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The Transcription Factor CCAAT/Enhancer-binding Protein β Regulates Gluconeogenesis and Phosphoenolpyruvate Carboxykinase (GTP) Gene Transcription during Diabetes*

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CCAAT/enhancer-binding protein (C/EBP) β and C/EBPα are members of the *c/ebp* gene family and are highly expressed in mammalian liver and adipose tissue. C/EBPα is essential for adipogenesis and neonatal gluconeogenesis, as shown by the C/EBPα knockout mouse. C/EBPβ binds to several sequences of the phosphoenolpyruvate carboxykinase (PEPCK) gene promoter with high affinity, and C/EBPβ protein is increased 200% in the livers of streptozotocin-diabetic mice, concurrent with increased PEPCK mRNA. To elucidate the role of C/EBPβ in the control of gluconeogenesis during diabetes, we studied the levels of plasma metabolites and hormones related to energy metabolism during diabetes in adult mice heterozygous and homozygous for a null mutation of the gene for C/EBPβ. We also examined the expression of PEPCK and glucose 6-phosphatase mRNAs and regulation of blood glucose, including the contribution of gluconeogenesis to blood glucose in *c/ebpβ*$^{-/-}$ mice. C/EBPβ was not essential to basal PEPCK mRNA levels. However, C/EBPβ deletion affected streptozotocin-diabetic response by: (*a*) delaying hyperglycemia, (*b*) preventing the increase of plasma free fatty acids, (*c*) limiting the full induction of PEPCK and glucose 6-phosphatase genes, and (*d*) preventing the increase in gluconeogenesis rate. Gel supershifts of transcription factor C/EBPα, bound to CRE, P3I, and AF-2 sites of the PEPCK promoter, was not increased in diabetic *c/ebpβ*$^{-/-}$ mouse liver nuclei, suggesting that C/EBPα does not substitute for C/EBPβ in the diabetic response of liver gene transcription. These results link C/EBPβ to the metabolic and gene regulatory responses to diabetes and implicate C/EBPβ as an essential factor underlying glucocorticoid-dependent activation of PEPCK gene transcription in the intact animal.

The CCAAT/enhancer-binding protein (C/EBP)[1] family includes nuclear transcription factors C/EBPα-, β-, δ-, ϵ-, and D-binding protein, encoded by intronless genes located on different chromosomes (1). The C/EBPs consist of homologous C-terminal basic DNA binding and leucine zipper dimerization domains, and less homologous N-terminal activation and attenuation domains (1). The enrichment of C/EBPs in liver and adipose tissue suggested their physiological role could be in the control of expression of genes for energy metabolism (2–4). In adipose cells, C/EBPα and C/EBPβ participate in the differentiation of pre-adipocytes, including the transcription of fat-specific genes (5–7). They also bind and transactivate a variety of genes encoding key metabolic enzymes in the liver, including (but not limited to) phosphoenolpyruvate carboxykinase (PEPCK) and tyrosine aminotransferase (8–11), fatty acid synthesis enzyme acetyl-CoA carboxylase (12), and the albumin gene (13). Additionally, C/EBPβ participates in the induction of cytokines (14–16) and liver acute phase response genes (17).

c/ebpα knockout mice die shortly after birth of profound neonatal hypoglycemia (18–21). The knockout of *c/ebpβ* results in a lethal phenotype following birth in a subset of the homozygous offspring (15, 21–22). Surviving adult *c/ebpβ*$^{-/-}$ mice display impaired macrophage activation and reduced induction of hepatic genes encoding acute-phase response proteins (15–16). *c/ebpβ*$^{-/-}$ females are infertile (15, 23). The epididymal fat pads and mammary glands demonstrated impaired differentiation (22, 24), but no overt disruption of glucose homeostasis was reported (15, 22). However, a more detailed analysis revealed that *c/ebpβ*$^{-/-}$ adult mice fail to regulate blood glucose during fasting and in response to glucagon stimulation, apparently due to lower cAMP levels (25).

In normal mice and cells, C/EBPβ expression, like gluconeogenesis, is stimulated by cAMP (26, 27) and glucocorticoids (28), and down-regulated by insulin (29). During streptozotocin (STZ)-diabetes, liver C/EBPβ mRNA is increased 3-fold while C/EBPα is decreased (29). This change is reverted by insulin treatment in diabetic animals, suggesting that glucagon, glucocorticoids, and possibly insulin action may be expressed in part through C/EBPβ. The hyperglycemia of diabetes results from impaired insulin-dependent glucose utilization and increased hepatic glucose output, via glycogenolysis, and in-

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[1] The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; PEPCK, phosphoenolpyruvate carboxykinase; STZ, streptozotocin; FFA, free fatty acid; Glc-6-Pase, glucose 6-phosphatase; GR, glucocorticoid receptor; HNF, hepatic nuclear factor; AP, activator protein; CREB, cAMP response element-binding protein; GRU, glucocorticoid response modular unit; Mops, 4-morpholinepropanesulfonic acid; IRS, insulin receptor sequence; CRE, cAMP response element; AF, accessory factor; HGP, hepatic glucose production; HMT, hexamethylenetetramine.

creased gluconeogenesis (30). The rate-limiting enzyme for gluconeogenesis, PEPCK (EC 4.1.1.32), is controlled exclusively at the transcription level in response to hormones and metabolites (31–32). Glucagon and catecholamines (via cAMP) and glucocorticoids are positive regulators, while insulin, in the presence of glucocorticoids, is a negative signal (for review, see Ref. 31). PEPCK gene transcription is regulated by several composite modular units in the promoter/enhancer region, comprising two or more *cis*-acting DNA elements (33–36). Mutational analysis of the sites *in vitro* and reporter gene expression driven from mutated promoter constructs in transgenic mice have identified the sites for tissue-specific and hormonally regulated transcriptional response (for review, see Ref. 37). The transacting factors that bind to these sequences include CREB, AP1, C/EBPs, HNFs, TR, RAR/RXR, and glucocorticoid receptor (GR) (see Fig. 3 and Refs. 36 and 37). Many of the genes encoding these transcription factors have been knocked out. HNF-4α and HNF-3β knockouts are embryonic lethal (38–40), and GR knockout mice die soon after birth (41), while HNF-1α knockout mice die within 1 month from birth (42).

Friedman *et al.* (43) showed that glucocorticoids are essential for increased PEPCK gene transcription during diabetes. Indeed, removal of the pituitary gland (44) or adrenalectomy (45) lessens or reverses many of the metabolic abnormalities of diabetic animals, including reducing blood glucose and PEPCK gene expression. At the molecular level, a deletion of the glucocorticoid response modular unit (GRU) of the PEPCK promoter prevents the increase in reporter gene transcription in STZ-diabetic transgenic mice (43). The diabetic response is also blocked in animals with the glucocorticoid receptor antagonist RU-486 (46). Glucocorticoid-stimulated PEPCK gene transcription is suggested to involve a cooperation of the GR with factors binding to accessory factor sites AF-1, AF-2, and AF-3, and through interaction of factors binding to the CRE (36). C/EBP isoforms bind to three major sites on the PEPCK gene promoter, the CRE, the P3I site, and the AF-2 element (26–27, 36–37, 47–50, Fig. 3). Because C/EBPβ has been found related to the main signals controlling PEPCK gene expression, we decided to test whether C/EBPβ inactivation *in vivo* would provide integrative data for understanding glucose homeostasis during diabetes. Our analysis suggests that homozygous *c/ebpβ*$^{-/-}$ mice display decreased blood lipids and impaired gluconeogenesis in response to diabetes. Our results indicate there is a selective increase in C/EBPβ protein in liver nuclei, which binds with greater affinity to DNA sequences within the PEPCK GRU during STZ-diabetes. In mice with a *c/ebpβ* deletion, however, the normal induction of PEPCK and Glc-6-Pase during STZ-diabetes is reduced, gluconeogenesis is decreased, and blood glucose is significantly lower. The impairment in gluconeogenesis in *c/ebpβ*$^{-/-}$ mice during diabetes suggests this transcription factor is an essential mediator of glucocorticoid signaling in the physiological context of the intact mouse.

MATERIALS AND METHODS

Experimental Animals—Mice used in this study were obtained by cross-breeding female mice heterozygous for a null mutation of the *c/ebpβ* gene with homozygous male *c/ebpβ*$^{-/-}$ mice. The generation and mixed genetic background as well as the methods used for genotyping have been described previously by Screpanti *et al.* (15). Adult male and female *c/ebpβ*$^{-/-}$, *c/ebpβ*$^{+/-}$, and *c/ebpβ*$^{+/+}$ mice were studied at 10–14 weeks of age. Mice were housed in microisolater cages and were maintained on a fixed 12-h light/dark cycle at Case Western Reserve University animal facility. Animals had free access to water and were fed regular animal chow (Harlan Teklad, Madison, WI) *ad libitum*. For experimental diabetes, food was removed overnight, mice received a single streptozotocin intraperitoneal injection (0.2 mg/g STZ in 0.05 M citrate, pH 5.0), and food was returned 4 h later. Development of hyperglycemia (> 250 mg%, One Touch II blood glucose meter; Lifescan Inc.) was considered as diabetic state. Animals were sacrificed 3–4 days after STZ treatment. Excised livers were used immediately for nuclear protein extraction or frozen in liquid nitrogen and kept at −80 °C.

Analytical Procedures—Blood was taken from the tails in the morning, centrifuged, and plasma separated and frozen. Plasma concentrations of non-esterified fatty acids and glucose were measured with diagnostic reagent kits from Wako and Sigma, respectively. Insulin and corticosterone levels in plasma were determined using radioimmunoassay kits from Linco Research (St. Charles, MO) and ICN Pharmaceuticals (Costa Mesa, CA). Glycogen was extracted from frozen livers by homogenization in 6% perchloric acid, precipitated in ethanol, hydrolyzed by boiling in 1 N HCl, and glucose measured as for plasmas (51). Statistical comparisons between groups were made using Student's *t* test.

Measurement of Gluconeogenesis Rate—The rate of gluconeogenesis was estimated *in vivo* in mice deprived of food 4 h (noon) prior to receiving intraperitoneal injection of ^2H_2O (0.4% of body weight), and drinking water was supplemented to 0.4% in ^2H_2O. Four hours later (8 p.m.), mice were anesthetized by an intraperitoneal injection of a solution containing ketamine HCl (65 mg/kg), acepromazine maleate (2 mg/kg), and xylazine HCl (13 mg/kg) (Henry Schein, Port Washington, NY) and blood collected from the abdominal aorta. Blood samples were centrifuged at 13,000 rpm at 4 °C for 30 min, the plasma separated, snap-frozen in liquid nitrogen, and stored at −80 °C until analyzed. Enrichment of ^2H at carbon 6 of glucose was assayed by mass spectrometry as hexamethylenetetramine (HMT) formed from formaldehyde by periodate oxidation of the plasma glucose C-6 (52). Briefly, 200 μl of plasma was deproteinized, the supernatant passed through columns AG1-X8 in the formate form and AG 50W-X8 in the H^+ form, and a neutral effluent evaporated to dryness. Glucose content of the effluent was quantified in an automatic analyzer (53). Formaldehyde obtained after periodate oxidation of glucose C-6 was treated with NH_4OH to form HMT. The residue after evaporation was taken up in methylene chloride. HMT formed was injected into the gas chromatograph-mass spectrometer (HP5985; Hewlett Packard, Palo Alto, CA) without further derivatization. The samples were run with standards of HMT of known deuterium enrichments (0.125–2.0 range). The deuterium enrichment of the samples was calculated in mole percent excess from a linear regression equation of the standard curve. The percent contribution of gluconeogenesis was calculated by comparing the ^2H enrichment of glucose with that of body water, as measured in mouse urine by isotopic exchange with [U-$^{13}C_3$]acetone (54).

Hepatic Glucose Production Analysis—Mice were fasted overnight before injecting 5 μCi of D-[3-^{3}H]glucose (NEN Life Science Products) in 100 μl of saline via tail vein. Blood samples (25 μl) for glucose and radioactivity determinations were obtained at 5, 15, and 30 min from the tip of the tail. Serum was obtained after centrifugation at 5,000 × *g* for 5 min, and glucose levels were determined using the glucose oxidase method (Sigma). For radioactivity determinations, 10 μl of blood was deproteinized with 200 μl of 20% trichloroacetic acid. Samples were centrifuged at 5,000 × *g* for 5 min, and the supernatants were evaporated to dryness overnight at 65 °C under a hood. The residue was reconstituted in 200 μl of water, 5 ml of scintillation fluid was added, and the samples were counted in a β-scintillation counter. The rate of hepatic glucose production (HGP) was calculated using steady-state equations (55). The gluconeogenesis rate during overnight fasting was obtained by multiplying the fraction of plasma glucose derived via gluconeogenesis times HGP. Statistical comparisons between groups were made using Student's *t* test.

Liver Nuclear Protein—Liver nuclear extracts were prepared using the combined livers of 2–4 mice by a slight modification of the method of Gorski *et al.* (27, 56). Freshly excised livers were minced, homogenized, nuclei pelleted and lysed, and ammonium sulfate-precipitated nuclear proteins recovered by centrifugation. After dialysis, nuclear proteins were quantitated by Bradford (27), aliquoted, and snap-frozen in liquid nitrogen. All buffers used contained a mixture of proteases and phosphatase inhibitors with the following final concentrations: 1 mg/ml antipain, chymostatin, pepstatin, and leupeptin; 10 mg/ml aprotinin; 2.5 mM benzamidine; 20 mg/ml trypsin inhibitor; 0.1 mM PMSF; 10 mM sodium fluoride, sodium orthophosphate, and sodium vanadate; and 5 nM Microcystin LR.

Gel Supershifting—Double-stranded oligodeoxynucleotides containing sequences of the PEPCK promoter: CRE (−94/−77), 5′-CC**CCTTACGTCAGAGGC**TCTAG-3′ (underlined consensus CRE); P3I (−249/-232), 5′-CTAG**ACGTTGTGTAAGGACTCA-**3′ (underlined C/EBP site homologous sequence); and AF-2 (−420/-406), 5′-GCG**GCTGTGGTGTTTTGA**AAC-3′(underlined consensus IRS) were synthesized using an Applied Biosystems 380A DNA synthesizer, and gel-purified before use (CWRU Core Laboratory). The annealed double-

TABLE I

Metabolic characteristics of c/ebpβ$^{+/+}$, c/ebpβ$^{+/-}$, and c/ebpβ$^{-/-}$ mice

Plasma glucose was measured in the fed state and after 12 h of fasting. Basal levels of plasma corticosterone and insulin were measured by radioimmunoassay. Liver glycogen was extracted from freeze-clamped tissue in fed mice and expressed as glycosyl unit concentrations. Hepatic glucose production rate was determined after overnight fasting and calculations for gluconeogenesis was determined as detailed under "Materials and Methods." Results are means ± S.E. of 6–12 animals per group. *, significantly reduced compared to +/+ mice; $p < 0.05$; **, $p < 0.01$.

Characteristics	c/ebpβ$^{+/+}$	c/ebpβ$^{-/+}$	c/ebpβ$^{-/-}$
Body weight (g)	26.2 ± 0.75	24.8 ± 1.83	24.9 ± 1.53
Plasma glucose (mM)			
Fed state	10.04 ± 0.37	9.41 ± 0.36	8.47 ± 0.31
12-h fasting	9.18 ± 0.35	7.80 ± 0.81	6.94 ± 0.70**
Plasma free fatty acids (mM)	0.81 ± 0.03	0.81 ± 0.03	0.79 ± 0.12
Plasma insulin (ng/ml)	0.89 ± 0.26	0.87 ± 0.37	0.45 ± 0.04*
Plasma corticosterone (ng/ml)	81.0 ± 21	94.1 ± 19	73.7 ± 15
Liver glycogen (μmol/g)	137.8 ± 20		78.8 ± 8.8**
Hepatic glucose production (mg/kg/min)	36.9 ± 5.0	29.6 ± 2.1	17.6 ± 3.6**
Gluconeogenesis (mg/kg/min)	25.1 ± 3.4	21.5 ± 1.4	11.5 ± 2.3**

stranded oligomers were labeled by filling-in the overhanging ends with the Klenow fragment of DNA polymerase and [α-^{32}P]dCTP, gel-purified, and used at 20,000 cpm/ml in the reaction mixture for nuclear protein binding assay. Binding reactions were carried out as described (27). Briefly, 20 μl of mixture consisted of 3–14 fmol of labeled probe (60,000 cpm), 10 mM Tris (pH 7.9), 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 50 mM NaCl, 10% glycerol, 50 μg of bovine serum albumin, and 1 μg of poly(dI-dC) as non-specific competitor. Following 15 min at room temperature, either antisera against C/EBPα, C/EBPβ, CREB, p-CREB, or preimmune serum were added. Binding reactions continued for another 10 min before electrophoresis. Protein-DNA complexes and free probe were resolved on a 20 × 20-cm^2 4% acrylamide (55:1 acrylamide:bisacrylamide) gel in 0.5× Tris borate/EDTA at 100 V for 2 h. The gel was dried and exposed to Kodak X-ARO5 film. The specific band intensities were quantitated by optical densitometry using a Digiscan scanner (U.S. Biochemical Corp.) and the autoradiographic signals integrated (27). The means from these experiments, including total binding and relative amount of C/EBPα and C/EBPβ binding to each separate oligonucleotide, on the same autoradiography were calculated as relative percentage of the control wild-type or C/EBPβ$^{-/-}$ signals integrated in arbitrary units. The values are means of samples in duplicates of three experiments. S.E. were 10–15% of the average values.

RNA Extraction and Northern Blot Analysis—Total RNA was extracted from mouse liver using the guanidine thiocyanate procedure as described previously (46). Solutions were made in diethyl pyrocarbonate-treated water and materials were rinsed in RNase-off solution (CPG Inc.). RNA was purified through gradient centrifugation in cesium chloride, resuspended, and the concentration determined with reference to absorbance at 260 nm ($A_{260/280}$ for purity). 20 μg of total RNA were placed in 37% deionized formamide, 0.66 M formaldehyde gel loading solution and size-fractionated by electrophoresis through a 1.4% agarose, 0.66 M formaldehyde gel in 1× Mops buffer. RNA was transferred overnight to a GeneScreen Plus membrane (NEN Life Science Products) and cross-linked by vacuum-baking at 80 °C. Prehybridization was done at 65 °C, for 3 h, in Church buffer. Probes used were a nick-translated ^{32}P-labeled cDNA (10^6 dpm × μg^{-1}) for PEPCK (1.1-kilobase pair fragment; kind gift of Dr. R. W. Hanson) and glucose 6-phosphatase (1.25-kilobase pair fragment; kindly provided by Dr. D. Massillon). After hybridization overnight at 65 °C, the filter was washed extensively in 2× sodium chloride/sodium citrate (SSC)/0.1% SDS at room temperature, and exposed to Kodak BioMax autoradiographic film at −80 °C. For re-probing, the blots were stripped at 80 °C in 0.1× SSC, 0.1% SDS during 15 min or until no counts. The specific band intensities were quantitated by optical densitometry using a Digiscan scanner (U. S. Biochemical Corp.) and the autoradiographic signals integrated (27). The relative levels of mRNA were expressed as a percentage of mRNA hybridization in liver from wild-type control mice detected on the same Northern blot after correction for ribosomal RNA (28 S) to account for loading differences.

Western Immunoblot Analysis—Purified nuclei from 2–3 mouse livers were resuspended in a lysis buffer, sonicated, and protein quantitated by Bradford (27). Nuclear proteins were precipitated with 10% trichloroacetic acid, resuspended by sonication in 2× SDS-Laemmli sample buffer, and electrophoresed in a 12% polyacrylamide (35:1 acrylamide:bisacrylamide) SDS gel along with molecular weight standards (Life Technologies, Inc.). Liquid electroblotting transfer to a polyvinylidene difluoride membrane (Millipore) was accomplished after 2.5 h at 200 V according to manufacturer's instructions (Bio-Rad). Transcription factors were detected by primary antisera (1:1,000) (anti-C/EBPα and anti-C/EBPβ, Santa Cruz Biotechnology, Santa Cruz, CA; anti-CREB and anti-p-CREB, from Dr. D. Ginty, Harvard Medical School), followed by goat anti-rabbit horseradish peroxidase conjugate (1:5,000) (Amersham Pharmacia Biotech). Detection was done with ECL detection system (Amersham Pharmacia Biotech) as per manufacturer's instructions and membranes exposed to Kodak X-ARO5 film. The specific band intensities were quantitated by optical densitometry using a Digiscan scanner (U.S. Biochemical Corp.) and the autoradiographic signals integrated (27). The relative levels of transcription factors were the means of three or four experiments as a percentage of the arbitrary densitometric units of wild-type or mutant control mice liver detected on the same blot.

RESULTS

Metabolic Profile of Adult c/ebpβ$^{-/-}$ Mice—The involvement of C/EBPβ in liver gene expression has been suggested by previous studies in adult liver and hepatoma cell lines. In order to determine the potential role of C/EBPβ on glucose homeostasis *in vivo*, we studied an animal model lacking the transcription factor. Morning plasma glucose from mice fed overnight *ad libitum* was not significantly different in the c/ebpβ$^{-/-}$ mice compared with the other groups, indicating lack of a role of C/EBPβ on euglycemia in the fed state (Table I). However, when food was withheld overnight (approximately 12 h), glucose levels decreased in mutant homozygous mice by 31% compared with wild-type controls ($p < 0.05$) (Table I). Corticosterone values were not different among the three groups of mice (Table I). Plasma insulin in the fed state was not significantly different between normal (0.89 ng/ml) and c/ebpβ$^{+/-}$ mice (0.87 ng/ml), but it was 47% lower ($p < 0.05$) in c/ebpβ$^{-/-}$ mice. Plasma insulin was similar in c/ebpβ$^{-/-}$ mice and wild-type mice during fasting (0.32 ng/ml; Ref. 25). The corticosterone and insulin concentrations agree with values reported for mice at the same time of the day (57).

The lower fasting glucose in c/ebpβ$^{-/-}$ mice suggested that either the rate of glucose uptake from the circulation is greater or that a decrease in glucose production exists. HGP was tested under steady-state conditions and calculated as mg/min × kg (as described under "Materials and Methods"). In agreement with published levels, HGP in wild-type mice was 36.9 ± 4.98 mg/min × kg (58), while c/ebpβ$^{-/-}$ and c/ebpβ$^{+/-}$ mice showed a significantly lower HGP of 47% and 21%, respectively ($p < 0.01$) (Table I). Hepatic glycogen concentration in the fed state was 43% lower in the c/ebpβ$^{-/-}$ mice compared with the wild type ($p < 0.05$), suggesting a possible deficiency in glycogen stores. However, the rate of gluconeogenesis (fraction of endogenous glucose production) was 53% lower ($p < 0.01$) in c/ebpβ$^{-/-}$ mice (Table I), indicating that decreased HGP was associated with a deficiency in gluconeogenesis.

Gluconeogenesis and PEPCK Gene Expression Are Lower in

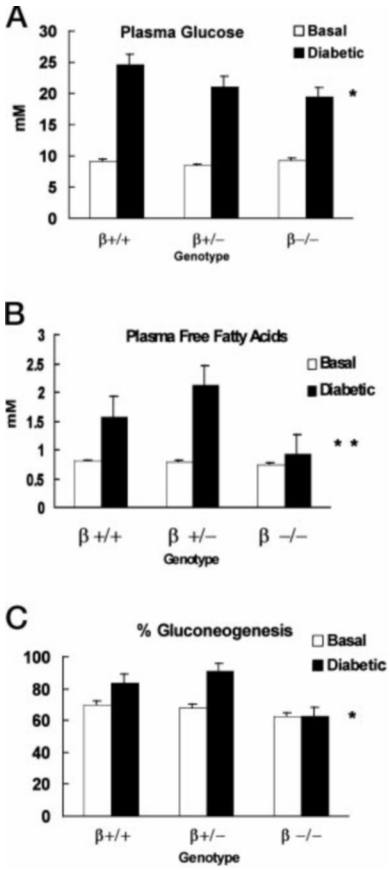

FIG. 1. **Metabolic response to STZ-diabetes in *c/ebpβ*-deficient mice.** *A* and *B*, plasma glucose and free fatty acids were measured in plasmas of mice mutated in none, one, or two alleles of the *c/ebpβ* gene. Mice received 0.2 mg/g STZ for diabetes and were sacrificed 3 days later. *C*, plasma glucose derived via gluconeogenesis (fractional percentage of endogenous glucose production) in +/+, +/−, and −/− mice was measured with ^2H_2O. Enrichment of ^2H in C-6 of glucose was assayed in plasmas by mass spectrometry as described under "Materials and Methods." Results are means ± S.E. from 6–12 animals per group. *, significantly reduced compared with +/+ controls, $p < 0.05$. **, significantly reduced compared with +/+ controls, $p < 0.01$.

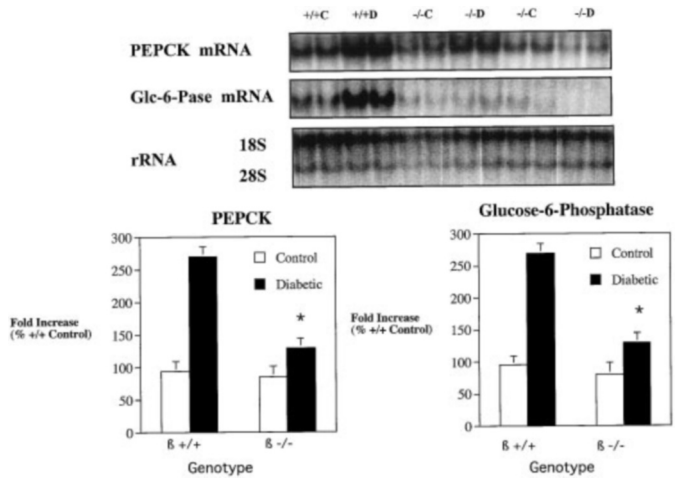

FIG. 2. **Autoradiograph of Northern blot reflecting changes in levels of hepatic PEPCK and Glc-6-Pase mRNAs in normoglycemic and diabetic *c/ebpβ*$^{+/+}$ and *c/ebpβ*$^{-/-}$ mice.** Total cellular RNA was isolated from livers of mice under fasting or diabetic conditions and PEPCK, Glc-6-Pase, and ribosomal RNA detected as described under "Materials and Methods." A representative hybridization from a Northern blot of six different mice is shown. The relative levels of mRNA were determined by densitometry and expressed as a percentage of mRNA hybridization in liver from +/+ control mice detected on the same Northern blot after correction for ribosomal RNA (28 S) to account for loading differences. Results are means ± S.E. of 4–6 animals per group. *, significantly reduced compared with +/+ control $p < 0.05$.

c/ebpβ$^{-/-}$ *Mice during Diabetes*—During streptozotocin-induced diabetes, a lack of insulin and an increase in glucagon and glucocorticoids cause a potent induction in liver gluconeogenic enzymes and hyperglycemia. To test the contribution of C/EBPβ to liver gluconeogenesis during diabetes, mice were treated with STZ and blood was taken from the tails everyday thereafter. The third day following injection, both STZ-treated wild-type and *c/ebpβ*$^{+/-}$ mice had reached hyperglycemic values of 24.7 and 19.5 mM, respectively, which represents 2.7-fold in normal mice (8.47 mM basal) and 2-fold in mutant *c/ebpβ*$^{+/-}$ (9.41 mM) ($p < 0.01$). Glucose levels in *c/ebpβ*$^{-/-}$ mice were also increased from 8.47 to 21.2 mM, which represented a 2.5-fold increase from mutant mice glucose basal levels ($p < 0.01$) (Fig. 1). The difference between STZ-*c/ebpβ*$^{-/-}$ and STZ-wild type mice blood glucose was close to 15% ($p < 0.05$).

The contribution of *in vivo* gluconeogenesis to hyperglycemia was estimated by administering ^2H_2O to control and diabetic mice and measuring incorporation of the stable isotope into plasma ^2H-C-6-glucose, as described under "Materials and Methods." Plasma glucose derived via gluconeogenesis (fractional percent of endogenous glucose production) in *c/ebpβ*$^{+/+}$, *c/ebpB*$^{+/-}$, and *c/ebpB*$^{-/-}$ mice shows that gluconeogenesis was 10% lower in *c/ebpβ*$^{-/-}$ compared with wild-type mice (NS) (Fig. 1). During diabetes, gluconeogenesis did not change significantly in *c/ebpβ*$^{-/-}$ mice (62.9% control *versus* 62.6% diabetes) but it did increase significantly by 20% in wild-type and mutant heterozygous mice ($p < 0.05$). These results support that the contribution of gluconeogenesis (via pyruvate) is over 80% of the total plasma glucose in wild-type mice, but remains constant at approximately 60% in the *c/ebpβ*$^{-/-}$ mice.

Failure to increase gluconeogenesis in the *c/ebpβ*$^{-/-}$ mice during diabetes could result from a failure to fully induce enzymatic machinery and/or from a decrease in availability of substrates and energy. Lipolysis from the adipose tissue is impaired in *c/ebpβ*$^{-/-}$ mice (25), and transcription factor C/EBPβ transactivates genes involved in lipid metabolism. When non-esterified plasma fatty acids were measured during diabetes, a 113% decrease in *c/ebpβ*$^{-/-}$ mice was found compared with the wild-type mice (Fig. 1). The expression of the key gluconeogenic genes PEPCK and glucose 6-phosphatase in diabetic mice was studied by Northern blot analysis. RNA was extracted from livers of control and STZ-treated *c/ebpβ*$^{-/-}$, *c/ebpβ*$^{+/-}$, and wild-type mice after 3–4 days of STZ injection. RNA was electrophoresed, blotted and probed with cDNAs encoding the enzymes as detailed under "Materials and Methods." A representative blot is shown in Fig. 2. Quantification of signals in bands from different blots of samples in duplicates from three or more independent experiments are shown as diagram in Fig. 2. PEPCK mRNA signal intensity was similar in wild-type and *c/ebpβ*$^{-/-}$ mice before STZ. However, following STZ, PEPCK mRNA was increased in normal mice livers, while the increase was significantly less by 35% in mutant mice ($p < 0.01$; Fig. 2). Changes in glucose 6-phosphatase mRNA levels paralleled those of PEPCK ($p < 0.01$; Fig. 2).

C/EBPβ Contributes to Nuclear Protein Binding to DNA Elements That Regulate the PEPCK Promoter Activity during Diabetes—The lower PEPCK mRNA in the STZ-diabetic mutant mice led us to investigate the effect of diabetes and lack of C/EBPβ on the PEPCK promoter activity. The sequences up to −500 base pairs of the PEPCK gene are sufficient to carry out the control of its expression by glucocorticoids, cAMP, and insulin. *In vitro*, C/EBPα and C/EBPβ have been shown to interact with CRE, P3I, and IRS sites of the proximal PEPCK

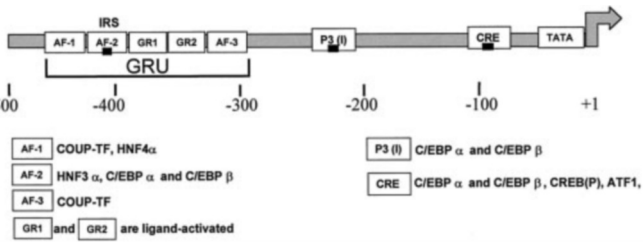

FIG. 3. **Schematic representation of the PEPCK GRU located between −455 and −349 base pairs upstream from the transcription start site.** The PEPCK GRU consists of three accessory factor binding sites (AF-1, AF-2, and AF-3) necessary for the full response to glucocorticoids (8, 36, 49, 50), and two adjacent glucocorticoid receptor binding sites, GR1 and GR2. The AF-2 site contains an IRS (−416 to −407), which mediates a negative effect of insulin on PEPCK gene transcription (49). The CRE site is more than 200 base pairs from the GRU and can cooperate with the GRU for glucocorticoid responsiveness of the PEPCK gene (59). The sequences that bind C/EBPs are indicated by ■ in the AF-2, P3I, and CRE elements (26, 27, 49, 50).

promoter (Fig. 3). Glucocorticoids signal through the composite element GRU containing two different glucocorticoid receptor binding elements together with sites for auxiliary factors, termed AF-1, AF-2, and AF-3 (Fig. 3). All of the sites in the GRU are necessary for maximal response to glucocorticoids (8, 35–36, 50) and to mediate synergism between factors binding at the CRE and GRU (8, 36, 59).

We tested whether nuclear protein binding to the PEPCK promoter sites was modified in the liver by diabetes. Electrophoretic mobility shift assays were performed as detailed under "Materials and Methods." Quantification of bands from different gels of samples in duplicates from three independent experiments was performed and presented in Fig. 7, and representative gel autorads are presented in Figs. 4–6. Liver nuclear proteins bound to the CRE, P3I, and AF-2 oligonucleotides with similar protein to DNA concentration ratios, and the banding profiles obtained agreed with previously reported by others and us for control mice (Figs. 4 and 5) (26, 27, 49, 50). Multiple bands shown correspond to homo- and heterodimers of the C/EBP isoforms (40 and 32 kDa for α, and 38, 34, and 20 kDa for β), together with other possible factors cross-dimerizing with C/EBPs or independently binding to the sites. As shown in Fig. 7, diabetes increased total binding of wild-type mice liver nuclear extracts by 150%, 170% and 150% to CRE, P3I, and AF-2 sites, respectively, which could be partially supershifted by antibodies specific to C/EBPβ but not to C/EBPα. Indeed, binding activity of C/EBPβ increased by diabetes 125%, 181%, and 171% over control to CRE, P3, and AF-2, respectively (Fig. 7*B*). Since C/EBPβ mRNA had been reportedly induced in rat liver by diabetes and repressed by insulin (29), the increase in the C/EBPβ binding to DNA is likely result of translation of a message increased by diabetes rather than a posttranslational event. Western blot analysis was performed with three different liver nuclear extracts obtained from the pooled livers of 2–3 mice of each condition and phenotype, and probed for C/EBPβ and C/EBPα. The results in Fig. 4 demonstrated that indeed, the increase in DNA binding with diabetes in wild-type mice was paralleled by a 187% enrichment in C/EBPβ protein. Scanning of silver-stained liver nuclear proteins, separated in SDS-PAGE, did not show differences in protein banding profile, or relative area of peaks, between control and diabetic state (data not shown), suggesting that the enrichment of transcription factor C/EBPβ in liver nuclei with diabetes was specific. Western blot analysis in Fig. 5 shows that C/EBPα nuclear protein is 25% decreased, in agreement with others results showing a down-regulation of the protein with diabetes (60). Accordingly, binding activity of C/EBPα decreased 17% to CRE and P3 and 40% to AF-2 site with diabetes (Fig. 7*C*). Neither binding activity of transcription factor CREB nor its Ser-133 phosphorylated form was detected in nuclear extracts from wild-type mice, independent of the diabetic or normal state. These results agree with previous results reported by Granner and colleagues (49). However, Western blots showed that CREB and p-CREB were present in the liver nuclei, although no changes in either form appeared with diabetes (results not shown). Hence, C/EBPβ was the major transcription factor responsible for the increase in DNA binding to the PEPCK promoter and, presumably, in transactivation of the PEPCK gene during diabetes in the liver *in vivo*.

The absence of C/EBPβ, however, did not totally prevent PEPCK expression or hyperglycemia after STZ-diabetes in the mice (Figs. 1 and 2). Next we studied whether STZ-diabetes had changed the nuclear protein binding to the PEPCK promoter sites in $c/ebp\beta^{-/-}$ mice. As shown in Fig. 6, liver nuclear proteins from control and STZ-treated $c/ebp\beta^{-/-}$ mice bound to CRE and AF-2 sites of the PEPCK promoter. The number of DNA-protein complexes decreased from 6, in wild-type mice (Figs. 4 and 5), to 2 in $c/ebp\beta^{-/-}$ mice (Fig. 6), but it was not changed by STZ-diabetes in any mice group. Whether the reduction of bands was due to the absence of C/EBPβ and/or of other proteins that necessitate this factor for DNA binding required further analysis. Total binding of diabetic nuclear extracts from mutant mice to the P3, CRE, and AF-2 sites was significantly reduced, by 32%, 38%, and 20%, respectively (Fig. 7*A*), as compared with extracts from livers of wild-type mice (170%, 150%, and 150%; $p < 0.01$) (Fig. 7). The increase in total binding did not appear to change the number of bands, but an increase in one of the two major complexes was detected (Fig. 6). The increased binding was supershifted by anti-C/EBPα antibody. In the mutant mice, binding of liver nuclear proteins to C/EBPα increased with diabetes to 130% (P3) and 131% (CRE), but it did not change for AF-2 (105%) (Fig. 7*C*). Antibody specific to CREB supershifted a band only in mutant mice extracts (Fig. 6). Together, antibodies to C/EBPα and CREB supershifted almost all of the protein-DNA complexes, and in both control and diabetic extracts from $c/ebp\beta^{-/-}$ mice (Fig. 6). The anti-p-CREB antibody used failed to supershift any bands on the sites tested (only AF-2 shown; Fig. 6), despite the addition of inhibitors of protein phosphatases during the nuclear protein preparation (see "Materials and Methods").

In summary, C/EBPβ is increased with diabetes in the liver, it can bind to three DNA sites that stimulate PEPCK gene transcription, and can only be partially substituted for by C/EBPα, and probably also CREB, to contribute to the induction of genes involved in the hepatic diabetic response, such as PEPCK and glucose 6-phosphatase (Fig. 2).

DISCUSSION

Our current understanding of the transcriptional regulation of the PEPCK gene promoter has derived from mutational analysis *in vitro* and *in vivo* using transgenic mice. Such procedures give clues as to the potential regulatory proteins involved in the control of gene expression. However, such results may be far from the native chromosomal and whole body physiological contexts. The liver-specific hormonal control of the PEPCK gene relies on a network of transcription factors responsive to various extracellular signals and integrated by coactivators. The necessity of each transcription factor in an intact physiological setting can only be discriminated by knocking out each family member. The knockout of the gene encoding C/EBPα resulted in a lethal failure to synthesize glycogen and to induce PEPCK and Glc-6-Pase mRNA at the time of birth (18). The knockout of $c/ebp\beta$ displayed a phenotype A, lethal for the same cause as the $c/ebp\alpha$ knockout, and a phenotype B, which survives by partial genetic complementation with no overt disruption of glycemia (21, 25). However,

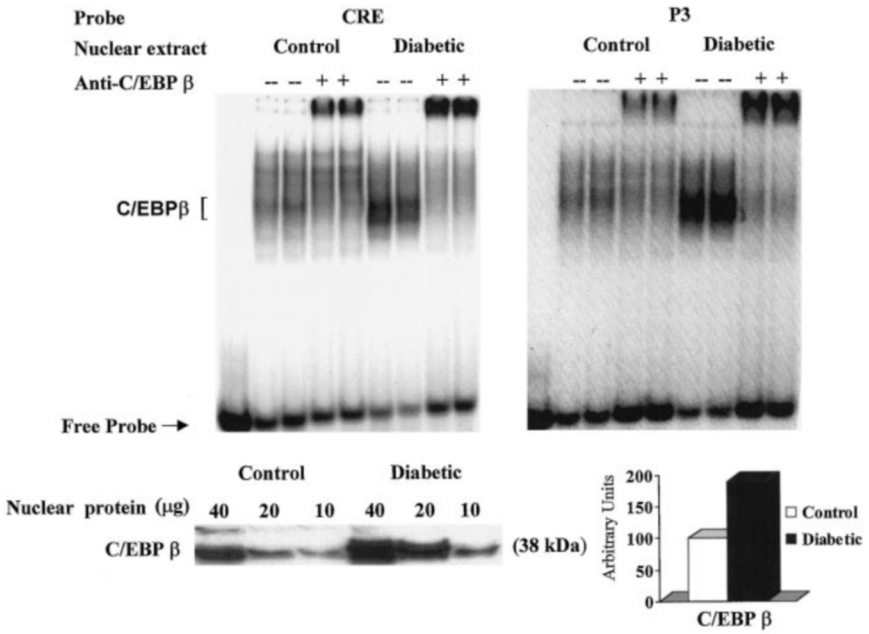

FIG. 4. **Increased C/EBPβ binding in nuclear proteins from wild-type diabetic mice to sites from the PEPCK promoter.** Mice received 0.2 mg/g streptozotocin for diabetes and were sacrificed 3 days later. Liver nuclear extracts were prepared from control and diabetic mice as outlined under "Materials and Methods." Mobility shift assays were performed using α-^{32}P-labeled oligonucleotides containing sequences CRE (−94 to −77) and P3 I (−249 to −232) from the PEPCK promoter. The complexes were supershifted using anti-C/EBPβ antiserum. *Lower panel*, 10–40 μg of liver nuclear protein from diabetic and control mice were electrophoresed, blotted and probed with anti-C/EBPβ antiserum. The major band corresponded to 38-kDa isoform as compared with molecular weight standards.

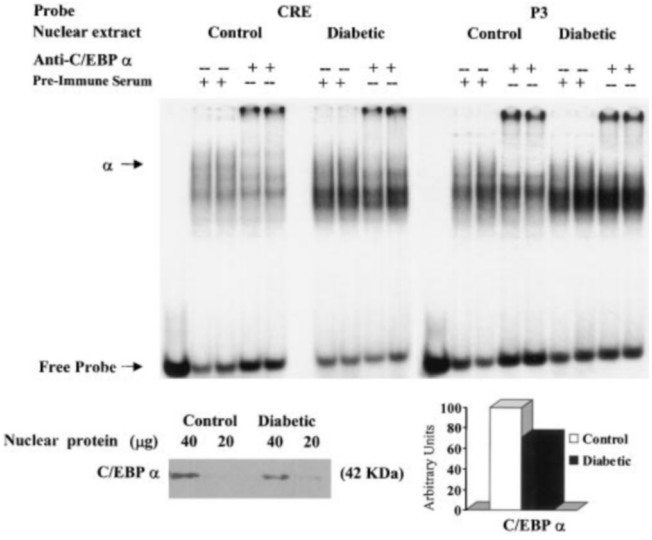

FIG. 5. **No effect of diabetes on C/EBPα binding in nuclear proteins from wild-type diabetic mice to sites from the PEPCK promoter.** Liver nuclear proteins were prepared from wild-type control and diabetic mice as outlined in the legend to Fig. 4. Mobility shift assays were performed with α-^{32}P-labeled oligonucleotides containing sequences CRE (−94 to −77) and P3 I (−249 to −232) from the PEPCK promoter and supershifted using specific anti-C/EBPα antiserum. *Lower panel*, 20–40 μg of liver nuclear protein from diabetic and control mice were electrophoresed, blotted, and probed with anti-C/EBPα antiserum. The major band corresponded to 42-kDa isoform as compared with molecular weight standards.

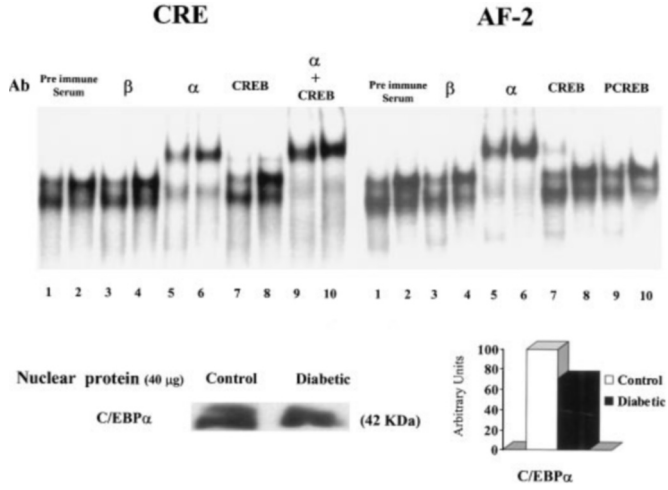

FIG. 6. **Binding of liver nuclear proteins from diabetic *c/ebpβ*$^{-/-}$ mutant mice to sites from the PEPCK promoter.** Mice were rendered diabetic and sacrificed 3 days later and nuclear extracts prepared as described under "Materials and Methods." Binding to α-^{32}P-labeled oligonucleotides of sequences CRE (−94 to −77) and AF-2 (−420 to −406) from the PEPCK promoter were supershifted by specific binding to anti-C/EBPβ, anti-C/EBPα, anti-CREB, and anti-p-CREB specific antibodies. *Lower panel*, 20–40 μg of liver nuclear protein from diabetic and control mice were electrophoresed, blotted, and probed with anti-C/EBPα antiserum. The major band corresponded to 42-kDa isoform as compared with molecular weight standards.

adult *c/ebpβ*$^{-/-}$ mice (B phenotype) had a 50% reduction in hepatic glucose production after an overnight fast, which caused hypoglycemia, demonstrating that C/EBPβ is essential for glucose homeostasis (Table I).

An increase in plasma glucagon concentration is the primary trigger of glycogenolysis and gluconeogenesis during overnight fasting (30). Recent results from our laboratory have demonstrated that, under glucagon or epinephrine stimulation, second messenger cAMP is diminished in the liver and adipose tissue from *c/ebpβ*$^{-/-}$ mice (25). In addition to having lower initial hepatic glycogen levels, liver glycogenolysis was impaired, consistent with reduced hepatic cAMP concentration (25). We have demonstrated here that C/EBPβ is essential for increasing gluconeogenesis during fasting and diabetes, despite the observation that PEPCK and Glc-6-Pase mRNA levels are similar after an overnight fast in wild-type and *c/ebpβ*$^{-/-}$ mice. Thus, both glycogenolysis, which is activated through phosphorylation by cAMP-regulated protein kinase A, and glu-

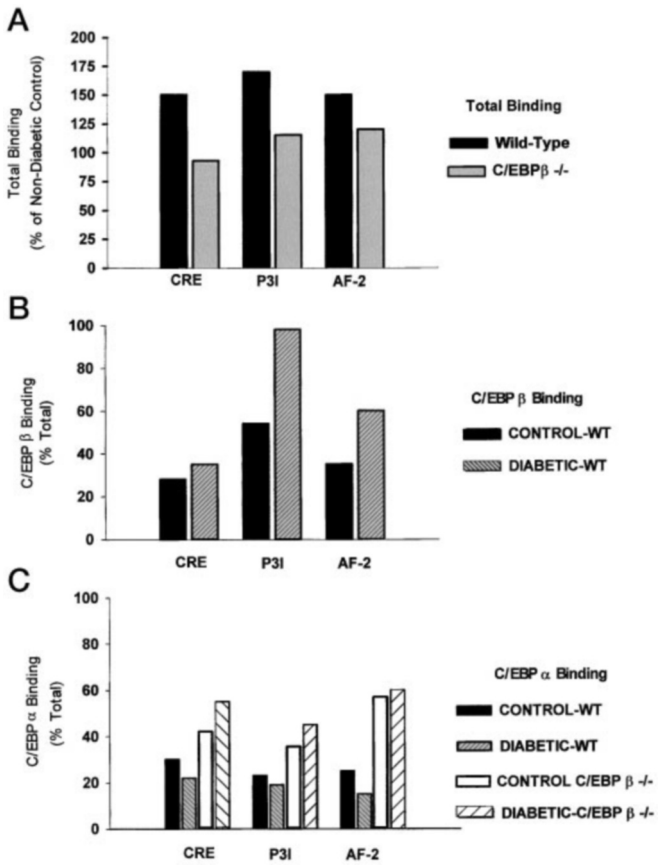

FIG. 7. **Diabetes-induced increase in C/EBPβ protein-DNA binding is reduced in *c/ebpβ*$^{-/-}$ mice and is partially substituted for by C/EBPα.** *A*, total binding to DNA sites was calculated relative to control wild-type or ***c/ebpβ***$^{-/-}$ **mice after integration of the scanned autoradiographs (represented in Figs. 4–6).** *B*, DNA binding due to C/EBPβ is increased by diabetes in wild-type mice liver nuclear extracts. Expressed relative percentage of total binding in *A*. *C*, DNA binding due to C/EBPα expressed relative to total binding in *A*. The means from these experiments include total binding and relative amount of C/EBPα and C/EBPβ binding in each separate oligonucleotide are presented. The values are means of samples in duplicates of three experiments. S.E. were 10–15% of the average values.

coneogenesis, controlled in part by cAMP at the level of gene expression, appear to be compromised. C/EBPβ has also been shown to repress insulin gene expression in cultured cell lines (61). However, *c/ebpβ*$^{-/-}$ mice present a compromised immune response (15), but do not show increased insulin levels. In contrast, *c/ebpβ*$^{-/-}$ mice demonstrated lower insulin levels and hypoglycemia that is only evident after overnight fasting. We also found that C/EBPβ is required for the normal increase in plasma non-esterified fatty acids (FFA) during diabetes and fasting. Gluconeogenesis is stimulated in part by increased oxidation of FFA and subsequent activation of pyruvate carboxylase by acetyl-CoA. The decreased plasma FFA levels are likely caused by decreased rate of FFA release from adipose tissue (25). Consequently, a significant decrease in FFA and glycerol from adipose tissue in *c/ebpβ*$^{-/-}$ mice may also contribute to lower gluconeogenesis. The observation that C/EBPβ deficiency impairs the rise in plasma FFA and gluconeogenesis demonstrates that C/EBPβ is required for cAMP-mediated responses in both liver and adipose cells. The putative target genes that respond to C/EBPβ and increase cAMP levels are currently under investigation in our laboratory.

Following STZ-diabetes, hyperglycemia results from a decrease in insulin combined with a rise in glucocorticoids and glucagon. These hormones synergistically stimulate increased PEPCK gene transcription and gluconeogenesis while reducing insulin-dependent glucose utilization. C/EBPβ gene expression is positively regulated by glucocorticoids and by cAMP, suggesting increased C/EBPβ could mediate the synergistic effects of cAMP and glucocorticoids on PEPCK gene transcription. C/EBPβ binds *in vitro* to the AF-2, CRE, and P3I sites in the PEPCK promoter responsible for glucocorticoid and cAMP activation (Figs. 4–6). During STZ-diabetes, C/EBPβ, and not C/EBPα, increases its binding to the composite glucocorticoid response unit of the PEPCK promoter (Figs. 4 and 5). In the *c/ebpβ*$^{-/-}$ mice, C/EBPα only partially substitutes for C/EBPβ binding to the AF-2 or CRE sites in the PEPCK promoter (Figs. 6 and 7), and does not mediate an increase in gluconeogenesis (Fig. 1). The presence of C/EBP binding sites in the C/EBPα promoter (62) suggests that induction of C/EBPα expression may depend on C/EBPβ. Furthermore, the increase in total binding to AF-2, CRE, and P3 sites from the PEPCK promoter by liver nuclear proteins during diabetes is significantly reduced in the absence of C/EBPβ. This suggests that no other transcription factors of the same or different family can substitute for C/EBPβ to fully stimulate PEPCK transcription during diabetes. However, the HNF-3 family members can also bind to AF-2 site *in vitro* (8, 50), and HNF-3α and HNF-3β are able to interact *in vitro* with the glucocorticoid receptor, thus being potential accessory factor-2 (36) for the full response of the PEPCK promoter to glucocorticoids. The knockout of hepatic nuclear factors HNF-3β and HNF-4α are both embryonic lethal (38–40). However, in HNF-3γ knockout mice, a 50–70% reduction in basal mRNA levels for gluconeogenic enzymes PEPCK and tyrosine aminotransferase occurred, despite an up-regulation of the expression of HNF-3α and HNF-3β (63). Thus, HNF-3γ could be an accessory factor in the physiological context. The fact that, in the absence of C/EBPβ, PEPCK gene expression and gluconeogenesis are impaired during diabetes is reasonable indirect evidence that HNF-3γ does not substitute for C/EBPβ in the glucocorticoid-mediated diabetic response of the PEPCK promoter. Natural mutations affecting genes encoding HNF-4α and HNF-1α have been found in humans with maturity onset diabetes of youth type 1 and 2, respectively (64), which result in a phenotype mainly affecting insulin secretion. Whether the rate of gluconeogenesis and/or transcription of gluconeogenic enzymes are also affected in these patients remains to be explored.

The main gene regulatory mechanisms for response to diabetes are governed by glucocorticoids (43, 46). *In vitro*, C/EBPβ gene expression is increased by glucocorticoids (28). C/EBPβ has displayed interaction, physically and functionally, with glucocorticoid receptor to synergistically mediate expression of genes involved in liver glucocorticoid responsiveness (65, 66). C/EBPβ, but not C/EBPα or C/EBPδ, has recently been shown to specifically interact with the glucocorticoid receptor (67, 68). However, neither dimerization nor DNA binding of the glucocorticoid receptor is required for the glucocorticoid response of the PEPCK promoter (68, 69). A putative coactivator protein, CBP/p300 binds functionally to the N-terminal sequences of C/EBPβ (and not C/EBPα or -δ) (70), in a manner that allows it to regulate transcription in response to dexamethasone (66, 71). If the mechanisms for glucocorticoid mediated PEPCK gene transcription involve interactions between CPB/p300 and C/EBPβ, this mechanism would be defective in C/EBPβ knockout mice. Indeed, we have found that induction of PEPCK mRNA in the *c/ebpβ*$^{-/-}$ mice is less responsive to administered glucocorticoids.[2] There is, however, no defect in the cAMP induction of PEPCK gene transcription when *c/ebpβ*$^{-/-}$ mice

[2] C. Croniger, J. E. Friedman, and R.W. Hanson, unpublished observations.

are treated with Bt_2-cAMP (25), indicating that C/EBPβ, although critical for the full response to hormones such as glucocorticoids, glucagon, and epinephrine, is not required for the response to cAMP. Overall, these results link C/EBPβ to the metabolic and gene regulatory responses to diabetes in liver and adipose tissue and indicate C/EBPβ is required for glucocorticoid-dependent activation of PEPCK gene transcription.

The phenomena that *c/ebpβ* knockout decreases gluconeogenesis and reduces circulating lipid and glucose levels reinforces the suggestion made by McKnight and colleagues in 1989 that C/EBPs are at the center of integration of molecular control of carbohydrate and lipid metabolism (4). Moreover, the fact that a single trans-acting factor can act globally to regulate both glucose and lipid concentration in the whole animal suggests that C/EBPβ could be a novel therapeutic target for treating multiple metabolic disorders. C/EBPβ is essential for expression of genes involved in the normal development of the ovaries and the mammary glands (23, 24), processes that involve steroid hormone receptor-mediated gene expression. It is temping to speculate that the unique activation domain of C/EBPβ (1) could provide interaction sites for multiple steroid hormone receptors. Understanding the interaction between C/EBPβ and glucocorticoid receptors would potentially be of therapeutic interest in the design of specific drugs based on abolition and/or selective increase of interactions involved in metabolic processes such as inflammation, diabetes, and obesity.

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