, V. Giguere, and R. Evans, unpublished data), unless otherwise noted. When specifically noted, some of the promoter fragments were inserted 1.2 kbp distant from the TK promoter, in an AatII site in the prokaryotic portion of the vector (S. Hollenberg, V. Giguere, and R. Evans, unpublished data).

Distant (upstream) region. 1. Promoter region −4.8/−2.2 kbp, correct orientation. 2. Promoter region −258/−181 bp, correct orientation. 3. Promoter region −258/−181 bp, inverted orientation. 4. Promoter region −988/−181 bp, correct orientation. 5. Promoter region −988/−181 bp, inverted orientation.

Proximate (downstream) region. 1. Promoter region −159/−130 bp, correct orientation. 2. Promoter region −128/−99 bp, correct orientation. 3. Promoter region −128/−62 bp, correct orientation. 4. Promoter region −128/−62 bp, two copies, correct orientation. 5. Promoter region −128/−62 bp, 1.2 kbp distant, correct orientation. 6. Promoter region −93/−62 bp, correct orientation. 7. Promoter region −93/−62 bp, two copies, correct orientation. 8. Promoter region −93/−62 bp, 1.2 kbp distant, correct orientation. 9. Promoter region −59/−47 bp, correct orientation.

Leader (5′ untranslated exon 1) domain tests. The following promoter and leader (that is, 5′ untranslated exon 1) domains were ligated in the correct orientation between the XhoI and HindIII sites in the polylinker of the promoterless luciferase reporter vector pXP1 (22): 1. Promoter region −181/−14 bp. 2. Promoter region −181/+42 bp. 3. Promoter region −181/+97 bp. 4. Promoter region −181/+158 bp. Note that the ATG initiation codon of mouse chromogranin A is at +158 bp (6).

Other plasmids. Control plasmids included the promoterless luciferase reporter vector pXP1 (25), a plasmid with luciferase expression controlled by the herpes simplex virus thymidine kinase promoter (pTK-LUC; S. Hollenberg, V. Giguere, and R. Evans, unpublished data), a plasmid with luciferase expression controlled by the SV-40 viral early promoter (pSV2ALΔ5; 27), and a plasmid with luciferase reporter expression controlled by the Rous sarcoma virus long terminal repeat (pRSV-LUC; 27).

To control for differences in transfection efficiency between cell lines and between plasmids, co-transfections were done with pRSV-CAT (28), encoding the chloramphenicol acetyltransferase (CAT) reporter controlled by the RSV long terminal repeat. Luciferase reporter results were then normalized as luciferase activity/CAT activity ratios.

Cell culture and transfections. Neuroendocrine cell lines were the rat adrenal medullary chromaffin cell line PC-12 (29), the rat anterior pituitary somatolactotrope line GH3 (30), and the mouse anterior pituitary corticotrope cell line AtT-20 (23). Control (non-endocrine) cell lines were the mouse fibroblast line NIH-3T3 and the T-antigen–transformed monkey kidney cell line COS (31).

Cell lines were grown in Dulbecco's modification of Eagle's medium (DME) with penicillin G (100 U/ml) and streptomycin sulfate (100 μg/ml). The medium for NIH-3T3 and AtT20 cells was supplemented with 10% fetal bovine serum. The medium for COS cells was supplemented with 5% fetal bovine serum. The medium for PC12 cells was supplemented with 5% fetal bovine serum and 10% horse serum.

Transfected plasmid DNAs were grown in *E. coli* strain HB101. After alkaline lysis, supercoiled plasmids were prepared by banding twice through gradients of CsCl in ethidium bromide (32).

Cells were transfected by the efficient lipofection method (Lipofectin; GIBCO-BRL, Gaithersburg, MD), at 50–70% cell confluence. 1–3 μg of total plasmid DNA were used per 6-cm plate. 48 h after transfection, cell extracts were prepared for assay of protein (33), luciferase (27), and chloramphenicol acetyltransferase (32).

DNaseI footprinting of promoter domains. After definition of functional promoter domains by reporter assay of promoter deletions, candidate domains were evaluated for interaction with nuclear proteins of appropriate cells (neuroendocrine versus control). Nuclear extracts were prepared (22) from AtT-20 and NIH-3T3 cell lines.

Plasmid pXP-258 (containing promoter fragment −258/+42 bp) was linearized with HindIII, and the minus strand was ^{32}P end-labeled by fill-in with Klenow fragment of DNA polymerase I. After heat inactivation of the polymerase, the plasmid was again digested with XhoI to remove one end label. The −258/+42 bp fragment, now labeled at only one end, was isolated by electrophoresis in a 2% agarose gel, followed by adsorption/elution from glass powder (Bio 101 Inc., San Diego, CA). Aliquots of the probe (10^4 dpm) were incubated with appropriate nuclear extracts (50 μg protein), followed by DNaseI digestion, and electrophoresis on denaturing polyacrylamide gels (Hot Footprint Kit; Stratagene). Additional aliquots of the probe were chemically cleaved (22) to yield a purine (G + A) size ladder for electrophoresis in an adjacent gel lane.

Electrophoretic gel mobility shift assays. Promoter fragments corresponding to regions footprinted by neuroendocrine nuclear extracts (see above) were synthesized (both strands), and ^{32}P-labeled by fill-in of 5′-overhangs with Klenow fragment of DNA polymerase. Labeled frag-

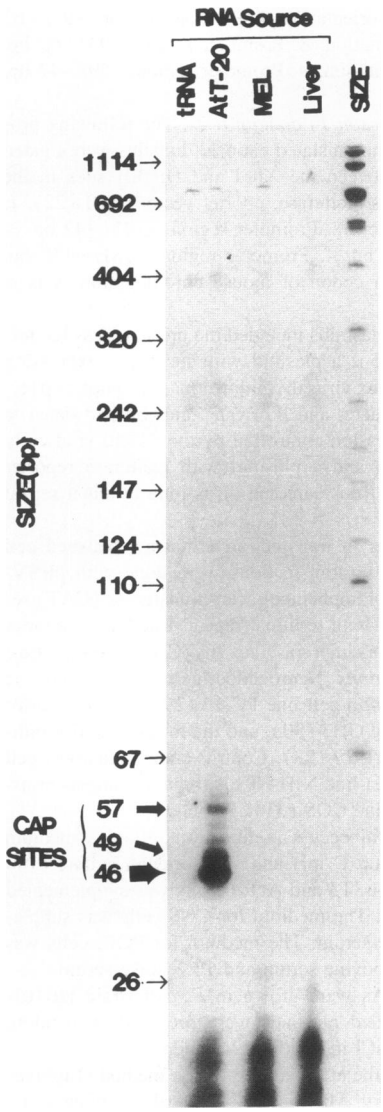

Figure 1. The cap (transcription initiation) site of the chromogranin A promoter, located by RNAse protection. A mouse chromogranin A promoter template was used to synthesize ^{32}P-labeled antisense RNA, which was then hybridized with (protected by) mRNA, digested by RNAse, electrophoresed, and autoradiographed. The quantitatively major cap site was located at position 46 on this KpnI-digested template (*broad arrow*). The most 5′ cap site (*medium arrow*) is at position 57 on this template, while another quantitatively minor cap site (*medium arrow*) is at position 49. tRNA: transfer RNA. AtT-20: mouse anterior pituitary corticotrope cell line. MEL: mouse erythroleukemia cell line. Liver: mouse liver.

ments (10^5 dpm) were incubated with nuclear extracts (4 μg protein) from either neuroendocrine cells (AtT-20 mouse anterior pituitary corticotropes) or control cells (NIH-3T3 mouse fibroblasts), with or without a 50-fold molar excess of cold (unlabeled) probe. Complexes were then electrophoresed through 5% acrylamide/0.4× TBE gels (22), which were dried and subjected to fluorography at −70°C.

The fragments used were (from 5′ to 3′): (*a*) −159 bp ACAAGCGGGATAGAGACAGCTGATGGAGAA −130 bp. (*b*) −128 bp CTGGAGGTGGGGGGCGGGACCCCGAAGGT −100 bp. (*c*) −93 bp AGGGCGCGGGGGGGCGGTCCTATGACGTAATT −62 bp. (*d*) −59 bp CTGGGTGTGTGCG −47 bp.

Results

Genomic clone isolation, transcriptional start site, and sequence homologies. Transcriptional initiation (cap) sites for the mouse gene were localized by RNAse protection with RNA from AtT-20 mouse anterior pituitary corticotropes. Three major sites were identified (Fig. 1) of which the most 5′ (indicated as 57 bp in Fig. 1) exactly matched the cap site determined previously by primer extension (6). Henceforth this start site is referred to as +1. The quantitatively major start site is at +11, with a second minor site at +8. No protected fragments were obtained with RNA from mouse liver or mouse erythroleukemia cells, neither of which express the chromogranin A (CgA)[1] gene.

Mouse and rat genomic clones were sequenced to > 1 kbp upstream of the transcriptional start site (Fig. 2). The aligned sequences are > 85% homologous. Sequence analysis of the first 1,135 bp upstream of the mouse cap site revealed numerous exact or near-perfect matches with binding sites for known transcription factors (see Appendix). A TATA box (TATAAA) (34) is located 22 bp upstream of the +1 start site. Other notable sites include (alphabetically): four AP-2 consensus matches (35); a Brn-2 (POU domain gene expressed in brain) site (36); a partial CREB (cyclic AMP response element box) site match (37); a *Drosophila* homeodomain site (38); 22 E box (CANNTG) core sites (eleven on each strand) (39, 40); a partial match for an Egr-1 site (41); three estrogen response element half-sites (42); eight GA boxes (four on each strand) (43); four GHF-1 matches (44); a glucocorticoid response element half-site (45); four homeodomain core matches (46); a nuclear factor kappa B match (palindrome on each strand) (47); a partial match with a 10 bp putative neuroendocrine element (48); a functional element found in the neuropeptide Y promoter (49); a Pit-1 match (50); three matches of the Sp1 consensus (51); a glucokinase upstream promoter element (UPE) (52); an element from the uteroglobin promoter (53); an Xpf-1 (exocrine pancreatic factor) site (54); and a Zeste site (55) (see Appendix for full listing).

Deletion analysis of the mouse CgA promoter. Having established the transcriptional start sites, a genomic fragment from −4.8 kbp to +42 bp was cloned upstream of a luciferase reporter. This construction was transfected into various cell lines to test for cell type-specific promoter activity. High-level expression of the luciferase reporter was observed in three neuroendocrine cell types: PC-12 rat adrenal medullary chromaffin cells (29), AtT-20 mouse anterior pituitary corticotropes (23), and GH3 rat anterior pituitary somatolactotropes (30). The endogenous CgA gene is expressed at high levels in all three of these cell types (56, 57). By contrast, two non-endocrine cell lines, mouse NIH-3T3 fibroblasts and COS monkey kidney cells, showed only trace levels of luciferase reporter activity.

A series of 5′ deletions was constructed to localize the regions of the promoter involved in cell type specificity. Cell-specific expression of the CgA promoter was preserved with promoter deletions down to −77 bp upstream of the cap site, beyond which point neuroendocrine specificity of expression was lost (Table I). In AtT-20 and PC12 cells, there was an apparent positive promoter domain between positions −2.2 and −4.8 kbp, deletion of which diminished reporter expression 2.4- and 3.6-fold, respectively, in these two cell types. An inhibitory promoter domain was suggested by 2.9- and 2.1-fold increases in reporter expression by deleting position −258 to −181 bp. A final positive domain was apparent upon deletions from −147 to −61 bp; activation by this domain was progressively lost with serial deletions from −147 to −77 to −61 bp. The proximate

1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyltransferase; CgA, chromogranin A.

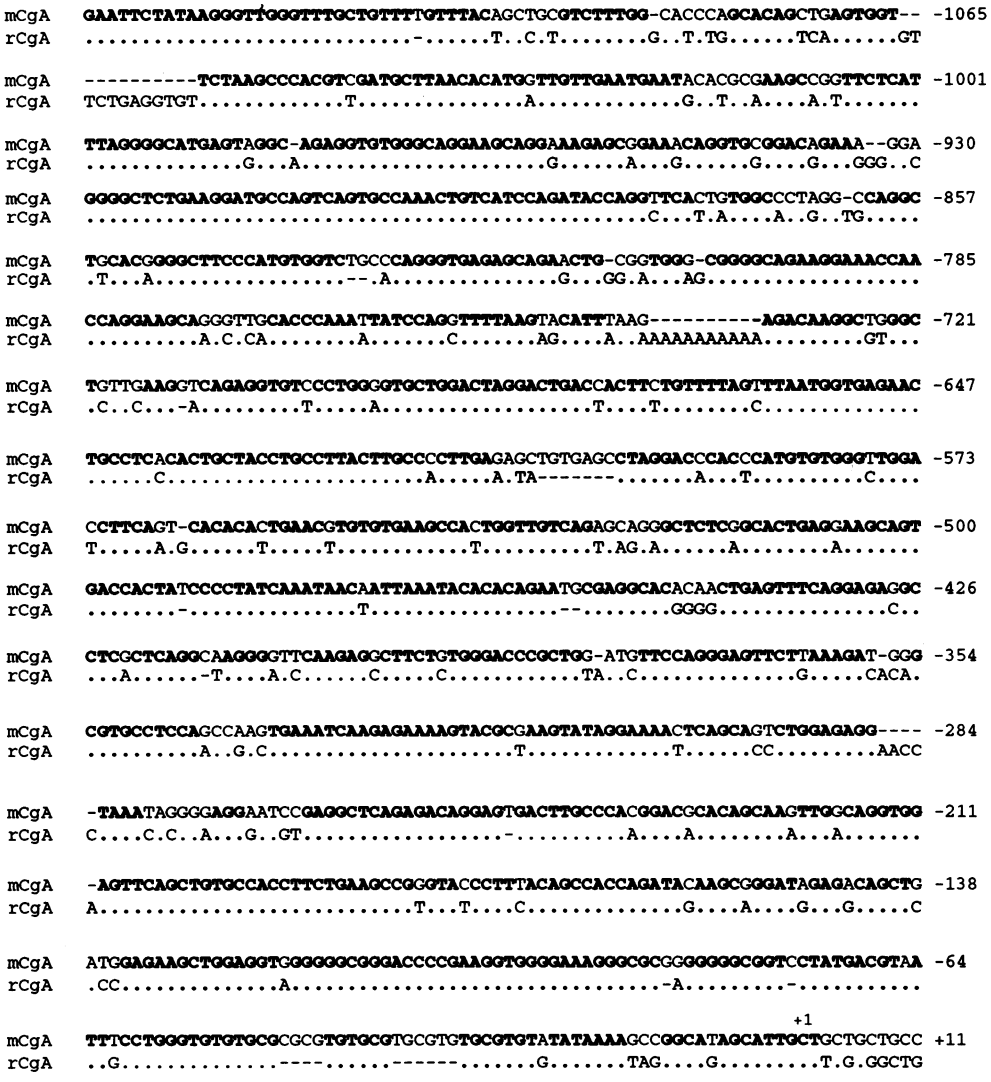

Figure 2. Mouse and rat chromogranin A (CgA) promoter sequences. The most 5′ mouse cap (transcription initiation) site is indicated by a +1. Mouse promoter positions are indicated (*right column*) by base number (*minus*) upstream of the cap site. Gaps are indicated by a hyphen (−). Bases conserved between rat and mouse sequences are indicated by a dot (·) in the rat sequence, and **bold** type in the mouse sequence. *m*, mouse, *r*, rat.

promoter to −147 bp had activity fully equivalent to the longer −4.8 kbp promoter.

A series of fine deletions was therefore created (Fig. 3) to localize more closely those domains necessary for neuroendocrine cell-specific expression. The deletions were tested by transfection into AtT-20 and NIH-3T3 cells as examples of expressing and non-expressing cells, respectively. At no point in the serial deletions, down to −77 bp, did CgA promoter expression in NIH-3T3 cells approach that seen in AtT-20 cells (Fig. 3). Cell type specificity was completely lost, however, by removal of a further 16 bases to −61 bp upstream of the start site. The 5′ end of the proximate domain conferring cell specificity thus lies downstream of −77 bp. The 3′ end has not yet been precisely positioned, and may extend further downstream (3′) than −61 bp; however, truncation at −61 bp is sufficient to abolish selective neuroendocrine transcriptional activation. Modest inhibitory domains were apparent from −529 to −426 bp, and from −258 to −181 bp in 3T3 cells; however, even deletion of these domains did not increase CgA promoter non-endocrine expression to levels seen in AtT-20 cells.

An internal deletion of −2.2 kbp to −426 bp was created to test the ability of the distal positive region to stimulate the proximate promoter. The distal region (−4.8 to −2.2 kbp) retained activity when fused to the proximate −426/+42 bp chromogranin A promoter region (Fig. 4).

A number of 3′ deletions of the CgA leader (5′ untranslated exon 1) region were constructed. When promoters with varying lengths of leader region were fused to luciferase, it became apparent that increasing the length of the leader (up to +158 bp, which marks the translation initiation site [ATG codon] for chromogranin A) augmented luciferase reporter expression (Table II).

Stimulation of a heterologous promoter by functional domains of the CgA promoter. Putative functional CgA promoter domains, suggested by deletion analysis, were tested for activity in neuroendocrine (AtT-20) cells by fusion to a heterologous thymidine kinase (TK) promoter in a TK promoter/luciferase reporter gene construction (pTK-LUC; Table III). Unlike the previous internal deletion, the distal region (−4.8 to −2.2 kbp) did not confer positive regulation to the heterologous TK promoter, in either orientation. Thus, the distal positive region was not a typical transcriptional enhancer, but instead seemed to cooperate specifically with proximate elements in the homologous chromogranin A promoter.

Table I. Analysis of Promoter Domains by Transfection of Chromogranin A Promoter/Luciferase Reporter Constructs into Neuroendocrine Versus Control Cells

		Relative luciferase activity of different promoters								
				Mouse CgA						
Cell type	Cell line	SV-40	RSV	−4.8kbp	−2.2kbp	−258bp	−181bp	−147bp	−77bp	−61bp	None (pXP1)
Neuroendocrine											
	PC-12	38.6	100	42.7	18.2	17.4	51.3	42.9	21.3	0.190	< 0.05
	GH3	—	100	6.90	—	—	—	18.0	14.8	—	< 0.01
	AtT-20	0.70	100	16.1	4.48	7.61	16.2	17.2	8.84	0.243	< 0.05
Control											
	NIH-3T3	117	100	0.72	0.69	0.54	2.49	0.82	0.81	0.239	< 0.01
	Cos	1158	100	0.34	0.68	—	1.30	1.38	0.49	—	< 0.05

The indicated promoter domains (from the specified 5′ end, to position +42) were ligated to the luciferase reporter in the vector pXP1, and then transfected into the indicated cell type. For each transfection, the plasmid pRSV-CAT, encoding the reporter chloramphenicol acetyltransferase (CAT), was co-transfected, to control for transfection efficiency differences between cell lines and between promoter lengths, and luciferase activity was normalized (ratioed) to CAT activity. Luciferase/CAT ratios were then normalized to the activity of the strong RSV promoter (pRSV-luciferase) in parallel transfections in each cell type, such that the RSV promoter result is 100% for each cell type. *SV-40,* SV-40 early promoter in the plasmid pSV2ALΔ5 (24). *RSV,* Rous sarcoma virus long terminal repeat. *PC-12,* rat adrenal medullary chromaffin cells. *GH3,* rat anterior pituitary somatolactotropes. *AtT-20,* mouse anterior pituitary corticotropes. *COS,* T antigen-transformed monkey kidney cells. *NIH-3T3,* mouse fibroblasts. *None,* the promoterless luciferase reporter vector pXP1. Results are luciferase/CAT ratios, normalized to pRSV-luciferase results (=100%) for each cell type.

The upstream negative promoter region (−988 to −181 bp) in both orientations conferred negative regulation on the heterologous TK promoter, although more effectively in the correct orientation (Table III). Since 5′ deletions suggested that the −258 to −181 bp region was the crucial negative promoter domain, we also tested the ability of this domain to confer negative regulation onto the TK promoter; however, repression was observed only in the correct orientation of this domain (Table III).

The ability of several more proximate promoter domains (from −159 to −47 bp) to influence the TK promoter was also tested. Neither the domain from −159 to −128 bp nor that from −128 to −99 bp affected TK/luciferase expression, but the domain from −128 to −62 bp augmented luciferase expression, in copy number-dependent fashion. This augmentation was lost when the element was moved 1.2 kbp away (further upstream) from the TK promoter. The domain from −93 to −62 bp also augmented luciferase expression in dose-dependent fashion (4.63-fold with two copies), and the effect was again lost at a distance. The proximate −59 to −47 bp element did not affect luciferase expression (Table III).

In vitro binding of neuroendocrine nuclear extracts to the

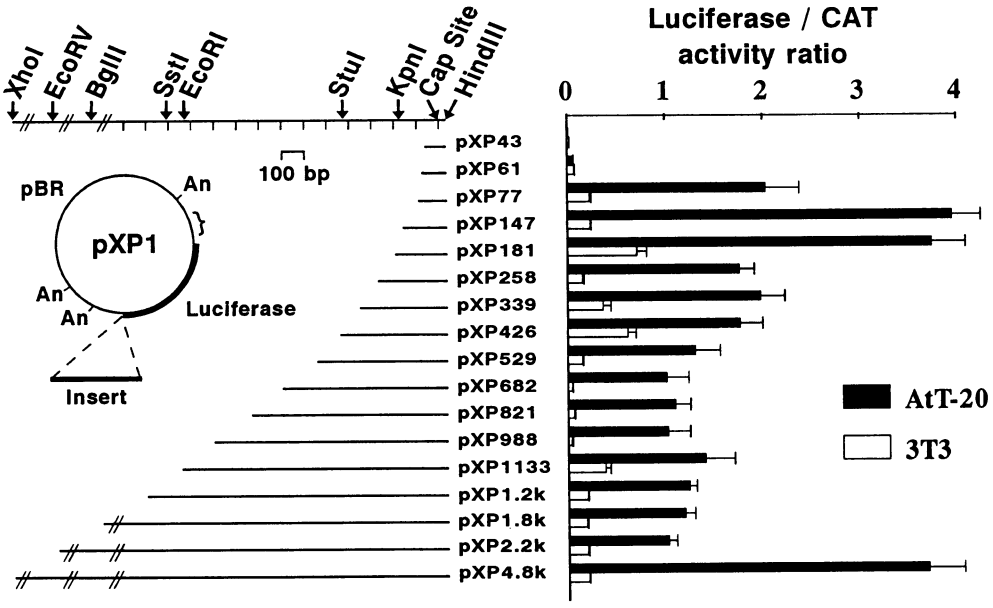

Figure 3. Deletion analysis of chromogranin A promoter domains in neuroendocrine (AtT-20 corticotrope) versus control (NIH-3T3 fibroblast) cells. Promoter deletions were created as described in Methods, and inserted into the promoterless luciferase reporter vector pXP1. The promoter deletion/luciferase reporter constructs were transfected along with the transfection control efficiency plasmid, pRSV-CAT. The results are expressed as ratios of luciferase/CAT activities.

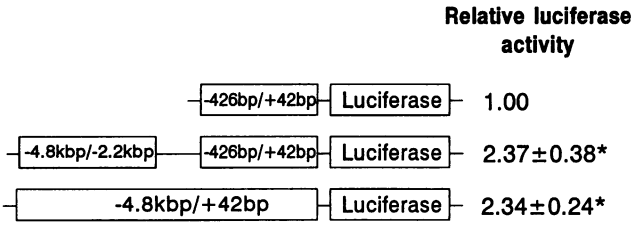

Figure 4. A distal positive promoter positive domain: promoter deletions and homologous promoter fusions. The indicated promoter domains were fused (after internal deletion of the −2.2 kbp/−426 bp region), then ligated to the luciferase reporter, and transfected into AtT-20 corticotropes. pRSV-CAT (encoding chloramphenicol acetyltransferase) was cotransfected in each plate. Luciferase/CAT activity ratios were normalized to the activity of the proximal (−426/+42 bp) promoter region alone (=1.0). $*P < 0.001$, compared with the proximal promoter region alone.

proximal promoter. DNAseI footprinting identified possible transcription factor binding sites in the proximal promoter region. Nuclear extracts from AtT-20 corticotropes protected the following regions from DNAseI digestion: −155 to −130 bp, −122 to −101 bp, −91 to −66 bp, and −57 to −49 bp (Fig. 5). The same nuclear extracts also induced DNAseI hypersensitivity adjacent to these footprints.

Confirmation that these footprints were bona fide binding sites for factors in the nuclear extract was derived from electrophoretic gel-mobility shift experiments. Double-stranded oligonucleotide probes corresponding to footprinted promoter regions were synthesized and end-labeled with ^{32}P. AtT-20 nuclear extracts bound each of the probes, as demonstrated by complexes with retarded mobility. Significantly different patterns of band shifts were obtained with control NIH-3T3 fibroblast nuclear extracts (Fig. 6). All complexes could be competed by a 50-fold molar excess of the unlabeled oligonucleotide.

Sequence homologies in the proximate promoter region. Alignment of the mouse CgA promoter sequence with that of rat and bovine (7) promoters in the proximate promoter region (−236 to +20 bp) revealed several areas of high homology (Fig. 7 *A*). Sequence similarity is most striking in the region from −110 to −53 bp. This region contains three of the four binding sites identified by DNaseI footprinting, as well as potential transcription factor binding sites such as Sp1 (two sites), CREB, GA box, and Xpf-1. A further region of homology was observed between −203 and −186 bp, an area implicated by deletion analysis in negative regulation.

The mouse chromogranin A proximate promoter (from −260 to −40 bp) was aligned to sequences in GenBank (Fig. 7 *B*). Several regions of sequence similarity were identified in promoter or untranslated regions of other genes expressed in brain or endocrine glands, including the neurotrophin 3 promoter (46/67 residues; GenBank document ID 98999) (58); the homeodomain Brn-1 promoter (42/58 residues; GenBank document ID 450151) (59); a ryanodine receptor intron (43/53 residues; GenBank document ID 138104) (60); the platelet-derived growth factor A chain promoter (52/71 residues; GenBank ID 320412) (61); and the insulin-like growth factor I

Table II. Function of the Leader (5′ Untranslated Exon 1) Region of the Chromogranin A Gene

Chromogranin A promoter or leader region	Relative luciferase activity
−181/−14 bp	0.31±0.01*
−181/+42 bp	1.0
−181/+97 bp	1.64±0.06*
−181/+158 bp	3.19±0.13*

The indicated promoter regions (numbered with reference to the cap site = +1) were ligated to the luciferase reporter, and transfected into AtT-20 mouse pituitary corticotropes. Cells were harvested 48 h later for assay of luciferase and protein. Luciferase results were first normalized to cell protein content, and then to the activity of the −181/+42 bp promoter. Results are mean±one standard error. $*P < 0.001$ versus control (−181/+42 bp promoter). The ATG initiation codon (translation start site) of endogenous mouse chromogranin A begins at position +158. Results are luciferase/CAT ratios, normalized to the activity of the −181/+42 bp promoter (=1.0).

Table III. Promoter/Enhancer Domain Transfer Experiments

CgA promoter				
Domain bp	Copies	Orientation	Relation to TK promoter	Relative luciferase activity
Distant (upstream)				
−4800/−2200	1	Correct	Adjacent (5′)	0.90±0.16
−2200/−4800	1	Inverted	"	1.31±0.24
−988/−181	1	Correct	"	0.32±0.06*
−181/−988	1	Inverted	"	0.65±0.09*
−258/−181	1	Correct	"	0.72±0.11‡
−181/−258	1	Inverted	"	1.02±0.18
Proximate (downstream)				
−159/−128	1	Correct	"	0.88±0.10
−128/−99	1	Correct	"	0.98±0.16
−128/−62	1	Correct	"	2.40±0.23*
−128/−62	2	Correct	"	2.89±0.31*
−128/−62	1	Correct	1.2 kbp away	1.18±0.20
−93/−62	1	Correct	Adjacent (5′)	3.67±0.31*
−93/−62	2	Correct	"	4.63±0.49*
−93/−62	1	Correct	1.2 kbp away	1.29±0.14
−59/−47	1	Correct	Adjacent (5′)	0.79±0.11

Luciferase reporter activity of chromogranin A promoter/enhancer upstream fragments linked to the heterologous herpes simplex virus thymidine kinase (TK) promoter, and transfected into AtT-20 mouse pituitary corticotropes. Chromogranin A promoter/enhancer fragments were cloned into the enhancerless promoter vector pTK-LUC, either adjacent (5′) to the TK promoter, or into an AatII site in the prokaryotic portion of the vector, 1.2 kbp distant from the TK promoter. Each transfection involved cotransfection with pRSV-CAT, to correct for differences in transfection efficiency of these plasmids of differing size. The results of duplicate transfections are reported as ratios of luciferase activity/chloramphenicol acetyl transferase (CAT) activity, with normalization by ratio to the activity of the original enhancerless vector into which the fragments were cloned, pTK-LUC (=1.0). $*P < 0.01$, $‡P < 0.05$ (versus pTK-LUC alone).

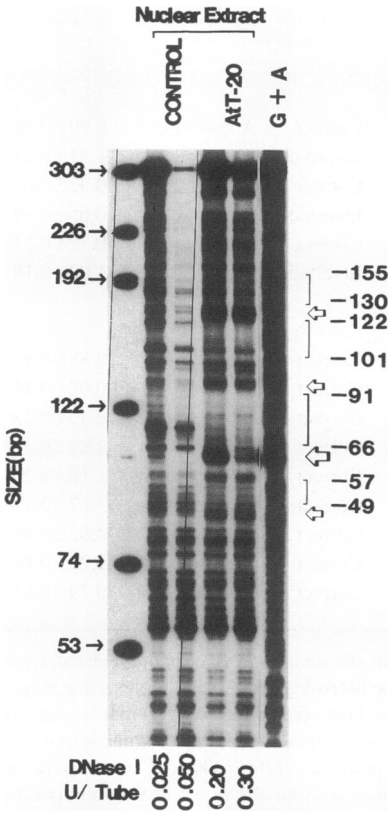

Figure 5. Footprint analysis of the minimal neuroendocrine cell type-specific region of the chromogranin A promoter. Promoter DNA templates, ^{32}P-labeled at one end, were incubated with extracts (50 μg nuclear protein) of AtT-20 neuroendocrine (corticotrope) nuclei, digested with DNaseI, and subjected to polyacrylamide gel electrophoresis, followed by gel drying and fluorography. Brackets indicate areas protected (footprinted) from DNaseI digestion by AtT-20 nuclear proteins. Open arrows indicate areas made hypersensitive to DNaseI digestion by AtT-20 nuclear proteins. (*AtT-20*) Mouse anterior pituitary corticotrope cell line. (*G + A*) Purine size ladder from Maxam-Gilbert sequencing reaction. (*Control*) Promoter DNA template not incubated with nuclear extract protein, prior to DNaseI digestion.

receptor promoter (41/57 residues; GenBank document ID 94696) (62). The sequence similarity extended from −137 to −66 bp, and included many of the potential transcription factor binding sites listed above.

Discussion

Clearcut universal or unifying factors underlying neuroendocrine gene expression have not yet been conclusively elucidated (63, 64).

Members of the chromogranin/secretogranin protein family are ubiquitous components in the core of secretory granules throughout the neuroendocrine system (3–5). Perhaps the most widely expressed such protein, especially in neuroendocrine neoplasia (15), is chromogranin A. We therefore analyzed its promoter region for clues to mechanisms universally governing gene expression in the neuroendocrine system, that is, elements controlling acquisition of the neuroendocrine secretory phenotype.

The 5′ flanking region of the mouse chromogranin A gene supported strong expression of a reporter gene in adrenal medullary chromaffin cells, anterior pituitary corticotropes, and somatolactotropes, but not in two fibroblast lines (Table I, Fig. 3).

Deletion analysis identified several functional promoter domains, both positive and negative, that governed chromogranin A expression in neuroendocrine cells. The minimal promoter that retained neuroendocrine specific expression was initially defined by the deletion construct pXP-147. Further deletion to position −77 bp reduced promoter activity by 50% but was still cell specific; deletion of a further 16 bp, down to position −61 bp, reduced promoter activity > 90%, to the level seen in non-endocrine (fibroblast) cells. Thus a neuroendocrine-specific element is disrupted by the deletion to −61 bp. These findings place the 5′ limit of this element at (or downstream of) −77 bp; the 3′ limit of this element is not yet completely defined, but deletion to −61 bp is sufficient to abolish activity.

Elements between −77 and −147 bp can augment the neuroendocrine-specific expression (Table I, Fig. 3), although this region may simply potentiate the effect of the downstream neuroendocrine-specific element (−77/−61 bp). Fragments from this proximate promoter (especially −93/−62 bp) also stimulated a heterologous TK promoter (Table III) in copy number-dependent fashion in pituitary corticotropes, but lost effect when moved 1.2 kbp away.

A distal domain (between −4.8 and −2.2 kbp) also stimulated the activity of a minimal chromogranin A promoter (though not a heterologous promoter) in corticotropes.

A negative domain, uncovered between −258 and −181 bp, conferred negative regulation onto the chromogranin A promoter and also repressed a heterologous TK promoter. Of note, this region contained a sequence, CACCTTC (at −197 to −191 bp), which is similar (6/7 bp) to a negative transcriptional regulatory element, CACCTCC, found in the rat collagen II gene (65). The CACCTTC sequence is found in mouse and rat chromogranin A genes (Fig. 7 *A*), while the bovine gene contains the sequence CACCTCC (identical to the collagen element).

Four areas in the minimal proximate positive promoter region were protected from DNaseI digestion by nuclear extracts from AtT-20 neuroendocrine cells (Fig. 5); the intervening areas showed hypersensitivity to DNaseI cleavage. All four "footprinted" areas interacted specifically with neuroendocrine nuclear proteins, as well as other non-neuroendocrine cell-specific factors in electrophoretic gel mobility shift assays (Fig. 6). These results suggest specific interactions between neuroendocrine nuclear proteins and these functionally important proximal promoter regions. The −59/−47 bp gel shift probe (Fig. 6) was designed to explore a footprinted promoter region (Fig. 5); even though promoter deletion to −61 bp seemed to abolish cell type-specific expression of the promoter (Fig. 3), neuroendocrine-specific shifted bands from the −59/−47 bp region (Fig. 6) may be functionally important, because we have not yet precisely defined the 3′ end of the chromogranin A promoter element conferring cell type-specific expression.

Sequence analysis of the minimal promoter highlighted several similarities to consensus binding sites for known transcription factors (Fig. 7 *B*). Some of these similarities coincide with the observed footprints, while others do not. Comparison of mouse, rat, and bovine proximal promoter regions (Fig. 7 *A*) showed conservation of several sites, including an Sp1 site (51) in the footprint at −122 to −101 bp, an Sp1 and a CREB site (37) in the footprint at −91 to −66 bp, a GA box and an Xpf-1 motif between the two footprints, and (as expected) the TATA box at −22 bp. The CREB site lies between −77 and −61 bp within the neuroendocrine-specific element. The two Sp1 sites lie between −77 and −147 bp. Although Sp1 is expressed ubiquitously (66), such sites might still potentiate an adjacent cell-specific element, perhaps synergizing the observed cell-specific

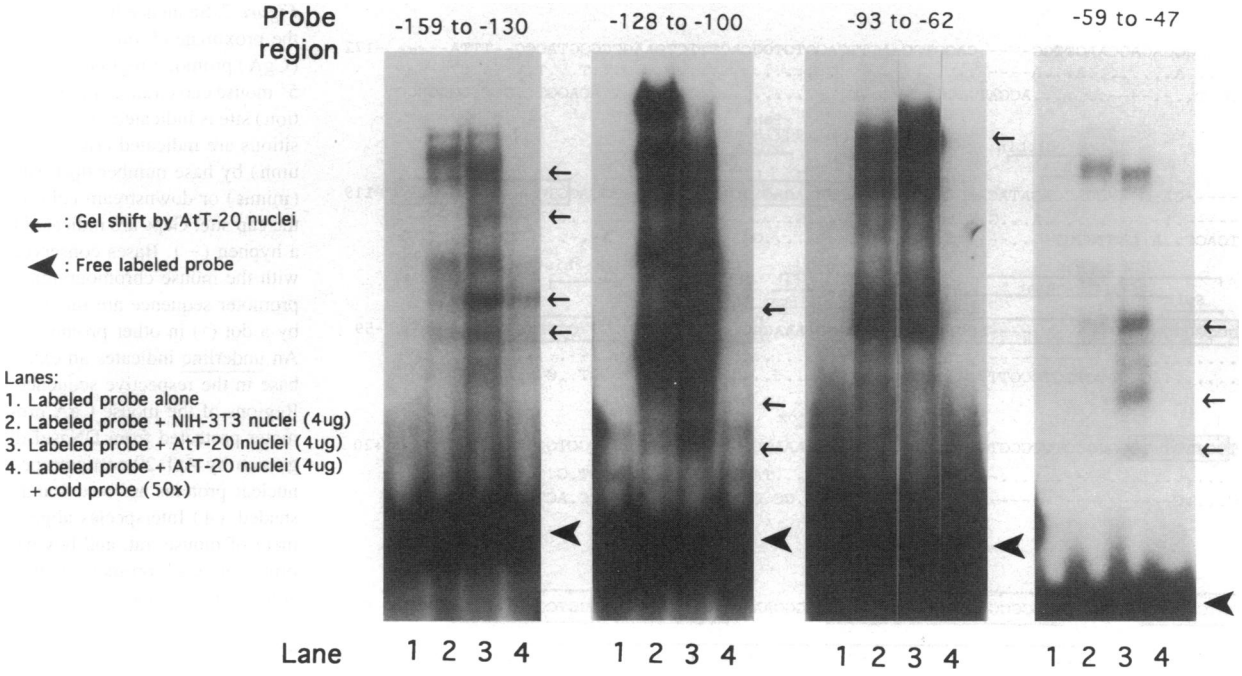

Figure 6. Electrophoretic gel mobility shift assays. Promoter fragments corresponding to regions footprinted by neuroendocrine nuclear extracts (see above) were synthesized (both strands), and ^{32}P-labeled by fill-in of 5′ overhangs with Klenow fragment of DNA polymerase. Labeled fragments (10^5 dpm) were incubated with nuclear extracts (4 μg protein) from either neuroendocrine cells (AtT-20 mouse anterior pituitary corticotropes) or control cells (NIH-3T3 mouse fibroblasts), with or without a 50× molar excess of cold (unlabeled) probe, and then subjected to gel electrophoresis (0.4× TBE gels), followed by autoradiography. Thin arrows indicate mobility of regions shifted to a new position by AtT-20 (but not NIH-3T3) nuclear proteins.

expression. Whether these particular *trans*-acting factors can activate this chromogranin A promoter region remains to be demonstrated.

Cyclic AMP stimulates a number of neuroendocrine genes, including chromogranins A (7), B (67), and C (67, 68), dopamine β-hydroxylase (69), phenylethanolamine–*N*-methyltransferase (70), pituitary glycoprotein hormone α subunit (71), prolactin (71), and renin (72). In some of these other genes, CREB sites have been implicated in the response to cAMP (73–75). Comparison of the proximate promoter region of chromogranin A with those of chromogranin B (76) and chromogranin C (77), two other secretory vesicle core proteins with similar tissue distribution to chromogranin A (5), shows conservation of the CREB site, despite very low homology in other regions of their proximate promoters. The presence of a CREB site in the neuroendocrine-specific region of the chromogranin A promoter element is thus quite suggestive of functional importance.

Footprinted proximate areas (Fig. 7 *A*) also included consensus matches for purine-rich GAGA regions (43) and an AP-2 match (35). Purine-rich areas have been implicated in control of insulin gene expression (43), while AP-2 is widely expressed in neural crest cell lineages (78).

Areas of extended homology between the proximate chromogranin A promoter region and nontranslated regions of other neuroendocrine-specific genes were identified by GenBank survey. These included an intron sequence in the ryanodine receptor gene (60), and sequences in the promoters of the neurotrophin-3 (58), Brn-1 (59), PDGF-A chain (61), and IGF-I receptor genes (62). Most of the sequence similarity occurs in the chromogranin A promoter's G/C-rich areas, which include two Sp1 sites. In the PDGF-A chain promoter, the similarity occurs in the middle of a G/C-rich element essential for promoter activity (79), while the similarity in the neurotrophin-3 promoter lies outside the area crucial for glial expression (58). Whether the similarities constitute functional promoter elements for the Brn-1 gene or the IGF-I receptor gene remains to be determined.

A number of other short consensus matches for neuroendocrine transcriptional control elements were noted in the chromogranin A 5′ flanking region (see Appendix), including one Brn-2 site (36), a *Drosophila* homeodomain site (38), several E box consensus matches (39, 40), four GHF-1 sites (44), a putative 10-bp neuroendocrine element (48), a neuropeptide Y expression element (49), a Pit-1 site (50), and a Zeste site (55). However, these recognition sites were generally located well outside of the minimal neuroendocrine-specific functional region (−77/−61 bp) defined by deletion analysis of the chromogranin A promoter (Table I, Fig. 3, and Appendix).

In conclusion, the chromogranin A 5′ flanking region confers neuroendocrine cell type-specific expression in diverse neuroendocrine cell types. A distal positive domain specifically activates the chromogranin A promoter. The proximate promoter contains both positive and negative elements. Cell type specificity is conferred primarily by a region from −61 to −77 bp which contains a conserved CREB site. The minimal proximate promoter contained four (''footprinted'') regions protected from DNAseI digestion by neuroendocrine nuclear pro-

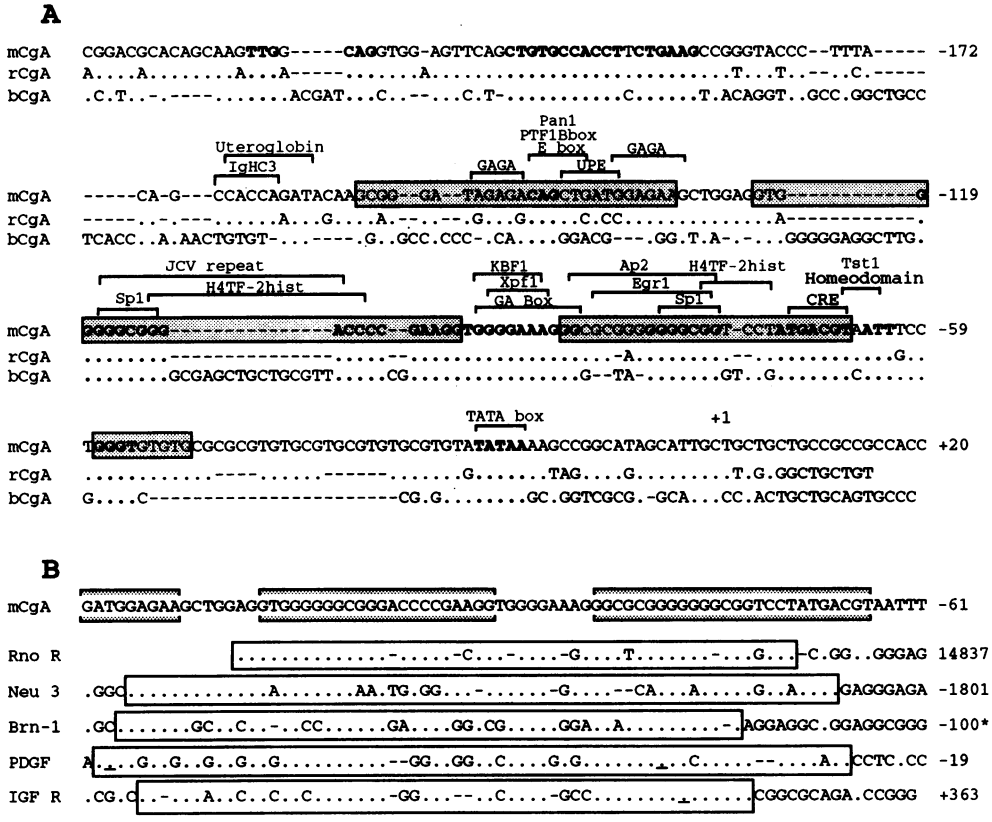

Figure 7. Sequence homologies in the proximate chromogranin A (CgA) promoter region. The most 5′ mouse cap (transcription initiation) site is indicated by a +1. Positions are indicated (right column) by base number upstream (minus) or downstream (plus) of the cap site. Gaps are indicated by a hyphen (−). Bases conserved with the mouse chromogranin A promoter sequence are indicated by a dot (·) in other promoters. An underline indicates an extra base in the respective sequence. Regions of the mouse CgA promoter protected from DNaseI digestion by AtT-20 corticotrope nuclear proteins are boxed and shaded. (*A*) Interspecies alignment of mouse, rat, and bovine proximate CgA promoter regions. Known transcription factor binding sites are indicated by the legend directly above the sequence. Bases conserved across all three species are indicated in **bold** type. *m*, mouse; *r*, rat; *b*, bovine. (*B*) Sequence homologies with other neural or endocrine genes in the proximate region of the mouse CgA promoter. A GenBank search of this region (−181/−61 bp) located areas of homology with promoter or nontranslated regions of other genes expressed in brain or endocrine glands, including the neurotrophin 3 promoter (Neu 3; 46/67 residues), the homeodomain Brn-1 promoter (Brn-1; 42/58 residues), a ryanodine receptor intron (Rno R; 43/53 residues), the platelet derived growth factor A chain promoter (PDGF; 52/71 residues) and the insulin-like growth factor I receptor promoter (IGF R; 41/57 residues). Regions with high homology are indicated in open-boxed areas. An asterisk to the right of the Brn-1 entry indicates numbering with reference to the position of translation (rather than transcription) initiation.

teins; these regions were also specifically complexed by such proteins in an electrophoretic gel mobility shift assay. Some of these areas corresponded to previously characterized transcription factor binding sites, while others were candidates for novel factors governing chromogranin A expression in particular, and the neuroendocrine secretory phenotype in general.

Appendix

Table AI. Transcriptional Control Region Sequence Homologies in the Mouse Chromogranin A (CgA) Gene 5′ Flanking Region (1135 Bp Upstream of Exon 1)

Site	Consensus sequence	Position(s) in the CgA promoter	Strand	Sequence in the CgA promoter	Match
AP2	YCSCCMNSSS	−801	−	CCGCCCACCG	10/10
		−81	−	CCCCCCGCGC	10/10
		−80	−	CCCCCCCGCG	10/10
		−77	−	CCGCCCCCCC	10/10
		+13	+	CCGCCACCGC	10/10
Bm-2	RATAATY	−756	−	GATAATT	7/7
CREB	ACGTCA	−66	−	ACGTCA	6/6
Drosophila homeodomain	CAATTAAA	−476	+	CAATTAAA	8/8
E box (consensus)	CANNTG	−1100	+	CAGCTG	6/6
		−1076	+	CAGCTG	6/6
		−1041	+	CACATG	6/6
		−948	+	CAGGTG	6/6
		−842	+	CATGTG	6/6
		−587	+	CATGTG	6/6
		−446	+	CAACTG	6/6

Table A1. (Continued)

Site	Consensus sequence	Position(s) in the CgA promoter	Strand	Sequence in the CgA promoter	Match
		−341	+	CAAGTG	6/6
		−217	+	CAGGTG	6/6
		−206	+	CAGCTG	6/6
		−143	+	CAGCTG	6/6
Egr-1	GCGGGGGCG	−88	+	GCGGGGGGCG	8/9
ERE half-site	GGTCA	−713	+	GGTCA	5/5
		−675	−	GGTCA	5/5
		−496	−	GGTCA	5/5
GA box	RRRRRRRR	−963	+	AGGAAAGAG	$(R)_9$
		−937	+	AGAAAGGAGGGG	$(R)_{12}$
		−797	+	AGAAGGAAA	$(R)_9$
		−434	+	AGGAGAGG	$(R)_8$
		−330	+	AAGAGAAAA	$(R)_9$
		−278	+	AGGGGAGGAA	$(R)_{10}$
		−99	+	GGGGAAAGGG	$(R)_{10}$
		+140	−	GGAAAGAG	$(R)_8$
GHF1	TAAAT	−995	−	TAAAT	5/5
		−734	−	TAAAT	5/5
		−475	+	TAAAT	5/5
		−286	+	TAAAT	5/5
HGRE.7	AGTCCT	−679	−	AGTCCT	6/6
Homeodomain	TAAT	−758	−	TAAT	4/4
		−659	+	TAAT	4/4
		−471	−	TAAT	4/4
		−66	+	TAAT	4/4
Neuroendocrine element	WGGGGWGTGR	−982	+	AGAGGTGTGG	9/10
NF-kB	GGGRNNYYCC	−851	+	GGGGCTTCCC	10/10
		−842	−	GGGAAGCCCC	10/10
NF1	AGCCAA	−344	+	AGCCAA	6/6
NPY element	CCCCTCC	−926	−	CCCCTCC	7/7
Pit-1	TATNCAT	−1021	−	TATTCAT	7/7
Sp1-hsp70	GGCGGG	−805	+	GGCGGG	6/6
		−116	+	GGCGGG	6/6
		+109	−	GGCGGG	6/6
TATA box	TATAAA	−22	+	TATAAA	6/6
UPE	CATYAS	−135	−	CATCAG	6/6
		+26	+	CATCAC	6/6
Uteroglobin	RYYWSGTG	−160	−	ATCTGGTG	8/8
Xpf-1	TTTCCC	−93	−	TTTCCC	6/6
Zeste-whit	TGAGTG	−1072	+	TGAGTG	6/6

Promoter/enhancer position numbers are determined in relation to the cap (transcription initiation) site as +1. Regions are listed in alphabetical order. Degenerate consensus sequence bases are symbolized by the IUPAC code. *AP2,* activator protein 2 (35); *Brn-2,* brain-2 member of the POU-domain gene family (36); *CRE,* cAMP response element (37); *E box* (39, 40), enhancer box (recognition site for helix-loop-helix factors); *Egr-1* (41), early growth response gene 1; *ERE* (42), estrogen response element; *GA box,* (43): purine rich region; *GHF1* (44), growth hormone factor 1; *HGRE* (45), human glucocorticoid response element; *NF-kB* (47), nuclear factor detected on kappa light chain enhancer gene in B cells; *NF1* (48), nuclear factor 1; *NPY element* (49), element important in neuropeptide Y gene expression; *Pit-1* (50), (growth hormone gene activating) pituitary factor 1; *Sp1* (51), stimulation protein 1; *UPE* (52), upstream promoter element in glucokinase gene; *Xpf* (54), exocrine pancreas transcription factor; and *Zeste-whit* (55), a transcription factor controlling *Drosophila* development.

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