

Loss of TR4 Orphan Nuclear Receptor Reduces Phosphoenolpyruvate Carboxykinase–Mediated Gluconeogenesis

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OBJECTIVE—Regulation of phosphoenolpyruvate carboxykinase (PEPCK), the key gene in gluconeogenesis, is critical for glucose homeostasis in response to quick nutritional depletion and/or hormonal alteration.

RESEARCH DESIGN/METHODS AND RESULTS—Here, we identified the testicular orphan nuclear receptor 4 (TR4) as a key PEPCK regulator modulating PEPCK gene via a transcriptional mechanism. TR4 transactivates the 490-bp PEPCK promoter-containing luciferase reporter gene activity by direct binding to the TR4 responsive element (TR4RE) located at –451 to –439 in the promoter region. Binding to TR4RE was confirmed by electrophoretic mobility shift and chromatin immunoprecipitation assays. Eliminating TR4 via knockout and RNA interference (RNAi) in hepatocytes significantly reduced the PEPCK gene expression and glucose production in response to glucose depletion. In contrast, ectopic expression of TR4 increased PEPCK gene expression and hepatic glucose production in human and mouse hepatoma cells. Mice lacking TR4 also display reduction of PEPCK expression with impaired gluconeogenesis.

CONCLUSIONS—Together, both in vitro and in vivo data demonstrate the identification of a new pathway, TR4 → PEPCK → gluconeogenesis → blood glucose, which may allow us to modulate metabolic programs via the control of a new key player, TR4, a member of the nuclear receptor superfamily. *Diabetes* 56:2901–2909, 2007

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AUC, area under the curve; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; DR, direct repeat; EMSA, electrophoretic mobility shift assay; HGP, hepatic glucose production; HOMA, homeostasis model assessment; HRE, hormone response element; PEPCK, phosphoenolpyruvate carboxykinase; PKA, cAMP-dependent protein kinase; QUICKI, quantitative insulin-sensitivity check index; TR4, testicular orphan nuclear receptor 4; TR4RE, TR4 responsive element.

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The cytosolic phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32) is the key enzyme controlling the rate of gluconeogenesis by its expression levels. PEPCK expression is suppressed during fetal liver development and then activated at birth. After birth, PEPCK is expressed mainly in the liver and kidney, and the tissues that participate in gluconeogenesis and glyceroneogenesis (1). Results from homologous recombination targeted disruption of the PEPCK gene in mice further confirmed that the gluconeogenesis mediated by PEPCK is essential for life (2). PEPCK activity can be controlled at the transcriptional level in response to various hormonal and nutritional conditions (3). The promoter of the PEPCK gene has been intensively studied, and many regulatory elements have been identified within 5'-flanking regions to control its spatial and temporal expression patterns under multiple hormonal regulation. Several hormone response elements (HREs) were determined by DNase I footprinting studies, including the cAMP response element (4), thyroid hormone regulatory element (5), glucocorticoid regulatory element (6), insulin regulatory element (7), and peroxisome proliferator-activated receptor regulatory element (8). A recent study found that genes involved in the gluconeogenic pathway in response to energy use and storage, including PEPCK, exhibit a rhythmicity in their mRNA levels and their corresponding regulatory hormones and/or transcriptional regulators (9).

Delayed PEPCK induction and impaired gluconeogenesis could cause hypoglycemia, and sustained or repetitive hypoglycemia in infants and children has been shown to have a major impact on somatic growth and normal brain development and function (10). On the contrary, excessive hepatic gluconeogenesis and glucose production are important factors for hyperglycemia in metabolic syndromes and type 2 diabetic patients. Several lines of evidence from animal studies also support the critical role of PEPCK in maintaining glucose homeostasis, where the gluconeogenesis and liver PEPCK messenger RNA (mRNA) are induced by two- to threefold in diabetic animals over nondiabetic controls (11). The same applies to PEPCK transgenic animals, in which mice had increased gluconeogenesis and hepatic glucose production (HGP) and attenuated insulin signaling and hepatic insulin sensitivity (12). In contrast, there were decreased blood glucose levels and increased glucose tolerance in mice with reduced liver PEPCK expression in the presence of short hairpin RNAs (13). These data suggest that intervention to control PEPCK expression in the liver might be a new therapeutic ap-

proach for the prevention of episodic hyperglycemia in type 2 diabetic patients.

The human and rat testicular orphan nuclear receptor 4 (TR4, NR2C2) cDNAs were initially cloned from human and rat hypothalamus, prostate, and testis libraries. The TR4 gene encodes a protein of 596 amino acids with a molecular mass of 67 kDa (14,15) and binds to a consensus HRE composed of an AGGTCA direct repeat (DR) with spacing from zero to six nucleotides (16,17). Molecular structure analyses indicate that TR4 belongs to the nuclear receptor superfamily, but its biological function remains largely unknown. To study the biological function of TR4, we generated TR4 knockout (TR4^{-/-}) mice via targeted gene disruption. TR4^{-/-} mice display significant growth retardation and abnormalities in maternal behavior (18). In addition, TR4^{-/-} mice display defects in spermatogenesis in testis (19) and in cerebellar development (20), where TR4 is highly expressed. Not limited to the testis and brain, TR4 is expressed modestly in four metabolic tissues, including liver, muscle, brown adipose, and white adipose. In vitro studies also found that TR4, as a transcriptional factor, regulated metabolic genes in the liver, such as apolipoprotein E, enoyl-CoA hydratase, and acyl-CoA oxidase (21,22), yet little is known about how TR4 regulates lipid and carbohydrate metabolism. According to recent studies in which nuclear receptor expression profiles in metabolic organs during circadian cycles were determined, TR4 was included in the "CNS, circadian, and basal metabolic functions" group (23). The circadian-like pattern of TR4 expression suggests that oscillation of TR4 may directly affect the rhythmicity of its target genes (24). It will be of interest to determine whether TR4 can regulate some circadian genes involved in metabolic pathways.

In this study, we demonstrate that TR4 regulates PEPCK, the key enzyme in gluconeogenesis, at the transcriptional level via binding to a newly identified TR4 responsive element (TR4RE) located in the 5'-flanking region of the gene. The roles of TR4 in mediating gluconeogenesis were further confirmed in vivo through analysis of TR4^{-/-} mice. Ectopic expression of TR4 in hepatic cells can stimulate the expression of the PEPCK gene and drive HGP. These results highlight a previously unrecognized role for TR4 in the transcriptional control of glucose homeostasis. Identification of modulator(s) and/or ligand(s) for TR4 might provide an approach for treatment of metabolic disease via this newly identified TR4-mediated pathway.

RESEARCH DESIGN AND METHODS

Cell culture, transfections, and luciferase assays. We separated and isolated the primary hepatocyte cultures from TR4^{+/+}, TR4^{+/-}, and TR4^{-/-} animals using the two-step collagenase perfusion technique (25). Hepa1-6 and HepG2 cells were cultured in 12-well plates (Falcon), at a concentration of 10⁵ cells/well, transfected with 1.2 μg DNA/well using SuperFect (Qiagen), and electroporated with MicroPulser Electroporator (Bio-Rad). The internal control plasmid pRL-TK (Promega) was cotransfected in all transfection experiments. After 48-h transfection, the cells were harvested, and luciferase assays were performed using the Dual-Luciferase kit (Promega). For knockdown of TR4 in cells, we used retroviral delivery system (pRetro-H1G; Cellogenetics), with AACGGGAGAAACCAAGCAATT as the targeted sequence.

Electrophoretic mobility shift assay. The electrophoretic mobility shift assay (EMSA) reaction was performed as described previously (26). Briefly, TR4 protein for EMSA was transcribed and translated in TNT reticulocyte lysates (Promega). For the antibody supershift assay, the mixtures were incubated for 15 min in the presence or absence of a mouse anti-TR4 monoclonal antibody (no. 15). The protein-DNA complexes were analyzed on

a 5% native polyacrylamide gel. The results were visualized by autoradiography (Storm PhosphorImaging System; Amersham Pharmacia).

DNA pull-down assay and immunoblotting. Oligonucleotides corresponding to TR4REs were synthesized, biotin-labeled at the NH₂ termini, and used in the pull-down assays. Sequences of the oligonucleotides were as follows: TR4RE1 (sense pull-down), 5'-biotin-CCTTCTCATGACCTTTGGCCGTGG GAGTA-3'; TR4RE1 (antisense pull-down), 5'-TACTCCCACGGCCAAAGGT CATGAGAAGG-3'; TR4RE2 (sense pull-down), 5'-biotin-TCACAACTGGG ATAAAGGTCTCGCTGCTC-3'; TR4RE2 (antisense pull-down), 5'-GAGCAG CGAGACCTTTATCCCAGTTGTGA-3'; AF3/RARE2 (sense pull-down), 5'-biotin-TCGGCTAGGCCTGCCCTTGACCCCACTC-3'; and AF3/RARE2 (antisense pull-down), 5'-AGGTGGGGTCAAGGGCAGGCCTAGCCGA-3'.

Nuclear extracts preparation and DNA pull-down assay were described previously (27,28). Proteins were separated by 8% SDS-PAGE and analyzed by immunoblot analyses as described previously (2). A sheep anti-PEPCK antiserum (a gift from D.K. Granner, Vanderbilt University) was used at a 1:5,000 dilution.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were performed in dissected livers and Hepa1-6 cells as previously described (29-31). Immunoprecipitations were performed at 4°C overnight, with 2 μg monoclonal antibody no. 15, and clones 6B9, 10D12, 9F4, and 9C10, which were generated using NH₂ termini of TR4 as an antigen (AndroScience). The primers used for the region encompassing mouse PEPCK TR4RE1, TR4RE2, and AF3 were as described previously (32). The sequences of primers used for PEPCK promoter 5' upstream region were as follows: PEPCK5' (sense), 5'-ATGGCTGGCTGTGTGATTAG-3'; and PEPCK5' (antisense), 5'-CGCATGGCTGGCTCTTGG-3'.

Real-time PCR quantification of gene expression. For RT-PCR and real-time PCR quantification analysis of TR2, TR4, and PEPCK mRNA expression, total RNA was isolated from TR4^{+/+} and TR4^{-/-} mouse liver and kidney and from primary hepatocyte cultures using TRIzol Reagent (Invitrogen). 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 and antisense strand PCR primer are as follows: PEPCK (sense)-220, 5'-AACTGTTGGCTGGCTCTC-3'; PEPCK (antisense)-390, 5'-GAACCTG GCGTTGAATGC-3'; mTR4 (sense), 5'-CATATTCACCACCTCGGACAAC-3'; mTR4 (antisense), 5'-TGACGCCACAGACCACAC-3'; mTR2 (sense), 5'-CCG CATCTAATCGCTGGAGAG-3'; mTR2 (antisense), 5'-GCATAGGAGAAG GCATGGTGAG-3'; β-actin (sense), 5'-TGTGCCCATCTACGAGGGGTATGC-3'; and β-actin (antisense), 5'-GGTACATGGTGGTCCGCCAGACA-3'.

Real-time PCR quantification amplifications of reverse-transcribed first-strand DNA samples were performed using the iCycler iQ PCR cycler (Bio-Rad). Relative quantification of PCR products were based on value differences between the target and β-actin control using the 2^{-ΔΔCT} method (33). Each sample was analyzed in triplicate in assays performed three separate times.

Glucose production assay. Primary hepatocytes and hepatoma cell lines were cultured in six-well plates (1.4 × 10⁶ cells/well) in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. The medium was then replaced with 1 ml glucose production buffer consisting of glucose-free DMEM (pH 7.4), without phenol red, supplemented with 20 mmol/l sodium lactate, 2 mmol/l sodium pyruvate, and 1 mmol/l glycerol and 1 mmol/l 8-Br-cAMP as positive control. After 4 h of incubation, 0.5 ml medium was collected, and the glucose concentration was measured with a colorimetric glucose assay kit (Molecular Probes). The readings were then normalized to the total protein content determined from the whole-cell lysates.

Mouse studies. For the fasting experiment, 8-week-old mice were either fed regular chow or fasted for 24 h, beginning during the dark cycle, with all mice having free access to water. We used age-matched littermates for all experiments. All experimental protocols were approved by the University Committee on Animal Resources and the Office of Environmental Health and Safety before implementation.

Analytical procedures. Blood glucose was monitored at the end of each fasting and fed state immediately before food withdrawal. Blood samples were collected from the tail vein, and glucose values were determined from whole blood using an automatic glucose monitor (One Touch; Lifescan). Plasma insulin levels were determined using a commercial insulin enzyme-linked immunosorbent assay kit (Crystal Chem). The liver glycogen content was measured by lysing the liver in 0.6 ml 1 N NaOH and heating the sample for 45 min at 65°C, after which 20 μl lysate was incubated for 30 min at 25°C in a buffer containing 1.14 mg/ml amyloglucosidase (34). Glucose levels were measured by Amplex Red Glucose/Glucose Oxidase Assay kit (Molecular Probes).

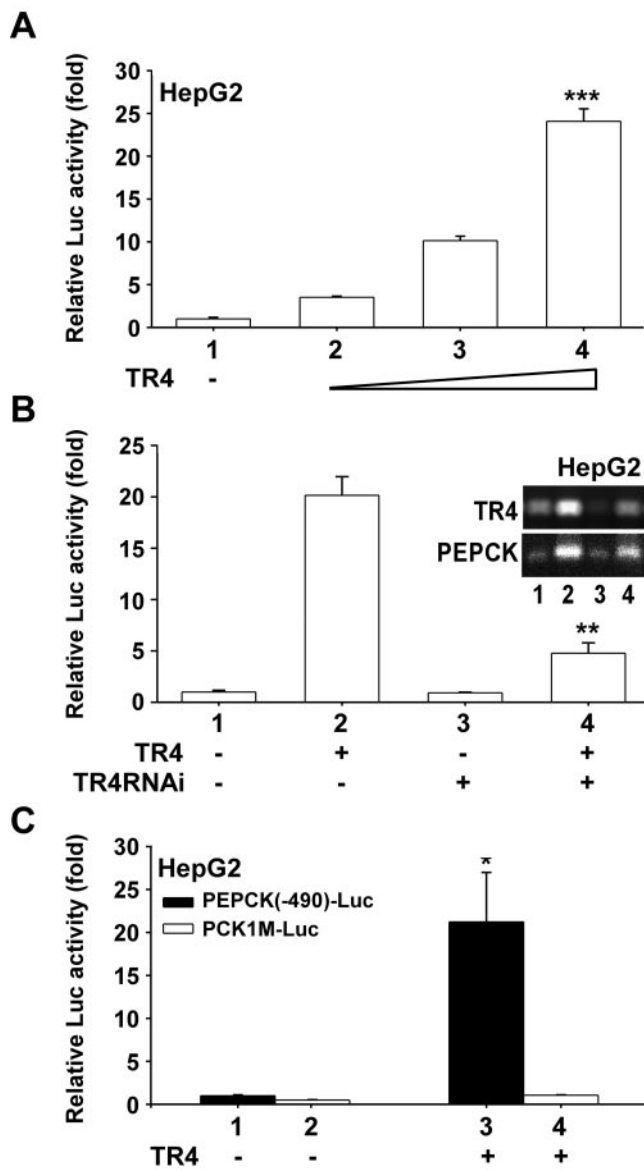


FIG. 1. Effect of TR4 on the transcriptional activity of the PEPCK promoter. HepG2 cells were cultured and transiently transfected with 0.6 μ g PEPCK(-490)-luciferase reporter without or with increasing amounts of TR4-expressing wild-type plasmid (**A**) as described in RESEARCH DESIGN AND METHODS. Bars represent the means \pm SE of three independent experiments ($***P < 0.001$ vs. vector control). **B:** Effect of pRetro-TR4 RNA interference (RNAi) (bar 2) on TR4-mediated PEPCK(-490)-luciferase activity ($***P < 0.01$ vs. bar 2). **C:** Both the wild type (■) and PCK1M (□) PEPCK reporters used to determine TR4 transactivation of the PEPCK promoter ($*P < 0.05$ vs. bar 4).

RESULTS

TR4 stimulates transcription of the PEPCK promoter. Because TR4 is a transcription factor and has the same circadian expression profile as PEPCK (9,24), TR4 might transcriptionally regulate PEPCK gene expression. To test this hypothesis, we investigated the effects of TR4 on hepatic PEPCK expression and conducted promoter analysis using a luciferase reporter linked to the 5' proximal promoter of the PEPCK gene, the PEPCK(-490)-luciferase plasmid (35–37), through transient transfections of HepG2 cells. As shown in Fig. 1A, TR4 dose dependently induced PEPCK(-490)-luciferase reporter activity, suggesting that TR4 regulates PEPCK gene expression at the

transcriptional level. Using vector-based RNA interference (RNAi) to knockdown TR4 expression, we evaluated the effect of reducing the expression of TR4 on PEPCK reporter activity in HepG2 cells. As shown in Fig. 1B, the suppression of TR4 gene expression by TR4 RNAi (pRetro-shTR4a) caused a dramatic reduction of the endogenous PEPCK mRNA expression (Fig. 1B, inset). Also, TR4-mediated PEPCK promoter transcriptional activation was significantly reduced in the TR4 RNAi knockdown cells (Fig. 1B, lane 4). This result further supported TR4 as a mediator that controls PEPCK gene expression via regulating its promoter activity and thus was consistent with the TR4 dose-dependent study.

Through analysis of the PEPCK gene sequence, we identified an AGGTCA DR 1 motif located at -439 to -451 bp as a putative TR4 binding site (PEPCK-TR4RE1 TGACCTT TGGCCG) (Fig. 2A), which is also known as gAF1/PCK1 (35,38). We then tested whether TR4 could positively regulate expression of the PEPCK promoter via this putative TR4RE in hepatocytes. Two PEPCK-luciferase reporter constructs (wild type and a TR4RE1/gAF1/PCK1 mutant, PCK1M TGACGATCGGCCG) (35) were transiently transfected in HepG2 cells to determine TR4 transactivation (Fig. 1C). Cotransfection of the wild-type reporter construct along with TR4 resulted in a 22-fold increase in luciferase activity (Fig. 1C, 3 vs. 1). In contrast, there was limited induction of luciferase activity when cotransfecting TR4 with the TR4RE1/gAF1/PCK1 mutant reporter (Fig. 1C, 4 vs. 2). These data suggest that TR4RE1/gAF1/PCK1 is a positive TR4RE that TR4 can bind to and transactivate PEPCK gene in hepatocytes.

TR4 binds to the regulatory sequence of the PEPCK gene. Through analysis of the PEPCK gene sequence, we identified another DNA fragment containing a DR 1 motif as a putative TR4 binding site (PEPCK-TR4RE2 GGGA TA_AAGGTCT, -1,006 to -1,018 bp), known as PCK2/RARE3 (39,40) (Fig. 2A). By performing EMSA using in vitro-translated TR4 proteins incubated with 32 P-labeled PEPCK-TR4RE1, PEPCK-TR4RE2, and AF3/RARE2 (40) DNA probes, we found a specific band between TR4 and PEPCK-TR4RE1 that could be supershifted in the presence of an anti-TR4 antibody, specifically (Fig. 2B). In contrast, no specific binding between TR4 and PEPCK-TR4RE2 or AF3/RARE2 was observed, suggesting that TR4 induces PEPCK gene expression via binding to the PEPCK-TR4RE1 in the 5' promoter of the PEPCK gene. To determine whether TR4 binding to PEPCK promoter is HepG2 cell line specific, DNA pull-down assays were performed in mouse hepatoma Hepa1-6 cells. The nuclear extracts from Hepa1-6 cells were incubated with biotin-labeled putative TR4REs of the PEPCK promoter, and then DNA-protein complexes were captured with streptavidin-agarose beads and analyzed by immunoblot analysis with an anti-TR4 antibody. As shown in Fig. 2C, lane 1, a positive input control was an indication of TR4 protein on SDS-PAGE after Western blot. Biotin-labeled oligonucleotides containing TR4RE1 were able to bind efficiently to TR4 (Fig. 2C, lane 2). However, TR4 fails to bind to biotin-labeled TR4RE2 or AF3/RARE2 (Fig. 2C, lanes 3 and 4). Together, we concluded that TR4 can bind to PEPCK-TR4RE1 specifically, but not to TR4RE2 or AF3/RARE2 in vitro.

ChIP has been demonstrated as a physiologically relevant way to identify the in vivo binding of transcription factors and to determine promoter occupancy in intact cells. Therefore, we applied the ChIP assay to confirm the

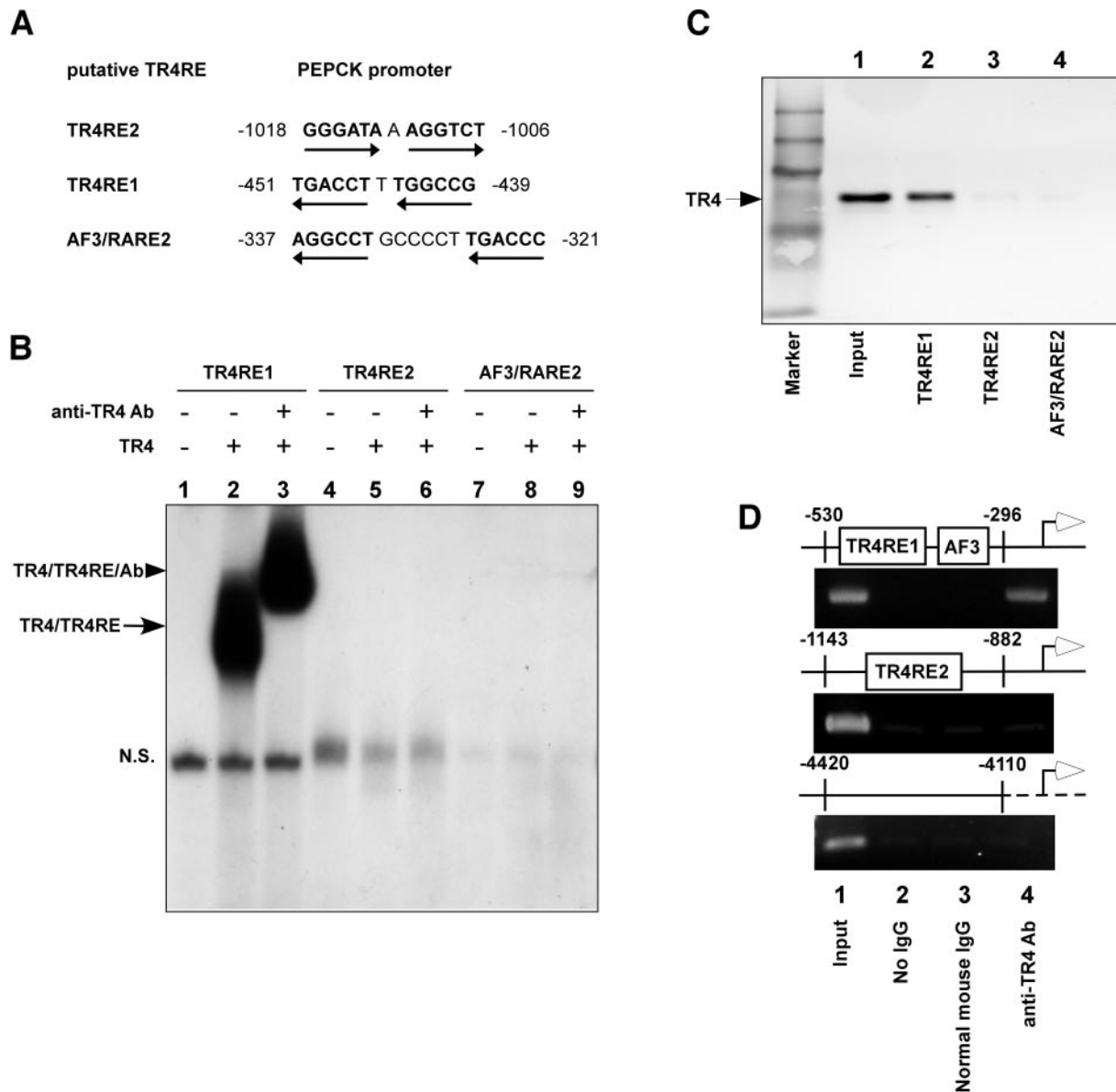


FIG. 2. Binding of expressed TR4 to PEPCK promoter TR4RE1, TR4RE2, and AF3/RARE2 sites. **A:** Locations of putative TR4RE(s) and AF3/RARE2 on the PEPCK gene promoter. **B:** ³²P-labeled TR4RE1, TR4RE2, and AF3/RARE2 oligomers were used in EMSA without (lanes 1, 4, and 7) or with in vitro-translated TR4 (lanes 2, 3, 5, 6, 8, and 9) and an anti-TR4 antibody (lanes 3, 6, and 9). The result was visualized by autoradiography. TR4 protein was found to bind the TR4RE1 probe (TR4/TR4RE, arrow). The presence of TR4 in the complex was confirmed by supershift of the complex using a TR4-specific antibody (TR4/TR4RE/Ab, arrowhead). N.S., nonspecific binding. **C:** DNA pull-down assay using biotin-labeled oligonucleotides containing the TR4RE1, TR4RE2, and AF3/RARE2. Nuclear protein extracts prepared from pCMX-TR4-transfected Hepa1-6 cells were incubated with biotin-labeled double-stranded oligonucleotides containing the putative TR4RE, and the bound proteins were pulled down with streptavidin-agarose and analyzed by immunoblotting with antibodies against TR4. Twenty-five micrograms of protein extract was loaded in the "input" lane as a control (lane 1); 100 μg was used in each pull-down (lanes 2, 3, and 4). **D:** ChIP assay of mouse hepatoma Hepa1-6 cells using TR4-specific antisera. PCR amplification of the mouse PEPCK promoter (-296 to -530) that includes the region containing the TR4RE1 and AF3 site, TR4RE2 site (-1,143 to -882), and the 5' upstream region (-4,110 to -4,420). Lane 1, input control; lane 2, control immunoprecipitation without normal mouse IgG; Lane 3, control immunoprecipitation with normal mouse IgG; Lane 4, PCR product was obtained from immunoprecipitates using TR4 antisera no. 15, which is specific for TR4 (anti-TR4 antibody).

binding of TR4 to the PEPCK promoter in intact Hepa1-6 cells. TR4 was recruited to the PEPCK-TR4RE1/AF3 region of the PEPCK promoter (Fig. 2D, top panel, lane 4) but not to the TR4RE2 and upstream region (Fig. 2D, bottom panel). No PCR product was observed using DNA immunoprecipitated with or without normal mouse IgG (Fig. 2D, lanes 2 and 3). In addition, primers to the β-actin structural gene did not show amplification, demonstrating the specificity of the antibodies (data not shown). These data demonstrate that TR4 can bind directly to the PEPCK promoter in vivo and in vitro.

TR4 increases glucose production in primary hepatocytes and hepatoma cells. To further determine whether TR4 regulation of PEPCK occurs in hepatocytes ex vivo, we isolated primary cells from TR4^{+/+} and TR4^{-/-} mouse livers. TR4^{+/+} hepatocytes had a robust increase in PEPCK gene expression after exposure to glucose-free medium for 4 h compared with TR4^{-/-} cells (Fig. 3A). We then examined the ability of TR4 to stimulate gluconeogenesis and HGP via the glucose production assay. As shown in Fig. 3B, TR4^{-/-} HGP was reduced by 68% in glucose-free medium containing pyruvate, lactate, and

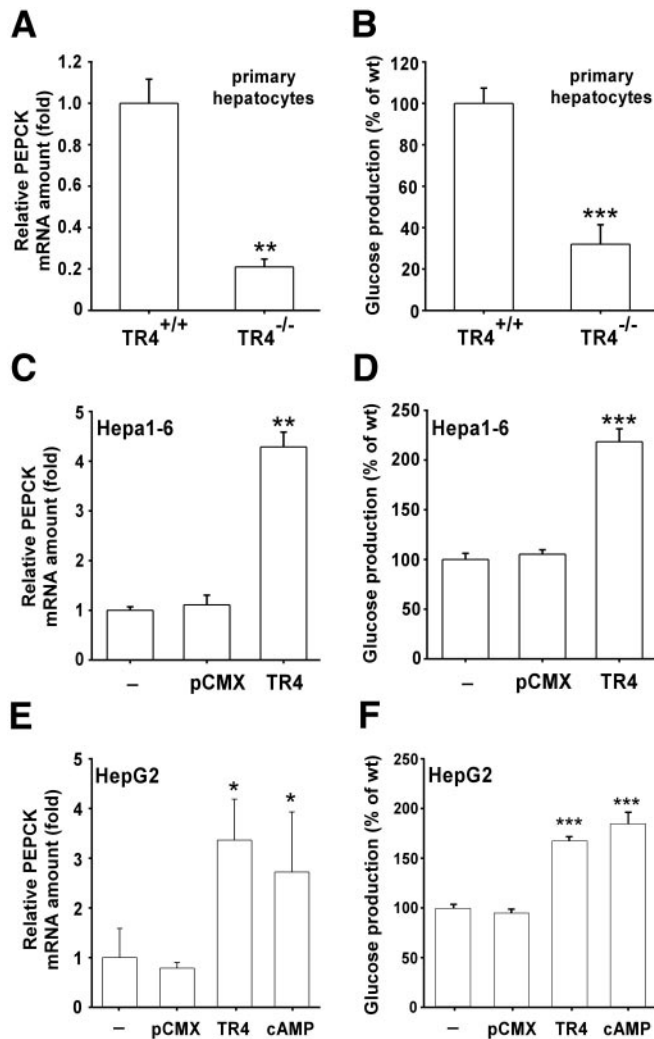


FIG. 3. TR4 is required for hepatic gluconeogenesis during fasting. Isolated primary hepatocytes from TR4^{+/+} and TR4^{-/-} mice were cultured in glucose-free medium for 4 h to measure PEPCK gene expression by real-time PCR quantification (A) and HGP (B) (***P* < 0.001; ***P* < 0.01 vs. TR4^{+/+}). C and E: Effect of TR4 on PEPCK gene expression in response to glucose depletion in Hepa1-6 and HepG2 cells (***P* < 0.01; **P* < 0.05 vs. vector control). D and F: TR4 or vector-transfected hepatoma cell lines, as indicated, were cultured in glucose-free medium for 4 h to measure glucose production (***P* < 0.001 vs. vector control). 8-Br-cAMP (1 mmol/l) served as positive control.

glycerol, which correlates with the reduction in PEPCK gene expression in primary hepatocytes derived from TR4^{-/-} mice. These data suggest that TR4 can modulate PEPCK gene expression to control glucose production.

To examine whether this regulatory pathway, TR4 → PEPCK → gluconeogenesis → glucose production, also occurred in mouse and human hepatoma cells, we electroporated Hepa1-6 and HepG2 cells with pCMX-TR4 or vector alone and measured endogenous PEPCK expression and glucose output. Cells transfected with TR4 have increased TR4 mRNA and PEPCK expression (Fig. 3C and E). Under glucose-free culture conditions, TR4-transfected cells had a higher glucose output compared with control cells; basal glucose output in TR4-transfected cells increased to 230 and 170% of control-transfected cells (Fig. 3D and F). The addition of 8-Br-cAMP served as positive control that can induce PEPCK gene expression and HGP (Fig. 3E and F). Results from PEPCK gene expression and

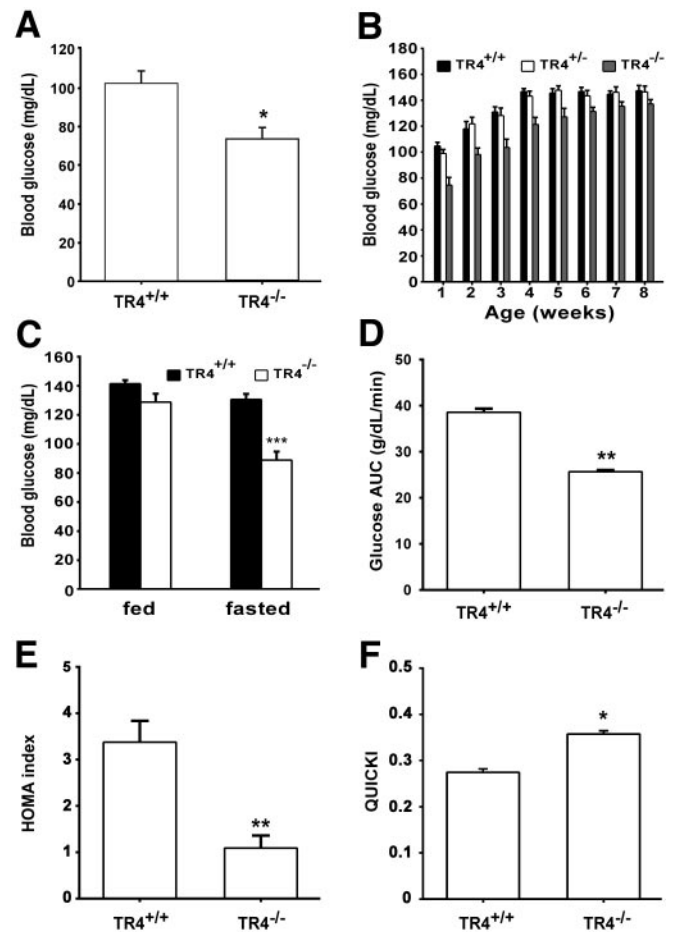


FIG. 4. Mice lacking TR4 display hypoglycemia. A: Blood glucose measurements of newborn TR4^{+/+} and TR4^{-/-} littermates 24 h after birth (means ± SE, *n* ≥ 6 per group; **P* < 0.05 vs. TR4^{+/+}). B: Fed blood glucose measurements of TR4^{+/+}, TR4^{+/-}, and TR4^{-/-} littermates from the ages of 1–8 weeks old. C: Blood glucose levels in 8-week-old TR4^{+/+} and TR4^{-/-} mice in fed and 24-h-fasted states (means ± SE, *n* = 17 per group; ****P* < 0.001 vs. fasted TR4^{+/+}). D: The glucose exposure with GTT (AUC) in adult TR4^{+/+} and TR4^{-/-} mice (means ± SE, *n* = 6). E: Insulin sensitivity assessed by the HOMA index in the control TR4^{+/+} and TR4^{-/-} mice. **P* < 0.05, significantly different from control. F: QUICKI in control TR4^{+/+} and TR4^{-/-} mice. ***P* < 0.01 vs. TR4^{+/+} mice.

HGP studies in hepatoma cell lines and primary hepatocytes from TR4^{+/+} and TR4^{-/-} mice strongly suggest that TR4 plays an important role in promoting the human and mouse hepatic gluconeogenesis in response to fasting signals through the modulation of PEPCK gene expression.

Altered glucose homeostatic response in TR4^{-/-} mice. To further determine whether loss of TR4 results in the suppression of gluconeogenesis *in vivo*, TR4^{-/-} mice were studied. Because the activation of PEPCK is essential for maintaining glucose homeostasis, especially in neonates and fasting adults, we examined TR4^{-/-} mouse blood glucose levels in newborn and young adult mice. As shown in Fig. 4A, 1-day-old newborn TR4^{-/-} pups were hypoglycemic with 73.5 mg/dl blood glucose concentrations compared with 102.2 mg/dl in newborn TR4^{+/+} pups. Hepatic glycogen content in 1-day-old TR4^{-/-} pups was 15% less than that of TR4^{+/+} or TR4^{+/-} pups (Table 1), although hepatic glycogen content of newborn mice before suckling did not differ among the three genotypes (data not shown). Plasma insulin levels of 1-day-old

TABLE 1
Plasma hormone concentrations and hepatic glycogen concentration in 1-day-old pups

Pup genotype	Concentration		
	Plasma		Hepatic
	Insulin (pg/ml)	Glucagon (pg/ml)	Glycogen ($\mu\text{mol} \cdot \text{l}^{-1} \cdot \text{g}^{-1}$)
TR4 ^{+/+}	427 ± 34	ND	146.20 ± 9.4
TR4 ^{+/-}	403 ± 69	ND	141.80 ± 13
TR4 ^{-/-}	278 ± 51*	ND	128.30 ± 16.8

Data are means ± SE. ND, not determined. **P* < 0.05 vs. TR4^{+/+}; *n* = 5.

TR4^{-/-} pups were 35% less than those of TR4^{+/+} pups (Table 1).

The lower blood glucose concentrations in fed TR4^{-/-} mice resolved to normal levels gradually. By 1 week of age, the blood glucose concentration of TR4^{-/-} mice increased to 70% that of TR4^{+/+} mice and continued to increase, reaching 90–95% that of TR4^{+/+} mice by 4 to 6 weeks (Fig. 4B) and remaining at the same level in 8-week-old mice. In contrast, in 24-h-fasted 8- to 10-week-old TR4^{-/-} mice, blood glucose levels were significantly lower compared with TR4^{+/+} mice, dropping from 128 ± 5 to 88 ± 6 mg/dl during the fasting period (Fig. 4C). These results suggest that TR4 is important for normal gluconeogenesis and glucose homeostasis during fasting.

After 24 h of fasting, plasma glucagon was increased in both TR4^{+/+} and TR4^{-/-} mice but was more markedly increased in TR4^{-/-} mice (Table 2), which is consistent with a reduction in hepatic glycogen content (Table 2). These results indicate that hepatic glycogenolysis in response to glucagon was unchanged in livers of TR4^{-/-} mice. There was no significant difference found in insulin concentrations of fed 8-week-old TR4^{+/+} and TR4^{-/-} mice; however, in 24-h-fasted TR4^{-/-} mice, insulin levels were significantly lower compared with those of TR4^{+/+} mice (Table 2). Therefore, the hypoglycemia observed in TR4^{-/-} mice after a 24-h fast was not due to high insulin levels, suggesting that the lower blood glucose in TR4^{-/-} mice was due to impaired gluconeogenesis.

To further investigate whether impaired glucose homeostasis in adult TR4^{-/-} mice alters insulin sensitivity, we performed the glucose tolerance and insulin sensitivity test. We observed that TR4^{-/-} mice had a significant reduction in blood glucose and area under the glucose concentration-time curve (glucose AUC) in glucose toler-

TABLE 2
Plasma hormone concentrations and hepatic glycogen concentration in fed and 24-h-fasted mice

Mouse genotype	Concentration		
	Plasma		Hepatic
	Insulin (pg/ml)	Glucagon (pg/ml)	Glycogen ($\mu\text{mol} \cdot \text{l}^{-1} \cdot \text{g}^{-1}$)
Fed			
TR4 ^{+/+}	1,914.3 ± 277.5	11.6 ± 3.4	381.7 ± 11.9
TR4 ^{-/-}	1,608.3 ± 65.7	10.8 ± 5	327.5 ± 23.6
24-h fasted			
TR4 ^{+/+}	738.3 ± 61.5	62.7 ± 8.3	64.4 ± 7.3
TR4 ^{-/-}	509.1 ± 37.4*	69.6 ± 3.7*	52.1 ± 8.9*

Data are mean ± SE. Mice were sex-matched and 8 weeks old. **P* < 0.05 vs. TR4^{+/+}; *n* = 5.

ance tests (Fig. 4D). To measure the systemic insulin sensitivity, homeostasis model assessment (HOMA) index and quantitative insulin-sensitivity check index (QUICKI) were accessed. Mean fasting insulin, HOMA index, and QUICKI for each group were calculated from data obtained from TR4^{+/+} and TR4^{-/-} mice. We found that TR4^{-/-} mice have lower HOMA index (Fig. 4E) and higher QUICKI (Fig. 4F). Collectively, we observed that TR4^{-/-} mice had fasting hypoglycemia and higher insulin sensitivity, suggesting that TR4 is not only important for gluconeogenesis but also influences systemic insulin signaling.

Reduced ability to induce PEPCK expression in TR4^{-/-} mice. The alterations observed in glucose metabolism in TR4^{-/-} mice further confirmed our hypothesis that TR4 is a key regulator controlling PEPCK-mediated gluconeogenesis. PEPCK needs to be activated at birth to produce glucose for newborn pup survival; we therefore examined newborn PEPCK expression levels. As shown in Fig. 5A, hepatic PEPCK mRNA levels in newborn TR4^{-/-} pups were less than 10% of that of newborn TR4^{+/+} pups. After weaning, PEPCK is expressed in several tissues and participates in gluconeogenesis to provide the organism with glucose in times of starvation. After a 24-h fast, blood glucose levels of 8-week-old TR4^{-/-} mice were significantly lower than those of TR4^{+/+} mice (Fig. 4C), and hepatic PEPCK protein was induced in TR4^{+/+} mice, whereas less induction was observed in TR4^{-/-} mice (Fig. 5B). PEPCK mRNA was also induced robustly in TR4^{+/+} mice after 24 h of fasting, whereas only a slight induction of hepatic PEPCK mRNA was found in TR4^{-/-} mice (Fig. 5B). The relative amount of PEPCK mRNA in TR4^{-/-} mice was 75% lower than that in TR4^{+/+} mice in the fasted state (Fig. 5C). Similar results were obtained when we measured PEPCK mRNA expression in the kidney, another organ that produces glucose via gluconeogenesis (data not shown).

Results from Fig. 5A–C demonstrate that the expression of PEPCK is lower in newborn and fasting young TR4^{-/-} mice, which suggests roles for TR4 in the induction of PEPCK-mediated gluconeogenesis. As shown in Fig. 5D, the expression of TR4 mRNA and PEPCK mRNA was induced in the liver and kidney of TR4^{+/+} mice after fasting, suggesting that induction of TR4 expression results in increased PEPCK gene expression in vivo. Unlike TR4, the expression of the nuclear orphan receptor TR2, which is highly homologous to TR4, was not altered by changes in fed and 24-h-fasted states (Fig. 5D). Because TR4 gene expression is upregulated after fasting, we would expect to see a difference in the relative association of TR4 with PEPCK promoter in the fed and food-deprived states in vivo. To determine the difference of TR4-PEPCK promoter associated complex between fed and fasting, we conducted liver tissue ChIP assay using chromatin isolated from TR4^{+/+} mice livers. As shown in Fig. 5E, there was three times greater TR4-PEPCK promoter DNA complex in fasting mice than in their fed counterparts. These data reveal that a sequence of events occurs during the fasted state: first, TR4 is increased during fasting and TR4 then stimulates PEPCK expression, which leads to increased glucose production. Through both in vitro and in vivo studies, we have demonstrated a novel pathway, TR4 → PEPCK → gluconeogenesis, important in the control of glucose homeostasis.

In summary, we have demonstrated 1) that both PEPCK expression and HGP were positively associated with TR4 expression (Fig. 4); 2) that hepatic TR4 expression was

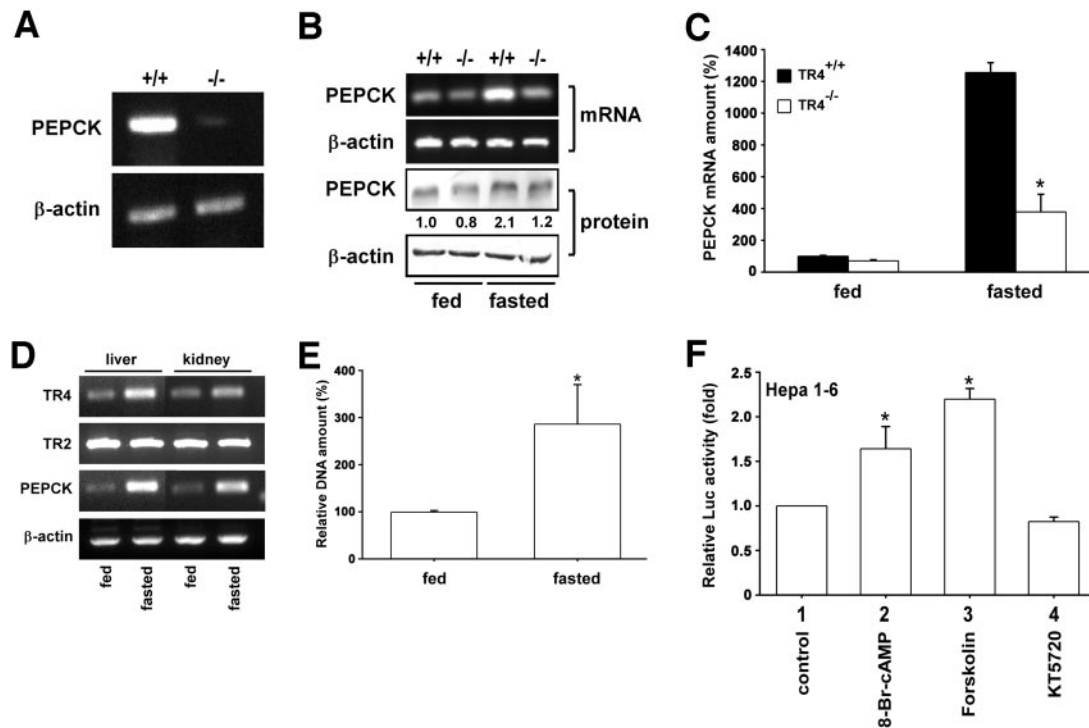


FIG. 5. TR4 and PEPCK expression levels in newborn and young mice. **A:** Semiquantitative RT-PCR analysis of PEPCK and β -actin mRNA expression in liver in TR4^{+/+} and in TR4^{-/-} pups. β -Actin levels were determined as a control for template amounts in PCRs. **B:** PEPCK gene mRNA and protein levels were determined by semiquantitative RT-PCR and Western blot from fed and 24-h-fasted mouse liver tissue. **C:** Using real-time PCR quantification analysis, the relative levels of PEPCK mRNA were based on value differences between the PEPCK and β -actin control, using the comparative $2^{-\Delta\Delta CT}$ method ($*P < 0.05$ vs. fasted TR4^{+/+}). **D:** TR4, TR2, and PEPCK gene mRNA levels were determined by semiquantitative RT-PCR from fed and 12-h-fasted TR4^{+/+} mouse liver and kidney tissues. **E:** Relative TR4-PEPCK promoter association in vivo in fed and fasting mice liver. The data shown are derived from real-time PCR quantification of each TR4-DNA complex immunoprecipitations. $*P < 0.05$ vs. fed state. **F:** TR4 promoter activity was accessed by transiently transfected in Hepa1-6 cells with pGL3-TR4-luciferase under 100 μ M 8-Br-cAMP, 50 μ M forskolin, or 1 μ M KT5720 treatment.

robustly induced by fasting (Fig. 5D); and 3) an increase in TR4 association with PEPCK promoter in fasting liver (Fig. 5E), which lead us to hypothesize that TR4 gene expression was regulated by fasting signals, such as glucagon. In vivo, PEPCK gene transcription is induced during a fast by glucagon via a cAMP-dependent pathway (1). The cAMP/cAMP-dependent protein kinase (PKA) signaling pathway is activated by the glucagon receptor, the epinephrine receptor, and their coupled G-proteins. To test the effect of activation of the cAMP/PKA signaling on TR4 gene expression, their activators and inhibitors were applied. pGL3-TR4-luciferase was transiently transfected in Hepa1-6 cells and subsequently exposed to cAMP/PKA modulators to test the effect of cAMP/PKA on the transcriptional activity of the TR4 gene promoter. 8-Br-cAMP and forskolin treatment caused a 1.6-fold and a 2.2-fold stimulation, respectively, of luciferase activity of TR4-luciferase, whereas KT5720 had no effect on TR4 promoter (Fig. 5F).

DISCUSSION

Using EMSA, DNA pull-down, and ChIP assays, we demonstrated that TR4 functions as a transcription factor via binding to the PEPCK-TR4RE1 sequence to regulate PEPCK expression in liver cells. Early studies also found that the same DNA response element (named gAF1/PCK1/RARE1) can be bound by several nuclear receptors, such as RAR/RAR, RAR/RXR, and HNF4. Through RT-PCR analysis, we found that the mRNA levels of RAR, RXR, and HNF4 in 8-week-old mice were slightly decreased in TR4^{-/-} mice compared with TR4^{+/+} mice (data not

shown), suggesting no compensation by these nuclear receptors for the loss of TR4. Furthermore, TR2, another orphan receptor that is highly homologous to TR4 and recognizes identical HREs in many targets, is expressed at similar levels in TR4^{-/-} and TR4^{+/+} mice. These results suggest that TR4-mediated regulation of PEPCK and the gluconeogenesis pathway is specific and essential for maintenance of glucose homeostasis. The effects of transcription factors on gene regulation have been studied extensively via in vitro binding and transfection assays. However, different transcription factors, including nuclear receptors, may act on multiple response elements within the promoter region of their target genes. Various nuclear receptors may also share identical binding sites in many genes and often compete or bind cooperatively with other nuclear receptors under certain circumstances; therefore, the environment/content-dependent regulation might be critical in determining the spatial and temporal specificity of PEPCK expression.

Liver-specific PEPCK knockout mice ($pck^{lox/lox} + Albcre$) in the fed state have normal glucose levels and are euglycemic at rest after a 24-h fast (2,41). Resting mice without hepatic PEPCK are capable of producing almost the same amount of glucose as $pck^{lox/lox}$ mice. Because the kidney is another gluconeogenic tissue, glucose production in the kidney appears adequate to compensate for the loss of PEPCK and gluconeogenesis in the liver. In our TR4^{-/-} mouse model, the TR4 gene was deleted in both liver and kidney. Therefore, the fasting-induced hypoglycemia in TR4^{-/-} mice could be due to the PEPCK deficiency in both liver and kidney. The TR4^{-/-} phenotype is

similar to that of PCK^{-/-} mice but not observed in liver-specific PEPCK-deficient mice. To dissect tissue-specific roles of TR4 in PEPCK-mediated gluconeogenesis, a tissue-specific TR4 knockout model, such as liver- and kidney-specific TR4^{-/-} mice, would help us to clarify the role of TR4 in these two gluconeogenic tissues. We have shown that TR4 and PEPCK were upregulated in kidney after 24 h of fasting; it is possible that TR4 also plays a role in regulating kidney PEPCK expression. Because PEPCK gene regulation is tissue specific (42), studying the effects of TR4 on PEPCK gene regulation in the kidney would provide useful information regarding control of PEPCK expression and its overall effects on glucose homeostasis in the whole animal. PEPCK is also an important enzyme in glyceroneogenesis in adipocytes, which is involved in fatty acid re-esterification. In TR4^{-/-} mice, we found slightly reduced PEPCK expression in white adipocytes, which suggested that TR4 could also play roles in the PEPCK gene transcription in adipocytes. However, this TR4-mediated PEPCK regulation in adipocytes needs further studies.

PEPCK mRNA and protein expression levels are induced dramatically right after birth to produce glucose for sustaining sufficient nutrient for newborn; therefore, the induction of PEPCK is critical for newborn survival. TR4^{-/-} pups had normal suckling behavior shown by a similar abdominal "milk spot" compared with wild-type and heterozygous mice (data not shown), which excludes the possibility of low milk intake-induced hypoglycemia. The hypoglycemia found in TR4^{-/-} pups may be one of the contributing factors for the high postnatal mortality in TR4^{-/-} mice. Moreover, the mRNA levels of the other two key enzymes in the control of gluconeogenesis, fructose-1,6-bisphosphatase and glucose-6-phosphatase, were also reduced in TR4^{-/-} mice compared with TR4^{+/+} mice (data not shown). It is possible that suppression of PEPCK in TR4-deficient mice might result in the suppression of fructose-1,6-bisphosphatase and glucose-6-phosphatase or that lack of TR4 might simultaneously suppress those key gluconeogenesis enzymes that control glucose production (Fig. 6).

Here, we show that TR4 is a downstream mediator of cAMP/PKA action in the control of gluconeogenesis. Hepatic TR4 promoter activity and gene expression are potentially induced by cAMP/PKA signaling in vitro and by fasting in vivo; and the expression of TR4 in mouse liver stimulates the expression of PEPCK in gluconeogenesis, drives HGP, and raises blood glucose levels. The relative association of TR4 with PEPCK promoter was also increased after fasting. These data leading to the inactivation of the TR4 signaling pathway in mice by blocking cAMP/PKA signaling or TR4 modulators might compromise HGP (Fig. 6). These data also highlight the potential ligand-independent TR4 activity in glucose metabolism and identify the TR4-PEPCK pathway as a potential target for intervention in metabolic disease.

In addition to hypoglycemia via impaired gluconeogenesis, TR4^{-/-} mice also developed insulin hypersensitivity, suggesting that modulation of TR4 may be a pathway to regulate insulin sensitivity. Because TR4 is a member of the nuclear receptor superfamily and may require ligand(s) or modulator(s) for activation, identification of a small molecule may provide a new therapeutic approach to regulate glucose metabolism and type 2 diabetes via modulation of TR4 activity (Fig. 6).

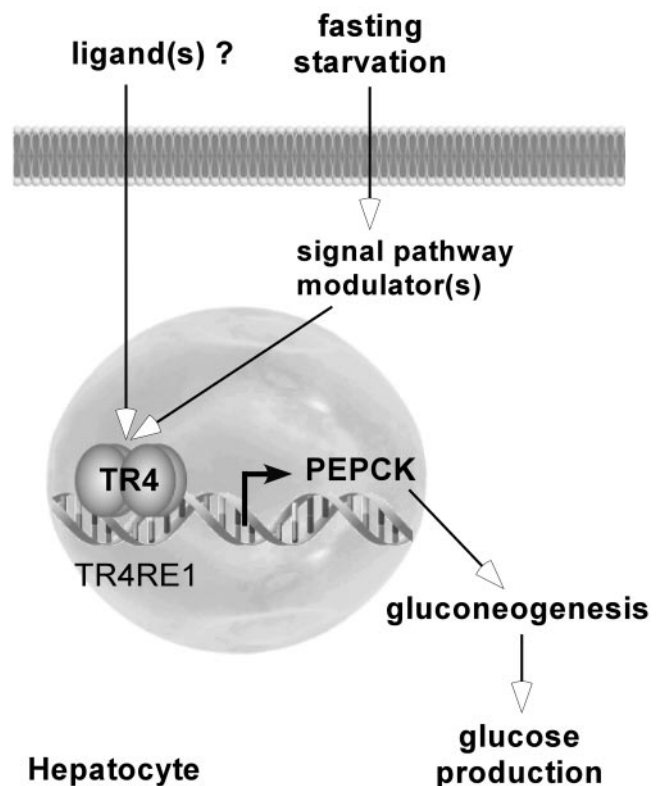


FIG. 6. TR4 acts as a key regulator of PEPCK gene expression and glucose metabolism in hepatocytes. TR4 binding to the PEPCK promoter activates PEPCK expression and stimulates gluconeogenesis and HGP.

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