RESEARCH COMMUNICATION

Expression cloning of a zinc-finger cyclic AMP-response-element-binding protein

Richard M. O'BRIEN,* Natasha HALMI, Per-Erik STROMSTEDT, Richard L. PRINTZ and Daryl K. GRANNER

Department of Molecular Physiology and Biophysics, Vanderbilt University Medical School, Nashville, TN 37232, U.S.A.

In response to specific extracellular signals, intracellular cyclic AMP levels increase, leading to a variety of responses including the alteration of transcription of many eukaryotic genes. This transcriptional effect is frequently mediated through the cyclic AMP-response element (CRE) motif T(T/G)ACGTCA. Using an expression screening approach we have cloned a yeast gene, *MSN2*, that encodes a 78 kDa protein that recognizes this consensus CRE motif. Phosphorylation of the MSN2 protein by the catalytic subunit of protein kinase A stimulates DNA binding *in vitro*. Two putative Cys_2His_2-type zinc fingers present in the C-terminal 79 amino acids of the MSN2 protein are sufficient to confer CRE-binding specificity. Therefore, MSN2 represents a novel CRE-binding protein distinct from the multiple previously characterized basic region-leucine zipper repeat CRE-binding proteins.

INTRODUCTION

The cyclic AMP-signalling pathway is one of the major intracellular signal transduction pathways in eukaryotic cells [1]. Stimulation of adenylate cyclase increases the intracellular concentration of cyclic AMP, resulting in the activation of protein kinase A (PKA), which, in turn, phosphorylates a number of substrates [1]. Multiple genes whose rate of transcription is regulated by cyclic AMP have been identified [2]. In most, but not all, cases the action of cyclic AMP has been shown to be mediated via a *cis*-acting sequence, designated the cyclic AMP-response element (CRE), that has the consensus sequence T(G/T)ACGTCA [2].

Three families of *trans*-acting factors, the CRE-binding protein/activating transcription factor family, the Fos/Jun family and the CCAAT/enhancer binding protein family have been shown to bind the CRE [3,4]. These families include both transcriptional activators and repressors; however, all the members share a structural motif known as the basic region-leucine zipper repeat (bZIP) that allows for DNA binding and dimerization respectively [3,4]. As yet, PKA has been shown to phosphorylate only a small number of these CRE-binding proteins [3,4].

This paper describes the identification of a yeast protein, MSN2, that recognizes the CRE motif through a Cys_2His_2 zinc-finger motif. As such, MSN2 may represent a novel class of CRE-binding proteins distinct from the bZIP families.

EXPERIMENTAL

Materials

The catalytic subunit of PKA [5] and the PKA inhibitor peptide [6] were generous gifts from Dr. Jackie Corbin and Dr. Sharron Francis (both of Vanderbilt University) respectively. A λGT11 rat liver 5′ stretch cDNA library was purchased from Clontech Laboratories.

Expression screening

The expression cloning strategy used was based on the method of Singh et al. [7] as modified by Vinson et al. [8] except that 0.2% polyoxyethylene–sorbitan monolaurate (Tween 20) was used instead of dry milk to block non-specific binding. Positive clones were plaque purified, and bacteriophage DNA was isolated by standard procedures [9].

Genomic screening

To isolate a complete MSN2 genomic clone, a YEp24 genomic plasmid library [10] transformed into *Epicurian coli* (Strategene) was screened by standard methods [9] using the 818 bp *Eco*RI–*Eco*RI fragment from the 18MC λGT11 recombinant phage as the labelled probe (Figure 1).

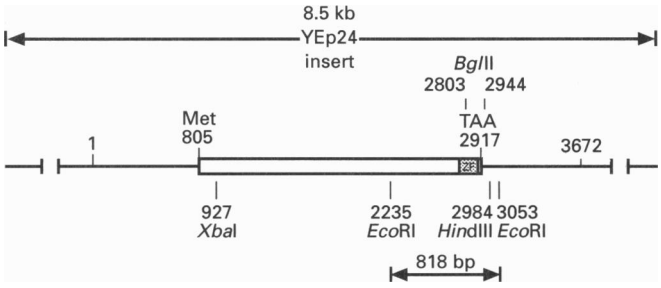

Figure 1 Partial restriction enzyme map of a YEp24 yeast genomic clone encoding MSN2

An 8.5 kbp yeast genomic clone containing the entire MSN2 open reading frame was isolated from a YEp24 plasmid library [10]. The partial restriction enzyme map shows the sites used in construction of the various MSN2 expression vectors. The 818 bp *Eco*RI–*Eco*RI fragment was contained in the 18MC phage identified by expression screening. Abbreviation used: ZF, zinc fingers.

Abbreviations used: bZIP, basic region-leucine zipper repeat; CRE, cyclic AMP-response element; IPTG, isopropyl β-D-thiogalactoside; PEPCK, phosphoenolpyruvate carboxykinase; PKA, protein kinase A; PKI, PKA catalytic subunit inhibitor.

* To whom correspondence should be addressed.

Plasmid construction

The *Eco*RI–*Eco*RI fragment from the 18MC λGT11 recombinant phage was subcloned into pGEX-2T (Pharmacia) such that it was not in frame with the glutathione S-transferase coding sequence; the resulting plasmid was designated pGEX-18MC. To delete the two putative zinc fingers, pGEX-18MC was digested with *Bgl*II (Figure 1) and re-ligated to create pGEX-ZM.

To determine whether a short (118 amino acid, ~ 12 kDa) C-terminal fragment of MSN2 that included the two putative zinc fingers was sufficient to confer DNA-binding activity, the DNA sequence encoding this peptide was synthesized using PCR and ligated into the T7 expression vector pET-15b (Novagen), in frame with the His·Tag linker, to create pET-12kDa.

To create an expression vector for the production of full-length MSN2, the YEp24 plasmid containing the MSN2 genomic clone was digested with *Xba*I and *Hin*dIII, and a fragment of 2050 bp was isolated (Figure 1). This was ligated into pGEM7 (Promega) to create pGEM-18MCP. A 150 bp fragment containing the missing 5′ MSN2 sequence (Figure 1) was synthesized by PCR and ligated into pGEM-18MCP to create pGEM-18MCT. The full-length MSN2 coding sequence was isolated from pGEM-18MCT and ligated into pET-15b (Novagen) to create pET-18MCT. The MSN2 coding sequence is in frame with the His·Tag linker. The orientation of all subcloned DNA fragments was determined by restriction enzyme analysis, and the absence of PCR-induced errors was confirmed by DNA sequencing (Sequenase kit, USB).

Transcription and translation *in vitro*

Coupled transcription and translation *in vitro* of pET-18MCT was performed using the TnT T7-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. Where appropriate (Figure 3b), 1 μl of [^{35}S]methionine (Tran ^{35}S-label, 1016 Ci/mmol, 12 mCi/ml; ICN) was included in a final 25 μl reaction volume.

Bacterial expression of MSN2

pGEX-18MC and pGEX-ZM were transformed into DH5-α cells, whereas pET-18MCT and pET-12kDa were transformed into BL21 (DE3) pLysS cells. After 2–4 h growth in LB/ampicillin (200 μg/ml) at 37 °C in the presence or absence of 1 mM isopropyl β-D-thiogalactoside (IPTG), 2.5 ml of cells were pelleted by centrifugation and resuspended in 250 μl of lysis buffer: 50 mM Hepes, pH 7.5, 100 mM NaCl and 2 mM PMSF. Cells were lysed by sonication [9] and, after precipitation of insoluble matter by centrifugation, the supernatant was assayed for DNA-binding activity as described below.

Gel retardation assay

Oligonucleotides representing the wild-type and mutant phosphoenolpyruvate carboxykinase (PEPCK) CRE and the wild-type somatostatin CRE have been previously described [11]. The analysis of protein binding to the labelled PEPCK CRE using the gel retardation assay was performed exactly as described [12].

MSN2 phosphorylation

To investigate whether MSN2 protein is a substrate for various kinases, unprogrammed reticulocyte lysate or lysate programmed with pET-18MCT (3 μl of each lysate) was incubated in the presence or absence of PKA catalytic subunit (0.5 μM final concentration) in a final volume of 20 μl containing 10 mM Tris, pH 7.4, 0.1 mM magnesium acetate, 0.3 mg/ml BSA, 1.2 μM calmodulin, 0.5 mM $CaCl_2$ and 0.2 mM [γ-^{32}P]ATP (specific activity ~ 2.5 μCi/nmol) for 5 min at room temperature. The reactions were terminated by the addition of SDS/PAGE sample buffer and analysed by PAGE [9].

To investigate whether phosphorylation of the MSN2 protein by the catalytic subunit of PKA has any effect on DNA-binding activity, unprogrammed reticulocyte lysate or lysate programmed with pET-18MCT (1μl of each lysate) was incubated in the presence or absence of PKA catalytic subunit (0.5 μM final concentration) or PKA catalytic subunit inhibitor (PKI, final concentration 11 μM) or both in a final volume of 10 μl containing 10 mM Tris, pH 7.4, 10 mM magnesium acetate, 0.2 mM ATP and 0.3 mg/ml BSA for 5 min at room temperature. A DNA-binding cocktail (10 μl) containing labelled PEPCK CRE oligonucleotide (~ 20 fmol, 75000 c.p.m.), 20 mM Hepes, pH 7.8, 2 mM spermidine. 20 mM dithiothreitol, 2 μg of poly(dI–dC)·poly(dI–dC), 10% glycerol and 0.2% Nonidet P40 (v/v) was then added for a further 10 min at room temperature before loading the mixture on a non-denaturing polyacrylamide gel, electrophoresis and subsequent analysis of DNA binding by autoradiography as described previously [12].

RESULTS

To identify novel CRE-binding proteins, an expression cloning strategy was used, based on the well-characterized method of Singh et al. [7] as modified by Vinson et al. [8]. A ^{32}P-labelled, catenated PEPCK CRE probe [11] was used to screen a Clontech rat liver cDNA library. About one million plaques were screened, and a recombinant λGT11 phage, designated 18MC, that encoded a protein that bound the probe was identified. The protein encoded by the 18MC phage specifically recognized the wild-type but not a mutant CRE probe (results not shown).

The plaque-purified 18 MC λGT11 recombinant phage contained an 818 bp *Eco*RI–*Eco*RI insert (Figure 1), the sequence of which was identical with part of the yeast protein MSN2 [13]. It was subsequently reported that Clontech libraries are prone to contamination with yeast genomic DNA [14]. Since all the known mammalian CRE-binding proteins identified to date contain the putative bZIP motif (see Introduction), and because *MSN2* encodes a 78 kDa Cys_2His_2-type zinc-finger protein [13], MSN2 may represent a new class of CRE-binding protein.

To prove that the two MSN2 zinc-finger motifs are sufficient to confer CRE-binding activity, an expression vector encoding only the C-terminal 118 amino acids of MSN2 with an additional N-terminal 24-amino-acid His·Tag extension, designated pET-12 kDa, was constructed as described in the Experimental section. When this plasmid was transformed into *E. coli* BL21(DE3) P. Lys cells, two IPTG-inducible CRE-binding proteins were detected (Figure 2a). An analysis of the MSN2 sequence reveals a perfect Shine–Dalgarno box (AGGAGA; [15]) at an appropriate distance (7 bp) from a methionine residue that could initiate internal translation of an approximately 8 kDa (79 amino acid) peptide that still contains both zinc fingers. Thus we would speculate that the two CRE-binding proteins observed in Figure 2(a) represent 118- and 79-amino-acid forms of MSN2. Supporting the potential for internal initiation of translation is the observation that ligation of the *Eco*RI–*Eco*RI fragment from the 18MC lGT11 recombinant phage (Figure 1) into pGEX-2T, such that it is not in frame with the glutathione S-transferase coding sequence, still leads to the production of an IPTG-inducible CRE-binding protein that co-migrates with the putative 79-amino-acid peptide (Figure 2b). Deletion of the DNA sequence encoding the two putative zinc fingers abolishes CRE-binding activity (Figure 2b).

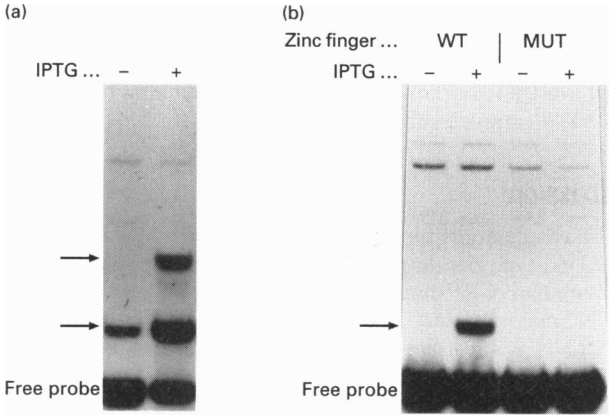

Figure 2 The putative zinc fingers in the MSN2 protein are required for CRE binding

(**a**) Two IPTG-inducible CRE-binding proteins (arrows) were detected in a gel retardation assay [12] when a 118-amino-acid C-terminal fragment of MSN2 was expressed in bacteria using the pET-12kDa plasmid. (**b**) IPTG-inducible CRE-binding activity (arrow) was expressed from the pGEX-18MC (WT) but not the pGEX-ZN (MUT) plasmid in which the DNA sequence encoding the two putative zinc fingers had been deleted.

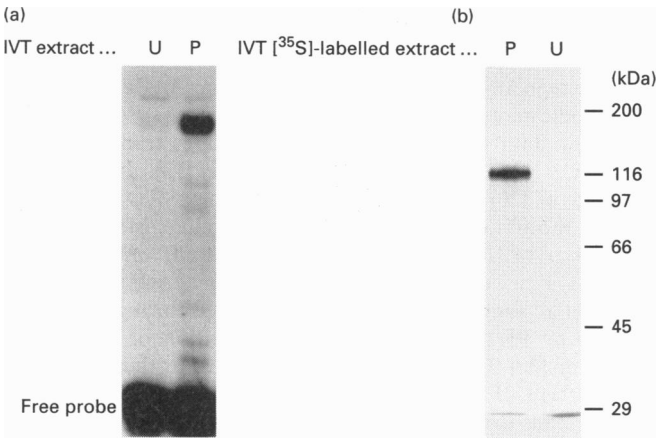

Figure 3 Expression *in vitro* of the full-length MSN2 protein

The ability of proteins present in unprogrammed reticulocyte lysate (U), or lysate programmed with the pET-18MCT plasmid (P), to bind to a labelled PEPCK CRE probe in a gel retardation assay (**a**) was assessed as described previously [12]. Incorporation of [^{35}S]methionine into MSN2 was assessed by SDS/PAGE and fluorography (**b**). For the gel retardation assay, 1 μl of each lysate was used, whereas for SDS/PAGE only 1 μl of the pET-18MCT lysate was loaded compared with 4 μl of the unprogrammed lysate. Abbreviation used: IVT, *in vitro* transcription and translation.

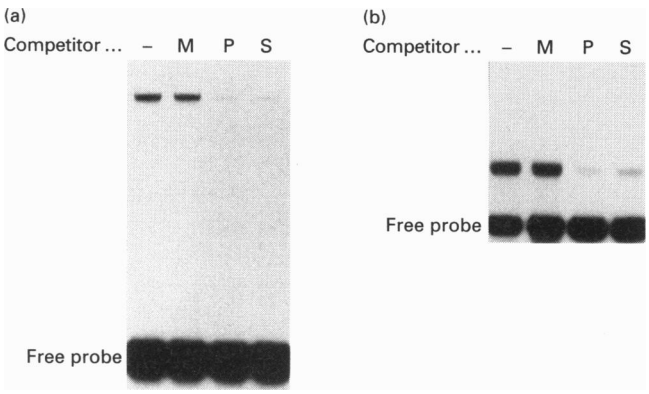

Figure 4 MSN2 specifically recognizes the CRE core motif

The pET-18MCT plasmid was used to express full-length MSN2 in reticulocyte lysate (**a**) and truncated MSN2 in bacteria (**b**). A 200-fold molar excess of double-stranded oligonucleotides representative of the wild-type (P) and mutant (M) PEPCK CRE and the wild-type (S) somatostatin CRE were incubated with a ^{32}P-labelled PEPCK CRE probe before addition of reticulocyte lysate (1 μl; **a**) or bacterial extract (0.25 μl; **b**) and analysis of protein binding using the gel retardation assay [12].

To investigate further the DNA-binding properties of MSN2, a YEp24 yeast genomic clone encoding MSN2 was isolated (Figure 1) and an expression vector, designated pET-18MCT, was constructed for the expression of the full-length MSN2 protein with an additional N-terminal 24-amino-acid His·Tag extension, as described in the Experimental section. When analysed in a gel retardation assay, reticulocyte lysate programmed with this plasmid produced a single major protein–DNA complex not seen in unprogrammed lysate (Figure 3a). When lysate programmed with pET-18MCT in the presence of [^{35}S]methionine was analysed by SDS/PAGE, a single major band was detected with a molecular mass of ~ 116 kDa (Figure 3b). This is much greater than the predicted molecular mass of 78 kDa and suggests anomalous migration on SDS/polyacrylamide gels.

Although the protein encoded by the 18MC phage recognized the wild-type but not mutant PEPCK CRE (results not shown), this result does not prove that the protein actually binds the consensus CRE core sequence [T(G/T)ACGTCA]. To confirm that it does, we performed a competition experiment comparing oligonucleotides representing the wild-type PEPCK and somatostatin CREs with an oligonucleotide containing mutated sequences that in functional assays abolish cyclic AMP responsiveness [11]. The somatostatin and PEPCK CREs have completely different flanking sequences on either side of the consensus core sequence [11]. When the PEPCK CRE was used as the labelled probe in a gel retardation assay, both the wild-type PEPCK (P) and somatostatin (S) CREs competed for binding of full-length MSN2 (expressed *in vitro* using the pET-18MCT plasmid) (Figure 4a). The mutant CRE (M) was ineffective (Figure 4a).

When the pET-18MCT plasmid was transformed into *E. coli* BL21(DE3) P. Lys cells, no full-length MSN2 could be detected. Similar problems using this vector to direct protein expression in bacteria have been reported previously [16]. However, an IPTG-inducible CRE-binding protein was produced that co-migrates with the putative 79-amino-acid MSN2 peptide generated by internal iniation of translation as described above. This maintained the same DNA-binding specificity as the full-length protein (Figure 4b). Additional studies will be required to determine the consensus sequence for MSN2 binding.

An analysis of the deduced amino acid sequence of MSN2 using the IntelliGenetics PROSITE 10 data bank revealed the presence of several possible phosphorylation sites for PKA. Since MSN2 binds the CRE consensus sequence, we wanted to determine whether it was a substrate for PKA and, if so, whether phosphorylation affected DNA binding. Since we were unable to express (and then purify) full-length MSN2 in bacteria, we used the crude *in vitro* translation lysate as a source of full-length protein. The catalytic subunit of PKA phosphorylates a number of proteins in the reticulocyte lysate (Figure 5). However, a comparison of unprogrammed lysate (U) with lysate pro-

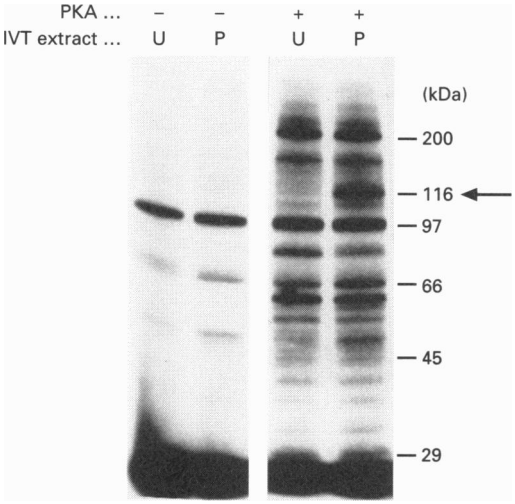

Figure 5 MSN2 is a substrate for the catalytic subunit of PKA

The ability of the catalytic subunit of PKA to phosphorylate proteins in unprogrammed reticulocyte lysate (U) or lysate programmed with the pET-18MCT plasmid (P) was assessed by SDS/PAGE as described in the Experimental section. Abbreviation used: IVT, *in vitro* transcription and translation.

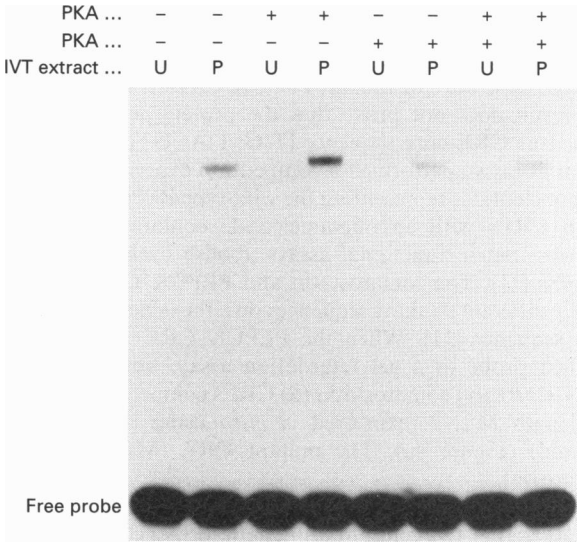

Figure 6 Phosphorylation of MSN2 by PKA stimulates DNA binding

The pET-18MCT plasmid was used to express full-length MSN2 in reticulocyte lysate. The effect of PKA-dependent phosphorylation on CRE DNA-binding activity, in the presence or absence of PKI, was analysed using the gel retardation assay as described in the Experimental section. Abbreviation used: IVT, *in vitro* transcription and translation.

grammed with pET-18MCT (P) reveals the presence of a unique band at approximately 116 kDa (Figure 5), a size that is compatible with the [^{35}S]methionine data (Figure 3b).

Having established that MSN2 is a substrate for PKA *in vitro* we next wanted to determine whether phosphorylation affected DNA binding. To answer this question we used the gel retardation assay and compared unprogrammed lysate (U) and lysate programmed with pET-18MCT (P). The catalytic subunit of PKA had no effect on DNA binding by proteins in the unprogrammed lysate (Figure 6). By contrast, the catalytic subunit of PKA stimulated binding of MSN2 to the PEPCK CRE ~ 2.5-fold [2.5±0.6 (S.D.); $n = 4$] and also induced a slight upward shift in mobility. Both effects were blocked by the specific PKA inhibitor PKI, at a concentration known to inhibit the activity of the PKA catalytic subunit ([6]; Figure 6).

DISCUSSION

We have demonstrated that the yeast gene MSN2 encodes a Cys_2His_2-type zinc-finger CRE-binding protein. Since all known mammalian CRE-binding proteins contain the bZIP motif, MSN2 may represent a new class of CRE-binding proteins [3,4]. MSN2 is a substrate for the catalytic subunit of PKA, and phosphorylation stimulates DNA binding. Additional studies will be required to demonstrate that MSN2 is phosphorylated in a cyclic AMP-dependent manner *in vivo* and that MSN2 binding activity correlates with the regulation of transcription of an endogenous yeast gene by cyclic AMP. PKA has previously been shown to phosphorylate and inactivate the yeast transcriptional activator ADR1 [17]. Unlike MSN2, ADR1 binds an upstream activating sequence in the ADH2 gene promoter that is distinct from the CRE consensus motif, and phosphorylation does not affect DNA binding [18].

MSN2 was initially identified by multicopy suppression of an SNF1 protein kinase mutant of *Saccharomyces cerevisiae* [13]. Two other genes from the cyclic AMP-dependent protein kinase pathway in yeast also suppress mutations in SNF1 when present in multiple copies [19]. These genes, MSI1 and PDE2, encode a negative regulator of the cyclic AMP pathway and a cyclic AMP phosphodiesterase respectively [19].

Genetic data suggest that MSN2 stimulates transcription of the SUC2 gene, which encodes invertase [13]. Interestingly, Nehlin et al. [20] identified another yeast gene, SKO1, that binds a CRE motif in the SUC2 promoter. Unlike MSN2, SKO1 contains a bZIP DNA-binding domain, and, unlike MSN2, SKO1 is a negative regulator of SUC2 gene expression [20]. Nehlin et al. suggest that SKO1 is inactivated through phosphorylation by PKA, thus relieving the inhibition of SUC2 gene expression [20]. This hypothesis is complicated by the observation that deletion of the SKO1-binding site in the SUC2 promoter actually leads to a decrease in SUC2 expression. Nehlin et al. [20] hypothesized that this result could be explained if an activator also bound the same CRE motif, much like the well-characterized antagonism between CRE-binding protein and cyclic AMP-responsive element modulator [3,4]. The MSN2 protein is an obvious candidate for such a role.

We thank P. Anthony Weil, David Poon, Roger J. Colbran, Sharron Francis and Jackie Corbin for advice and encouragement during the course of this project. We also thank Deborah Caplenor for preparing the manuscript. This work was supported by an HHS grant (DK35107) and the Vanderbilt Diabetes Research and Training Center (DK20593).

REFERENCES

1 Scott, J. D. (1991) Pharmac. Ther. **50**, 123–145
2 Roesler, W. J., Vandenbark, G. R. and Hanson, R. W. (1988) J. Biol. Chem. **263**, 9063–9066
3 Meyer, T. E. and Habener, J. F. (1993) Endocr. Rev. **14**, 269–290
4 de Groot, R. P. and Sassone-Corsi, P. (1993) Mol. Endocrinol. **7**, 145–153
5 Flockhart, D. A. and Corbin, J. D. (1984) in Brain Receptor Methodologies, Part A (Marangos, D. J., Campbell, I. C. and Cohen, R. M., eds.), pp. 209–215, Academic Press, Orlando
6 Scott, J. D., Fischer, E. H., Demaille, J. G. and Krebs, E. G. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4379–4383
7 Singh, H., LeBowitz, J. H., Baldwin, A. S., Jr. and Sharp, P. A. (1988) Cell **52**, 415–423

8 Vinson, C. R., LaMarco, K. L., Johnson, P. F., Landschulz, W. H. and McKnight, S. L. (1988) Genes Dev. **2**, 801–806
9 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor
10 Carlson, M. and Botstein, D. (1982) Cell **28**, 145–154
11 Quinn, P. G. and Granner, D. K. (1990) Mol. Cell. Biol. **10**, 3357–3364
12 O'Brien, R. M., Lucas, P. C., Yamasaki, T., Noisin, E. L. and Granner, D. K. (1994) J. Biol. Chem. **269**, 30419–30428
13 Estruch, F. and Carlson, M. (1993) Mol. Cell. Biol. **13**, 3872–3881
14 Gersuk, V. H. and Rose, T. M. (1993) Science **260**, 605
15 Shine J. and Dalgarno, L. (1975) Nature (London) **254**, 34–38
16 Furlong, J., Meighan, M., Conner, J., Murray, J. and Clements, J. B. (1992) Nucleic Acids. Res. **20**, 4668
17 Cherry, J. R., Johnson, T. R., Dollard, C., Shuster, J. R. and Denis, C. L. (1989) Cell **56**, 409–419
18 Taylor, W. E. and Young, E. T. (1990) Proc. Natl. Acad. Sci U.S.A. **87**, 4098–4102
19 Hubbard, E. J. A., Yang, X. and Carlson, M. (1992) Genetics **130**, 71–80
20 Nehlin, J. O., Carlberg, M. and Ronne, H. (1992) Nucleic Acids Res. **20**, 5271–5278

Received 1 August 1995/1 September 1995; accepted 13 September 1995