Different Transcription Factor Binding Arrays Modulate the cAMP Responsivity of the Phosphoenolpyruvate Carboxykinase Gene Promoter*

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The cAMP responsiveness of the phosphoenolpyruvate carboxykinase (PEPCK) gene promoter is mediated by a cAMP response unit, which includes three CCAAT/enhancer-binding protein (C/EBPs) sites, and a CAMP response element (CRE). Because both the CRE-binding protein and several C/EBP isoforms can bind to the CRE with similar affinity, a variety of transcription factor bindings arrays in the CAMP response unit are possible that may affect the protein kinase A (PKA) responsiveness of the promoter. To explore this issue, we have designed PEPCK promoter variants that have the native cis-elements within the CAMP response unit replaced with one or more LexA- and/or GAL4-binding sites. We also engineered the corresponding C/EBP and CRE-binding protein chimeras, which have their basic region leucine zipper domains replaced with LexA or GAL4 DNA-binding domains. Using this approach, we have reconstituted the PKA responsiveness of permissive PEPCK promoters in hepatoma cells and have characterized the PKA responsiveness of the promoter under defined transcription factor occupancy patterns. Furthermore, analysis of deletion mutants of C/EBPα indicated that the domains that mediate its constitutive and PKA-inducible activities vary depending on which cis-element it occupies on the PEPCK promoter. These results suggest that promoter context may influence which domains within a transcription factor are employed to mediate transactivation.

The gene coding for phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme involved in gluconeogenesis, is an archetype for transcriptional regulation and has been used extensively to study hormonal regulation of gene transcription (1). Its promoter is composed of a complex array of cis-elements, and different combinations of these cis-elements appear to functionally cooperate to form “hormone response units” that mediate the effects of thyroid hormone, glucocorticoids, and cAMP in liver (2). The CAMP response unit (CRU) consists of five cis-elements, including three binding sites for CCAAT/enhancer-binding proteins (C/EBPs), a binding site for activator protein-1 (AP-1), and a typical CAMP response element (CRE), which can bind both CREB and C/EBPα (3–5). A mutation in any one of the five cis-elements results in a severe abrogation of CAMP responsiveness in liver-derived cells (6). It has been proposed that the advantage that hormone response units confer to promoters is the ability to broaden the range of responses to a hormonal signal as well as to fine-tune the response (7).

C/EBPs play an important role in mediating the CAMP responsiveness of the PEPCK promoter in liver, particularly the α-isoform. For example, inhibition of C/EBPα expression in hepatoma cells by antisense methodology impairs the ability of the PEPCK gene to respond to CAMP (8). Structure/function analysis of C/EBPα identified domains that mediate the constitutive activity of C/EBP as well as the cAMP-inducible activity, and although the domains that mediate these two activities overlap to some degree, mutational analysis suggests that they are distinct from one another (9, 10). It is also interesting to note that C/EBPs can compete with CREB for binding to the CRE in the PEPCK promoter, and indeed a significant level of CAMP responsiveness is maintained when C/EBPα replaces CREB at the CRE (9). Thus, the presence of CREB on the PEPCK promoter does not appear to be an absolute requirement for CAMP responsiveness.

Based on the knowledge that (i) C/EBPα and β co-exist in the liver along with CREB, and all three factors can bind to the CRE with similar affinity in vitro (11), and (ii) C/EBPα and β appear to be able to compete for binding to the three distal C/EBP-binding sites that form part of the CRU (11), it is reasonable to hypothesize that a number of different transcription factor binding arrays are possible on the PEPCK promoter. It is further conceivable that the cAMP responsiveness of the promoter might vary depending on the precise nature of transcription factor occupancy. In this study, we examined this issue using a combination of LexA and GAL4 chimeras of CREB and C/EBPs, along with appropriate changes in cis-elements within the PEPCK promoter to make them permissive for binding by these chimeras. This approach allowed us to assess the PKA responsiveness of the PEPCK promoter under defined transcription factor occupancy as well as to examine the relative importance of specific domains in C/EBPs when bound to different sites on the promoter.

EXPERIMENTAL PROCEDURES

Materials—HepG2 (ATCC HB8065) cells were purchased from American Type Culture Collection (Manassas, VA). Synthetic oligonucleotides were made by ID Labs Inc. (London, Canada) and the University of Calgary DNA Labs (Calgary, Canada). DNA modifying enzymes were purchased from New England Biolabs (Mississauga, Canada) and Pro-
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**Materials**

- Histone (Madison, WI), [acetetyl-3H]CoA (10 Ci/mmol) was purchased from PerkinElmer Life Sciences. Taq polymerase and T4 DNA ligase were purchased from Invitrogen, and tissue culture supplies were obtained from Invitrogen and Fisher. Antibodies against GAL4, LexA, and the hemagglutinin epitope were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**C/EBP-based Expression Plasmids**

The construction of the plasmids expressing the various GAL4-C/EBPα fusion proteins and the hemagglutinin-tagged C/EBPβ proteins were described in Ref. 12. The construction of GAL4-C/EBPβ was described in Ref. 13 and consists of amino acid residues 1–108 of C/EBPβ, linked to the GAL4 DNA-binding domain.

The general strategy for the construction of the plasmids expressing the three different C/EBPs involved introducing restriction sites by site-directed mutagenesis (14) at similar positions within the open reading frames of both C/EBPα and C/EBPβ cDNAs such that the coding regions for the transcription activation domains and bZIP domains of each isoform could be swapped or rejoined to form the following proteins: C/EBPα (transactivation domain of C/EBPα and the bZIP domain of C/EBPβ) and C/EBPβ (transactivation domain of C/EBPβ and bZIP domain of C/EBPα). These proteins were expressed with a hemagglutinin tag at their N terminus as described previously (15).

The plasmids expressing the LexA-C/EBPα fusions were based on that described (16). The LexA-C/EBPβ fusion vector was prepared essentially by replacing the corresponding C/EBPα DNA-binding domain with the coding region for the bZIP domain of LexA. The LexA-C/EBPα expression vector was created by linking the region corresponding to the transcription activation domain of C/EBPβ by the expression plasmid GAL4/C/EBPβ 1–108 (described in Ref. 13) and introduced into the pcDNA3.1 vector in frame with the LexA DNA-binding domain.

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**Transfection Experiments**

The pMT-C plasmids were verified by diagnostic restriction analysis and sequencing. The remaining PEPCK promoter derivatives were created within the context of the promoter region. The resulting pMT-C and C/EBPα or LexA-C/EBPβ sites were overexpressed in HepG2 cells and found to be transactivating their respective permissive promoter, PCK-V1 or PCK-V2, both in the absence and presence of PKA. The data indicate that the PKA-inducible activities of these two fusion proteins were similar, whereas the constitutive activity of LexA-C/EBPβ was consistently greater than its GAL4 counterpart. It should be pointed out that in the case of LexA-C/EBPα, fusion of the LexA domain to the N terminus resulted in a chimera with no transactivation capacity (data not shown), and activity was only observed when the LexA domain was fused to the C terminus. Conversely, we observed that flipping the position of the GAL4 domain to the C terminus had no effect on the activity of GAL4-C/EBPβ (data not shown). However, the important observation is that both fusion proteins retained constitutive and PKA-inducible activity. We also compared the activity of GAL4 and LexA chimeras of C/EBPβ in a similar fashion, and the results demonstrated that the transactivation potentials of these two fusion proteins are similar (Fig. 2B), although the LexA derivative possesses greater PKA-inducible activity.

These GAL4 and LexA fusions were then expressed in various combinations in conjunction with PEPCK promoter variant V4 (Fig. 1A) to examine the PKA responsivity of the promoter in HepG2 cells under conditions where the transcription factor occupancy at the CRE and the three distal C/EBP-binding sites could be controlled. PCK-V4 has a GAL4 site introduced in the CRE and LexA sites introduced in the three C/EBP sites, which bind the corresponding GAL4 and LexA fusion proteins. There were three experimental groups, based on which the GAL4 derivative was directed to the CRE location (Fig. 3). These experiments were designed based on the observation that C/EBPβ and both the α- and β-isoforms of C/EBPβ are capable of binding to the CRE (3, 5, 18, 24). In the absence of any fusion protein expressed, there was little basal or PKA-induced activity of PCK-V4. In the group where C/EBPα was directed to the CRE via a GAL4 domain, a small amount of basal and PKA-inducible promoter activity could be observed; however, both of these activities were greatly enhanced by co-expression of either LexA-C/EBPα or LexA-C/EBPβ, which bound to the three distal sites in the PCK-V4 promoter. Very similar observations were made in the next experimental group, which examined promoter activities when C/EBPβ was directed to the CRE location. In general, the data suggest that the basal and PKA-inducible level of promoter activity is somewhat greater under conditions where C/EBPβ is bound to the three distal sites. However, these data indicate that the PEPCK promoter retains responsivity to CAMP regardless of the C/EBP isoform bound to either the CRE or the distal sites, thus supporting earlier observations that there is a relative requirement for CREB in this response. The third experimental group examined promoter activities under conditions where CREB was directed to the CRE. Under this condition, the basal activity of the promoter was significantly lower in the presence of LexA-C/EBPα or LexA-C/EBPβ; however, the level of PKA-induced promoter activity achieved was similar to the other two experimental groups. Thus, the responsiveness of the PEPCK promoter to PKA appears to be greatest when CREB is bound to the CRE, although the absolute level of promoter activity achieved in the presence of PKA is similar when any one of the three proteins is bound to this site.

**Preparation of Nuclear Extracts and Western Blot Analysis**

Primary hepatocytes were prepared from rat liver as previously described (21). Nuclear extracts were prepared from primary hepatocytes and HepG2 cells using the method of Waite et al. (22). Western blot analysis was performed under nonreducing conditions using antibodies against CREB (New England Biolabs) and C/EBPα, C/EBPβ, and TFIIIEα (Santa Cruz Biotechnology).
the natural CRE sequence, and overexpression of full-length CREB from a transfected plasmid should promote CREB occupancy of this site. As can be observed, regardless of whether full-length CREB or GAL4-CREB was used, the PKA responsiveness of the promoter was very similar when either LexA-C/EBP or -C/EBP was tethered to the distal sites on the promoter. These data provide further validation of our model system, as well as indicate that the natural DNA-binding domain of CREB plays little if any role in its transactivation properties, except to direct it to the CRE.

The data in Fig. 3 showing that PKA responsiveness of the PEPCK promoter variant was reconstituted when C/EBP is tethered to the CRE location was surprising, because previous studies from our laboratory had suggested that PKA respon-
expression vector (1). Overexpression of chimera or PKA as described in the legend of Fig. 2.

![Graph showing relative CAT activity](Image)

**Fig. 3.** Reconstituting PKA responsiveness of the PEPCK promoter with different combinations of GAL4 and LexA chimeras of C/EBPs and CREB. HepG2 cells were transfected with 3.5 μg of PKC-V4 in the absence (white bars) or presence (solid bars) of a PKA expression vector (1 μg), 500 ng of each GAL4 fusion expression vector, and 100 ng of LexA fusion expression vector, respectively. The values shown are the means ± S.E. of at least three experiments and are relative to the activity of the reporter gene in the absence of overexpression of chimera or PKA as described in the legend to Fig. 2.

![Graph comparing the ability of GAL4-CREB and wild-type CREB to mediate PKA responsiveness of the PEPCK promoter](Image)

**Fig. 4.** Comparison of the ability of GAL4-CREB and wild-type CREB to mediate PKA responsiveness of the PEPCK promoter. HepG2 cells were transfected with 3.5 μg of either the PKC-V4 or PKC-V2 reporter gene in the absence (white bars) or presence (solid bars) of a PKA expression vector (1 μg), 500 ng of GAL4-CREB or CREB expression vector, and 100 ng of LexA-C/EBPα or LexA-C/EBPβ expression vector. The values shown are the means ± S.E. of at least three experiments and are relative to the activity of the reporter gene in the absence of overexpression of chimera or PKA as described in the legend to Fig. 2.

siveness was inhibited when C/EBPβ was bound to this location (9). However, the precise nature of the GAL4-C/EBPβs used in that study and the present one were different. In the earlier study, the C/EBPβ domain fused to the GALA DNA-binding domain included amino acids 8–315, which included part of the basic region. In the present study, the GAL4-C/EBPβ used contained amino acids 1–108, shown previously to contain a PKA-inducible domain (13). Thus, it appears that the domain extending from amino acids 109–315 contains a region that attenuates the PKA-inducible activity of C/EBPβ when it is in the GAL4 context. We decided to re-examine the potential for C/EBPβ to reconstitute PKA inducibility of the PEPCK promoter from the CRE using the 1–108 fusion protein. In Fig. 5, the results of transfection experiments using the PKC-V3 promoter, a promoter variant containing a GAL4 sequence replacing the CRE, are shown. In the presence of any of the three GAL4 fusion proteins, including C/EBPβ, PKA inducibility was reconstituted. Once again, the absolute degree of responsiveness was greatest when GAL4-CREB was located at the CRE, consistent with the data in Fig. 3.

Three data in Figs. 3 and 5 indicate that the transactivation domain of C/EBPβ contains a potential PKA-inducible domain; however, studies have previously shown that overexpression of full-length C/EBPβ either inhibited or was without any positive effect on PKA responsiveness of the PEPCK promoter, whereas overexpression of C/EBPs enhanced the responsiveness of the PEPCK promoter (9). Taken together, these studies suggest that the bZIP domains of these two isoforms, although highly conserved, may exert unique regulatory properties onto their respective proteins. To test this hypothesis, we engineered hemagglutinin-tagged C/EBP proteins in which the bZIP domains of the α and β isoforms were swapped, creating chimeras with either the transactivation domain of C/EBPα fused to the bZIP domain of C/EBPβ (C/EBPαβ) or vice versa (C/EBPβα) (Fig. 6, top panel). These chimeras were tested, along with the wild-type versions of these isoforms, for their effect on the PKA responsiveness of the PEPCK promoter when overexpressed in HepG2 cells. Overexpression of C/EBPα enhanced the PKA responsiveness of the PEPCK promoter, as did overexpression of either of the two swapped mutants, C/EBPαβ and C/EBPβα (Fig. 6, bottom panel). Overexpression of C/EBPβ, however, showed no ability to enhance PKA responsiveness. These data suggest that the attenuation domain within C/EBPβ that inhibits its PKA-inducible activity may indeed lie outside of the bZIP domain, perhaps in the region between the transactivation domain (amino acids 1–108) and the bZIP domain.

Previous studies from our laboratory identified domains within C/EBPα that are important for mediating its constitutive and PKA-inducible activities (12, 20). This analysis was performed on C/EBPα and related mutants that were bound to the distal sites in the CRU. However, as demonstrated above, C/EBPs can also bind the CRE, which can lead to reconstitution of the PKA responsiveness of the promoter. The PEPCK promoter has a complex array of cis elements that can be occupied...
by multiple transcription factors (25), resulting in very distinct and different promoter "environments" in the vicinity of the CRE compared with that in the distal region of the promoter. Therefore, we were interested in determining whether different motifs within C/EBPα/H9251 were used to mediate its constitutive and PKA-inducible activities when bound to these two different locations.

In Fig. 7, the results of experiments examining the constitutive (panel A) and PKA-inducible (panel B) activities of GAL4-C/EBPα and related mutants when bound to the CRE location (PCK-V3) or to the distal sites (PCK-V5) are shown. GAL4-C/EBPα and the corresponding mutants 50–217, ΔCR1, and ΔCR3 had similar constitutive activities when bound to the LSR (PCK-V5) (Fig. 7A). Only the ΔCR2 mutant had significantly lower activity relative to GAL4-CEBPα. However, when bound to the CRE as measured using the PEPCK reporter gene PCK-V3, the effect of the mutations was very different. The ΔCR2 mutant had similar activity relative to the GAL4-C/EBPα, whereas the mutants 50–217, ΔCR1, and ΔCR3 had substantially reduced activity.

When the PKA-inducible activity of these various mutants was assessed, there again was evidence that the location of the transcription factor on the promoter influenced the relative effect of the mutations. When assessed using the PCK-V5 reporter gene, where the C/EBPα derivatives were bound to the LSR, the ΔCR2 mutant had less than 15% activity of the parent protein, whereas the other three mutants, 50–217, ΔCR1, and ΔCR3, had activities that ranged from 50 to 60% of the parent protein (Fig. 7B). However, when assessed using the PCK-V3 reporter gene, on which the GAL4 fusions are bound to the CRE location, the ΔCR2 mutation had the least effect on the PKA-inducible activity of C/EBPα, retaining about 75% of its activity relative to GAL4-C/EBPα. The 50–217 mutant retained only 20% of its activity relative to the parent molecule, whereas the ΔCR1 and ΔCR3 mutations had similar effects on the PKA-inducible activity, regardless of which of the two locations on the promoter, i.e., on PCK-V3 or -V5, this assessment was made (Fig. 7A).

Finally, we examined the relative abundance of CREB, C/EBPα, and C/EBPβ in HepG2 cells and primary hepatocytes...
Fig. 8. Relative abundance of C/EBPα, C/EBPβ, and CREB in HepG2 cells compared with primary hepatocytes. Nuclear extracts were prepared from HepG2 cells (H) and primary hepatocytes (P), and equivalent amounts of protein were resolved by SDS-PAGE and subjected to Western blot analysis using antibodies against C/EBPα (Mr = ~43 kDa), C/EBPβ (Mr = ~39 kDa), CREB (Mr = ~42 kDa), and TFIIIEα (Mr = ~57 kDa) as described under “Experimental Procedures.”

for comparison purposes. Western analysis of nuclear extracts from these two cell types indicated that the levels of C/EBPα were significantly higher in primary hepatocytes compared with HepG2 cells (Fig. 8), consistent with previous data (26). However, the levels of C/EBPβ and CREB were both substantially greater in HepG2 cells compared with hepatocytes. As a control, we also assessed the levels of the general transcription factor TFIIIEα and observed that they were severalfold higher in HepG2 cells compared with primary hepatocytes. It should be noted that Western analysis of whole cell extracts from these two cell types showed similar relative levels of the transcription factors (data not shown).

DISCUSSION

The CRU of the PEPCK promoter offers a unique model system to examine how the response of a gene to a hormonal signal can be modulated as well as integrated with other signals that affect its rate of transcription. The CRU consists of five cis-elements, some of which are used in other hormone response units within the promoter, thereby suggesting a mechanism whereby integration of signals can occur. For example, the CRE is an important component of both the CRU and the glucocorticoid response unit (27), whereas one of the C/EBP-binding sites in the LSR is a component of both the CRU and the thyroid hormone response unit (28). To fully understand the mechanisms whereby these hormone response units function, as well as to better appreciate the properties they confer onto the promoter, it is necessary as a first step to determine the transcription factors that can bind to the various cis-elements.

In the case of the cis-elements that make up the CRU, in vitro binding assays have indicated that more than one transcription factor can bind to most if not all of the cis-elements involved. The nature of the AP-1-binding protein(s) has not been investigated to any great detail and will not be discussed further. However, the nature of the proteins that can bind to the CRE has received a great deal of attention, and it is clear that in addition to CREB (3), C/EBPα (4) and β (27), some ATF's (29), D-site protein (30), and AP-1-related proteins (31) can all bind to this sequence with similar affinity. Moreover, both C/EBPα (3) and β (6) as well as D-site-binding protein (31) can all bind in vitro to the three distal C/EBP-binding sites in the promoter. We have focused our attention primarily on CREB and C/EBP isoforms. CREB is an obvious candidate because it is a well characterized mediator of cAMP responsiveness and binds only to CRE-related sequences. C/EBP's have been considered to be likely regulators of PEPCK for a number of reasons, including the fact that they bind to several sites in the promoter, as well as the fact that mice with a disruption in the gene for either C/EBPα or C/EBPβ show alterations in the expression and regulation of the PEPCK gene (32–34). We have also observed that cAMP responsiveness can be observed under several different patterns of transcription factor binding. For example, cAMP responsiveness can be observed when either CREB or C/EBPα or β is bound to the CRE, and when either of the two C/EBP isoforms is bound to the three C/EBP-binding sites in the LSR. These studies were limited in that the analysis focused on transcription factor involvement at either the CRE or at the three distal C/EBP-binding sites but never assessed the degree of cAMP responsiveness under conditions where the transcription factors bound to both regions simultaneously were controlled. Nonetheless, the data suggested that different levels of cAMP responsiveness can be achieved with different patterns of transcription factor binding, a mechanism that has been proposed to explain the benefits of hormone response units (7).

In the present study, we attempted to overcome this limitation by using both GAL4 and LexA fusion proteins, along with corresponding sequence changes in the CRE- and C/EBP-binding sites to GAL4 and/or LexA sites, to allow us to assess the cAMP responsiveness of the promoter under conditions of defined transcription factor occupancy. We and others have shown previously that GAL4 derivatives of CREB and C/EBP isoforms retained their transactivation potential, both in terms of constitutive and cAMP-inducible activity (9, 11, 12, 18, 20), and in this paper and elsewhere (16) it was demonstrated that LexA fusions of C/EBP's also retain their activity. Although some differences in the absolute activity of GAL4 and LexA fusions of C/EBP were observed (Fig. 2), they were similar in their PKA-inducible activities, allowing their use to examine the effect of different transcription factor occupancy patterns on the PKA responsiveness of the PEPCK promoter.

The results from the experiments incorporating the use of both GAL4 and LexA fusion proteins to attempt to reconstitute PKA responsiveness (Figs. 3 and 4) served both to confirm the findings of earlier studies and offer new insights as well. In line with earlier studies, the data indicated clearly that in the absence of expressing proteins that occupy both the CRE and the distal sites in the LSR, PKA responsiveness is low or absent. The present studies also confirm previous findings that PKA responsiveness can be observed when CREB, C/EBPα, or C/EBPβ is bound to the CRE and when either C/EBPα or C/EBPβ bind to the distal sites. Taken together, these confirmatory observations provide a considerable degree of validation of our model system.

A major issue that needs addressing is the relevancy of the model system we have used (i.e. transfected PEPCK promoter-CAT vectors in HepG2 cells along with GAL4 and LexA fusions of the relevant transcription factors) and whether it is reflective of the natural regulatory aspects of this gene promoter. This transformed, human hepatoma cell line has been used extensively to characterize the specific cis-elements that make up the CRU of the PEPCK promoter and the corresponding transcription factors that bind to them. It needs to be appreciated and emphasized, however, that the information obtained from this cell line regarding the components of the CRU and the respective transcription factors has been confirmed in a variety of other model systems, including other hepatoma cell lines, transgenic animals, knockout animals, and antisense inhibition models. For example, the importance of the different cis-elements for their contribution in the CRU has been confirmed in transgenic mice where different promoter mutants driving expression of a reporter gene were characterized for their responsiveness to cAMP (35). This transgenic model verified the importance of the CRE and the distal C/EBP sites for the responsiveness of the promoter to cAMP. Expression of a dominant negative C/EBP was shown to inhibit PKA responsiveness of the PEPCK promoter in HepG2 cells (13, 20). Moreover, the particular importance of C/EBPα for participating in this response, which was first demonstrated by our group in HepG2 cells using GAL4 fusions of this protein (9, 20), was
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subsequently confirmed in two different model systems. Cronson and Roesler (8) demonstrated, in the rat hepatoma H4IE cell line, that antisense to C/EBPβ inhibited induction of the endogenous PEPCK gene by cAMP, whereas antisense to C/EBPα was without effect. Furthermore, Croniger et al. (32) showed that in livers of fetal C/EBPα−/− mice, cAMP responsiveness of the PEPCK gene was reduced by 80%, whereas no defect in this responsiveness was observed in C/EBPβ−/− mice. Thus, there is evidence from several model systems that supports the use of HepG2 cells to characterize the CRU and the pertinent transcription factors.

Given this evidential support of the appropriateness of the HepG2 cells for the analysis of the PEPCK promoter and its regulation by the CREB and C/EBP proteins, the Western analysis showing the significant differences in the relative abundance of these factors in HepG2 cells compared with hepatocytes (Fig. 8) was surprising. Despite HepG2 cells having significantly lower levels of C/EBPs but much higher levels of C/EBPβ and CREB relative to hepatocytes, the cAMP responsiveness of the PEPCK promoter assessed by transfection studies or transgenic methodology is relatively similar. These data would seem to suggest that the absolute level of these transcription factors may not be the determining factor for establishing the cAMP responsivity of this promoter, but rather the cAMP responsiveness is determined by the cumulative concentration of two or more of these factors that may have similar/redundant effects on a particular cis-element. On the other hand, the data are also consistent with the hypothesis that the promoter might be able to actively recruit specific transcription factors to particular cis-elements by a “jigsaw puzzle” approach, even when their relative concentrations fluctuate significantly.

There are several new insights that were gained by the present studies. First, it would appear that a similar, maximal level of PKA-inducible activity of the promoter can be achieved by several different transcription factor binding patterns on the cis-elements making up the CRU. Regardless of whether GAL4-CREB, -C/EBPα, or -C/EBPβ was bound to the CRE location of the promoter, the PKA-induced activity was similar when either LexA-C/EBPα or LexA-C/EBPβ was expressed, which directed these proteins to the three distal sites (Fig. 3).

A second insight, suggested from the data just discussed, is that C/EBPα and C/EBPβ appear to be equally effective in their participation of the PKA responsivity from their binding to the three distal sites, regardless of which transcription factor is bound to the CRE. Third, the data indicate that although the maximal PKA-induced promoter activity is similar under several occupancy patterns, the promoter appears to be most responsive to PKA under conditions where CREB is bound to the CRE (Fig. 3) because of the low promoter activity present when CREB is bound to the CRE in the absence of PKA. Thus, the binding of CREB to the promoter is a CREB basal promoter activity low but in a state most sensitive to changes in cAMP levels and thus PKA activation. On the other hand, when C/EBPα is bound to the CRE, the basal activity of the promoter is higher than when CREB is bound, but the fold responsiveness to PKA is lower.

The responsiveness of the promoter when C/EBPβ is bound to the CRE deserves special comment. The present studies show that a GAL4 fusion of C/EBPβ containing amino acids 1–108 can reconstitute PKA responsiveness from the CRE location in the promoter, allowing the conclusion that either CREB, C/EBPα, or C/EBPβ are at least to some extent functionally exchangeable. This observation, however, is inconsistent with previous studies. Our laboratory reported previously that a GAL4 fusion containing amino acids 8–315 of C/EBPβ could not reconstitute PKA responsiveness (13). Moreover, Park et al. (11) showed that overexpression of full-length C/EBPβ inhibited PKA responsiveness of a truncated PEPCK promoter that contained only the CRE and TATA box, whereas overexpression of CREB or C/EBPα enhanced PKA responsiveness. One possible conclusion from all of these studies could be that C/EBPβ contains an attenuation domain extending from amino acids 109–315 that includes part of the DNA-binding domain that modulates the PKA-inducible domain. However, the fact that the PKA-inducible activity can be artificially “un-masked” by deletion of a large portion of the protein would appear to be physiologically irrelevant. Rather, we believe the data are consistent with the conclusion that C/EBPβ does not facilitate PKA responsiveness from the CRE location but rather suppresses it. This conclusion is in fact consistent with antisense studies showing that inhibition of C/EBPs expression in hepatoma cells reduced fold cAMP responsiveness of the endogenous PEPCK gene, whereas inhibition of C/EBPβ expression left cAMP responsiveness intact (8). It should be noted that similar concerns do not exist for CREB and C/EBPα regarding discrepancies between the effects of GAL4 fusions and full-length versions of the molecules. These data are supported by the studies discussed above and by the data in Fig. 4 demonstrating that a similar degree of PKA responsivity is achieved when either the CREB or its GAL4 version is bound to the CRE location.

Precisely what feature of the “attenuation” domain within C/EBPβ provides it with a different activity than C/EBPs is unknown. Because the attenuation domain contains part of the bZIP domain, we hypothesized that it was conferred by distinct features of this domain. Although the bZIP domain is highly conserved between C/EBP isoforms, some amino acid positions differ between the two isoforms, some of which can be potentially phosphorylated and thus may confer unique regulatory properties (reviewed in Ref. 2). Our domain swapping experiments were designed to explore whether the bZIP domain of C/EBPβ provided the attenuation activity. However, we observed that a chimera consisting of the transactivation domain of C/EBPs linked to the bZIP domain of C/EBPβ was still able to reconstitute PKA responsiveness (Fig. 6). Thus, the explanation for the different abilities of these two isoforms to facilitate PKA responsiveness remains elusive.

A fourth and particularly important insight offered by the present studies is that promoter context appears to influence the specific domain in a transcription factor that is used to mediate transactivation. The ability of promoter context to regulate transcription by modifying the precise activity of a transcription factor has been appreciated for some time and can involve a number of mechanisms including transcription factor-induced DNA bending, overlapping binding sites for transcription factors, protein-protein interactions, the structure of the core promoter, and the particular cellular environment (36). Our studies suggest an additional mechanism that is perhaps linked to one or more of those listed above. The conclusion that promoter context can modify the domains used by a transcription factor to mediate transactivation was drawn from the studies shown in Fig. 7. In this experiment, we showed that some mutations in the transactivation domain of C/EBPα had significantly different effects, depending on whether the analysis was performed when the GAL4 fusion was bound to the distal LSR region of the PEPCK promoter or from the CRE location. The promoter context around these two sites is very different (26). The CRE, for example, is immediately juxtaposed to a binding site for nuclear factor-1 on its upstream side and has a TATA box 50 nucleotides downstream from it. The LSR, on the other hand, has three C/EBP-binding
sites along with an AP-1 site, all present in a certain arrangement, as well as a thyroid hormone response element sandwiched in its midst and a binding site for hepatic nuclear factor 1 just downstream of it. Clearly there is the opportunity for unique protein-protein interactions to occur between C/EBPβ and other transcription factors, and these interactions could cause some conformation change that would unmask, or make more accessible, a specific domain of C/EBPβ, which would be used to mediate transactivation. The presentation of different domains might, for example, result in the recruitment of different co-activators and/or contacts with different general transcription factors that may confer a unique transcriptional response. This hypothesis could explain the inability to observe some protein-protein interactions between co-activators and transcription factors, even though a functional interaction can be detected. Regardless of the mechanism involved, our studies provide compelling evidence for an additional level of transcriptional regulation that needs to be taken into account when performing structure/function analysis of transcription factors and argues strongly that analysis of both cis-elements and the corresponding transcription factors should be performed in the context of the natural promoter.

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