The cAMP-response element mediates induction of secretogranin II by CHX and FSK in GH4C1 cells

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J ones, Letetia C., and Jonathan G. Scammell. The cAMP-response element mediates induction of secretogranin II by CHX and FSK in GH4C1 cells. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E656–E664, 1998.—The effect of cAMP on secretogranin II (SgII) gene transcription in GH4C1 (GH) cells is not observed unless protein synthesis is inhibited. We have identified elements in the SgII promoter that mediate regulation by cycloheximide (CHX) and forskolin (FSK) and characterized the nuclear proteins that interact with them. GH cells were transfected with p2774Luc, p351Luc, p242Luc, and p223Luc containing 2,612, 189, 80, and 61 bp of the SgII promoter upstream of the luciferase gene, respectively. Treatment with CHX and FSK increased promoter activity 8- to 12-fold in cells transfected with p2774Luc, p351Luc, and p242Luc but had no effect in cells transfected with p223Luc. The same 19-bp element (−80 to −62) mediates regulation by CHX alone, as CHX caused a 3.8-fold increase in activity in GH cells transfected with p242Luc but not p223Luc. Gel mobility shifts using sequences −84 to −53 resulted in three complexes, which contained cAMP response element-binding protein heterodimerized with cAMP response element modulator or activating transcription factor-1. No differences were observed in complex formation when cells were treated with either CHX, FSK, or CHX and FSK. Thus CHX affects the response to FSK in GH cells by inhibiting the synthesis of a protein, which does not itself interact with DNA or affect the binding of CRE-binding proteins with the SgII promoter, but likely interferes with the interaction of CRE-binding proteins with the general transcriptional machinery.

SECRETOGRAFIN II (SgII) is a member of the gramin family of secretory proteins (8). The expression of these proteins is limited to endocrine and neuronal cells that concentrate their secretory products into storage granules and release them upon stimulation (33). Although the function of SgII is not completely understood, both intracellular and extracellular roles have been proposed for the protein. Intracellularly, it has been suggested that SgII has a role in the packaging of peptide hormones and neuropeptides into newly forming secretory granules (13, 25). However, most recent studies support an extracellular role for the protein, serving as a precursor to the biologically active peptide secretoneurin (17).

Our interests have focused on the regulation of SgII biosynthesis. Our laboratory has shown that SgII expression is under negative control in rat pituitary tumor GH4C1 (GH) cells (27). When GH cells were treated with the protein synthesis inhibitor cycloheximide (CHX), SgII mRNA levels increased threefold above control. The extent of induction of SgII mRNA correlated with the level to which protein synthesis was inhibited, and induction was observed with other protein synthesis inhibitors. We also found that SgII mRNA was increased by elevated cAMP in GH cells. However, the effect of cAMP was not observed unless protein synthesis was first inhibited (27). These increases in the steady-state levels of SgII mRNA in GH cells were likely transcriptional because they were completely blocked by either of the transcriptional inhibitors actinomycin D or 5,6-dichlorobenzimidazole riboside.

Regulation of SgII gene expression by cAMP in GH cells differs from that described for many other genes for which cAMP-induced expression occurs independently of the level of protein synthesis. In these cases, a transcriptional response requires activation of an intracellular signaling cascade that ultimately converges on the cAMP response element (CRE; see Ref. 10). The final mediators of cAMP regulation of gene expression are members of the cAMP response element-binding protein (CREB)/activating transcription factor (ATF) family of transcription factors, which activate transcription by binding to CREs in cAMP-responsive genes (10). The mechanism by which ongoing protein synthesis inhibits the induction of SgII gene expression by cAMP in GH cells is not known, although we have demonstrated the presence of a CRE within the rat SgII gene promoter (14). However, its role in the induction of SgII gene expression by elevated cAMP in the presence of CHX has yet to be established.

The goal of this study was to define elements in the SgII promoter that mediate its regulation by CHX and cAMP in GH cells. First, we asked whether upstream elements in the SgII promoter repress cAMP-inducible transcription. Such a silencer module was recently shown to specifically repress cAMP-induced transcription of the lactate dehydrogenase A promoter (2). We concluded that upstream elements were not involved and next focused on downstream sequences that might mediate this regulation. We identified a 19-bp sequence in the SgII promoter (including the CRE) that mediates the response to cAMP and CHX and characterized the nuclear proteins in GH cells that interact with this region.

MATERIALS AND METHODS

Materials. Culture medium was obtained from GIBCO-BRL (Grand Island, NY). Pregnant mare serum (PMS) was purchased from Central Biowaste (Irwin, MO). Tissue culture dishes were purchased from Costar (Cambridge, MA). Forskolin (FSK), CHX, and reagents for RNA isolation and hybridization and nuclear isolation were from Sigma Chemical (St. Louis, MO). [γ-32P]ATP (6,000 Ci/mmol) and [α-
GAGCTC) or 5-162 of the rat SgII gene. Primers for amplification contained either a Sac I (GAGCTC) or Bam H I (GGATCC) restriction site at the 5'-end of the SgII sequences. The forward primer used in the construction of p242Luc was 5'-GAGCTCTGAGCCGGTGACGTCAGCG-3'. The oligonucleotide 5'-GGATCCCTAAGGTGAGACCTAGC-3' was used for reverse priming. After digestion with Sac I and Bam H I, each PCR fragment was subcloned into the Sac I-Bam H I cut pLuc-Link reporter construct. To determine the proper orientation and to confirm the nucleotide sequence, the resulting constructs were sequenced by the dyeoxy chain termination method (24) using the Sequenase version 2.0 DNA sequencing kit (US Biochemical, Cleveland, OH).

Transfection and luciferase assays. Transfected plasmid DNAs were generated in the M109 strain of Escherichia coli and prepared using the Wizard Megaprep DNA Purification System (Promega) followed by phenol-chloroform extraction and ethanol purification. Transfection studies were performed using lipofectin (400 U/ml) at 37°C for 30 min with occasional vortexing. The levels of luciferase and cyclophilin mRNA were analyzed by HybSpeed Ribonuclease Protection Assay (Ambion) according to the manufacturer’s instructions. Fifty micrograms of the transfected RNA were hybridized with cyclophilin and luciferase riboprobe (50,000 counts/min (cpm) each) and digested with RNase A (10 U/ml) and RNase T1 (400 U/ml) at 37°C for 30 min with occasional vortexing. Protected RNA fragments were precipitated at −20°C for 15 min and resolved on 6% polyacrylamide-8 M urea gels, and their lengths were determined by comparison with RNA standards.

Preparation of nuclear extracts. GH cells were plated in 100-mm tissue culture dishes at a density of 5 × 10^6 cells/dish and were incubated overnight. Cells were transfected in serum-free medium with 12 µg plasmid/dish using 10 µg lipofectin. After an 8-h incubation, the medium containing lipofectin and plasmids was aspirated, and complete medium was added to the cultures. After an additional 24-h incubation, cells were treated with 10 µM FSK, 1 µg/ml CHX, or a combination of the two for 4 h. Total cytoplasmic RNA was extracted using RNA STAT-60, and residual input plasmid DNA was removed by DNase I (Promega). The levels of luciferase and cyclophilin mRNA were analyzed by HybSpeed Ribonuclease Protection Assay (Ambion) according to the manufacturer’s instructions. Fifty micrograms of the transfected RNA were hybridized with cyclophilin and luciferase riboprobe (50,000 counts/min (cpm) each) and digested with RNase A (10 U/ml) and RNase T1 (400 U/ml) at 37°C for 30 min with occasional vortexing. Protected RNA fragments were precipitated at −20°C for 15 min and resolved on 6% polyacrylamide-8 M urea gels, and their lengths were determined by comparison with RNA standards.

Preparation of nuclear extracts. GH cells were plated in 100-mm tissue culture dishes at a density of 5 × 10^6 cells/dish and were incubated for 2–3 days. Before harvesting, the cells were treated with 10 µM FSK, 1 µg/ml CHX, or a combination of the two for 4 h. After treatment (four 100-mm dishes/group), the cells were collected by scraping into 1 ml PBS, GH cell nuclear extracts were isolated by a micropreparation technique described by Andrews and Faller (1). Briefly, cell pellets were resuspended in 400 µl of a buffer containing 10 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml leupeptin, and 1 µg/ml pepstatin on ice for 10 min. Nuclei were pelleted by centrifugation at 5,000 g for 10 s and resuspended in 80 µl of a buffer containing 20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 20% DTT, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 1 µg/ml leupeptin, and 1 µg/ml pepstatin on ice for 20 min. Cellular debris was removed by centrifugation at 12,000 g for 2 min, and the supernatant fraction was stored in aliquots at −70°C.

Gel mobility shift analysis. DNA oligonucleotides were synthesized in the University of South Alabama Biopolymer Core Facility Laboratory. Sense and antisense oligonucleotides were annealed and used as blunt-ended, double-stranded DNA molecules. The sense sequences of the probes...
used in the gel shift analyses were as follows: rat SgII-CRE, 5'-AGGCTGAGCCGCTAGTCAAGGAGATTCCG-3' (14); tandem CRE, 5'-GATCTAGACGTATATGCTACGCTTAGACTGACGTAC-3'; and activator protein (AP)-2 element, 5'-GATCGAAGTCGCTCAGGCTCGCCCGTGGC-3' (the consensus CRE is underlined, and the AP-2 element is in bold). When used as a probe, 40 ng of double-stranded DNA were labeled using 100 μCi [γ-32P]ATP and 10 units T4 polynucleotide kinase (Promega) in a reaction buffer containing 70 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, and 5 mM DTT. The labeled oligonucleotide was purified over two successive Probe Quant G-50 spin columns (Pharmacia Biotech, Piscataway, NJ), and the radioactive incorporation was determined by liquid scintillation counting. Five micrograms of GH cell nuclear extract were incubated for 15 min at room temperature in binding buffer consisting of 10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% (vol/vol) glycerol, 80 μg/ml sonicated salmon sperm DNA, and 2 μg poly(dI-C)] before the addition of radiolabeled probe. This incubation was performed in the absence or presence of 500-fold molar excess of competing oligonucleotides (unlabeled probe, tandem repeat of CRE, or the AP-2 motif as a nonspecific competitor). Radiolabeled probe (50,000 cpm/reaction) was then added to the preincubation mixture for a final volume of 20 μl and allowed to incubate at room temperature for 20 min. Protein-DNA complexes were fractionated on 4.5% nondenaturing polyacrylamide gels in 0.25× Tris-borate-EDTA buffer, pH 8.5. After electrophoresis, gels were dried and autoradiographed. For antibody supershift experiments, a standard gel shift procedure was performed as described above except that 2–5 μg of antibody were added per 20-μl reaction volume subsequent to the addition of 32P-labeled oligonucleotide probe. Antibodies to c-j un/AP-1, AP-2, cAMP response element modulator (CREM)-1, CREB-2, and ATF-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), whereas CREB and phosphorylated CREB antibodies were from Upstate Biotechnology (Lake Placid, NY). Incubation with antibody was carried out for 15 min at room temperature, after which multimeric complexes were electrophoresed as described above.

RESULTS

Regulation of the SgII promoter by CHX and FSK. We previously showed that the steady-state levels of SgII mRNA are elevated by CHX and FSK in GH cells, an effect that was blocked by transcription inhibitors (27). In identifying sequences in the 5′-flanking region of the SgII gene that mediate its regulation by CHX and FSK in GH cells, it was first necessary to determine that the SgII promoter conferred regulated expression of a reporter gene in response to CHX and FSK. Because protein synthesis was inhibited in these experiments, the level of luciferase mRNA was quantified using RNase protection analysis. In addition, the level of endogenous cyclophilin mRNA was also quantified by RNase protection and was used as an internal standard as it is unaffected by treatment with CHX and FSK in these cells (27). Luciferase and cyclophilin riboprobes were synthesized in an in vitro transcription reaction, and run-off transcription produced antisense probes for cyclophilin and luciferase mRNAs that were 195 and 293 nt in length, respectively (Fig. 1, lanes 2 and 3). These cRNA probes were hybridized with total RNA from GH cells that had been transfected with the construct p2774Luc and treated with a combination of 1 μg/ml CHX and 10 μM FSK for 4 h. GH cell mRNA protected 210- and 103-nt fragments of luciferase and cyclophilin riboprobes, respectively (Fig. 1, lanes 4-7). Degraded excess probe migrated to the bottom of the gel. Densitometric analysis of bands representing the luciferase protected fragment (standardized to cyclophilin) revealed a robust increase (12-fold in this experiment) in luciferase mRNA expression in GH cells treated with CHX and FSK (Fig. 1, lanes 6 and 7) compared with control (Fig. 1, lanes 4 and 5). The results of this study showed that the SgII promoter confers regulation of a luciferase reporter plasmid by CHX and FSK.

Effect of FSK on the expression of deletional constructs of the SgII promoter in GH cells. We previously showed that increases in cellular cAMP did not affect SgII mRNA levels in GH cells (27). Rather, induction of SgII mRNA by FSK was not observed until after protein synthesis was inhibited. A possible explanation for the regulation of SgII expression by FSK in the presence of CHX is that an upstream silencer module in the SgII promoter confers regulation of a luciferase reporter plasmid by CHX and FSK.
cells were transfected with constructs containing either 1,032 (p1194Luc), 189 (p351Luc), 80 (p242Luc), or 61 bp (p223Luc) of the SgII promoter upstream of the luciferase gene. Transfected cells were treated with 10 µM FSK for 4 h and then collected for determination of luciferase activity. The level of activity seen in FSK-treated cells was compared with that of control GH cells transfected with the same construct. The levels of luciferase activity in each dish of control cells transfected with the various constructs were 4,473 ± 45 (p1194Luc), 5,230 ± 270 (p351Luc), 5,567 ± 218 (p242Luc), and 2,270 ± 199 (p223Luc) relative light units (rlu)/µM β-Gal, consistent with the results of our previous study (14). FSK had no effect on the expression of constructs containing 1,032, 189, 80, or 61 bp of SgII promoter sequence (Fig. 2). However, in the same experiments, FSK increased the expression of a plasmid containing 2,500 bp of rat prolactin promoter sequence upstream of the luciferase gene (pPRLLuc) by twofold, consistent with studies by Liang et al. (18). The basal activity of pPRLLuc in dishes of untreated GH cells was 156,900 ± 3,800 rlu/µM. Therefore, progressive 5′-deletions in the SgII promoter do not appear to uncover an FSK-mediated stimulation of SgII promoter-luciferase reporter gene expression. These results do not support the presence of a silencer module upstream in the 5′-flanking region of the rat SgII gene that selectively represses cAMP-induced transcription. Rather, a common downstream sequence likely mediates regulation by CHX and FSK in GH cells.

Identification of promoter sequences involved in regulation of SgII gene expression by CHX and FSK. To identify sequences in the 5′-flanking region of the rat SgII gene that mediate regulation by CHX and FSK, we evaluated the effect of CHX and FSK on SgII promoter activity in deletional constructs. GH cells were transfected with the constructs p351Luc, p242Luc, and p223Luc, which correspond to 189, 80, and 61 bp of the SgII promoter upstream of the luciferase gene, and regulation of these constructs by CHX and FSK was compared with p2774Luc. The constructs p351Luc and p242Luc contain the CRE sequence (located at −72 to −65), whereas p223Luc does not (Fig. 3, top). To assess regulation of each construct by CHX and FSK, total RNA was harvested from transfected GH cells under control and treated conditions, and the levels of luciferase and cyclophilin mRNAs were analyzed by RNase protection analysis of total RNA isolated from the cells. The level of luciferase mRNA was standardized to that of cyclophilin, and the data were expressed as a percent of control for each plasmid (set at 100%; bottom). Each bar represents the mean ± SE of 3 independent experiments.

Fig. 3. Deletion of a 19-bp fragment of the SgII promoter results in loss of reporter gene regulation by CHX and FSK. GH cells were transfected with the constructs p2774Luc, p351Luc, p242Luc, and p223Luc (top) and incubated in the absence or presence of 1 µg/ml CHX and 10 µM FSK (CHX + FSK) for 4 h. Levels of luciferase and cyclophilin mRNAs were analyzed by RNase protection analysis of total RNA isolated from the cells. The level of luciferase mRNA was standardized to that of cyclophilin, and the data were expressed as a percent of control for each plasmid (set at 100%; bottom). Each bar represents the mean ± SE of 3 independent experiments.
ments, GH cells were transfected with p242Luc and p223Luc (Fig. 3, top) and treated with either 1 µg/ml CHX or a combination of 1 µg/ml CHX and 10 µM FSK for 4 h. The effect of each treatment on reporter gene expression was evaluated by RNase protection. Treatment with CHX increased promoter activity 3.8-fold in cells transfected with p242Luc compared with an 8-fold increase observed when CHX was combined with FSK (Fig. 4). On the other hand, CHX had no effect on promoter activity in GH cells transfected with p223Luc. As expected, treatment with CHX and FSK did not affect promoter activity in these cells. These results suggest that deletion of promoter sequences corresponding to -80 to -62 of the SgII promoter led to loss of reporter gene regulation by CHX as well as the combination of CHX and FSK. Thus a common sequence mediates reporter gene regulation by CHX alone and CHX and FSK together.

In vitro binding of neuroendocrine nuclear proteins to the SgII CRE motif. Once a common regulatory sequence within the SgII promoter had been identified, our first goal was to evaluate its interaction with putative inhibitory or stimulatory proteins from GH cells. Gel mobility shift analysis was performed using a double-stranded oligonucleotide probe corresponding to SgII promoter sequences -84 to -53, which includes this regulatory sequence. The pattern of proteins binding to this probe was determined using nuclear extracts isolated from GH cells under control conditions. Two prominent protein-DNA complexes and one less prominent complex, which migrated more rapidly, were identified using this technique (Fig. 5A, lane 2). To determine whether the complexes represented specific interactions of nuclear proteins with this probe, we tested the effect of several competitor oligonucleotides on these interactions. The addition of a 500-fold molar excess of unlabeled probe resulted in disappearance of all three complexes (Fig. 5A, lane 3). Likewise, addition of an excess of an oligonucleotide containing a tandem

![Fig. 4. Common sequence mediates reporter gene regulation by both CHX alone and CHX and FSK in combination. After transfection with p242Luc or p223Luc, GH cells were treated with either 1 µg/ml CHX or 1 µg/ml CHX and 10 µM FSK together for 4 h. The effect of each treatment on reporter gene expression was evaluated by RNase protection analysis; the level of luciferase mRNA was standardized to that of cyclophilin, and the data were expressed as a percent of control for each plasmid (set at 100%). Each bar represents the mean ± SE of 3 independent experiments.](http://ajpendo.physiology.org/)

![Fig. 5. Analysis of SgII cAMP response element (CRE)-binding proteins. A: gel mobility shift analysis using the SgII CRE. A labeled, double-stranded oligonucleotide probe spanning the SgII CRE region (-84 to -53) was incubated with nuclear extracts prepared from untreated GH cells. A binding experiment without extract is shown in lane 1. In lane 2, GH cell nuclear extract was incubated with labeled probe in the absence of competing oligonucleotides. Competitors [unlabeled probe (a), lane 3; tandem CRE (b), lane 4; activator protein (AP)-2 motif (c), lane 5] were added to the binding reaction at 500-fold molar excess of the labeled probe. B: antibody supershift analysis of SgII CRE-binding proteins. The labeled SgII oligonucleotide probe was incubated with no GH cell nuclear extract (lane 1), with nuclear extract in the absence of antibodies (lane 2), or with nuclear extract in the presence of antibodies (lanes 3–9). Antibodies specific for the following proteins were added to the in vitro binding reactions: cAMP response element-binding protein (CREB; lane 3), phosphorylated CREB (P-CREB; lane 4), activating transcription factor (ATF)-1 (lane 5), cAMP response element modulator (CREM)-1 (lane 6), CREB-2 (lane 7), c-Jun/AP-1 (AP-1, lane 8), or AP-2 (lane 9). Arrows indicate supershifted complexes.](http://ajpendo.physiology.org/)
tandem CREs to compete suggests that the complexes formed between GH cell nuclear extract and the SgII promoter fragment represent CRE-binding proteins. CRE-binding transcription factors belong to a superfamily of proteins that contain highly related basic DNA-binding domains and homologous leucine zipper (bZip) domains responsible for dimerization (reviewed in Ref. 21). Both homo- and heterodimers of the bZip superfamily can interact with the CRE. To help characterize the protein makeup of the SgII promoter CRE complexes, we used supershift analysis with antibodies to members of the bZip family of proteins: two CREB antibodies (one that recognizes both phosphorylated and non-phosphorylated forms of CREB and one that recognizes only phosphorylated CREB) and antibodies to ATF-1, CREM-1 (CRE modulator), CREB-2, c-Jun/AP-1, and AP-2. Consistent with Fig. 5A, three protein-DNA complexes (designated complexes I, II, and III) were observed when the SgII CRE was incubated with GH cell nuclear extracts in the absence of antibody (Fig. 5B, lane 2). Incubation with a CREB antibody resulted in a complete disappearance of complex I, partial loss of complexes II and III, and the appearance of a supershifted complex (Fig. 5B, lane 3). This suggests that all three bands include CREB-containing complexes. An antibody to phosphorylated CREB had a similar effect on complex migration (Fig. 5B, lane 4), further supporting the presence of CREB in these complexes and suggesting that the CREB protein interacting with the SgII CRE is phosphorylated. Addition of an anti-ATF-1 antibody resulted in the disappearance of the less prominent, fastest migrating complex, complex III, whereas the most slowly migrating complex, complex I, was supershifted by the addition of an antibody to CREM-1 (Fig. 5B, lanes 5 and 6, respectively). Antibodies to CREB-2, c-j un/AP-1, or AP-2 had no effect on the migration of the complexes (Fig. 5B, lanes 7–9, respectively). Identical gel mobility shift and supershift patterns were observed in three other experiments using different preparations of GH cell nuclear extract. Thus complex I likely contains a CREB-CREM heterodimer, whereas complex III likely contains a CREB-ATF-1 heterodimer. Complex II appears to contain CREB, although it is not clear whether it is homo- or heterodimeric in nature. All of the CRE-B-binding proteins associated with the SgII promoter fragment appear to be phosphorylated in these in vitro binding studies.

CHX does not alter protein-DNA interactions at the SgII CRE. Removal of upstream DNA did not reveal a cAMP response (Fig. 2), arguing against the existence of an upstream silencer element in the rat SgII gene. Rather, our results have shown that a common sequence including a CRE, which forms at least three complexes with GH cell nuclear extracts, mediates the induction of SgII promoter activity not only in response to CHX and FSK but also in response to CHX alone. We reasoned that, if CHX inhibited the synthesis of a repressor protein that is contained in one of the DNA-protein complexes, a different pattern of protein binding would be expected in gel mobility shift analysis using extracts from CHX-treated cells compared with control cells. To examine this possibility, we analyzed the pattern of proteins binding to the SgII CRE using nuclear extracts from control GH cells and GH cells treated with CHX, FSK, and CHX and FSK together. Our results show that the various treatments had no effect on the pattern of proteins binding to the SgII CRE (Fig. 6, compare lanes 2, 6, 10, and 14). Formation of the complexes was eliminated when an excess of unlabeled probe (Fig. 6, lanes 3, 7, 11, and 15) or of a tandem repeat of the CRE (Fig. 6, lanes 4, 8, 12, and 16) was included in the incubation, regardless of the prior treatment of the cells. An oligonucleotide containing an AP-2 motif had no effect on complex formation in any of
the treatment groups (Fig. 6, lanes 5, 9, 13, and 17). No differences between control and treated cells were observed when gel mobility supershifts were performed using nuclear extracts derived from them (data not shown). We conclude that the complexes formed between an oligonucleotide containing the rat SgII CRE and nuclear extracts derived from control GH cells and GH cells treated with CHX, FSK, and the two together are identical.

**DISCUSSION**

The goal of this study was to investigate the molecular basis for the regulation of the rat SgII gene by CHX and FSK in GH cells. First, we found that we could reproduce this effect using an SgII promoter-luciferase fusion gene. Furthermore, a 19-bp sequence that includes a consensus CRE within 80 bp of the transcription start site conferred regulation by CHX and FSK in GH cells. The CRE has been shown to mediate cAMP inducibility of genes of other members of the granin family, chromogranin A and chromogranin B, in neuroendocrine PC-12 and AtT-20 cells and HIT cells, respectively (34, 15), as well as the mouse SgII gene in SN56 and HIT cells (3). cAMP has also been shown to upregulate SgII mRNA in primary cultures of rat hypothalamic neurons (26) and bovine chromaffin cells (7). The difference between regulation of the rat SgII gene promoter in GH cells and the regulation of the chromogranin A, chromogranin B, and the mouse SgII genes in other cell types is that regulation in GH cells by cAMP is only seen after protein synthesis is inhibited (27). We also observed this phenomenon in the regulation of the SgII gene in rat PC-12 cells (31). The effect of CHX is likely related to inhibition of protein synthesis, as the level of induction of SgII transcription was related to the extent of protein synthesis inhibition and was reproduced with other inhibitors, which act by different mechanisms (27, 31). Thus direct nuclear actions of CHX that occur at concentrations below those required to inhibit protein synthesis (6) do not mediate this effect of CHX. Likewise, we can discount an effect of CHX on mRNA stability (12) as the effect of CHX was dependent on specific promoter elements. Rather, the data support a role for a labile repressor that affects the ability of FSK to activate transcription of the SgII gene in GH cells.

The mechanism by which a labile repressor affects SgII gene expression in GH cells is apparently not due to an upstream silencer suppressing cAMP-inducible transcription. This has been described in the lactate dehydrogenase A promoter (2). In their study, deletion of a 19-bp sequence >800 bp upstream of the start site unmasked regulation of a lactate dehydrogenase A promoter-chloramphenicol acetyltransferase fusion gene by cAMP (2). In our study, we failed to uncover an FSK regulation of the SgII promoter, even when nearly 1 kb of DNA upstream of the CRE was deleted. These studies, however, do not exclude the possibility that a silencer module that selectively represses cAMP-induced transcription is present downstream of the SgII CRE. It would be impossible to localize such a sequence using deletional analysis because of coincident deletion of the CRE. However, no sequence within the downstream region of the rat SgII gene resembles the palindromic sequence thought to repress the lactate dehydrogenase A promoter (2, 14).

The identity of this labile repressor is unknown, but we know that it neither directly interacts with a 32-bp fragment of the SgII promoter containing the CRE nor alters the interaction of nuclear factors with the SgII promoter CRE. The patterns of protein binding to the SgII CRE were not different when the activity of nuclear extracts from control and CHX-treated GH cells were compared by gel mobility shift analysis. It is possible that a ternary complex is formed between a repressor and the CRE-binding complexes, but it is either unstable, in very low abundance, or is difficult to maintain in an in vitro binding assay. Examples of such repressors might be inducible cAMP early repressor (ICER) proteins (10), although there is no evidence that they fulfill the property of labile repressors. On the other hand, repression of CRE-mediated transcription of the SgII gene in GH cells may be mediated by a nuclear protein such as YY1 (28). YY1 is thought to repress CREB-mediated transcription in a promoter-specific manner, not by direct association with CREB but rather by interfering with the interaction of CREB with the preinitiation complex (9). Thus changes in the expression of a YY1-like protein would not be detected by gel mobility shift analysis. A role for YY1 or other proteins acting by the same mechanism in repression of SgII expression in GH cells remains to be investigated.

During the course of these experiments, we have identified nuclear factors from GH cells that interact with the rat SgII CRE. We demonstrated sequence-specific binding of CREB, confirming the results of Cibelli et al. (3) using the mouse SgII promoter sequence. They also showed that overexpression of the CRE-binding protein CREB-2 impaired cAMP-mediated transcription of the SgII gene in HIT cells, likely resulting from displacement of CREB from the CRE. We did not detect any CREB-2 in nuclear extracts from GH cells using gel mobility shift analysis. Thus, although CREB-2 has been shown to be expressed in a number of human tumor cell lines and mouse tissues (16), it likely does not play a functional role in the regulation of SgII gene expression in GH cells. On the other hand, we found that one of the protein-DNA complexes formed with the rat SgII CRE likely contains CREM. We are aware of the potential cross-reactivity of each of the antibodies we used with other members of the bZIP family of proteins. Furthermore, the phosphorylated CREB antibody is made to a peptide sequence that is shared with phosphorylated CREM (21). However, the specific nature of the complex I supershift with the CREM antibody strongly suggests that a CREM-like protein from GH cells participates in complex formation with the rat SgII CRE. The nature of this CREM-like protein is intriguing, as to our knowledge the only product of the CREM gene so far detected in GH cells (specifically GH3 cells) is an ICER protein (22). However, as discussed above, it likely does not
play a role in suppression of the FSK response, and we observed no differences in its gel mobility shift pattern with treatment. Regardless of which CREB-like protein mediates the response of the SgII promoter to FSK, it is unlikely that it functions alone. Regulation of gene transcription by cAMP is thought to require the synergistic interaction of tissue-specific transcription factors and the cAMP signal transduction system (23, 30).

We were surprised to find that most of the CREB and CREM associated with the SgII CRE is phosphorylated under control conditions. The extent of phosphorylation of these bZIP proteins in GH cells has not been previously reported. Our finding suggests that the DNA-binding proteins that mediate activation of the SgII gene after treatment with CHX and FSK may not be CREB or CREM. However, they may heterodimerize with another CRE-binding protein whose phosphorylation state (i.e., transactivation potential) increases after FSK treatment. For example, ATF-1 was identified as a component of complex III, but the antibody to ATF-1 does not discriminate between the nonphosphorylated and phosphorylated forms of ATF-1. It is also possible that the target of activation by FSK is not a CRE-binding protein but rather a coactivator protein (10). Another possibility is that the level of phosphorylated CREB and CREM in nuclear extracts from control GH cells may represent an artifact of the isolation protocol, which disrupts the normal spatial arrangement of kinases and target proteins.

On the other hand, the high basal level of phosphorylation of a transcription factor such as CREB in GH cells is consistent with the effect that we observe when cells are treated with CHX alone. We had previously shown that treatment of GH cells with protein synthesis inhibitors such as CHX results in increased transactivation of the SgII gene (27). We have now shown that this effect is mediated by a 19-bp element containing the CRE. CHX response elements have been described only rarely in the literature but include the serum response element of the c-fos gene (29). The only proteins in GH cell nuclear extracts that interact with this 19-bp element of the SgII gene are CRE-binding proteins, suggesting that they mediate the effect of CHX, although probably not directly for reasons already discussed. Conversely, the SgII gene in PC-12 cells is completely unaffected by administration of protein synthesis inhibitors (31). An apparent difference between PC-12 and GH cells in this regard is that CREB in untreated PC-12 cell nuclei resides predominantly in the nonphosphorylated state (31). It is possible that the selective induction of SgII gene transcription in GH cells after CHX treatment results from the decay of a labile repressor in the presence of an already activated transcriptional factor (e.g., CREB). However, induction of the SgII gene in PC-12 cells in the presence of CHX is only observed after activation of CREB or other transcription factors with FSK (31).

In summary, we have identified the CRE as the promoter sequence that mediates induction of the SgII gene by CHX and FSK in GH cells. We have identified proteins that interact with this sequence in control GH cells. Protein-DNA binding activity did not change after treatment of cells with CHX and/or FSK, suggesting that changes in protein-protein interactions mediate the responses of the SgII gene to CHX and FSK in GH cells. The regulation of SgII expression by the mechanism described here is unlikely to apply to in vivo regulation of this gene by physiological stimuli, such as salt loading or lactation (19, 20). Rather, it may provide insight into the tissue-specific regulation of SgII expression. Although gonadotrophs, corticotrophs, and somatotrophs express SgII to varying degrees, the SgII gene in mammothrophs is silent at every point of development (32). The mechanism by which the SgII gene is selectively silenced in PRL cells of the pituitary gland is not known.

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