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Disruption of the *c/ebp*α Gene in Adult Mouse Liver

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The liver-enriched transcription factor C/EBPα has been implicated in the regulation of numerous liver-specific genes. It was previously reported that mice carrying a homozygous null mutation at the *c/ebp*α locus died as neonates due to the absence of hepatic glycogen and the resulting hypoglycemia. However, the lethal phenotype precluded further analysis of the role of C/EBPα in hepatic gene regulation in adult mice. To circumvent this problem, we constructed a conditional knockout allele of *c/ebp*α by using the Cre/*loxP* recombination system. Homozygous *c/ebp-loxP* mice, (*c/ebp*α$^{fl/fl}$; *fl*, flanked by *loxP* sites) were found to be indistinguishable from their wild-type counterparts. However, when Cre recombinase was delivered to hepatocytes of adult *c/ebp*α$^{fl/fl}$ mice by infusion of a recombinant adenovirus carrying the *cre* gene, more than 80% of the *c/ebp*α$^{fl/fl}$ genes were deleted specifically in liver and C/EBPα expression was reduced by 90%. This condition resulted in a reduced level of bilirubin UDP-glucuronosyltransferase expression in the liver. After several days, the knockout mice developed severe jaundice due to an increase in unconjugated serum bilirubin. The expression of genes encoding phosphoenolpyruvate carboxykinase, glycogen synthase, and factor IX was also strongly reduced in adult conditional-knockout animals, while the expression of transferrin, apolipoprotein B, and insulin-like growth factor I genes was not affected. These results establish C/EBPα as an essential transcriptional regulator of genes encoding enzymes involved in bilirubin detoxification and gluconeogenesis in adult mouse liver.

C/EBPα is a liver-enriched transcriptional regulator (12) that controls the expression of numerous hepatic genes encoding enzymes involved in energy metabolism, including insulin-responsive glucose transporter and phosphoenolpyruvate carboxykinase (PEPCK) (29, 30, 44), and of genes encoding factors that maintain liver-specific functions, such as transferrin, factor IX, insulin-like growth factor I (IGF-I), and IGF-II (13, 24, 32, 36). The essential role of C/EBPα in development was recently examined with C/EBPα-deficient mice. These studies confirmed the involvement of C/EBPα in energy homeostasis in neonates. However, the C/EBPα-null mice did not survive beyond the first day after birth (40). This lethal phenotype precludes further study of the role of C/EBPα in energy metabolism in liver and adipose tissues at later stages of postnatal development and the possible role of this factor in other tissues, such as those of the brain and skin.

Here we report the generation of a *c/ebp*α$^{fl/fl}$ (*fl*, flanked by *loxP* sites) mouse strain for use at any developmental stage to conditionally inactivate the *c/ebp*α gene in different tissues by using the Cre/*loxP*-mediated DNA recombination system (35). We used the *c/ebp*α$^{fl/fl}$ mouse line to disrupt the gene at two different developmental stages: at the zygote stage by crossing with an EIIa-*cre* transgenic mouse (17) and at the adult stage in the liver by use of a Cre recombinant adenovirus delivery vehicle (2). The latter animals developed severe jaundice due to markedly decreased expression of bilirubin UDP-glucuronosyltransferase (UGT), the principal enzyme responsible for bilirubin detoxification and clearance (3). Thus, the deficiency of C/EBPα in the postnatal liver results in adult-onset jaundice in mice. In addition, among numerous genes documented by in vitro *trans*-activation and DNA-binding studies as being potentially regulated by C/EBPα, there are several genes encoding enzymes involved in gluconeogenesis whose expression is selectively abolished by a deficiency of C/EBPα in adult liver.

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MATERIALS AND METHODS

Targeting vector and generation of *c/ebp*α$^{fl/fl}$ mice. A *c/ebp*α genomic clone was obtained from a mouse 129SVJ Lambda DASH genomic library (Stratagene), and a 9.5-kb *Sal*I-*Bam*HI fragment containing the C/EBPα coding region was isolated and used for preparing the targeting construct. The first *loxP* site was inserted into the 5′ untranslated region at a *Dra*III site located 40 bp downstream of the transcriptional start site of the *c/ebp*α gene such that it would not interfere with the expression of C/EBPα. A *Hin*dIII site was also incorporated along with the first *loxP* site (see Fig. 1A). The second and third *loxP* sites flanking the PGK-*neo* gene were inserted into an *Eco*RI site 2 kb downstream of the polyadenylation signal and in the same orientation as that of the first *loxP* site. The targeting vector contained 4 kb of homologous DNA upstream of the first *loxP* site and 1.3 kb of homologous DNA downstream of the *loxP*-PGK-*neo* cassette.

The ES cells (RW4; GenomeSystem) were propagated and electroporated with the linearized targeting vector DNA. G418-resistant ES clones were selected, expanded, and analyzed by Southern blotting with both a 5′ probe and a coding region probe to identify specific homologous recombinants (see Fig. 1A). The correctly targeted ES cells were injected into C57BL/6 blastocysts to generate chimeric founder mice as described previously (10, 31). Chimeric male founders with close to 100% agouti coat color were bred with C57BL/6 females. The F_1 mice having germ line transmission of the *loxP*-targeted *c/ebp*α allele were interbred to generate F_2 mice.

Genotyping of the wild-type and *loxP*-targeted (*fl*) alleles. Isolated mouse tail DNA was digested with the indicated restriction enzymes (*Bam*HI or *Hin*dIII), electrophoresed in a 0.5% agarose gel, transferred to a nylon membrane (GeneScreen; Dupont), and hybridized with a 5′ probe derived from the *c/ebp*α gene or the C/EBPα coding region as indicated in Fig. 1B to D.

Culture of recombinant adenovirus. Recombinant adenovirus Ad.cre was a generous gift from Frank Graham. Since this virus is an *EIa* deletion recombinant, it was propagated in 293 cells as previously described (6). Adenovirus was purified by double banding on CsCl gradients and stored at −80°C in 10% glycerol. Titers of the purified viral stock were determined by a plaque assay (6).

Animal studies. All mice used in this study were F_2 siblings from the same five mating pairs of *c/ebp*α$^{fl/fl}$ F_1 heterozygotes. Mice were administered adenovirus 6 weeks after birth. Purified Ad.cre was diluted in phosphate-buffered saline

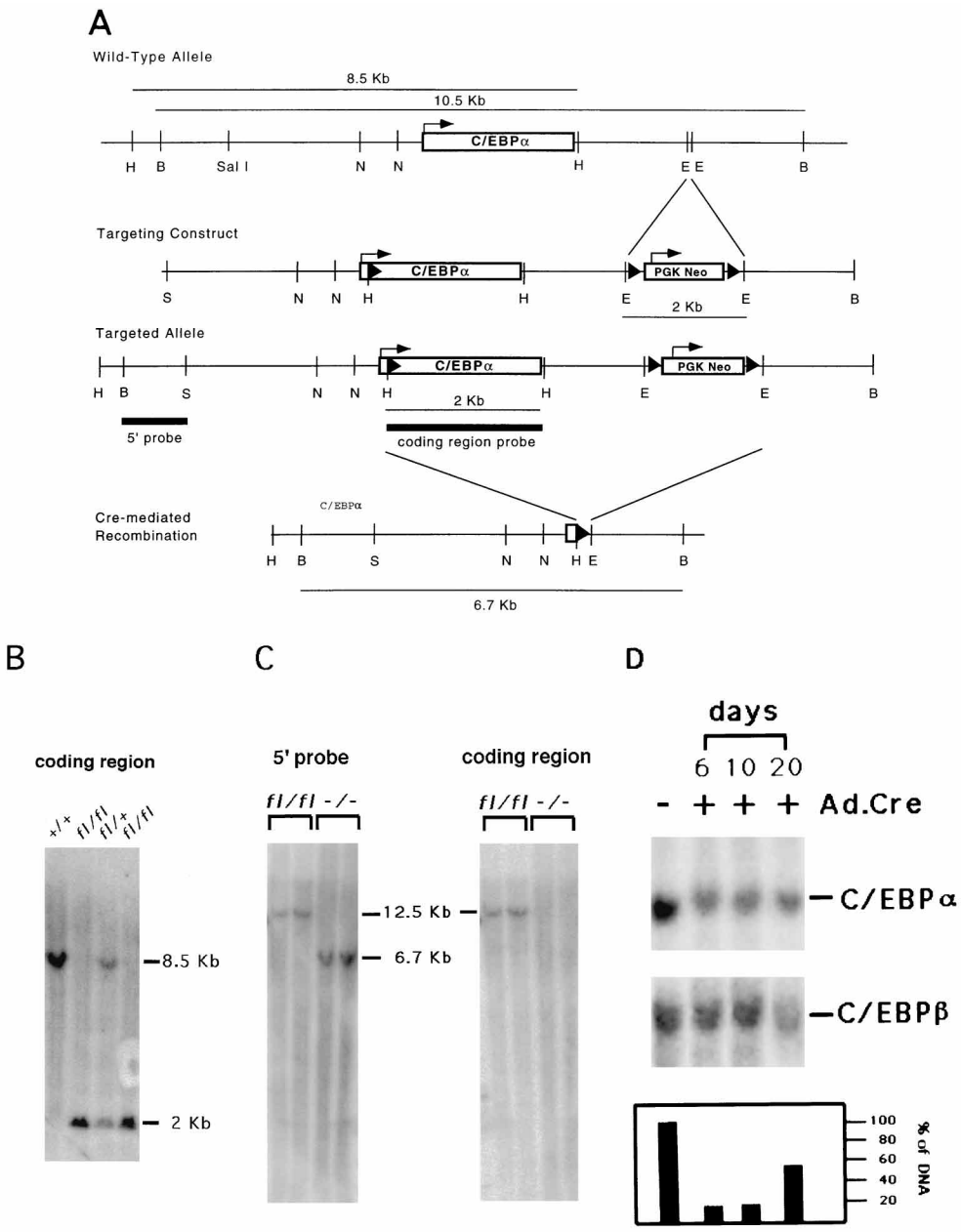

FIG. 1. Targeted modification of the *c/ebp*α gene locus. (A) Diagram of the *c/ebp*α gene (wild-type allele), targeting construct, and targeted allele and schematic of the expected Cre/*loxP*-mediated deletion of the *c/ebp*α gene. Solid triangles represent the *loxP* sites, and the arrows show the direction of transcription. The restriction sites (B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Not*I; S, *Sal*I) are shown. (B and C) Results of Southern blot analysis of representative mouse tail biopsies. Tail DNA was digested with *Hin*dIII (B) or *Bam*HI (C) and probed with the 1.2-kb 5′ probe or the 2-kb C/EBPα-coding regions indicated in the tops of the panels. The sizes of the expected bands are indicated for DNA from the wild-type allele (+), the *loxP*-targeted allele (*fl*), and the *c/ebp*α-deleted homozygote (−/−). (D) Southern blot analysis of representative mouse liver biopsies after Ad.cre infusion. Liver DNA from Ad.cre-infused *c/ebpα*$^{fl/fl}$ mice was digested with *Hin*dIII and probed for the C/EBPα-coding region (top) and the C/EBPβ-coding region (bottom). Each lane represents liver DNA from an individual animal. The signal was scanned and quantified with a PhosphorImager detector. The signal for the C/EBPα-coding region was normalized with the respective C/EBPβ signal.

(PBS) just prior to infusion, and each animal received either 100 μl of PBS or diluted virus by tail vein injection. After Ad.cre administration, the mice were monitored for changes in body weight every 2 days and for any sign of illness or distress. Blood samples were taken from the tail veins at various days after Ad.cre infusion. Selected tissues were snap frozen in liquid nitrogen or fixed in 10% buffered formalin phosphate.

Immunohistochemistry. Formalin-fixed livers were used for histological sections and for immunohistochemical staining with antibodies specifically against mouse C/EBPα, p21 (Waf-1), and proliferating cell nuclear antigen (PCNA). The monoclonal antibodies against C/EBPα and p21 were purchased from Santa Cruz Biotechnology.

Liver RNA extraction and Northern blot analysis. Frozen mouse liver was homogenized in Ultraspec RNA reagent (Biotecx, Houston, Tex.), and total RNA was isolated according to the manufacturer's protocol. RNA (10 μg) was denatured, electrophoresed, transferred to a nylon membrane and probed with cDNA or oligonucleotide probes as previously described (19). The membrane was scanned with a Molecular Dynamics PhosphorImager, and the signal was quantified. The UGT1 cDNA (15) was kindly provided by I. S. Owens and T. A. N. Kong, and the p21 (Waf-1) cDNA was a generous gift from K. Huppi (11). The oligonucleotides used to probe liver RNA in this study are as follows: albumin, 5′-CACTACAGCACTTGGTAACATGCTCACTC; PEPCK, 5′-CA GACCATTATGCAGCTGAGGAGGCATT; glycogen synthase (GS), 5′-GCTC

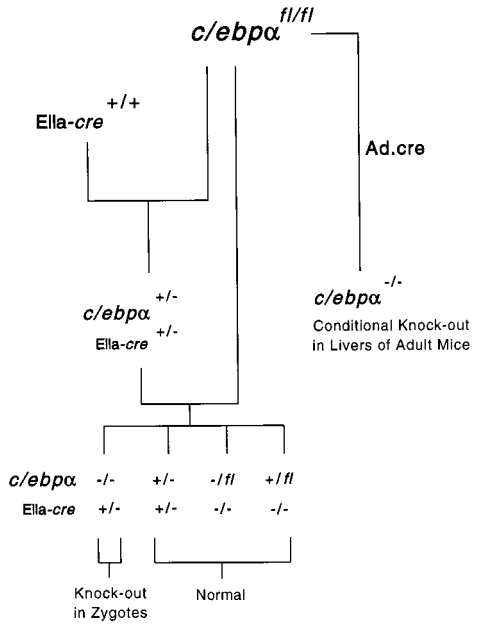

FIG. 2. Generation of the $c/ebp\alpha^{-/-}$ mice at the zygote and adult stages from the $c/ebp\alpha^{fl/fl}$ mice. *fl*, flanked by *loxP* sites; +/+, present in both alleles; +/−, present in one allele only; −/−, absent in both alleles.

TGGATCACAGTACAGATGC; IGF-I, 5′-CATCCACAATGCCTGTCTGA GGTGCC; apolipoprotein B (Apo B), 5′-GTACTGATTGAATCTGGTACTC GCTTG; factor IX, 5′-CTGCACATTCGGTACTGAGTAGATATC; transferrin, 5′-CATCCAAGGTCATAGCATCGGCTTCAC; 18S rRNA, 5′-GTGCGT ACTTAGACATGCATG.

RESULTS

Generation of the $c/ebp\alpha^{fl/fl}$ mice. In order to construct a *c/ebp*α gene-targeting vector in which the entire *c/ebp*α coding region and 1.8 kb of the 3′ sequences were flanked by two *loxP* sites, a *loxP* site was first integrated into the 5′ untranslated region 40 bp downstream of the transcriptional start site of the *c/ebp*α gene. The *loxP*-PGK-*neo* cassette carrying the second and the third *loxP* sites was then inserted 1.8 kb downstream of the *c/ebp*α gene (Fig. 1A). After transfection of the linearized targeting vector DNA into ES cells, 5% of the G418-resistant ES clones were found to carry a mutant allele in which all *loxP* sites were present. The targeted ES cells were then injected into blastocysts to generate mice carrying the *loxP*-targeted allele. The presence of the *loxP*-targeted allele was determined with a probe containing the *c/ebp*α coding region and a diagnostic *Hin*dIII site introduced into the *loxP* sequence. In the wild-type and targeted alleles, 8.5- and 2-kb *Hin*dIII fragments, respectively, were detected (Fig. 1A and B).

Generation of *c/ebp*α germ line and conditional knockout mice from the $c/ebp\alpha^{fl/fl}$ homozygotes. To generate conventional knockout mice (in which the gene is permanently inactivated at the germ cell stage) from the $c/ebp\alpha^{fl/fl}$ mice, the $c/ebp\alpha^{fl/fl}$ mice were bred with a homozygous *cre* transgenic mouse line, EIIa-*cre* (17). The EIIa-*cre* mice carry the *cre* transgene under the control of the adenovirus EIIa promoter and express Cre recombinase only in early mouse embryos (17). Two crosses were required to obtain the conventional *c/ebp*α knockout homozygotes as illustrated in Fig. 2. In $c/ebp\alpha^{-/-}$ mice, a 3.8-kb fragment flanked by the *loxP* sites along with the PGK-*neo* marker (Fig. 1A) was deleted from the *c/ebp*α gene locus by Cre recombinase, as indicated by the appearance of the truncated 6.7-kb genomic fragment hybridizing to the 5′ probe (Fig. 1A and C). Deletion of the *c/ebp*α coding region was confirmed by probing the genomic DNA with the *c/ebp*α coding region (Fig. 1C). As reported previously (40), the $c/ebp\alpha^{-/-}$ mice developed to term but did not survive beyond the first day after birth.

To inactivate the *c/ebp*α gene in adult liver, we infected the $c/ebp\alpha^{fl/fl}$ mice with a recombinant adenovirus carrying the *cre* gene under the control of the cytomegalovirus major immediate-early promoter (2). Human adenovirus targets mouse hepatocytes in vivo with high efficiency following intravenous infusion, and up to 100% of the cells can be transduced in vivo to express a transgene carried by a recombinant adenovirus vector (20, 22, 23). Cre recombinase delivered to hepatocytes by the recombinant adenovirus achieves high site-specific DNA recombination efficiency in fully differentiated and nondividing hepatocytes (41).

The $c/ebp\alpha^{fl/fl}$ mice were infected with 2×10^9 PFU of Cre recombinant adenovirus (Ad.cre) by tail vein infusion. At this dose, more than 80% of the liver hepatocytes can be transduced (20). In our experiments, control mice given this dose of the vector showed no signs of sickness during the period of experimentation. However, the $c/ebp\alpha^{fl/fl}$ mice given Ad.cre lost 10% of their body weight by day 10 after viral infusion, whereas the wild-type ($c/ebp\alpha^{+/+}$) and the heterozygote ($c/ebp\alpha^{fl/+}$) littermates given the same viral dose maintained their body weight, as did control mice administered only PBS (data not shown). Animals were sacrificed at various times after viral infusion, and their tissues were examined for the *c/ebp*α gene deletion. By 6 days after Ad.cre infusion, the level of the C/EBPα-coding region in liver was reduced to about 20% of that in control littermates (Fig. 1D). This reduced level remained constant for 10 days after viral infusion. However, by 20 days after infusion the signal of the C/EBPα-coding region increased to close to 60% of the control level. DNA was extracted 6 days after infusion from other tissues, including lung and adipose tissues, and examined for deletion of the *c/ebp*α gene. No reduction of the C/EBPα signal was detected in these tissues (data not shown). In addition to that from the genomic Southern analysis of liver DNA, evidence for loss of the *c/ebp*α gene was provided by examination of C/EBPα mRNA in liver (see below; Fig. 3).

The Ad.cre vector specifically abolishes the C/EBPα mRNA in the $c/ebp\alpha^{fl/fl}$ mice. RNA extracted from livers of the control and $c/ebp\alpha^{fl/fl}$ mice infused with the Ad.cre was used to examine the specificity of the Ad.cre effects. The level of C/EBPα mRNA was reduced by Ad.cre to 10% of that in saline-infused livers only in the $c/ebp\alpha^{fl/fl}$ mice (Fig. 3). In wild-type control mice, the liver C/EBPα mRNA level was not affected by the Ad.cre administration. In addition, the viral infection did not change the mRNA levels of β-actin and C/EBPβ, a transcription regulator closely related to C/EBPα, in either genotype (Fig. 3).

We also determined the effect of the virus on cell division by examining levels of mRNA encoding cyclin-dependent kinase inhibitor p21 (Waf-1/CIP-1/SDI-1) (8, 9, 11), whose mRNA level is barely detectable in mouse liver but is strongly elevated by partial (70%) hepatectomy (1). The p21 mRNA level was significantly elevated by Ad.cre infection to a similar degree in the livers of mice of both genotypes (Fig. 5). Although p21 was reported to be regulated by C/EBPα (38), our results indicate that the loss of the C/EBPα did not alter the effects of the virus on p21 expression in liver. Immunohistochemical staining with antibodies specifically against p21 and C/EBPα confirmed the observations from Northern analysis (Fig. 4); Ad.cre infusion resulted in loss of the C/EBPα protein from more than 80% of the hepatocytes only in the $c/ebp\alpha^{fl/fl}$ animals 10 days after

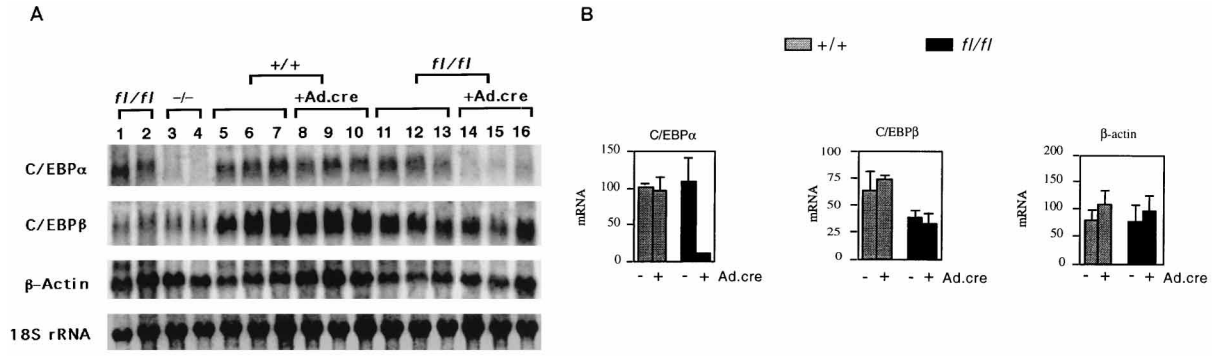

FIG. 3. The infusion of Ad.cre vector specifically abolishes the C/EBPα mRNA levels in the *c/ebpα*$^{fl/fl}$ mice. (A) Northern blotting analysis of representative liver biopsies after Ad.cre infusion. Ten micrograms of total RNA from *c/ebpα*$^{fl/fl}$ neonates (lanes 1 and 2), the *c/ebpα*$^{-/-}$ neonates (lanes 3 and 4), and saline- (lanes 5–7 and 11–13) or Ad.cre-infused (lanes 8–10 and 14–16) adult mouse livers 10 days after infusion was denatured and electrophoresed on a formaldehyde-containing 1% agarose gel, blotted to nylon membranes, and probed with the indicated cDNA and oligonucleotide probes. Each lane presents RNA from an individual animal. (B) The signal of mRNA from each sample was quantified with a Molecular Dynamics PhosphorImager and normalized with its respective 18S rRNA signal. The value for each group is the average of the three different mRNA samples shown in panel A and is presented as a vertical bar in the figure. The standard deviation for each group is shown as a vertical line.

infusion, while strongly elevating p21 protein expression in livers of the wild-type control or *c/ebpα*$^{fl/fl}$ mice (Fig. 4E to H). For either genotype, p21 protein was not detectable in the livers of saline-infused mice, whereas C/EBPα protein was easily detected (Fig. 4E and F). The Ad.cre-infused livers were also examined with an antibody against PCNA, a marker for cell division. Livers from Ad.cre-infected animals of both genotypes contained similar percentages of PCNA-positive hepatocytes, ca. 10% (Fig. 4A to D). Taken together, these results establish that Ad.cre markedly abolishes expression of C/EBPα only in *c/ebpα*$^{fl/fl}$ mice but does not affect levels of C/EBPβ or β-actin. Although the virus stimulated hepatic cell division, this response was not affected by C/EBPα.

C/EBPα deficiency in adult mice reduces expression of PEPCK, GS, and factor IX, but not that of transferrin, Apo B, and IGF-I. On the basis of promoter *trans*-activation assays and DNA-binding studies, numerous liver-specific genes were reported to be regulated at the transcription level by C/EBPα (13). We therefore examined mRNA levels for several hepatic genes in the *c/ebpα*$^{fl/fl}$ conditional knockout mice (Fig. 5). The PEPCK and GS mRNA levels were reduced by about 10-fold in the *c/ebpα*$^{fl/fl}$ mice infused with Ad.cre. The reduced levels for both transcripts correlated well with the reduction in level of C/EBPα. The factor IX mRNA level was also reduced only in the *c/ebpα*$^{fl/fl}$ mice, but to a lesser degree (about twofold) than was observed for PEPCK and GS mRNAs (Fig. 5). In agreement with the results for adult liver, both GS and factor IX mRNAs were not detectable in the germ line *c/ebpα*$^{-/-}$ neonates (generated by germ cell knockout as shown in Fig. 2). In contrast, the PEPCK mRNA levels of the *c/ebpα*$^{-/-}$ and *c/ebpα*$^{fl/fl}$ neonates appeared to be similar (Fig. 5A, lanes 1 to 4). The transferrin, apo B, albumin, and IGF-I mRNA levels were not affected by Ad.cre in either genotype, although the transferrin mRNA level was reduced in the livers of *c/ebpα*$^{-/-}$ neonates. Taken together, these results suggest that in adult mouse liver, C/EBPα is essential for the transcriptional activation of the PEPCK and GS genes but is dispensable for the expression of genes encoding transferrin, albumin, IGF-I, and apo B, even though their promoter/enhancer regions contain functional C/EBP binding sites.

Unconjugated hyperbilirubinemia in the *c/ebpα* conditional knockout mice. To examine the effects of C/EBPα deficiency on liver function, blood chemistry was analyzed for the virally infused mice (Table 1). Mice of both genotypes administered Ad.cre displayed elevated levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), indicating virus-associated liver damage. The elevated serum AST and ALT concentrations reached maximal levels on day 6 after viral infusion, significantly declined on day 10 (Table 1), and returned to the normal range on day 17 (data not shown). The adenovirus-associated changes in the serum chemistry also included low glucose and high cholesterol and triglyceride levels. However, while the glucose levels remained low, the abnormal levels of cholesterol and triglyceride became insignificant in both genotypes 10 days after infusion, as compared to the levels in saline-infused mice (Table 1). Serum albumin levels were not altered by the Ad.cre regardless of the genotype. Surprisingly, all of the 28 *c/ebpα*$^{fl/fl}$ mice given Ad.cre developed jaundice by 6 days after infusion. Some mice showed signs of jaundice as early as 3 days after infusion, while none of the 19 wild-type *c/ebpα* littermates given the same dose of Ad.cre developed symptoms. Jaundice was evident on the basis of elevated levels of serum bilirubin on day 6 after infusion (Fig. 6). The majority (>60%) of the elevated serum bilirubin was unconjugated. Elevated bilirubin was not found in any of the various control animals, including wild-type mice injected with Ad.cre and *c/ebpα*$^{fl/fl}$ mice injected with saline. Unlike the elevated levels of ALT and AST caused by viral infection, the elevated unconjugated bilirubin levels in the serum of the *c/ebpα* deletion homozygotes persisted and even increased 10 days after viral infusion. These results indicate that the unconjugated hyperbilirubinemia caused by Ad.cre is a direct result of C/EBPα deficiency and not the result of viral infection.

Many factors can cause hyperbilirubinemia, such as excess production of bilirubin by hemolytic anemias, reduced hepatic uptake by drugs, and impaired bilirubin conjugation by the action of UGT. UGT facilitates the clearance of bilirubin through the bile by converting the heme biproduct to a more water-soluble derivative (16). Bilirubin UGT is the principal enzyme involved in bilirubin conjugation and clearance (3). Since the serum chemistry results did not suggest hemolytic anemia and because C/EBPα is a liver-enriched transcription factor involved in regulating the transcription of numerous liver-specific enzymes, we examined whether the transcription of genes involved in bilirubin conjugation was affected by the deficiency of C/EBPα. Bilirubin UGT mRNA from the livers

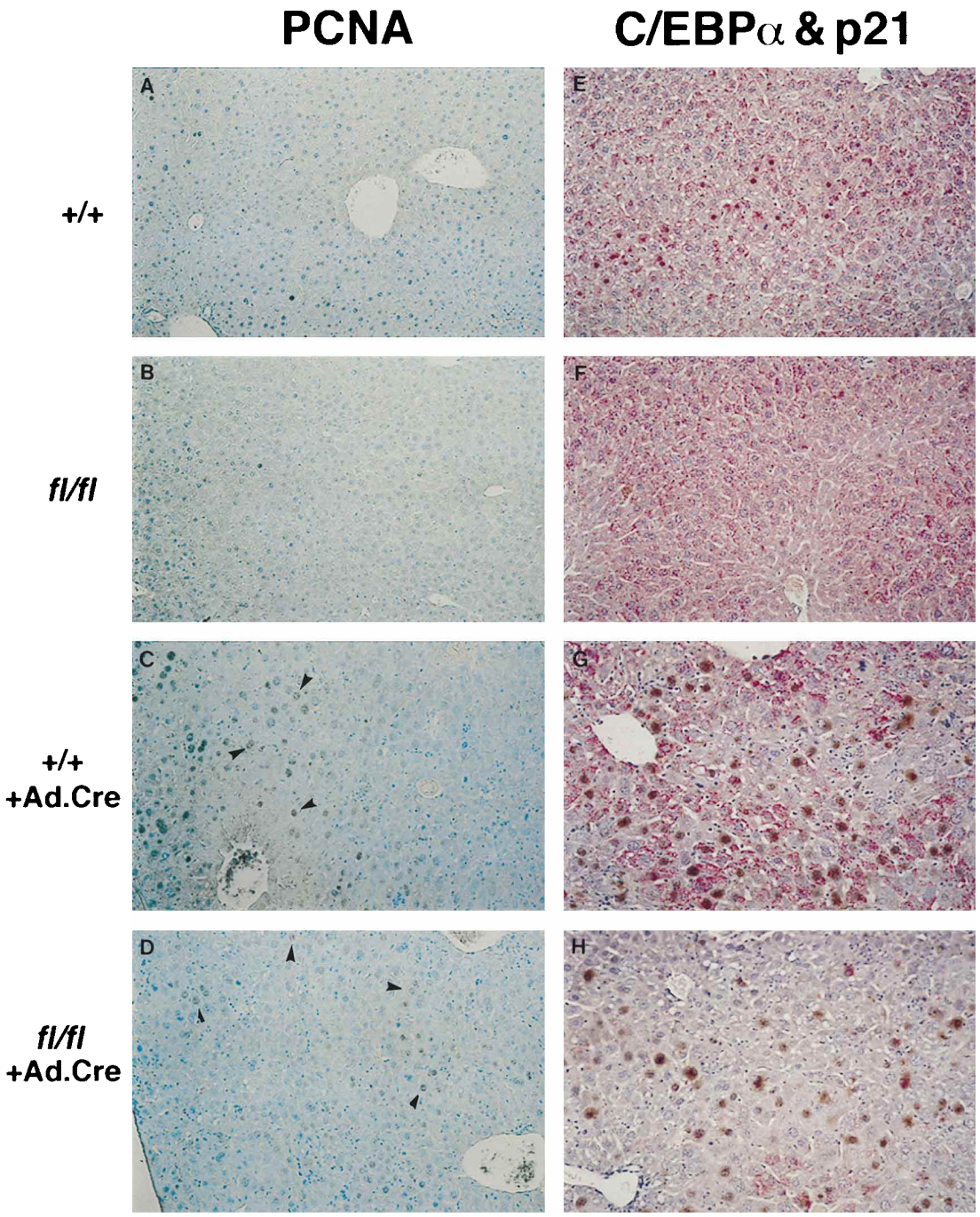

FIG. 4. The infusion of Ad.cre ablates C/EBPα protein expression specifically in the *c/ebpα*$^{fl/fl}$ mice and increases PCNA and p21 protein expression in either genotype. (A to D) Immunostaining against PCNA. Formalin-fixed, paraffin-embedded liver sections were stained with antibody to PCNA (peroxidase–3,3-diaminobenzidine [DAB] labeled and brown; some are shown by arrowheads). Magnification, ×137. (E to H) Double immunostaining against C/EBPα and p21. Formalin-fixed, paraffin-embedded liver sections were stained with monoclonal antibody to C/EBPα (alkaline phosphatase method labeled and red) and monoclonal antibody to p21 (peroxidase-DAB method labeled and brown). The sections were counterstained with hematoxylin. Magnification, ×182.

of the *c/ebpα*$^{fl/fl}$ mice given Ad.cre was reduced to 13% of that of the *c/ebpα*$^{fl/fl}$ saline-treated controls or wild-type mice administered Ad.cre (Fig. 7). In addition, bilirubin UGT mRNA was absent in the livers of *c/ebpα*$^{-/-}$ neonates, whereas it was easily detected 5 h after birth in the livers of untreated *c/ebpα*$^{fl/fl}$ mice (Fig. 7A, lanes 1 to 4). These findings indicate that C/EBPα is essential for the expression of the liver bilirubin UGT gene at both the neonate and adult stages.

The bilirubin *UGT* gene is located within the *UGT1* gene complex locus, which contains four exons (exons 2 to 5) in the 3′ region. These four exons encode a shared carboxyl-terminal region for all the UGT1 isoforms. In the 5′ region of the *UGT1* locus, more than seven different first exons exist, each with a 5′ proximal promoter element and each encoding a unique amino-terminal region that confers the unique substrate specificity of each isoform (28). Bilirubin UGT is the major isoform of

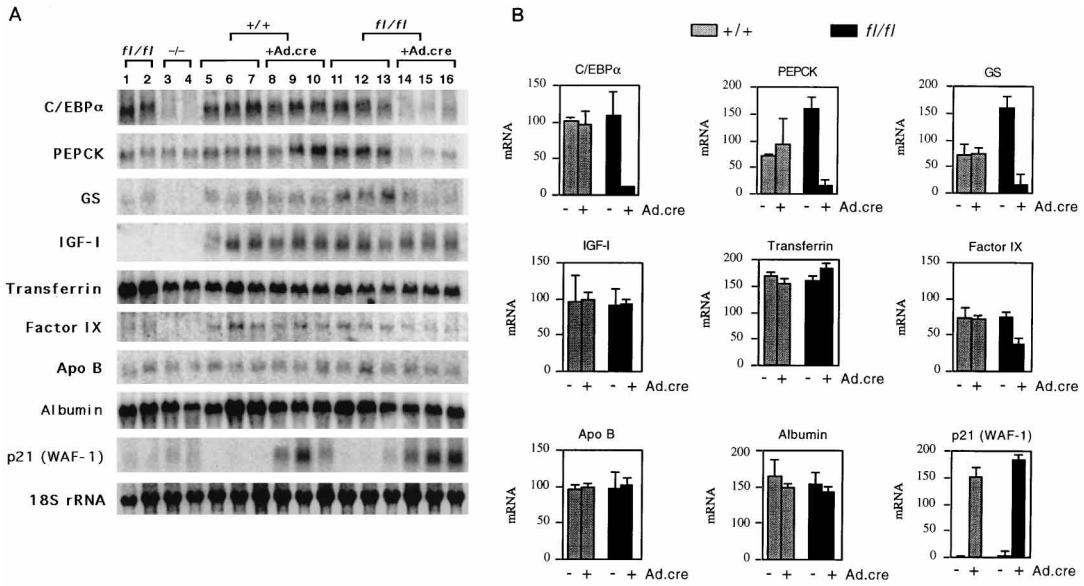

FIG. 5. The infusion of Ad.cre selectively reduces the PEPCK, GS, and factor IX transcript levels in the *c/ebpα*$^{fl/fl}$ mice. (A) Northern blotting analysis of representative liver biopsies after Ad.cre infusion. Ten micrograms of total RNA from *c/ebpα*$^{fl/fl}$ neonates (lanes 1 and 2), the *c/ebpα*$^{-/-}$ neonates (lanes 3 and 4), and saline- (lanes 5–7 and 11–13) or Ad.cre-infused (lanes 8–10 and 14–16) adult mouse livers 10 days after infusion was denatured and electrophoresed on a formaldehyde-containing 1% agarose gel, blotted to nylon membranes, and probed with the indicated cDNA and oligonucleotide probes. Each lane presents RNA from an individual animal. (B) The signal of mRNA from each sample was quantified with a Molecular Dynamics PhosphorImager and normalized with its respective 18S rRNA signal. The value for each group is the average of the three different mRNA samples shown in panel A and is presented as a vertical bar in the figure. The standard deviation for each group is shown as a vertical line.

the UGT1 enzymes. However, since each exon 1 has its own proximal promoter, we examined whether the expression of other UGT1 isoforms was affected by C/EBPα deficiency. By using a cDNA fragment as the probe that detects the portion of the mRNA encoding the common C termini of the UGT1 isoforms, including bilirubin UGT, we observed that the signal for the UGT1 transcripts was reduced by about 50% in *c/ebpα*$^{fl/fl}$ mice given Ad.cre (Fig. 7). This result suggests that expression of some, if not all, of the other UGT1 isoforms was unaffected by *c/ebpα* deletion. A similar result is suggested by the presence of UGT1 mRNA in the livers of germ line *c/ebpα*$^{-/-}$ mice (Fig. 7A, lanes 3 and 4).

DISCUSSION

As a first step in exploring the regulatory function of C/EBPα in defined tissues at different developmental stages, and to avoid the lethal neonatal phenotype in the *c/ebpα*$^{-/-}$ mice reported earlier, we generated a mouse strain, *c/ebpα*$^{fl/fl}$, in which the *c/ebpα* gene locus was modified by gene targeting to contain flanking *loxP* sites. This allows regulated deletion of the C/EBPα-coding region by the Cre/*loxP*-mediated DNA recombination system (35). Recombinant adenovirus Ad.cre (2) was used to deliver the Cre recombinase specifically to the livers of *c/ebpα*$^{fl/fl}$ adult mice. Infection resulted in efficient site-specific DNA excision of the C/EBPα-coding region, as evidenced by an 80% loss of the gene and a 90% reduction of C/EBPα mRNA in liver. The loss of C/EBPα was evenly distributed throughout the liver, as assessed by immunohistochemical determination. By using this conditional knockout strategy, we demonstrated that C/EBPα is necessary for transcription of the bilirubin UGT, PEPCK, and GS genes, which encode enzymes crucial to the detoxification of serum bilirubin and to the gluconeogenesis pathway.

Bilirubin UGT is the major isoform of the UGT1 enzymes in liver. It is expressed constitutively in liver at a level higher than those of any of the other UGT1 isoforms (3, 28). Bilirubin UGT was found to be the only conjugating enzyme for serum bilirubin, the majority of which is derived from the daily breakdown of erythrocytes (3). Unconjugated bilirubin is highly hydrophobic and cannot be excreted in the urine (16). It is also toxic, especially in the central nervous system. Therefore, the regulation of bilirubin UGT is of critical importance to cellular and organismal physiology. Alteration of its expression may impair the steady-state level of toxic, unconjugated bilirubin, which is normally maintained at an undetectable level in serum. The mechanisms regulating bilirubin UGT gene expression, as well as that of other UGT1 members, at the transcriptional level have not yet been determined. However, the structure of the UGT1 gene complex and the distinct (liver-specific) expression pattern of the bilirubin UGT isoform indicate that the UGT1 genes are regulated independently of each other (28). Our findings support this possibility. Bilirubin UGT appears to be mainly controlled by C/EBPα, while the expression of other UGT1 mRNAs is less affected by the reduced level of C/EBPα.

Results from in vitro DNA-binding studies and transfection of intact cells with reporter gene constructs strongly implicate C/EBP proteins as one of several *trans*-activators for the PEPCK and GS gene promoters (30, 44) as well as for those of many other genes, such as IGF-I (24, 27, 37). In addition to C/EBPα, other liver-enriched transcription factors involved in controlling the PEPCK gene promoter include HNF-1 and HNF-4 (29, 39). PEPCK expression is also under multihormonal control; it is inhibited by insulin and increased by glucocorticoid hormone (7, 34). C/EBPα and HNF-3 may be involved in mediating these hormonal effects on PEPCK (26, 27). Similarly, in the case of IGF-I gene regulation, C/EBPβ, HNF-1α, and HNF-3β, in addition to C/EBPα, were all reported to be strong *trans*-activators of the IGF-I gene promoter (24, 25,

TABLE 1. Changes of serum component levels after Ad.cre infusion[a]

Serum component	Concn[b] for indicated mouse type and infusion[c] at indicated day after infusion											
	+/+			+/+ + Ad.cre			*fl/fl*			*fl/fl* + Ad.cre		
	2	6	10	2	6	10	2	6	10	2	6	10
AST	ND[d]	122 ± 25	105 ± 9	ND	3,080 ± 120	752 ± 440	ND	103 ± 40	118 ± 11	ND	2,400 ± 450	977 ± 210
ALT	24 ± 7	16 ± 2	21 ± 12	53 ± 30	1,672 ± 49	690 ± 354	25 ± 4	25 ± 11	16 ± 8	80 ± 73	1,680 ± 550	645 ± 285
Glucose	162 ± 13	243 ± 37	175 ± 24	145 ± 20	144 ± 24 ($P = 0.018$)	67 ± 25 ($P = 0.040$)	224 ± 42	208 ± 28	268 ± 40	148 ± 28	136 ± 26 ($P = 0.007$)	78 ± 16 ($P = 0.001$)
Albumin	3.3 ± 0.5	3.3 ± 0.2	3.2 ± 0.2	3.2 ± 0.2	3.4 ± 0.1	3.7 ± 0.3	3.7 ± 0.3	3.5 ± 0.5	3.5 ± 1	3.1 ± 0.2	3.6 ± 0.2	4.0 ± 0.5
Cholesterol	ND	94 ± 14	67 ± 25	ND	182 ± 56 ($P = 0.058$)	100 ± 21 ($P = 0.113$)	ND	94 ± 16	93 ± 16	ND	115 ± 23 ($P = 0.215$)	83 ± 67 ($P = 0.768$)
Triglycerides	ND	44 ± 4	67 ± 27	ND	105 ± 21 ($P = 0.007$)	81 ± 13 ($P = 0.375$)	ND	38 ± 53	34 ± 12	ND	73 ± 15 ($P = 0.005$)	51 ± 22 ($P = 0.214$)

[a] Six-week-old mice were given 2×10^9 PFU of Ad.cre in 100 μl of PBS by tail vein injection. Control mice received 100 μl of saline. Blood samples were taken from the tail vein at the indicated days after infusion. Values presented are the means ± standard errors of at least four serum samples. The Student's *t* test was performed with unpaired data for the virus-infused and saline-infused samples. *P* values indicate the significance of viral treatment with respect to control treatment.

[b] Concentration units are as follows: AST and ALT, units per liter; glucose, cholesterol, and triglycerides, milligrams per deciliter; albumin, grams per deciliter.

[c] For the definitions of +/+ and *fl/fl*, see the legend for Fig. 6. Mice given Ad.cre are indicated by "+ Ad.cre"; the other mice received saline.

[d] ND, not determined.

FIG. 6. Change of serum unconjugated bilirubin level after adenovirus infusion. The values presented as vertical bars are the averages of four serum samples. The vertical line on each bar represents the standard error for each group. +/+, wild-type *c/ebp*α homozygotes; *fl/fl*, *c/ebp*$\alpha^{fl/fl}$ homozygotes. +Ad.cre, Ad.cre infusion; the other bars are the results for saline-infused mice.

27). These findings implicate several liver-enriched transcription factors as being involved in the regulation of PEPCK and IGF-I gene expression in liver. Indeed, synergism and cooperativity between transcription factors, either by direct interaction or elicited through DNA regulatory elements, have been well documented in recent years (13, 18, 19, 39, 44). Nevertheless, our conditional knockout mice established an essential role for C/EBPα in regulating the constitutive expression of PEPCK in hepatocytes of adult mice. These results suggest that in adult mice, other transcription factors are not capable of supporting PEPCK transcription after the loss of C/EBPα. On the other hand, we show that C/EBPα is not essential for IGF-I gene expression in adult liver. In this regard, HNF-1α was found to be primarily responsible for *trans*-activating IGF-I, as determined by using a new *hnf-1*$\alpha^{-/-}$ mouse line generated by the Cre/*loxP* recombination system (17a).

C/EBPα appears to be essential for GS expression in both the neonatal and adult stages. On the other hand, the C/EBPα deficiency does not affect the PEPCK mRNA level in liver of the germ line *c/ebp*$\alpha^{-/-}$ neonate (reference 40 and this study), while it appears to be essential for PEPCK expression in the adult mouse liver. These results suggest that transcriptional activation of the PEPCK gene might be differentially regulated during postnatal development as compared to its regulation in the adult stage. Another possibility is that deletion of the *c/ebp*α gene at the germ cell stage alters the regulation of PEPCK gene expression during development, resulting in more involvement of other transcription factors in *trans*-activating PEPCK expression. Consequently, the deficiency of C/EBPα has less effect on PEPCK expression.

The EIa-deficient adenovirus vector has been used as a gene delivery vehicle in many gene therapy studies (5, 14, 20, 21). Hepatic cell metabolism and integrity were shown not to be compromised after efficient adenovirus-mediated gene transfer (4). However, the effects caused by adenovirus infection itself, such as an enhanced immune response, have been documented (42, 43). With the 2×10^9-PFU dose of Ad.cre used in our system, we inferred a mild level of liver damage from the elevation of the AST and ALT activities and increased cell

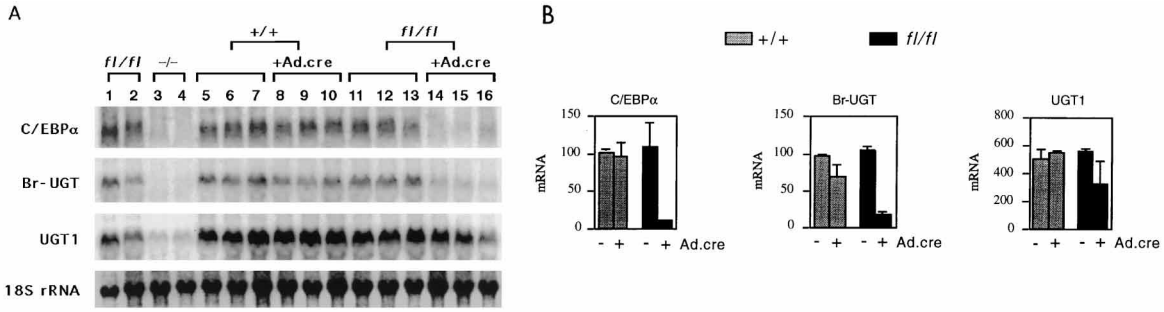

FIG. 7. The infusion of Ad.cre vector specifically reduces bilirubin UGT transcript levels in the *c/ebpα*$^{fl/fl}$ mice. (A) Northern blotting analysis of representative liver biopsies after Ad.cre infusion. Ten micrograms of total RNA from *c/ebpα*$^{fl/fl}$ neonates (lanes 1 and 2), the *c/ebpα*$^{-/-}$ neonates (lanes 3 and 4), and saline- (lanes 5–7 and 11–13) or Ad.cre-infused (lanes 8–10 and 14–16) adult mouse livers 10 days after infusion was denatured and electrophoresed on a formaldehyde-containing 1% agarose gel, blotted to nylon membranes, and probed with the indicated cDNA and oligonucleotide probes. Each lane presents RNA from an individual animal. (B) The signal of mRNA from each sample was quantified with a Molecular Dynamics PhosphorImager and normalized with its respective 18S rRNA signal. The value for each group is the average of the three different mRNA samples shown in panel A and is presented as a vertical bar in the figure. The standard deviation for each group is shown as a vertical line.

division, as indicated by elevated PCNA levels in the virus-infused mice. However, we also demonstrated that the liver damage and the viral effect on cell division for the C/EBPα-deleted mice were not different from those for wild-type mice receiving the same amount of Ad.cre. Thus, the deletion of the *c/ebpα* gene did not exacerbate liver cell damage and the increased cell division caused by the adenovirus, nor did the virally induced liver changes affect expression of the C/EBPα-dependent genes.

DNA recombination presumably could occur in most liver cells, if sufficiently large amounts of recombinant adenovirus particles (i.e., $\geq 10^{10}$ PFU) were infused intravenously (20, 33, 41). C/EBPα was ablated in 80 to 90% of hepatocytes with the low dose of Ad.cre infused in our studies; we chose to limit the amount of virus used out of concern that the effects of *c/ebpα* gene loss in liver might be masked by massive liver damage. By this approach, we established the crucial role of C/EBPα in bilirubin detoxification. Cre-mediated gene ablation of *c/ebpα* in these mice provides the opportunity to further explore the underlying regulatory mechanisms; such exploration cannot be achieved by conventional gene knockout technology or cell culture systems.

In conclusion, by using the Cre/*loxP* conditional gene knockout system, the role of C/EBPα in regulating liver-specific expression of bilirubin UGT, GS, and PEPCK, the key enzymes in bilirubin metabolism and gluconeogenesis, was established in an intact animal model. In addition, the potential use of Ad.cre in the conditional knockout of hepatic genes is demonstrated by this study. Conditional deletion of the *c/ebpα* gene in a tissue- and stage-specific manner can be achieved in *c/ebpα*$^{fl/fl}$ mice by using a variety of Cre recombinase delivery systems. This strategy will allow examination of the roles of *c/ebpα* in both tissue-specific and developmental gene regulation.

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