Increased Hepatic Cell Proliferation and Lung Abnormalities in Mice Deficient in CCAAT/Enhancer Binding Protein α*

(Received for publication, January 19, 1996, and in revised form, July 3, 1996)

Per Flodby, Carrolee Barlow, Helen Kylefjord, Lars Åhlund-Richter, and Kleanthis G. Xanthopoulos

From the §Karolinska Institute, Department of Biosciences at Novum S-141 57 Huddinge, Sweden and the $National Center for Human Genome Research, CGTB, National Institutes of Health, Bethesda, Maryland 20892-1852

CCAAT/enhancer binding protein α (C/EBPα) is a transcription factor that has been implicated in the regulation of cell-specific gene expression mainly in hepatocytes and adipocytes but also in several other terminally differentiated cells. It has been previously demonstrated that the C/EBPα protein is functionally indispensable, as inactivation of the C/EBPα gene by homologous recombination in mice results in the death of animals homozygous for the mutation shortly after birth (Wang, N., Finegold, M. J., Bradley, A., Ou, C. N., Abdelsayed, S. V., Wilde, M. D., Taylor, L. R., Wilson, D. R., and Darlington, G. J. (1995) Science 269, 1108–1112). Here we show that C/EBPα+−/− mice have defects in the control of hepatic growth and lung development. The liver architecture is disturbed, with acinar formation, in a pattern suggestive of either regenerating liver or pseudoglandular hepatocellular carcinoma. Pulmonary histology shows hyperproliferation of type II pneumocytes and disturbed alveolar architecture. At the molecular level, accumulation of glycogen and lipids in the liver and adipose tissue is impaired, and the mutant animals are severely hypoglycemic. Levels of c-myc and c-jun RNA are specifically induced by several fold in the livers of the C/EBPα−/− animals, indicating an active proliferative stage. Furthermore, immunohistologic detection with an antibody to proliferating cell nuclear antigen (PCNA) demonstrates that proliferation in vivo is restricted and differential expression pattern of the C/EBPα gene (22, 23).

Studies in 3T3-L1 adipoblasts have provided evidence for reciprocal expression patterns of C/EBPα and c-myc (24). It has been suggested that this is the result of negative regulation of the C/EBPα gene exerted by c-myc. Repression is mediated through interactions of c-myc with the initiator element of the C/EBPα promoter (25, 26).

There are an increasing number of genes known to be subjected to transcriptional regulation by C/EBPα. Several of the target genes are expressed in a cell-specific manner, e.g. albumin in hepatocytes (27, 28) and uncoupling protein in brown adipocytes (29). Furthermore, many target genes such as apo2 and SCD-1 (30, 31), GLUT-4 (32), PEPCK (33), aldolase B (34), and acetyl-CoA carboxylase (35) are involved in carbohydrate and lipid metabolism pathways. In addition, it has been demonstrated by two different sets of experiments that C/EBPα contributes to the maturation of hepatocytes and dimerization (5). Several members of this family have been cloned from different species, for a review see Refs. 6, 7, that are able to both homodimerize and heterodimerize with each other and to bind the same C/EBP consensus DNA sites. These properties are reflected in the high degree of homology of the bZIP domain (8). By contrast, the transactivation and attenuation domains are located in the N-terminal part (9–12), where a relatively low level of homology exists (8). Thus, the members of the C/EBP family are able to bind the same DNA sites but differ in their transacting and attenuating properties.

Expression of C/EBPα in rodents is restricted to certain tissues and cell types (8, 13, 14). High expression levels are detected in liver, white and brown adipose tissue, and placenta. C/EBPα is also expressed in the lung, mainly in differentiated type II cells (15), in the intestine where it is found in differentiated enterocytes (16), in the ovary during follicular development (17), and in myeloid cells (18). Furthermore, expression has been described in adrenal gland and skin (13), and at least in humans, also in pancreas and prostate (19). The basis for the restricted and differential expression pattern of the C/EBPα mRNA is regulation at the transcriptional level (14). The cellular mechanisms that control the expression of the C/EBPα gene have not been fully elucidated (20); however, the organization of the promoter has been studied in detail (21–23). The results from these studies suggest an autoregulatory mechanism that contributes to the control of the expression of this gene (22, 23).

CCAAT/enhancer binding protein α (C/EBPα)1 is the prototype member of the C/EBP family and belongs to the basic leucine zipper class (bZIP) of transcription factors (2–4). All members of the C/EBP family have a C-terminally located basic leucine zipper domain that is responsible for DNA binding and
the maintenance of the differentiation state. Results from studies on regenerating liver after partial hepatectomy clearly show a dramatic decrease of the expression of C/EBPα, of at least 5-fold, during the proliferative phase (41, 42). Recently, growth arrest by C/EBPα was demonstrated by co-transfection of a C/EBPα expression vector in a variety of cell lines (12). The multiplicity of interactions of C/EBPα with a variety of regulatory elements of genes suggests a central role for C/EBPα in cell proliferation and differentiation and in key metabolic pathways.

To ascertain the role of the C/EBPα protein in vivo, we have generated C/EBPα-deficient mice by homologous recombination in ES cells. Here we report that C/EBPα is critical for the proper development of both the liver and the lung since animals deficient in C/EBPα display gross abnormalities in these organs and die within 10 h after birth. In accordance to a previous report (1), we also show that C/EBPα is indispensable for postnatal maintenance of systemic energy homeostasis and lipid metabolism. Furthermore, in nullizygous livers c-fos, c-jun, and β-actin steady state RNA levels and PCNA/cyclin protein levels are increased suggesting an active proliferative state.

**EXPERIMENTAL PROCEDURES**

**Construction of the Targeting Vector**—A genomic clone including the C/EBPα gene was derived from an OLA 129-a GEM-12 genomic library by screening with a PstI/Sst1 100-bp fragment representing the bZIP region of C/EBPα as a probe. A 7.3-kb EcoRI fragment from the 12.6-kb genomic clone was subcloned into a PBS þ vector (Stratagene). The subcloned fragment contains sequences 3.4 kb upstream and 1.2 kb downstream of the C/EBPα gene. The 5’ EcoRI site in the fragment is derived from the λ GEM-12 linker, whereas the 3’ site represents a genomic EcoRI site. An 1133-bp Xhol/Hin3II fragment, containing the neoR gene driven by a TK promoter, was derived from the vector pMC1neo poly(A) (Stratagene), optimized for neoR expression in ES cells (43). This fragment was blunt-end ligated into the MluI site within C/EBPα in the opposite direction compared with the transcriptional direction of the C/EBPα gene. To enable negative selection against random integration, a 1854-bp Xhol/Hin3III fragment containing the HSV thymidine kinase gene driven by a polyoma virus enhancer (44) was inserted into the Hin3III/SauI site of linker, 5’ of the homologous genomic sequence.

**Electroporation, Selection, and Screening of ES Cells**—The targeting vector was linearized by PvuI digestion. 20 μg was used to electroporate 20 × 10⁶ R1 ES cells (45) with a Bio-Rad Gene Pulser at 260 V and 500 microfarads. Transfected R1 cells were then plated on gamma-irradiated G418-resistant mouse embryo fibroblast feeder cells. Around 48 h after transfection selective media containing 675 μg of G418/ml (50% active substance) was added. After another 24 h new media also containing 2 μg/ml Ganciclovir (Cymevene, Syntex Inc.) was added. New selective media (G418 + Ganciclovir) was added daily, and after 10 days surviving clones were transferred individually to round-bottomed 96-well plates, trypsinized, and then seeded onto two different flat-bottomed 96-well plates, one with feeder cells and one without feeder cells. The cells on the former plate were trypsinized prior to confluence, suspended in fetal calf serum, 10% dimethyl sulfoxide, and frozen in the plate, whereas the cells on the latter plate were allowed to reach confluence and then used for DNA extraction according to the microextraction method described by Ramirez-Solís et al. (46). DNA was then analyzed by Southern blot to screen for homologous recombination. In brief, microextracted genomic DNA was digested by 15 units/well of BamHI (high concentration, Life Technologies, Inc.) in a total volume of 50 μl in a digestion mix including 100 μg of bovine serum albumin/ml, 1 mM spermidine, and RNase A at 50 μg/ml. Digested DNA was separated on 0.6% agarose gels, transferred to Hybond N membranes (Amersham Corp.), and hybridized to a 1.3-kb EcoRI/BamHI fragment (E1.3B), representing the region 3’ of the genomic sequence in the targeting construct (Fig. 1D). The 1133-bp Xhol/Hin3II TK promoter-Neo-poly(A) fragment derived from pMC1neo poly(A), was used as a probe to verify that only one copy of the targeting construct had been inserted into the genome.

**Generation of C/EBPα −/− Mice**—R1 ES cells with one targeted C/EBPα allele were injected into C57BL/6 blastocysts and implanted into F1 (CBA × C57BL/6) foster mothers. Male chimeras were mated to C57BL/6 females to verify germ line transmission by coat color. Agouti offspring was screened for presence of the targeted C/EBPα allele by Southern blot on tail DNA, as reported to Laird et al. with the E1.3B probe described above. Heterozygous offspring was then intercrossed to obtain homozygous mice with both C/EBPα alleles targeted.

**Analysis of Serum Glucose and Lipid Levels**—Blood was obtained by decapitation and bleeding onto heparinized 35-mm cell culture plates. The blood was collected with a small rubber policeman, and 20 μl was added to 400 μl of 0.2% NaF, 0.9% NaCl solution. Blood was centrifuged, and the glucose concentration in the supernatant was measured in a Hitachi 917 spectrophotometer (Huddinge University Hospital) according to standard methods.

**Northern and Western Blot Analysis**—Total RNA was isolated from liver using the Ultraspec RNA isolation system (Biotex, Houston, TX), according to the manufacturer’s instructions. 20 μg of total RNA/well was fractionated on 1% agarose/formaldehyde gels and transferred to Hybond N filters. Prehybridization was performed at +42 °C in 50% formamide, 1.5 × SSPE, 10 × Denhardt’s, 1% SDS, 0.5 mg/ml fragmented and denatured salmon sperm DNA, 0.2 mg/ml tRNA. Hybridization was performed at +42 °C with same buffer composition as above with the exception that Denhardt’s was lowered to 5 × and dextran sulfate was added to 10%. Probe concentration in the hybridization was 2 × 10⁶ cm²/ml buffer. Densitometric analysis of the Northern blot signals was performed using a Molecular Dynamics instrument and software.

Liver nuclear lysates for Western blot analysis were prepared by adding an equal volume of 2 × polyacrylamide gel electrophoresis reducing sample buffer to purified nuclei pellets (48) and then carefully sonicated to shear DNA and heated at +100 °C for 3 min. After correction for differences in protein concentrations between the samples, 20 μg of the nuclear lysates were loaded on SDS-polyacrylamide gel electrophoresis minigels. Western blot analysis was then performed as described (42). Detection of total protein on the transfer membranes was used as a control of protein loading and was performed with the enhanced chemiluminescence protein biotinylation system (Amersham Corp.) according to the manufacturer’s recommendations.

**Morphological and Immunohistologic Analysis**—Tissues were fixed in 10% neutralized formalin or frozen in liquid nitrogen. Sections for analysis were prepared either with a cryostat or a microtome after paraffin embedding. Fixed and paraffin-embedded sections were stained with hematoxylin and eosin using standard protocols. Oil Red O staining of fat in liver and white and brown adipose tissue, periodic acid-Schiff staining of glycogen, and PCNA immunostaining (monoclonal antibody number 19A2, Innovex Biosciences) in liver were performed using standard protocols.

**RESULTS**

**Generation of C/EBPα −/− Mice**—To inactivate the C/EBPα gene, a mutation was generated by inserting a HSV-TK promoter-driven neoR poly(A)⁺ gene into the unique MluI site at position +701 in C/EBPα. Within the C/EBPα gene there are several in frame AUG translation start codons, but only two AUG codons (+130 and +491) are used in vivo, giving rise to two proteins, p42 and p30 (49, 50). Another AUG codon further downstream of the MluI site was shown to be used only when the other upstream AUG codons were deleted. By inserting the neoR gene into the MluI site in the opposite direction compared with the C/EBPα gene, we introduced stop codons in all three reading frames downstream of both AUG start sites. Thus, only truncated protein products, lacking the DNA binding and dimerizing domain (bZIP), are expected to be translated from the transcript of the targeted C/EBPα gene. Furthermore, since the nuclear localization signal resides in the bZIP domain, the produced truncated proteins will not be imported into the nucleus and will probably be rapidly degraded in the cytoplasm. Fig. 1A and B, shows the construction of the targeting vector, including a flanking tk gene to reduce the number of ES cell clones with randomly integrated vector (44). Correct insertion of the neoR gene was verified by sequencing of the NeoR/C/EBPα boundaries (data not shown). As shown in Fig. 1C and D, homologous recombination, targeting vector and the C/EBPα gene will result in the introduction of a new BamHI site within C/EBPα, resulting in a BamHI fragment of decreased size (5.7 kb) compared with the wild type.
Fig. 1. Strategy for disruption of the C/EBPα gene. A, restriction map of the 12.6-kb genomic fragment containing the C/EBPα gene. The 7.3-kb EcoRI fragment used for targeting vector construction and the 1.3-kb EcoRI/BamHI probe (E1.3B) are indicated. C/EBPα is an intronless gene. The restriction sites indicated are as follows: N, NruI; M, MluI; H, HindIII; E, EcoRI; Bg, BglII, and B, BamHI. B, targeting vector construction. The neoR gene was inserted into the MluI site in the opposite direction compared with the C/EBPα gene, and the tk gene was inserted 5′ of the genomic sequence. The neoR gene is driven by a HSV-TK promoter, whereas the tk gene is driven by a duplicated mutant polyoma virus enhancer (PYF441 Enh). C–D, homologous recombination between the targeting construct and the C/EBPα allele and the targeted allele resulting from homologous recombination. Small x in the mRNA represents stop codons introduced in the C/EBPα transcript by the insertion of the neoR gene. E, Southern blot analysis of transfected and selected R1 ES cell clones. The 10.5-kb wild type BamHI fragment from the nontargeted allele and the 5.7-kb fragment from the mutated allele are shown. F, Southern blot analysis of a litter from a heterozygote intercrossing. DNA was digested with BamHI and probed with E1.3B. The genotypes are indicated above the lanes. +/+, wild type; +/−, heterozygous; and −/−, nullizygous animals. G–H, Northern blot analysis of liver RNA from a litter resulting from a heterozygote intercrossing (same individuals as in F). Hybridization with a 400-bp probe of the C/EBPα bZIP region detects both the 2.8-kb wild type C/EBPα transcript and the 3.9-kb C/EBPα:Neo fusion transcript (G). A neoR probe detects the 1.1-kb Neo(+) mRNA, transcribed by the TK promoter and the fusion transcript in +/− and −/− animals (H).

10.5-kb fragment. A representative Southern blot analysis of microextracted DNA from transfected and double selected R1 ES cell clones is shown in Fig. 1E. In total, seven positive ES clones were obtained with a frequency of homologous recombination of 1:38. The positive clones were expanded and analyzed further with a neoR probe. To substantiate that the event of homologous recombination had been correct, EcoRI-digested DNA was probed with an Xhol/HindIII neoR fragment from pMC1Neo poly(A) that resulted in the expected hybridizing 3′ fragment of 3.25 kb and one 5′ fragment of 7.3 kb. In addition, BamHI digestion resulted in the expected 5.7-kb fragment, verifying that only one copy of the targeting vector was present in the genome (data not shown). Two positive clones (S12 and S18) were used for blastocyst injections and generation of chimeric males. Germ line transmitting chimeric animals were derived from both targeted ES clones and were used to establish two independent heterozygous lines. The same phenotypic effects were observed in −/− animals derived from both lines.

Heterozygous animals were interbred to obtain mice homozygous for the mutated alleles. Fig. 1G shows a Southern blot analysis, using the E1.3B probe, of a litter resulting from a representative heterozygote intercross. The outcome of the intercrossings shows that there is no significant negative selection against the mutation during embryogenesis since C/EBPα −/− animals were born approximately at the expected 1:3 Mendelian ratio. A small reduction in the numbers of −/− animals was observed, but this decrease was not statistically significant (27% +/+, 51.8% +/−, 21.2% −/−, n = 425; χ² test, p > 0.10).

Transcription of the Inactivated C/EBPα Locus Occurs in the Absence of the C/EBPα Protein—In addition to inactivation of the locus, our targeting strategy with insertion of the neoR gene in the opposite orientation within the C/EBPα gene was designed to test whether efficient and sustained transcription of the locus is possible without the positive autoregulation by C/EBPα protein. In such case a 3.9-kb transcript should be detected in C/EBPα −/− mice using either a C/EBPα or a neoR probe. The data presented in Fig. 1G, demonstrate that a neoR:C/EBPα fusion transcript of 3.9 kb appears as a result of transcription of the targeted C/EBPα allele, not only in the +/− mice but also in the −/− animals. As expected the fusion transcript is also detected by the neoR probe as shown in Fig. 1H, suggesting that C/EBPα gene transcription occurs in the absence of the C/EBPα protein. Perhaps other C/EBP family members are able to compensate for the loss of C/EBPα. This notion is supported by transient transfection experiments that
show transactivation of the C/EBPa promoter by C/EBPβ (22). Finally, the appearance of both bands in the heterozygous animals shows that both C/EBPa alleles are transcribed.

To demonstrate that the targeted event resulted in total elimination of the C/EBPa protein, liver nuclear lysates were analyzed by Western blot analysis that was performed on pups from several litters. The results from one such experiment are shown in Fig. 2A, verifying that the C/EBPa protein is completely absent in livers from nullizygous animals. Two other members of the family, C/EBPβ and C/EBPδ, were analyzed in parallel. As shown in Fig. 2, B and C, no overt differences could be demonstrated between the three genotypes.

Most C/EBPa Nullizygous Mice Die in the First 10 h after Birth, but Some Die at Birth—Littermates in most litters showed no obvious differences at birth. However, in about 20% of the litters, most of the C/EBPa −/− pups died virtually at delivery. The majority of these born almost dead (BAD) mice died immediately after birth, while a few were able to survive for a shorter period of approximately 30 min. Dissection of these animals showed the presence of bubbles in their stomachs. BAD mice are, in general, born in large litters (9.4 ± 1.6 pups, n = 10 litters). The reason why this phenomenon only occurs in about 20% of the C/EBPa −/− mice and only in large litters remains unclear.

As shown in Table I, there was a small but significant difference in body weight at birth between the pups. Normal and heterozygous pups had the same weight, whereas nullizygous pups weighed 10% less than their littermates. This weight difference is further augmented at 7–10 h where the +/+ and +/− pups have increased their body weight by 15–20%, while the C/EBPa −/− animals have not gained any weight at all. These nullizygous animals, although apparently normal at birth, gradually become weaker, and most of them were never able to start feeding. After 7–10 h severe symptoms of hypoglycemia, such as lethargy and shivering, were manifested, and these animals died soon after. Although some −/− pups clearly were able to start feeding, they did not survive more than 20 h. Dissection revealed that these mice had milk in their stomachs. Finally, in very few cases (less than 1%) nullizygous mice were able to survive for a considerably longer period of up to 4 weeks of age. These long term survivors are severely retarded in development. At around 2 weeks of age they are about half the size of their littermates. These animals are very thin and skin problems were observed with flaking from large areas of the body before fur outgrowth. This is a very rare but reproducible phenotype.

Since C/EBPa has been shown to regulate several genes involved in carbohydrate and lipid metabolism, the dramatic effects on the early postnatal survival in nullizygous mice could be due to low blood glucose levels, similar to the phenotype displayed by the C14Cos albino deletion mice (51). We therefore tested the blood serum levels of glucose in newborn litters and in litters at the time point when knockout animals usually die (7–10 h). The results from these tests, shown in Table I, clearly demonstrate that there is a dramatic drop in blood glucose levels some hours after birth in the −/− animals compared with their +/+ and +/− littermates. Low glucose levels between 0.1 and 0.2 mmol/l were detected within 1 h postpartum (data not shown), but the animals survive for an additional 6–10 h. This is consistent with the results of an earlier report (1). We have also investigated the serum lipid levels at birth and around 10 h postnatally. No statistically significant differences for either cholesterol or triglyceride levels were detected.

C/EBPa Nullizygous Mice Have Disturbed Liver Architecture, Immature Pulmonary Phenotype, and Fail to Accumulate Lipids—Histologic analysis of livers from heterozygous intercrosses, performed either at birth or at 7–10 h postpartum, revealed striking differences in liver, lung, and adipose tissues when −/− animals were compared with the other two genotypes. The hepatic architecture of the nullizygous mutants was severely distorted with acinar formation. The liver morphology also had a clear resemblance to regenerating liver following partial hepatectomy or pseudoglandular hepatocellular carcinoma. The number of biliary canaliculi in the C/EBPa −/− liver is considerably higher compared with both +/+ and +/− liver (Fig. 3, upper lane). Hepatocytes from nullizygous animals appear to have a smaller cytoplasm/nucleus ratio, which may explain the dilated bile canaliculi. However, no cholestasis or bile thrombi were observed. Liver sections of littermates using periodic acid-Schiff stained for glycogen at birth and 10 h postpartum showed that the normal and heterozygous animals contained substantial amounts of glycogen, whereas the −/− mice had drastically decreased but detectable levels (data not shown). It has been suggested that the distorted architecture may be due to the deficiency in fat and glycogen stores in the cytoplasm that results in smaller hepatocyte volumes (1). An alternative explanation may be that genes involved in the cytoskeleton formation in hepatocytes are targets of C/EBPa regulation resulting in an imperfect three-dimensional structure.

Lipid accumulation in both white and brown adipose tissue is dependent on C/EBPa (36, 37). To investigate the effects of C/EBPa deficiency in lipid storage, we performed histologic analysis of adipose tissue. In newborn mice there are detecta-
ble fat depots in white adipose tissue localized to the inguinal region and considerable amounts in the brown adipose tissue. Although cells mass and general histologic appearance of the brown adipose tissue was not altered, the fat depot was greatly reduced in the C/EBPα−/− mice, as seen by Oil Red O staining (data not shown). Thus, unlike the situation in the liver, C/EBPα deficiency does not affect the overall brown adipose tissue morphology but is rather specific for the accumulation of lipids.

All C/EBPα−/− mice displayed irregular pulmonary histopathology. Although location of the lungs of C/EBPα−/− mice was comparable with the normal littermates, embryonic, fetal, and neonatal development of the airways was abnormal (Fig. 3). In mutant mice the lungs showed hyperproliferation of type II pneumocytes and bronchiolization of the alveoli. The primitive-appearing lung resembles the appearance of the lungs of premature human infants. C/EBPα−/− mice, particularly of the BAD type, had clinical symptoms of a respiratory pathology. Although location of the lungs of C/EBPα−/− mice and results in reduction in the alveolar airspace (arrow, middle right panel) and interstitial thickening (arrow, lower right panel).

**Fig. 3.** Liver architecture and lung morphology are distorted in C/EBPα−/− animals. A representative series of histologic sections of liver and lung tissues stained with hematoxylin and eosin. Note the acinar formation in the livers of C/EBPα−/− mice. Pulmonary histology of newborn littermates indicates h. Hyperproliferation of type II pneumocytes in C/EBPα−/− mice and results in reduction in the alveolar airspace (arrow, middle right panel) and interstitial thickening (arrow, lower right panel).

| Wild Type +/+ | Heterozygous +/- | Nullizygous -/- |
|---------------|-----------------|-----------------|
| Liver (Hematoxylin and E osin, original magnification, x 50) | ![Liver Wild Type](image) | ![Liver Heterozygous](image) | ![Liver Nullizygous](image) |
| ![Liver Wild Type](image) | ![Liver Heterozygous](image) | ![Liver Nullizygous](image) |
| Lung (Hematoxylin and E osin, original magnification, x 50) | ![Lung Wild Type](image) | ![Lung Heterozygous](image) | ![Lung Nullizygous](image) |
| ![Lung Wild Type](image) | ![Lung Heterozygous](image) | ![Lung Nullizygous](image) |

**Induction of c-myc and c-jun in the Liver of C/EBPα−/− Mice.** The distorted liver architecture of the C/EBPα−/− mice is indicative of an active proliferative state. To investigate the molecular mechanisms that may underlie these differences, we analyzed the transcription rates and steady state levels of genes that may be important in maintenance of the balance between proliferation and differentiation in hepatocytes. We chose to test the expression levels of albumin and α-fetoprotein as markers for hepatocyte differentiation and tumor development, respectively. In addition, we tested β-actin, c-myc, and c-jun that correlate well with active cellular proliferation. Total liver RNA isolated at birth and several hours later was compared among littermates. A representative Northern analysis is shown in Fig. 4, and densitometric analysis of these experiments is shown in Table II. mRNA levels of albumin were reduced in C/EBPα−/− animals, especially in the livers of newborn animals. By contrast, α-fetoprotein expression levels were increased about 2-fold. This is indicative of a more differentiated state of the C/EBPα−/− hepatocytes. Levels of the β-actin RNA were induced by 3-fold suggesting a proliferative state. However, the most predominant change, in addition to changes in the expression of genes involved in glycogenesis that were demonstrated in an earlier report (1), was the induction of c-myc and c-jun RNA (Fig. 4 and Table II). Steady state c-jun RNA levels from livers of both newborn and 7-h-old C/EBPα−/− mice were increased by about 10-fold. Induction of c-myc RNA was pronounced in livers of 7-h-old −/− mice (Fig. 4 and Table II). These findings are consistent with the patterns of expression observed in the early regenerating mouse liver. In addition, early stages of experimentally induced hepatocellular...
carcinoma in rodents display a similar expression pattern for these genes (55).

Since C/EBPα and c-myc are reciprocally regulated in adipocytes (24) and hepatocytes (26), it is possible that c-myc induction is a direct effect of C/EBPα deficiency. However, a more likely explanation is that c-myc induction reflects the critical role of this molecule in mitogenesis and transformation (56) and, together with the c-jun induction, is indicative of the proliferative stage of the C/EBPα−/− mice.

Because proliferating hepatocytes and hepatocellular carcinoma cells have induced levels of proliferating cell nuclear antigen (PCNA/cyclin) (57), we performed immunohisto- staining with an antibody to PCNA/cyclin. The results presented in Fig. 5 illustrate a 5–10 times higher frequency of positively stained hepatocytes in C/EBPα−/− liver, further supporting the notion that an increased portion of the nullizygous hepatocytes are in the G1/S phase of the cell cycle. Thus, loss of C/EBPα has an effect on the proliferative potential of hepatocytes in vivo.

**DISCUSSION**

C/EBPα Is Essential for Postnatal Survival—We have inactivated the C/EBPα gene in mice by the introduction of stop codons downstream of the two AUG translation start sites used in the C/EBPα gene. These stop codons are situated within the neoR gene sequence that was inserted in the opposite direction to the C/EBPα transcription unit. Thus, this manipulation will not result in a gene inactivation at the transcriptional level but rather at the translational level. This targeting strategy has enabled us to obtain new information about the mechanisms of transcription of the C/EBPα gene. First, both alleles of the C/EBPα are actively transcribed since two transcripts of different sizes are detected in heterozygote animals. Thus, we conclude that the C/EBPα gene is not imprinted in mice. Second, the presence of a C/EBPα:NeoR fusion transcript in the nullizygous liver indicates that the previously suggested autoregulatory mechanism of the C/EBPα gene can be substituted by other C/EBP members. It is likely that C/EBPβ is responsible, since this C/EBP family member has been shown to transactivate C/EBPα promoter-reporter constructs in vitro (22) and is expressed at high levels in nullizygous liver (Fig. 2). The steady state level of the C/EBPα:NeoR fusion transcript is lower compared with the C/EBPα wild type transcript in heterozygote liver. This may be due to a less efficient transcription of the mutated allele or the fusion transcript is more unstable. Alternatively, C/EBPα may not be required for its own expression, but rather may augment transcription from its own promoter through interactions with other transcription factors, as suggested for the human gene (23). C/EBPα deficiency may result in lower levels of C/EBPα gene expression.

Mice nullizygous for C/EBPα are born to term. We have not found any significant deviation from the expected Mendelian ratio of nullizygous mice in the litters. This is different compared with a recent report (1). Perhaps this reflects the different genetic background of the back crossings of the two nullizygous mutants. Another remote possibility is that in the study of Wang et al. (1) C/EBPα gene inactivation was accomplished by the deletion of the entire C/EBPα coding region plus 2.4 kb of upstream sequences. This deletion could in turn have affected important regulatory elements associated with other genes important for embryogenesis, particularly since a strong DNAse I-hypersensitive site is located in this region (20).

**Disturbed Liver Architecture—**The effects of the C/EBPα gene inactivation are dramatic but are not manifested until after birth, when metabolic functions characteristic for the differentiated liver are initiated in the newborn animal. C/EBPα nullizygous mice begin to runt and die shortly after birth. About 20% of the mutant mice die consistently at birth or within 30 min postpartum while the rest survive only for a period of 7–10 h. All mice show gross liver histologic abnormalities and hypoglycemia and failure to accumulate lipids or fat. The drastically reduced amounts of stored glycogen and fat in liver and adipose tissues observed in newborn nullizygous mice are highly likely to be an important reason for the weakness of these animals and is probably the reason for the lower body weight at birth. Stored energy fuels, which are normally built up prior to birth in liver and adipose tissues, are very important glucose sources for the newborn animal before the sucking period. Furthermore, the ability to realize gluconeogenesis is necessary for maintaining energy homeostasis. Genes coding for PEPCK, glucose-6-phosphatase, and tyrosine aminotransferase, all highly expressed in liver, are crucial for gluconeogenesis. We have found that the transcription rate of the PEPCK gene, a known C/EBPα target gene, is reduced to approximately 30% in C/EBPα−/− liver (data not shown). Thus, inadequate gluconeogenesis is likely to be another reason for the rapidly appearing low blood glucose levels in C/EBPα−/− mice. This hypothesis is further substantiated by the fact that another gene involved in the reversible gluconeogenic/
glycolytic pathways, aldolase B, displays a transcription rate in C/EBPα−/− liver that is only about 30% of the rate found in +/+ and +/− liver (data not shown). Interestingly, aldolase B has recently been shown to be a target gene for C/EBPα (34). The maternal glucose in the blood of the newborn C/EBPα−/− animals is likely consumed very rapidly, since we detect very low blood glucose levels in these mice already 1-h postpartum. The apparent lack of energy makes the nullizygous mice so weak that they very often are unable to start suckling. Even individuals that are able to start some feeding do not generally survive for any longer periods. This might indicate metabolic dysfunctions other than storage of energy fuels and gluconeogenesis. It has been suggested that severe hypoglycemia is the primary reason for death (1). However, glucose injections cannot rescue the mutant mice for more than a couple of days (1). Problems with absorption of nutrients do not appear to be likely, because histology of the gut does not reveal any abnormalities (3), a view that is supported by a previous analysis (1).

Problems with absorption of nutrients do not appear to be likely, because histology of the gut does not reveal any abnormalities, a view that is supported by a previous analysis (1). Thus, the exact mechanisms underlying the death of C/EBPα−/− animals is not completely clear.

The fact that triacylglycerol stores are not found in either liver or in adipose tissue suggests that genes important for lipid accumulation, expressed in both these tissues, may be the targets of C/EBPα regulation. We envisaged that the identification of such target genes regulated by C/EBPα will reveal the mechanisms of action of this molecule in lipid storage.

The effects of C/EBPα gene inactivation are pleiotropic. As mentioned under “Results,” in very rare cases C/EBPα−/− animals are able to survive for longer periods of up to 4 weeks. These mice will be the subject for further analysis, enabling studies of the effects of the C/EBPα gene inactivation in later stages of mouse development. For instance, the brain would be a tissue of interest since C/EBPα expression has been shown to appear first a few weeks postpartum (14, 58) and the Aplysia C/EBP has been implicated in long term potentiation of neurons (59). In addition, it has been postulated that C/EBPα may play a role in keratinocyte development (13). The long term C/EBPα−/− survivors may provide some information on this aspect since the few individuals that survived that long appear to have skin problems.

Retarded Pulmonary Development—The C/EBPα nullizygous mice also differ from their littermates in that they exhibit retarded pulmonary development. In particular, hyperproliferation of type II pneumocytes is clearly visible in neonatal lungs. About 20% of the C/EBPα nullizygous mice die within 20–30 min after birth, apparently from respiratory failure. However, all nullizygous animals showed histologic evidence of delayed maturation of type II epithelial cells. This phenotype is analogous to the pulmonary effects of targeted disruption of a homeodomain gene, GSH-4 (60), TGF-β3, and GM-CSF nullizygous mice (52, 53). Recent evidence suggested a role for C/EBPα in the development and maintenance of the surfactant system in lung type II cells (15). Nonetheless, since both C/EBPβ and C/EBPδ are expressed in the lung, it is possible that these proteins compensate for the loss of C/EBPα in proper regulation of surfactant protein genes in lung epithelial cells. By contrast, deficiency of lipid production in alveoli, due to the absence of C/EBPα, may interfere with inadequate production of functional surfactant molecules. This could contribute to the respiratory problems associated with the nullizygous mice and may be the primary reason for the cause of the immediate death observed in C/EBPα−/− animals of the BAD type.

Induced Hepatic Proliferation in C/EBPα−/− Mice—Earlier experiments suggested that the C/EBPα gene product may be a component of the balance between proliferation and differentiation (24, 40–42). The loss of C/EBPα results in a dramatic induction of c-myc, c-jun, and β-actin RNA in the liver. These genes correlate well with active cellular proliferation. Histology of the liver shows that hepatocytes in the nullizygous mice appear healthy with smaller cytoplasm/nuclei ratio. The morphology of the −/− liver is indicative of either regenerating liver or pseudoglandular hepatocellular carcinoma. PCNA/cyclin immunostaining experiments that demonstrate excessive accumulation of PCNA/cyclin in C/EBPα−/− hepatocytes further support the notion that a substantial portion of the nullizygous hepatocytes are in the G1/S phase of the cell cycle. Taken together these data suggest a role for C/EBPα as “orthogene” necessary for acquisition and maintenance of the differentiated hepatocyte phenotype. However, heterozygous mutants do not show, so far, any evidence of hepatocellular

3 P. Flodby and K. G. Xanthopoulos, unpublished data.
carcinoma formation. It is clear that some of the activities of the C/EBPα gene are compensated for by other members of the C/EBP family (i.e. as is the case of activation of C/EBPα gene promoter). However, the severity of the C/EBPα knockout line will greatly facilitate our understanding of the function of this critical molecule in vivo.

Acknowledgments—We thank Irma Jansson for culturing and transfecting ES cells. We also thank José Inzunza for excellent technical assistance and contribution at the early stages of this work. Dr. Andreas Nagy is acknowledged for the R1 ES cell line. Drs. Mark Brantly, David Kleiner, Sylvia Fojo, and Melissa Rosenfeld have provided expert advice and critical input.

REFERENCES

1. Wang, N., Finegold, M. J., Bradley, A., Ou, C. N., Abdelayed, S. V., Wilde, M. D., Taylor, L. R., Wilson, D. R., andDarlington, G. J. (1995) Science 269, 1108–1112
2. Johnson, P. F., Landschulz, W. H., Graves, B. J., and McKnight, S. L. (1987) Nature 325, 133–146
3. Grayson, D. R., Costa, R. H., Xanthopoulos, K. G., and Darnell, J. E. (1988) Science 239, 786–788
4. Landschulz, W. H., Johnson, P. F., Adashi, E. Y., Graves, B. J., and McKnight, S. L. (1988) Science 240, 1759–1764
5. Johnson, P. F. (1990) Cell Growth Differ. 1, 47–51
6. Antonson, P., and Xanthopoulos, K. G. (1995) Cell 80, 4070–4079
7. Antoson, P., Pray, M. G., Jacobsson, A., and Xanthopoulos, K. G. (1995) Eur. J. Biochem. 232, 397–403
8. Friedmann, A. D., Landschulz, W. H., and McKnight, S. L. (1989) Genes Dev. 3, 1314–1322
9. Costa, R. H., Grayson, D. R., Xanthopoulos, K. G., and Darnell, J. E., Jr. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3840–3844
10. Ueyama, T., Manchado, C., Gadut-Doucet, A. M., Hambel, T., villaflores, O., Inglesias, R., Giralt, M., and Villarroya, F. (1994) Biochem. Biophys. Res. Commun. 196, 653–659
11. Nerlov, C., and Ziff, E. B. (1994)Nature 368, 85–87
12. Hendricks-Taylor, L. R., and Darlington, G. J. (1995) Mol. Cell. Biol. 15, 4295–4298
13. Berns, A. (1991) Nucleic Acids Res. 19, 4293
14. Hattori, M., Togore, A., Veloz, L., Karin, M., and Brenner, D. A. (1990) DNA Cell Biol. 9, 777–783
15. Li, H., Witte, D. P., Branford, W. W., Aronow, B. J., Weinstein, M., Kaur, S., Heisterkamp, N., and Groffen, J. (1995) Nat. Genetics 11, 415–421
16. D nan, G., Crawford, A. D., Sadelain, M., Ream, B., Rashid, A., Bronson, R. T., Dickerson, G. R., Bachurski, C. J., Mark, E. L., Whitsett, J. A., and Mulligan, R. C. (1994) Science 264, 713–716
17. Smith, C. I., Rosenberg, E., Reisher, S. R., Li, F., Kefalides, P., Fisher, A. B., and Feinstein, S. I. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8224–8228.