MOLECULAR AND CELLULAR BIOLOGY, Dec. 1990, p. 6799–6804
0270-7306/90/126799-06$02.00/0

Vol. 10, No. 12

Transcriptional Activation of the Rat Glucagon Gene by the Cyclic AMP-Responsive Element in Pancreatic Islet Cells

WILLHART KNEPEL,* JAMES CHAFITZ, AND JOEL F. HABENER

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

Received 5 June 1990/Accepted 30 August 1990

The 5′-flanking region of the rat glucagon gene contains, from nucleotides −291 to −298, a sequence (TGA CGTCA) which mediates cyclic AMP (cAMP) responsiveness in several genes (cAMP-responsive element [CRE]). However, because of nonpermissive bases surrounding the CRE octamer, the glucagon CRE does not confer cAMP responsiveness to an inert heterologous promoter in placental JEG cells that do not express the glucagon gene. This report describes transient transfection experiments with glucagon-reporter fusion genes that show that glucagon gene expression is activated by cAMP-dependent protein kinase A in a glucagon-expressing pancreatic islet cell line. This activation is mediated through the glucagon CRE.

Cyclic AMP (cAMP) is the second messenger of one of the major signal transduction pathways and induces the transcription of various eucaryotic genes through *trans*-acting factors binding to the control region of those genes (20, 25). The base sequence TGACGTCA (the cAMP-responsive element [CRE]) has been identified in the control regions of some of those genes and is essential for cAMP regulation of transcription of those genes (20, 25). However, the functional activity of the CRE depends on the bases surrounding the CRE octamer motif, some being permissive and others being less so or even nonpermissive (4). The multiplicity of CRE contextual sequences and the multiplicity of CRE-binding proteins which have already been cloned (1, 9, 10, 14 18) generate a diversity which has the potential of establishing differential regulation of genes that contain CREs.

Three control elements (G1, G2, and G3) which confer pancreatic A cell-specific expression to the rat glucagon gene have been characterized (17, 22). In addition, the 5′-flanking region of the rat glucagon gene contains, from nucleotides (nt) −291 to −298, the perfect CRE octamer sequence, TGACGTCA (22). However, cAMP induction of glucagon gene transcription has not been demonstrated. Furthermore, the glucagon CRE is unable to confer cAMP responsiveness to an inert heterologous promoter in placental JEG cells, which do not express the glucagon gene (4). This suggests that because of nonpermissive bases surrounding the core CRE octamer, the glucagon CRE might be functionally inactive. This study addressed the question of whether glucagon gene transcription can be activated by cAMP-dependent protein kinase A in a glucagon-expressing pancreatic islet cell line.

Activation of glucagon gene transcription by the cAMP-dependent protein kinase A. A total of 350 bp of the 5′-flanking region of the rat glucagon gene was fused to the chloramphenicol acetyltransferase (CAT) reporter gene (−350CAT [22]) and transiently transfected into the glucagon-expressing pancreatic islet cell line InR1-G9 (27) as described previously (22), except that pRSV-luciferase (1 μg) (2, 5) was used to check for transfection efficiency. Cotransfection (2 μg per dish, if not indicated otherwise) of an expression vector coding for the catalytic subunit of the cAMP-dependent protein kinase A (19) enhanced CAT activity about 3.5-fold (Fig. 1A). By contrast, cotransfection of an expression vector encoding an inactive catalytic subunit of cAMP-dependent protein kinase A (single-base mutant [19]) did not enhance but somewhat decreased glucagon gene transcription (Fig. 1A), suggesting that the protein kinase A-induced increase in CAT activity is specific and related to protein kinase A activity. The protein kinase A-induced increase in CAT activity was correlated with an increase in correctly initiated transcripts, as shown by primer extension analysis (Fig. 1B), performed as described previously (21).

In contrast to protein kinase A, the cAMP analog 8-Br-cAMP (1 mM) caused only a slight (about 1.5-fold) enhancement of CAT activity in InR1-G9 cells (Fig. 1A). Previous studies with InR1-G9 cells suggested a functional defect in the cAMP-induced signal transduction pathway (7, 16, 24), which may be bypassed by transfecting an expression vector coding for the catalytic subunit of the cAMP-dependent protein kinase. We therefore tested for cAMP regulation of glucagon gene transcription by using another pancreatic islet cell line, HIT-T15, which is well known to be cAMP responsive (11, 26). HIT cells are a predominantly insulin-producing cell line, and the basal expression of an insulin-CAT fusion gene, InsCAT (22), is about 50-fold higher than that of the glucagon-CAT fusion gene, −350CAT (Fig. 1C). 8-Br-cAMP caused about a fivefold increase in −350CAT expression (Fig. 1C), indicating that rat glucagon gene transcription is regulated by cAMP in a cAMP-responsive pancreatic islet cell line. The cAMP levels in pancreatic A cells are regulated (15, 23). Thus, the induction of glucagon gene transcription by cAMP-dependent protein kinase A offers a means by which the biosynthesis of glucagon in pancreatic A cells could be adjusted to changing demands during the regulation of glucose homeostasis. Glucagon gene transcription may also be induced by cAMP in intestinal L cells (3, 6).

Functional characterization of the protein kinase A-responsive element. To localize the *cis*-acting DNA sequences of the rat glucagon gene involved in transcriptional activation by cAMP-dependent protein kinase A, either the 5′-flanking region of the rat glucagon gene was deleted from its 5′ end up to nt −292 (−292CAT [22]), or 4 bases in the CRE octamer motif (from nt −296 to −293) were selectively deleted with the restriction enzyme *Aat*II and T4 DNA polymerase (construct −350[−297/−292]CAT). Basal reporter activity was

* Corresponding author.

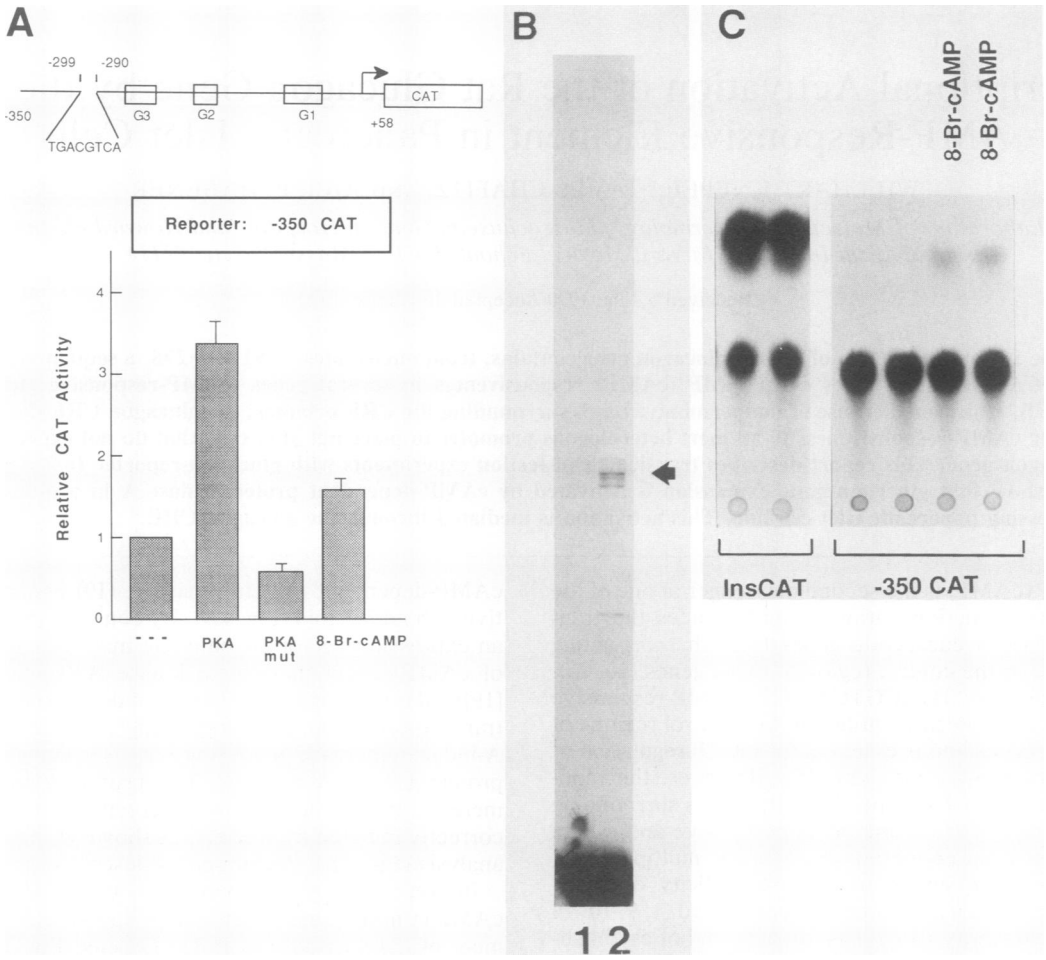


FIG. 1. Induction of rat glucagon gene transcription by the cAMP-dependent protein kinase. (A) The plasmid −350CAT was transiently transfected into InR1-G9 cells. PKA, Cotransfection with an expression vector containing the coding sequence of the catalytic subunit of the cAMP-dependent protein kinase; PKA mut, cotransfection with an expression vector encoding an inactive catalytic subunit mutant (single-base mutant); and 8-Br-cAMP, cAMP analog (1 mM) added to the medium for 24 h. CAT activities are expressed relative to the mean value in each experiment of the activity observed with the CAT reporter plasmid without cotransfection or treatment (---). Values are means (plus the standard error of the mean) of seven independent experiments, each done in duplicate. (B) Primer extension analysis. CAT mRNA transcripts from InR1-G9 cells transfected with −350CAT and either with (lane 2) or without (lane 1) cotransfection of an expression vector coding for the catalytic subunit of protein kinase A were analyzed. The arrow points to extension products expected for correctly initiated transcripts. (C) The plasmids InsCAT and −350CAT were transiently transfected into the cAMP-responsive pancreatic cell line HIT. Lanes 8-Br-cAMP, cAMP analog (1 mM) added to the medium for 24 h. The autoradiogram (CAT assay) of an experiment which is representative of four independent experiments is shown.

not changed by these deletions (Fig. 2A). However, the protein kinase A-induced increase in glucagon gene transcription was abolished by the 5′ deletion and markedly decreased by the 4-base deletion in the CRE octamer motif (Fig. 2A), suggesting that the glucagon CRE is a *cis*-acting DNA control element involved in the effect of protein kinase A on glucagon gene transcription. The remaining catalytic subunit-induced activation of the −350(−297/−292)CAT construct may be explained by either residual activity of the mutated glucagon CRE or an additional responsive element between nt −297 and −350.

To test whether the identified element is capable of conferring protein kinase A inducibility, the synthetic oligonucleotide Glu-CRE-27 containing the rat glucagon CRE (from nt −280 to −306) with 5′ GATC overhangs was cloned into the *Bam*HI site of −136CAT (22). The promoter alone was not responsive to the catalytic subunit, whereas the plasmid with the glucagon CRE cassette in front of the promoter responded with about a fivefold enhancement of CAT activity to cotransfection of the cayalytic subunit (Fig. 2B). Cotransfection of the catalytic subunit mutant did not stimulate CAT activity (data not shown). The ability of the oligonucleotide to confer protein kinase A responsiveness depended on the presence of the core CRE octamer motif, since after a single-base deletion (converting the core CRE octamer, TGACGTCA, into the heptamer TGAGTCA without altering the 19 surrounding bases), no induction by the catalytic subunit was found (not shown). Thus, by 5′ deletion, internal deletion, and oligonucleotide cassette insertion, this study shows that the glucagon CRE confers protein kinase A responsiveness to rat glucagon gene transcription. Remarkable is its distant location. The characterized CREs usually lie within the first 150 bp of the 5′-flanking regions of their genes (25), whereas in the rat glucagon gene the CRE octamer extends from nt −291 to −298.

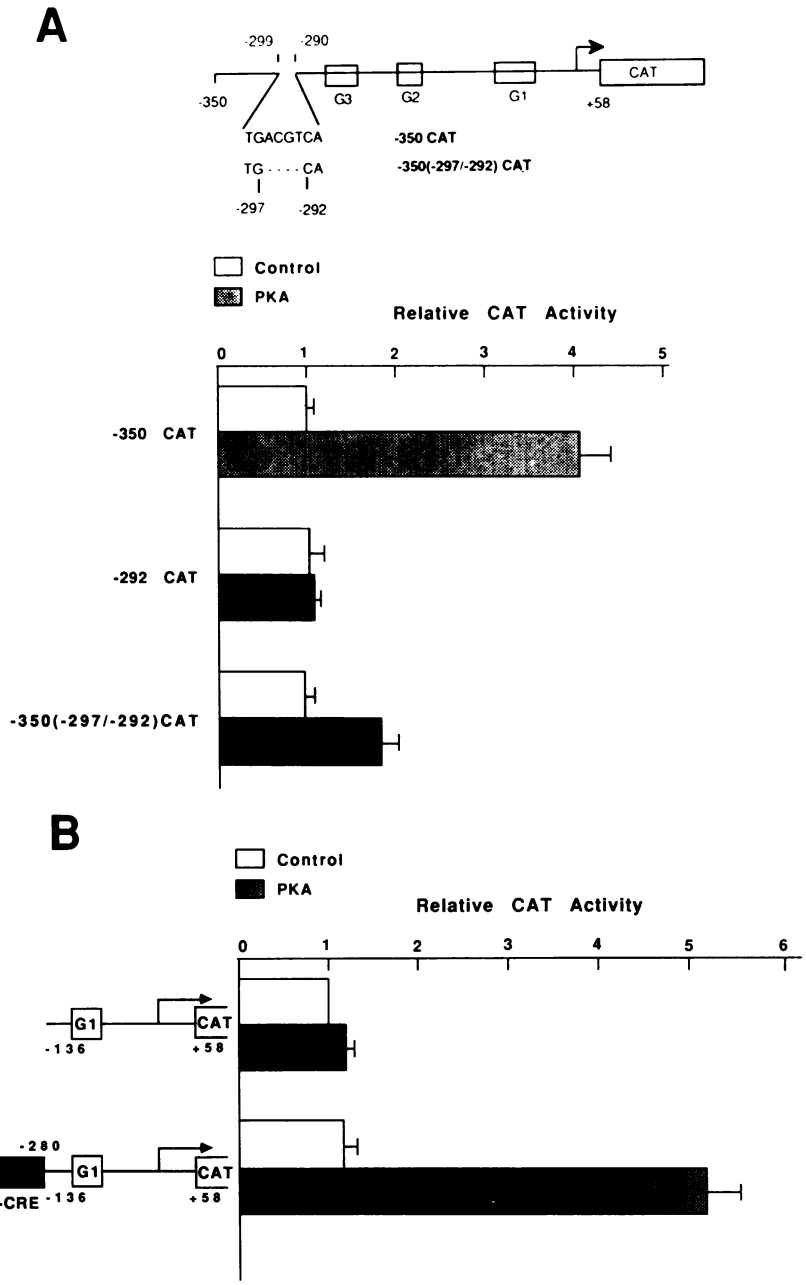


FIG. 2. The glucagon CRE confers protein kinase A responsiveness to rat glucagon gene transcription. (A) Effect of a 5′ deletion or an internal 4-bp deletion on the transcriptional induction by the catalytic subunit of protein kinase A. (B) An oligonucleotide containing the rat glucagon CRE (Glu-CRE-27) confers protein kinase A inducibility to the nonresponsive glucagon promoter (−136CAT). Transient transfections into InR1-G9 cells with (PKA) or without (control) cotransfection of an expression vector coding for the catalytic subunit of protein kinase A. CAT activities are expressed relative to the mean value in each experiment of the activity observed with −350CAT (A) or −136CAT (B) controls. Values are means (plus the standard error of the mean) of three or four independent experiments, each done in duplicate.

Comparison of the transcriptional activities conferred by the CRE octamer in distinct base contexts inserted into the rat glucagon gene 5′-flanking region. The CRE octamer consensus motif in the base contexts of the rat somatostatin or human choriogonadotropin (CG) alpha gene are well described, functionally active CREs (4, 25). To compare the transcriptional activity conferred by the glucagon CRE with that conferred by well-characterized CREs, 10 bp upstream (from nt −299 to −308) and 11 bp downstream (from nt −290 to −280) of the CRE octamer motif were converted to the corresponding ones surrounding the CRE octamer motif in the rat somatostatin or human CG alpha genes without otherwise disrupting the organization of the rat glucagon gene 5′-flanking region, creating the plasmids −350(SMS-CRE)CAT and −350(alpha-CRE)CAT, respectively (Fig. 3A). Starting with plasmid −350CAT, the 21 bases were mutated by site-specific mutagenesis with the polymerase chain reaction technique and a strategy outlined elsewhere

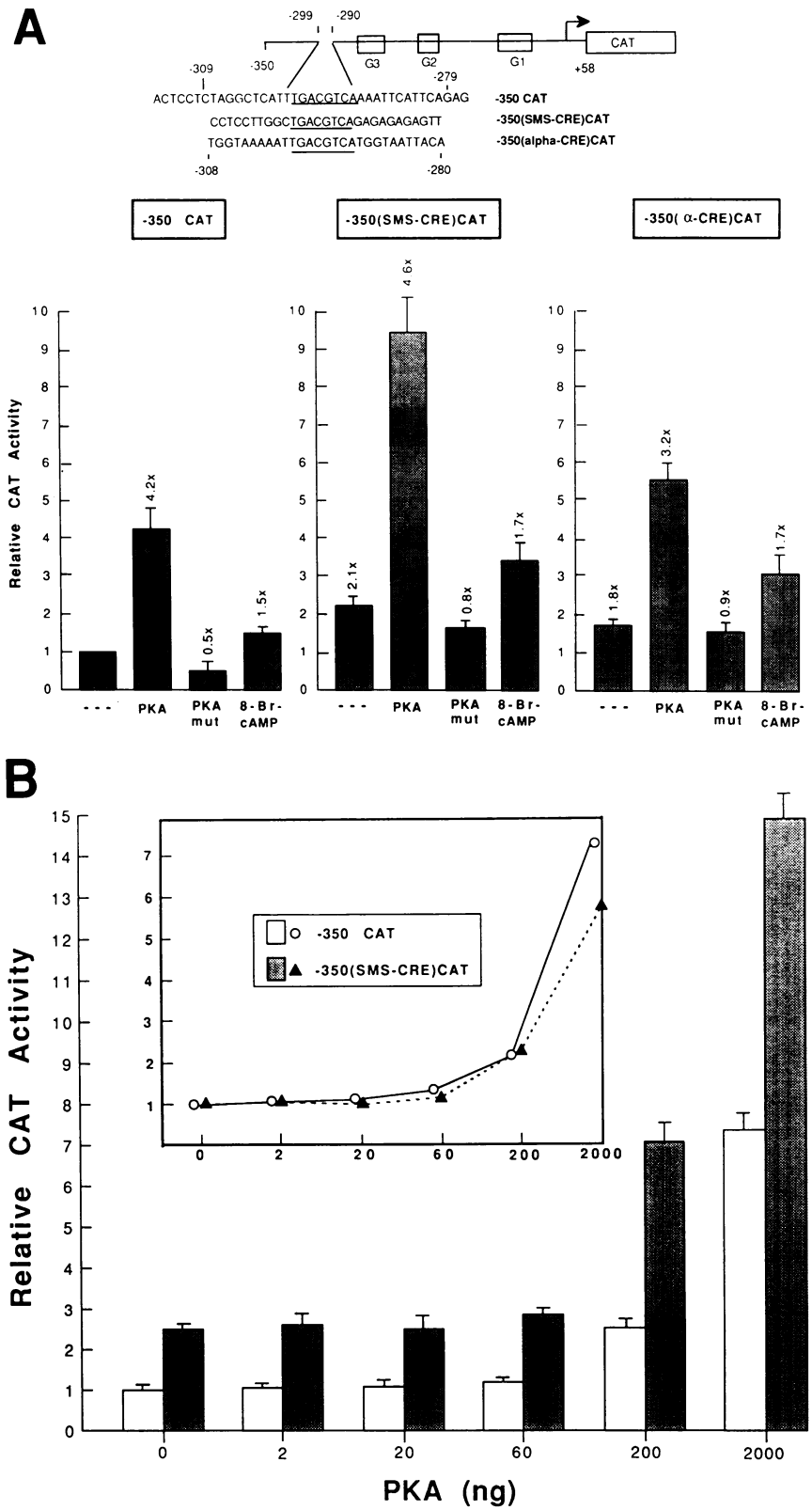
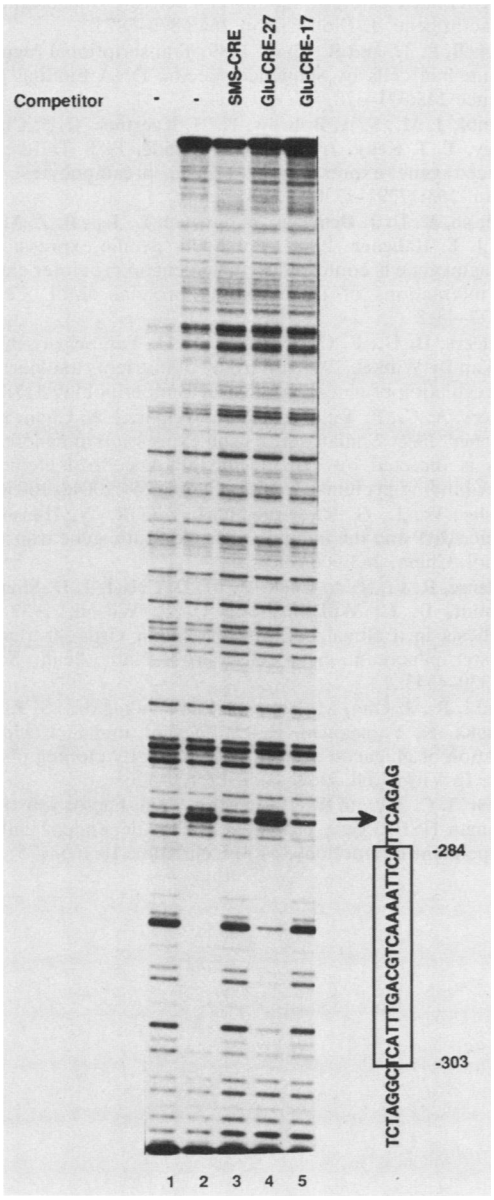


FIG. 3. Effect of the CRE context on transcriptional induction by the catalytic subunit of protein kinase A. (A) The plasmids −350CAT, −350(SMS-CRE)CAT, and −350(alpha-CRE)CAT were transiently transfected into InR1-G9 cells. CAT activities are expressed relative to the mean value in each experiment of the activity observed with −350CAT without cotransfection or treatment. Values are means (plus the standard error of the mean) of four independent experiments, each done in duplicate. The numbers above each bar give the fold increase in the noncotransfected and nontreated groups relative to the value for −350CAT and in all other groups relative to the value for the noncotransfected and nontreated group with the same CAT reporter. For further explanations, see the legend to Fig. 1. (B) Cotransfection

of increasing amounts of an expression vector coding for the catalytic subunit of protein kinase A (PKA) result in parallel induction of −350CAT and −350(SMS-CRE)CAT transcription in InR1-G9 cells. CAT activities are expressed relative to the mean value in each experiment of the activity observed with −350CAT without cotransfection or (inset) relative to the value for its own noncotransfected group of −350CAT or −350(SMS-CRE)CAT, respectively. Values are means of three independent experiments, each done in duplicate.

(12). Changing the glucagon CRE context into the somatostatin or CG alpha context moderately elevated basal CAT activity about 2.1- or 1.8-fold, respectively (Fig. 3A). All three fusion genes responded to cotransfection of the catalytic subunit of protein kinase A (Fig. 3A). Although the CAT activity levels reached under the stimulation by the catalytic subunit were higher than that of −350CAT, particularly for −350(SMS-CRE)CAT and less so for −350(alpha-CRE)CAT (Fig. 3A), the protein kinase A-induced stimulation of transcriptional activity was similar for the rat glucagon wild-type construct (4.2 fold) and the mutants with the somatostatin (4.6-fold) or CG alpha (3.2 fold) CRE contexts (Fig. 3A). Cotransfection of an inactive catalytic subunit mutant did not stimulate reporter activity of these constructs (Fig. 3A). When increasing amounts of the expression vector coding for the catalytic subunit of protein kinase A were cotransfected with −350CAT or −350(SMS-CRE)CAT, transcription of the wild-type and mutant CRE constructs increased in parallel (Fig. 3B). These data suggest that the rat glucagon CRE at its native position mediates protein kinase A induction as effectively and with the same sensitivity as does the CRE octamer in the base context of the rat somatostatin or human CG alpha gene.



Recombinant CREB-327 binds to the glucagon CRE. CREB-327 (14) is a CRE-binding protein and activates gene transcription in a cAMP-dependent manner (8). To test whether CREB-327 can bind to the glucagon CRE, the cDNA encoding CREB-327 (13, 14) was cloned into the procaryotic expression vector pPROK-1 (Clontech Lab. Inc., Palo Alto, Calif.). After transformation of JM109 with pPROK-1 or pPROK-CREB-327, the bacteria were grown under isopropyl-β-D-thiogalactopyranoside induction, and the bacterial lysates were subjected to 50% ammonium sulfate precipitation. As revealed by in vitro DNase I footprinting (Fig. 4) performed as described previously (i.e., 72 μg of protein [17, 22]), bacterially expressed CREB-327 does bind to the rat glucagon CRE. Because of a less permissive contextual sequence, CREB-327 binds to the glucagon CRE with a lower affinity than it does to the somatostatin CRE, as indicated by competition experiments (Fig. 4).

While this study clearly indicates that the rat glucagon CRE is a functional protein kinase A-inducible enhancer element of the glucagon gene in a glucagon-expressing pancreatic islet cell line, it is unable to confer cAMP responsiveness to a heterologous CG alpha promoter in placental JEG cells (4). This marked difference in functional activity of the rat glucagon CRE in InR1-G9 and JEG cells raises the possibility that the effect of cAMP and protein kinase A on gene expression may depend on the cell type and/or the linked promoter, a variability which may result from the differential regulation by a multiplicity of homo- and heterodimeric CRE-binding proteins able to selectively interact with DNA (1, 9, 10, 13, 14, 18). Transcriptional activation by the CRE octamer motif has been shown to be promoter dependent (28), and some CRE-binding proteins

FIG. 4. CREB-327 binds to the rat glucagon CRE as revealed by DNase I in vitro footprinting. The rat glucagon 5′-flanking sequence (nt −350 to +50, 5′ end labeled on the coding strand) was incubated with protein extracts from bacteria transformed with pPROK-1 (vector only; lane 1) or pPROK-CREB-327 (lanes 2 through 5). The protected sequence is shown on the right; the hypersensitivity site is indicated by an arrow. Competitors (350-fold molar excess) were added as indicated on top of the lanes. Note that an oligonucleotide with the sequence of the CRE octamer and 19 bases of the rat glucagon gene context (Glu-CRE-27, from nt −306 to −280) was unable to block the hypersensitivity site and was less effective in inhibiting the protection than oligonucleotides spanning the rat somatostatin CRE (SMS-CRE, from nt−62 to −32) or the sequence of the CRE octamer and 9 bases of the rat glucagon gene context (Glu-CRE-17, from nt −302 to −286). An oligonucleotide with an unrelated sequence did not compete (data not shown).

seem to be expressed at various levels in different tissues (18). Although this study shows that CREB-327, which is expressed in InR1-G9 cells (G. Waeber and J. F. Habener, unpublished observation), does bind to the glucagon CRE, additional glucagon CRE-binding proteins may exist.

We thank R. A. Maurer (Iowa City, Iowa) for the generous gift of the wild-type and mutant catalytic subunit expression vectors. We also thank James P. Hoeffler for helpful discussions.

Willhart Knepel was supported by the Deutsche Forschungsgemeinschaft, Bonn, Federal Republic of Germany. This work was supported in part by Public Health Service grant DK30834 from the National Institutes of Health.

LITERATURE CITED

1. **Benbrook, D. M., and N. C. Jones.** 1990. Heterodimer formation between CREB and JUN proteins. Oncogene **5**:295–302.
2. **Brasier, A. R., J. E. Tate, and J. F. Habener.** 1989. Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. BioTechniques **7**:1116–1122.
3. **Brubaker, P. L.** 1988. Control of glucagon-like immunoreactive peptide secretion from fetal rat intestinal cultures. Endocrinology **123**:220–226.
4. **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.
5. **De Wet, J. R., K. V. Wood, M. De Luca, D. R. Helinski, and S. Subramani.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7**:725–737.
6. **Drucker, D. J., and P. L. Brubaker.** 1989. Proglucagon gene expression is regulated by a cyclic AMP-dependent pathway in rat intestine. Proc. Natl. Acad. Sci. USA **86**:3953–3957.
7. **Drucker, D. J., J. Philippe, and S. Mojsov.** 1988. Proglucagon gene expression and posttranslational processing in a hamster islet cell line. Endocrinology **123**:1861–1867.
8. **Gonzalez, G. A., and M. R. Montminy.** 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell **59**:657–680.
9. **Gonzalez, G. A., K. K. Yamamoto, W. H. Fischer, D. Karr, P. Menzel, W. Biggs III, W. W. Vale, and M. R. Montminy.** 1989. A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. Nature (London) **337**:749–752.
10. **Hai, T., F. Liu, W. J. Coukos, and M. R. Green.** 1989. Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. Genes Dev. **3**:2083–2090.
11. **Hammonds, P., P. N. Schofield, and S. J. H. Ashcroft.** 1987. Glucose regulates preproinsulin messenger RNA levels in a clonal cell line of simian virus 40-transformed B cells. FEBS Lett. **213**:149–154.
12. **Higuchi, R.** 1989. Using PCR to engineer DNA, p. 61–70. *In* H. A. Erlich (ed.), PCR technology. Stockton Press, New York.
13. **Hoeffler, J. P., T. E. Meyer, G. Waeber, and J. F. Habener.** 1990. Multiple adenosine 3',5'-cyclic monophosphate response element DNA-binding proteins generated by gene diversification and alternative exon splicing. Mol. Endocrinol. **4**:920–930.
14. **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.
15. **Iversen, J.** 1973. Adrenergic receptors and the secretion of glucagon and insulin from the isolated, perfused canine pancreas. J. Clin. Invest. **52**:2102–2116.
16. **Jameson, J. L., A. C. Powers, G. D. Gallagher, and J. F. Habener.** 1989. Enhancer and promoter element interactions dictate cyclic adenosine monophosphate mediated and cell-specific expression of the glycoprotein hormone alpha-gene. Mol. Endocrinol. **3**:763–772.
17. **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.
18. **Maekawa, T., H. Sakura, C. Kanei-Ishii, T. Sudo, T. Yoshimura, J. Fujisawa, M. Yoshida, and S. Ishii.** 1989. Leucine zipper structure of the protein CRE-BP1 binding to the cyclic AMP response element in brain. EMBO J. **8**:2023–2028.
19. **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.
20. **Mitchell, P. J., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245**:371–378.
21. **Ntambi, J. M., S. A. Buhrow, K. H. Kaestner, R. J. Christy, E. Sibley, T. J. Kelly, Jr., and M. D. Lane.** 1988. Differentiation-induced gene expression in 3T3-L1 preadipocytes. J. Biol. Chem. **263**:17291–17300.
22. **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.
23. **Pipeleers, D. G., F. C. Schuit, C. F. H. Van Schravendijk, and M. Van De Winkel.** 1985. Interplay of nutrients and hormones in the regulation of glucagon release. Endocrinology **117**:817–823.
24. **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.
25. **Roesler, W. J., G. R. Vandenbark, and R. W. Hanson.** 1988. Cyclic AMP and the induction of eukaryotic gene transcription. J. Biol. Chem. **263**:9063–9066.
26. **Santerre, R. F., R. A. Cook, R. M. D. Crisel, J. D. Sharp, R. J. Schmidt, D. C. Williams, and C. P. Wilson.** 1981. Insulin synthesis in a clonal cell line of simian virus 40-transformed hamster pancreatic beta cells. Proc. Natl. Acad. Sci. USA **78**:4339–4343.
27. **Takaki, R., J. Ono, M. Nakamura, Y. Yokogawa, S. Kumae, T. Hiraoka, K. Yamaguchi, K. Hamaguchi, and S. Uchida.** 1986. Isolation of glugacon-secreting cell lines by cloning insulinoma cells. In Vitro Cell. Dev. Biol. **22**:120–126.
28. **Taylor, I. C. A., and R. E. Kingston.** 1990. Factor substitution in a human HSP70 gene promoter: TATA-dependent and TATA-independent interactions. Mol. Cell. Biol. **10**:165–175.