Transcription of the Rat Glucagon Gene by the Cyclic AMP Response Element-Binding Protein CREB Is Modulated by Adjacent CREB-Associated Proteins

CHRISTOPHER P. MILLER,* JULIA C. LIN, AND JOEL F. HABENER

Laboratory of Molecular Endocrinology, Massachusetts General Hospital, and Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts 02114

Received 6 May 1993/Returned for modification 7 July 1993/Accepted 23 August 1993

The cyclic AMP (cAMP) response element (CRE) of the rat glucagon gene (Glu-CRE, 5'-TGACGTCA-3') mediates transcriptional responses to 8-bromo-cAMP and protein kinase A (PKA) in a glucagon-producing hamster islet cell line (InR1G9). By several different DNA-protein binding assays, we show that the transcription factor CREB binds to the CRE octamer and that additional nuclear proteins bind to sequences adjacent to the CRE. Mutation of the Glu-CRE octamer attenuates both the binding of CREB and cAMP-dependent PKA-stimulated transcriptional activity in transient transfection experiments but does not affect the binding of adjacent CREB-associated proteins. Progressive deletions and clustered point mutations of the sequences flanking the Glu-CRE identify sequences (5'-TCATT-3') located both 5' and 3' to the core CRE octamer that bind several proteins. Two proteins with molecular masses of 80 and 100 kDa bind to each of the 5' and 3' TCATT sites. Formation of additional protein-DNA complexes containing 45- and 20-kDa proteins depends upon the integrity of both TCATT sequences. Deletion or point mutation of the TCATT motif located on the 3' side of the CRE octamer results in enhanced transcriptional responses to PKA, suggesting that the CREB-associated proteins decrease the ability of CREB to mediate PKA-stimulated transcription. Results from these studies demonstrate that nucleotides flanking the core CRE octamer can influence the activity of the CRE by serving as binding sites for proteins that modulate the function of CREB and suggest a mechanism to explain why some consensus palindromic CREs are less responsive to cAMP stimulation than others.

The second messenger cyclic AMP (cAMP) regulates the transcription of many genes by a mechanism involving the activation of protein kinase A (PKA) and subsequent phosphorylation and activation of nuclear transcription factors such as CREB (12, 13, 15, 24). CREB recognizes and binds to DNA sequences typified by the consensus palindromic cAMP response element (CRE), 5'-TGACGTCA-3'. Many genes whose transcription is regulated by cAMP contain a CRE, or close variants of it, in their 5' flanking regions (5, 17, 18, 27, 31, 33, 40).

Earlier studies demonstrated variations in the relative responsiveness of different CREs to cAMP-induced transcriptional activation (6). The rat somatostatin and human gonadotropin alpha-subunit CREs are highly responsive to 8-bromo-cAMP when inserted into the p α 100CAT reporter and transfected into JEG-3 human choriocarcinoma cells. Conversely, the rat glucagon and bovine parathyroid hormone CREs are much less active (6). These four CREs share the identical 5'-TGACGTCA-3' sequence but differ in the nucleotides flanking this sequence. The observation of different transcriptional activities of these CREs suggested that the bases flanking the core octamer may modulate their transcriptional activity. The ability of CRE-binding proteins to bind DNA has been shown to be regulated by nucleotides flanking the CRE. For example, nucleotides flanking AP-1 sites and CREs have been shown to modulate the stability of Jun- and Fos-DNA complexes (35). Alternatively, bases flanking CREs may bind additional proteins. Overlapping or adjacent DNA regulatory elements in which the DNA-binding or function of one protein is modulated by the

presence of another protein at the adjacent or overlapping site have been described. Examples of these include the overlapping AP-1 and glucocorticoid receptor binding sites in the rat α -fetoprotein gene (43), the adjacent CRE and CCAAT motifs in the human fibronectin promoter (28), the adjacent NFY and C/EBP binding sites in the mouse serum albumin promoter (25), and the overlapping AP-1 and CCAAT motifs and AP-1 site and vitamin D response element in the rat osteocalcin gene (29).

Cell-specific expression of the glucagon gene is dependent upon DNA regulatory elements located at nucleotides -65 to -100, -175 to -195, and -240 to -268 relative to the transcription start site (9, 19, 30). These regulatory elements are termed G1, G2, and G3, respectively. The G2 and G3 elements confer pancreatic islet-specific expression, whereas the G1 element mediates cellular specificity by restricting expression of the glucagon gene to the α cells of the pancreatic islets (30). The CRE of the glucagon gene, located between nucleotides -291 and -298, is necessary for stimulation of transcription by the cAMP-dependent signalling pathway in hamster islet InR1G9 cells (18). The studies reported here examine the proteins that bind to the glucagon gene CRE in InR1G9 cells and describe the roles that they may play in the cAMP-dependent transcriptional regulation of the glucagon gene. Earlier work showed that bacterially-expressed recombinant CREB binds to the glucagon CRE *in vitro* (18). We now show that endogenous CREB present in InR1G9 cells binds to the CRE and mediates cAMP-stimulated transcription. Furthermore, we describe proteins that bind to TCATT sequences adjacent to the core CRE and provide evidence that these proteins modulate the actions of CREB to mediate cAMP-induced transcription.

* Corresponding author.

MATERIALS AND METHODS

Materials. DNA-modifying enzymes were purchased from New England BioLabs (Beverly, Mass.) or Boehringer Mannheim (Indianapolis, Ind.). Radioactive compounds ($[^{14}\text{C}]$ chloramphenicol, $[\alpha\text{-}^{32}\text{P}]\text{dATP}$, and $[\alpha\text{-}^{35}\text{S}]\text{dATP}$) were obtained from DuPont-New England Nuclear (Boston, Mass.). All other reagents were of molecular biology grade and were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Cell culture. Hamster glucagon-producing InR1G9 cells (39) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (GIBCO, Grand Island, N.Y.), 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Nuclear extracts. Cells were grown to approximately 80% confluence in 100- or 150-mm-diameter culture dishes (Falcon Co., Oxnard, Calif.), and nuclear extracts were prepared by the method of Dignam et al. (8). Extracts were stored at -70°C until use. Protein concentrations in the extracts were determined by the Bio-Rad (Richmond, Calif.) Bradford assay using bovine serum albumin (Fraction V; Sigma) as a standard.

DNA-protein-binding assays. Electrophoretic mobility shift assay (EMSA) and Southwestern (DNA-protein) blot procedures were performed as described by Knepel et al. (19). In situ DNA-protein UV cross-linking was performed as described by Molitor et al. (26).

Antisera. The R1090 anti-CREB serum was produced by immunization of rabbits with a synthetic peptide consisting of amino acids 92 to 124 of CREB-327 (15). The antiserum to C/EBP- β (LAP) (4) was raised in rabbits immunized with a synthetic peptide corresponding to amino acids 54 to 70 (34). These antisera were used in EMSA supershift experiments. The R1090 antiserum was also used in Western blot (immunoblot) analysis of InR1G9 nuclear proteins fractionated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and electroblotted onto nitrocellulose membranes (Micron Separations Inc., Westborough, Mass.). CREB was detected with a chemiluminescent detection system (ECL; Amersham, Inc.) using R1090 antiserum (diluted 1:20,000) and an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Bio-Rad) (1:10,000).

Plasmid constructs. For the reporter constructs used in these studies, double-stranded oligonucleotides corresponding to the glucagon CRE region (either wild-type, truncated, or mutated [Table 1]) were inserted into the *Bam*HI site of the plasmid -136GluCAT (18). This plasmid contains nucleotides -136 to +58 of the rat glucagon gene 5' flanking region linked 5' to the coding sequence of the bacterial chloramphenicol acetyltransferase (CAT) gene. The oligonucleotides were inserted in the forward orientation, 5' to the glucagon promoter sequence. The sequences and orientations of the inserted oligonucleotides were confirmed by dideoxy-chain termination sequencing (Sequenase; U.S. Biochemicals). Cloning and isolation of plasmids were performed according to standard procedures (22). The eukaryotic expression vectors for wild-type and mutant catalytic subunits of PKA were obtained from R. Maurer (University of Iowa) (23). The bacterial expression vector for full-length CREB-327 has been described previously (14).

DNA transfections. InR1G9 cells were transfected with 2 μg of reporter DNA and 1 μg of mutant or wild-type PKA expression plasmids (23). Transfections were performed with a modified DEAE-dextran procedure as described by Vallejo et al. (41). Harvesting of cells, preparation of cell

TABLE 1. Coding strand sequences of glucagon CRE oligonucleotides used in these studies^a

Oligonucleotide	Coding sequence
Glu-CRE-27 (WT)	5'-GATCGGGCTCATTGACGTCAAAATTCATTCA-3'
Glu-CRE-24	5'-GATCGGGCTCATTGACGTCAAAATTCATA-3'
Glu-CRE-21	5'-GATCGGGCTCATTGACGTCAAAATTA-3'
Glu-CRE-14	5'-GATCGGGCTCATTGACGTA-3'
Glu-CRE-5'	5'-GATCGGGCTCATTGAA-3'
Glu-CRE-24B	5'-GATCGCATTTGACGTCAAAATTCATTCA-3'
Glu-CRE-21B	5'-GATCCTTGACGTCAAAATTCATTCA-3'
Glu-CRE-16	5'-GATCCACGTCAAAATTCATTCA-3'
Glu-CRE-3'	5'-GATCCTCAAAATTCATTCA-3'
Glu-CRE-17	5'-GATCCCATTTGACGTCAAAATTA-3'
Glu-CRE-27Mut1	5'-GATCGGGCTCATTGACGTCAAAATTCATTCA-3'
Glu-CRE-27Mut2	5'-GATCGGGCTCATTGACGTCAAAATTTGGGTCA-3'
Glu-CRE-27Mut3	5'-GATCGGGCTCATTGACGTCAAAACCTGCCCA-3'
Glu-CRE-27Mut4	5'-GATCGGGCTCTGCGTGACGTCAAAATTCATTCA-3'
Glu-CRE-27Mut3/4	5'-GATCGGGCTCTGCGTGACGTCAAAACCTGCCCA-3'

^a All of the oligonucleotides have 5' GATC overhangs. The wild-type Glu-CRE-27 (WT) oligonucleotide comprises the sequence of the rat glucagon gene from nucleotide -279 to -305 relative to the transcription start site. The mutated bases in Glu-CRE-27Mut1, 2, 3, 4, and 3/4 are underlined.

extracts, and CAT assays were performed as described previously (30). For each transfection, CAT activities were normalized relative to the basal values obtained for the -136GluCAT reporter.

RESULTS

Multiple InR1G9 nuclear protein binding to the Glu-CRE region. EMSAs and Southwestern binding assays were used to examine InR1G9 nuclear proteins that bind to the glucagon CRE and surrounding bases. In initial experiments, two different ^{32}P -labelled double-stranded oligonucleotide probes were compared (Fig. 1). The Glu-CRE-27 oligonucleotide corresponds to nucleotides -305 to -279 relative to the transcription start site of the rat glucagon gene. Glu-CRE-27 contains the consensus palindromic core CRE, flanked by 9 nucleotides 5' and 10 nucleotides 3' to the CRE (Table 1). The shorter Glu-CRE-17 oligonucleotide corresponds to nucleotides -301 to -284 relative to the transcription start site. Incubation of InR1G9 nuclear extracts with the Glu-CRE-27 probe results in the appearance of six distinct protein complexes by EMSA (Fig. 1A). Complexes 1A to 1D (Fig. 1A) appear to migrate as a single protein band when the EMSA gels are run for shorter times but are reproducibly resolved into four bands under the conditions used in these experiments. Incubation of InR1G9 nuclear extracts with the Glu-CRE-17 probe leads to the appearance of two complexes corresponding to bands 1B and 2 (Fig. 1A). The difference in the EMSA patterns of the Glu-CRE-27 and Glu-CRE-17 probes suggests that binding of complexes 1A, 1C, 1D, and 3 is dependent upon the flanking nucleotides present in Glu-CRE-27 but absent in Glu-CRE-17, while binding of complexes 1B and 2 occurs within the central region that is common to both probes.

Since the consensus palindromic CRE within the Glu-CRE-27 probe represents a canonical CREB-binding site, experiments were conducted to determine whether CREB in InR1G9 nuclear extracts binds to this probe. Preincubation of the EMSA binding reaction with CREB antiserum (R1090) causes complex 1B to be selectively supershifted (Fig. 1B, SSB), indicating that CREB is a component of this complex. Neither normal rabbit serum nor a C/EBP- β (LAP) anti-

FIG. 1. Binding of InR1G9 nuclear proteins to ^{32}P -labelled oligonucleotides corresponding to the rat glucagon gene CRE region. (A) EMSA comparing binding to the Glu-CRE-27 and Glu-CRE-17 probes (Table 1). InR1G9 nuclear extracts were incubated with the indicated probes, then DNA-protein complexes were resolved on nondenaturing polyacrylamide gels. (B) Effects of preincubating InR1G9 nuclear extracts with either normal rabbit serum (NRS), R1090 CREB antiserum (anti-CREB), or a LAP (C/EBP- β) antiserum (anti-LAP) prior to EMSA binding reactions. SSB, bands supershifted because of incubation with the CREB antiserum. (C) Southwestern binding assay with InR1G9 nuclear extracts and the Glu-CRE-27 or Glu-CRE-17 probes. The approximate molecular masses of the protein bands are shown on the left. The Glu-CRE-27 probe detected protein bands of 20, 43, 46, and 110 kDa. (The faint 20-kDa band, which is always detected with this probe, is more apparent with longer autoradiography exposures.) The Glu-CRE-17 probe binds only to the 43-kDa CREB band. (D) Western immunoblot analysis of InR1G9 nuclear proteins with the R1090 CREB antiserum. The R1090 antiserum detects the 43-kDa CREB-327 protein in InR1G9 nuclear extracts.

serum (4) used as a negative control produces alterations of the EMSA pattern (Fig. 1B).

Southwestern binding assays and Western immunoblot analysis also showed that CREB in InR1G9 cells binds to the Glu-CRE and that other nuclear proteins bind to the bases surrounding the core CRE (Fig. 1C and D). The Glu-CRE-27 probe binds to four InR1G9 nuclear proteins (110, 46, 43, and 20 kDa) in Southwestern assays (Fig. 1C). The 43-kDa protein is likely to be CREB, as it binds to the Glu-CRE-17 probe (Fig. 1C) and is also recognized by the R1090 CREB antiserum in Western immunoblot analysis (Fig. 1D). Binding of the 110-, 46-, and 20-kDa proteins to the Glu-CRE-27 probe appears to be dependent upon the presence of nucleotides flanking the core CRE, because these proteins are not bound by the shorter Glu-CRE-17 probe. The results from the Southwestern binding assay (Fig. 1C) are consistent with the findings from the EMSA (Fig. 1A) in that for both assays the Glu-CRE-17 probe binds fewer complexes than does Glu-CRE-27. On repeated EMSA, the protein(s) in complex 2 binds Glu-CRE-17 more weakly than Glu-CRE-27, to which complex 2 proteins consistently bind strongly.

Mutations that disrupt CREB binding decrease PKA-stimulated transcription. Experiments were next conducted to determine whether binding of CREB in InR1G9 cells to the Glu-CRE mediates PKA-stimulated transcriptional activity.

This was done by assaying the protein-binding activities and the transcriptional responsiveness of wild-type and mutated Glu-CRE-27 oligonucleotides to the activation of the cAMP signalling pathway in InR1G9 cells. A single base mutation in the core CRE octamer (TGACGTCA to TGAGGTCA [Table 1, Glu-CRE-27Mut1]) eliminates binding to the 43-kDa CREB band in Southwestern assay with InR1G9 nuclear extracts (Fig. 2A). In contrast, binding to the 100- and 20-kDa proteins is unaffected by this mutation. In support of these findings, binding of bacterially expressed CREB to the Glu-CRE-27Mut1 probe is greatly diminished when compared with that of the wild-type probe in EMSA (Fig. 2B).

The wild-type and mutant Glu-CRE-27 oligonucleotides were inserted into the reporter construct -136GluCAT, consisting of the minimal promoter of the rat glucagon gene (contains the α -cell-specific G1 element [30]) fused to the bacterial reporter CAT (Fig. 3A). These constructs were cotransfected into InR1G9 cells with eukaryotic expression vectors encoding either the wild type or an enzymatically inactive mutant catalytic subunit of PKA (23), the latter of which provided a measure of basal transcriptional activity. Forty-eight hours after transfection, cells were harvested for assay of CAT activity. The -136GluCAT reporter itself has a small but reproducible twofold response to overexpression of PKA. In contrast, the reporter bearing the wild-type

FIG. 2. Mutation of the core CRE octamer of Glu-CRE-27, decreasing binding to CREB. (A) Southwestern binding assay with InR1G9 nuclear proteins and probes corresponding to the wild-type (WT) Glu-CRE-27 or Glu-CRE-27Mut1 (Mut1). The approximate molecular masses of the protein bands are shown on the left. The autoradiograms shown are from two different gels run concurrently with the same nuclear extracts. Note that the Glu-CRE-27Mut1 probe does not detect the 43-kDa CREB protein. (B) EMSA with bacterially expressed human CREB-327 (Bact. CREB) and the wild-type Glu-CRE-27 or Glu-CRE-27Mut1 probes.

Glu-CRE-27 gives a 17-fold stimulation (Fig. 3B) in these experiments. Importantly, the Glu-CRE-27Mut1 oligonucleotide has a substantially diminished (threefold) response to cotransfection and expression of PKA when compared with the wild-type Glu-CRE-27. These findings suggest that most, if not all, of the PKA-stimulated transcriptional activity mediated through the CRE is dependent upon the binding of CREB or CREB-related proteins. The little residual PKA-stimulated transcriptional activity that remains after mutation of the core CRE may be mediated by non-CREB DNA-binding proteins that respond only weakly to PKA.

Deletions and mutations of Glu-CRE-27 identifying proteins that bind adjacent to the core CRE. To characterize further the interactions between InR1G9 nuclear proteins and the Glu-CRE-27 sequence, oligonucleotides were designed in which progressive 3' or 5' deletions or clustered point mutations were introduced (Table 1). These oligonucleotides were used either as probes or as competitors in EMSAs with InR1G9 nuclear extracts or bacterially expressed CREB. In the deletion analysis with InR1G9 nuclear extracts, EMSA complexes 1B and 1C are eliminated when the deletions extend into the core CRE octamer from either the 5' or 3' side (probes Glu-CRE-16 and Glu-CRE-14, respectively) (Fig. 4A). Complex 1D is also disrupted by deletions into the 3' end of the core CRE but appears more resistant to alterations of the 5' side of the core CRE. Complex 1D differs slightly from complexes 1B and 1C in that base substitution of the 5' two nucleotides (CCACGTCA) of the core CRE octamer (TGACGTCA) does not decrease binding of complex 1D (probe Glu-CRE-16 [Fig. 4A]). Further deletion of the core CRE from the 5' end (Glu-CRE-3') disrupts complex 1D binding. Tentatively, complex 1D consists of a

FIG. 3. Mutation of the core CRE octamer of Glu-CRE-27 to disrupt CREB binding, decreasing PKA-stimulated transcriptional activity. (A) Schematic representation of the reporter constructs used. The wild-type or mutant Glu-CRE-27 oligonucleotides were inserted into the -136GluCAT reporter construct and then transfected into InR1G9 cells. The arrows indicate the direction of transcription. (B) Relative CAT activities of Glu-CRE-27(WT or Mut1) and -136GluCAT reporter constructs after transfection into InR1G9 cells. Cells were cotransfected with an expression vector for the wild-type catalytic subunit of PKA or with an expression vector for an inactive, mutant catalytic subunit of PKA (BASAL). CAT activities were normalized relative to the basal activity for the -136GluCAT reporter for each experiment. The fold stimulations (PKA versus basal) for each reporter construct are shown in parentheses. Values represent the means from four separate transfections, each done with duplicate culture plates of InR1G9 cells.

CRE-binding protein(s) that recognizes asymmetric CREs such as CCACGTCA or TGACGTGG in the opposite strand. Deletions into the core CRE octamer of Glu-CRE-27 also decrease binding of bacterially expressed CREB (Fig. 4B). EMSA complex 1B most likely consists of the CREB homodimers, because it is perturbed by the CREB antiserum (Fig. 1B), comigrates with bacterially expressed CREB (Fig. 4A, lane 11), and is disrupted by deletions into the core CRE (Fig. 4A). EMSA complexes 1C and 1D represent additional CRE-binding proteins that interact with Glu-CRE-27 at the core CRE. Presumably, these could either be CRE-binding proteins unrelated in structure to CREB or alternately spliced isoforms of CREB or CREM (10) that are not recognized by the R1090 CREB antiserum.

Complex 3 in Fig. 4A is altered when bases are removed from either end of the Glu-CRE-27 oligonucleotide. However, this complex does not bind to probes corresponding to the isolated CRE-flanking sites (Glu-CRE-3' or Glu-CRE-5') or to a probe with sequences removed from both the 3' and 5' ends of Glu-CRE-27 (the Glu-CRE-17 probe in Fig. 1A).

FIG. 4. EMSA with Glu-CRE probes and InR1G9 nuclear extracts or bacterially expressed CREB-327. (A) EMSA with InR1G9 nuclear extracts and progressive 5' and 3' deletions into the Glu-CRE-27 probe. The sequences of the oligonucleotide probes are shown in Table 1. Lanes 1 to 5, progressive deletions into the 3' end of Glu-CRE27; lanes 6 to 10, progressive deletions into the 5' end of Glu-CRE-27; lane 11, bacterially expressed CREB-327 (Bact. CREB) migration when bound to the Glu-CRE-27 probe. N, nonspecific binding. (B) EMSA with bacterially expressed CREB-327 and progressive 5' and 3' deletions into the Glu-CRE-27 probe. The ³²P-labelled oligonucleotide probes are the same as described for panel A. (C) Cross-competition of binding for EMSA complexes 1A and 2 in InR1G9 nuclear extracts with the Glu-CRE-5' and Glu-CRE-3' probes. Lanes 1 to 7, EMSA with a Glu-CRE-5' probe; lanes 8 to 14, EMSA with a Glu-CRE-3' probe. The results are from competition with 30- and 300-fold molar excesses of unlabelled Glu-CRE-5', Glu-CRE-3', and an idealized NF-κB site (17). (D) EMSA with InR1G9 nuclear extracts and the wild-type (WT) Glu-CRE-27 probe. Competition was performed with clustered point mutations within Glu-CRE-27. The sequences of the competitor oligonucleotides are shown in Table 1.

Therefore, the formation of EMSA complex 3 appears to be dependent upon the integrity of sequences located both 5' and 3' to the core CRE for binding to the Glu-CRE-27 probe.

The oligonucleotides containing the 5' and 3' flanks of the core CRE alone (Glu-CRE-5' and Glu-CRE-3') contain no overlapping sequences, yet both probes bind complexes 1A and 2 (Fig. 4A), albeit probe Glu-CRE-16 appears to bind complexes 1A and 2 more avidly than does probe Glu-CRE-14. These observations suggest that the proteins in these complexes bind to sites on both sides of the core CRE and that binding affinity to the 3' site is greater than to the 5' site. The two oligonucleotides each contain a common motif, PuPyTCATTPyPu, and exhibit cross-competition in the gel retardation binding assay (Fig. 4C). On the Glu-CRE-3' probe, Glu-CRE-3' is a more effective competitor than is Glu-CRE-5', suggesting that the affinity of the 3' sequence site is greater than that of the 5' sequence site. However, unlabelled Glu-CRE-5' competes with the labelled oligonucleotide used as a probe more effectively for binding to complex 2 than does unlabelled Glu-CRE-3'. Complex 1A competes only weakly with the Glu-CRE-5' probe in these experiments, suggesting that its interaction with these oligonucleotide probes is of lower affinity than for complex 2. An oligonucleotide used as a negative control (NF- κ B binding site [26]) does not compete with the binding of Glu-CRE-3' or Glu-CRE-5' to complexes 1A or 2. The observation that the Glu-CRE-3' and Glu-CRE-5' oligonucleotides cross-compete with each other for binding to complexes 1A and 2 supports the above proposal that the same or similar proteins can bind to sites on both sides of the core CRE.

Oligonucleotides with nucleotide substitution mutations were used as competitors in EMSAs using InR1G9 nuclear extracts and the wild-type Glu-CRE-27 probe (Fig. 4D). Compared with the wild-type Glu-CRE-27, an oligonucleotide with a C-to-G mutation in the core CRE octamer (Glu-CRE-27Mut1) is a less efficient competitor for binding to complexes 1B to 1D but still effectively competes for binding to complexes 1A, 2, and 3. These results are consistent with data shown in Fig. 2 and 3, in which this same mutant oligonucleotide was shown not to bind to CREB in EMSAs and Southwestern binding assays and has a greatly diminished transcriptional response to PKA stimulation. Block mutation of the CTCATT motif located on the 5' side of the core CRE (Glu-CRE-27Mut4) does not alter competition for binding to any of the EMSA complexes. However, mutation of the TTCATT sequence in the 3' end (Glu-CRE-27Mut3) decreases competition for binding to complexes 1A and 2. Mutation of both of the TCATT motifs (Glu-CRE-27Mut3/4) decreases competition for binding of Glu-CRE-27 to complexes 1A, 2, and 3. In isolation, sequences flanking the CRE on both the 5' and 3' sides appear to bind complexes 1A and 2, but within the context of the Glu-CRE-27 oligonucleotide, the 3' TTCATT sequence appears to be a more effective binding site than the 5' CTCATT sequence.

In situ UV-cross-linking analysis of proteins bound to the Glu-CRE-27 sequence. Additional information about the proteins that bind to the Glu-CRE region was obtained by in situ UV-cross-linking analysis using InR1G9 nuclear extracts and double-stranded oligonucleotides (Glu-CRE-27, Glu-CRE-5', or Glu-CRE-3') that were uniformly substituted with Br-dUTP and labelled with [32 P]dATP. Labelled protein complexes were excised from preparative EMSA gels and were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Complexes 1A and 1B were analyzed together, as were complexes 1C and 1D, whereas complexes 2 and 3

FIG. 5. In situ UV cross-linking of InR1G9 nuclear proteins to a Br-dUTP-substituted Glu-CRE-27 probe. (A) Preparative EMSA gel with InR1G9 nuclear extract and the 32 P-labelled Br-dUTP-substituted Glu-CRE-27 probe. The UV-irradiated wet gel was exposed to autoradiography film overnight at 4°C, and then the complexes were excised and fractionated by SDS-PAGE. Bands 1A and 1B were excised together, as were bands 1C and 1D. Bands 2, 3, and N were excised individually. (B) Autoradiogram of the analytical SDS gel showing the proteins detected in the EMSA complexes shown in panel A. The positions of protein molecular markers are shown along the left margin. On the far right is a shorter exposure of the SDS gel for EMSA complex 2 from a separate experiment with InR1G9 nuclear extract and the Br-dUTP-substituted Glu-CRE-27 probe. This more clearly shows the triplet of bands between 85 and 110 kDa. N, nonspecific binding.

were analyzed independently. The band labelled N, a presumed nonspecific band with regard to binding to the Glu-CRE-27 probe, was also excised and analyzed. EMSA complexes 1A and 1B and 1C and 1D give rise to similar patterns of proteins in the SDS gel with molecular masses of between 40 and 55 kDa (Fig. 5B). The major proteins from complex 2 are 80 to 100 kDa. Shorter exposures of the autoradiograms reveal that complex 2 is resolved into three proteins (Fig. 5B). The complex labelled N did not produce any detectable cross-linked proteins, whereas complex 3 gives rise to proteins of approximately 45 and 20 kDa. To investigate further the characteristics of the proteins that form complexes 1A and 2, in situ UV cross-linking was performed using Br-dUTP-substituted Glu-CRE-5' and Glu-CRE-3' probes. Complexes 1A and 2 for each probe were excised and analyzed independently. EMSA complex 1A does not produce any detectable cross-linked bands in the SDS gel, even after longer autoradiography exposures. However, EMSA complex 2 cross-linked to either the Glu-CRE-5' or Glu-CRE-3' probes gives rise to two proteins that are electrophoretically indistinguishable, migrating at 80 to 90 kDa (Fig. 6B).

The three proteins seen by in situ UV cross-linking of EMSA complex 2 with the Glu-CRE-27 probe have a slower mobility in the SDS gel than does the doublet detected by cross-linking with the Glu-CRE-3' or 5' probes (Fig. 6C).

FIG. 6. In situ UV cross-linking of InR1G9 nuclear proteins to Br-dUTP-substituted Glu-CRE-3' and Glu-CRE-5' probes. (A) Preparative EMSA gel with Br-dUTP-substituted Glu-CRE-3' and Glu-CRE-5' probes. Complexes 1A and 2 were excised individually for each probe. (B) Autoradiogram of the analytical SDS gel showing the proteins detected in the EMSA complexes shown in panel A. The positions of molecular markers are shown along the left margin. (C) Autoradiogram of an SDS gel showing proteins detected in EMSA complex 2 for the Glu-CRE-27, Glu-CRE-5', and Glu-CRE-3' probes.

This circumstance may reflect the different sizes of the probes that are cross-linked to the proteins and/or alterations in the conformations of the proteins induced by binding to the probes. At present, it is not known whether the multiple bands represent distinct proteins or different conformations of the same protein. In any event, the observation that

proteins of indistinguishable electrophoretic mobilities on SDS-PAGE are cross-linked to both the Glu-CRE-5' and the Glu-CRE-3' sequences further supports the proposal that the same proteins can bind to DNA sequences flanking either side of the core CRE in Glu-CRE-27.

Proteins that bind adjacent to the core CRE of Glu-CRE-27 modulate PKA-stimulated transcriptional activity. Deletions and mutations of the bases flanking the core CRE octamer lead to alterations in the proteins that bind to the Glu-CRE probes (Fig. 4A to D). To determine whether these deletions and mutations alter transcriptional responsiveness to cAMP, the truncated or mutated oligonucleotides were ligated into the -136GluCAT reporter construct and cotransfected into InR1G9 cells with the expression vector for either the active wild-type or the inactive mutant catalytic subunit of PKA. In these experiments, the wild-type Glu-CRE-27 oligonucleotide confers a 14-fold PKA-induced transcriptional responsiveness to the -136GluCAT reporter (Fig. 7B). Deletions of 3 and 6 nucleotides from the 3' end of Glu-CRE-27 to create Glu-CRE-24 and Glu-CRE-21, respectively, result in 1.5- and 4.5-fold increases in PKA-stimulated transcriptional activities. The actual increases in stimulation (PKA versus basal) for these reporter plasmids are 28- and 48-fold, respectively. The deletion of 6 nucleotides removes most of the TTCATT motif located 3' to the CRE octamer. Deletion of 3 nucleotides from the 5' end of Glu-CRE-27 (to create Glu-CRE-24B) results in a twofold enhancement of PKA-stimulated CAT activity. Because of decreased basal activity for Glu-CRE-24B, PKA stimulation causes a 63-fold increase in CAT activity. Removal of an additional 3 bases (Glu-CRE-21B) leads to a 50% suppression of PKA-stimulated CAT activity but no change in the fold response relative to Glu-CRE-27. The Glu-CRE-17 reporter construct appears to have a similar response to that of PKA, when compared with Glu-CRE-27. However, the twofold lower basal activity of the Glu-CRE-17 results in a twofold enhancement relative to Glu-CRE-27 of CAT activity by cotransfection and expression of the catalytic subunit of PKA.

Clustered point mutations of the bases flanking the core CRE of Glu-CRE-27 also cause alterations in PKA-responsive transcription. Mutations of the 3' flanking sequences have a greater effect on transcription than do mutations of the 5' flanking region (Fig. 7C). Mutations of the TTCATT motif on the 3' side of the core CRE (Glu-CRE-27Mut2 and Mut3) result in 2.5-fold increases in PKA-stimulated transcriptional activity. These mutations also cause increases in the basal activities of the reporter constructs so that the fold stimulation by PKA is similar to that observed for the Glu-CRE-27WT reporter. Mutation of the CTCATT sequence on the 5' side of the CRE octamer (Glu-CRE-27Mut4) does not substantially alter CAT activity. When both 5' and 3' sequences are mutated (Glu-CRE-27Mut3/4), the activity of the reporter construct is the same as that observed when only the 3' TCATT motif is mutated. Both deletion and mutation of the TCATT sequence located 3' to the core CRE lead to increases in PKA-stimulated CAT activity, although the 3' deletions appear to cause greater increases than the 3' mutation. Deletions into the 5' flanking sequences first decrease and then slightly increase transcription, while mutations of this region are without demonstrable effect. Results from these transient transfection experiments are most consistent with a model in which one of the proteins responsible for complexes 1A or 2 (that bind[s] Glu-CRE-27WT and Glu-CRE-27Mut4 but not Glu-CRE-27Mut3) represses both basal and PKA-activated transcription in InR1G9 cells.

FIG. 7. Deletions or mutations of nucleotides flanking the core CRE of Glu-CRE-27 modulate responsiveness to PKA. (A) Schematic representation of deletions and mutations of nucleotides flanking the core CRE of Glu-CRE-27. (B) Relative CAT activities of -136GluCAT reporter constructs bearing deletion mutant versions of Glu-CRE-27 after transfection into InR1G9 cells. (C) Relative CAT activities of -136GluCAT reporter constructs bearing clustered point mutations within the Glu-CRE-27 oligonucleotide. Cells were cotransfected with an expression vector for the wild-type (WT) catalytic subunit of PKA or with an expression vector for an inactive, mutant catalytic subunit of PKA (BASAL). CAT activities were normalized relative to the basal activity for the -136GluCAT

DISCUSSION

The results from these experiments help to explain why there are differences in cAMP responsiveness among consensus palindromic CREs. Deutsch et al. (6) noted that the rat glucagon CRE was relatively unresponsive to cAMP stimulation when compared with several other consensus CREs and proposed that the sequences flanking the core CRE octamer were responsible for the weak cAMP responses observed with reporter constructs containing the glucagon CRE. We show here that nucleotides flanking the core octamer of the glucagon CRE bind CREB-associated proteins that inhibit CREB-mediated cAMP-stimulated transcription. Attenuation of CREB activity by CREB-associated inhibitory proteins could be one mechanism to confer gene-specific differences in transcriptional responsiveness to cAMP. Genes that contain consensus CREs with adjacent binding sites for inhibitory proteins would be predicted to be less responsive to cAMP than genes containing consensus CREs without adjacent inhibitory binding sites. The findings of potential inhibitory sequence motifs and DNA-binding proteins in the glucagon gene promoter are consistent with the known physiological regulation of glucagon secretion and production. Unlike the insulin gene, which responds by 5- to 10-fold increases in expression in response to feeding versus fasting states, the glucagon gene is regulated by only 1.2- to 0.8-fold during fasting versus feeding. Thus, the relative excursions of glucagon gene expression in response to glucose and cAMP signals generated by nutrients are dampened compared with the excursions of insulin gene expression. The CREB-associated proteins that inhibit cAMP-stimulated expression of the glucagon gene reported in our studies may be wholly or partially responsible for this dampening effect.

CREB-associated protein binding to sequences adjacent to the core CRE of Glu-CRE-27. Results from gel retardation and Southwestern binding assays indicate that nucleotides flanking the core CRE octamer are important for binding of CREB-associated proteins. In both assays, the shorter Glu-CRE-17 probe binds fewer protein complexes than the longer Glu-CRE-27 probe, and in EMSAs the flanking sequences alone bind at least two complexes. By in situ UV cross-linking of EMSA gels, several proteins that have apparent molecular weights similar to those detected by Southwestern blot are detected. Both assays detect bands of 20, 43, and 46 kDa that may represent the same InR1G9 nuclear proteins. However, several proteins between 50 and 55 kDa are detected by UV cross-linking but not by Southwestern blot. Furthermore, higher-molecular-weight proteins are detected by both assays but differ in apparent molecular masses (110 kDa by Southwestern and 80 to 100 kDa by UV cross-linking) and probably represent different proteins. The Southwestern blot detects proteins bound to DNA as monomers or homodimers and whose binding activities are not destroyed by denaturation and renaturation, whereas in situ UV cross-linking detects proteins bound to DNA under non-denaturing conditions in EMSA. The two assays are not necessarily expected to yield identical results with regard to interpretations of apparent estimated molecular weights.

reporter for each experiment. The fold stimulations (PKA versus Basal) for each reporter construct are shown in parentheses. Values are the means from three (B) or four (C) separate transfections with duplicate culture plates of InR1G9 cells.

FIG. 8. Hypothetical model for binding of InR1G9 nuclear proteins to the Glu-CRE region. This scheme is based upon the results from EMSA, Southwestern and in situ UV-cross-linking analyses presented in Fig. 1 through 6. The numbered proteins correspond to the EMSA complexes in Fig. 1 through 4 and are described in the text. CAP, CREB-associated protein.

In EMSA experiments, complexes 1B, 1C, and 1D bind to the core CRE of Glu-CRE-27 because mutations or deletions of the CRE octamer disrupt the formation of these complexes and decrease competition for binding (Fig. 4A and D). Complexes 1A and 1B but not 1D are disrupted by base substitution of 2 nucleotides from the 5' end of the core CRE octamer. This base substitution still leaves an intact CRE half-site. Formation of complex 1D is prevented by deletion of an additional 3 nucleotides from the 5' end of the core CRE, which destroys the remaining CRE half-site. The differential sensitivities to substitution and deletion within the CRE octamer suggest that proteins in complex 1D may bind to the CRE differently from those in complexes 1B and 1C. The 2-base substitution in the 5' end of the CRE creates a motif, CCACGTCA, that may bind ATF-CREB-like proteins represented by those in complex 1D but not in complexes 1B and 1C. Complex 1B appears to be CREB or CREM, because it is supershifted by CREB antiserum (Fig. 1B) that also recognizes CREM (41a) and comigrates with bacterially expressed CREB (Fig. 4A). In the deletion analyses, complexes 1A and 2 bind to nonoverlapping oligonucleotides containing sequences from each side of the core CRE (Fig. 4A and C). However, mutations of the TCATT sequence 3' but not 5' to the core CRE decrease competition for complexes 1A and 2. Thus, within the context of the Glu-CRE-27 oligonucleotide, the TCATT sequence 3' to the CRE octamer is considered to be more important for binding proteins in complexes 1A and 2 than the TCATT sequence 5' to the CRE although both 5' and 3' TCATT sequences bind complexes 1A and 2 (Fig. 4A, C, and D), albeit with different effectiveness. Formation of complex 3 is perturbed by deletions into either end of Glu-CRE-27 and is not observed with the Glu-CRE-17, Glu-CRE-5', or Glu-CRE-3' probe (Fig. 1A and 4A and C), suggesting that sequences on both sides of the core CRE are required for binding. Also, since an oligonucleotide with the core CRE mutated (Glu-CRE-27Mut1) still effectively competes with complex 3 (Fig. 4D), the intact CRE octamer appears not to be required for binding to complex 3 proteins.

CREB-associated protein modulation of CREB transcriptional transactivation activity. We show here that in InR1G9 islet cells CREB binds to and transactivates the Glu-CRE and that adjacent CREB-associated proteins modulate the transcriptional transactivation activity of CREB. Because

PKA-stimulated CAT activities increase as deletions or block mutations are made within the 3' end of Glu-CRE-27 (Fig. 7B and C), we hypothesize that proteins binding to this region attenuate transcriptional responses mediated by CREB through the CRE. As for the EMSA mutation studies (Fig. 4D), alterations of nucleotides 5' to the CRE octamer appear not to have as great an effect on transcriptional activation by PKA as do alterations of sequences 3' to the CRE (Fig. 7B and C). Differences in transcriptional activities of the truncated Glu-CRE oligonucleotides are not likely to be due to differences in the affinities of CREB binding, since InR1G9 CREB (EMSA complex 1B in Fig. 4A) and bacterially expressed CREB (Fig. 4B) bind equivalently to CREs with different activities (i.e., Glu-CRE-24B and Glu-CRE-21B).

The mechanism by which these proteins interact with CREB remains to be elucidated. A hypothetical model depicting how the various protein complexes might interact with the Glu-CRE-27 element is given in Fig. 8. EMSA complexes 1C and 1D bind to the same region of the Glu-CRE-27 probe as does CREB and might be expected to compete with CREB for binding to the core CRE octamer. Alternatively, proteins in complexes 1C and 1D may contribute to PKA-stimulated transcriptional activity. EMSA complexes 1A and 2 bind to sites on both sides of the core CRE octamer. Since EMSA complex 2 contains a relatively large protein, there could be steric hindrance of CREB binding by proteins in complex 2. However, some of the deletion mutant Glu-CRE oligonucleotides bind to CREB and to EMSA complex 2 and exhibit different transcriptional activities, suggesting that simple competition for binding sites is not the only mechanism by which CREB and the proteins present in EMSA complex 2 interact. Because EMSA complex 3 requires sequences on both sides of the core CRE for binding, it may also impede the binding of CREB to the CRE.

Modulation of the transcriptional activity of CREB may involve direct protein-protein interactions between the CREB-associated proteins and the transactivation domain of CREB. Several domains within the amino-terminal region of CREB, including the kinase-inducible domain and an adjacent glutamine-rich region, have been implicated in transactivation (11). Phosphorylation by PKA of Ser-133 within the kinase-inducible domain and integrity of the glutamine-rich domain appear to be required for transcriptional activity (11). It is possible that interactions of CREB-associated proteins with the kinase-inducible domain or the glutamine-rich region modulate the interaction of the transactivation domain with protein kinases or phosphatases or with components of the basal transcriptional apparatus.

Transcriptional activities of other CREs are influenced by adjacent DNA sequences that may serve as binding sites for CREB-modulatory proteins. Mutations of sequences adjacent to the CREs of the human glycoprotein hormone alpha-subunit gene (2, 3) and the rat somatostatin gene (32) suppress cell-specific transcriptional enhancer activities of these CREs, whereas transcriptional activity of a CRE-like sequence in the promoter of the rat thyrotropin receptor gene is enhanced by mutations of nucleotides located adjacent to the CRE-like element (16). For these examples, the effects of mutations of flanking nucleotides were examined in relation to basal promoter activities but not for activation of cAMP signalling pathways. Our studies indicate that basal and cAMP-stimulated transcriptional activities of the glucagon CRE are suppressed by adjacent inhibitory DNA-binding proteins.

cAMP induction of the phosphoenolpyruvate carboxykinase and glycoprotein alpha-subunit gene promoters has been shown to be inhibited by glucocorticoid receptors, which bind sequences adjacent to or overlapping the CREs of these two genes (1, 37). Glucocorticoid receptors also suppress 12-*O*-tetradecanoylphorbol-13-acetate-inducible expression of collagenase and proliferin promoter reporter constructs by mechanisms involving either direct protein-protein interactions between glucocorticoid receptor and Jun-Fos proteins bound to the simple DNA response element of the collagenase gene, so called tethering, or by cobinding of certain Jun-Fos proteins and glucocorticoid receptor to composite DNA-response elements of the proliferin gene (7, 20, 42). Neither of these two mechanisms involves competitive displacement of DNA-binding proteins. Interactions of the Jun-Fos proteins and glucocorticoid receptor in the composite element can either activate or suppress transcription, depending on the particular isoforms of Jun-Fos that are involved (7). It is tempting to speculate that the Glu-CRE-27 is such a composite element and that in the glucagon-producing islet cell line, the CREB-associated proteins described in this report suppress transcriptional activation mediated by CREB and/or CREB-like proteins through protein-protein interactions similar to those of Jun-Fos and the glucocorticoid receptor. The possibility that glucocorticoid receptors or related proteins may inhibit CREB activity at the glucagon CRE is currently under investigation.

The TCATT motif. The sequence 5'-TCATT-3' was shown by deletions and block mutations to be important for binding of CREB-associated proteins to the Glu-CRE-27 probe. Although present on both sides of the CRE octamer of Glu-CRE-27, the TCATT sequence located 3' to the core CRE appears to be more important than the TCATT located 5' to the CRE. The TCATT motif is repeated eight times within the -100 to -550 portion of the rat glucagon promoter, including the two copies adjacent to the CRE, and one copy in the α -cell-specific enhancer element G1 (30). The TCATT motif is also found within the B-element of the rat elastase I promoter (21, 38), and is repeated 14 times within 315 nucleotides upstream of the transcription start site of the rat nerve growth factor-inducible gene *VGF* (36), including one copy positioned immediately adjacent to a consensus CRE with exactly the same spacing as for the TCATT sequence located 3' to the Glu-CRE. It will be of interest to determine whether some of these other TCATT motifs also bind to functionally important proteins, especially the 80- to 110-kDa protein complex identified in our studies.

ACKNOWLEDGMENTS

We are grateful to M. Vallejo and W. Walker for helpful discussions and to M. LeSieur for expert experimental assistance.

These studies were supported in part by USPHS grant DK30834. J.F.H. is an investigator for the Howard Hughes Medical Institute.

REFERENCES

- Akerblom, I. E., E. P. Slater, M. Beato, J. D. Baxter, and P. L. Mellon. 1988. Negative regulation by glucocorticoids through interference with a cAMP responsive enhancer. *Science* **241**: 350-357.
- Andersen, B., G. Catignani-Kennedy, and J. H. Nilson. 1990. A *cis*-acting element located between the cAMP response elements and CCAAT box augments cell-specific expression of the glycoprotein hormone α subunit gene. *J. Biol. Chem.* **265**: 21874-21880.
- Catignani-Kennedy, G., B. Andersen, and J. H. Nilson. 1990. The human α subunit glycoprotein hormone gene utilizes a unique CCAAT binding factor. *J. Biol. Chem.* **265**:6279-6285.
- Descombes, P., M. Chojkier, S. Lichtsteiner, E. Falvey, and U. Schibler. 1990. LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. *Genes Dev.* **4**:1541-1551.
- Deutsch, P. J., J. P. Hoeffler, J. L. Jameson, and J. F. Habener. 1988. Cyclic AMP and phorbol ester-stimulated transcription mediated by similar DNA elements that bind distinct proteins. *Proc. Natl. Acad. Sci. USA* **85**:7922-7926.
- Deutsch, P. J., J. P. Hoeffler, J. L. Jameson, J. C. Lin, and J. F. Habener. 1988. Structural determinants for transcriptional activation by cAMP-responsive DNA elements. *J. Biol. Chem.* **263**:18466-18472.
- Diamond, M. I., J. N. Miner, S. K. Yoshinaga, and K. R. Yamamoto. 1990. Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. *Science* **249**:1266-1272.
- Dignam, J. D., R. M. Lebovitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**:1475-1489.
- Drucker, D. J., J. Philippe, L. Jepeal, and J. F. Habener. 1987. Glucagon gene 5'-flanking sequences promote islet cell-specific gene transcription. *J. Biol. Chem.* **262**:15659-15665.
- Foulkes, N. S., E. Borrelli, and P. Sassone-Corsi. 1991. CREM gene: use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription. *Cell* **64**:739-749.
- Gonzalez, G. A., P. Menzel, J. Leonard, W. H. Fischer, and M. R. Montminy. 1991. Characterization of motifs which are critical for activity of the cyclic AMP-responsive transcription factor CREB. *Mol. Cell. Biol.* **11**:1306-1312.
- Gonzalez, G. A., and M. R. Montminy. 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59**:675-680.
- Habener, J. F. 1990. Cyclic AMP response element binding proteins: a cornucopia of transcription factors. *Mol. Endocrinol.* **4**:1087-1094.
- Hoeffler, J. P., J. W. Lustbader, and C. Y. Chen. 1991. Identification of multiple nuclear factors that interact with cyclic adenosine 3',5'-monophosphate response element-binding protein and activating transcription factor-2 by protein-protein interactions. *Mol. Endocrinol.* **5**:256-266.
- Hoeffler, J. P., T. E. Meyer, Y. Yun, J. L. Jameson, and J. F. Habener. 1988. Cyclic AMP-responsive DNA-binding protein: structure based on a cloned placental cDNA. *Science* **242**:1430-1433.
- Ikuyama, S., H. Shimura, J. P. Hoeffler, and L. D. Kohn. 1992. Role of cyclic adenosine 3',5'-monophosphate response element in efficient expression of the rat thyrotropin receptor promoter. *Mol. Endocrinol.* **6**:1701-1715.
- Jameson, J. L., P. J. Deutsch, G. D. Gallagher, R. C. Jaffe, and J. F. Habener. 1987. *trans*-acting factors interact with a cyclic AMP response element to modulate expression of the human gonadotropin α gene. *Mol. Cell. Biol.* **7**:3032-3040.
- Knepel, W., J. Chafitz, and J. F. Habener. 1990. Transcriptional activation of the rat glucagon gene by the cyclic AMP-responsive element in pancreatic islet cells. *Mol. Cell. Biol.* **10**:6799-6804.
- Knepel, W., L. Jepeal, and J. F. Habener. 1990. A pancreatic islet cell-specific enhancer-like element in the glucagon gene contains two domains binding distinct cellular proteins. *J. Biol. Chem.* **265**:8725-8735.
- Konig, H., H. Ponta, H. J. Rahmsdorf, and P. Herrlich. 1992. Interference between pathway-specific transcription factors: glucocorticoids antagonize phorbol ester-induced AP-1 activity without altering AP-1 site occupation *in vivo*. *EMBO J.* **11**:2241-2246.
- Kruse, F., S. D. Rose, G. H. Swift, R. E. Hammer, and R. J. MacDonald. 1993. An endocrine-specific element is an integral component of an exocrine-specific pancreatic enhancer. *Genes Dev.* **7**:774-786.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular

- cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. Maurer, R. A. 1989. Both isoforms of the cAMP-dependent protein kinase catalytic subunit can activate transcription of the prolactin gene. *J. Biol. Chem.* **264**:6870–6873.
 24. Meyer, T. E., and J. F. Habener. 1993. Cyclic AMP response element binding protein (CREB) and related transcription activating DNA binding proteins. *Endocr. Rev.* **14**:269–290.
 25. Milos, P. M., and K. S. Zaret. 1992. A ubiquitous factor is required for C/EBP-related proteins to form stable transcription complexes on an albumin promoter segment *in vitro*. *Genes Dev.* **6**:991–1004.
 26. Molitor, J. A., W. H. Walker, S. Doerre, D. W. Ballard, and W. C. Greene. 1990. NF- κ B: a family of inducible and differentially expressed enhancer-binding proteins in human T cells. *Proc. Natl. Acad. Sci. USA* **87**:10028–10032.
 27. Montminy, M. R., K. A. Sevarino, J. A. Wagner, G. Mandel, and R. H. Goodman. 1986. Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proc. Natl. Acad. Sci. USA* **83**:6682–6686.
 28. Muro, A. F., V. A. Bernath, and A. R. Kornblihtt. 1992. Interaction of the -170 cyclic AMP response element with the adjacent CCAAT box in the human fibronectin gene promoter. *J. Biol. Chem.* **267**:12767–12774.
 29. Owen, T. A., R. Bortell, S. A. Yocum, S. L. Smock, M. Zhang, C. Abate, V. Shalhoub, N. Aronin, K. L. Wright, A. J. Van Wijnen, J. L. Stein, T. Curran, J. B. Lian, and G. S. Stein. 1990. Coordinate occupancy of AP-1 sites in the vitamin D-responsive and CCAAT box elements by Fos-Jun in the osteocalcin gene: model for phenotype suppression of transcription. *Proc. Natl. Acad. Sci. USA* **87**:9990–9994.
 30. Philippe, J., D. J. Drucker, W. Knepel, L. Jepeal, Z. Misulovin, and J. F. Habener. 1988. Alpha-cell-specific expression of the glucagon gene is conferred to the glucagon promoter element by the interactions of DNA-binding proteins. *Mol. Cell. Biol.* **8**:4877–4888.
 31. Philippe, J., and M. Missotten. 1990. Functional characterization of a cAMP-responsive element of the rat insulin I gene. *J. Biol. Chem.* **265**:1465–1469.
 32. Powers, A. C., F. Tedeschi, K. E. Wright, J. S. Chan, and J. F. Habener. 1989. Somatostatin gene expression in pancreatic islet cells is directed by cell-specific DNA control elements and DNA-binding proteins. *J. Biol. Chem.* **264**:10048–10056.
 33. Roesler, W. J., G. R. Vandebark, and R. W. Hanson. 1988. Cyclic AMP and the induction of eukaryotic gene expression. *J. Biol. Chem.* **263**:9063–9066.
 34. Ron, D., A. R. Brasier, R. E. McGehee, and J. F. Habener. 1992. Tumor necrosis factor-induced reversal of adipocytic phenotype of 3T3-L1 cells is preceded by a loss of nuclear CCAAT/enhancer binding protein (C/EBP). *J. Clin. Invest.* **89**:223–233.
 35. Ryseck, R.-P., and R. Bravo. 1991. c-JUN, JUN B, and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins. *Oncogene* **6**:533–542.
 36. Salton, S. R. J., D. J. Fischberg, and K.-W. Dong. 1991. Structure of the gene encoding VGF, a nervous system-specific mRNA that is rapidly and selectively induced by nerve growth factor in PC12 cells. *Mol. Cell. Biol.* **11**:2335–2349.
 37. Short, J. M., A. Wynshaw-Boris, H. P. Short, and R. W. Hanson. 1986. Characterization of the phosphoenolpyruvate carboxykinase (GTP) promoter-regulatory region. II. Identification of cAMP and glucocorticoid regulatory domains. *J. Biol. Chem.* **261**:9721–9726.
 38. Swift, G. H., F. Kruse, R. J. MacDonald, and R. E. Hammer. 1989. Differential requirements for cell-specific elastase I enhancer domains in transfected cells and transgenic mice. *Genes Dev.* **3**:687–696.
 39. Takaki, R., J. Ono, M. Nakamura, Y. Yokogawa, S. Kumaie, T. Hiraoka, K. Yamaguchi, K. Hamaguchi, and S. Uchida. 1986. Isolation of glucagon-secreting cell lines by cloning insulinoma cells. *In Vitro Cell. Dev. Biol.* **22**:120–126.
 40. Tsukada, T., J. S. Fink, G. Mandel, and R. H. Goodman. 1987. Identification of a region in the human vasoactive intestinal polypeptide gene responsible for regulation by cyclic AMP. *J. Biol. Chem.* **262**:8743–8747.
 41. Vallejo, M., C. P. Miller, and J. F. Habener. 1992. Somatostatin gene transcription regulated by a bipartite pancreatic islet D-cell-specific enhancer coupled synergetically to a cAMP response element. *J. Biol. Chem.* **267**:12868–12875.
 - 41a. Walker, W. Unpublished data.
 42. Yang-Yen, H.-F., J.-C. Chambard, Y.-L. Sun, T. J. Schmidt, J. Drouin, and M. Karin. 1990. Transcriptional interference between c-jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. *Cell* **62**:1205–1215.
 43. Zhang, X.-K., J.-M. Dong, and J.-F. Chiu. 1991. Regulation of α -fetoprotein gene expression by antagonism between AP-1 and the glucocorticoid receptor at their overlapping binding site. *J. Biol. Chem.* **266**:8248–8254.