

Regulation of the Mouse Protein Targeting to Glycogen (PTG) Promoter by the FoxA2 Forkhead Protein and by 3',5'-Cyclic Adenosine 5'-Monophosphate in H4IIE Hepatoma Cells

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The scaffolding protein, protein targeting to glycogen (PTG), orchestrates the signaling of several metabolic enzymes involved in glycogen synthesis. However, little is known concerning the regulation of PTG itself. In this study, we have cloned and characterized the mouse promoter of PTG. We identified multiple FoxA2 binding sites within this region. FoxA2 is a member of the forkhead family of transcription factors that has recently been implicated in the cAMP-dependent regulation of several genes involved in liver metabolism. Using luciferase reporter constructs, we demonstrate that FoxA2 transactivates the PTG promoter in H4IIE hepatoma

cells. Nuclear extracts prepared from mouse liver and H4IIE cells were able to bind a FoxA2-specific probe derived within the PTG promoter region. Chromatin immunoprecipitation experiments further demonstrate that FoxA2 binds to the PTG promoter *in vivo*. Finally, we show that treatment with cAMP analogs activates the PTG promoter and significantly increases PTG levels in H4IIE cells. Our results provide a framework to investigate how additional transcription factors may regulate PTG expression in other cell types. (*Endocrinology* 147: 3606–3612, 2006)

IN MAMMALS, GLYCOGEN is the main storage form of glucose and is found primarily in liver and muscle tissues (1). Its metabolism is regulated primarily by the enzymes glycogen synthase and phosphorylase. Both are allosterically controlled by metabolites and phosphorylation events that affect activity and/or subcellular localization (2). Glycogen synthase, the rate-limiting enzyme in glycogen synthesis, is phosphorylated on up to nine regulatory serine residues by several kinases, resulting in progressive inactivation. The protein kinases that catalyze these reactions, which include cAMP-dependent protein kinase A, calmodulin-dependent kinases, glycogen synthase kinase 3, and AMP-activated kinase, are stimulated by hormones such as glucagon as well as by energy deprivation. In contrast, glycogen phosphorylase, which promotes glycogenolysis, is activated by phosphorylation on a single serine residue by phosphorylase kinase.

During the fed state, the release of insulin triggers increased glycogen synthesis in liver and muscle. Insulin promotes the net dephosphorylation of glycogen synthase via

inactivation of the regulatory kinases protein kinase A and glycogen synthase kinase 3 and by activation of the protein phosphatase 1 (PP1) family of protein phosphatases (3). Insulin also inactivates glycogen phosphorylase and phosphorylase kinase by promoting their dephosphorylation. Although PP1 has been found in virtually every cellular compartment, only discrete pools localized to glycogen particles are activated during glycogen synthesis. This specific localization of PP1 is accomplished by several glycogen-targeting subunits. In humans, six subunits have been identified to date: G_M, G_L, G_C, G_D, G_E, G_F, and G_G.

We and others previously identified protein targeting to glycogen (PTG)/G_C/R5 as a PP1-interacting protein from 3T3-L1 adipocytes and rat liver (4, 5). PTG localizes exclusively to the glycogen-enriched fraction in cells, and its overexpression causes an increase in the amount of glycogen-associated PP1. We have shown that PTG can bind to the enzymes glycogen synthase, glycogen phosphorylase, and phosphorylase kinase (4, 6, 7). Deletion analyses revealed that these proteins bind to a single domain located in the C-terminal region of PTG, distinct from that of the PP1- and glycogen-binding regions. PTG thus acts as a molecular scaffold that significantly enhances the ability of PP1 to dephosphorylate and regulate activities of the glycogen regulatory enzymes. Indeed, it is well established that overexpression of PTG in several cell types leads to increased glycogen synthesis by activation of glycogen synthase and inactivation of glycogen phosphorylase (8–10).

The physiological importance of PTG in glycogen metabolism has been validated by studies using gene-targeted mice (11). Compared with their wild-type (WT) counterparts, PTG heterozygous knockout mice exhibit decreased glycogen

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Abbreviations: ChIP, Chromatin immunoprecipitation; CPT, 8-(4-chlorophenylthio)-cAMP; DTT, dithiothreitol; EBNA, Epstein-Barr nuclear antigen; HNF, hepatocyte nuclear factor; PP1, protein phosphatase 1; PTG, protein targeting to glycogen; SREBP, sterol-regulatory element-binding protein; TORC2, transducer of regulated cAMP response element binding protein activity 2; USF, upstream stimulatory factor; WT, wild type.

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stores and glycogen synthase activity in several tissues, including fat, liver, heart, and skeletal muscle. Although glucose homeostasis is initially normal, progressive glucose intolerance occurs in older PTG heterozygous mice. Interestingly, the levels of PTG seem to be decreased in diabetic rats (12) as well as in diabetic patients undergoing cardiopulmonary bypass and cardioplegic arrest (13). This suggests that PTG may be crucial in maintaining whole-body glucose metabolism.

Despite the clear involvement of PTG in glycogen metabolism, how its function is regulated is not known. PTG is not phosphorylated in response to insulin or forskolin (6), suggesting the absence of a posttranslational modification. Whereas glycogen synthase relocalizes within cells in response to insulin, PTG does not (14, 15). In contrast, recent evidence suggests that transcriptional regulation may play an important role in PTG function. For example, both noradrenaline and adenosine dramatically up-regulate PTG mRNA levels in astrocytes, concomitant with increased glycogen synthesis (16, 17). These effects are thought to be mediated through a cAMP signaling cascade.

Recently, it has been demonstrated that the transcription factor FoxA2/hepatocyte nuclear factor (HNF)-3 β mediates gene activation of the gluconeogenic program by cAMP in hepatocytes (18). FoxA(1–3) belong to the forkhead family of transcription factors that were originally identified as liver-enriched proteins (19, 20). In addition to other processes, FoxA2 is well known to be critically involved in liver and pancreas development and metabolism. There is also evidence to suggest that FoxA2 regulates glycogen metabolism (21–23). Stable overexpression of FoxA2 in hepatocytes leads to diminished glycogen levels. This is a result, in part, of decreased expression of glycogen synthase. However, whether FoxA2 directly regulates the glycogen synthase promoter is unclear.

The purpose of the present study was to identify the transcriptional regulatory elements of PTG. We cloned and characterized the mouse promoter region and identified binding sites for the FoxA2 forkhead transcription factor. Studies using H4IIE hepatoma cells demonstrate that FoxA2 binds to and transactivates the PTG promoter. The effect of cAMP on the promoter was also investigated.

Materials and Methods

Antibodies and reagents

The FoxA1 and FoxA2 rabbit antiserum and plasmids were generous gifts of Dr. Robert H. Costa and have been previously described (24, 25). An additional FoxA2 antibody (mouse monoclonal 4C7) was developed by Thomas M. Jessell and Susan Brenner-Morton (26) and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa. Rabbit IgG was purchased from Sigma Chemical Co. (St. Louis, MO). The H4IIE cell line, previously derived from the Reuber hepatoma H-35 (27), was obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum. Theophylline (1,3-dimethylxanthine) and 8-(4-chlorophenylthio)-cAMP (CPT) were purchased from Sigma.

Luciferase constructs

Promoter fragments were amplified by PCR using mouse genomic DNA as a template. PCR products with engineered restriction sites were

then subcloned into the pGL3 vector (Promega, Madison, WI) using *KpnI-SmaI* sites. The sequence of the 3' oligo (*SmaI* site *underlined*) used is 5'-GCC CCG GGC TTC GCA CTC AGT ACT TA-3'. For the -784 to +36 fragment, the 5' oligo (*KpnI* site *underlined*) used is 5'-GCG GTA CCG CCT AAG GAT GTC ATA AC-3'. For the -351 to +36 fragment, the 5' oligo (*KpnI* site *underlined*) used is 5'-GCG GTA CCG CTC AGA TTC AAC TCT GC-3'.

Luciferase reporter assays

H4IIE cells were transfected with pGL3 constructs and *Renilla* luciferase (Promega; 100:1 ratio) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells cultured in 10-cm dishes were transfected at a confluency of 50–60%, with a maximum of 20 μ g DNA. The total amount of DNA in each experiment was kept constant by using vector DNA controls. Cells were harvested 48 h later in Passive Lysis Buffer (Promega). Samples were subjected to two freeze-thaw cycles and then precleared by centrifugation at 14,000 \times g for 1 min. The supernatant was assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

EMSA

Nuclear extracts were prepared from mouse liver and H4IIE cells as previously described with minor modifications (28). Briefly, cells were homogenized in buffer A [20 mM HEPES (pH 7.9), 100 mM NaCl, 1.5 mM MgCl₂, 0.5 mM EDTA, 0.7% Nonidet P-40, 0.5 mM dithiothreitol (DTT), 10% glycerol, and Complete EDTA-free protease inhibitor]. Lysates were then centrifuged for 10 min at 2000 \times g, and the pellet was resuspended in buffer B [20 mM HEPES (pH 7.9), 500 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 25% glycerol, and Complete EDTA-free protease inhibitor]. The sample was incubated for 30 min at 4 C and then precleared by centrifugation at 14,000 \times g for 30 min. EMSAs were performed with the Lightshift Chemiluminescent EMSA kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Briefly, nuclear lysates from liver (3 μ g) and H4IIE cells (1 μ g) were incubated with a 5'-biotinylated 39-bp DNA oligonucleotide duplex with sequence corresponding to the FoxA2 binding site derived from the PTG promoter: 5'-TTC GTT TCA TGG GCT CAC GTG TTT TCT TAA CAT TTG AGT-3'. A control duplex, derived from the Epstein-Barr nuclear antigen (EBNA), was also used and provided with the kit (Pierce). The reaction consisted of 20 fmol biotinylated duplex incubated with nuclear lysate in 10 mM Tris (pH 7.5), 50 mM KCl, and 1 mM DTT for 20 min at room temperature. In some samples, 4 pmol unlabeled duplex was used for competition experiments. To disrupt potential FoxA2 complexes, 0.2 μ g FoxA2 antibody (4C7) was added to the mix. After the incubation, the samples were separated by electrophoresis in 6% polyacrylamide Tris-borate-EDTA gels. The gels were then transferred to Biotodyne B nylon membranes (Pierce) and cross-linked. Protein/DNA complexes were then detected by chemiluminescence according to the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP) experiments

H4IIE cells were treated with 1% formaldehyde for 10 min at 37 C, washed with PBS, and then collected by centrifugation at 500 \times g. For liver, 0.2 g tissue was cut into small pieces and incubated in 10 ml DMEM with 1% formaldehyde for 15 min. Cross-linking was stopped by addition of glycine to a final concentration of 0.125 M for 5 min. After several washes with cold PBS, nuclear lysates were obtained using the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce). Samples were then processed with a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's instructions. For the immunoprecipitations, 2 μ g of antibodies were used. After elution and proteinase K treatment, DNA was recovered and purified by using a QIAquick column (QIAGEN, Valencia, CA). An aliquot of DNA was then used for PCR analysis using the following primers corresponding to the rat PTG genomic sequence: 5'-GAA GTC AGA ACT CTG TCT AGG GAT CT-3' and 5'-CCG CAC CCA GCG CAG CTT CGC ACC AC-3'. For the liver samples, the primers used for the mouse sequence are 5'-GTT GGC CTA TAG TTC TCT CTC CC-3' and 5'-CAG TAC TTA AGA GGA TGC AGC GC-3'.

Results

Characterization of the PTG promoter region

The mouse genomic sequence of PTG (National Center for Biotechnology Information Gene ID/Locus Link ID: 53412) was obtained from the National Center for Biotechnology Information. According to their numbering system, the transcriptional start site occurs at sequence number 29803859, and the initiating methionine at sequence number 29803772. Using this information, a fragment of the promoter region was obtained by PCR from mouse genomic DNA and thoroughly sequenced (Fig. 1). Potential transcriptional regulators of PTG were then searched by performing a TRANSFAC analysis using a stringent threshold score of 90.0. Among the candidates, we were particularly interested in FoxA, sterol-regulatory element-binding protein (SREBP), and upstream stimulatory factor (USF) sites, given their importance in liver nutrient metabolism. In addition, we also noted a microsatellite (AC)_n repeat that may have a structural purpose for this region.

Little is known concerning the transcriptional regulation of PTG. There have been reports indicating that cAMP can increase PTG transcription in astrocytes (16, 17). Interestingly, FoxA2 was recently demonstrated to be essential in cAMP-mediated transcription of several liver metabolic genes (18). Therefore, we decided to focus our study on the potential of FoxA2 to transcriptionally regulate PTG in liver cells.

Transcriptional activation of the PTG promoter region by FoxA2

Two regions of the promoter, from –784 to +36 and from –351 to +36, were subcloned into the pGL3 luciferase reporter plasmid for transfection experiments in H4IIE hepatoma cells. The region from –784 to +36 contains three putative FoxA binding sites, whereas the region from –351 to +36 lacks all of them (Fig. 1). *Renilla* luciferase, driven under the control of the cytomegalovirus promoter, was co-transfected to normalize for efficiency. When the level of FoxA2 was steadily increased in the transfection assays, the promoter region from –784 to +36 demonstrated a gradual increase in activity (Fig. 2). In contrast, FoxA2 expression had substantially less effect on the transcriptional activity of the promoter region from –351 to +36. A statistically significant increase for this region was observed at high levels of FoxA2 (5–10 μ g in the transfection). This could be a secondary or nonspecific effect. Taken together, this suggests that the promoter region from –784 to –351 (containing the three FoxA2 binding sites) is transactivated by FoxA2.

Next, we tested whether transactivation occurred for other FoxA family members. H4IIE cells were transfected with the promoter region from –784 to +36 and either FoxA2 or FoxA1. The DNA-binding domain of both forkhead proteins are nearly identical and are expected to have similar targets; however, previous experiments using adenoviral-mediated expression of FoxA1 or FoxA2 in hepatocytes have suggested that distinct targets may exist (22). In our experiments, FoxA1

TGCCCTAAGGATGTCATAACTCAGTCTAGGGATTTGGGCTG –745

–744 TCTGCTGCAATTGGTGACAATGAGCAAGGACAGTAACTTCATGTTGGCCTATAGTTCCT –685

–684 CTCCCAAGCAGAGAAGTGAATAGGTCAGCACATAGTCTCCATTTGCTTATTCAGAGGCAG –625

–624 AGTCTCGCTAGGCTGTACAGACTGGCCCTCTCTGCCTCTGTCTTCAGAGGGAAACACACA –565

Poly-AC region **USF/SREBP/FoxA2**

–564 CACACACACACACACACACACACACACACACCGGCCACTTCGTTTTCATGGGCTCACGTGTT –505

FoxA2

–504 TTCTTAACATTTGAGTGGCTGTGTGACTTTATTGTGGAGGAAGAAGAGCCAGCCAACTT –445

FoxA2

–444 CTCTTTGCTTGGGCCAAGGTGCTTGGACACAAGCAAGGAGCATCACAGCATTTCAAAT –385

–384 AACACAGACACCGGGCGGAGCCACAACCTTCTGAGCTCAGATTCAACTCTGCACAGACCT –325

–324 ACGCGTTTCCATAGAAGACCCCGCCAGGCTCGCACAAGCTGCAGAAGGCCAGCTGCGAGC –265

–264 CGTGAGCTACGTGCTGCACGCCAGGCTCCGGGAACACGGCTGGATCCCGTGGCCAGGG –205

–204 CTCAGGGACTCTACGGCCGCCTTCCAGCACGCTCTGGTCACATCCAGCCCCGCGGTGATC –145

–144 ACGTTCAGGGGCGAGGGCTCGTGCCCCAGCTGCACAGTGGTTGGTCAGGGCGCACGGCC –85

SREBP/USF

–84 TTTGATTGGTCGGAGGGACCGGTTACAGTGATCTGGCTTTGATAAGCTGCCTCCCGGTTG –25

→ Transcriptional Start

–24 CCTGCGGTCAGTCGGCCGGCTGGACCGCGGCTGCATCCTCTTAAGTACTGAGTGCGAA 36

FIG. 1. Sequence of the mouse PTG promoter. Nucleotide sequence of the 5' flanking region, including part of the first exon of the PTG gene. The *underlined* cytosine (C) at the +1 position indicates the transcriptional start site. TRANSFAC analysis revealed potential regulatory elements and transcription factors within this region. Putative protein binding sites for USF, SREBP, and FoxA2 are *underlined*. A poly-AC region is also found within the region –570 to –535.

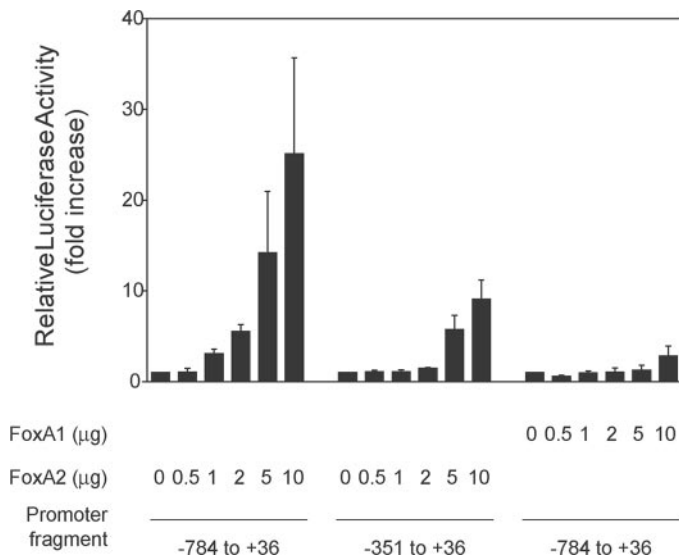


FIG. 2. FoxA2 transactivates the PTG promoter within the -784 to -351 region. H4IIE cells cultured in 10-cm dishes were cotransfected with $10 \mu\text{g}$ of the indicated luciferase reporter constructs and an increasing dose of FoxA2 or FoxA1 (up to $10 \mu\text{g}$). Empty vector was added to keep the total amount of plasmid at $20 \mu\text{g}$. Cells were harvested 48 h after transfection and assayed for luciferase activity. Results were normalized (Firefly/*Renilla* luciferase) and expressed as fold increase over the basal value of each construct. The data represent mean \pm SE of three independent experiments in each case. The promoter region -784 to $+36$ was transactivated by FoxA2 in a dose-dependent manner. In contrast, the -351 to $+36$ region required the highest doses of FoxA2 for transactivation. FoxA1 did not have a significant effect on the promoter region from -784 to $+36$.

was unable to transactivate the PTG promoter, even when expressed to high levels.

The promoter region from -784 to $+36$ contains putative binding sites for several transcription factors, including FoxA2, SREBP, and USF (Fig. 3). Thus, we mutated the binding sites and assessed their activity in H4IIE cells. Compared with the WT -784 to $+36$ region, mutation of the SREBP/USF (M1, M2) or FoxA2 (M3) binding sites significantly decreased promoter activity.

Binding of FoxA2 to the promoter region

To establish the binding of FoxA2 to the PTG promoter, we performed EMSAs using a 39-bp oligonucleotide duplex probe containing a FoxA2 binding site derived from the PTG promoter region (Fig. 4A). We isolated nuclear lysates from H4IIE cells (lanes 1–5) and mouse liver (lanes 6–10) and analyzed the complexes formed with the probe. Several complexes were observed, and most were disrupted with unlabeled probe (lane 2 *vs.* lane 3; lane 7 *vs.* lane 8). A high-molecular-weight complex in the liver samples that was not affected by addition of unlabeled probe (lane 8) probably represents nonspecific binding to the biotin moiety. To determine which complexes might involve FoxA2, we used an anti-FoxA2 antibody to disrupt the complexes (lanes 4 and 9). In the H4IIE and liver samples, a FoxA2 complex with similar molecular weight was disrupted (as indicated in the figure). As a control, rabbit IgG had no effect on any of the complexes (lanes 5 and 10). To further confirm the specificity of the probe, we performed an EMSA with liver lysates using a

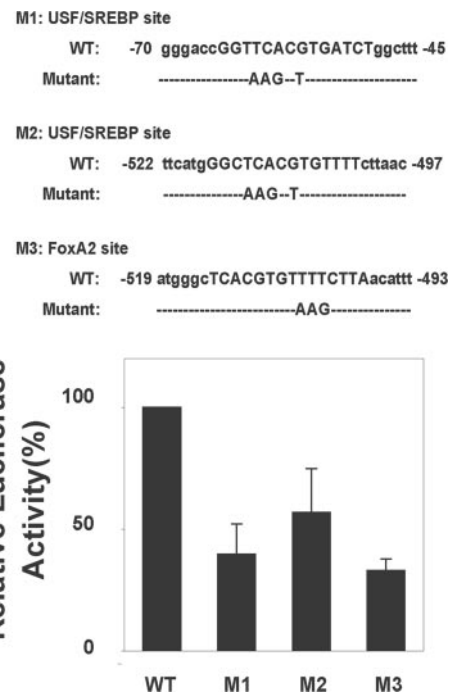


FIG. 3. Effect of binding site mutations on promoter activity. Consensus binding sites of various transcription factors were mutated by PCR-directed mutagenesis. H4IIE cells were transfected with WT (-784 to $+36$) or the indicated mutant reporter constructs and assayed for luciferase activity. Data were obtained as described in Fig. 2, except that the normalized activity of the WT construct was set to 100, and the value of each mutant was expressed as a percentage of that of the WT. The data represent mean \pm SE of five independent experiments.

biotinylated probe derived from the EBNA as another control (Fig. 4B). Only the high-molecular-weight nonspecific complex was formed.

We also used a ChIP assay to demonstrate the *in vivo* binding of FoxA2 to the PTG promoter region (Fig. 4C). Nuclear lysates from formaldehyde-cross-linked H4IIE cells and mouse liver were isolated and subjected to immunoprecipitations using anti-FoxA1, anti-FoxA2, or control (rabbit IgG) antibodies. PCR analysis was then performed on the immunoprecipitates using primers spanning the mouse or rat PTG promoter region (rat sequence obtained from the National Center for Biotechnology Information database (Gene ID: 309513). Sequence and TRANSFAC analysis demonstrated conservation of the FoxA2 binding sites between rat and mouse (data not shown). Nevertheless, only anti-FoxA2 immunoprecipitates contained the DNA target sequence. Thus, we demonstrate that FoxA2 binds to the PTG promoter *in vivo*.

cAMP regulates PTG levels in H4IIE cells

In astrocytes, the transcriptional up-regulation of PTG has been observed in response to several cAMP mimetics, leading to a massive increase in glycogen resynthesis (16, 17). A recent report demonstrated that FoxA2 was critical for the cAMP-induced transcription of several genes in hepatocytes (18). We were therefore interested to determine whether

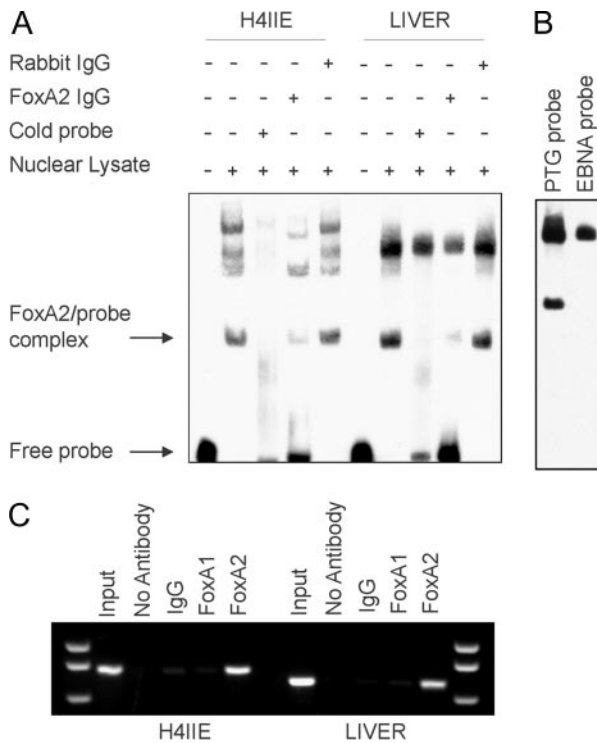


FIG. 4. FoxA2 binds to the PTG promoter region. **A**, Nuclear lysates from H4IIE cells (lanes 1–5) or mouse liver (lanes 6–10) were subjected to an EMSA using a biotin-labeled oligonucleotide probe containing a FoxA2 binding site derived from the PTG promoter region. Lanes 1 and 6 show free probe. The addition of lysate results in the formation of multiple complexes (lanes 2 and 7) that are essentially disrupted by the inclusion of unlabeled probe (lanes 3 and 8). In liver, the high-molecular-weight complex that is not disrupted probably represents nonspecific binding to the biotin moiety. An anti-FoxA2 antibody (lanes 4 and 9) was used to identify FoxA2 complexes (indicated by an *arrow*). Rabbit IgG (lanes 5 and 10) was used as a control. **B**, Nuclear lysates from mouse liver were subjected to an EMSA using the above PTG probe or a control probe derived from the EBNA sequence. **C**, A ChIP assay was performed in H4IIE cells and mouse liver. Cross-linked chromatin was immunoprecipitated with the indicated antibodies and extensively washed before elution and precipitation of DNA. PCR was performed with primers flanking the rat PTG promoter to amplify the sequence from –800 to +40. For the mouse liver samples, the primers used to amplify the sequence span from –702 to +28.

cAMP could cause the transactivation of the PTG promoter in H4IIE cells.

Cells were transfected with the PTG promoter region from –784 to +36 and then stimulated with the cell-permeable cAMP analog CPT and/or the phosphodiesterase inhibitor theophylline. Stimulation with CPT alone had a negligible effect, whereas theophylline induced an approximate 3-fold increase in activity compared with unstimulated controls (Fig. 5A). However, the combination of both compounds synergistically induced the promoter activity by 7-fold.

We also tested the ability of cAMP to transcriptionally regulate endogenous PTG levels. Stimulation of H4IIE cells with CPT caused a gradual increase in PTG levels up to 8 h (Fig. 5B). Furthermore, this effect was dose dependent (Fig. 5C). However, unlike what was observed in the luciferase experiments mentioned above, we noted that CPT alone produced an insignificant effect on promoter activity. We

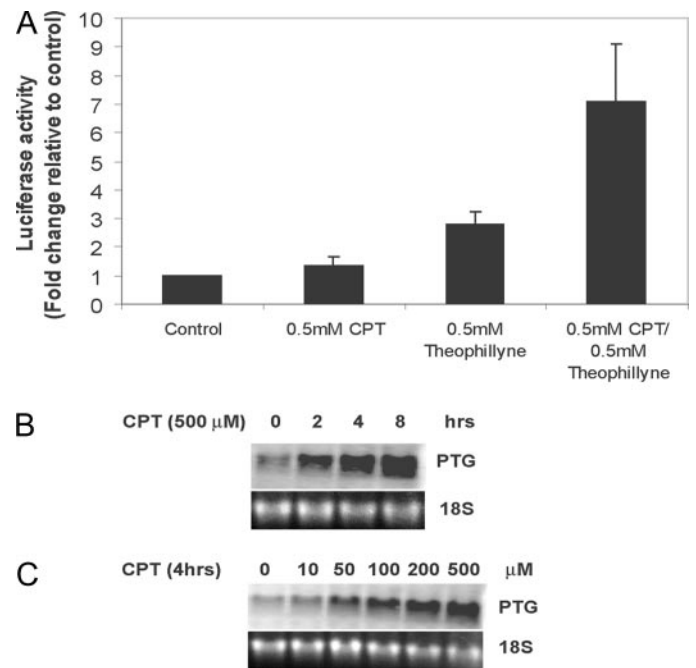


FIG. 5. cAMP increases PTG levels. **A**, H4IIE cells were transfected with 10 μ g of the reporter construct containing promoter region –784 to +36. Forty hours after transfection, cells were either left untreated (control) or treated with 500 μ M CPT and/or theophylline for 4 h and then assayed for luciferase activity. Data were obtained as described in Fig. 2 and expressed as fold increase over control. The data represent mean \pm SE of six independent experiments. **B**, H4IIE cells were treated with 500 μ M CPT for the indicated time, and total RNA was isolated with Trizol reagent (Invitrogen) and subjected to Northern blot analysis for the expression of PTG mRNA levels. **C**, H4IIE cells were treated with the indicated amount of CPT for 4 h and again subjected to Northern blot analysis for PTG mRNA expression.

suspect that our transfection conditions may have decreased the responsiveness of cells to cAMP and/or increased phosphodiesterase activity. Nevertheless, these results demonstrate that cAMP increases PTG levels in H4IIE cells.

Discussion

PTG is a scaffolding protein that assembles several glycogen-metabolizing enzymes with the serine/threonine phosphatase PP1 in the process enhancing their dephosphorylation (2). Both overexpression and knockout studies in cell culture and animal models have clearly established the physiological importance of PTG in regulating glycogen synthesis (8–11). However, how PTG itself is regulated remains poorly understood. In the present study, we cloned and analyzed the mouse PTG promoter to gain a better understanding of its transcriptional regulation.

TRANSFAC analysis of the promoter sequence identified putative FoxA2 binding sites (Fig. 1). To demonstrate that FoxA2 binds to the promoter, we performed EMSAs with nuclear lysates derived from mouse liver and H4IIE cells (Fig. 3A). Moreover, ChIP analysis demonstrated that FoxA2, but not FoxA1, bound to the PTG promoter *in vivo* (Fig. 3C). When expressed in H4IIE cells, FoxA2, but not FoxA1, readily transactivated the PTG promoter (Fig. 2). Only the region containing the FoxA2 sites was readily transactivated.

Taken together, these results demonstrate that expression of FoxA2 transactivates the PTG promoter.

The DNA-binding domains within the FoxA family are highly conserved, and all their targets possess an identical core sequence (19, 20). Nevertheless, FoxA1 did not bind the PTG promoter in the ChIP assay and did not transactivate the PTG promoter. In a recent study, the overexpression of FoxA1 or FoxA2 in hepatocytes was found to have different biological effects (22). For example, FoxA2, but not FoxA1, expression resulted in a decrease in glycogen synthase mRNA levels. One possibility for the differences in the ability of FoxA proteins to transactivate promoters may be because of the presence of accessory transcription factors. For example, HNF-6 has been shown to stimulate FoxA2 but not FoxA1 or FoxA3 transcriptional activity (24).

Recently, FoxA2 has also been shown to affect hepatic glycogen levels. Overexpression of FoxA2 in mouse liver or hepatocytes dramatically diminishes glycogen levels. This is attributable in part to a reduction in glycogen synthase (23). It would be interesting to determine whether PTG levels in these mice were also affected. However, even if they were up-regulated, the decrease of glycogen synthase would still be expected to predominate in the regulation of glycogen synthesis. It is also important to note that the stable overexpression of FoxA2 in hepatocytes or liver seems to disrupt the balance in the transcriptional network. For example, overexpression of FoxA2 in these cells leads to decreased expression of endogenous FoxA and HNF-6 proteins (23). Interestingly, rescue with adenovirus expressing HNF-6 was able to restore glycogen levels (21). Thus, it seems that the role of FoxA2 in glycogen metabolism is complex and is likely to involve multiple targets.

FoxA2 was recently demonstrated to be crucial for cAMP-dependent transcription of gluconeogenic genes in liver cells (18). We therefore asked whether cAMP would similarly regulate PTG levels in liver cells. We show here that cAMP transactivates the PTG promoter and also increases PTG mRNA levels (Fig. 5). At first glance, this seems counterintuitive. It is well established that cAMP induces glycogenolysis in liver, yet overexpression of PTG is known to increase glycogen levels. In addition, PTG levels have been shown to be decreased in starved rats (12). One possibility for this discrepancy is that in whole organisms, other hormones or growth factors may act in concert to regulate PTG expression. Also, organisms are able to maintain their blood glucose levels in a narrow range, whereas cells in culture cannot significantly adjust the level of glucose in the media. These factors may ultimately decide whether the PTG promoter becomes activated.

In astrocytes, the cAMP-mediated induction of PTG levels coincides with a massive glycogen resynthesis phase (16, 17). It is possible that increased PTG levels may prime cAMP-treated hepatocytes for glycogen resynthesis upon the removal of starvation signals, acting as a potential negative feedback loop. One recent example of such a scenario in cAMP signaling involves the transcription factor transducer of regulated cAMP response element binding protein activity 2 (TORC2) (29, 30). During fasting, cAMP activates the gluconeogenic program through TORC2. However, TORC2 also stimulates the expression of insulin receptor substrate 2,

resulting in enhanced insulin signaling to shut down gluconeogenesis (31). Thus, the balance of these two pathways serves to fine tune glucose output.

It is well established that depletion of muscle or liver glycogen stores leads to a resynthesis phase that exceeds the basal level, a phenomenon commonly referred to as glycogen supercompensation (32). It is clear that the initial phase of this glycogen repletion involves the activation of glycogen synthase and inactivation of glycogen phosphorylase through changes in protein phosphorylation (33, 34). Additional studies will be required to carefully determine the potential role of PTG during this phenomenon.

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