Activating Transcription Factor-2 Regulates Phosphoenolpyruvate Carboxykinase Transcription through a Stress-inducible Mitogen-activated Protein Kinase Pathway*

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Several protein-nucleic acid complexes are observed when nuclear extracts from hepatoma cells are assayed for binding to the cAMP response element found in the phosphoenolpyruvate carboxykinase-cytosolic (PEPCK-C) promoter. Although cAMP response element-binding protein and CCAAT/enhancer binding proteins α and β have been identified as liver factors that bind this motif, an uncharacterized, slower migrating complex was also observed. We identify activating transcription factor-2 (ATF-2) as the factor in this complex and show that ATF-2 stimulates expression from the PEPCK-C promoter. ATF-2 is a basic-leucine zipper transcription factor and a target for stress-activated protein kinases. We demonstrate that p38 β mitogen-activated protein (MAP) kinase augments ATF-2 transcription activity on the PEPCK-C promoter, which is consistent with the interpretation that PEPCK-C promoter activity is maintained under stress through a p38 MAP kinase dependent pathway. In this regard, we show that treatment with sodium arsenite, a known activator of p38 MAP kinases, also stimulates expression from the PEPCK promoter. These results show that ATF-2 can stimulate transcription of the PEPCK-C promoter and support a role for stress inducible kinases in the maintenance of PEPCK-C expression.

The enzyme phosphoenolpyruvate carboxykinase-cytosolic (PEPCK-C) catalyzes a regulatory step in gluconeogenesis and is regulated primarily at the level of transcription initiation (1, 2). The PEPCK-C promoter is a model for metabolic regulation of gene expression. It is expressed primarily in liver, kidney, small intestine, and adipose tissue, where it integrates cues arising from diverse signaling pathways. For example, PEPCK-C transcription in liver is induced by the action of glucagon, thyroid hormone, and glucocorticoids (3–5); whereas the action of a single hormone, insulin, exerts dominant negative control (6). PEPCK-C transcription also responds to nutritional status, where starvation signals act inductively and a carbohydrate-rich meal results in repression (3).

The minimal sequence of the PEPCK-C promoter sufficient for reduplication of hormonal regulation in liver has been mapped to the region encompassing −460 to +73 (7–10), and many of the transcription factors that bind elements in this region have been identified (2, 11). For example, glucagon secretion leads to an increase in cAMP levels, which exerts effects by inducing factors that bind to the element denoted cyclic AMP response element I (CRE-1) and may also induce factors that bind to the element denoted P3. Prima facie, CREB might be anticipated as the primary factor impacted by increased cAMP levels. However, PEPCK-C expression was reported to be normal in CREB knockout mice (12). Binding to CRE-1 has also been reported for C/EBPα (13), C/EBPβ (14), AP1 (15), and D-site binding protein (16). In fact, evidence from gene deletion experiments supports a role for C/EBPs α and β in the regulation of PEPCK-C expression (17).

The PEPCK-C gene is essential in humans (18), and its expression is not only maintained but, in fact, induced following partial hepatectomy (19). We uncovered the PEPCK-C CRE-1 motif TTACGTCA during a search of promoter sequences as a perfect match to the consensus reported for ATF-2 homodimers (20). ATF-2, a basic-leucine zipper transcription factor, is expressed in liver tissue (21) and exhibits increased DNA binding and transcriptional activation activities following phosphorylation of specific residues by p38 MAP kinases (22) or c-Jun N-terminal MAP kinases (23, 24). Because PEPCK-C transcription increases after partial hepatectomy (19), a stress condition, we considered that ATF-2 may contribute to regulation through the CRE-1 element. We show here that ATF-2 binds the PEPCK-C CRE-1 element, that sense ATF-2 expression correlates with increased promoter activity, and that antisense ATF-2 expression correlates with decreased promoter activity. Furthermore, expression of p38 β MAP kinase, a known modifier of ATF-2, correlates with increased reporter activity, while cotransfection of p38 β MAP kinase and ATF-2 shows augmented transcriptional activation. These results support a role for ATF-2 in the maintenance of PEPCK-C expression in the liver that may have special relevance under stress conditions.

EXPERIMENTAL PROCEDURES

Cell Lines, Culture Conditions, and Plasmid Constructions—Fao cells are a hepatoma derivative that exhibit many of the characteristics of differentiated hepatocytes and were kindly provided by Dr. Mary Weiss (Institut Pasteur) (25). Fao cells were maintained in Coon's modified, Ham's F-12 medium (Life Technologies, Inc.) supplemented with 5% fetal calf serum, penicillin, streptomycin, and glucose. The ATF-2 expression plasmid has been described (21). The human p38 β expression plasmid was provided by Jiahuai Han (The Scripps Research Institute) (26). The Reporter plasmid PEPCK – 275 was prepared by polymerase chain reaction of rat genomic DNA using 5’ primer GG-
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TACC ACA GTC AGCG GTC AAA GTT TAG TCA ATC 3′ and 3′ primer
GCTCGA GAG ATC TCA GAG CGT CTC GCC GG, which encompasses.
positions −275 through +73 of the PEPPCK-C promoter using number-
ning according to Roessler et al. (27).

For sodium arsenite treatment, 1 × 10⁶ Fao cells were plated on a
10-cm dish, transfected by standard calcium phosphate treatment,
and fed complete medium for 48 h. Medium was supplemented
with 50 μM sodium arsenite (Sigma) for 6 h prior to cell harvest.

Gel Shift Analysis—Full-length C/EBPα and ATF-2 proteins were
prepared in vitro transcription-coupled translation (TNT; Promega,
Madison, WI) under conditions described by the manufacturer. A dou-
ble-stranded oligonucleotide encoding the PEPPCK-C CRE-1 sequence
(promoter positions −99 to −76) was used for gel shift analysis: 5′
CCGGCCCC TTACGTCA GAGGCG. Binding reactions were assembled
without probe and held 5 min on ice followed by 5 min at room tem-
perature. Probe was added with further room temperature incubation for
30 min. Samples were separated in 4% acrylamide, 0.5× TBE (0.045 M
Tris, 0.045 M boric acid, 1.0 mM EDTA (pH 8.0) gels run at 200 V
constant voltage (28).

RESULTS AND DISCUSSION

ATF-2 Binds the PEPPCK-C CRE-1 Sequence—The transcrip-
tion factors CREB (29), C/EBPα (13), C/EBPβ (14), AP1 (15), and
D-site-binding protein (16) have been reported to bind the
PEPPCK-C CRE-1 element (Fig. 1A) (13). Notably, the CRE-1
site matches the consensus sequence reported for ATF-2 ho-
modimers (Fig. 1A) (20) rather than the CREB or AP1 consensus
sequences. Because ATF-2 expression was recently demonstra-
ted in rat liver (21), we wondered if it bound the CRE-1 site.

As shown in Fig. 1B, the slowest migrating shift produced with
hepatoma nuclear extracts (lane 1) is supershifted when anti-
ATF-2 serum is included in the binding reaction (lane 3). By
comparison, C/EBPα, a factor reported to bind this site, also
produces a supershift complex (lane 2) upon inclusion of spe-
cific antisera. However, the C/EBPα supershift complex is
not abundant and the band from which the supershift complex
derives is not obvious. To demonstrate the expected migration
positions for these protein-nucleic acid complexes, shift reac-
tions were formed with full-length C/EBPα and ATF-2 pre-
bred by in vitro transcription-coupled translation (lanes 4 and
5, respectively). These results demonstrate that ATF-2, like
C/EBPα, binds the CRE-1 element in the PEPPCK-C promoter.

Modulation of ATF-2 Expression Levels Correlates with Re-
porter Gene Activity—To determine what effect, if any, ATF-2
might have on PEPPCK-C promoter activity, we transfected Fao
hepatoma cells with a luciferase reporter construct containing an
enhancer transcribed from the PEPPCK-C promoter (Fig. 1A) (27).
This region contains the CRE-1 motif as well as the P3(I) motif, both
reported binding sites for C/EBPα. As shown in Fig. 2A, lucif-
erase activity increased up to 4-fold with increasing amounts of
transfected sense ATF-2 expression plasmid.

To extend these observations, we cotransfected the reporter
vector with increasing amounts of antisense ATF-2 expression
plasmid. As shown in Fig. 2B, basal reporter activity decreased
about 2-fold when cells were cotransfected with plasmid encod-
ing antisense ATF-2. The Western blot (Fig. 2C) shows nuclear
extracts from a representative transfection experiment and
demonstrates that ATF-2 protein levels decrease upon anti-
sense ATF-2 expression (lane 2) and increase upon sense ATF-2
expression (lane 3) relative to steady state ATF-2 levels (lane 1).
These results are consistent with a role for ATF-2 in the
regulation of PEPPCK-C gene expression.

p38 MAP Kinase Augments ATF-2-dependent Transcrip-
tional Activation—ATF-2 is a known substrate for p38 MAP
kinases (26). The p38 MAP kinase family of stress-activated protein
kinases in the MAP kinase family (30–33). Therefore, we tested the effect of p38β expression on a reporter construct containing the
minimal thymidine kinase promoter modified by proximal inser-
tion of two ATF-2 consensus sites (2× CRE-1-Luc) (21). As
shown in Fig. 3A, p38β MAP kinase expression correlated with an
8-fold increase in 2× CRE-1-Luc reporter activity. This is
comparable with expression of ATF-2 alone, which stimulates activity approximately 10-fold. When ATF-2 and p38 MAP kinase are cotransfected, reporter activity increased about 24-fold. In contrast, expression of a transfected p38 MAP kinase "dead" mutant showed activity that was comparable with basal levels (data not shown). These results suggest that p38 MAP kinase augments ATF-2 activity in Fao hepatoma cells.

We next tested the PEPCK reporter, which shows much higher basal levels of transcription despite the fact that cultures were propagated in the presence of complete medium (serum and glucose), repressing conditions for PEPCK-C expression. As shown in Fig. 3B, p38 MAP kinase alone stimulates transcription about 2-fold. This result is comparable with the 2-fold effect observed upon expression of ATF-2 alone. Upon cotransfection, ATF-2 and p38 MAP kinase stimulate PEPCK reporter activity about 5-fold. These results are analogous to those obtained using the minimal promoter/reporter construct, although they show lower levels of transcriptional activation. This is likely due to endogenous factors that contribute to regulation of the PEPCK-C promoter. Given the level of induction observed, the results are consistent with the notion that ATF-2 functions in maintenance of PEPCK-C transcription, likely during conditions that activate a stress-inducible MAP kinase pathway(s).

As a specificity control for the effects we observed with p38 MAP kinase, we cotransfected protein kinase A or p42 ERK-1 with ATF-2. As shown in Fig. 4, expression of protein kinase A alone showed reporter activity twice basal. This is not surprising, as PEPCK-C is a known target for cAMP mediated effects. When ATF-2 is cotransfected, reporter activity increases 2.6-fold, again, equivalent to ATF-2 alone. These observations argue that the transcriptional augmentation we observed with ATF-2 and p38 MAP kinase is not generalizable to any protein kinase, but is restricted to stress activated protein kinases.

An Oxidative Stress That Activates p38 MAP Kinases Also Stimulates PEPCK Reporter Activity—Because sodium arsenite activates p38 MAP kinases (32), a finding we confirmed in Fao cells using antipeptide sera specific for phosphorylated p38 MAP kinase (data not shown), we wondered if PEPCK-C pro-
ATF-2 is expressed in most tissues, is a substrate for stress-inducible MAP kinases (both the p38 and the stress-activated protein/JNK subfamilies), and exhibits increased transcription activating and DNA binding activities in the phosphorylated form (23, 24). The p38 MAP kinases and the JNKs modify the same residues in ATF-2, Thr68/71, and Ser90 (35). Although the studies presented here were limited to p38 MAP kinases, we expect that JNK MAP kinases would result in a similar augmentation of ATF-2-dependent transcriptional starting. From conditions that are repressing for endogenous PEPCK-C activity, serum and high glucose, we observed stimulation of PEPCK-C promoter activity by transfection of ATF-2 alone or p38β MAP kinase alone. When ATF-2 and p38β MAP kinase were cotransfected, augmented transcriptional activation was observed. Failure to observe augmented transcription with PKA and ATF-2 or with ERK-1 and ATF-2 indicates that the transcriptional increase was specific. In addition, treatment of Fao cells (in the presence of serum) with sodium arsenite, an activator of p38 MAP kinases, resulted in a similar increase over basal promoter activity. The fact that this effect was eliminated upon expression of antisense ATF-2 again indicates specificity. These results support a role for ATF-2 in the maintenance of PEPCK-C expression in hepatocytes in response to extracellular signals that activate stress-inducible MAP kinases.

Our studies are consistent with available results from gene deletion experiments. Homozygous deletion of the genes encoding transcription factors CREB, CREM, C/EBPβ, and D-site-binding protein show no obvious defect in glucose homeostasis (2). In contrast, deletion of the gene for C/EBPα results in hypoglycemia, failure to store liver glycogen, failure to develop fat tissue, and lethality (17). However, C/EBPα is neither an acute phase-reactive protein (36), nor does it’s expression increase following partial hepatectomy (19). In fact, C/EBPα decreases under both of these conditions, consistent with observations regarding its antiproliferative effects in cell lines (37–39). Although C/EBPβ expression increases after partial hepatectomy (19), it is not a known substrate for stress-induced MAP kinases, suggesting that C/EBP proteins and CREB/CREM proteins are not likely activators of PEPCK-C expression during conditions of stress. Although transcriptional effects observed in cell culture systems do not always faithfully reflect regulation as it occurs in vivo (2), our studies support a role for ATF-2 as a regulator of the hepatocyte PEPCK-C promoter during conditions of stress.

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FIG. 5. Sodium arsenite, an activator of p38 MAP kinases, stimulates ATF-2 reporter activity in Fao transfecteds. Fao cultures were transfected with the indicated expression plasmid (ATF-2 or antisense ATF-2), allowed to recover, and then induced with 50 μM sodium arsenite for 6 h prior to harvest. Transfection results were normalized to β-galactosidase activity and represent the mean of three independent experiments (error bars show S.D. of the mean).
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