Two CGTCA Motifs and a GHF1/Pit1 Binding Site Mediate cAMP-dependent Protein Kinase A Regulation of Human Growth Hormone Gene Expression in Rat Anterior Pituitary GC Cells*

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We established the cis-acting elements which mediate cAMP responsiveness of the human growth hormone (hGH) gene in transiently transfected rat anterior pituitary tumor GC cells. Analysis of the hGH 5′-flanking DNA (5′-FR) coupled to the hGH cDNA or chloramphenicol acetyltransferase or luciferase genes, indicated that cAMP primarily stimulated hGH promoter activity. Cotransfection of a protein kinase A inhibitory protein cDNA demonstrated that the cAMP response was mediated by protein kinase A. Mutational analysis of the hGH promoter identified two core cAMP response element motifs (CGTCA) located at nucleotides -187/-183 (distal cAMP response element; dCRE) and -95/-91 (proximal cAMP response element; pCRE) and a putative-specific transcription factor (GHF1/Pit1) binding site at nucleotides -123/-112 (dGHF1) which were required for cAMP responsiveness. GHF1 was not a limiting factor, since overexpression of GHF1 in cotransfections increased basal but not forskolin induction levels. Gel shift analyses indicated that similar, ubiquitous, thermostable protein(s) specifically bound the pCRE and dCRE motifs. The CGTCA motif-binding factors were cAMP response element binding protein (CREB)/activating transcription factor-1 (ATF-1)-related, since the DNA-protein complex was composed of unalabeled CREB consensus oligonucleotide, specifically supershifted by antisera to CREB and ATF-1 but not ATF-2, and was bound by purified CREB with the same relative binding affinity (pCRE < dCRE < CREB) and mobility as the GC nuclear extract. UV cross-linking and southwestern blot analyses revealed multiple DNA-protein interactions of which 400- and 45-kDa proteins were present. Antibody supershift analyses indicated that CREB/ATF-1-related factors act coordinately with the cell-specific factor GHF1 to mediate cAMP-dependent regulation of hGH-1 gene transcription in anterior pituitary somatotrophs.

Human growth hormone (hGH)1 belongs to a family of hormones which includes chorionic somatomamotropin (hCS) by the hGH-1 gene; rGH, rat growth hormone; GHRH, hypothalamic growth hormone releasing hormone; GHP1/Pit1, pituitary-specific transcription factor; pGHF1 and dGHF1, proximal and distal GHF1 binding sites, respectively; hCS, human chorionic somatomamotropin (encoded by the hCS-1 gene); PRL, prolactin; CAT, chloramphenicol acetyltransferase; LUC, luciferase; 5′-FR, 5′-flanking region; CRE, CAMP response element; pCRE and dCRE, proximal and distal CREs, respectively; CRU, CAMP response unit; CREB, CAMP response element binding protein; ATF, activating transcription factor; PKI, protein kinase A inhibitory peptide; hCG, human chorionic gonadotropin; PKImut, protein kinase A mutant inhibitory peptide; bp, base pair; nt, nucleotide.

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1 The abbreviations used are: hGH, human growth hormone (encoded by the hGH-1 gene); rGH, rat growth hormone; GHRH, hypothalamic growth hormone releasing hormone; GHP1/Pit1, pituitary-specific transcription factor; pGHF1 and dGHF1, proximal and distal GHF1 binding sites, respectively; hCS, human chorionic somatomamotropin (encoded by the hCS-1 gene); PRL, prolactin; CAT, chloramphenicol acetyltransferase; LUC, luciferase; 5′-FR, 5′-flanking region; CRE, CAMP response element; pCRE and dCRE, proximal and distal CREs, respectively; CRU, CAMP response unit; CREB, CAMP response element binding protein; ATF, activating transcription factor; PKI, protein kinase A inhibitory peptide; hCG, human chorionic gonadotropin; PKImut, protein kinase A mutant inhibitory peptide; bp, base pair; nt, nucleotide.
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(1990), and Roessler et al. (1988)). These genes contain common CGTCA motifs yet their flanking sequences differ considerably which may be important in dictating binding specificity (Deutsch et al., 1988). CREB/ATF-related factors may mediate the actions of CAMP by binding as homodimers or heterodimers to these various CGTCA motifs (reviewed in Meyer and Habener (1993)). Alternatively, these sites may bind heterodimers, consisting of members of the CREB/ATF family and unrelated factors, as shown by Maguire et al., (1991) for the hepatitis B virus X protein.

Previous efforts to identify the CAMP-responsive elements in the rat and human GH genes have focused on the 5'-flanking region (5'-FR) (Copp and Samuels, 1989; Brent et al., 1988; Dana and Karin, 1989). These studies showed that the rGH and hGH 5'-FR mediate CAMP responsiveness; however, precise identification of the CRE5s was not achieved. Brent et al., (1988) narrowed the CAMP responsive region of the hGH 5'-FR to within nt -212/-83. In contrast, Dana and Karin (1989) reported the hGH promoter CRE to lie within 82 bp upstream of the transcriptional start site. Both studies suggested GHF1 involvement in the CAMP response. GHF1's role in mediating the CAMP response was supported by the identification of two CREB/ATF binding sites in the 5'-FR of the GH-1 gene (McCormick et al., 1990; Chen et al., 1990) and by the regulation of GHF1 gene transcription by forskolin (McCormick et al., 1990). GHF1 may also be phosphorylated in vitro by protein kinase A (Kapiloff et al., 1991). Accordingly, intranuclear increases in GHF1 levels or posttranslational modification of GHF1 might account in part for the CAMP-mediated increase in hGH gene transcription.

We determined the CAMP regulation of hGH gene expression by analyzing the intact hGH gene or hGH 5'-FR coupled to the hGH cDNA or chloramphenicol acetyltransferase (CAT) or luciferase (LUC) genes in transiently transfected rat anterior pituitary GC cells exposed to the CAMP-elevating agent forskolin (Seamon and Daly, 1986). Our results demonstrate that CAMP regulation of the hGH promoter is protein kinase A-mediated at the level of transcription. The elements controlling CAMP responsiveness of the hGH gene are localized to the promoter region and require two CGTCA motifs and a GHF1 binding site. The two CGTCA motifs are located distally (dCRE; nt -187/-180) and proximally (pCRE; nt -99/-95) to the distal-most GHF1 binding site (dGHF1; nt -123/-112). DNA binding studies and immunoprecipitation revealed that the protein(s) binding to the CGTCA motifs were CREB/ATF-1-related. UV cross-linking and Southwestern blot analyses revealed multiple DNA-protein interactions of which ~100- and ~45-kDa proteins were predominant; the ~45-kDa protein may represent CREB. Thus CREB/ATF-1-related factors may act coordinately with the tissue-specific factor GHF1 to mediate CAMP/protein kinase A regulation of hGH-1 gene transcription in anterior pituitary somatotrophs.

**EXPERIMENTAL PROCEDURES**

**Materials**—Oligonucleotides were synthesized by the Molecular Biology Core Facility, Mayo Clinic, [H]acetyl-CoA (2-10 Ci/mmol), [γ-32P]ATP (5000 Ci/mmol), and [α-32P]dATP (1000 Ci/mmol) were obtained from Amersham Corp.

**Plasmid Constructions**—Standard recombinant DNA techniques were used for most DNA manipulations (Sambrook et al., 1989). In this study hGH and hCS refer to the hGH-1 and hCS-1 genes exclusively. All nucleotide numbering is relative to the transcription start site. The hGH promoter (nt -492/EcoRI to +6/BamHI) is designated GHP. The hCS promoter (nt -329/EcoRI to +24/BamHI) is designated GHP. The human β-actin promoter (4.3-kilobase EcoRI to AluI fragment) is designated ACTP. The hGH cDNA (nt +1/BamHI to +689/SmaI) is designated GHP. The cDNA sequence (nt +1/BamHI to +2651/EcoRI) is designated GHP. The cDNA fragment (nt +1029 to +2651) and SV40 (HindIII/EcoRI) 3'-untranslated/3'-FR are designated GHS' and SV3', respectively.

The construction of the GHP.Hs.GHS', GHP.Gc.GH3', Csp.Gh.GH3', AC.Tp.Gh.GH3', GHP.Gh-SV3', and Csp.Gh.GH3' plasmids was described in detail by Zhang et al. (1992). The GHP and CS hybrid promoters in plasmids Csp/HGF.SV3 GHP.HG.SV3' and GHP.Csp.GSV3' were fused at their common NsiI site (nt -83) as described by Nachtigal et al. (1989). Deletions of the hGH 5'-FR were constructed by Bal31 digestion and subcloning described by Eberhardt et al. (1989). The -1015-1717 thymidine kinase promoter/CAT construct (Tk(CAT)) was described by Cattini and Eberhardt (1987). The Rous sarcoma virus promoter (Rsvp) fused to the rat GHFL cDNA (Rsvp.GHFL) was a gift from Dr. Michael Karin (University of California, San Diego). Rsvp fused to the β-galactosidase gene (Rsvp.β-Gal) was described by Walker et al. (1983). Plasmids expressing wild-type (Rsvp) and mutant (RSVPmut) protein kinase A inhibitory proteins were described by Grosveld et al. (1987).

Site-specific mutagenesis of the hCS and hGH 5'-FR was done with minor modifications of the protocol of Hemaley et al. (1989). The hGH 5'-FR (nt -492/EcoRI to +6/BamHI) in pUC8 (hGHpu.Cuc) was modified by insertion of a SaclI linker into the EcoRI site. Five ng of the hGHpu.Cuc plasmid was subjected to inverse polymerase chain reaction with 10-100 pmol of each primer (listed below) in a 100-μl reaction consisting of 20 mm Tris-HCl (pH 8.2), 10 mm KCl, 1.5-2.0 mm MgCl2, 6 mm (NH4)2SO4, 0.1% Triton X-100, 200 μm each dNTP, and 1.25 units of Pfu DNA polymerase (Stratagene). The reaction underwent 25 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C with a final cycle 10 min. In the PCR reaction, we used an inverse polymerase chain reaction (all sequences are written 5' to 3'; ψ = sense strand, − = antisense strand):

GHP(cCRE)p.LUC, CCCTGTTAAGG-GAAAGATG (+); CCCTGATTATTGTGAGGGTT (-); GHP(cP)p.LUC, ACGCGGGCCCATCATGAAT (+); ACGCGTGTCTCAATGGAT (-);

GHP(hGHp1).p.LUC, TATACACATGTACAAACCGCTATGCCC (+); GHP(hGHp2).p.LUC, CTCTCCTGCTATGCCC (-); GHP(hGHp3).p.LUC, TATACACATGTACAGGCGCTATGCCC (+); GHP(hGHp4).p.LUC, CTCTCCTGCTATGCCC (-); GHP(hGHp5).p.LUC, TATACACATGTACAGGCGCTATGCCC (+).

The modified pA3.LUC vector was first digested with HindIII, blunt-ended with Klenow and then digested with SaclI to accommodate the DNA fragments, containing the mutated promoter sequences, upstream of the LUC gene. Plasmid DNA was purified by two sequential CsCl gradients and the DNA concentration measured spectrophotometrically. Plasmids were also checked for purity, concentration, supercoiling and restriction digestion pattern by agarose gel electrophoresis. In most cases, at least two separate plasmid preparations were tested in the transfection experiments.

**Cell Culture and DNA Transfections**—GC cells were grown in monolayers at 37°C, 5% CO2 and 100% humidity in Dulbecco’s modified Eagle’s medium (MEM, high glucose, Celox) supplemented with 10% fetal bovine serum (FBS, Whittaker), 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Inc.), and 1 mm l-glutamine (Celox). One to two days prior to transfection, GC cells were rinsed with Dulbecco’s phosphate-buffered saline containing MgCl2 and CaCl2 (PBS-Mc, Celox) and deinduced with DMEM containing 4% horse serum (FBS; Ster-FBS; Fetal White Serum, Biotechnologies Inc.), rinsed twice with 4% STR-FBS/DMEM, and divided to 4 6-cm2 dishes. Forskolin was added at 2.5 μM final concentration for the entire 20–24-h transfection period. For the LUC reporter gene transfections, GC cells were seeded at 3000 cells/cm2 in monolayers and transfected essentially as described by Zhang et al. (1992). Briefly, GC cells were harvested and transfected (4 × 104 cells/0.4 ml) at 350 V at 960 microfarads (Gene Pulser, Bio-Rad) with either 15 μg of CAT plasmid or 15 μg of hGH reporter plasmid and 3 μg of a human actin promoter driven human choric gonadotropin (hCG) cDNA expression plasmid to control for transfection efficiency. Transfected cells were seeded in 6 STR-FBS/DMEM plates of 24-mm dishes. Forskolin was added at 2.5 μM final concentration for the entire 20–24-h transfection period. For the LUC reporter gene transfections, deinduced cells were harvested in 0.5× Trypein-EIDTA (Life Biotechnologies Inc.), rinsed twice with 4% STR-FBS/DMEM, resuspended at 5 × 104 cells/ml in 0.1% glucose/PBS-Mc solution, and aliquoted at 0.2 ml/4-mm gap electroporation cuvette (Bio-Rad). Plasmid DNA (3.5 pmol, −15 μg) was added, and the cell suspension was electroporated at 350 V at 500 microfarads at room temperature. The electroporated cells were plated at room temperature for 7–10 min, resuspended in 22 ml of 4% STR-FBS/DMEM, and divided to 4 × 6-mm dishes (Bacterial). After a 16–20-h incubation, two of the four dishes were
adjusted to 10 µM forskolin or 0.2% ethanol vehicle for a final 6-h exposure.

Reporter Assays—CAT activity was measured by a modified two-phase liquid scintillation assay (Neumann et al., 1987; Eastman, 1987; Zhang et al., 1992) and normalized per mg of protein (counts/min x min⁻¹ x mg⁻¹). β-Galactosidase assays were performed essentially as previously described (Sambrook et al., 1989). hGH and hCG protein assays were performed with commercial solid-phase radioimmunoassay kits (Hybritech) from media collected 20–24 h post-transfection. The hGH radioimmunoassay is specific for human but not rat GH. The final sample was then dried onto Whatman No. 3 filter paper for later analysis.

RESULTS

CAMP Stimulates hGH-1 Gene Expression by a Transcriptional Mechanism—CAMP-AMP-mediated regulation of hGH gene expression was analyzed through a series of plasmids containing various segments of the hGH gene (Zhang et al., 1992). These plasmids were transiently transfected into GC cells and exposed to forskolin. The first series of plasmid constructions utilized radioimmunochemical detection of secreted hGH protein as a measure of forskolin stimulation of hGH gene expression. Initially, ~500 bp of the hGH 5'-FR was coupled to the hGH structural gene containing introns and exons (designated HGH) and ~500 bp of the hGH 3'-FR (designated GH3') to form GHP.HGS.HG3' (Fig. 1). GHP.HGS.HG3' expression was stimulated ~5-fold in forskolin versus ethanol treated cells (Fig. 1). The hGH structural gene was replaced with the hGH cDNA (designated HGC) to form GHP.HGC.HG3'. Forskolin regulation of GHP.HGC.HG3' was not significantly different from GHP.HGS.HG3' regulation (Fig. 1). Thus, intrinsic sequences did not significantly contribute to CAMP regulation of the hGH gene. Next we determined if the ~93% identical hCS 5'-FR may have dampened any hCS 5'-FR mediated forskolin responsiveness of the hGH gene. Conversely, since the hCS promoter was not significantly different from the hGH promoter, the hCS promoter mediated forskolin responsiveness. The possibility that the hGH 5'-FR required the hGH 3'-FR to mediate forskolin responsiveness was explored by replacing the hGH 3'-FR in GHP.HGC3' with the heterologous SV40 3'-FR (designated SV3') to form GHP.HGC.SV3'. The GHP.HGC3' and GHP.HGC.SV3' plasmids were regulated similarly by forskolin (Fig. 1). Further supporting the concept that the hGH promoter mediated forskolin responsiveness.

The approximate location of the CREs in the hGH promoter
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**TABLE I**

| Gene* | Expression vectorb | Basal activity | Forskolin induction | n^c |
|-------|-------------------|----------------|---------------------|-----|
| GHp.CAT | RSVp.PKImut | 18,621 ± 2812 | 5.3 ± 0.4 | 9 |
| GHp.CAT | RSVp.PKI | 13,339 ± 2549 | 1.1 ± 0.1 | 9 |
| CSp.CAT | RSVp.PKImut | 13,687 ± 685 | 2.7 ± 0.3 | 3 |
| CSp.CAT | RSVp.PKI | 15,409 ± 667 | 1.2 ± 0.0 | 3 |

* GHp.CAT and CSp.CAT plasmin (15 μg) were co-transfected with 15 μg wild-type (RSVp.PKI) or mutant (RSVp.PKImut) PKA inhibitory protein cDNA expression vectors (Grove et al., 1987) and 5 μg of RSVp.β-Gal as a transfection efficiency control.

b Basal CAT activity was determined as counts/min × min⁻¹ and normalized to β-galactosidase activity (Zhang et al., 1992).

c Forskolin induction ratios were determined as in the legend to Fig. 1.

d Number of independent transfections.

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**FIG. 1.** cAMP regulation of the hGH-I gene. The left panel schematically represents the various gene constructs as described in detail under “Experimental Procedures.” The promoter region of the growth hormone, actin, and choricionic somatomammotropin genes are abbreviated GHp, ACTp, and CSp. GHs and GHe represent the structural gene (exons and introns) and cDNA of the growth hormone gene. GHp and SV3’ represent the 3’-untranslated/3’-flanking DNA of the growth hormone and SV40 genes. GC cells were transfected with 15 μg of the diagrammed hGH expression plasmids and 3 μg of hCG plasmid as described under “Experimental Procedures.” Forskolin was added to the medium at 2.5 μM final concentration for 20–24 h. The medium was assayed for immunoreactive hGH and hCG by radioimmunoassay kits (Hybritech). Basal secreted hGH levels were expressed as nanograms of secreted hGH normalized to mIU hCG (mean ± S.E., n = number of independent transfections) as follows: GHp.GHC.GH3’ = 247 ± 5 (n = 55), GHp.GHC.GH3’ = 56 ± 8 (n = 18), CSp.GHC.GH3’ = 228 ± 58 (n = 12), ACTp.GHC.GH3’ = 377 ± 91 (n = 2), GHp.GHC.SV3’ = 53 ± 7 (n = 12), CSp.GHC.SV3’ = 107 ± 31 (n = 10), CSp/GHp.GHC.SV3’ = 450 ± 135 (n = 6), GHp/CSp/GHC.SV3’ = 136 ± 11 (n = 8). Background levels from a promoterless HGH3 constructs transfected into GC cells were equivalent to buffer background controls lacking hCG and were 5 ng of hGH/mIU hCG. Forskolin induction ratios given in the right panel represent the mean ± S.E. of reporter levels of forskolin-treated cells divided by ethanol-treated cells. Statistics were determined by t test.

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was determined by hybrid promoter plasmid constructions. Replacement of the -493/-83 hCS 5'-FR in CSp.GHC.SV3' with the -492/-83 hGH 5'-FR to form GHp/-83CSp.GHC.SV3' yielded forskolin regulation which was not significantly different from Gp.GHC.SV3' (Fig. 1), suggesting that the forskolin responsiveness was localized upstream of nt -83. The reciprocal experiment replacing the -492/-83 hGH 5'-FR in GHp.GHC.SV3' with the -493/-83 hCS 5'-FR to form CSp/-83 GHp.GHC.SV3' yielded forskolin regulation which was not significantly different from CSp.GHC.SV3', thus supporting the upstream localization of the hGH 5'-FR cAMP responsive region.

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**cAMP Stimulation of the hGH Promoter Is Mediated by Protein Kinase A**—Forskolin is known to stimulate adenylate cyclase, elevate cAMP levels, and activate protein kinase A (Seamon and Daly, 1986) but reports suggesting that forskolin has other specific effects (Hoshi et al., 1988; Wagoner and Pallotta, 1988) prompted us to verify forskolin specificity in GC cells. The hGH or hCS promoter was cloned upstream of the CAT gene (GHp.CAT or CSp.CAT) and cotransfected into GC cells with either wild-type or nonfunctional mutant protein kinase A inhibitory protein cDNA driven by the Rous sarcoma virus long terminal repeat (RSVp.PKI or RSVp.PKImut) and assayed for forskolin inducibility. Homodimers of the protein kinase A catalytic subunit and protein kinase A inhibitory protein can effectively block CAMP stimulation of protein kinase A activity (Grove et al., 1987). Cotransfection of RSVp.PKI led to complete loss of cAMP stimulation but did not affect basal GHp.CAT and CSp.CAT activity in transfected GC cells (Table I). Cotransfection of RSVp.PKImut was without effect (Table I). This suggested that the forskolin regulation of the hGH promoter, and even the marginally regulated hCS promoter, were mediated by the cAMP-dependent protein kinase A pathway.

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**CAMP Responsive Elements in the hGH Promoter Localize to at Least Two Distinct Regions**—To localize the hGH 5'-FR CREs, the hGH promoter in GHp.CAT was progressively deleted from its 5' end (Fig. 2). Deletion of the hGH 5'-FR from nt -190 to -163 led to a small, but significant (p < 0.05) drop in forskolin inducibility (Fig. 2A). A second, more pronounced drop (p < 0.001) in forskolin inducibility was evident upon deletion from nt -135 to -87 (Fig. 2). This suggested that CREs were located in two regions, nt -190/-163 and -135/-87, of the hGH promoter. These results confirm the hybrid promoter results obtained in Fig. 1 which suggested localization of CREs upstream of nt -83. Deletion of the hGH 5'-FR to nt -45 resulted in no further loss of forskolin stimulation (Fig. 2). However, complete removal of the hGH 5'-FR further reduced forskolin responsiveness, suggesting that the hGH 5'-FR nt -45/+46 may contain a partial CRE. Transfection of the heterologous thymidine kinase (TK) promoter (nt -109/+51)/CAT plasmid (Tkp.CAT) (Cattini and Eberhardt, 1987) resulted in an ~2-fold forskolin induction. This is essentially the same stimulation as seen with -45 HGH.CAT and suggests that this regulation may be mediated by common elements.

**Two CGTCA Motifs Are Required for cAMP Regulation of the hGH Promoter**—Sequence inspection of the two cAMP-responsive regions of the hGH promoter (nt -190/-163 and -135/-87) revealed a CGTCA motif at nt -188/-184 (antisense strand; designated dCRE) and nt -100/-96 (sense strand; designated pCRE) (Fig. 3A). To determine if the CGTCA motifs represented the CREs, these sequences were mutated within the 500-bp hGH promoter and cloned upstream of the more sensitive firefly luciferase reporter gene (designated Ghp.LUC) (deWet et al., 1987). The dCRE sequence was substituted with a NotI restriction enzyme site to form GH(dCRE)p.LUC whereas the pCRE sequence was substituted with a KpnI restriction enzyme site to form GH(pCRE)p.LUC (Fig. 3A). A combination of the above pCRE and dCRE substitution mutations was also constructed to form GH(pdCRE)p.LUC. Elimination of one or both CGTCA motif(s) resulted in a significant drop in GH promoter forskolin inducibility relative to Ghp.LUC (p < 0.0005, Fig. 3B). The pCRE is apparently more critical than the dCRE since the loss in forskolin inducibility is more pronounced with the mutated pCRE than the mutated dCRE (Fig. 3B). This is consistent with the 5'-deletion data (Fig. 2) which showed a smaller drop in forskolin induction from -190 to -163 than from -135 to -87. The promoterless (pA3.LUC) and RSV proximal promoter (RSV13,p.LUC) control plasmids were slightly negatively regulated by forskolin, which suggested that there was specificity to the residual forskolin inducibility seen with the hGH promoter mutants. Nonetheless, these data indicate that maximal cAMP stimulation of the
hGH promoter requires at least two CGTCA elements.

**Distal GHFl Binding Site Is Also Required for cAMP-regulated hGH Gene Transcription**—The hGH promoter 5' deletion data suggested that the -135/-87 region was necessary for forskolin responsiveness. In addition to the pCRE site, this region contains the distal-most GHFl binding site (designated dGHFl) of the two known hGH 5'-FR GHFl binding sites. To test GHFl involvement in cAMP regulation, we mutated the dGHFl binding site in Ghp.LUC to form Ghp(GHFl)p.LUC and assessed its forskolin responsiveness in transiently transfected GC cells. The GhpFl site was mutated by substitution in the core GHFl binding site from TAAATTATCCAT to **tggATTATCCAT** (Fig. 3A). Mutation of the TAAAT motif has been shown to reduce GHFl binding affinity about 4-fold in a linker-scanning mutation (Lefever et al., 1987; Bodner et al., 1988) and the T to C transition at nt -112, which is a natural difference between hGH and hCS, resulted in reduced GHFl binding (data not shown). Forskolin induction of Ghp(dGHFl)p.LUC was significantly reduced (p < 0.0005) relative to Ghp.LUC (Fig. 3B), indicating that the dGHFl site is critical for cAMP responsiveness. The incomplete loss in forskolin response seen with Ghp(dGHFl)p.LUC, Ghp(CRE)p.LUC, and Ghp(dCRE)p.LUC may be due to the presence of response elements downstream of nt -45 as seen in Figs. 1 and 2. Based on the above deletion and mutation results it appears that at least three distinct cis elements are necessary for maximal forskolin-regulated hGH transcription.

To further assess GHFl involvement in cAMP regulation of the hGH promoter, we overexpressed GhpFl in Ghp.LUC transfected GC cells. Ghp.LUC basal activity was elevated ~10-fold but forskolin inducibility was unaffected (Fig. 3C). The elevated Ghp.LUC basal activity in GhpFl versus control α-β-galactosidase cotransfected cells suggested that GhpFl was indeed being overexpressed and that GhpFl levels were limiting Ghp.LUC activity. The unaffected forskolin response, however, suggests that a mechanism other than modulation of GhpFl protein levels or posttranslational modification status is responsible for cAMP regulation of the hGH promoter.

**Specific Binding of CREB/ATF-related Proteins to the Proximal and Distal CGTCA Motifs**—To support the functional role of the CGTCA motifs in the hGH promoter cAMP response, we attempted to show specific binding of protein to these sites. Numerous DNase I footprinting analyses of the hGH 5'-FR with crude and partially purified GC nuclear extracts by us and others have failed to show protection over either CGTCA motif (data not shown) (Lefever et al., 1987; Bodner and Karin, 1987; Nickel et al., 1991). However, given the relative insensitivity of DNAse I footprinting we performed the more sensitive gel shift analysis using end-labeled oligonucleotides spanning the hGH dCRE and pCRE CGTCA motifs. As a positive control we included a synthetic, palindromic TGACGTCA-containing oligonucleotide (CREB consensus, Promega). Increasing concentrations of crude GC nuclear extract incubated with end-labeled pCRE, dCRE, or CREB oligonucleotides, generated specific DNA-protein complexes (Fig. 4A). GC nuclear extract bound the radiolabeled probes with the relative binding affinity order pCRE < dCRE < CREB (Fig. 4A, compare lanes 3, 8, and 13). The pCRE and dCRE DNA-protein complexes migrated at the same relative mobility as the CREB DNA-protein complex suggesting that similar CREB/ATF-related factors may bind these sites.

We next assessed the specificity of the complexes formed with the pCRE, dCRE, and CREB probes by oligonucleotide competition gel shift analysis. The CREB DNA-protein complex (Fig. 4B, lane 1) was specifically competed for by unlabeled CREB (Fig. 4B, lane 2), dCRE (Fig. 4B, lane 3), and CREB (Fig. 4B, lane 4) oligonucleotides but not by mutated pCRE (pCREm; Fig. 4B, lane 5) oligonucleotide. The same pattern was evident with the dCRE DNA-protein complex (Fig. 4B, lane 6), where the cold dCRE (Fig. 4B, lane 7), pCRE (Fig. 4B, lane 8), andCREB (Fig. 4B, lane 9) oligonucleotides specifically competed binding but the mutated dCREm oligonucleotide did not (Fig. 4B, lane 10). The CREB DNA-protein complex (Fig. 4B, lane 11) was effectively competed for by cold CREB oligonucleotide (Fig. 4B, lane 12) but not mutated CREBm oligonucleotide (Fig. 4B, lane 15) and reduced with cold pCRE (Fig. 4B, lane 13) or dCRE (Fig. 4B, lane 14) oligonucleotide consistent with their relative binding affinities (Fig. 4A). An alternative approach to demonstrate specific binding of the GC nuclear extract to the CGTCA motifs was to end-label the mutated pCREm, dCREm, and CREBm oligonucleotides and run them in parallel with the radiolabeled wild-type probes (Fig. 4E, lanes 1-6). DNA-protein complex formation was reduced or eliminated with the mutated probes (Fig. 4E, compare lanes 1 to 2, 3 to 4, and 5 to 6) reinforcing the specificity of the CGTCA motifs.

Since CREB and ATF-1 are known positive CAMP-responsive TGACGTCA-binding proteins (reviewed in Meyer and Habener (1993), Montminy et al. (1990), and Rehfuss et al. (1991)), we determined whether the pCRE and dCRE binding factors were CREB/ATF-related. CREB and ATF-1 belong to the bZIP family of proteins of which CREB has been shown to be thermostable (Hurst et al., 1990). Exposure of GC nuclear extract to elevated cAMP Regulation of hGH-1 Gene Expression
Fig. 3. Site-specific mutagenesis of the hGH-1 promoter. A, detailed diagram of substitution mutations introduced into the 493-bp GH promoter fused to the luciferase reporter gene (GH-p.LUC). Only the sense strand sequence is shown. Bold, lowercase letters represent homology to the CREB/ATF binding site. Underlined nucleotides represent the consensus GHI1 binding site. Broken underline represents the pu.

temperature (24–100 °C) prior to incubation with end-labeled pCRE (Fig. 4C, lanes 1–5), dCRE (Fig. 4C, lanes 6–10), or CREB (Fig. 4C, lanes 11–15) oligonucleotides led to no significant reduction in the DNA-protein complex (Fig. 4C). This is consistent with the GCTCA-binding proteins being thermostable and CREB-like.

Immunoreactivity of the complexed pCRE, dCRE, and CREB oligonucleotides was tested with polyclonal antisera to ATF-1, ATF-2, and CREB glutathione S-transferase fusion proteins. The pCRE (Fig. 4D, lane 1), dCRE (Fig. 4D, lane 6), and CREB (Fig. 4D, lane 11) DNA-protein complexes were supershifted or blocked from forming with the ATF-1 (Fig. 4D, lanes 3, 8, and 13) and CREB (Fig. 4D, lanes 5, 10, and 15) antisera but not with ATF-2 (Fig. 4D, lanes 4, 9, and 14) or control glutathione S-transferase antisera (Fig. 4D, lanes 2, 7, and 12). Thus the pCRE, dCRE, and CREB probes bound protein antigenically related to CREB and ATF-1.

The ability of the pCRE, dCRE, and CREB probes to specifically bind purified CREB was tested. Bacterially purified CREB327 was incubated with either wild-type pCRE (Fig. 4E, lane 7), dCRE (Fig. 4E, lane 9), or CREB (Fig. 4E, lane 11) probe or mutated pCRE (Fig. 4E, lane 8), dCRE (Fig. 4E, lane 10), or CREB (Fig. 4E, lane 12) probe. The relative affinity (pCRE < dCRE < CREB) and mobility of CREB binding was similar to that with GC nuclear extract (Fig. 4E, compare lanes 1 with 7, 3 with 9, and 5 with 11). This suggests that at least part of the GCTCA-binding protein in GC nuclear extracts might be CREB.
FIG. 4. Gel shift analyses of pCRE and dCRE oligonucleotides. Gel shift analyses were performed as described under "Experimental Procedures." Essentially, each gel shift was incubated for 10 min at 24 °C with protein and competitor DNA followed by a 10-min, 24 °C incubation with 10,000 cpm (1 fmol) end-labeled probe. A, end-labeled pCRE, dCRE, or CREB oligonucleotide was incubated with increasing concentrations...
To determine the tissue specificity of the CGTCA-binding proteins we tested nuclear extracts prepared from human placental choriocarcinoma (BeWo and JEG-3) and human cervical carcinoma (HeLa) cell lines in the gel shift assay. JEG-3 and HeLa nuclear extracts formed a complex with pCRE (Fig. 4F, lanes 3 and 4, respectively), dCRE (Fig. 4F, lanes 7 and 8, respectively), and CREB (Fig. 4F, lanes 11 and 12, respectively) oligonucleotide which migrated to the same relative position as that with GC nuclear extract (Fig. 4F, lanes 1, 5, and 9). This suggested that the CGTCA-binding proteins were present in a non-tissue-specific fashion. BeWo nuclear extract was unable to form this specific complex with the end-labeled DNA (Fig. 4F, lanes 2, 6, and 10) which may indicate that the CGTCA-binding proteins are not present in these cells or that the nuclear extract was degraded upon preparation. The significance of the other DNA-protein complexes formed with JEG-3, HeLa, and BeWo nuclear extracts is unclear but may represent specific or nonspecific complexes or proteolytic degradation products.

Proteins of ~100 and ~45 kDa Bind Predominately to the Proximal and Distal CGTCA Motifs—The molecular weight of the proteins bound to the pCRE and dCRE sites was determined by two methods. We first attempted in situ UV cross-linking of DNA-protein complexes by UV irradiating a gel shift gel containing pCRE or dCRE probe shifted with GC or HeLa nuclear extract, isolating the specifically shifted bands, and separating the cross-linked from unlinked products by SDS-PAGE. This yielded many cross-linked species of which an ~130-kDa complex was predominate for both the pCRE and dCRE oligonucleotides (Fig. 5A). The specificity of the cross-linked products is implicit to in situ UV cross-linking since the isolated gel shift bands were shown previously (Fig. 4B) to be specific. Due to the low efficiency of UV cross-linking, some of the ~130-kDa band probably consists of a 1:1 DNA:protein complex. Subtraction of the oligonucleotide molecular weight (~15 kDa) from the complex cross-linked bands yields a protein of ~115 kDa.

To more accurately estimate the molecular weight of the proteins binding the pCRE and dCRE oligonucleotides we performed Southwestern blotting (Vinson et al., 1988). Filter strips were probed with end-labeled, nonmonomeric wild-type or mutated pCRE or dCRE oligonucleotides. We also probed with wild-type CREB oligonucleotide as a positive control. The pCRE (Fig. 5F, lanes 1 and 3) and dCRE (Fig. 5B, lanes 5 and 7) oligonucleotides predominately bound ~100- and ~45-kDa proteins in both GC and HeLa nuclear extracts. These interactions were specific for the GC TGF motifs since the mutated pCRE (Fig. 5B, lanes 2 and 4) and dCRE (Fig. 5B, lanes 6 and 8) oligonucleotides did not bind these proteins or bound them with reduced affinity. The other bands present on the blots were probably nonspecific since they bound both wild-type and mutated DNA. The positive control CREB probe (Fig. 5B, lanes 9 and 10) also bound an ~100-kDa protein and a protein in the ~45-kDa range.

DISCUSSION

We have demonstrated that two CGTCA motifs, designated pCRE and dCRE, and the distal GHF1 binding site comprise a functional cAMP responsive unit (CRU) on the hGH promoter. The pCRE, dCRE, and dGHFl sites are located between nt -190 and -85 and are required for most of the forskolin responsiveness of the hGH promoter (Figs. 1-3). The remaining forskolin responsiveness is located downstream of these elements and may be part of the general transcriptional machinery. Our results support and extend those of Brent et al. (1988) who localized the cAMP-responsive region of the hGH promoter to within nt -212-83. The slight forskolin responsiveness seen downstream of hGH 5' FR nt -85 also partially supports the conclusions of Dana and Karin (1989) who localized the CRE downstream of nt -85. However, the bulk of cAMP regulated activity arises from elements upstream of nt -85. The reason for this discrepancy is unclear, but may have to do with the less sensitive CAT constructs used by Dana and Karin (1989). Both reports suggested GHF1 involvement in the cAMP response which is consistent with our finding with the dGHFl site. The cAMP regulation of the hGH promoter is fully dependent on protein kinase A, since it is completely blocked by coexpression of a protein kinase A inhibitory protein (Table I).

The hGH promoter contains high affinity (nt -285-278) and low affinity (nt -163-156) AP-2 binding sites that are potentially capable of mediating cAMP responsiveness (Imagawa et al., 1987). However, our deletion data (Fig. 2A) indicate that in GC cells these sites are not functional in the hGH promoter, confirming the findings of Dana and Karin (1989). Copp and Samuels (1989) reported similar results with the homologous rGH promoter. Thus AP-2 probably does not contribute to cAMP stimulation of the hGH promoter in GC cells, possibly due to the relatively low abundance of AP-2 in these cells (Dana and Karin, 1989).

The exact identity of the hGH promoter CGTCA-binding proteins is unknown. We have shown that the CGTCA-binding proteins are CREB/ATF-1-related by thermostability, competition with consensus CREB oligonucleotide, and immunoactivity with CREB and ATF-1 antisera (Fig. 4). Our UV cross-linking and Southwestern blot data (Fig. 5) suggest that ~45- and ~100-kDa proteins may be conferring cAMP responsiveness on the hGH promoter. The ~100-kDa CGTCA-binding factor may be related to a previously identified ~120-kDa TGACGTCA-binding factor purified from HeLa cells (Andrisani and Dixon, 1990). The ~100-kDa CGTCA-binding factor may also be related to the hPRL promoter CGTCA-binding factor (Peers et al., 1992) especially given the homology between the GH and PRL genes. Cloning of the hGH and hPRL promoter ~100-kDa CGTCA-binding proteins and HeLa cell ~120-kDa TGACGTCA-binding factors will be necessary to determine their relatedness. The ~45-kDa CGTCA-binding protein may correspond to the CREB factor (~43-kDa (Gonzalez et al., 1989) but is unlikely to represent ATF-1 since its molecular mass is only ~38-kDa (Liu et al., 1993). The similarity between the pCRE, dCRE, and CREB oligonucleotide-bound proteins (Figs. 4 and 5) supports this possibility. The ability of polyclonal antisera to both ATF-1 and CREB to react with the pCRE, dCRE, and CREB DNA-protein complexes is not surprising given the immunological-relatedness of the CREB/ATF family (Hai et al., 1988). By the same reasoning it is perhaps
Fig. 5. UV cross-linking and Southern blot analysis of the pCRE and dCRE motifs. A, in situ UV crosslinking analysis was performed as described under "Experimental Procedures." Standard gel shift reactions (Fig. 4F) using GC and HeLa nuclear extracts and \(^{32}\)P-labeled pCRE or dCRE were scaled up 5-fold. After separating the free from the bound probe on a native polyacrylamide gel, the gel was exposed to UV light for 1 h. DNA-protein complexes were visualized, excised, equilibrated in SDS sample buffer, and resolved on an SDS-polyacrylamide gel. Molecular mass marker locations are indicated between gel lanes and sizes (kDa) are indicated in the left margins. Specific DNA-protein complexes are indicated by an arrow in the right margins. Uncross-linked probe migrated off the gel. B, Southern blot analysis was performed as described under "Experimental Procedures." GC or HeLa nuclear extracts (100 μg) were resolved on an SDS-polyacrylamide gel and electrottransferred to nitrocellulose. The nitrocellulose was cut into strips, denatured/renatured with guanidine hydrochloride, and probed with end-labeled pCRE, pCREm, dCRE, dCREm, or CREB oligonucleotides. Molecular mass marker sizes (kDa) and locations are indicated in the left margin. Specific DNA-protein complexes are indicated by arrows in the right margins.
surprising that ATF-2 antisera is unable to react with the CGTCA-binding factors, but underscore the specificity of the antisera.

Based on oligonucleotide competition gel shift experiments, Peers et al. (1991) concluded that CREB does not bind the CGTCA motif in the hPRL gene. Liang et al. (1992) concluded that CREB is not responsible for the CAMP regulation of the homologous rPRL promoter, since CREB antibodies did not react with rPRL gel-shifted proteins and co-transfection of a dominant negative inhibitor of CREB had little effect on CAMP-mediated rPRL promoter activity. Using DNase I protection analysis, Keech et al. (1992) demonstrated that affinity purified CREB from GC cells did not interact with the proximal rPRL promoter. Nevertheless, their results suggest that a CREB-related factor might be involved in regulating the hGH promoter CAMP response. Our finding that the hGH gene is transcriptionally up-regulated by CAMP parallels findings with the rGH gene (Barinaga et al., 1983, 1985). This raises the possibility that the rat and human GH genes could be similarly regulated by CAMP. However, the rGH gene does not have CGTCA motifs in its 5′-FL. Interestingly, it does contain two tandemly repeated CGTCA motifs at nt 2255 and 2276 on the antisense strand of the 3′-FL downstream of the polyadenylation signal but these have not been characterized. Based on these similarities it is likely that the rGH and hGH genes may be transcriptionally regulated by CAMP but by different mechanisms. The homologous hCS 5′-FL does contain a CGTCA motif, analogous to the hGH CRE, which represses basal activity but not CAMP-responsiveness on this marginally CAMP responsive promoter (data not shown).

Multiple CGTCA motifs are a common feature of many CAMP-responsive promoters. CGTCA motifs spaced by 10 bp are present in the human vasopressin intestinal polypeptide gene (Fink et al., 1988). Tandem repeats of the palindromic TGAACGTCA sequence with 1-bp intervening DNA are found in the human glycoprotein hormone α gene (Drust et al., 1991). Three CGTCA repeats are present in the HTLV-1 long terminal repeat (Beilming and Moelling, 1992; Zhao and Giam, 1992). Multimers of the rat PRL promoter CGTCA motif support CAMP regulation (Liang et al., 1992). Palindromic TGACGTCA and non-palindromic GTACGTCA sequences with 35-bp intervening DNA are present in the GHFl promoter (McCormick et al., 1993). Three CGTCA sites may allow cooperative interactions between related or unrelated proteins, thereby stabilizing individual CGTCA-binding protein interactions and providing more efficient CAMP responsiveness. The hGH promoter CGTCA motifs are unusual given their relatively long separation. The center-to-center spacing of the CGTCA motifs corresponds to 8.5 turns (88 bp) of B form DNA helix, which places these motifs out of phase with one another. However, the proximal CGTCA motif is on the sense strand and the distal CGTCA motif is on the antisense strand which might consequently place their cognate factors in phase. This would allow interaction of these factors with their respective binding factors, with each other or with the basic transcriptional apparatus, provided that the DNA could bend to allow protein-protein contacts.

Our results overexpressing GHFl indicate that GHFl levels affect the basal Ghp-LUC activity but not its CAMP responsiveness (Fig. 3C). Perhaps GHFl plays a structural role on the hGH promoter and needs merely be present for CAMP inducibility. In fact, Verrijzer et al. (1991) showed that GHFl induces DNA bending when bound to its cognate sequence in vitro, suggesting that GHFl binding might enhance interaction between the distant CGTCA-binding proteins. Further studies will be required to test this model.

The hGH CRU appears to be similar to the GHFl CRU (McCormick et al., 1990), hPRL CRU (Peers et al., 1991) and rPRL CRU (Liang et al., 1992; Keech et al., 1992) which consists of one or more CGTCA motifs and a GHFl binding site. The hGH and hPRL genes are structurally related members of the same gene family (Miller and Eberhardt, 1983), are expressed in pituitary cells, and are dependent on GHFl for their cell-specific expression. The GHFl gene is positively autoregulated by GHFl and requires both CREB and GHFl for CAMP responsiveness (McCormick et al., 1990). Coordinated control by GHRH of GHFl and GH gene expression is therefore possible. Cooperative interactions between GHFl and the factors that recognize the hGH or rPRL CGTCA motifs may account for the cell-specific, CAMP regulation of these promoters. Similar cooperative mechanisms have been proposed for the repression of tyrosine aminotransferase gene expression in non-liver cells by the tissue-specific extinguisher Tse-1 which has an absolute dependence on a CRE for its function (Boshart et al., 1990).

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