Activating Transcription Factor-2 Mediates Transcriptional Regulation of Gluconeogenic Gene PEPCK by Retinoic Acid

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All-*trans*-retinoic acid (RA) is known to increase the rate of transcription of the PEPCK gene upon engagement of the RA receptor (RAR). RA also mediates induction of specific gene transcription via several signaling pathways as a nongenomic effect. Here we show that RA upregulation of PEPCK promoter activity requires the cAMP response element (CRE)-1 in addition to the RA-response element and that activating transcription factor-2 (ATF-2) binds the CRE element to mediate this effect. Furthermore, we show that RA treatment potentiates ATF-2–dependent transactivation by inducing specific phosphorylation of ATF-2 by p38β kinase. ATF-2 activation by RA blocked the inhibitory intramolecular interaction of ATF-2 amino and carboxyl terminal domains in a p38β kinase–dependent manner. Consistent with these results, RA treatment increased the DNA binding activity of ATF-2 on the PEPCK CRE-1 sequence. Taken together, the data suggest that RA activates the p38β kinase pathway leading to phosphorylation and activation of ATF-2, thereby enhancing PEPCK gene transcription and glucose production. *Diabetes* 51:3400–3407, 2002

The PEPCK promoter is a well-defined model for metabolic regulation of gene expression (1). PEPCK, which catalyzes a regulatory step in gluconeogenesis, is expressed primarily in liver, kidney, small intestine, and adipose tissue, where its synthesis is regulated at the level of transcriptional initiation. In liver, dysfunctional regulation of the PEPCK promoter is associated with the pathophysiology of type 2 diabetes (2,3). The PEPCK promoter integrates cues arising from diverse signaling pathways. PEPCK mRNA is induced by glucocorticoids, thyroid hormone, or glucagon (4), whereas insulin results in a repression of the promoter activities in a dominant manner (5). The PEPCK promoter fragment encompassing −460 to +73 was demonstrated to be sufficient for hormonal regulation in liver, and many of the transcription factors that bind elements in this region have been identified (6,7). Proteins demonstrated to bind and impact regulation of the PEPCK promoter include cAMP response element–binding protein (CREB), CCAAT/enhancer-binding protein (C/EBP)α, C/EBPβ, activating transcription factor-2 (ATF-2), nuclear factor-1 (NF-1), hepatocyte nuclear factor (HNF)3, glucocorticoid receptor (GR), thyroid hormone receptor (TR), retinoic acid receptor (RAR), and retinoid X receptor (RXR). The energy-balance state can affect signals for the PEPCK gene regulation through activating CREB, C/EBPα, and C/EBPβ, whereas ATF-2 mediates the stress response signals (8).

Although the physiological role of RA in the regulation of PEPCK gene transcription has not been fully characterized, all-*trans*-retinoic acid (RA)-deficient rats exhibit impaired gluconeogenesis. Given that PEPCK catalyzes a rate-limiting step in gluconeogenesis, it is possible that RA is required for maintenance of basal PEPCK expression in hepatocytes. RA levels apparently do not change appreciably in healthy adult animals (9), suggesting that it does not acutely affect PEPCK transcription. RA may be permissive for both, generating relatively high basal levels of transcription and elevating hormonal responsiveness of the PEPCK gene (10). Indeed, when RA stimulation is accompanied by glucocorticoids or cAMP, an additive or even synergistic effect on PEPCK gene expression is observed (11).

It is well established that RA binds to the RAR and RXR nuclear receptors, resulting in the formation of RAR-RXR heterodimers or RXR homodimers (rev. in 12,13). These complexes have the ability to induce gene transcription via binding to the RA response element (RARE) in the promoters of RA-inducible genes (13,14). Other mechanisms through which retinoids induce their biological effects in malignant cells include inhibition of activation of the AP-1 protein via a CREB-binding protein–regulated mechanism (15,16), inhibition of the c-Jun NH_2-terminal kinase (JNK) (17), modulation of histone acetylation (18), and upregulation of transforming growth factor-2 and IGF-binding protein-3 expression (19). Recent evidence also indicates that the extracellular signal–regulated kinase (ERK)-2

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ATF, activating transcription factor; bZIP, basic-leucine zipper; C/EBP, CCAAT/enhancer-binding protein; CRE, cAMP response element; CREB, cAMP response element–binding protein; ERK, extracellular signal–regulated kinase; GR, glucocorticoid receptor; HNF, hepatocyte nuclear factor; JNK, c-Jun NH_2-terminal kinase; MAP, mitogen-activated protein; NF-1, nuclear factor-1; RA, all-*trans*-retinoic acid; RAR, RA receptor; RARE, RA-response element; RXR, retinoid X receptor; TR, thyroid hormone receptor.

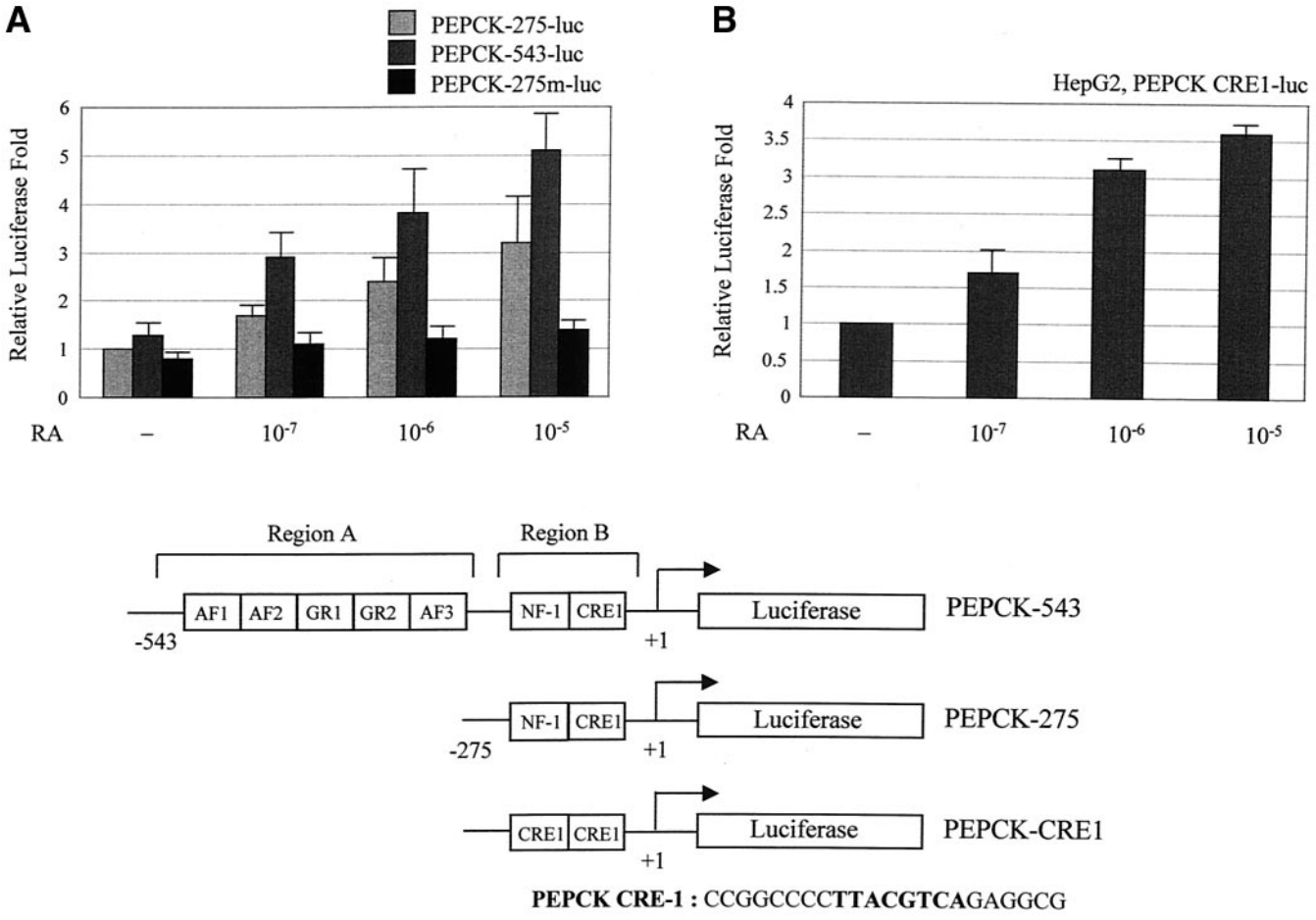


FIG. 1. RA increases CRE-dependent PEPCK promoter activity. The hepatoma cell line HepG2 was transfected with 20 ng of reporter plasmid, PEPCK-275-luc/PEPCK-543-luc and PEPCK-275 *m*-luc (*A*), and PEPCK-CRE-1-luc (*B*) with increasing concentrations of RA. Transfection results were normalized to β-galactosidase activity and represent the average of three independent experiments, with fold induction indicated relative to reporter alone. A schematic representation of the two luciferase reporter constructs is shown below. Region A contains the GRU composed of two glucocorticoid regulatory elements, three accessory factor-binding sites, and a CRE. This region provides several factor-binding sites for RAR, RXR, GR, TR, C/EBP, and HNF-3 (12). Region B contains a CRE (CRE-1) (−99 to −76) and is immediately adjacent to a NF-1–binding site (12).

mitogen-activated protein kinase is activated by RA in HL-60 cells and mediates the induction of cell differentiation and growth arrest (20).

p38β kinase leads to ATF-2 modulation of gene expression (8). ATF-2 stimulates transcription of the PEPCK promoter, supporting a role for stress inducible kinases in the maintenance of PEPCK expression. In this study, we show that RA impacts the transactivation activity of ATF-2 by activation of p38β kinase, ultimately leading to upregulated expression of the PEPCK gene.

RESEARCH DESIGN AND METHODS

Plasmids. A full-length rat ATF-2 cDNA was generated by RT-PCR and verified by sequencing. ATF-2 was subcloned into pCMX1 as *Bam*HI (21) and *Bam*HI/*Kpn*I fragments, respectively. pCMX1 was a gift from Catherine Thompson (Carnegie Institution of Washington) and was used in coupled transcription-translation reactions. In vitro translation products were verified by [^{35}S]Met incorporation and SDS-PAGE analysis. The human p38β expression plasmid was provided by Jiahuai Han (The Scripps Research Institute) (22). The reporter plasmids PEPCK-275 and PEPCK-543 were constructed by PCR amplification of rat genomic DNA encompassing positions –275 or −543 through +73 of the PEPCK promoter. The cAMP response element (CRE)-1 mutant PEPCK-275 reporter was prepared by standard mutagenesis, changing CCGGCCCCT**TACG**TCAGAGGCG to CCGGCCCCT**TTTT**CAGAGGCG.

Western blot analysis. HepG2 cells were harvested in Nonidet P-40 lysis buffer, and 30–50 μg nuclear protein was fractionated by SDS-PAGE. Proteins were electroblotted to Immobilon-P (Millipore), and membranes were blocked in Tris-buffered saline, 0.02% Tween 20, containing 5% nonfat milk. Specific antibody for ATF-2 was previously described (23), and antibodies for phospho–ATF-2, p38β, and phospho-p38β were obtained from Santa Cruz Biotechnology. Secondary detection utilized goat anti-rabbit–conjugated horseradish peroxidase (Amersham Pharmacia Biotech) visualized with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) essentially as described (23).

Gel mobility shift analysis. Nuclear extracts were prepared from HepG2 cells following stimulation with hormones or kinase inhibitors as indicated in the figure legends. Approximately 10 μg nuclear extract was immunoprecipitated with an ATF-2–specific antibody and incubated with a probe. A double-stranded oligonucleotide encoding the PEPCK CRE-1 sequence (promoter positions −99 to −76) was used for gel shift analysis: 5′ CCGGCCCCTT ACGTCAGAGGCG (6). Binding reactions were assembled without probe and held 5 min on ice followed by 5 min at room temperature. Probe was added with further room temperature incubation for 30 min. Samples were separated in 4% acrylamide, 0.5× TBE (0.045 mol/l Tris, 0.045 mol/l boric acid, and 1.0 mmol/l EDTA [pH 8.0]) gels run at 200 V constant voltage (24).

Transient transfection and luciferase assays. HepG2 cells were transfected by the standard calcium phosphate method (24). Cells were incubated with DNA precipitates for 16 h, washed, and maintained in complete medium 48 h before harvest. Relative luciferase and β-galactosidase activities were determined as described (23). Basal promoter activity is reported as the activity observed after transfection of the reporter plus an appropriate amount of empty expression vector. In all cases, transfection data represent the mean of three independent experiments.

Mammalian two-hybrid assay. Cells were seeded with growth medium supplemented with 10% fetal bovine serum and 1% antibiotics and cotransfected with expression vectors encoding Gal4-DNA–binding domain fusions

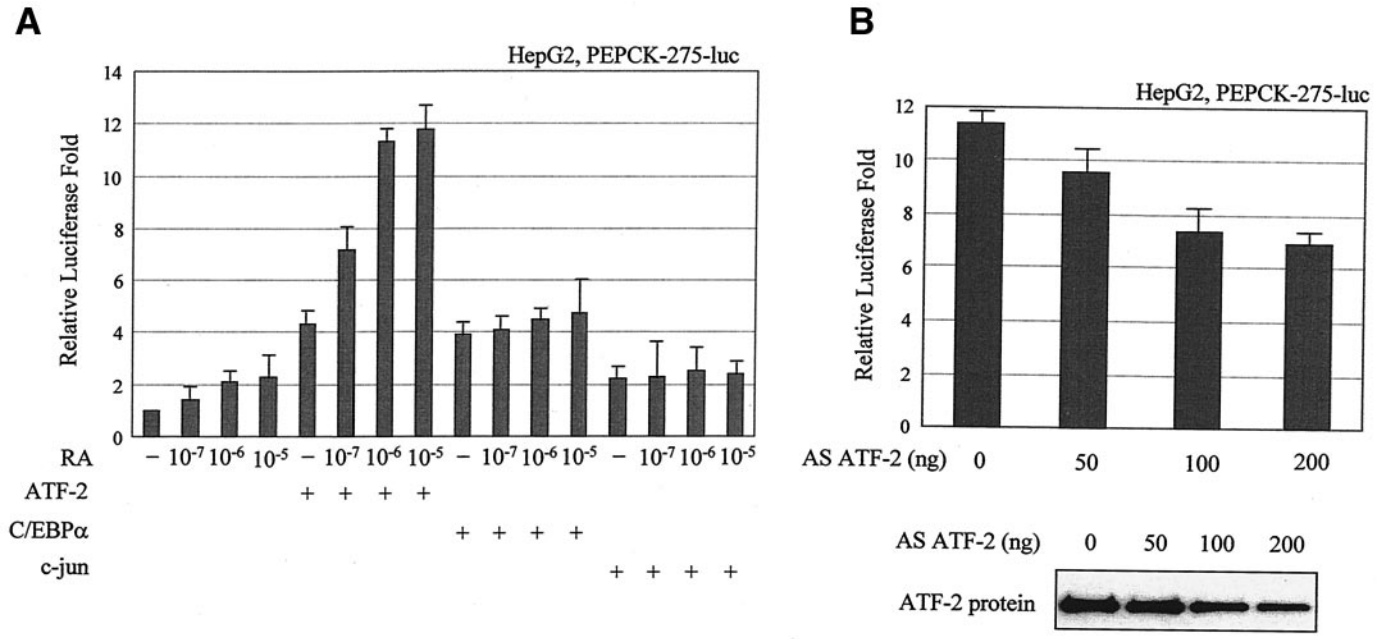


FIG. 2. RA enhances ATF-2–dependent transactivation of PEPCK promoter. *A*: HepG2 cells were transfected with the indicated expression plasmids and assayed for reporter activity with the PEPCK-275 reporter construct. A total of 20 ng reporter vector was transfected into HepG2 cells along with 50 ng of the indicated expression plasmid, either in the absence or presence of the indicated concentrations of RA. Then, 48 h after transfection, cells were harvested for luciferase activities. All the transfection results were normalized to β-galactosidase activity, and the presented results represented the average of four independent experiments, with fold induction over the level observed with the reporter alone. *B*: Antisense expression of ATF-2 affects RA-induced activation of PEPCK promoter activity. HepG2 cells were transfected with the indicated expression plasmids and assayed for reporter activity with the PEPCK-275 reporter construct. A total of 20 ng reporter vector was transfected into HepG2 cells along with the indicated amounts of antisense ATF-2 expression plasmids in the presence of 1 μmol/l RA treatment. Then, 48 h after transfection, cells were harvested for luciferase activities. The protein expression of ATF-2 was determined by using Western blotting with ATF-2–specific antibody.

(pCMX/Gal4N-ATF-2N) and VP16-activation domain fusions (pCMX/VP16/-, pCMX/VP16-ATF-2C, or pCMX/VP16-ATF-2-full) as well as the previously described Gal4-tk-luc reporter plasmid. After 48 h, cells were harvested and the luciferase activity was normalized to the β-galactosidase internal standard. All the results represent the average of at least three independent experiments.

RESULTS

RA increases CRE-1–dependent PEPCK promoter activity. In a previous study, we showed that the HBx transactivator encoded by hepatitis B virus increased RXR-dependent transactivation of the PEPCK promoter in an RA-dependent fashion through direct protein-protein interaction (45). To extend these studies, we have examined the effect of additional nuclear receptor ligands (RA, 9-*cis*-RA, dexamethasone, and thyroid hormone [T3]) on transactivation activity of the PEPCK-275 and PEPCK-543 promoters. A schematic representation of these luciferase reporter constructs is shown at the bottom of Fig. 1. Region A contains the glucocorticoid response unit (GRU) composed of two glucocorticoid regulatory elements, three accessory factor-binding sites, and a CRE. This region provides several factor-binding sites for RAR, RXR, GR, TR, C/EBP, and HNF-3 (12). Region B contains a CRE (CRE-1) (−99 to −76) and is immediately adjacent to a NF-1–binding site (12).

The hepatoma cell line HepG2 was transfected with reporter plasmids PEPCK-275-luc and PEPCK-543-luc (Fig. 1*A*) or PEPCK-CRE-1-luc (Fig. 1*B*) with increasing concentrations of RA. RA treatment stimulated the activity of PEPCK-275 and PEPCK-543 reporters in a dose-dependent manner (Fig. 1*A*). It is noteworthy that the promoter region of PEPCK-275 does not have an RARE sequence for RAR/RXR binding. Interestingly, Scott et al. (44) also showed that RA increased transactivation of a PEPCK-306 promoter in H4IIE cells ~3.3-fold.

To determine whether the CRE-1 sequence was responsible for RA-induced transactivation of the PEPCK-275 reporter, a mutant PEPCK –275 reporter was constructed by changing the CRE-1 sequence (RESEARCH DESIGN AND METHODS). The CRE-mutant PEPCK –275 promoter was not responsive to increasing concentrations of RA (Fig. 1*B*). However, transient transfection (Fig. 1*B*) shows that RA activated the PEPCK CRE-1 promoter essentially equivalently to activation of the PEPCK –275 reporter (Fig. 1*A*). These results indicate that RA can stimulate PEPCK gene transcription through the CRE-1 site in addition to the RARE-1 site.

RA enhances ATF-2–dependent transactivation of PEPCK promoter. To identify the transcription factor mediating RA responsiveness on the PEPCK-275 promoter, expression plasmids encoding ATF-2, C/EBPα, and c-Jun were cotransfected with the PEPCK-275 reporter in the presence or absence of RA. As shown in Fig. 2*A*, transfection of ATF-2 largely increased PEPCK promoter activity in a dose-dependent manner. In contrast, transfection of C/EBPα or c-Jun failed to show significant RA-dependent changes in transactivation. To extend these observations, we cotransfected the reporter vector with increasing amounts of antisense ATF-2 expression plasmid. As shown in Fig. 2*B*, basal reporter activity decreased approximately two- to fourfold when cells were cotransfected with plasmid encoding antisense ATF-2. The West-

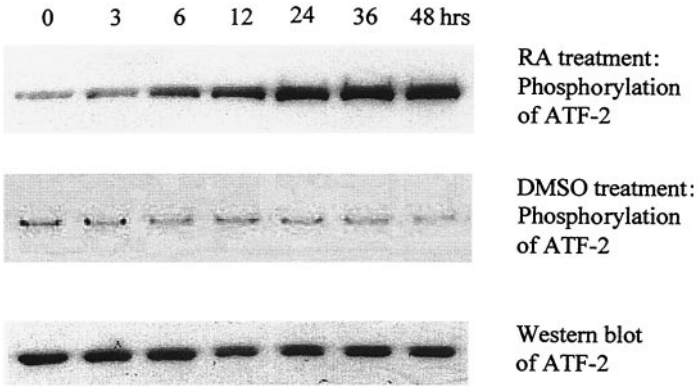


FIG. 3. Phosphorylation of ATF-2 by RA. HepG2 Cells were incubated with 1 μmol/l RA or DMSO (as vehicle) for the indicated times. Total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated/activated form of ATF-2. The protein amount of ATF-2 was probed with ATF-2–specific antibody as a loading control.

ern blot (Fig. 2*B*) shows nuclear extracts from a representative transfection experiment and demonstrates that ATF-2 protein levels decrease upon antisense ATF-2 expression (Fig. 2*B*, *lanes 2–4*) relative to steady-state ATF-2 levels (Fig. 2*B*, *lane* 1). These results are consistent with a role for ATF-2 in the regulation of PEPCK gene expression following RA treatment.

Phosphorylation of ATF-2 following RA treatment. Transcriptional activation by ATF-2 is enhanced by phosphorylation of NH_2-terminal threonine residues through several mitogen-activated protein kinases. We tested whether ATF-2 phosphorylation is increased by RA treatment by Western blot analysis with an anti-phospho-ATF-2–specific antibody. Hepatoma cells were incubated with 1 μmol/l RA for varying times (3–48 h), lysed, and total cell lysates analyzed by SDS-PAGE immunoblots with an antibody specific for phosphorylated/activated ATF-2. As shown in Fig. 3, RA treatment potentiated strong phosphorylation of ATF-2 in a time-dependent manner, reaching a maximum at ~24 h. Hyperphosphorylation is still observable, even after 48 h (Fig. 3). In contrast, the amount of total ATF-2 protein is similar in RA-stimulated and -unstimulated cells. These results show that ATF-2 phosphorylation is induced after RA treatment.

p38β kinase partly mediates ATF-2 activation after RA treatment. RA treatment of cells induces a time- and dose-dependent phosphorylation of p38 kinase, and such phosphorylation results in activation of its catalytic domain (25). To identify whether the increased phosphorylation of ATF-2 after RA treatment results from activation of the p38β kinase pathway, we cotransfected either a wild-type p38β kinase or an inactive p38β kinase mutant (T188A, Y190F), which cannot be phosphorylated by p38β kinase kinase (22), into cells. As shown in Fig. 4*A*, CRE-dependent transactivation after RA treatment increased with p38β kinase overexpression but not by JNK overexpression. In addition, we used a p38β-mutant (kinase dead) expression vector in the transient transfection assay, which shows that the RA effect was abrogated upon expression of the dominant interfering kinase (Fig. 4*A*). Finally, a p38 kinase selective chemical inhibitor, SB203580 (20 μmol/l), blocked the RA-dependent transactivation of –275 PEPCK, whereas an ERK inhibitor, PD98059, essentially showed no effect (Fig. 4*A*). To further confirm that p38β kinase activation is involved in the RA response, we assayed for the activation of p38β kinase itself by phosphorylation of the kinase after RA treatment. Total cell extracts were prepared at the times indicated and assayed for expression and phosphorylation of p38β kinase. As shown in Fig. 4*B*, although p38β kinase expression levels did not change, its phosphorylation state increased from 6 to 12 h after RA treatment. This time course correlates with the phosphorylation of ATF-2, as shown in Fig. 3. Hence, these data provide direct evidence that RA stimulation leads to p38β kinase activation and ATF-2 phosphorylation, potentiating the transactivation activity of ATF-2.

RA signaling blocks the inhibitory intramolecular interaction of ATF-2. Previous studies showed that the basic-leucine zipper (bZIP) region of ATF-2 interacts with the amino-terminal region intramolecularly (26), inhibiting the transactivation activity of ATF-2. From this result we proposed that RA signaling might block this intramolecular interaction through phosphorylation of ATF-2. To test this possibility, we investigated the effect of RA on the intramolecular interaction between the amino- and carboxyl-terminal regions of ATF-2 by using the mammalian two-hybrid assay. Coexpression of Gal4-ATF-2N and VP16-ATF-2C or VP16-ATF-2-full enhanced Gal4-tk-luc–dependent transactivation, whereas RA treatment clearly inhibited the transactivation mediated by association of amino-terminal ATF-2 sequences (Gal4-ATF-2N) with carboxy-terminal ATF-2 sequences (VP16-ATF-2-full) (Fig. 5*A*). Given that p38β kinase modulates ATF-2–dependent transactivation of the PEPCK promoter after RA treatment (Fig. 4*A*), we tested the effect of constitutively active p38β kinase in the mammalian-2 hybrid assay. The assay shown in Fig. 5*A* was repeated with p38β kinase coexpression, the expression level of which is shown by p38β kinase–specific antibody (Fig. 5*B*, *bottom*). As shown in Fig. 5*B*, p38β kinase expression largely inhibited transactivation by ATF-2 in the mammalian-2 hybrid assay. These results strongly suggest that RA signaling inhibits the intramolecular interaction between the NH_2-terminal and COOH-terminal regions of ATF-2 by a mechanism involving the p38β kinase pathway.

RA treatment potentiates DNA binding by ATF-2. The results shown in Fig. 5 suggest that the DNA binding domain of ATF-2 would be more exposed after RA treatment, possibly leading to more efficient binding to its cognate DNA element. To analyze whether increased DNA binding activity of ATF-2 may be involved in the RA-dependent stimulation of the PEPCK promoter activity, electromobility shift assay analysis was used. Nuclear extracts were prepared from control cells, RA-treated cells, or cells treated with phosphatase inhibitors and/or

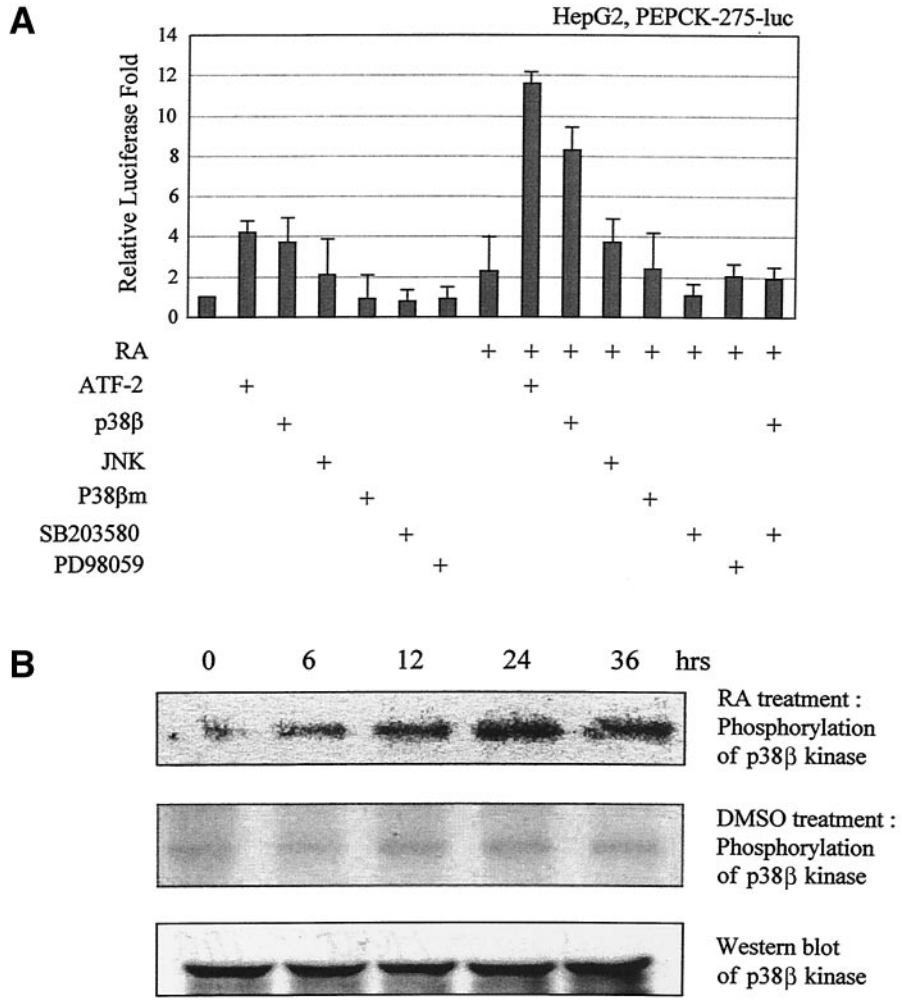


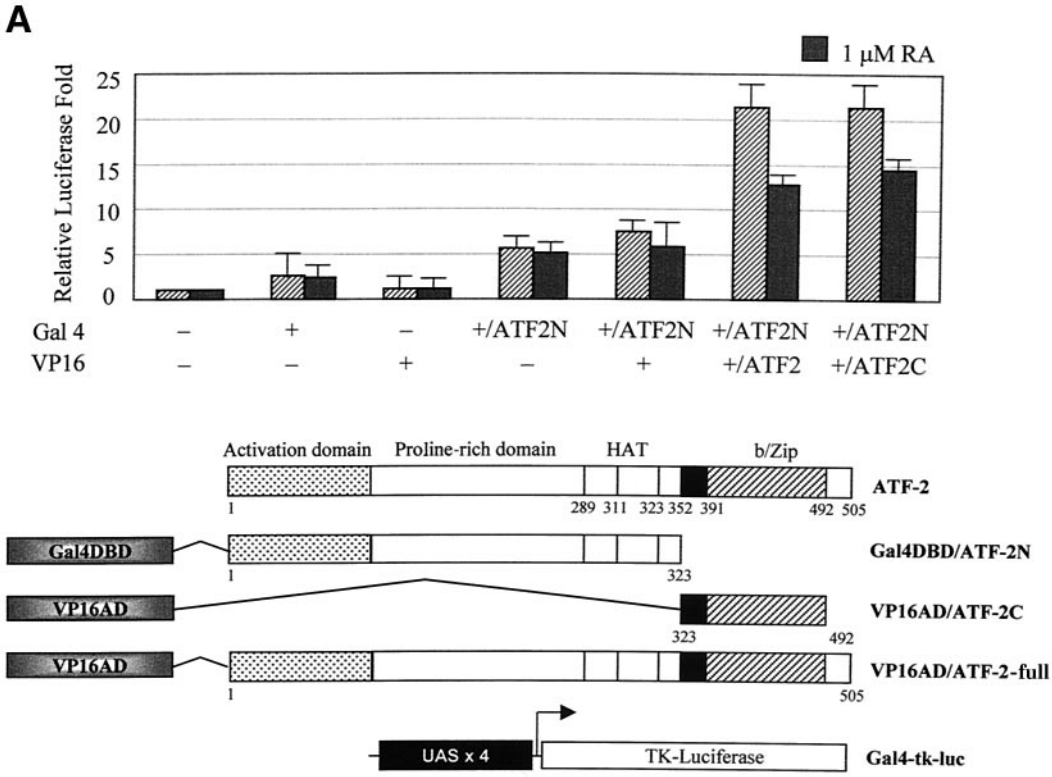
FIG. 4. p38β kinase partly mediates ATF-2 activation by RA. *A*: HepG2 cells were transfected with the indicated expression plasmids and assayed for reporter activity using a native promoter reporter containing the PEPCK-275 reporter construct in the absence or presence of 1 μmol/l RA together with kinase inhibitors SB203580 (20 μmol/l) and PD98059 (25 μmol/l). A total of 5 μg reporter vector was transfected with 1 μg of the indicated expression plasmid. All results were normalized to β-galactosidase activity and represent the mean of three independent experiments. *B*: RA induces phosphorylation of p38β kinase in cells. Cells were incubated with 1 μmol/l RA or DMSO for the indicated times. Total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of p38β kinase. The blot shown below was stripped and reprobed with an antibody against p38β kinase to control for loading.

protein kinase inhibitors. Immunoprecipitates for ATF-2 from 10 μg of nuclear extract, as indicated, were incubated with a ^{32}P-labeled probe spanning the PEPCK CRE-1 promoter region between −99 and −76. As shown in Fig. 6, increasing concentrations of RA led to increased DNA binding activity by ATF-2. As would be predicted, ATF-2 binding was inhibited by the p38β kinase inhibitor SB203580, but not by the ERK inhibitor PD98059. This result is also consistent with induced DNA binding activity for ATF-2 through a p38β kinase pathway (Fig. 6). Similarly, the phosphatase inhibitor okadaic acid weakly potentiated CRE–ATF-2 complex formation. These results are consistent with a mechanism whereby the DNA binding activity of ATF-2 is induced through activation of a p38β kinase pathway downstream of RA treatment.

DISCUSSION

RA-transduced signals induce specific gene transcription by activating RAR binding to its cognate DNA element as a heterodimer with RXR. In addition, RA has been reported to mediate signal transduction through what has been termed a nongenomic mechanism (25). But, the mechanism of activation of Rac by RA is not clear and whether it involves a classical RARE-mediated genomic effect or occurs through some nongenomic action of RA will need to be determined in future studies. In nonsmall cell lung cancer, RARs and RXRs are expressed but are not transcriptionally activated by RA, such that RA does not increase the expression of genes typically activated by RXR-RAR heterodimers (20). Thus, these cells exhibit a transcriptional defect specific to retinoid nuclear receptors. In this study, we show that RA activates a p38β kinase pathway, subsequently phosphorylating ATF-2 for increased transcriptional regulation of the PEPCK promoter.

The transcription factors CREB (27), C/EBPα (28), C/EBPβ (29), AP1 (30), and D-site binding protein (31) have been reported to bind the PEPCK CRE-1 element. We previously noted that the CRE-1 site matches the consensus sequence reported for ATF-2 homodimers (8), rather than CREB or AP1 consensus elements previously noted (32). ATF-2, a bZIP transcription factor, is highly expressed in liver and brain, tissues central to glucose homeostatic mechanisms. Most bZIP transcription factors including Jun, CREB, C/EBPβ, ATF-2, and C/EBP homology protein are known substrates for mitogen-activated protein (MAP) kinases, which leads to induction of transcriptional activity. There are three main classes of MAP kinases, ERKs, stress-activated protein kinases, JNKs, and p38-MAPKs. ERKs are preferentially activated in response to growth factors, cytokines, and mitogens via the well-known Ras/Raf-1/MEK pathway. JNKs and p38-MAPKs are activated in response to a variety of cell stresses, including ultraviolet irradiation, proinflammatory cytokines, protein



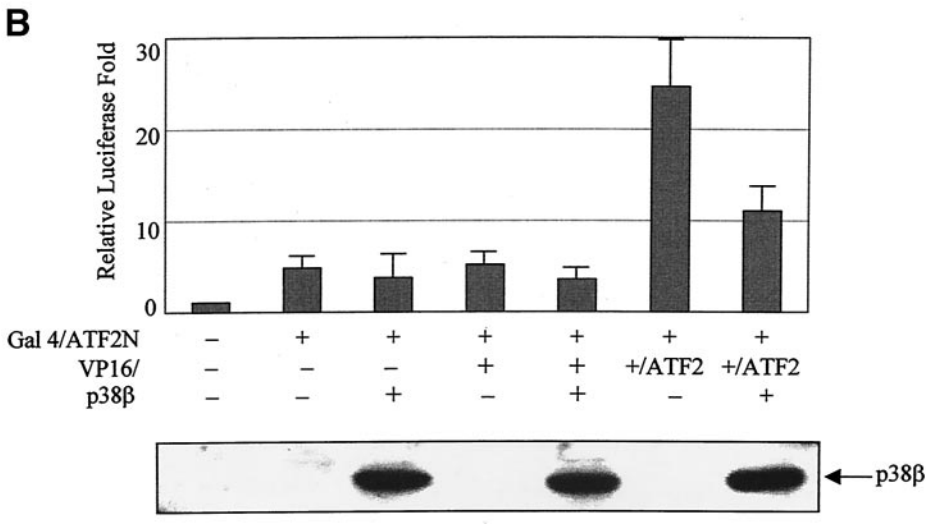


FIG. 5. RA treatment inhibits the intramolecular interaction of ATF-2. *A*: RA inhibits the intramolecular interaction of ATF-2. The mammalian expression plasmids encoding GAL-ATF-2N and VP16-ATF-2-full were transfected into HepG2 cells, as indicated. Then, 48 h after transfection in the absence or presence of 1 μmol/l RA, cells were harvested for luciferase activities. All the transfection results were normalized to β-galactosidase activity, and the presented results represented the average of three independent experiments, with fold induction over the level observed with the reporter alone. *B*: p38β kinase inhibits the intramolecular interaction of ATF-2. RA inhibits the intramolecular interaction of ATF-2. The mammalian expression plasmids encoding GAL-ATF-2N and VP16-ATF-2-full were transfected with p38β kinase expression plasmid into HepG2 cells, as indicated. Then, 48 h after transfection, cells were harvested for luciferase activities. The transient protein expression of p38β kinase was confirmed by using Western blotting with p38β kinase-specific antibody.

synthesis inhibitors, osmotic shock, and chemical stress, such as that induced by H_2O_2 and other reactive oxygen species. These kinases are activated in response to a variety of stimuli and mediate signals important for the generation of various biological responses (33). Two members of the MAP kinase family, ERK2 and JNK, have been previously shown to be involved in the generation of retinoid responses (34). ERK2 is activated in response to RA treatment in the HL-60 acute myelogenous leukemia cell line (20), where activation is required for the induction of RA-dependent cell differentiation and growth arrest. The engagement of the p38 signaling cascade by RA is of considerable interest, as this pathway is critical for the generation of signals required for important biological activities in response to stress and/or engagement of certain cytokine receptors. These include activation of transcription factors (35), transcriptional regulation (36), and induction of cytokine production (35). Therefore, our finding that the p38β kinase pathway is activated during RA treatment of cells led us to further studies to determine the functional role of p38β kinase and ATF-2 activation in the induction of RA responses for regulating PEPCK gene transcription.

Transcription of the PEPCK gene is enhanced by glucagon and glucocorticoids and inhibited by insulin in a dominant manner (37). These actions of insulin are phosphatidylinositol-3 kinase dependent and are not blocked by inhibition of the p70 S6 kinase or the ERK pathways (38–40). Interestingly, activation of the p38β kinase pathway led to increased PEPCK gene transcription after RA-dependent phosphorylation and activation of ATF-2 (Fig. 4). It is therefore important to determine whether the

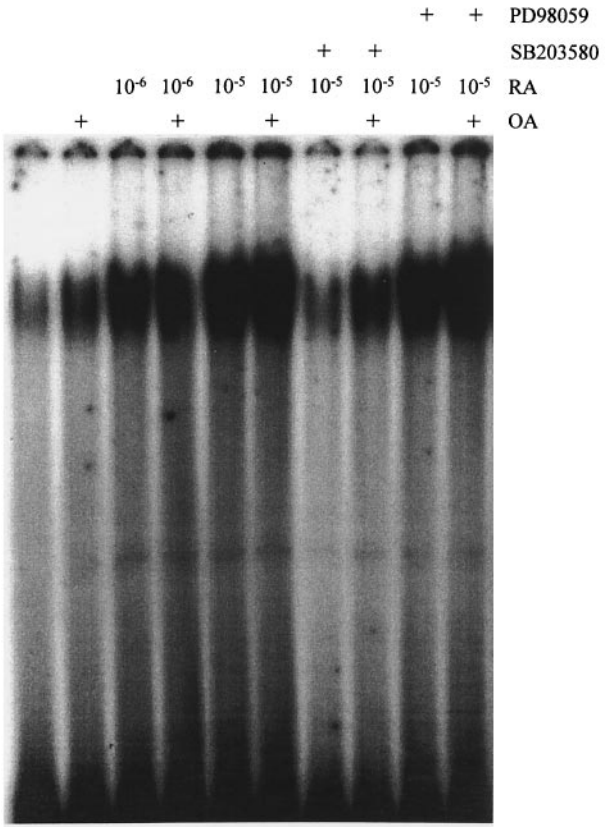


FIG. 6. RA treatment increases the DNA binding of ATF-2 on PEPCK promoter. A double-stranded oligonucleotide probe containing the CRE-1 site (from –99 to –76) of the PEPCK promoter was used in electrophoretic mobility shift analysis. HepG2 cells, which were treated with RA (10^{-6} mol/l or 10^{-5} mol/l), SB203580, PD98059, or phosphatase inhibitor okadaic acid (OA), were harvested and applied to immunoprecipitation using ATF-2–specific antibody. Each 10 μg of immunoprecipitated ATF-2 proteins was added in a binding reaction.

p38β pathway affects the insulin-dependent regulation of the PEPCK gene.

Gel shift analysis is commonly used to evaluate the phosphorylation status of ATF-2 (Fig. 6). It is important to note that RA induces a conformational change in the ATF-2 protein, such that homodimers form inefficiently, resulting in avid formation of heterodimers and binding a cognate DNA element, leading an efficient inducible transcription of PEPCK gene in a dominant manner. As shown in Fig. 5, RA signaling decreased the inactive ATF-2 conformation by inhibiting the intramolecular interaction. We already presented the formation of ATF-2:C/EBPα heterodimers, describing cross-family dimerization with ATF-2, a possible general property for C/EBP family proteins (23). The RA-induced inhibition of the intramolecular interaction of ATF-2 may allow the heterodimerization of ATF-2 with a variety of transcription factors and the functional interaction with coactivators. Heterodimer formation of ATF-2 with other transcription factors is appealing from a regulatory viewpoint because of the asymmetry that is generated. By selecting for asymmetric DNA elements, heterodimers bind their target in an orientation-dependent fashion, presenting distinct surfaces for interaction with proteins bound to adjacent DNA sites. Both the position and the orientation of protein-binding sites within some enhancer elements are important for the formation of what has been termed the "stereospecific" complex (41). Although the function of heterodimers comprising ATF-2 as a dimeric partner is not known, it has been proposed that cross-family heterodimers serve as a common target for the integration of signals arising from different extracellular stimuli. In this model, each subunit of the heterodimer would be modified by a unique protein kinase, p38β kinase, in response to one signal, RA, of the signals being transduced. Because each subunit of the heterodimer is independently modified in this scenario, signals from two pathways converge, leading to the appropriate changes in the pattern of PEPCK gene expression (42,43).

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REFERENCES

1. Hanson RW, Reshef L: Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. *Annu Rev Biochem* 66:581–611, 1997
2. Valera A, Pujol A, Pelegrin M, Bosch F: Transgenic mice overexpressing PEPCK develop NIDDM. *Proc Natl Acad Sci U S A* 91:9151–9154, 1994
3. Rosella G, Zajac JD, Baker L, Kaczmarczyk SJ, Andrikopoulos S, Adams TE, Proietto J: Impaired glucose tolerance and increased weight gain in transgenic rats overexpressing a non-insulin-responsive PEPCK gene. *Mol Endocrinol* 9:1396–1404, 1995
4. Loose DS, Cameron DK, Short HP, Hanson RW: Thyroid hormone regulates transcription of the gene for cytosolic phosphoenolpyruvate carboxykinase (GTP) in rat liver. *Biochemistry* 24:4509–4512, 1985
5. Kioussis D, Reshef L, Cohen H, Tilghman SM, Iynedjian PB, Ballard FJ, Hanson RW: Alterations in translatable messenger RNA coding for phosphoenolpyruvate carboxykinase (GTP) in rat liver cytosol during deinduction. *J Biol Chem* 253:4327–4332, 1978
6. McGrane MM, Yun JS, Moorman AF, Lamers WH, Hendrick GK, Arafah BM, Park EA, Wagner TE, Hanson RW: Metabolic effects of developmental, tissue-, and cell-specific expression of a chimeric phosphoenolpyruvate carboxykinase (GTP)/bovine growth hormone gene in transgenic mice. *J Biol Chem* 265:22371–22379, 1990
7. Short MK, Clouthier DE, Schaefer IM, Hammer RE, Magnuson MA, Beale EG: Tissue-specific, developmental, hormonal, and dietary regulation of rat phosphoenolpyruvate carboxykinase-human growth hormone fusion genes in transgenic mice. *Mol Cell Biol* 12:1007–1020, 1992
8. Cheong J, Coligan JE, Shuman JD: Activating transcription factor-2 regulates phosphoenolpyruvate carboxykinase transcription through a stress-inducible mitogen-activated protein kinase pathway. *J Biol Chem* 273: 22714–22718, 1998
9. Blomhoff R, Green MH, Berg T, Norum KR: Transport and storage of vitamin A. *Science* 250:399–404, 1990
10. Lucas PC, Forman BM, Samuels HH, Granner DK: Specificity of a retinoic acid response element in the phosphoenolpyruvate carboxykinase gene promoter: consequences of both retinoic acid and thyroid hormone receptor binding. *Mol Cell Biol* 11:5164–5170, 1991
11. Pan C, Hoeppner W, Chou JY: Induction of phosphoenolpyruvate carboxykinase gene expression by retinoic acid in an adult rat hepatocyte line. *Biochemistry* 29:10883–10888, 1990
12. Croniger C, Leahy P, Reshef L, Hanson RW: C/EBP and the control of phosphoenolpyruvate carboxykinase gene transcription in the liver. *J Biol Chem* 273:31629–31632, 1998
13. Chambon P: The retinoid signaling pathway: molecular and genetic analyses. *Semin Cell Biol* 5:115–125, 1994
14. Kastner P, Mark M, Chambon P: Nonsteroid nuclear receptors. What are genetic studies telling us about their role in real life? *Cell* 83:859–869, 1995
15. Kamei Y, Xu L, Heinzel T, Tochia J, Kurokawa R, Gloss B, Lin SC, Heyman RA, Rose DW, Glass CK, Rosenfeld MG: A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* 85:403–414, 1996
16. Chen JY, Penco S, Ostrowski J, Balaguer P, Pons M, Starrett JE, Reczek P, Chambon P, Gronemeyer H: RAR-specific agonist/antagonists which dis-

sociate transactivation and AP1 transrepression inhibit anchorage-independent cell proliferation. *EMBO J* 14:1187–1197, 1995
17. Lee HY, Walsh GL, Dawson MI, Ki Hong W, Kurie JM: All-trans-retinoic acid inhibits Jun N-terminal kinase-dependent signaling pathways. *J Biol Chem* 273:7066–7071, 1998
18. Nagy L, Kao HY, Chakravarti D, Lin RJ, Hassig CA, Ayer DE, Schreiber SL, Evans RM: Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* 89:373–380, 1997
19. Han GR, Dohi DF, Lee HY, Rajah R, Walsh GL, Hong WK, Cohen P, Kurie JM: All-trans-retinoic acid increases transforming growth factor-beta2 and insulin-like growth factor binding protein-3 expression through a retinoic acid receptor-alpha-dependent signaling pathway. *J Biol Chem* 272:13711–13716, 1997
20. Yen A, Roberson MS, Varvayanis S, Lee AT: Retinoic acid induced mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase-dependent MAP kinase activation needed to elicit HL-60 cell differentiation and growth arrest. *Cancer Res* 58:3163–3172, 1998
21. Friedman AD, McKnight SL: Identification of two polypeptide segments of CCAAT/enhancer-binding protein required for transcriptional activation of the serum albumin gene. *Genes Dev* 4:1416–1426, 1990
22. Jiang Y, Chen C, Li Z, Guo W, Gegner JA, Lin S, Han J: Characterization of the structure and function of a new mitogen-activated protein kinase (p38beta). *J Biol Chem* 271:17920–17926, 1996
23. Shuman JD, Cheong J, Coligan JE: ATF-2 and C/EBPalpha can form a heterodimeric DNA binding complex in vitro: functional implications for transcriptional regulation. *J Biol Chem* 272:12793–12800, 1997
24. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual*. 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989
25. Alsayed Y, Uddin S, Mahmud N, Lekmine F, Kalvakolanu DV, Minucci S, Bokoch G, Platanias LC: Activation of Rac1 and the p38 mitogen-activated protein kinase pathway in response to all-trans-retinoic acid. *J Biol Chem* 276:4012–4019, 2001
26. Li XY, Green MR: Intramolecular inhibition of activating transcription factor-2 function by its DNA-binding domain. *Genes Dev* 10:517–527, 1996
27. Quinn PG, Wong TW, Magnuson MA, Shabb JB, Granner DK: Identification of basal and cyclic AMP regulatory elements in the promoter of the phosphoenolpyruvate carboxykinase gene. *Mol Cell Biol* 8:3467–3475, 1998
28. Park EA, Roesler WJ, Liu J, Klemm DJ, Gurney AL, Thatcher JD, Shuman J, Friedman A, Hanson RW: The role of the CCAAT/enhancer-binding protein in the transcriptional regulation of the gene for phosphoenolpyruvate carboxykinase (GTP). *Mol Cell Biol* 10:6264–6272, 1990
29. Park EA, Gurney AL, Nizielski SE, Hakimi P, Cao Z, Moorman A, Hanson RW: Relative roles of CCAAT/enhancer-binding protein beta and cAMP regulatory element-binding protein in controlling transcription of the gene for phosphoenolpyruvate carboxykinase (GTP). *J Biol Chem* 268:613–619, 1993
30. Gurney AL, Park EA, Giralt M, Liu J, Hanson RW: Opposing actions of Fos and Jun on transcription of the phosphoenolpyruvate carboxykinase (GTP) gene: dominant negative regulation by Fos. *J Biol Chem* 267:18133–18139, 1992
31. Roesler WJ, McFie PJ, Dauvin C: The liver-enriched transcription factor D-site-binding protein activates the promoter of the phosphoenolpyruvate carboxykinase gene in hepatoma cells. *J Biol Chem* 267:21235–21243, 1992
32. Benbrook DM, Jones NC: Different binding specificities and transactivation of variant CRE's by CREB complexes. *Nucleic Acids Res* 22:1463–1469, 1994
33. Schaeffer HJ, Weber MJ: Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol Cell Biol* 19:2435–2444, 1999
34. Wang X, Studzinski GP: Inhibition of p38MAP kinase potentiates the JNK/SAPK pathway and AP-1 activity in monocytic but not in macrophage or granulocytic differentiation of HL60 cells. *J Cell Biochem* 82:68–77, 2001
35. Raingeaud J, Whitmarsh AJ, Barrett T, Derijard B, Davis RJ: MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol Cell Biol* 16:1247–1255, 1996
36. Uddin S, Lekmine F, Sharma N, Majchrzak B, Mayer I, Young PR, Bokoch GM, Fish EN, Platanias LC: The Rac1/p38 mitogen-activated protein kinase pathway is required for interferonα-dependent transcriptional activation but not serine phosphorylation of stat proteins. *J Biol Chem* 275:27634–27640, 2000
37. O'Brien RM, Granner DK: Regulation of gene expression by insulin. *Biochem J* 278:609–619, 1991
38. Sutherland C, O'Brien RM, Granner DK: Phosphatidylinositol 3-kinase, but not p70/p85 ribosomal S6 protein kinase, is required for the regulation of phosphoenolpyruvate carboxykinase gene expression by insulin. *J Biol Chem* 270:15501–15506, 1995
39. Gabbay RA, Sutherland C, Gnudi L, Kahn BB, O'Brien RM, Granner DK, Flier JS: Insulin regulation of PEPCK gene expression does not require activation of the Ras/MAP kinase signaling pathway. *J Biol Chem* 271:1890–1897, 1996
40. Sutherland C, Waltner-Law M, Gnudi L, Kahn BB, Granner DK: Activation of the Ras-MAP kinase-RSK pathway is not required for the repression of PEPCK gene transcription by insulin. *J Biol Chem* 273:3198–3204, 1998
41. Tjian R, Maniatis T: Transcriptional activation: a complex puzzle with few easy pieces. *Cell* 77:5–8, 1994
42. van Dam H, Wilhelm D, Herr I, Steffen A, Herrlich P, Angel P: ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-jun induction in response to genotoxic agents. *EMBO J* 14:1798–1811, 1995
43. Hsu W, Kerppola TK, Chen PL, Curran T, Chen-Kiang S: Fos and Jun repress transcription activation by NF-IL6 through association at the basic zipper region. *Mol Cell Biol* 14:268–276, 1994
44. Scott DK, Mitchell JA, Granner DK: Identification and characterization of a second retinoic acid response element in the phosphoenolpyruvate carboxykinase gene promoter. *J Biol Chem* 271:6260–6264, 1996
45. Kong HJ, Hong SH, Lee MY, Kim HD, Lee JW, Cheong J: Direct binding of hepatitis B virus X protein and retinoid X receptor contributes to phosphoenolpyruvate carboxykinase gene transactivation. *FEBS L* 483:114–118, 2000