Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 2198–2202, March 1996
Medical Sciences

Prevention of diabetic alterations in transgenic mice overexpressing Myc in the liver

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Communicated by C. R. Park, Vanderbilt University Medical Center, Nashville, TN, November 20, 1995 (received for review September 1995)

ABSTRACT Recent studies have demonstrated that the overexpression of the c-*myc* gene in the liver of transgenic mice leads to an increase in both utilization and accumulation of glucose in the liver, suggesting that c-Myc transcription factor is involved in the control of liver carbohydrate metabolism *in vivo*. To determine whether the increase in c-Myc might control glucose homeostasis, an intraperitoneal glucose tolerance test was performed. Transgenic mice showed lower levels of blood glucose than control animals, indicating that the overexpression of c-Myc led to an increase of blood glucose disposal by the liver. Thus, the increase in c-Myc might counteract diabetic hyperglycemia. In contrast to control mice, transgenic mice treated with streptozotocin showed normalization of concentrations of blood glucose, ketone bodies, triacylglycerols and free fatty acids in the absence of insulin. These findings resulted from the normalization of liver metabolism in these animals. While low glucokinase activity was detected in the liver of diabetic control mice, high levels of both glucokinase mRNA and enzyme activity were noted in the liver of streptozotocin-treated transgenic mice, which led to an increase in intracellular levels of glucose 6-phosphate and glycogen. The liver of these mice also showed an increase in pyruvate kinase activity and lactate production. Furthermore, normalization of both the expression of genes involved in the control of gluconeogenesis and ketogenesis and the production of glucose and ketone bodies was observed in streptozotocin-treated transgenic mice. Thus, these results suggested that c-Myc counteracted diabetic alterations through its ability to induce hepatic glucose uptake and utilization and to block the activation of gluconeogenesis and ketogenesis.

c-*myc* is part of a gene family encoding nuclear phosphoproteins that can bind to DNA in a sequence-specific manner and act as transcription factors. The c-Myc protein is involved in the control of cell proliferation, differentiation, neoplasia, apoptosis, and energy metabolism (1–3). This transcription factor recognizes an "E-box" motif with the central consensus sequence CACGTG (1–3), which is also contained in the glucose/carbohydrate regulatory elements located in the promoter of genes coding for some of the enzymes of glycolysis and lipogenesis (4, 5). A glucose-responsive element (GlcRE) was identified in the promoter of the L-pyruvate kinase gene (6–8), which consists of two imperfect E boxes, CACGGG, which differ from the c-Myc family E box by a single nucleotide. This GlcRE is closely related functionally to the carbohydrate response element (ChoRE) described in the S_{14} and the fatty acid synthase genes, which also contains a c-Myc family E-box motif, CACGTG (9, 10). We have recently shown that an increase in c-Myc protein in liver nuclei of transgenic animals that overexpress c-*myc* under control of the phospho*enol*pyruvate carboxykinase (PEPCK) gene promoter (11) leads to an induction of hepatic glycolysis by increasing both the expression of genes and the activity of the enzymes that control the glycolytic pathway, in the absence of cell proliferation and transformation. Thus, c-Myc transcription factor appears to be involved in the control of liver carbohydrate metabolism *in vivo*.

Glucose phosphorylation by glucokinase is an initial event in glucose utilization by the liver (12). Regulation of glucokinase activity is mainly due to changes in the transcription of its gene (13, 14). In contrast to the transcriptional activation of most hepatic glycolytic and lipogenic genes, which requires the presence of both glucose and insulin (5, 9, 15), the transcription of the glucokinase gene is increased by insulin and does not require the presence of glucose (13, 14). Concentrations of insulin within the physiologic range increase the transcription of the c-*myc* gene in rat hepatoma cells (16), suggesting that c-Myc might be involved in the mechanism(s) by which insulin regulates gene expression. Despite the decrease in circulating insulin caused by starvation, fasted transgenic mice overexpressing c-Myc showed an increase in glucokinase mRNA and in enzyme activity, reaching the levels observed in fed control mice (11). A major role of insulin in the activation of glycolytic genes appears to be the stimulation of glucokinase synthesis (4). In this regard, transduction of rat hepatoma cells, which lack endogenous glucokinase gene expression, with a retroviral vector carrying a PEPCK/glucokinase chimeric gene, results in the rescue of glucokinase activity and in the induction of glucose uptake and utilization (17).

Since c-*myc* was overexpressed in the liver of transgenic mice under control of the PEPCK promoter, which is induced during diabetes (18, 19), we aimed to study whether the increase in c-Myc was able to mimic the effects of insulin on hepatic glucose utilization and counteract diabetic alterations after streptozotocin (Stz) treatment.

MATERIALS AND METHODS

Treatment of Mice. The development and characterization of transgenic mice expressing the PEPCK/c-*myc* chimeric gene were described previously (11). Mice were fed ad libitum with a standard diet (Panlab, Barcelona) and maintained under a light/dark cycle of 12 hr (lights on at 8:00 a.m.). Diabetes was induced by injection through the jugular vein of doses of 2 mg of Stz per 10 g of body weight on two consecutive days. Stz (Sigma) was dissolved in a 10 mM sodium citrate solution with 0.9% NaCl, pH 4.5, immediately before administration. Mice were used 7 days after Stz treatment. Diabetes was assessed by measuring glycemic, glucosuric, and ketonuric levels (Accutrend and Gluketur Test; Boehringer Mannheim), and insulin blood levels. Animals were killed and samples were taken between 9 and 10 a.m. In the experiments described below male mice, F_2 generations from line 1 (11), of age 4–8 weeks were used.

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Abbreviations: CPT, carnitine palmitoyltransferase; GlcRE, glucose-responsive element; HMG, 3-hydroxy-3-methylglutaryl; PEPCK, phospho*enol*pyruvate carboxykinase; Stz, streptozotocin.

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RNA Analysis. Total RNA was obtained from liver by the guanidinium isothiocyanate method (20), and RNA samples (30 μg) were electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde. Northern blots were hybridized to the ^{32}P-labeled cDNA probes indicated in ref. 11, and also to the following probes: PEPCK, a 1.1-kb *Pst* I–*Pst* I fragment from the 3′ end of the rat PEPCK cDNA, (kindly provided by R. W. Hanson, Case Western Reserve University, Cleveland); tyrosine aminotransferase, a 0.6-kb *Pst* I–*Pst* I fragment that includes the 3′ end of the rat cDNA (kindly provided by G. Schütz, Institut für Zell und Tumorbiologie, Heidelberg); mitochondrial 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase, a 1.43-kb *Eco*RI–*Eco*RI fragment of the rat cDNA (kindly provided by F. G. Hegardt, University of Barcelona); carnitine palmitoyltransferase (CPT)-I, a 2.6-kb *Eco*RI–*Eco*RI fragment of the rat cDNA (kindly provided by J. D. McGarry, University of Texas, Dallas); CPT-II, a 1.8-kb *Eco*RI–*Bam*HI fragment of the rat cDNA (from J. D. McGarry); and a β-actin probe corresponding to a 1.3-kb *Eco*RI–*Eco*RI fragment of the rabbit cDNA. These probes were labeled with [α-^{32}P]dCTP by the method of random oligonucleotide priming as described by the manufacturer (Boehringer Mannheim). The specific activity of the DNA probe thus labeled was $\sim 10^9$ cpm/μg of DNA. Membranes were placed in contact with Kodak XAR-5 films. The β-actin signal was used to correct for loading inequalities.

Preparation and Incubation of Hepatocytes. Hepatocytes were isolated between 10 and 11 a.m. from fed normal and diabetic mice as described (21). After nonparenchymal cells and debris were removed, hepatocytes were suspended in Dulbecco's modified Eagle's medium (DMEM; GIBCO) containing 0.2% (wt/vol) albumin and 10% (vol/vol) fetal bovine serum (Boehringer Mannheim). Then 5.5×10^6 cells were plated in 10 ml of this medium on collagen-coated dishes and maintained at 37°C under a 5% CO_2 atmosphere. After 4 hr, the medium was removed and cells were washed three times in DMEM without serum and glucose. To measure glucose and ketone body production, hepatocytes were incubated in 10 ml of DMEM without glucose and supplemented with 16 mM lactate plus 4 mM pyruvate for up to 24 hr. Aliquots of 100 μl of medium were taken at different times, and glucose and β-hydroxybutyrate concentrations were determined.

Hormone, Enzyme, and Metabolite Assays. The concentrations of insulin and glucose in serum; glycogen, glucose 6-phosphate and lactate in liver extracts; and glucose in the incubation medium of hepatocytes were measured as described (11). Glucokinase and pyruvate kinase activities were analyzed in liver samples (11). Glucose was also determined in 20 μl of blood by using an Accutrend analyzer (Boehringer Mannheim). The β-hydroxybutyrate levels in serum and in the incubation medium of hepatocytes were measured by the β-hydroxybutyrate dehydrogenase technique (Boehringer Mannheim). Serum triacylglycerols were determined enzymatically (GPO-PAP; Boehringer Mannheim). Serum-free fatty acids were measured by the acyl-CoA synthase and acyl-CoA oxidase method (Wako Chemicals, Neuss, Germany). Enzyme activities and metabolite concentrations are expressed as the means ± SEM. Statistical differences between data were analyzed by the Student–Newman–Keuls test. Differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

This study was undertaken to determine the effects of overexpression of c-Myc transcription factor on glucose disposal by the liver in transgenic mice. When an intraperitoneal glucose tolerance test was performed in 24-hr-starved mice, transgenic mice showed a marked reduction (about 40%) in the blood glucose levels compared with control mice (Fig. 1). The increase in circulating glucose was transient and the level

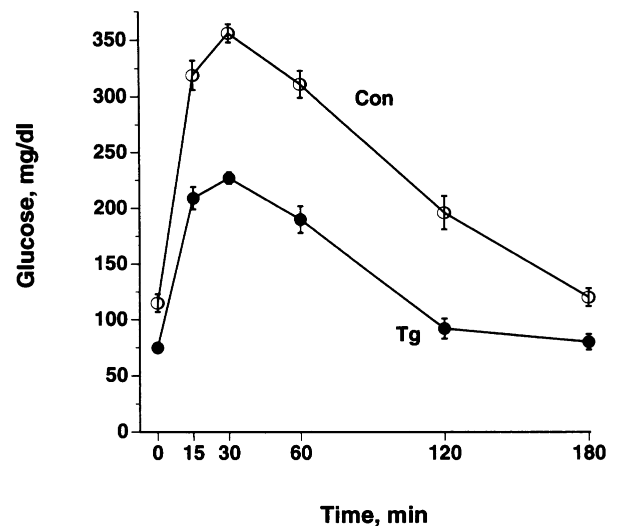

FIG. 1. Intraperitoneal glucose tolerance test. Overnight-starved transgenic (Tg, ●) and control mice (Con, ○) were given an i.p. injection of 1 mg of glucose per g of body weight. Blood samples were taken from the tail vein of the same animals at the times indicated. Glucose was determined as indicated in *Materials and Methods*. Results are means ± SEM of 10 transgenic and 10 control mice.

gradually returned to basal, by 120 min in transgenic mice and by 180 min in control mice. This increased glucose disposal noted in the transgenic mice overexpressing the PEPCK/c-*myc* chimeric gene most likely resulted from increased glucose uptake and utilization in the liver. We have previously reported that the expression of the transgene is increased during starvation, and an increase in both the expression and the activity of glucokinase and pyruvate kinase was observed in starved c-Myc transgenic mice (11). Increased peripheral glucose uptake in transgenic mice expressing the glucose transporter GLUT4 or GLUT1 in skeletal muscle also leads to an enhanced glucose tolerance *in vivo* (22, 23). Skeletal muscle comprises a large body mass, and thus, an increase in membrane transporters should lead to the use of high levels of glucose. Although the liver is smaller, its metabolic capacity enables it to control homeostasis.

To investigate whether an increase in c-Myc in the liver might lead to prevention of diabetic hyperglycemia, control and transgenic mice were treated with Stz. As expected, diabetic control mice had high levels of blood glucose (about 4-fold increase over healthy controls), whereas Stz-treated transgenic mice showed a dramatic reduction of hyperglycemia (Table 1). All these mice had very low levels of circulating insulin (Table 1). In addition, the concentration of serum β-hydroxybutyrate was normalized in diabetic transgenic mice compared with diabetic controls (Table 1). Transgenic mice also showed normal levels of circulating triacylglycerols and free fatty acids, which were markedly increased during diabetes (Table 1). Furthermore, diabetic control mice lost body weight, and a 25% reduction was noted 12 days after Stz treatment (from 23.3 ± 0.6 g to 17.4 ± 0.8 g; $n = 19$). The normalization of serum parameters in the Stz-treated transgenic mice was also accompanied by the maintenance of body weight (from 20.8 ± 0.6 g to 21.7 ± 0.9 g; $n = 22$). These results indicated that transgenic mice developed resistance to the establishment of the common alterations noted in diabetic animals, probably as a consequence of the effects of c-Myc on hepatic glucose metabolism.

The expression of c-*myc* in the transgenic mice was under control of the PEPCK promoter. As previously observed in starved mice (11), two mRNA transcripts, of 2.2 and 1.7 kb, were detected in the liver of Stz-treated transgenic mice, when Northern blots were hybridized with a mouse c-*myc* probe (24)

Table 1. Serum parameters in diabetic PEPCK/c-*myc* transgenic mice

Parameter	Con	Stz-Con	Stz-Tg
Glucose, mg/dl	189 ± 8	>600	243 ± 12
Insulin, ng/ml	1.92 ± 0.25	<0.2	<0.2
β-Hydroxybutyrate, mmol/liter	0.39 ± 0.08	3.22 ± 0.34	0.56 ± 0.11
Triacylglycerols, mg/dl	134 ± 10	352 ± 31	138 ± 18
Free fatty acids, mmol/liter	0.72 ± 0.11	2.08 ± 0.35	0.69 ± 0.13

Serum parameters are from nontreated control (Con), Stz-treated control (Stz-Con), and Stz-treated transgenic (Stz-Tg) mice. Results are means ± SEM of 15 animals in each group.

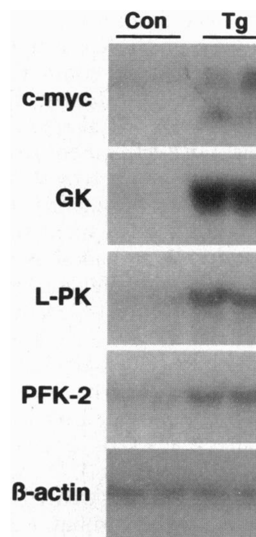

FIG. 2. Expression of c-*myc* and key genes in the regulation of glycolysis in the liver of Stz-treated control (Con) and transgenic (Tg) mice was determined by Northern analysis of total hepatic RNA. A representative Northern blot hybridized with c-*myc*, glucokinase (GK), L-type pyruvate kinase (L-PK), and 6-phosphofructo-2-kinase (PFK-2), and β-actin probes is presented.

(Fig. 2). The 2.2-kb transcript came from the expression of the endogenous gene and the 1.7-kb transcript came from the expression of the transgene, since it lacked the first exon of the c-*myc* gene (11). Thus, the overexpression of c-Myc after Stz treatment in the liver of transgenic mice might lead to the counteraction of diabetic alterations through the induction of the expression of key genes in the control of hepatic glucose utilization. Glucose transport and phosphorylation are the first steps in glucose utilization. Glucose transport does not appear to be rate-limiting for glucose uptake by the liver, and thus glucose phosphorylation by glucokinase is the major factor in the regulation of glucose utilization in this tissue (12). While no glucokinase mRNA transcripts were detected in Stz-treated control mice, Stz-treated transgenic mice expressed high levels of glucokinase mRNA (Fig. 2). Thus, in the absence of insulin, the increase in c-Myc appeared to mimic the effects of the hormone on the expression of glucokinase, acting either directly or through the activation of other transcription factor/s. However, neither a regulatory element responsive to insulin nor an E-box motif that binds transcription factors of the c-Myc family has yet been described in the glucokinase promoter. The increase in the expression of glucokinase was parallel to the activation of the enzyme. The enzyme activity in diabetic control mice was extremely low. However, glucokinase activity of Stz-treated transgenic mice was even higher than that noted in healthy control mice (Table 2). The reduction of glucokinase activity led to a decrease (about 70%) in the intracellular concentration of glucose 6-phosphate in diabetic control mice compared with healthy controls. In contrast, Stz-treated transgenic mice presented high levels of this metabolite, similar to those of healthy control mice (Table 2).

Glucose 6-phosphate is a substrate for the synthesis of glycogen as well as an allosteric activator of glycogen synthase (25, 26). We have previously reported that fed and starved transgenic mice accumulate ~2-fold more glycogen than control mice under the same feeding conditions (11). During diabetes, because of the lack of insulin and the increase in glucagon, glycogen synthase is phosphorylated and inactive (25, 26). As expected, no glycogen was stored in the liver of diabetic control mice. However, Stz-treated transgenic mice showed levels of glycogen similar to those in control healthy mice (Table 2), probably resulting, at least in part, from the increase of glucose 6-phosphate. Thus, the expression of the PEPCK/c-*myc* chimeric gene prevented the loss of glucokinase activity and glucose storage during diabetes in the transgenic mice. These results suggested that glucokinase activity may be rate-limiting for glycogen synthesis in hepatic cells. In agreement with this hypothesis, we have already shown that FTO-2B and H4IIE hepatoma cells, which are unable to store glycogen, express glucokinase and accumulate high levels of glycogen when infected with a retroviral vector carrying a PEPCK/glucokinase chimeric gene (17). Nevertheless, an effect of c-Myc inducing the expression of glycogen synthase and/or specific protein phosphatase genes cannot be ruled out.

Glucose utilization was also increased in these transgenic mice in both fed and starved conditions (11). The L-type pyruvate kinase (L-PK) is a main regulatory enzyme of glycolysis (12). When L-PK gene expression was analyzed, a 3-fold increase in the 3.2-kb mRNA transcript (27) was observed in Stz-treated transgenic mice compared with control mice (Fig. 2). The increase in L-PK mRNA was concomitant with an induction of L-PK activity, which was even higher than that noted in fed control mice (Table 2). Moreover, when the concentration of 6-phosphofructo-2-kinase (PFK-2) mRNA was analyzed in the liver of Stz-treated transgenic mice, a 2-fold increase over the levels in Stz-treated controls was detected (Fig. 2), suggesting that the concentration of fructose 2,6-bisphosphate was probably increased in these transgenic mice. In agreement with these findings, Stz-treated transgenic mice showed an increase of intrahepatic content of lactate compared with healthy control mice (Table 2). The increase in L-PK gene, expression, and perhaps PFK-2 gene expression, might be the result of a direct effect of c-Myc on the GlcREs in the L-PK promoter. However, other mechanism/s involving an unknown "signal" resulting from the increase in glucose 6-phosphate after the induction of glucokinase gene expression cannot be ruled out. Nevertheless, the results in the PEPCK/

Table 2. Glucose storage and utilization in the liver of nontreated control (Con), Stz-treated control (Stz-Con), and Stz-treated transgenic (Stz-Tg) mice

Parameter	Con	Stz-Con	Stz-Tg
Glucokinase, mU/mg of protein	24.6 ± 3.3	4.8 ± 2.9	39.5 ± 3.6
Glucose 6-*P*, nmol/g of liver	211 ± 15	68 ± 9	218 ± 17
Glycogen, mg/g of liver	41.4 ± 5.6	5.1 ± 2.7	37.9 ± 6.4
Pyruvate kinase, U/mg of protein	0.21 ± 0.03	0.12 ± 0.03	0.35 ± 0.06
Lactate, μmol/g of liver	0.16 ± 0.03	0.04 ± 0.02	0.23 ± 0.4

The concentrations of glucose 6-phosphate, glycogen, and lactate and the activities of glucokinase and pyruvate kinase were measured in liver extracts as indicated in *Materials and Methods*. Results are means ± SEM of 12 animals in each group. U, unit(s).

c-*myc* mice suggested that c-Myc prevented the development of diabetic alterations after Stz treatment, at least in part through its ability to induce hepatic glucose uptake and utilization.

The effects of Stz treatment on hepatic gluconeogenesis and ketogenesis in these animals were also analyzed. In contrast to diabetic control mice, Stz-treated transgenic mice showed a reduction in the expression of the gene for PEPCK, which was induced by the diabetic process (Fig. 3). Similarly, the expression of the gene encoding tyrosine aminotransferase, another enzyme involved in gluconeogenesis (28, 29), was also lower in the liver of Stz-treated transgenic mice than in controls (Fig. 3). In agreement with these findings, glucose production from gluconeogenic precursors by hepatocytes in primary culture from Stz-treated transgenic mice was lower than that of Stz-treated control mice and similar to that of healthy control mice (Fig. 4). The decrease in hepatic glucose production by the liver of Stz-treated transgenic animals was also related with a decrease in the glucose transporter GLUT2 mRNA (Fig. 3), which was increased in the liver of Stz-treated control mice probably as the result of hyperglycemia (30, 31). No changes were observed in the expression of GLUT1 between control and transgenic mice under the same conditions (data not shown). These results obtained in diabetic transgenic mice overexpressing c-*myc* were similar to those reported after treatment of diabetic animals with insulin or vanadate (31) and suggested that this transcription factor might be involved in the mechanism/s by which these effectors modulate hepatic glucose metabolism. In addition, in the Stz-treated transgenic mice gluconeogenesis appeared to be blocked in the absence of insulin, suggesting that c-Myc was able to overcome the effects of glucagon on this pathway. Similarly, it has been described that the insulin effect is dominant over the stimulatory effect of cAMP on PEPCK gene transcription (32). Four cis-acting elements in the PEPCK promoter that serve as cAMP response elements have been identified (CRE from −93 to −86, and P3[I], P3[II], and P4 located between −285 and −238) which are required for cAMP-stimulated PEPCK gene transcription (33). Moreover, at least two cis-acting elements are involved in insulin's action on PEPCK gene transcription. One element, designated the distal insulin response sequence (IRS), has been identified between −416 and −407, and one other IRS is known to be present in the proximal promoter region (between −271 and +69), but it has not yet been located (34). No c-Myc consensus sequence CACGTG has been described in the PEPCK promoter that could mediate the inhibitory effects on PEPCK gene expression observed in Stz-treated transgenic mice. Since the increase in c-Myc resulted in an insulin-like action in the liver of these transgenic mice, this transcription factor might induce or block the expression of other specific factors that bind to these regulatory regions of the PEPCK promoter, and thus counteract the effects of diabetes. However, other indirect effects resulting from the increase in glucose 6-phosphate in the hepatic cells as a consequence of activation of glucokinase in the diabetic transgenic mice cannot be ruled out.

An induction of ketogenesis is a common feature of untreated insulin-dependent diabetes mellitus (35, 36). CPT I and CPT II), two membrane-bound enzymes responsible for the entrance of acyl-CoA into mitochondria, and the mitochondrial HMG-CoA synthase, a regulatory enzyme of the ketone-body pathway, have been considered to control liver ketogenesis (35–39). These enzyme activities increase during diabetes. Stz-treated transgenic mice showed a decrease in the expression of the genes for these enzymes when compared with Stz-treated control mice (Fig. 3). The decrease in CPT I, CPT II, and HMG-CoA synthase led to a decrease in ketone-body production by hepatocytes from Stz-treated transgenic mice cultured in the presence of gluconeogenic precursors, compared with diabetic control mice (Fig. 4). The concentration of ketone-bodies in the incubation medium of Stz-treated transgenic mice was similar to that of healthy control mice. These results indicated that ketogenesis was blocked in Stz-treated mice expressing the PEPCK/c-*myc* chimeric gene. The de-

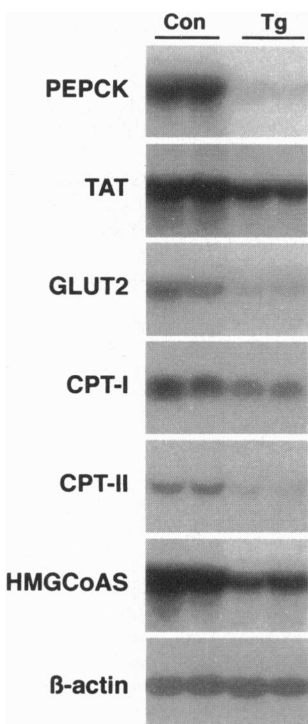

FIG. 3. Expression of key genes in the regulation of gluconeogenesis, glucose transport, and ketone-body metabolism in the liver of Stz-treated control (Con) and transgenic (Tg) mice was determined by Northern analysis of total hepatic RNA. A representative Northern blot hybridized with cDNA probes specific for PEPCK, tyrosine aminotransferase (TAT), glucose transporter GLUT2, CPT-I, CPT-II, mitochondrial HMG-CoA synthase (HMGCoAS), and β-actin is presented.

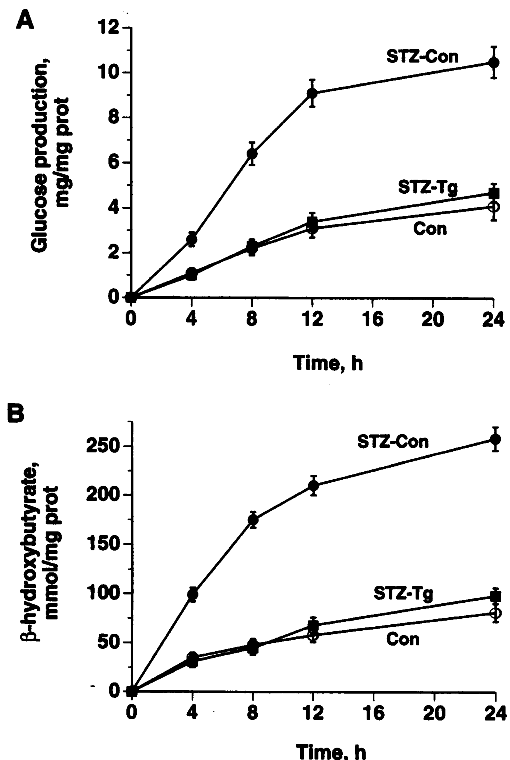

FIG. 4. Glucose and ketone-body production by hepatocytes from nontreated control (Con, ○), Stz-treated control (STZ-Con, ●), and Stz-treated transgenic (STZ-Tg, ■) mice. For glucose (*A*) and β-hydroxybutyrate (*B*) production, primary cultures of hepatocytes were incubated in DMEM without glucose and with 16 mM lactate and 4 mM pyruvate. At the indicated times an aliquot of the incubation medium was removed and concentrations of each metabolite were determined. Results are means ± SEM of three experiments.

crease in hepatic ketone-body production was reflected in the normalization of the serum concentration of β-hydroxybutyrate. A key role of the liver regulating ketogenesis has also been shown in transgenic mice overexpressing the mitochondrial HMG-CoA synthase gene, since these mice had increased levels of blood ketone bodies and normal levels of triacylglycerols, free fatty acids, and glucose (40). A decrease in mitochondrial HMG-CoA synthase gene expression was observed in diabetic rats after administration of insulin or treatment with vanadate (31, 41). These rats showed normalization of ketone-body levels in serum (31). However, the mechanism/s through which c-Myc might regulate in an insulin like manner the expression of these genes controling ketogenesis is not clear, but, since both gluconeogenesis and ketogenesis are blocked in the Stz-treated transgenic mice, a similar mechanism might be shared in the regulation of both metabolic pathways.

In summary, all these results indicated that c-Myc overexpression in the liver of transgenic mice prevented the development of diabetic alterations after Stz treatment not only by inducing hepatic glucose uptake and utilization, but also by blocking the diabetic activation of gluconeogenesis and ketogenesis. In addition, the normalization of hepatic metabolism appeared to compensate for the effects of insulinopenia on lipid movilization from adipose tissue, since the levels of circulating triacylglycerols and free fatty acids were not increased in Stz-treated transgenic mice. Further studies are needed to investigate whether c-Myc, and in turn liver glucose metabolism, might regulate lipid metabolism in the whole animal. Nevertheless, these results in transgenic mice overexpressing c-*myc* reinforce the role of the liver in maintaining normoglycemia and suggest that engineering the liver to increase glucose uptake and utilization may be a useful approach to the treatment of diabetes mellitus.

We thank J. E. Feliu for helpful suggestions, R. Casamitjana for insulin measurements, and D. Navarro and C. H. Ros for technical assistance. E.R. was a recipient of predoctoral fellowships from Direccio General D'Universitats, Generalitat de Catalunya, Spain. This work was supported by grants from Comisión Interministerial de Ciencia y Tecnología (Sal 91/635), Dirección General de Investigación Científica y Técnica (PB94-0668), and the Juvenile Diabetes Foundation International.

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