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ORIGINAL PAPER

Activation of TR4 orphan nuclear receptor gene promoter by cAMP/PKA and C/EBP signaling

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Abstract In earlier studies, we had suggested that the fasting signal induces TR4 orphan nuclear receptor expression in vivo. The detailed mechanism(s), however, remain unclear. In this study, we found that cAMP/PKA, the mediator of fasting and glucagon signals, could induce TR4 gene expression that in turn modulates gluconeogenesis. Mechanistic dissection by in vitro studies in hepatocytes demonstrated that cAMP/PKA might trigger C/EBP α and β binding to the selective cAMP response element, which is located at the TR4 promoter, thus inducing TR4 transcription. We also demonstrated that the binding activity of C/EBPs to the TR4 promoter is increased in response to cAMP treatment. Together, our data identified a new signaling pathway from the fasting signal → cAMP/PKA → C/EBP α and β → TR4 → gluconeogenesis in hepatocytes; and suggested that TR4 could be an important regulator to control glucose homeostasis. The identification of activator(s)/inhibitor(s) or ligand(s) of TR4 may provide us an alternative way to control gluconeogenesis.

Keywords TR4 · Orphan nuclear receptor · Cyclic AMP · Protein kinase A · CAAT/enhancer binding protein · Gene promoter · Chromatin immunoprecipitation

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Introduction

Expression of many genes is modulated by the intracellular second messenger, cyclic adenosine monophosphate (cAMP), in response to different signals from the extracellular environment. In the absence of cAMP, protein kinase A (PKA) is an inactive tetramer; when the cAMP level in the cell rises; it can bind to the regulatory subunit of PKA, and this causes dissociation of active catalytic subunits [1]. The regulation of cAMP/PKA axis is mediated via a cAMP response element (CRE) [2]. Phosphoenolpyruvate carboxykinase (PEPCK) is strongly activated by cAMP in liver. It contains several CREs, and is regulated by CRE binding protein (CREB) and CAAT/enhancer binding protein (C/EBP) [3]. Both C/EBPα and C/EBPβ isoforms can activate the PEPCK promoter via different C/EBP binding sites, acting alone or together with a coactivator(s) [4]. In C/EBPα-deficient mice, pups died within 2 h after birth because of delayed induction of gluconeogenesis. C/EBPβ-deficient mice (C/EBP$\beta^{-/-}$), on the other hand, exhibit two phenotypes: a lethal phenotype following birth and surviving adult mice display reduced induction of hepatic PEPCK and other gluconeogenic genes [5].

In the TR4 knockout (TR4$^{-/-}$) mouse model, the ability to induce PEPCK gene expression was impaired. TR4 has also been demonstrated to bind to PEPCK promoter and regulate PEPCK gene expression to control glucose homeostasis [6]. This regulation of PEPCK by TR4 may be through a ligand-independent manner. For some orphan nuclear receptors, ligand identification, such as bile acids for farnesoid X receptor and oxysterols for liver X receptor (LXR), has been achieved [7]. However, a greater proportion of orphan nuclear receptors, including TR4, remain without an identified ligand. The ligand-independent pathway for the activation of a nuclear receptor or orphan

nuclear receptor has been demonstrated. It goes through signaling transduction cascades from plasma membrane molecules such as cAMP, and then activates receptor's transcription [8].

Previous studies showed that TR4 expression is induced by some signals, such as retinoic acid and changes of calcium concentration [9]. The increased intracellular levels of calcium may activate several signal transduction pathways resulting in distinct patterns of gene expression via the Ca^{2+} calmodulin-dependent kinase (CaMK) [10]. C/EBPβ serine 276 can be phosphorylated by CaMK, and the phosphorylated C/EBPβ had higher CRE-binding ability on the target gene and activated its expression [11]. Hepatic expression of TR4 is also induced by fasting in vivo and by cAMP/PKA second messenger pathway in vitro. Collectively, we hypothesize that TR4 is one of the regulators, which mediates the cAMP/PKA and C/EBP action leading to PEPCK gene expression and glucose metabolism. Therefore, studying the pathways that regulate TR4 expression would help to clarify the ligand-independent mechanism(s) by which the cellular environment modulates TR4 transcription and function, and the identification of the cAMP/PKA → C/EBP → TR4 → PEPCK pathway could be a potential target for intervention in metabolic diseases.

Results

cAMP/PKA increases TR4 mRNA levels

A previous study demonstrated that hepatic TR4 expression is robustly induced by fasting, and that both PEPCK expression and hepatic glucose production are positively associated with TR4 expression [6]. TR4 promoter activity also can be induced by cAMP/PKA signaling activators. These data lead us to hypothesize that TR4 gene expression was regulated by fasting or starvation signals, such as glucagon. In vivo, PEPCK gene transcription is induced during a fast by glucagon via a cAMP-dependent pathway [12]. The cAMP/PKA signaling pathway is activated by the glucagon receptor, epinephrine receptor, and their coupled G-proteins. In order to test the role of cAMP/PKA signaling on TR4 gene expression, we performed studies using activators and inhibitors of the pathway. In order to test the effect of cAMP/PKA on endogenous TR4 mRNA amount, Hepa 1-6 cells were exposed to cAMP/PKA modulators. Application of 100 μM stable cAMP analog 8-Br-cAMP and 50 μM forskolin significantly increased the TR4 mRNA expression. The PKA inhibitor, KT5720, (1 μM) had little effect on TR4 expression (Fig. 1a). In order to determine whether the cAMP/PKA action on TR4 is Hepa 1-6 cell-specific or not, TR4 and PEPCK gene expressions were also examined in human hepatoma HepG2 cells. As shown in Fig. 1b, the

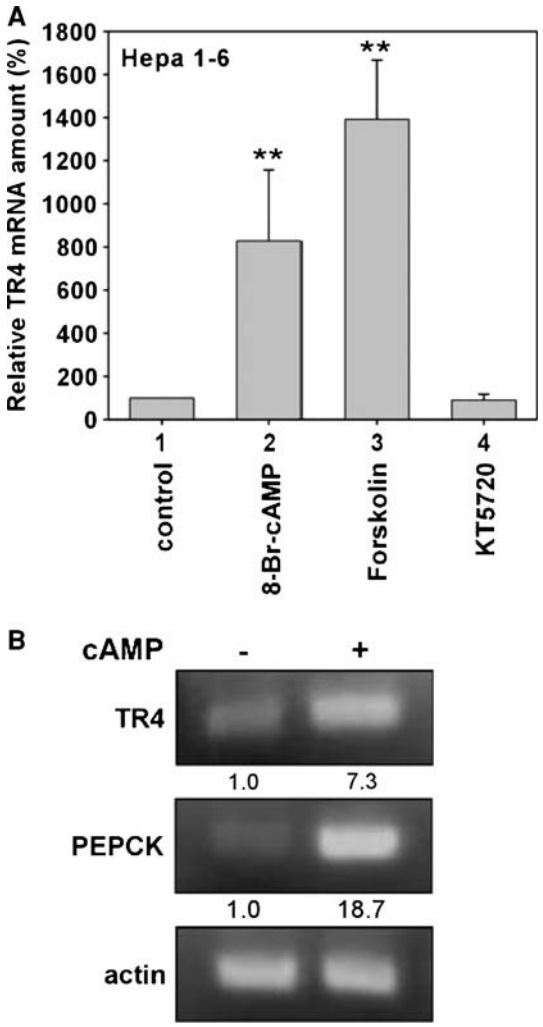

Fig. 1 Induction of TR4 mRNA levels in response to the cAMP/PKA axis. **a** TR4 gene expression was assayed by Q-PCR in Hepa 1-6 cells treated with 8-Br-cAMP, forskolin, and KT5720. Bars represent the mean ± SEM of three independent experiments (**$P < 0.01$ vs. mock control). **b** Semiquantitative RT-PCR of Hepa 1-6 cells. Induction of TR4 and PEPCK gene expression in vitro in the presence of cAMP. β-Actin gene expression was used as an internal control

significantly induced expression of TR4 gene by cAMP may cause a dramatic induction of endogenous PEPCK mRNA expression, although the increased PEPCK gene expression might be partially due to a direct cAMP effect through the CRE on its promoter. Taken together, these results suggest that cAMP/PKA positively regulates TR4 mRNA expression, and indicate that the TR4 promoter is accountable for the cAMP sensitivity, and in turn activates PEPCK gene expression.

C/EBP stimulates TR4 and PEPCK gene expression

C/EBP has been known to mediate cAMP/PKA signaling with effects on PEPCK induction [13]. We hypothesize that the up-regulation of TR4 transcription by cAMP/PKA may

be mediated through the binding of C/EBPs to TR4 promoter. We then addressed the question of whether C/EBP could positively regulate the activity of TR4 promoter in hepatocytes. Transient transfection of C/EBPα, C/EBPβ, and C/EBPβ dominant negative mutant (C/EBPβ-DN) with TR4-Luc reporter into Hepa 1-6 cells demonstrated that both C/EBP isoforms activated TR4 reporter activity, but not the C/EBPβ-DN (Fig. 2a). C/EBPβ more strongly induced TR4 promoter activity compared with C/EBPα (Fig. 2a). Figure 2b also showed the dose-dependent induction of TR4-Luc reporter activity by C/EBPβ. Similar results were obtained in endogenous TR4 mRNA expression studies, in Hepa 1-6 cells where C/EBP induced TR4 mRNA expression, while C/EBPβ-DN failed (Fig. 2c). In order to verify that the TR4 mRNA induced by C/EBP is universal in hepatocytes, we repeated the experiment in human hepatic cell line. Similar patterns were also shown in HepG2 cells, with TR4 and PEPCK gene expression induced in cells transfected with C/EBPα and C/EBPβ (Fig. 2d). Collectively, our data suggest that C/EBPs are the downstream mediators of cAMP/PKA action on the induction of TR4 gene transcription, and consequently, they activate TR4 target genes, such as PEPCK.

C/EBPs bind to TR4 promoter in vivo

Through sequence analysis of the TR4 gene promoter, we identified putative C/EBP binding sites located at the positions from −801 to −816 and from −884 to −897 in human and from −123 to −138 in mouse (Fig. 3a). We then applied the ChIP assay to confirm the binding of C/EBPs to the TR4 promoter in intact HepG2 cells. The C/EBP antibody used in ChIP has been demonstrated to detect C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε in human cells. As shown in Fig. 3b, C/EBPs were recruited to the TR4 region of the TR4 promoter (upper panel, lane 4), but

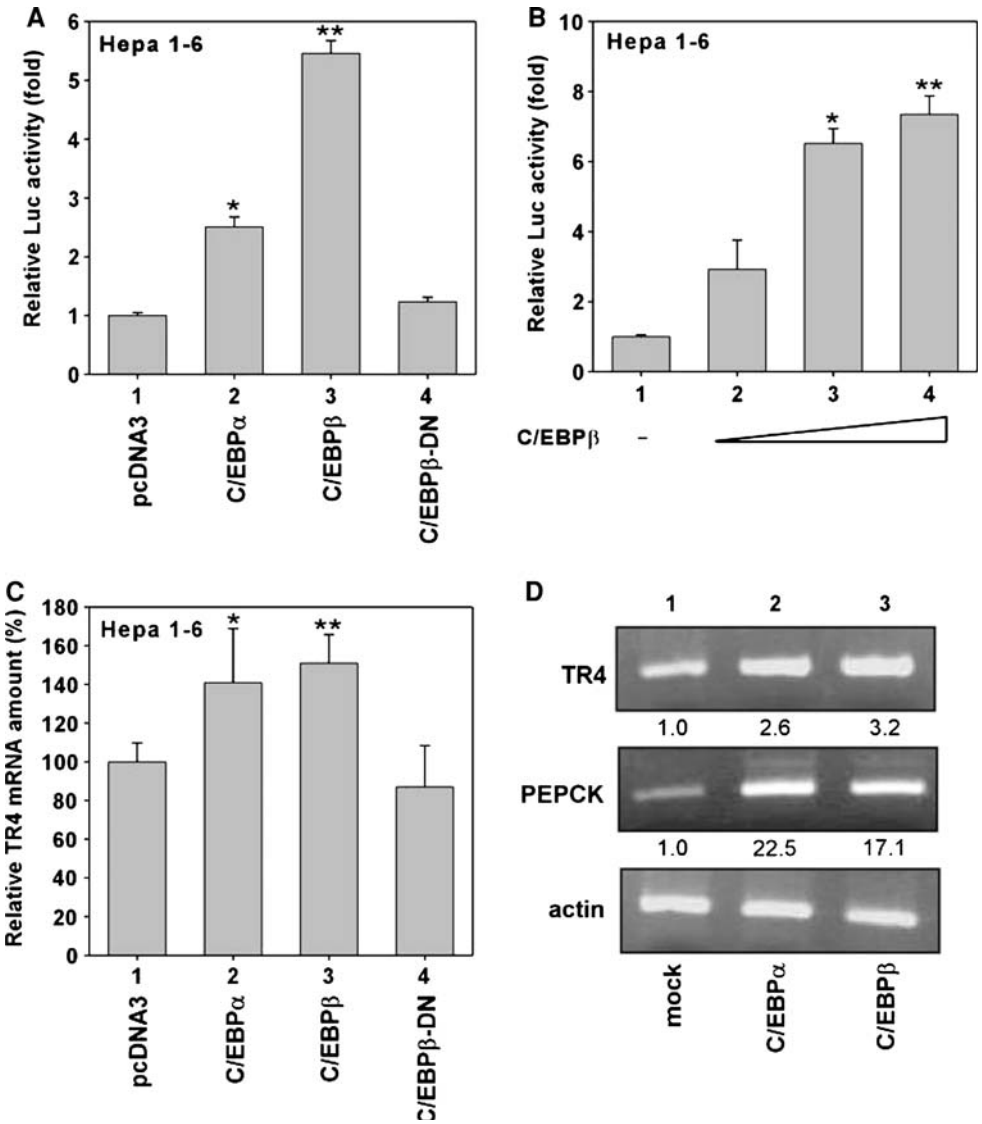

Fig. 2 Cotransfection studies to determine transactivation of TR4 promoter by C/EBPs: **a** pcDNA-C/EBPα, C/EBPβ, and C/EBPβ-DN were cotransfected with TR4 reporter plasmid in Hepa 1-6 cells (**$P < 0.01$; *$P < 0.05$ vs. vector control); **b** Increasing amounts of pcDNA-C/EBPβ expression plasmids were cotransfected with TR4 reporter in Hepa 1-6 cells (**$P < 0.01$; *$P < 0.05$ vs. vector control); **c** Endogenous TR4 gene expression was assayed by Q-PCR in Hepa 1-6 cell transfected with pcDNA-C/EBPα, C/EBPβ, and C/EBPβ-DN individually (**$P < 0.01$; *$P < 0.05$ vs. vector control). **d** Semiquantitative RT-PCR of HepG2 cells: Induction of TR4 and PEPCK gene expression in vitro in the presence of C/EBPα and C/EBPβ. β-Actin gene expression was used as an internal control

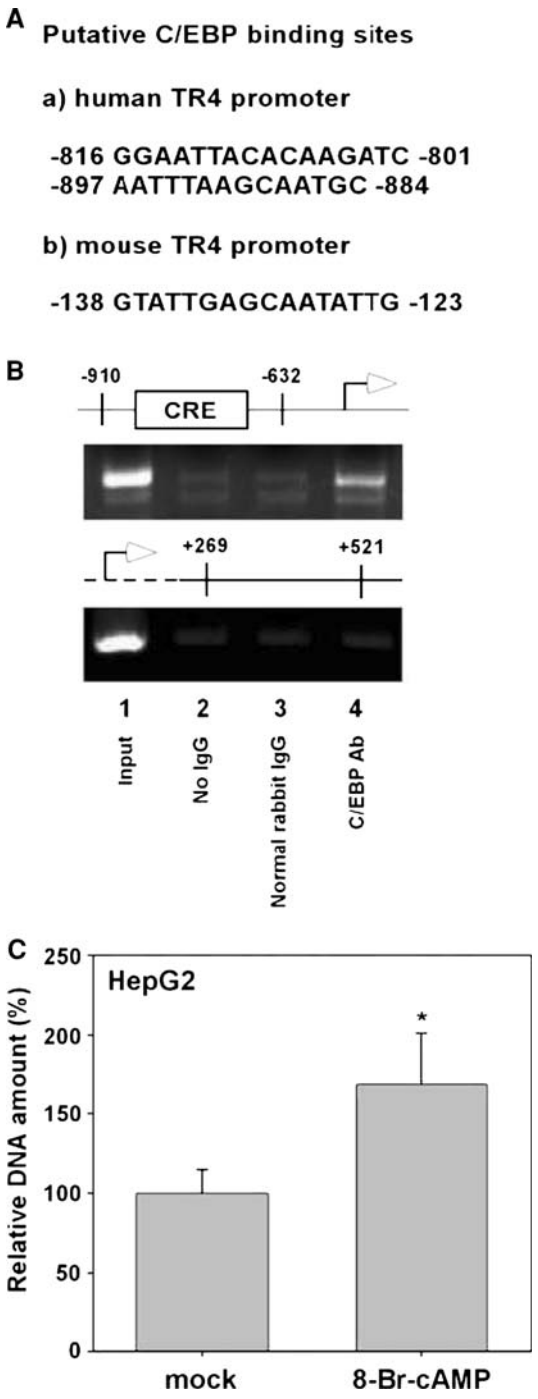

Fig. 3 Binding of expressed C/EBP to CRE on TR4 promoter; **a** Locations of putative CRE(s) on the human and mouse TR4 gene promoter; **b** ChIP assay of human hepatoma HepG2 cells using polyclonal antibody C/EBP β (Δ 198). PCR amplification of the human TR4 promoter (-632 to -910) that includes the region containing the CRE, as well as the coding region ($+269$ to $+521$). Lane 1, input control. Lane 2, control IP without normal rabbit IgG. Lane 3, control IP with normal rabbit IgG. Lane 4, PCR product was obtained from immunoprecipitates using polyclonal antibody C/EBP β (Δ 198); **c** Relative C/EBP-TR4 promoter association in vivo in response to cAMP. The data shown are derived from Q-PCR of each C/EBP–DNA complex immunoprecipitations ($^*P < 0.05$ vs. mock control)

not to the negative control region (lower panel). No PCR product was observed using DNA immunoprecipitated with or without normal rabbit IgG (Fig. 3b, lanes 2 and 3). These data demonstrate that C/EBPs can directly bind to the TR4 promoter in vivo. Because C/EBP activity is upregulated upon cAMP/PKA signaling, we would like to see a difference in the relative association of C/EBP with TR4 promoter with or without cAMP treatment. When HepG2 cells were treated with or without cAMP and compared, there was 1.7 times greater C/EBP and TR4 promoter association in 8-Br-cAMP-treated cells than in their counterparts (Fig. 3c). These data reveal that a sequential event occurs during the cAMP signaling: first, C/EBP activity is increased during cAMP/PKA action and the enhanced C/EBP then stimulates TR4 expression, which leads to activation of PEPCK and glucose production. Through both in vitro and in vivo studies, we have demonstrated a novel pathway, cAMP/PKA → C/EBP → TR4 → PEPCK → gluconeogenesis, which is important in the control of glucose homeostasis (Fig. 4).

Discussion

TR4 functions downstream of the glucagon-cAMP/PKA axis. Other nuclear receptors that can regulate gluconeogenic gene expression can also be regulated by cAMP/PKA, such as glucocorticoid receptor, RARβ, and NR4A [14–16]. cAMP/PKA signaling could directly induce gluconeogenic gene expression through the CRE on their promoters; it can also indirectly activate gluconeogenic gene expression through regulating nuclear receptor gene expressions. These effects could amplify the signals from cAMP/PKA to downstream targets. During a feeding period, glucagon levels drop, and blood insulin levels elevate. A crucial question for future study is where insulin fits into the TR4 pathway. Previous studies have shown that insulin is dominant over gluconeogenic stimuli and inhibits hepatic glucose production. We have observed that there is little effect of insulin on TR4 mRNA expression in hepatocytes in vitro. A remaining possibility is that TR4 may be modulated by either cAMP/PKA or insulin signaling pathways, and thus altering TR4's protein stability, subcellular location, and DNA-binding ability. Posttranslational modification of TR4 by phosphorylation and acetylation has also been reported previously [17], which could be another way to regulate TR4 by metabolic signaling pathways. Alternatively, cAMP/PKA signaling also might modulate TR4 interacting proteins, and thus influence TR4 activity.

C/EBPα and C/EBPβ are involved in regulating PEPCK gene expression in response to cAMP/PKA. Both C/EBP isoforms can activate the PEPCK promoter via different

C/EBP-binding sites, acting alone or together with a coactivator(s). C/EBPα-deficient mice die within 2 h after birth because of the delayed induction of gluconeogenesis. C/EBP$\beta^{-/-}$ mice; on the other hand, exhibit two phenotypes: mice with phenotype A survive to adulthood but have hypoglycemia; mice with phenotype B are unable to maintain normal blood glucose levels and die within 2 h after birth [5]. Interestingly, these two distinct phenotypes also occurred in TR4$^{-/-}$ mice: some TR4$^{-/-}$ mice live to adulthood with hypoglycemia, while others died before puberty. In this study, we uncovered the new linkages between cAMP/PKA signaling, C/EBP, and PEPCK. Elevated TR4 expression in fasting mice is due to enhanced cAMP/PKA pathways under hormonal regulation. Through activated C/EBP, TR4 transcription is induced, and consequently PEPCK expression is promoted by increased TR4 levels in liver. C/EBPβ regulates its target gene expression through direct and indirect mechanisms [18], suggesting that additional factors might participate in the fine tuning of PEPCK regulation. We have shown that C/EBP activates TR4 transcription activity and TR4 binding to PEPCK promoter triggers gene transcription, protein expression, and enhanced glucose production (Fig. 4). These data implicate TR4 as a factor partially mediating PEPCK expression through cAMP/PKA and C/EBP responsiveness.

A recent study showed that the phosphorylation of C/EBPα by p38 mitogen-activated protein kinase (MAPK) results in an increased transactivation activity in the context of PEPCK gene transcription [19]. TR4 can also be phosphorylated by MAPK at the activation function 1 domain. The phosphorylation of TR4 leads to the recruitment of corepressor RIP140 and inhibits TR4 target gene expression. [17]. MAPK, on one hand, activates C/EBPα, and the activated C/EBPα could bind to TR4 promoter and induce TR4 gene expression; on the other hand, MAPK could phosphorylate and inactivate TR4. The regulation of MAPK via C/EBPα resulting in TR4 activation and via direct phosphorylation resulting in TR4 inactivation still needs further study.

In this study, we identified TR4 as a mediator that activates PEPCK gene expression in response to fasting, cAMP/PKA, and C/EBP signaling. In previous studies, ApoE has also been identified as a target gene of TR4. cAMP/PKA action and C/EBP could induce ApoE gene expression, which supports our observation that the induced TR4 gene expression may also mediate the effect of cAMP/PKA on ApoE gene expression [20–23]. In a previous report, we also identified another TR4 target gene, S14 [24], which is influenced by cAMP as well. However, cAMP plays a dominant negative role in regulating hepatic S14 gene expression in vivo [25]. These results suggest that cAMP regulates S14 gene expression via different mechanisms; the TR4-mediated regulation may be just one of them. The negative effect of cAMP through other mechanisms may overpower the positive effect of TR4 on S14 regulation. The elucidation of the possible mechanisms under cAMP/PKA axis on TR4, ApoE, S14, and other TR4 target genes will require further investigation.

Materials and methods

Cell culture, transfections, and plasmids

Hepa 1-6 and HepG2 cells were cultured in 24-well plates (BD Falcon, Franklin Lakes, NJ), at a concentration of 4×10^4 cells/per well, and transfected with 1 μg DNA/well using SuperFect^{TM} (Qiagen, Valencia, CA) or electroporated with MicroPulser Electroporator (Bio-Rad, Hercules, CA). 8-Br-cAMP, forskolin, and PKA inhibitor KT5720 were purchased from Calbiochem (San Diego, CA). C/EBPα, C/EBPβ, and C/EBPβ-dominant negative

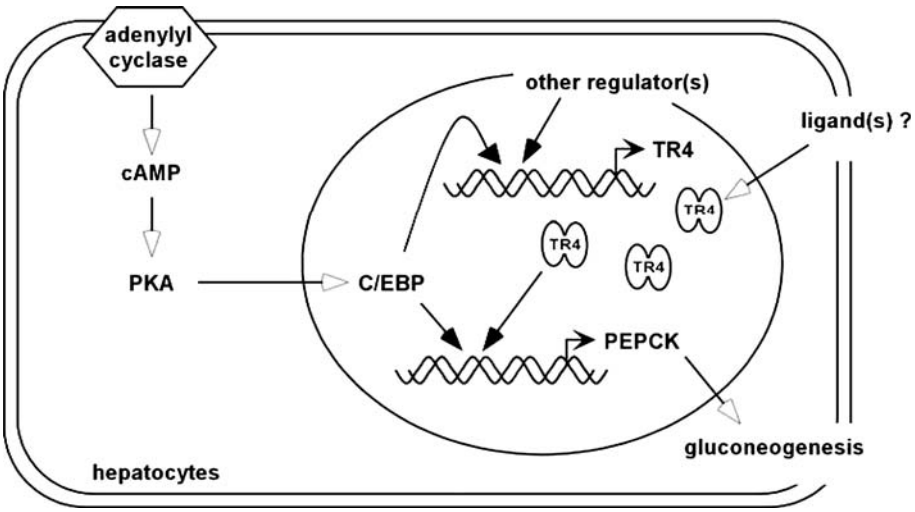

Fig. 4 TR4 works downstream of the cAMP/PKA axis. cAMP and PKA modulators and C/EBPs regulate TR4 gene expression in hepatocytes. C/EBPs binding to the TR4 promoter activate TR4 expression and TR4 target genes, such as PEPCK. C/EBPs may also regulate PEPCK through direct binding and activate PEPCK gene expression

(C/EBPβ-DN) mutant plasmids [26, 27] were compared with empty pcDNA3.1. The TR4 promoter luciferase reporter (pGL-TR41920) contains a total of 2.0 kb of human genomic sequence spanning 1920 bp of the TR4 promoter and 95 bp of the native transcript [28]. The plasmid pRL-TK (Promega, Madison, WI) for internal control was co-transfected in all the transfection experiments. After 48-h transfection, the cells were harvested, and luciferase assays were performed using the Dual-Luciferase kit (Promega, Madison, WI).

Chromatin immunoprecipitation assay (ChIP)

ChIP was performed in HepG2 cells as previously reported [6, 29–31]. Immunoprecipitations (IPs) were performed at 4°C overnight, with 2 μg rabbit polyclonal antibody C/EBP β (Δ 198) and normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). The primers used for the region encompassing human TR4 putative CRE and negative control were: hTR4 CRE (S): 5′-AAGAGTTTAAGAGCAAGACAGG-3′, hTR4 CRE (AS): 5′-GGTTGTATTGGGTGGGTATTG-3′; hTR4 negative control (S): 5′-ACCCGACCCTTTTACCTTTG-3′, hTR4 negative control (AS): 5′-GGAGGAGGAGGAGGATAAGG-3′.

Gene expression analysis by quantification PCR

For reverse transcriptase PCR (RT-PCR), semiquantitative RT-PCR, and Q-PCR analysis of TR4 and PEPCK mRNA expression, total RNA was isolated from HepG2 and Hepa 1-6 hepatoma cells using TRIzol® Reagent (Invitrogen, Carlsbad, CA). The relative abundance of target mRNA was quantified relative to the control β-actin gene expression from the same reaction. The sequences for sense strand (S) and antisense strand (AS) PCR primer are: PEPCK(S)-220: 5′-AACTGTTGGCTGGCTCTC-3′, PEPCK(AS)-390: 5′-GAACCTGGCGTTGAATGC-3′; mTR4(S): 5′-CATATTCACCACCTCGGACAAC-3′, mTR4(AS): 5′-TGACGCCACAGACCACAC-3′; β-actin(S): 5′-TGTGCCCATCTACGAGGGGTATGC-3′, β-actin(AS): 5′-GGTACATGGTGGTGCCGCCAGACA-3′.

Q-PCR amplifications of reverse-transcribed first strand DNA samples were performed using the iCycler iQ^{TM} PCR cycler (Bio-Rad, Hercules, CA). Relative quantification of PCR products was based on value differences between the target and β-actin control using the $2^{-\Delta\Delta CT}$ method [32]. Each sample was analyzed in triplicate, in assays performed three separate periods.

Statistical analysis

The P values calculated from the Student's t-test and one way ANOVA <0.05 are interpreted as statistically significant.

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