Chicken Ovalbumin Upstream Promoter-Transcription Factor II, a New Partner of the Glucose Response Element of the L-type Pyruvate Kinase Gene, Acts as an Inhibitor of the Glucose Response*

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Transcription of the L-type pyruvate kinase (L-PK) gene is induced by glucose in the presence of insulin and repressed by glucagon via cyclic AMP. The DNA regulatory sequence responsible for mediating glucose and cyclic AMP responses, called glucose response element (GIRE), consists of two degenerated E boxes spaced by 5 base pairs and is able to bind basic helix-loop-helix/leucine zipper proteins, in particular the upstream stimulatory factors (USFs). From ex vivo and in vivo experiments, it appears that USFs are required for correct response of the L-PK gene to glucose, but their expression and binding activity are not known to be regulated by glucose. A genetic screen in yeast has allowed us to identify a novel transcriptional factor binding to the GIRE, i.e. the chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII). Binding of COUP-TFII to the GIRE was confirmed by electrophoretic mobility shift assays, and COUP-TFII-containing complexes were detectable in liver nuclear extracts. Neither abundance nor binding activity of COUP-TFII appeared to be significantly regulated by diets. In footprinting experiments, two COUP-TFII-binding sites overlapping the E boxes were detected. Overexpression of COUP-TFII abrogated the USF-dependent transactivation of an artificial GIRE-dependent promoter in COS cells and the glucose responsiveness of the L-PK promoter in hepatocytes in primary culture. In addition, a mutated GIRE with increased affinity for USF and very low affinity for COUP-TFII conferred a dramatically decreased glucose responsiveness on the L-PK promoter in hepatocytes in primary culture by increasing activity of the reporter gene in low glucose condition. We propose that COUP-TFII could be a negative regulatory component of the glucose sensor complex assembled on the GIRE of the L-PK gene and most likely of other glucose-responsive genes as well.

Carbohydrates are the major source of energy for mammalian cells. High carbohydrate intake results in the conversion of excess carbohydrates to triglycerides in the liver and adipocyte. This process is accompanied by the induction of many enzymes of the glycolytic and lipogenic pathways. This glucose response has been found to be, at least in part, at the transcriptional level, e.g. in the case of the L-pyruvate kinase (L-PK) (1), aldolase B (2), spot 14 (S14) (3), acetyl-CoA carboxylase ACC (4), hormone-sensitive lipase HSL (5), and fatty acid synthase (6) genes. However, little information has been reported so far on the interacting factors that confer transcriptional glucose responsiveness on these genes.

Our laboratory has extensively studied the regulation of the L-PK gene by glucose. Dioron et al. (7) suggested that glucose is metabolized into active intermediates that in turn activate specific transcriptional factors that enhance the L-PK gene promoter. We defined a sequence responsible for mediating the positive response to glucose and the negative responses to cAMP in the proximal L-PK promoter from the position −172 to −142 bp, ex vivo by transient expression assays in hepatocytes in primary culture and in vivo in transgenic mice (8–11). This DNA-binding site was termed the glucose response element (GIRE). This GIRE is closely related to the carbonate response element (ChoRE) described by Towle’s group (12) in the regulatory region of the S14 gene and also to the glucose response element recently identified upstream of the glucagon receptor gene (13). In the context of the wild-type L-PK promoter, the complex that binds to this element cooperates with an adjacent DNA-binding site, the auxiliary site L3 (8, 10), to confer a strong glucose responsiveness. This auxiliary site has been shown to bind mainly hepatocyte nuclear factor 4 (HNF4) in the liver but has also some affinity for chicken ovalbumin upstream promoter transcription factor (COUP-TF) (10, 14) and nuclear factor 1 (15). However, our group and Towle’s group showed that a GIRE multimer is sufficient to confer both carbohydrates and cAMP responses in the absence of the auxiliary site (8, 10). The GIRE consists of two palindromic noncanonical E boxes (CANNTG) separated by 5 bp (12). The full activity of the L-PK GIRE seems to require the cooperation between these two E boxes (8, 10). We have found that these E boxes are crucial for the regulation of the L-PK gene by glucose.

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Glucose-responsive plasmids were transformed and then tested for specificity by transforming yeast cells containing either the GIRE-HIS3 or the unrelated MEPF3-HIS3 gene construct integrated in the genome. From this second round of selection, only one plasmid was shown to result in the specific activation of the GIRE-HIS3 reporter gene. The cDNA insert selected within this plasmid was sequenced using the Sanger dideoxy termination method adapted to the automated sequencer 373A from Applied Biosystems.

Plasmid Constructions—All plasmids were constructed by using standard DNA cloning procedures. The constructs were verified by nucleotide sequencing.

Using COUP-TFII DNA cloned into an eukaryotic expression vector, pMT2, driven by the adeno virus major late promoter and the simian virus 40 enhancer region (kindly provided by Dr. S. Karathanasis). Human USF2a was cloned into an eukaryotic expression vector, pCR3, driven by the cytomegalovirus immediate-early promoter region (18). The USF2 gene gives rise predominantly to full-length USF2a subunits, associated with two minor forms, USF2b (resulting from an alternative splicing out of an exon in the NH2 domain) and mini-USF2, a NH2-truncated form.

COUP-TFII used for in vitro transcription/translation was obtained by inserting the COUP-TFII EcoRI DNA fragment into the pCR3 vector. The recombinant rat COUP-TFII expression vector pGEX-KG-COUP-TFII was a gift from J.-M. Boutin. The NcoI-XhoIII fragment containing the entire coding frame, was inserted in the same sites in the pGEX-KG vector (32). All plasmids were purified with a Qiagen kit.

Animals—Three-month-old Harlan Sprague-Dawley rats were subjected to different previously described nutritional and hormonal treatments to study L-PK expression in the rat liver (1). Animals were fasted for 48 h, and then separated into three groups and fed for 24 h with different diets: high carbohydrate for the first group, high protein for the second group, and high fat for the third group. As previously demonstrated, we observed that the endogenous L-PK mRNA was abundant in the animals of the first group and scarcely detectable in the animals of the other two groups (data not shown). Other rats were fed with a regular chow ad libitum.

In Vitro Transcription and Translation—The plasmids pCR3/COUP-TFII and pCR3/USF2a were linearized with NotI and XhoI, respectively, and transcribed with T7 RNA polymerase in the presence of m[7G]5[ppp]5'G (Roche Molecular Biochemicals). The resulting mRNAs were translated in vitro with rabbit reticulocyte lysate according to the manufacturer's instructions (Promega).

Recombinant Protein Production—GST-COUP-TFII protein was expressed in Escherichia coli BL-21 (DE3). Overnight cultures of bacteria that were newly transformed with the plasmid pGEX-KG-COUP-TFII were diluted with 10 volumes of medium, cultured for several hours to an optical density of 0.6 at 600 nm, and induced with 1% isopropyl-

β-D-thiogalactopyranoside at 37 °C for 3 h. Bacteria from 500 ml of culture were harvested and resuspended in 10 ml of NTEN (0.2 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 10 μg of leupeptin/ml, 10 μg of pepstatin/ml, 10 μg of aprotinin/ml, 1 mM phenylmethylsulfonyl fluoride). The lysates were sonicated, and after centrifugation, the supernatants were mixed with glutathione-Sepharose 4B beads (500 μl, Amersham Pharmacia Biotech) at 4 °C for 30 min in NTEN buffer. After two washes with TG buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol), the GST-COUP-TFII was eluted with TG buffer containing 30 mM glutathione. GST-COUP-TFII protein was expressed in E. coli tagged with histidine residues at its N terminus, allowing purification over Ni2+ affinity resin as described (18).

Extract Preparation and EMSA—Nuclear extracts for EMSA were prepared at a concentration of 0.1 mg/mL. Different methods of nuclear extract preparation were tested. Results present nuclear extract preparation according to the procedure described in Hasegawa.

**MATERIALS AND METHODS**

**Yeast Strain**—The Saccharomyces cerevisiae strain used in this study was the CD156 strain (MATa, ade2, his3, leu2, lys2, trp1, ural3, gal4, gal80, cfl1Δ). The CYC1-HIS3 gene fusion used for the one-hybrid screen was as described in Blaiseau et al. (28). Wild-type GIRE motifs of the L-PK gene was cloned as two oriented copies, 200 bp upstream of the TATA box owing to the unique cloning site Stu I (within the TATA box) and was first identified as a homodimer that binds to a direct repeat regulatory element (DR1) in the chicken ovalbumin gene promoter (27). Indeed, COUP-TFII is able to bind to the GIRE in vitro, and COUP-TFII-containing complexes interacting with the GIRE are detected in liver nuclear extracts. DNA binding activities of these complexes do not seem to be modulated by diets. COUP-TFII- and USF-binding sites are overlapping, and consequently binding of these factors mutually interferes one on the other. Here, we demonstrate that overexpression of COUP-TFII not only inhibits USF-dependent transactivation of the L-PK promoter but also represses its stimulation by glucose in hepatocytes. Furthermore, a mutant GIRE with very low affinity for COUP-TFII conferred an impaired glucose response, because of increased activity under low glucose conditions. We propose that the glucose responsiveness mediated by the GIRE could involve a complex interaction between USF transactivators and COUP-TFII, probably in an as-yet-undefined manner, mediated by the GlRE could involve a complex interaction between USF transactivators and COUP-TFII, probably in an as-yet-undefined manner.
et al. (25) in the presence of phosphatase inhibitors, as described in Ref. 33.

EMSA were performed at room temperature with 1 ng of double-stranded oligonucleotides as probes, end-labeled and blunted-ending with Klenow enzyme with the appropriate radionucleotide or labeled in the presence of [γ-32P]ATP and polynucleotide kinase. Incubations were done on ice in the presence of 10 mM Tris- HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 5% (v/v) glycerol, 1 μg of poly(dI-dC) (100 ng for recombinant proteins), and either 5 μg of liver nuclear extracts or 1–4 μl of reticulocyte lysate extracts and recombinant proteins. In Fig. 5B, the reaction buffer used was 20 mM Hepes/ KOH, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 5 mM DTT, 0.5% (v/v) nonfat dry milk, 10% (v/v) glycerol as reported by Hasegawa et al. (25).

For competition assays, 10–50 ng of different oligonucleotides were used as specific competitors, and 50 ng of MEF3 oligonucleotide was used as an unrelated competitor. For antibody supershift analysis, 0.5–1 μl of the antisera were included in the binding reactions.

Each reaction mixture was then electrophoresed in a native 6% (w/v) polyacrylamide gel in 0.25 × Tris-borate-EDTA (or 5% in the case of the in vitro translated product and recombinant proteins experiments or 4.5% in Fig. 5B). Gels were prerun for 30 min and run for 3–4 h at 140 V.

The sequences of L-PK/GIRE (L4 or LL), MM, L-PK, MLP, NF-Y, and MEF3 oligonucleotides were as follows: GIRE-LPK site (1172 to 12–12) GGCGGCCCAGGACCCTCCTCGTGTTGTCGTCAGGTTGTCGCTCGG; MM site (bp –172 to –142) TGGGGCCACGGGGCACTCCCGTGGTTCGCTCGG; L-PK site (bp –150 to –121) TGGTTCTCCGACTCGTCCTGGCCCCAGGTGATCAGGTTGATAGCCGGCCGACGGTGCTCGTCTCGG; MLP site (bp –94 to –65) GGGGAGTGACACATGTAAACGTTGACGGAGGGCCGACGGCTCGTGCTCGG.

Anti-COUP-TFI antibody, used to characterize the retarded complexes in supershift or Western blotting experiments, was provided by Dr. S. Karathanasis. EKαx and TRα antibodies were purchased from Santa Cruz. Anti-USF (USF1 and L22) antibodies were produced in our laboratory (18).

**Results**

**Molecular Cloning of COUP-TFI by One-hybrid Selection in Yeast**—To identify transcriptional factors other than USF that could recognize the L-PK GIRE (also named L4 or LL boxes), we used the one-hybrid method, which allows, in yeast cells, the cloning of eukaryotic DNA-binding factors. First, we observed by mobility shift assays that wild-type yeast cell extracts do contain proteins capable of binding to the L-PK GIRE with a high affinity (data not shown). It was previously established that, in *S. cerevisiae*, the major CAGGTG binding activity is accounted for by the multifunctional factor *cbf1* (35). To increase the sensitivity of our one-hybrid screen, we decided to perform the screen in cells that do not express *CBF1*, thus avoiding competition between the endogenous GIRE binding activity and the factors encoded by the transfected cDNA libraries. The *CBF1* gene was disrupted by using the *hisg-URA3-hisg* marker construct (36). In a second step, the *URA3* marker was removed from the *cbf1* locus because of recombination between the two *hisg* direct repeats by selecting for uracil auxotroph cells on 5-fluoroorotate-containing medium (37), yielding the CD156 strain (*cbf1::hisg*). As expected, the *cbf1::hisg* cells were methionine auxotroph and exhibited only a residual GIRE DNA binding activity (data not shown). A GIRE-HIS3 reporter gene was then integrated into the genome of the CD156 strain at the *URA3* locus. The GIRE-HIS3 fusion consisted of two tandem copies of the GIRE inserted 200 bp upstream of the CYTA gene, which is the catalytic C domain of the CytA complex, with its own UAS deleted and placed in front of a *hisg* gene. The multicistronic (YM1) exhibited a leaky His+ phenotype that was suppressed by the addition of 10 mM AT to the medium. The YM1 strain was transfomed by an adult rat liver cDNA expression library that coded for oriented poly(A) tail protein domains fused to the Gal4p transcriptional activation domain. Transformsants were selected directly for growth in the presence of 10 mM AT. From a screen of about 2 × 10^6 transfor-
mants, 30 colonies capable of growing in the presence of 10 mm AT appeared over a course of 8 days. Plasmid DNA was recovered from these colonies and used to retransform the YMV1 and the YMEF3 control strains (the latter comprising the unrelated integrated MEF3-HIS3 gene fusion). Only one plasmid gave rise to AT-resistant clones when transformed in the YMV1 strain but not in YMEF3 control strain. Sequencing of this plasmid revealed that it encodes an USF2 cDNA deleted from exons 1 and 2 and fused in frame to the Gal4p activation domain. This result thus confirmed that in yeast cells, USF is indeed capable of binding to the L-PK GIRE and provided us with a positive control of our screening.

To account for the fact that this first screen did not reveal a new GIRE-binding factor, we next surmised that if such proteins exist, they might be more abundant in tissues other than liver. Therefore, we performed a new one-hybrid screen in the YMV1 strain with another library that expresses short fragments of mouse embryo cDNA fused to the VP16 activation domain (29). In this case, the transformants were selected directly for growth in the presence of 30 mm AT. From a screen of about 2 × 10^6 transformants, 60 colonies capable of growing in the presence of 30 mm AT appeared over a course of 10 days, and plasmids were recovered. After retransformation of both the YMV1 and YMEF3 strains, only one plasmid was shown to encode a fusion protein capable of specifically activating transcription of the GIRE-HIS3 reporter gene. Sequencing revealed that this plasmid expressed the VP16 activation domain fused to an 80-amino acid open reading frame comprising the COUP-TFII DNA-binding domain flanked by additional residues.

**COUP-TFII Protein Binds to the L-PK GIRE**—The DNA binding capacity of the entire COUP-TFII protein was assessed by EMSA with in vitro translated COUP-TFII. Double-stranded oligonucleotide containing wild-type L-PK GIRE was retarded by in vitro translated COUP-TFII (Fig. 1A, lane 1). A polyclonal antiserum raised against COUP-TFII specifically recognized the protein-DNA complex (Fig. 1A, lane 2). We also demonstrated that in vitro translated COUP-TFII bound to a DR1 probe was competed for by the L-PK GIRE oligonucleotide (data not shown). In addition, as previously demonstrated USF2a was retarded and displaced by anti-USF2 (Fig. 1A, lanes 3 and 4). The faint band migrating faster was also displaced by anti-USF2 antibody (Fig. 1A, lanes 3 and 4). This USF protein was described as mini-USF (ΔUSF2) (18) and was supposed to be generated by internal translation initiation from internal methionines.

Binding of COUP-TFII to the GIRE was also evaluated by DNase I protection assays, using recombinant COUP-TFII protein and the −196 to −96 region of the L-PK gene promoter as probe. A protection pattern was observed between bases −172 and −142 encompassing the GIRE site (Fig. 2A for coding strand and Fig. 2B for noncoding strand). A second region was strongly protected between bases −142 and −126 corresponding to the L3 box. This L3 region contains a DR1 site that has been previously described to bind not only HNF4 but also COUP-TF (10). Examination of the GIRE area suggested that it could encompass at least two COUP-TFII-binding sites. COUP-TFII strongly protected a central half-site between −162 and −157 and more weakly 5′ and 3′ half-sites, generating direct repeats DR1 or DR7 in the GIRE (half-sites are delineated by arrows in Fig. 2C). These two DR1 and DR7 sites have been shown by EMSA to have similar affinity for COUP-TFII (data not shown). A G to T mutation of the second position of the central half-site (mutant MM) practically abolished any affinity for COUP-TFII (Fig. 1B, lane 1), whereas this mutation created a consensus E box (CACG TG) with high affinity for USF (Fig. 1B, lane 2). These results suggested that COUP-TFII and USF binding to the GIRE could interfere one with the other.

**Interference between COUP-TFII and USF in Binding to the GIRE**—To understand more precisely the interaction between COUP-TFII and USF transcription factors and the GIRE, we performed band shift experiments using COUP-TFII and USF2 recombinant proteins. In these experiments, a combination of both proteins in various ratios was used. Recombinant COUP-TFII and USF2a proteins formed two major distinct complexes (Fig. 3, lanes 1 and 6). In lane 6 of Fig. 3, the major faster migrating complex was a USF2a homodimer, and the slower complex corresponded to two homodimers, each binding to one of the two palindromic E boxes separated by 5 bp. Accordingly, both can be competed for by a USF-binding site and displaced by anti-USF antibodies (data not shown). In lane 1 of Fig. 3, COUP-TFII corresponded to a major fast migrating complex (truncated, tCOUP-TFII), whereas a minor slower migrating band (full-length, fCOUP-TFII) was, on this gel, at the limit of detection. Fig. 3B shows a Western blot analysis of recombinant COUP-TFII, demonstrating that the major form was a proteolytic product. The same result has been previously reported by Malik and Karathanasis (26), namely that recombinant full-length COUP-TFII (rARP-1) preparations were very sensitive to proteolytic cleavage, leading to a truncated form retaining DNA binding activity.

Upon addition of increasing amounts of USF2a, an increase in USF2a-DNA complexes and a corresponding decrease in the COUP-TFII retarded complex was observed (Fig. 3, lanes 2–4).
used in our laboratory (18) and the one described by Hasegawa et al. (25). This buffer is characterized, in particular, by increased EDTA concentration (to 1 mM) and absence of KCl. In this new binding conditions, up to seven DNA-protein complexes were detected with the GIRE probe (Fig. 4, lane 1). Identification and specificity of these complexes were analyzed by competition with unlabeled oligonucleotides and depletion or supershift by specific antibodies. All of these complexes were specific because they were displaced by 10 ng of unlabeled GIRE site (Fig. 4, lane 3). The complex 2 corresponded to binding of the USF1/USF2 dimers because depletion by anti-USF antibodies (Fig. 4, lane 2) and competition with an unlabeled MLP site (data not shown and Ref. 18) suppressed formation of this complex. Complexes 1 were likely to belong to the Sp1 family of transcriptional factors as demonstrated by competition with a GC box-containing oligonucleotide (lane 4). Finally, the complex 3 binding activities remaining after USF depletion and Sp1 DNA binding activity displacement were competed for by an excess of a DR1 motif (Fig. 4, lanes 12 and 13) and of the L3 element of the L-PK gene promoter, which also contains a DR1 site (Fig. 4, lanes 14 and 15) (10); in contrast the complex 3 binding activities were not displaced by an unrelated probe (Fig. 4, lane 11). These results suggest that the complexes 3 could contain members of the nuclear receptor superfamily. To determine whether the complex 3 binding activities actually corresponded to COUP-TFII, we used an antibody toward COUP-TFII. The addition of this antibody to liver nuclear extracts, depleted of USF DNA binding activity, supershifted the complex 3 binding activities (Fig. 4, lane 5), whereas the addition of anti-RXRα or anti-TRα antibodies did not affect them (Fig. 4, lanes 6 and 7). After depletion of USF and Sp1 DNA binding activities, we observed that the addition of COUP-TF supershifted both complexes 3 (Fig. 4, lane 8), whereas the addition of anti-RXRα or anti-TRα antibodies did not affect them (Fig. 4, lanes 9 and 10). All other antibodies specific to various members of the nuclear receptor superfamily tested so far were without any effect on the complex 3-DNA binding activities (data not shown). These results confirm that the two COUP-TFII containing complexes binding to the GIRE can be easily detected in liver nuclear extracts, provided that binding conditions are optimized.

In Vitro Binding of COUP-TFII-containing Complexes Is Not Significantly Modulated by Diets—In Fig. 5A, we compared the GIRE binding activities in liver nuclear extracts of rats fed either L-PK gene inducing diet (i.e. high carbohydrate, lanes 1 and 2) or L-PK gene inhibiting diet (i.e. high protein in lane 3 and high fat in lane 4). The complexes described in Fig. 4 were still observed, in particular complex 2, i.e. USF, supershifted by anti-USF antibodies (Fig. 5A, lane 5), and complexes 3 supershifted by anti-COUPTFII antibodies (Fig. 5A, lane 6) but not by anti-RXRα antibodies (Fig. 5A, lane 7) and competed for by a DR1 motif (Fig. 5A, lane 8). With respect to the NF-Y (rat albumin CAAT box) (Fig. 5C) and nuclear factor 1 (data not shown) complexes characterized in parallel to control quality and quantity of the nuclear extracts, none of the GIRE binding activities seemed to be detectably affected by diets. We also found that the relative binding activity of the different GIRE-binding complexes depends on the binding conditions; Fig. 5B shows the patterns obtained with extracts from carbohydrate-refed and protein-rich diet-refed rats using the binding buffer described by Hasegawa et al. (25). Here, as in our previous publication (18), USF is the major binding activity, whereas COUP-TF-containing complexes and GC-box binding activities are scarcely detectable (compare lanes 1 and 3 of Fig. 5A and lanes 1 and 2 of Fig. 5B).

In Fig. 5D, we evaluated the presence of COUP-TFII and

**Fig. 2.** DNase I footprinting protection of the rat L-PK promoter by COUP-TFII. A 3′-end-labeled L-PK promoter fragment extending from −196 to −96 bp was subjected to partial digestion by DNase I in the absence (Free) or in the presence of increasing amounts of recombinant COUP-TFII protein, either on the coding (A) or on the noncoding strand (B) as outlined under "Materials and Methods." A sequencing reaction performed on this fragment (Maxam and Gilbert) was run in parallel (G + A). The protected regions are indicated by either a solid or a dotted line (indicating partial protection), and arrows indicate hypersensitivity to digestion relative to the free DNA. The numbers alongside refer to the positions with respect to the transcriptional initiation site. C, the oligonucleotides containing the wild-type L-PK GIRE (LL) or mutated L-PK GIRE (MM) used in the experiments are shown. Arrows indicate COUP-TF half-binding sites, and brackets indicate the DR1 or the DR7 COUP-TF DNA-binding sites. E-boxes are in boxes. Mutated nucleotides are indicated by vertical lines.

Conversely, upon addition of increasing amounts of COUP-TFII, an increase in COUP-TFII-DNA complex and a corresponding decrease in the USF2a retarded complexes was observed (Fig. 3, lanes 7–9). These results suggest a competition between USF and COUP-TFII for binding to the GIRE. However, we could observe a minor complex detected only when both COUP-TFII and USF are present. This complex was clearly detectable at a ratio of 1 COUP-TFII/2 USF2 (Fig. 3, lanes 4 and 7), displaced by DR1 (Fig. 3, lane 5) and MLP sites (data not shown). This result suggests that a ternary complex consisting of the GIRE probe, one USF dimer, and one COUP-TF dimer can be detected in vitro in the presence of concentrated recombinant factors. Fig. 2C shows that, indeed, binding of a USF dimer on the downstream E box and a COUP-TF dimer on the DR1 overlapping the upstream E box is in principle conceivable.

**Liver Nuclear Extracts Contain Different Factors Able to Bind the L-PK GIRE—**We performed band shift experiments using the GIRE oligonucleotide and liver nuclear extracts from rats fed a regular diet ad libitum. EMSA were performed with a novel binding buffer as compared with the one previously

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**COUP-TFII, a New Partner of the Glucose Response Complex**

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In Fig. 5D, we evaluated the presence of COUP-TFII and
USF2 in carbohydrate and protein-refed liver nuclear extracts by Western blot analysis with specific antibodies. The amounts of COUP-TFII and USF2 proteins were similar under both dietary conditions used.

COUP-TFII Represses USF2-dependent Transcription of the L-PK Promoter in COS-7 Cells—COUP-TFII has been demonstrated to be a repressor of transcription of many genes (27). Therefore, we explored the possibility that COUP-TFII homodimers might counteract the activity of USFs on a promoter depending on the GIRE element. To test this hypothesis, COS-7 cells were transiently transfected with L-PK reporter plasmids and both USF2a and COUP-TFII expression vectors. The (LL)_2-54-PK/CAT reporter plasmid was directed by two copies of the wild-type GIRE, while the (MM)_2-54-PK/CAT construct was directed by two copies of the MM mutant GIRE. This latter mutant was characterized by very low affinity for COUP-TFII and high affinity for USF (Fig. 2C). Fig. 6A showed that expression of USF2a increased dramatically the CAT activity of the LL reporter construct (lane 3), as previously reported (19). As expected USF2a also increased activity of the MM mutant reporter gene (Fig. 6A, lane 10) because canonical E boxes were created. COUP-TFII overexpression in transfected COS-7 cells inhibited this USF2a-mediated activation of the reporter genes in a dose-dependent fashion, but this inhibition was more important on the LL (Fig. 6A, lanes 4–7) than on the MM construct (lanes 11–14). Western blot controls allowed us to assure that the COUP-TFII inhibitory effect was not due to inhibition of the USF2a expression vector; USF2a abundance was constant in cells transfected with a same amount of USF2a expression vector and increasing amounts of COUP-TFII expression vector (Fig. 6A, lower panels). These results demonstrate that COUP-TFII is capable of antagonizing transactivation by USF2a of a reporter gene directed by the wild-type GIRE but that this antagonism effect is strongly reduced by a GIRE mutation decreasing affinity for COUP-TFII.

COUP-TFII Represses GIRE-mediated Glucose Induction in Hepatocytes—To determine whether COUP-TFII could play a role in mediating the glucose responsiveness, we used the natural −183 L-PK promoter and a reporter gene carrying three copies of the GIRE placed upstream of the −54 L-PK minimal promoter, (LL)_3-54-PK/Luc in hepatocytes in primary culture. To increase sensitivity of the assay after transient transfection in hepatocytes, we constructed different pGL3 luciferase chimeric L-PK constructs. As can be seen in Fig. 7, both the natural −183 L-PK promoter and the artificial promoter with oligomerized GIRE (LL)-binding sites were specifically stimulated by glucose, 8- and 50-fold, respectively, whereas a construct directed by oligomerized box L3, (L3)_3-54-PK/Luc was insensitive to glucose. Cotransfection of hepatocytes with both glucose-responsive L-PK/Luc constructs and COUP-TFII expression vector repressed the response to glucose, strongly for the (LL)_3-54-PK construct and totally for the −183-PK construct. Because the inhibitory effect of COUP-TFII overexpression on the glucose-dependent promoter activation was observed with the (LL)_3-54 plasmid devoid of the L3 element, which was found to bind HNF4 and COUP-TF factors (10), as well as with the −183-PK construct, this effect could not be ascribed to competition of COUP-TFII for binding of HNF4 to element L3 but rather to interaction with the GIRE. Accordingly, COUP-TFII did not repress activity of the (L3)_3-54 construct in hepatocytes. To confirm these data, we also studied the effect of HNF4 overexpression on the inhibition by COUP-TFII of the glucose-dependent −183-PK/Luc activation; although HNF4 overproduction increased the glucose responsiveness, as already described (38), it did not block the inhibitory action of COUP-TFII (data not shown). Therefore, these experiments demonstrate that COUP-TFII is an inhibitor of glucose responsiveness that acts through the GIRE.

This conclusion was supported by the decreased glucose responsiveness of a L-PK/Luc construct directed by three copies of the MM GIRE mutant with decreased affinity for COUP-TF and increased affinity for USF (Fig. 6B). Although a (LL)_9-96-PK/Luc construct (directed by three copies of the wild-type GIRE upstream of the L-PK HNF1-binding site) (16) was activated 26 ± 6-fold by glucose in hepatocytes, the MM construct was only activated 3.2 ± 1.2-fold. This decreased glucose re-
COUP-TFII, a New Partner of the Glucose Response Complex

**Fig. 4.** EMSA analysis of nuclear GIRE-binding proteins in the liver. Liver nuclear extracts (6 μg) were prepared from rats fed ad libitum a regular diet. Competition experiments were performed with either 25 or 50 ng unlabeled oligonucleotides. Lane 1, nuclear extract; lane 2, same as lane 1 plus 0.3 μl of anti-USF1 and 0.3 μl of USF2 antibodies; lane 3, same as lane 2 plus GIRE competitor; lane 4, same as lane 2 plus Sp1 competitor; lane 5, same as lane 2 plus 1 μl of anti-COUPTFII antibody; lane 6, same as lane 2 plus 1 μl of anti-RXRα antibody; lane 7, same as lane 2 plus 1 μl of anti-TRα antibody; lane 8, same as lane 4 plus 1 μl of anti-COUPTFII antibody; lane 9, same as lane 4 plus 1 μl of anti-RXRα antibody; lane 10, same as lane 4 plus 1 μl of anti-TRα antibody; lane 11, same as lane 4 plus MEF3 competitor; lane 12, same as lane 4 plus 25 ng of DR1 competitor; lane 13, same as lane 4 plus 50 ng of DR1 competitor; lane 14, same as lane 4 plus 25 ng of L3 competitor; lane 15, same as lane 4 plus 50 ng of L3 competitor. Position of the complexes of types 1, 2, and 3 are indicated on the left. F indicates free probe.

**Fig. 5.** EMSA analysis of the nuclear GIRE-binding proteins in the liver of rats fed different diets. A, EMSA analysis using the same binding buffer as in Fig. 4. 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 5% (v/v) glycerol. C1 and C2 are extracts from different animals. Lanes 1 and 2, nuclear extract from a rat refed a carbohydrate-rich diet (C); lane 3, nuclear extract from a rat refed a protein-rich diet (P); lane 4, nuclear extract from a rat fed a fat-rich diet (F); lane 5, same as lane 1 plus 0.25 μl of anti-USF1 and 0.25 μl of USF2 antibodies; lane 6, same as lane 1 plus 1 μl of anti-COUPTFII antibody; lane 7, same as lane 1 plus 1 μl of anti-RXRα antibody; lane 8, same as lane 1 plus DR1 competitor. B, EMSA analysis using the binding buffer described by Hasegawa et al. (25): 20 mM Hepes/KOH, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10% (v/v) glycerol. Lane 1, same as lane 1 of A; lane 2, same as lane 3 of A. C, EMSA analysis with the NF-Y probe. The extracts C2, C1, P, and F are the same as in lanes 1–4 of A. D, Western blot analysis of COUPTFII and USF2 proteins in extracts C1 and P (same as lanes 2 and 3 in A). The retarded complexes of types 1, 2, and 3 are indicated on the left; the asterisk indicates the position of the supershifted complexes, and F indicates the position of free probe.

The most intensively investigated glucose/carbohydrate response elements are the GIRE of the L-PK gene promoter (8–10, 12, 39) and the ChoRE located 1448 bp upstream of the S14 gene (12, 24). Both are able to bind USF transactivators, but this property alone does not easily explain the glucose responsiveness because USF-binding sites exist in regulatory regions of numerous genes insensitive to nutrient regulation. Moreover, Kaytor et al. (24) reported that a mutant ChoRE that had lost its ability to bind USFs still retained its glucose responsiveness. However, in our hands, this mutant conserved a clearly detectable affinity for USFs.2 In any case, affinity for USFs is clearly not parallel to the efficacy as a glucose response element. Although our results in cell culture (19) and in vivo in knock-out mice indicate that endogenous USFs are important for a kinetically normal activation of various dietary-dependent genes by glucose (20–22), they cannot explain by themselves the transcriptional regulation of glucose-responsive genes by glucose. Because both S14 and L-PK gene ChoRE/GIRE have the same structure, characterized by two more or less degenerated E boxes separated by a 5-bp spacer, it could be hypothesized that this unique arrangement was needed for binding of both USF dimers and other components of a multimolecular glucose sensor component. Therefore, we undertook a search for additional GIRE-binding proteins that could act as components of the glucose sensor system.

COUP-TFII Binds to the GIRE, in Vivo and in Vitro—Our one-hybrid screen in yeast allowed us to detect two GIRE-binding proteins: USF2, starting from an adult rat liver cDNA library, and COUP-TFII from a mouse embryo cDNA library. The liver-derived library was constructed using oligo(dT) as the primer for first strand cDNA synthesis, which probably explains why COUP-TFII was not found here. Indeed, the DNA-binding domain for this factor is very upstream from the

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2 M. Vasseur-Cognet, unpublished results.
poly(A) tail in the mRNA and was therefore highly underrepresented in the liver library. In contrast, the embryo library was derived from cDNAs primed with random hexamers, such that cDNAs for 5' parts of the messengers had more chance to be present.

COUP-TFII protein synthesized in a cell-free transcription-translation system binds efficiently to the GlRE and also to the S14 ChoRE (data not shown). EMSA of the GlRE incubated with liver nuclear extracts revealed complexes displaced by a DR1 oligonucleotide in excess and supershifted by anti-COUP-TF antibody. Finally, footprinting experiments localize two COUP-TF DNA-binding sites within the GlRE that overlap the E boxes: a DR1 in 5' (that does not bind HNF4; data not shown) and a DR7 in 3'. Both contained a common central half-site (Fig. 2C).

In addition to USF and COUP-TF-containing complexes, the GlRE also gave Sp1-type complexes displaced by a Sp1-specific oligonucleotide competitor. Because binding of Sp1 has been proposed to be involved in the response of the ACC gene to glucose in cultured adipocyte cell line (40), we asked whether Sp1 binding might be a component of the GlRE/ChoRE. In fact, the S14 ChoRE has no affinity for Sp1, and we never cloned Sp1-cDNAs in our one-hybrid screen, which could indicate that in vivo, in yeast, the GlRE does not interact with members of

**FIG. 6.** Compared cis-acting effects of the wild-type (LL) and mutant (MM) GIREs on COUP-TFII-dependent inhibition and glucose responsiveness of L-PK reporter constructs. A, COS-7 cells were transfected with 2 μg of different reporter constructs: (LL)2-54PK/CAT and (MM)2-54PK/CAT. In addition, cells were co-transfected with different amounts of expression vectors, as indicated below the figure: pSV2/COUP-TFII and CMV/USF2a. The CAT activity was standardized by the luciferase activity generated by 200 ng of co-transfected RSV/Luc plasmid and expressed in arbitrary units. The data are taken from two separate studies involving triplicate samples. The results are represented as the means ± S.D., n = 6. B, hepatocytes were cultured in the presence of either 5 or 25 mM glucose. They were transfected with 4 μg of different reporter constructs: (LL)3-96PK/Luc, (MM)3-96PK/Luc. Results are calculated from the ratio of luciferase/Renilla activities expressed in arbitrary units. The results are obtained from five separate experiments. Each bar represents the mean ± S.D., n = 10.

**FIG. 7.** Effect of COUP-TFII overexpression on the glucose responsiveness of L-PK-GIRE-dependent promoters in hepatocytes in primary culture. Cells were cultured in the presence of either 5 or 25 mM glucose. The hepatocytes were transfected with 4 μg of different reporter constructs: (LL)3-54PK/Luc, (LL)3-54PK/Luc, or 183-PK/Luc. In addition, the cells were co-transfected with 500 ng of pSV2/COUP-TFII (+) or of the empty vector (−). These amounts of expression vectors were previously determined as the highest amounts before occurrence of nonspecific squelching. Results are calculated from the ratio of luciferase/Renilla activities expressed in arbitrary units. The results were obtained in triplicate experiments. Each bar represents the mean ± S.D., n = 10.
the Sp1 family. In addition, we previously showed that a GIRE construct devoid of the Sp1 site conserved its property to mediate the glucose responsiveness (19). Finally, the putative GC-rich Sp1-binding site in the L-PK GIRE is not conserved between rat, human, and mouse (GenBankTM accession numbers are X05684 for rat and Z18922 for Homo sapiens; for mouse, the sequence is unpublished data).

**COUP-TF II Counteracts Transactivation by USF**—

COUP-TF proteins are orphan nuclear receptors that are most generally considered to be transcription inhibitors (27). Accordingly, COUP-TFII has been shown to interact with corepressors such as N-CoR and SMRT (41). COUP-TF can also inhibit gene transcription by heterodimerization with RXR, the common partner of several nuclear receptors, and by competing with them for binding to common sites (27). However, several reports also show that COUP-TFs act as gene activators, either by directly binding to DNA elements and cooperating with contiguous factors (27, 42, 43) or by interacting with other transcription factors (e.g. HNF4 or Sp1) through protein-protein contacts (44, 45). COUP-TFII is widely expressed, in particular in hepatocytes (46); its defect in knock-out mice results in perinatal death (48).

When COS cells are co-transfected with COUP-TFII and USF2a expression vectors, USF-dependent transactivation through the GIRE was inhibited. Footprinting experiments demonstrate that COUP-TF-binding sites overlap E boxes and therefore are expected to compete with basic helix-loop-helix/leucine zipper family proteins for binding to the GIRE. Accordingly, there is competition between USF2 and COUP-TFII for binding to this element in EMSA experiments. However, at high concentration of recombinant COUP-TFII and USF2 proteins, a ternary complex consisting of the GIRE binding both factors could be documented; it is likely that in this complex, USF binds the downstream E box while COUP-TFII binds the upstream one overlapping with the DR1 site. It is noteworthy that we were unable to detect any direct protein-protein interaction between USF2a and COUP-TFII in a two-hybrid test in yeast (data not shown).

**COUP-TFII Inhibits the Glucose Responsiveness of the L-PK Promoter**—The −183 L-PK promoter confers a good glucose responsiveness on a reporter gene in hepatocytes in primary culture. Overexpression of COUP-TFII in these cells inhibited the glucose response. This inhibition was found again on an artificial promoter in which three GIRE motifs were oligomerized and therefore are expected to compete with basic helix-loop-helix/leucine zipper family proteins for binding to the GIRE. Accordingly, there is competition between USF2 and COUP-TFII for binding to common sites (27). However, several reports also show that COUP-TFs act as gene activators, either by directly binding to DNA elements and cooperating with contiguous factors (27, 42, 43) or by interacting with other transcription factors (e.g. HNF4 or Sp1) through protein-protein contacts (44, 45). COUP-TFII is widely expressed, in particular in hepatocytes (46); its defect in knock-out mice results in perinatal death (48).

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In transient transfection experiments, overexpression of COUP-TFII counteracts the transactivating effect of USF and inhibits the glucose response of the L-PK promoter in hepatocytes in primary culture; in addition, intact COUP-TFII DNA-binding site is required for correct glucose response. Therefore, COUP-TFII is obviously a very good candidate to participate in the transcriptional regulation by glucose.

Recently, different groups reported new data concerning GIRE DNA-binding proteins. Hasegawa et al. (25) reported that carbohydrate refeeding resulted in increase of a GIRE binding activity in the liver and cultured hepatocytes and excluded USF, Sp1, and c-Myc factors as being involved in this activity. These assumptions were based exclusively on the use of commercial antibodies. We reproduced the results of these authors and found that under their described nuclear extract preparations (precipitation by 25% polyethylene glycol, eventually after discarding of the 10% polyethylene glycol precipitate and use of the nuclear extract precipitates without dialysis) and EMSA conditions, Sp1 family members, USF, and COUP-TF-containing complexes were the only GIRE-binding proteins detected (Fig. 5B). In the proteins precipitating between 10 and 25% polyethylene glycol, we found that the major GIRE binding activity was USF, displaced by anti-USF1, anti-USF2 antibodies, and an excess of the MLP USF-binding site. We are currently determining with a large number of nuclear extract samples, conditions which may modulate these binding activities.

Yamada et al. (50) described two novel purified GIRE-binding proteins that are different from USF. The DNA binding activity of these 24- and 26-kDa proteins disappeared after preincubation for 5 min at 60 °C. We found that COUP-TFII...
either translated in vitro or from liver nuclear extract is labile after a preincubation at 60 °C for 5 min, whereas USFs were stable. Yamada et al. (50) observed that the binding profile of their purified proteins was apparently migrating faster than that from the starting materials and indicated that purification of this GI5 binding activity was very difficult because it corresponded to an extremely unstable protein. All these results suggest that this novel activity is proteolytically unstable as described for COUP-TF (26). Therefore, we propose that this novel GI5 binding activity could be COUP-TFII.

Finally, Ferre and co-workers (51) recently proposed that the basic helix-loop-helix/leucine zipper family SREBP-1c/ADD1 factor could be responsible for the transcriptional activation of lipogenic genes, including the L-PK gene, by glucose. However, the SREBP gene is activated by insulin, regardless of the presence of glucose (51) and binds very poorly to the L-PK GI5. In fact, we have shown that SREBP-1c was a very inefficient transactivator of a reporter gene directed by oligomerized GI5s in hepatoma mAhT3F cells (38).

The regulation of the transcriptional activity by glucose through the GI5 could be due to competition between glucose-inducible activators and the inhibitor COUP-TFII for binding to the response element. This hypothesis is in line with the observation that under low glucose conditions the GI5 behaves rather as a negative cis-acting element, probably because it binds mainly COUP-TFII, which could be displaced by activators upon glucose refeeding. Such an alternative binding of COUP-TFII or basic helix-loop-helix activators on the GI5E boxes could still explain why this element seems to be always occupied in in vitro footprinting experiments in fasted as well as refed animals. In addition, the increased activity at low glucose and the decreased activation by glucose conferred by the oligomerized GI5 mutant consisting of two canonical E boxes with high affinity for USF and very low affinity for COUP-TFII are also consistent with our hypothesis.

The putative role of COUP-TFII in the glucose response complex as a binding competitive inhibitor of glucose-dependent activators is reminiscent of its reported capability to antagonize different members of the nuclear receptor family, either ligand-dependent (estrogen receptor) (52, 53), peroxisome-proliferator-activated receptor (54, 55) or still orphan but sensitive to cAMP (steroidogenic factor) (56). In conclusion, although we are aware that confirmation or invalidation of these hypotheses still require extensive studies, we have demonstrated in this paper that COUP-TFII does bind to the L-PK GI5 and inhibits the glucose response and is therefore likely to be an important player of the glucose sensor system of glucose-sensitive genes.

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