Activating transcription factor 3 (ATF3), a member of the ATF/cAMP-responsive element-binding protein family of transcription factors, is a transcriptional repressor, and the expression of its corresponding gene, ATF3, is induced by many stress signals. In this report, we demonstrate that transgenic mice expressing ATF3 in the liver had symptoms of liver dysfunction such as high levels of serum bilirubin, alkaline phosphatase, alanine transaminase, aspartate transaminase, and bile acids. In addition, these mice had physiological responses consistent with hypoglycemia including a low insulin:glucagon ratio in the serum and reduced adipose tissue mass. Electrophoretic mobility shift assays indicated that ATF3 bound to the ATF/cAMP-responsive element site derived from the promoter of the gene encoding the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK). Furthermore, transient transfection assays indicated that ATF3 repressed the activity of the PEPCK promoter. Taken together, our results are consistent with the model that the expression of ATF3 in the liver results in defects in glucose homeostasis by repressing gluconeogenesis. Because ATF3 is a stress-inducible gene, these mice may provide a model to investigate the molecular mechanisms of some stress-associated liver diseases.

The liver is a critical organ for homeostasis. Two metabolic processes in the liver, glycojenolysis and gluconeogenesis, help to maintain glucose homeostasis (1–5). In newborns, to adjust to the discontinued supply of glucose by maternal blood, glycojenolysis is turned on to degrade glycogen to glucose, and gluconeogenesis is turned on to synthesize glucose from non-carbohydrate precursors. In adults, these two metabolic processes also function to maintain the glucose homeostasis of the body. During the fasting state, glycojenolysis and gluconeogenesis are stimulated to produce glucose, whereas during the fed state these processes are inhibited. The main regulator of these processes is the ratio of two hormones secreted by the endocrine pancreas, insulin and glucagon. When the blood glucose level is low, the insulin:glucagon ratio is low, resulting in the activation of the intracellular cAMP signaling pathway that in turn stimulates glycojenolysis and gluconeogenesis. However, when the blood glucose level is high, the insulin:glucagon ratio is high and these metabolic processes are repressed. In addition to maintaining glucose homeostasis, the liver plays an important role in the other functions including (a) metabolism of xenobiotic toxins and endogenous substances such as heme, steroids, and biogenic amines, (b) synthesis of plasma proteins such as albumin and clotting factors, and (c) carrying out the acute phase responses upon inflammation, infection, or trauma (6).

Because of its metabolic functions, the liver is the primary target for a variety of stress signals such as carcinogens and toxins. Therefore, to understand many liver diseases, it is important to understand the effects of stress signals on the liver. We have been investigating a stress-inducible gene, activating transcription factor 3 (ATF3), the corresponding protein product of this gene, is a member of the ATF/CREB family of basic region leucine zipper transcription factors (for reviews see Refs. 7–10). Although cDNA encoding ATF3 was isolated from a human library (11), homologous cDNAs from rats and mice with ~95% identity to ATF3 at the amino acid level have been identified as liver-regenerating factor-1 in the rat (12) and lipopolysaccharide responsive gene-21 (13), cytokine-responsive gene-5 (14), or TI-241 (15) in the mouse. For the convenience of discussion, we use the ATF3 nomenclature in the rest of this report.

Overwhelming evidence from many laboratories indicates that ATF3 is induced by a variety of stress signals in the liver (for review see Ref. 16). By in situ hybridization, we demonstrated that the mRNA level of ATF3 greatly increases in the liver after exposing the animals to chemicals such as carbon tetrachloride, alcohol, acetaminophen, and cycloheximide (16, 17). Others have demonstrated that ATF3 is induced in the liver by hepatic ischemia (18) and partial hepatectomy (12). In addition, ATF3 is induced in cultured hepatocytes by cycloheximide, endothelial growth factor, and human growth factor (19). Importantly, ATF3 is also induced in many other tissues by stress signals (for reviews see Refs. 7 and 16) such as the heart by ischemia coupled with reperfusion (ischemia reperfusion) (17), the peripheral nerves by axotomy (20), and the pancreas by partial pancreatectomy, ischemia reperfusion, and streptozotocin treatment (21). Therefore, ATF3 is induced in many tissues by a variety of stress signals, suggesting that it is a key regulator in cellular stress responses.

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Although the induction of ATF3 by stress signals is neither tissue-specific nor stimulus-specific, one common theme of all the signals that induce ATF3 is that they induce cellular damage (for reviews see Refs. 7 and 16). This correlation raises an important question. Is the induction of ATF3 a protective response for the cells to cope with stress or a part of the cellular response that leads to detrimental outcomes? To address this question, we took a gain-of-function approach and generated transgenic mice expressing ATF3 in selective tissues. Our results are consistent with the interpretation that the expression of ATF3 leads to detrimental outcomes. Transgenic mice expressing ATF3 in the heart under the control of the α-miosin heavy chain promoter exhibit conduction abnormality and contractile dysfunction (22). Transgenic mice expressing ATF3 under the control of the transthyretin (TTR) promoter, a promoter that is predominantly active in the liver but could also be active in the pancreas (23), have defects in glucose homeostasis and reduced expression of the gene encoding the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) (21). In this report, we refer to these mice as the TTR-ATF3 mice.

As described previously (21) and under “Experimental Procedures,” the TTR-ATF3 transgenic founders did not express detectable levels of the ATF3 transgene, presumably due to mosaicism or silencing of the transgene. However, they could pass on the transgene to their progeny, and the F1 mice expressed the ATF3 transgene. These mice had growth retardation and died within hours after birth (21). The analyses of phenotypes were facilitated by the generation of “F1 hybrid” between the transgenic founders (FVB/N mice) and wild-type BALB/c mice. The F1 hybrid had the advantage of hybrid vigor and survived longer (for up to 7 days). Consistent with the observations that the TTR promoter is predominantly active in the liver (24–26), F1 transgenic mice derived from all 24 founders expressed the ATF3 transgene in the liver (21). However, F1 mice derived from some founders also expressed the transgene in the pancreas, presumably due to the differences in the integration sites and/or copy numbers of the transgene.

Previously, we described that the expression of ATF3 in the transgenic ductal epithelium impairs endocrine pancreas development (21). In this report, we describe the hepatic phenotypes of these mice.

Because ATF3 is a stress-inducible gene and because expression of ATF3 leads to detrimental outcomes in other tissues (heart and pancreas as described above), we hypothesized that transgenic mice expressing ATF3 in the liver have hepatic dysfunction. Furthermore, because ATF3 is a transcriptional repressor and because the PEPCK promoter contains an ATF/CRE site, we hypothesized that ATF3 represses the PEPCK promoter, providing a mechanistic explanation for the reduced expression of PEPCK gene in the TTR-ATF3 transgenic mice. Below we present evidence supporting these hypotheses.

**EXPERIMENTAL PROCEDURES**

The TTR-ATF3 Transgenic Mouse—The generation of these mice was described previously (21). The human ATF3 open reading frame was cloned into the pTTRex3 vector to make the transgene, and the transgenic mice were generated in the FVB/N background. The transgenic founders did not express the transgene, presumably because of mosaicism or silencing of the transgene but could pass on the transgene to their progeny. The F1 mice expressed the gene and died within hours after birth. Because of this perinatal lethality, it was not possible to establish transgenic lines. Repeated injections were carried out to generate 24 founders and thus sufficient F1 mice for analysis. To facilitate the analyses of F1 mice, we crossed FVB/N transgenic founders with BALB/c non-transgenic mice to generate F1 hybrid mice. These F1 mice had the advantage of hybrid vigor and survived longer. Most of them survived for 4 days, and some survived for up to 7 days. Because the F1 hybrid mice are genetically uniform except at the locus of the transgene, a comparison of the transgenic mice with the non-transgenic littersmates allowed the assessments of phenotypes attributed to ATF3 expression. As described previously (21), F1 hybrids derived from all 24 founders expressed ATF3 transgene in the liver, but F1 hybrids derived from some founders also expressed ATF3 in the pancreas. The pancreatic phenotypes were described previously (21). In this report, we describe the hepatic phenotypes. To avoid hepatic phenotypes resulting from indirect effects of pancreatic expression of ATF3 (see “Discussion”), we only describe the phenotypes observed in mice with hepatic but not pancreatic expression of ATF3. Because not all founders gave rise to progeny efficiently, detailed analyses were limited to a subset of founders. The phenotypes described in this report were observed in F1 hybrid mice derived from >8 founders. This reproducibility strongly underlines the fact that these phenotypes were not because of the artifacts of integration sites.

**Preparation and Staining of Tissue Sections**—Newborn mice between postnatal days (P) 1 and 7 were sacrificed by decapitation. Tissues were removed and rinsed in ice-cold phosphate-buffered saline (PBS). For samples from embryos, the appearance of a vaginal plug was considered to be day 0.5 of gestation. Embryos at embryonic day 18.5 were dissected in ice-cold PBS. Tissues or embryos were fixed for 1–3 days in 10% phosphate-buffered formalin (Fisher Scientific), pH 7.0, at 4 °C. Paraffin sections were prepared and stained by hematoxylin and eosin or periodic acid-Schiff stain according to established procedures by the Ohio State University Veterinary Histology Laboratory.

**Serum Chemistry and Hormone Assays**—Newborn mice were decapitated with scissors, and the blood was immediately collected in microhematocrit capillary tubes (Fisher Scientific) and transferred to MicrotainerR serum-separator tubes (BD Pharmingen). The tubes were centrifuged according to the instructions from the manufacturer, and the top layer (serum) was analyzed on the same day or after storage. Serum β-hydroxybutyrate levels were determined using the β-hydroxybutyrate dehydrogenase method (Sigma). All other serum tests were performed according to the instructions from the manufacturer (Roche Molecular Biochemicals) on an automated discrete random access multianalysis clinical chemistry analyzer (Hitachi 911 Analyzer, Roche Molecular Biochemicals). Analyses were described previously (27) and performed at the Ohio State University Veterinary Teaching Hospital Clinical Chemistry Laboratory. Serum insulin and glucagon levels were measured by radioimmunoassay at Linco (St. Charles, MO). Because of the low body weight and low blood volume of the transgenic mice, sera from multiple mice were combined to generate each data point. Student’s t test was used to analyze the data, and the p values are indicated in the figure legends.

**Electrophoretic Mobility Shift Assay**—ATF3 protein was generated using the vaccinia virus expression system and isolated from HeLa cells as described previously (27). ATF3 generated by this system specifically binds to an ATF/CRE site as demonstrated previously (27) and by the competition and supershift experiments used in this study (see Fig. 5A). DNA-binding reactions and gel electrophoresis were carried out as described previously (11) using 32P-labeled double-stranded oligonucleotides containing the ATF/CRE site derived from the PEPCK promoter, 5'-AGGGCCCGCTTACGCTAGCAGGAGA-3' (ATF/CRE site is underlined).

**Transient Transfection and Chloramphenicol Acetyltransferase (CAT) Assay**—CAT reporter driven by the PEPCK promoter (−600 to +69 region) was transfected into HepG2 cells with effector DNAs (pcG, pCG-ATF3, or pCG-ATF4) using the calcium phosphate method with modifications. 500 μl of 2× Hepes-buffered saline (28) were gradually added to 350 μl of solution containing 12 μg of PEPCK promoter reporter and 2 μg of effector in 0.25 M CaCl2 with constant mixing to make total 700 μl of DNA mixture. The mixture was incubated to room temperature for 30 min to form DNA precipitates before being added to the cell suspension prepared as follows. The cells from two 10-cm plates at 70% confluence were washed with PBS, trypsinized, and resuspended in 800 μl of medium. The cell suspension with the DNA mixture was incubated at room temperature for 15 min and divided into three 6-cm plates containing 2 ml of medium. These plates were incubated at 37 °C with 5% CO2, 16 h later, cells were shocked with 20% glycerol for 2 min, washed with PBS three times, and incubated with media containing 1 μM dexamethasone (0.5 mM stock prepared in ethanol, Sigma) and 20 μM forskolin (10 mM stock prepared in ethanol, Sigma) to induce the PEPCK promoter. 48 h later, cells were washed with PBS, and the CAT activity was measured by a phase extraction method (28). A mock transfection was carried out using 12 μg of pGEM3 to make the DNA mixture.
RESULTS

Because F1 mice derived from some TTR-ATF3 founders also expressed ATF3 in the pancreas, we only describe phenotypes obtained from mice with hepatic but not pancreatic expression of ATF3 to exclude hepatic phenotypes resulting from indirect effects of pancreatic expression of ATF3. Importantly, the phenotypes described below were observed in F1 hybrid mice derived from >8 founders. This reproducibility strongly suggests that these phenotypes were not attributed to the artifacts of integration sites.

Physiological and Biochemical Analyses of Transgenic Mice Expressing ATF3 in the Liver—Because the liver is the major organ that maintains homeostasis of many endogenous substances (6), we carried out a serum profile analysis to address whether the expression of ATF3 in the liver affects its functions. As shown in Figs. 1 and 2, serum levels of alkaline phosphatase, bile acids, alanine transaminase, aspartate transaminase, and bilirubin were significantly higher ($p < 0.001$) in the transgenic mice than in the non-transgenic mice, indicating general liver dysfunction. An analysis of conjugated versus non-conjugated bilirubin indicated that both forms of bilirubin were increased ($p < 0.001$) in transgenic mice (Fig. 2, B and C), suggesting defects in the uptake or conjugation of bilirubin by the liver and the release of bilirubin via the biliary ducts. Because of the low body weight of the transgenic mice, the volumes of blood collected from the newborn transgenic mice were very low. Therefore, we combined sera from several mice for each assay, and the results shown in Figs. 1 and 2 were derived from multiple samples (from 12 to 35) with each sample containing sera combined from 3 to 10 mice as indicated in the legends. We also examined the serum for other compounds and found no significant differences between the transgenic and non-transgenic mice in the concentrations of sodium, potassium, calcium, chloride, phosphorus, creatine kinase, serum urea nitrogen, cholesterol, lipase, albumin, and total protein (Table I).

As described previously, the serum glucose levels in these mice were consistently lower than in the non-transgenic littermates (64 ± 7 versus 128 ± 26 mg/dl) (21). Radioimmunoassays showed that these mice had lower insulin but higher glucagon levels than the non-transgenic littermates (Table II), indicating that the transgenic mice had a proper hormonal response to low serum glucose levels (low insulin:glucagon ratio). To assay insulin, glucagon, and glucose in parallel from each sample, we combined sera from several mice to obtain enough sera for all three assays. The results shown in Table II were derived from five samples with each sample containing combined sera from multiple mice as described in the legend.

To determine whether the mice could respond to these hormonal changes, we examined the adipose tissue and serum ketone bodies. As shown in Fig. 3A, the transgenic mice had lower adipose tissue mass than the non-transgenic mice. This result was reproduced in multiple mice; however, the relative degree of decrease in white adipose tissue varied among the mice. Despite this variation, the combined white adipose tissue and brown adipose tissue was consistently lower in the transgenic mice than that in the non-transgenic mice. The serum β-hydroxybutyrate levels were slightly higher in the transgenic mice than that in the non-transgenic mice; however, the difference was not statistically significant (Fig. 3B). The presence of β-hydroxybutyrate indicates that the transgenic mice could carry out β-oxidation to produce ketone bodies. Because of the low blood volume described above, the results of β-hydroxybutyrate were derived from samples with combined sera as detailed in the figure legends. Taken together, these results indicate that the transgenic mice had a proper hormonal response to low serum glucose levels (low insulin:glucagon ratio) and could respond to the
To determine whether ATF3 represses the PEPCK promoter, we analyzed the ability of ATF3 to bind to the PEPCK CRE site derived from the PEPCK promoter (PEPCK ATF/CRE site). As shown by electrophoretic mobility shift assay, recombinant ATF3 isolated from a vaccinia virus expression system bound to the PEPCK ATF/CRE site (Fig. 5A, lane 1). The specificity of the binding was demonstrated by the observation that an ATF3-specific antiserum shifted the mobility in the DNA-protein complex (Fig. 5A, lane 2). The specificity of the binding was demonstrated by the observation that an ATF3-specific antiserum shifted the mobility in the DNA-protein complex (Fig. 5A, lane 2). The specificity of the binding was demonstrated by the observation that an ATF3-specific antiserum shifted the mobility in the DNA-protein complex (Fig. 5A, lane 2).
we transfected HepG2 hepatocytes with DNA expressing ATF3 and a CAT reporter driven by the −600 to +69 region of the PEPCK promoter. This region of the PEPCK promoter contains the ATF/CRE site and has been demonstrated to mediate the up-regulation of PEPCK gene expression by the cAMP pathway (29, 30, 32). To address transcriptional repression, we activated the PEPCK promoter with dexamethasone and forskolin and asked whether ATF3 could repress the promoter. As shown in Fig. 5B, ATF3 repressed the PEPCK promoter. However, ATF4, a transactivator that also binds to the ATF/CRE site (33), further activated the promoter. Therefore, not all members of the ATF/CREB family could repress the PEPCK promoter.

**DISCUSSION**

**ATF3 in Stress-associated Liver Diseases**—In this report, we demonstrated that transgenic mice expressing ATF3 in the liver have decreased adipose tissue mass, disturbed glucose homeostasis, and show signs of hepatic dysfunction, i.e., elevated levels of aspartate transaminase, alanine transaminase, alkaline phosphatase, bile acids, and bilirubin. Because ATF3 is a stress-inducible gene and because many of the hepatic phenotypes observed in the TTR-ATF3 mice resemble those observed in stress-associated liver diseases, transgenic mice expressing ATF3 may provide a good model to investigate how stress-induced gene expression affects hepatic functions. Because of the perinatal lethality and the small size of the transgenic mice, repeated transgenic injections were necessary to obtain the results described in this report. Therefore, transgenic mice expressing ATF3 in an inducible manner would greatly facilitate further mechanistic studies. As an example, one could investigate the expression of genes encoding bile acid transporters and bilirubin-conjugating enzymes to elucidate the mechanism for elevated serum bile acids and bilirubin, two commonly observed changes in liver diseases. In addition, one could make primary hepatocytes or nuclear extracts from the mice to investigate the transcriptional programs in the transgenic livers with ATF3 expression versus those without ATF3 expression.

In this context, we note the differences between induction of ATF3 and release of epinephrine and norepinephrine during a stress response. We showed in this report that ATF3 represses PEPCK gene expression, which would result in reduced gluconeogenesis. However, epinephrine and norepinephrine release under acute stress response activates the cAMP pathways and leads to increased gluconeogenesis and glycogenolysis (5). Therefore, different types of stress response have different physiological significance. The release of epinephrine and norepinephrine increases the blood glucose level that is critical for the organism to fight or flee in an acute response. The induction of ATF3 affects the transcriptional programs at the cellular level, which in turn sets things in motion and contributes to the long term detrimental consequences observed in many stress-associated diseases.

**Repression of PEPCK Gene Expression by ATF3**—Previously, we showed by Northern blot analysis that the PEPCK mRNA level is lower in the livers of the TTR-ATF3 transgenic mice than those in the non-transgenic mice (21). In this report, we showed that ATF3 binds to the CRE site derived from the PEPCK promoter in an in vitro assay and represses the PEPCK promoter in a transient transfection assay. Taken together, these results support a model in which ectopic expression of ATF3 in the transgenic livers represses the expression of PEPCK, providing an explanation for the disturbed glucose homeostasis observed in these mice. The PEPCK promoter has a consensus ATF/CRE site, which plays a key role in the up-regulation of PEPCK by the cAMP pathway (for review see Refs. 34 and 35 and references therein). When a PEPCK promoter with a mutated ATF/CRE site was used in the assay, ATF3 did not repress the promoter (data not shown), suggesting that ATF3 represses the PEPCK promoter via the ATF/CRE site. However, we can not rule out the possibility that ATF3 may repress the PEPCK promoter by other mechanisms such as forming dimers with CCAAT/enhancer-binding protein or activating protein-1, which has been demonstrated to play important roles in the regulation of the PEPCK promoter (for reviews see Refs. 34, 36, and 37). Because ATF3 is a stress-inducible gene, our results are consistent with a previous report by Graner and colleagues (38) that the expression of PEPCK gene is repressed by stress signals in cultured hepatocytes. It is possible that stress signals inhibit PEPCK expression, at least in part, by inducing ATF3. However, more work is required to determine the validity of this speculation. Recently, Magnuson and colleagues (39) generated liver-specific PEPCK knock-out mice and demonstrated that PEPCK plays a critical role in the integration of multiple pathways in energy metabolism, a previously unidentified function for PEPCK. Therefore, the decrease in PEPCK gene expression in our transgenic mice may also contribute to impairments in other metabolic pathways not examined in the current study.

**Disturbance of Hepatic Gene Expression and Its Effects on Liver Function**—In this study, we used transgenic mice expressing ATF3, a stress-inducible transcription factor gene, in the liver to investigate how disturbance in transcriptional programs may affect liver function. As described earlier, there are two groups of TTR-ATF3 mice: group 1 had expression of ATF3 in the liver, and group 2 had expression in the liver and pancreas. The difference was “founder-specific.” Founders giving rise to F1 mice with the group 1 expression pattern always gave rise to F1 mice with the group 1 expression pattern, and the same was true for the group 2 expression pattern. Presumably, the difference between groups 1 and 2 mice was because of the differences in the sites of integration and/or copy numbers of the transgene. The hepatic phenotypes presented under “Results” were all derived from the group 1 mice, which did not express ATF3 in the pancreas. Therefore, they are not because of the indirect effects of pancreatic defects. As an example of this indirect phenotype, osmium tetroxide stain for lipid indicated that the group 2 mice had fatty liver, but the group 1 mice did not. We suspect that the fatty liver phenotype in the group 2 mice was because of the deficiency of insulin, an important regulator of lipid metabolism, in these mice (21).

Other genetically altered mice have been used to elucidate how disturbance in transcriptional programs may affect liver function. Examples are knock-out mice deficient in C/EBPα (40) or C/EBPβ (41). These studies indicate that the disturbance in transcriptional program impairs liver function and affects glucose homeostasis. Therefore, our results are consistent with previous findings. However, our results add a dimension of stress. Because ATF3 is a stress-inducible gene, our results indicate that stress signals may affect hepatic functions, at least in part, by inducing ATF3 and perturbing the normal hepatic gene expression patterns.

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The Roles of ATF3 in Liver Dysfunction and the Regulation of Phosphoenolpyruvate Carboxykinase Gene Expression
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