Glucose-dependent Liver Gene Expression in Upstream Stimulatory Factor 2 −/− Mice*

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Virginie S. Vallet‡§, Alexandra A. Henrión‡§, Danielle Bucchini**, Marta Casado‡,
Michel Raymondjean‡, Axel Kahn‡, and Sophie Vaulont‡ ‡

From the Institut Cochin de Génétique Moléculaire, ‡U.129 INSERM Unité de Recherches en Physiologie et Pathologie Génétiques et Moléculaires and **U.257 INSERM Laboratoire de Génétique Cellulaire et Moléculaire, Université René Descartes, 24 rue du Faubourg Saint-Jacques, 75014 Paris, France

Upstream stimulatory factors (USF) 1 and 2 belong to the Myc family of transcription factors characterized by a basic/helix loop helix/leucine zipper domain responsible for dimerization and DNA binding. These ubiquitous factors form homo- and heterodimers and recognize in vitro a CACGTG core sequence termed E box. Through binding to E boxes of target genes, USF factors have been demonstrated to activate gene transcription and to enhance expression of some genes in response to various stimuli. In particular, in the liver USF1 and USF2 have been shown to bind in vitro glucose/carbohydrate response elements of glycolytic and lipogenic genes and have been proposed, from ex vivo experiments, to be involved in their transcriptional activation by glucose. However, the direct involvement of these factors in gene expression and nutrient gene regulation in vivo has not yet been demonstrated. Therefore, to gain insight into the specific role of USF1 and USF2 in vivo, and in particular to determine whether the USF products are required for the response of genes to glucose, we have created, by homologous recombination, USF2 −/− mice. In this paper, we provide the first evidence that USF2 proteins are required in vivo for a normal transcriptional response of L-type pyruvate kinase and Spot 14 genes to glucose in the liver.

Glucose is a major source of metabolic energy for all mammalian cells. In the liver, glucose metabolism begins or ends with the movement of glucose into or out of the hepatocyte. When hormonal conditions favor glucose utilization (high insulin, low glucagon), glucose entry is facilitated along a downhill gradient through the specific GLUT2 transporter, and intracellular glucose is rapidly phosphorylated (1, 2).

In response to the glucose signal, transcription of several genes implicated in glucose metabolism is activated (for review see Refs. 3 and 4). Two such glucose-responsive genes have been extensively studied in the liver, i.e. the genes for L-type pyruvate kinase (L-PK), a glycolytic enzyme, and Spot 14 (S14), a protein associated with lipogenesis. Transient transfection assays in primary hepatocytes have allowed for the identification of a common glucose response element, termed GIRE by our group and ChoRE, for carbohydrate response element, by the group of H. Towle, in the regulatory regions of the L-PK and S14 genes (5–8). The functional importance of the GIRE has been further demonstrated in vivo using transgenic mice (9).

The L-PK GIRE (located between nucleotides −168 and −144, with respect to the liver-specific start site of transcription) and the S14 ChoRE (between nucleotides −1448 and −1242) present a similar arrangement consisting of two E boxes separated by 5 base pairs (10). The so-called E box consensus CACGTG is the recognition site for members of the Myc family of transcription factors characterized by a highly conserved C-terminal basic/helix-loop-helix/leucine-zipper domain responsible for dimerization and DNA binding (11).

Through binding to their DNA target as homo- or heterodimers, these factors are known to regulate a variety of genes (12, 13). In the liver, the predominant members of this family able to recognize the L-PK GIRE in vitro are USF factors (14). These USF factors were originally described as a binding activity able to interact and to transactivate the adenovirus major late promoter (15). Purification of this activity revealed the presence of two polypeptides of 43 (USF1) and 44 kDa (USF2a) (16). These two forms of USF are encoded by distinct genes that have been cloned and characterized in the mouse (12, 13). In the liver, these genes are expressed in all liver tissues, and we have assessed that, in the liver, the USF1/USF2a heterodimer accounted for more than 65% of the USF binding activity (14). Besides, we have shown that, when transfected into hepatoma cells, USF isoforms were able to interfere with the glucose response of the L-PK gene (19).

To test for the possible involvement of USF2 in the glucose-mediated induction of hepatic gene expression in vivo and to fully reveal its biological role, we sought to examine mice with a null mutation for USF2. To this end, we have created a mouse USF2 −/− line by disrupting the USF2 locus by gene targeting. In this paper, we present the first model of USF2 −/− mice and provide evidence that, in the liver, USF2 proteins are needed in vivo for a normal transcriptional response of the L-PK and S14 genes to glucose.

EXPERIMENTAL PROCEDURES

Gene Targeting and Generation of USF2 −/− Mice—The USF2 genomic fragments were all generated from 4 clones isolated from a murine ES-129 embryonic stem cell line genomic library (12). ES culture and embryo manipulation were performed as described (20).

Spot 14; USF, upstream stimulatory factor; kb, kilobase pair(s); ES, embryonic stem.

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A 7.5-kb XhoI-No1 fragment, encompassing part of exon 7 and the almost totality of intron 7, was blunted, modified by the addition of No1 linkers, and subcloned into the 3’ No1 site of the previously reported ribosomal internal entry site-βgeo containing vector (21). This plasmid was SrfI-digested, and the 5’ USF2 homologous fragment, consisting of a 1.8-kb fragment spanning intron 1 to exon 7, was inserted. The resulting targeting construct was linearized at the unique XhoI site, and 60 μg was used to electroporate 1 × 10^7 CCB-ES cells derived from 129Sv/J mice (kindly provided by Martin Evans, Cambridge, UK), which were cultured on mitomycin-treated embryonic fibroblast feeder layers. DNA from clones surviving G418 selection (200 μg/ml) was individually analyzed on Southern blot. DNA of clones EcoRI-digested and hybridized with a 5’ external probe consisting of a genomic EcoRV-BamHI fragment in the 5’ regulatory sequences of the USF2 gene (see Fig. 1A) or EcoRI-digested and hybridized with a 3’ external probe consisting of a polymerase chain reaction-amplified fragment encompassing exon 8 to exon 10. ES cells from positive clones were injected into blastocysts derived from C57BL/6J mice (20). Chimeric males, as judged by the agouti coat color, were mated to wild-type C57BL/6J female for the germ line transmission. Mice heterozygous for the gene targeting event were then used to generate homozygous mutant USF2−/− mice. Genotyping of offspring was performed by Southern blot analysis with 10 μg of EcoRI-digested mouse tail DNA. 

**Nutritional Treatment and Metabolic Parameters of the Animals:** For metabolic studies, animals were fed a high carbohydrate diet, for 10 min at 17,000 g was used to electroporate 1 × 10^7 CCB-ES cells derived from 129Sv/J mice (kindly provided by Martin Evans, Cambridge, UK), which were cultured on mitomycin-treated embryonic fibroblast feeder layers. DNA from clones surviving G418 selection (200 μg/ml) was individually analyzed on Southern blot. DNA of clones EcoRI-digested and hybridized with a 5’ external probe consisting of a genomic EcoRV-BamHI fragment in the 5’ regulatory sequences of the USF2 gene (see Fig. 1A) or EcoRI-digested and hybridized with a 3’ external probe consisting of a polymerase chain reaction-amplified fragment encompassing exon 8 to exon 10. ES cells from positive clones were injected into blastocysts derived from C57BL/6J mice (20). Chimeric males, as judged by the agouti coat color, were mated to wild-type C57BL/6J female for the germ line transmission. Mice heterozygous for the gene targeting event were then used to generate homozygous mutant USF2−/− mice. Genotyping of offspring was performed by Southern blot analysis with 10 μg of EcoRI-digested mouse tail DNA. 

**Liver Samples Assayed for USF2 Content**—Liver samples assayed for USF2 content were homogenized in 20 mM Hepes, pH 8.5, 15 mM MgCl₂, 0.2 mM EDTA, 1 mM diethiothreitol, 25% (v/v) glycerol, 0.2% (v/v) Nonidet P-40, and protease inhibitor mixture tablets “COMPLETE” (Boehringer Mannheim). 

Western blot analyses were performed as described above, using affinity purified USF2 1–49 antibodies at a 1:200 dilution (14). For Western blotting, protein samples were electrophoresed on 10% SDS-PAGE gels under non-reducing conditions. The Western blot was then immunoblotted and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. The resulting bands were visualized by ECL+ (Amersham Corp.). 

**RESULTS**

**Generation of USF2-deficient Mice**—To inactivate the USF2 gene, a replacement vector was constructed with 9.3 kb of ES-129 derived genomic fragment and a selection cassette disrupting exon 7 (Fig. 1A). Ensured that the USF2 gene was active in embryonic stem (ES) cells, we carried out a promoter trap strategy to maximize the targeting frequency. To this end, the promoterless internal ribosomal entry site-βgeo cassette was chosen as the selection cassette. This cassette allows, via an internal ribosomal entry site element, for efficient translation of the βgeo fusion transcript which leads to the synthesis of a fusion protein with both β-galactosidase and neomycin activities (21). Successful targeting should abolish the transcriptional function of USF2 by disrupting the basic domain, encoded by exon 7, responsible for its DNA binding. After electroporation and selection of recombinant ES cells, homologous recombinants were detected by Southern analysis. Among them, one targeted stem cell clone contributed particularly to the germ line of chimeric mice. After transmission of the mutation, heterozygous progeny, which were normal and fertile, were intercrossed to produce homozygous offspring. The presence of the mutated gene in the F2 offspring was routinely checked by Southern analysis of tail DNA. As shown in Fig. 1B, hybridization of EcoRI-digested genomic DNAs with the indicated 5’ external probe led to wild-type alleles of 12.5 kb and mutated alleles of 8.5 kb. 

**Characterization of Homozygous USF2−/− Knock-out Mice**—Of the 110 F2 offspring analyzed after cesarean section at 19 days post-coitum, 21.8% were homozygous (−/−) for the disrupted allele and 25.4% were wild type (+/±), indicating that USF2 deficiency did not affect embryonic development. However, after birth, survival of homozygous mice was markedly affected by disruption of the USF2 gene. Indeed, only 20% homozygous mice survived a few hours after birth. The USF2−/− mice were significantly smaller in size than their littermates. This decrease in body weight (approximately 20%) was already evident at the embryo stage of 17.5 days and either stabilized or dramatically increased after birth. Based on this body size criterion, we were able to significantly improve survival of −/− mice, by reducing the litter size. Still, the lifespan of surviving mice was variable. This increase in the survival of USF2−/− mice suggests that the developmental delay of the lethal phenotype could be due at least, to competition for food as homozygotes seemed more apathetic than their littermates. Phenotype of the knock-out mice will be presented in detail elsewhere. 

**USF Binding Activity in USF2−/− Liver Cells**—Disruption of the USF2 gene resulted in the complete absence of USF2 protein in −/− animals. This was ascertained by immunoblot-
ting of liver nuclear proteins of \(-/-\), \(-/+\), and \(+/+\) animals with an anti-USF2 specific antibody (Fig. 2A). The 44-kDa USF2a protein was detected only in extracts from heterozygous and wild-type animals. The reduced level of USF2 in \(-/1\) animals, as compared with the \(1/1\) animals, suggested the failure of the wild-type USF2 allele to compensate for the mutation. To determine the changes in the composition of USF binding activity, the same nuclear extracts were used in electrophoretic mobility shift assays with a double-stranded oligonucleotide containing the canonical E box from the adenovirus major late promoter.

As visualized in Fig. 2B, in \(+/+\) extracts, a major band migrating as the control USF1/USF2 heterodimer of the rat liver nuclear extract was observed. The same complex, although less intense, was detected in the \(-/+\) extracts. A fainter and slightly lower band was observed in the \(-/-\) extracts which was reminiscent to that observed in rat liver USF2-immunodepleted extracts (14). To precisely determine the nature of this complex, we used USF1 and USF2 antibodies (Fig. 2C). As previously observed, USF1 and USF2 homodimers, visualized as weak bands after supershift with either USF1 or USF2 antibody, appeared to be minor complexes in the liver nuclear extracts of the \(+/+\) and \(-/+\) mice. On the contrary, in \(-/-\) nuclear extracts, the use of USF1 antibody fully displaced the observed complex, and the USF2 antibody had no effect. This revealed that, in vitro, the only detectable binding activity on the E boxes, in extracts from animals devoid of USF2, was the USF1 homodimer. By assessing the quantity of USF1 homodimer in \(-/-\) extracts, we could speculate that the expression of USF1 gene was poorly affected by the absence of USF2. To test this, RNA samples from the liver of \(-/-\) and \(+/+\) mice were assayed by Northern blot using specific USF1 probe. This analysis revealed indeed no change in the USF1 gene expression (data not shown). This result was consistent with our previous observation indicating the absence of E box in the USF1 gene sequence (13).

**Hepatic Content of L-PK Protein in Fetal Liver and L-PK and Spot 14 mRNAs in Adult Liver of USF2-deficient Mice**—To determine the impact of USF2 deficiency on L-PK gene expression, our first experiments were conducted on fetuses of several litters. After cesarean section at the end of gestation, each fetal liver was homogenized, and the content of L-PK protein was assessed by Western blot analyses using specific L-PK antibody. The signals were quantitated and averaged out into \(-/-\), \(-/+\), and \(+/+\) groups after genotyping. As shown on the histogram of Fig. 3, the amount of L-PK protein was significantly reduced in liver of \(-/-\) fetuses.

To study further the glucose responsiveness of hepatic gene expression, a series of metabolic analyses was performed on wild-type and surviving USF2 \(-/-\) mice. After an 18-h refeeding period with a high carbohydrate diet, animals were sacrificed, and total liver RNAs were prepared and analyzed by Northern blot to assess the content of L-PK and S14 messenger RNAs. This analysis revealed that, in the liver of all the \(-/-\)
animals tested, the amount of both L-PK and S14 mRNAs was markedly lowered as compared with the +/- animals (Fig. 4A). In ad libitum fed animals, the amount of both L-PK and S14 mRNAs was not significantly different (data not shown).

After a 40-h feeding period with a high carbohydrate diet, the difference in the level of L-PK and S14 mRNAs was still obvious except for the 17.1 mouse that did contain relatively high level of mRNAs (Fig. 4B). However, after 5 days on this high carbohydrate diet, -/- and +/- animals displayed similar levels of L-PK and S14 mRNAs (Fig. 4C).

**Glucose Metabolism in USF2 -/- Mice Refed a High Carbohydrate Diet for 18 Hours**—To assign the diminution in the level of L-PK and S14 mRNAs, observed 18 h after glucose refeeding, to the transcriptional state of the genes rather than to a putative alteration of glucose metabolism, several steps, from blood glucose disposal to cellular glucose utilization, were carefully checked.

Blood glucose disposal was first assessed by performing an oral glucose tolerance test in fasted mice. As shown in Fig. 5, after the glucose load, glucose accumulation was quite similar in +/- and -/- animals. To further challenge glucose homeostasis in -/- animals, blood glucose concentration was assayed in fasted and refeed animals. The absence of USF2 seemed to have no effect on the glycemia of either fasted animals (series not shown) or refeed animals (1.8 ± 0.5 (n = 7) and 1.6 ± 0.4 (n = 10) g/liter glucose for the -/- and +/- groups, respectively, Table 1). The next step was to confirm that insulin was correctly synthesized and secreted in response to oral glucose, as suggested from the normal plasma glucose levels. As monitored, neither the amount of pancreatic insulin messenger RNA (not shown) nor the plasma insulinemia (Table I) was modified in USF2 -/- refeed mice.

The second part was to evaluate glucose entry and phosphorylation, which in hepatocytes involve the specific GLUT2 transporter, and hexokinase IV or glucokinase (1, 2). This coupled transport/phosphorylation system forms part of a glucose sensing apparatus that responds to subtle changes in blood concentration. Therefore, GLUT2 gene expression was assessed by Northern blot analysis and, in fact, as shown in Fig. 6, the hepatic amount of GLUT2 mRNA remained unchanged in USF2 -/- mice. To monitor glucose phosphorylation into glucose 6-P, we measured directly the intracellular concentration of glucose 6-P in the liver of the animals. The results, presented in Table I, demonstrated that the glucose 6-P concentration was roughly similar in both -/- and +/- groups, 6.2 ± 2.0 (n = 10) and 5.9 ± 1.6 (n = 7) nmol/mg proteins, respectively. Furthermore, the presence of glycogen in liver section of USF2 -/- mice was also noted by histochemical staining (data not shown), indicating a normal capacity of the liver for glycogen replenishment after refeeding.

Taken together, these results were indicative of an apparent normal glucose uptake and utilization by the liver of the knockout mice.

**Discussion**

In the USF2 -/- mice presented in this paper, we have primarily studied glucose metabolism and expression of glucose-responsive genes in the liver. In the adult USF2 -/- mice refeed a high carbohydrate diet, we have assessed that glucose was available, normally transported, and phosphorylated in hepatocytes. Indeed, in these mice, glycemia was normal, the specific facilitative transporter GLUT2 that provides glucose to the hepatocytes was present, and glucose phosphorylation to glucose 6-P was normal as judged from the normal accumulation of glucose 6-P in the liver of -/- mice.

Nevertheless, in the liver of these animals, we observed a significant diminution of the L-PK and S14 gene expression 18 h after glucose refeeding, whereas in ad libitum fed mice, no difference was detected. This result, obtained in adult liver of USF2 -/- mice, was consistent with the 64% reduction in the amount of L-PK protein noticed in the liver of -/- fetuses at the end of gestation, when the L-PK promoter begins to be active (27). We conclude therefore that, in vivo, USF2 proteins

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**Fig. 2. Analysis of USF2 content and USF binding activity in liver nuclear extracts from -/-, +/-, and +/- animals.** A, Western blot analysis of the USF2 protein using an anti-USF2 peptide antibody. Liver nuclear extracts were from -/-, +/-, and +/- animals refed a high carbohydrate diet for 18 h. Each animal is referenced with a double number as indicated above each lane. The first number indicates the litter and the second number indicates the animal figure. C, electrophoretic mobility shift assays using the radiolabeled major late promoter oligonucleotide in the presence of liver nuclear extracts from -/-, +/-, and +/- mice. Rat liver nuclear extracts were used as a control. C, electrophoretic mobility shift assays were performed as above, and selective depletion of the complexes was realized by adding specific anti-USF antibodies (Ab) in the binding reactions. The asterisk indicates nonspecific protein complexes.

**Fig. 3. L-PK content in liver of -/-, +/-, and +/- fetuses as determined by Western blot analysis using anti-L-PK antibody.** Results are expressed as means ± S.D. of five different fetuses in each group, on three separate blots. The values obtained in USF2 -/- mice are statistically different from those in USF2 +/- and USF2 +/- lines (*p < 0.034).
are required for a normal kinetics of transcriptional induction of the two genes by glucose. This induction is thought to depend on the nature of the glucose-response complex assembled on the GlRE/ChoRE of the L-PK and S14. Although the exact role of USF proteins in this complex remains disputed (28), evidence argues for their important involvement, in cooperation with other still unknown factors (19). Very recently, Kennedy et al. (29) were able to demonstrate that microinjection of USF antibodies specifically inhibited the glucose responsiveness of the L-PK promoter in INS-1 rat insulinoma cells. Therefore, we carefully characterized USF binding activity in the liver of animals refed a high carbohydrate diet for 18 h and then refed a carbohydrate-rich diet for 18 h (A) or fed during 40 h (B) or 5 days (C) with this diet. Characteristics (figure, weight, age, and sex) of each animal is indicated above each lane.

**USF2 Knock-out Mice**

**FIG. 4.** L-PK and S14 mRNA content in liver of −/− and +/+ animals as determined by Northern blot analysis. Animals were either fasted for 24 h and then refed a carbohydrate-rich diet for 18 h (A) or fed during 40 h (B) or 5 days (C) with this diet. Characteristics (figure, weight, age, and sex) of each animal is indicated above each lane.

**FIG. 5.** Oral glucose tolerance test in −/− and +/+ animals. Wild-type (open triangles) and −/− (open squares) animals were loaded with 40 mg of glucose, and blood glucose level (ng/liter) was determined at the indicated time points. The data points indicate means ± S.D. (n = 4).

When mice were fed for 5 days a high carbohydrate diet, as well as an ad libitum regular diet, L-PK and S14 mRNAs content in the liver of USF2 −/− mice were not significantly different from USF2 +/+ mice. This result seems to indicate **2 S. Lopez, unpublished data.**
that the intrinsic transcriptional capabilities of glucose-responsive genes are normal in USF2−/− mice but that kinetics of glucose-dependent activation is slow. Alternatively, an altered transcriptional rate could be compensated by progressive accumulation of stable mRNAs. Indeed, we previously reported that L-PK mRNAs appear to be very stable in carbohydrate refed animals (30).

In conclusion, we confirm in this paper that USF factors are involved in the normal response of hepatic glucose-responsive genes to glucose and that USF1 homodimers cannot totally replace the predominant USF1/USF2 heterodimers normally present on the GIRE/ChoRE of the L-PK and S14 genes. Further investigations will determine the respective role of USF1 and USF2 in the transcriptional response to glucose. The mechanisms of the other phenotypic abnormalities associated with deficiency in these factors will be investigated in USF2−/− mice, for instance, growth delay, relatively apathetic and increased post-natal lethality which is not obviously related to the decreased transcriptional activation by glucose in the liver.

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TABLE I

Concentration of glucose, insulin, and glucose-6-P in USF2+/+ and −/− animals

| Animals | +/+    | −/−    |
|---------|--------|--------|
| Glucose | 4.1    | 12.1   |
| Insulin | 7.6    | 9.0    |
| Glucose 6-P | 6.2 | 7.4 |

Note: ND, not determined

FIG. 6. GLUT2 mRNAs content in the liver of −/− and +/+ animals as determined by Northern blot analysis. Animals were fasted for 24 h and then refed a carbohydrate-rich diet for 18 h.