Intestinal maturation in mice lacking CCAAT/enhancer-binding protein α (C/EPBα)

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In rodents, there is a surge of intestinal expression of CCAAT/enhancer-binding protein α (C/EBPα) in the late fetal phase just before morphological maturation and the onset of expression of numerous epithelial genes. To investigate directly the hypothesis that C/EBPα plays a causal role in the latter phenomena, we have assessed both structural and functional maturation in neonatal intestine from C/EBPα-null mice and their littermates. No effects of C/EBPα genotype were observed on mucosal architecture or on the size of the proliferative zone in the intestinal crypts. Likewise, the mRNA levels for the glucose transporter 2 (GLUT2), intestinal and liver fatty acid-binding proteins, and apolipoprotein A-IV in newborn intestine were similar in all genotypes. Paradoxically, Na^+/glucose co-transporter (SGLT1), lactase phlorizin-hydrolase and apolipoprotein B mRNAs were more abundant in the C/EBPα-deficient animals. In wild-type intestines, C/EBPβ and C/EBPδ mRNAs were detectable throughout the late fetal period and increased toward term in parallel with C/EBPα mRNA. In newborn intestine, there was no compensatory up-regulation of these isoforms in the C/EBPα-deficient mice. We conclude that C/EBPα has no essential role in morphological maturation of the intestine, the pattern of proliferation of the epithelium, or the onset of expression of this cluster of epithelial mRNAs. However, since other C/EBP isoforms are present in the developing intestine, it is possible that there is a generic requirement for a member of the C/EBP family.

INTRODUCTION

CCAAT/enhancer binding proteins (C/EBPs) are a family of transcription factors that play critical roles in the differentiation of a number of tissues [1–4]. Although the relative importance of the isoforms varies from tissue to tissue, C/EBPα has consistently been found to be associated with the differentiated phenotype [1–4]. In adipose tissue, for example, C/EBPα is induced as adipocytes differentiate and, in turn, stimulates the transcription of a group of genes associated with the mature phenotype of this tissue [2,5,6]. Likewise, in liver, not only have C/EBP sites been found in many liver-specific genes, but in addition, the *in vivo* expression of C/EBPα is repressed in proliferating hepatocytes and induced during their differentiation [1,7,8]. A causal role for C/EBPα in cessation of proliferation in several cell types *in vitro* has been reported [9]. The *in vivo* significance of C/EBPα in the maturation of both liver and adipose tissue has been demonstrated dramatically by the recent generation of C/EBPα-null mice [10,11]. These mice are born with serious derangements of energy metabolism resulting from a lack of triacylglycerol storage in adipose tissue and glycogen storage in the liver. If left untreated, they become severely hypoglycaemic and die within 8 h of birth [10,11]. They can be maintained until postnatal day 2 by glucose injections but, despite suckling, rarely survive beyond 40 h [10].

Previous studies of C/EBPα expression in the intestine have pointed to the possibility that, as in adipose tissue and liver, this transcription factor may be associated with the process of differentiation. For example, in adult mice, C/EBPα is expressed in the differentiated cells of the villus epithelium and not in the proliferating cells found in the intestinal crypts [12]. Likewise, during ontogeny, the onset of C/EBPα expression in the intestine of both rats and mice occurs during the late fetal phase [7,13], just before morphological maturation and the onset of expression of several genes encoding proteins associated with functional maturation of the epithelium [13,14]. Examples of the latter include lactase-phlorizin hydrolase (LPH), intestinal and liver fatty acid-binding proteins (I-FABP and L-FABP respectively), apolipoprotein A-IV (apo A-IV) and apolipoprotein B (apoB) [14]. In addition to the temporal correlation of expression during intestinal maturation, the involvement of C/EBPα in the transcriptional control of these genes is supported by the presence of C/EBP-binding sites in the promoters of several of them, specifically LPH [15], apoB [16], I-FABP [17] and L-FABP [18]. Taken together, these previous findings have led to the hypothesis that the onset of C/EBPα expression plays a causal role in the differentiation of the intestinal epithelium during the late fetal period [13]. To date, however, there has been no definitive test of this hypothesis. Just as in classical endocrinology, to get beyond temporal correlations it is necessary to perturb the system in order to investigate causality. The availability of mice in which C/EBPα expression in the developing intestine is ablated [10] provides an ideal experimental paradigm for such investigation.

The goals of the current study were to use newborn mice lacking C/EBPα to investigate directly the *in vivo* role of this transcription factor in perinatal intestinal maturation. To this end we first assessed morphological maturation by histological comparison of C/EBPα-null mice with control littermates.

Abbreviations used: C/EBP, CCAAT/enhancer-binding protein; SGLT1, Na^+/glucose co-transporter; GLUT2, glucose transporter 2; I-FABP, intestinal fatty acid-binding protein; L-FABP, liver fatty acid-binding protein; apo A-IV, apolipoprotein A-IV; LPH, lactase-phlorizin hydrolase; apoB, apolipoprotein B; ANOVA, analysis of variance; EF-1α, elongation factor 1α.

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This comparison included quantitative assessment of the size of the proliferative zone in the intestinal crypts. Subsequently, we quantified the expression of mRNAs for seven genes associated with the functional maturation of the intestinal epithelium. These genes fell into two categories. The first was a group of genes encoding proteins concerned with digestion and absorption of the carbohydrate components of milk, namely LPH, the Na^+/glucose co-transporter 1 (SGLT1) and glucose transporter 2 (GLUT2). The second category comprised genes encoding proteins associated with lipid absorption, namely apo A-IV, apoB, I-FABP and L-FABP. Finally, we assessed the possibility that the expression of other C/EBP isoforms (namely β and δ) was up-regulated in the intestines of C/EBPα-null mice and examined the patterns of expression of C/EBPβ and C/EPBδ during late fetal development in wild-type mice.

MATERIALS AND METHODS

Animals and tissue collection

The C/EBPα-null mice were generated as described elsewhere [10]. To rescue the null offspring, all littermates born to the heterozygote dams received glucose injections within 8 h of birth, and were killed within 2.5 h of this injection. The entire small intestine (duodenum, jejunum and ileum) was removed and immediately placed in liquid nitrogen for RNA isolation or in 10% formalin for histological examination. Tails were used to prepare genomic DNA. Genotyping was performed by Southern blotting of *Hin*cII-digested DNA. As a result of the mutation strategy, the null allele yields a 9 kb *Hin*cII fragment whereas the wild-type allele yields a 6.5 kb fragment [10]. After genotyping, we chose three or four samples of each genotype (+/+, +/− and −/−) from which to prepare RNA. Two samples of each genotype were used for histology.

To study the intestinal expression of C/EBP isoforms during normal mouse development, we used C57B1/6J mice, *i.e.* the background strain of the C/EBPα-null mice [10]. After overnight breeding, dams showing a vaginal mucus plug were considered pregnant and the fetal litter was designated embryonic age E0.5. On days E15.5, E16.5, E17.5 and E18.5, dams were anaesthetized with isoflurane and fetuses were removed one at a time. Fetuses were then killed, and the entire small intestines were removed and placed in liquid nitrogen for RNA preparation. On days E15.5 and E16.5, intestines from entire litters were pooled, and on days E17.5 and E18.5 intestines from each litter were subdivided into two pools.

Histology

A portion of the midsection of the duodenum, jejunum and ileum was dissected from the formalin-fixed tissue and embedded in paraffin. Sections (4 μm) were stained with either haematoxylin and eosin, periodic acid/Schiff for goblet cells, or the Grimelius silver method for enteroendocrine cells. To identify actively dividing cells, other sections were subjected to immunohistochemistry using antibodies to Ki-67 (Novocastra Laboratories, Newcastle-upon-Tyne, U.K.). Detection was via a peroxidase-labelled secondary antibody, routine aminoethylcarbazole staining and haematoxylin counterstaining. To generate quantitative data, the number of crypt cells staining positive for Ki-67 were counted at 400× magnification independently by two observers who did not know the genotype of the sections they were examining. Representative crypts from each quadrant of each intestinal cross-section were selected and scored. The total number of crypts scored per section ranged from 9 to 29, the difference reflecting variation in the intensity of the stain. Preliminary studies indicated that weakly stained regions could not be reliably scored. The number of Ki-67-positive cells along one side of each scored crypt was recorded. The average number of positive cells was computed independently for each section and each observer. As there was no significant difference between the data sets from the two observers, only one set is presented.

RNA isolation and Northern-blot analysis

Intestinal RNA was prepared as described by Leeper and Henning [19]. Procedures for Northern blotting were also as previously described [19] except that formamide concentration during hybridization was increased to 40% for GLUT2 and SGLT1 and to 50% for all other probes. In addition, the temperature of the last two washes was increased to 65 °C. Blots were probed with ^{32}P-labelled cDNAs for rat GLUT2 [20], rat L-FABP [21], rat apoB [22], rat C/EBPα [23], mouse C/EBPβ [24] and mouse C/EBPδ [24], and with linearized plasmids from the cDNA clones for rat LPH [25], rat I-FABP [26], rat apo A-IV [27] and mouse elongation factor 1α (EF-1α) [28]. For SGLT1, since the available cDNA was from rabbit [29], the 1.3 kb *Bam*HI–*Sna*BI fragment was used as the probe to maximize the signal for mouse RNA. This fragment is from a region of the cDNA that has been shown to have very high interspecies homology [30]. After each probing, the blot was stripped with 0.1 × SSC containing 0.5% SDS (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) at 75–80 °C for 15–30 min. Hybridization signals were quantified by a Molecular Dynamics PhosphorImager, and the relative abundance of each mRNA was expressed as the ratio of its hybridization signal to that of the constitutive marker EF-1α on the respective blot. Approximate band sizing was performed by comparison with the 28S and 18S rRNA bands.

RESULTS

Morphological maturation of small intestine from C/EBPα-null mice and their littermates

Tissue sections from wild-type, heterozygote and null mice were examined for representation of all intestinal cell lineages. In the haematoxylin- and-eosin-stained sections, well-developed villi were seen in the duodenum, jejunum and ileum of mice of all genotypes. Enterocytes appeared to be morphologically similar in all animals, and there were no differences in villus height. The intervillus region displayed rudimentary crypts typical of those seen in other strains of neonatal mice [31,32]. Again, there were no apparent differences between the three genotypes. Paneth cells were not observed in any genotype. Periodic acid/Schiff staining was used to assess the presence of goblet cells. These were present in both villi and crypts of mice of all three genotypes. There was a definite longitudinal distribution, with higher numbers seen in the ileum than in the duodenum and jejunum, but there were no differences between C/EBPα-null mice and their littermate controls. Enteroendocrine cells visualized by the Grimelius stain were found to be equal in number and distribution in all genotypes.

In view of the role of C/EBPα in cessation of cell division in various other tissues [2,5,9,33], we hypothesized that the C/EBPα-null mice would display increased proliferation of the intestinal epithelium. As this would not necessarily have been detected in the regular histological examination described above, further studies were performed using the antigen Ki-67 to identify proliferating cells [34,35]. Typical staining patterns are shown in Figure 1. Examination of sections from C/EBPα-null mice and control mice showed no apparent effect of genotype on the size

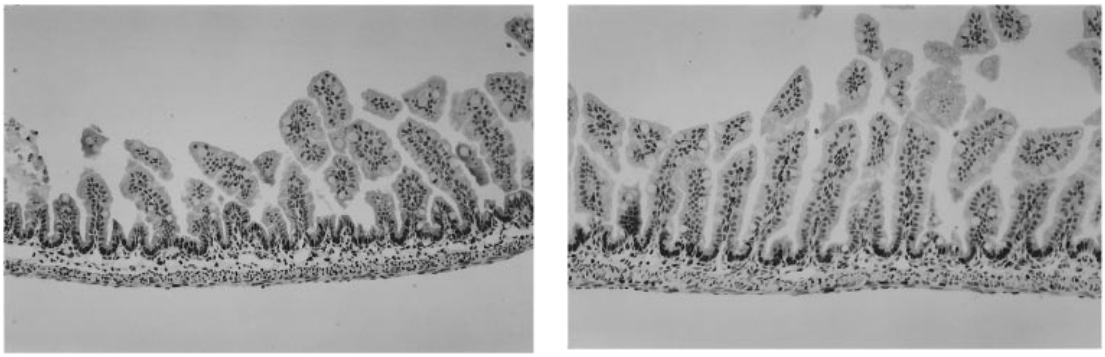

Figure 1 Identification of proliferating cells in ileum of newborn mice

Ileal cross-sections from a C/EBPα-null mouse (left) and a control littermate (right) were stained with antibodies to the proliferation marker Ki-67 as described in the Materials and methods section. Positive staining produces a reddish-brown nuclear precipitate which is readily distinguished against the blue counterstain on the original slides and is seen here as a black deposit. Magnification 200 ×.

Table 1 Assessment of epithelial proliferation in the duodenum, jejunum and ileum of newborn mice

The number of proliferating cells was identified by counting Ki-67-positive cells along one side of each crypt as described in the Materials and methods section. Data shown are the average cell counts obtained for individual animals from two different litters. Analysis of variance (ANOVA) using a general linear model with repeated measures showed no significant effect of either genotype or region.

	Number of proliferating cells			
	C/EBP-null mice		Littermate controls	
	1	2	1	2
Duodenum	5.2	5.2	5.6	5.9
Jejunum	5.4	4.8	4.7	5.4
Ileum	5.4	5.1	5.5	4.5

of the zone of proliferation in the duodenum, jejunum or ileum. This was confirmed by the quantitative analysis shown in Table 1. As can be seen, the average number of Ki-67-positive cells per half-crypt ranged from 4 to 6 in all regions and in all animals. This number agrees well with the study by Calvert and Pothier [32] in which [^{3}H]thymidine labelling of late fetal mouse intestine led to the conclusion that the proliferative component is confined to the first five cell positions within the primitive crypts.

Northern blots of selected intestinal mRNAs in mice of the three C/EBP*α* genotypes

The first panel of Figure 2 shows the mRNA for C/EBPα and in essence confirms the genotypes. As expected from previous analyses in liver [10], the heterozygotes displayed reduced levels of C/EBPα mRNA in their intestines as compared with the wild-type animals. No C/EBPα mRNA was detected in the −/− intestines.

Panels 2–4 of Figure 2 shows mRNAs for the proteins concerned with lactose digestion and absorption. The LPH probe detected a single mRNA in intestines from all animals. Levels of LPH mRNA were, if anything, lower in the wild-type

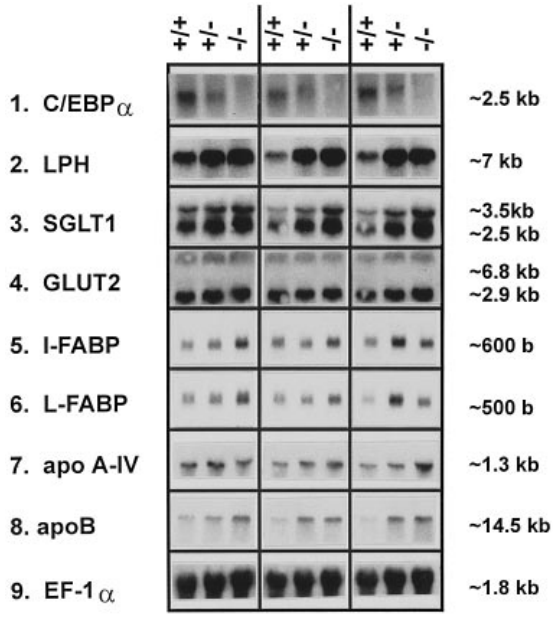

Figure 2 Northern blots of various intestinal mRNAs from newborn C/EBPα-deficient mice and their littermates

Each lane represents 20 μg of total RNA from the small intestine of an individual mouse. Samples were run in three groups with each group consisting of the three genotypes, wild-type (+/+), heterozygote (+/−) and null for C/EBPα (−/−). Blots were sequentially hybridized with various probes as described in the Materials and methods section. Approximate sizes of the mRNAs are shown on the right.

animals than in the heterozygotes and the C/EBPα-null animals. For both SGLT1 and GLUT2, two transcripts were detected in all animals, with sizes as indicated (Figure 2). These mRNAs displayed a similar pattern to LPH, with wild-type animals seeming to have weaker signals than the other two genotypes.

Panels 5–8 of Figure 2 show mRNAs for a group of proteins involved in lipid absorption, which have been previously reported

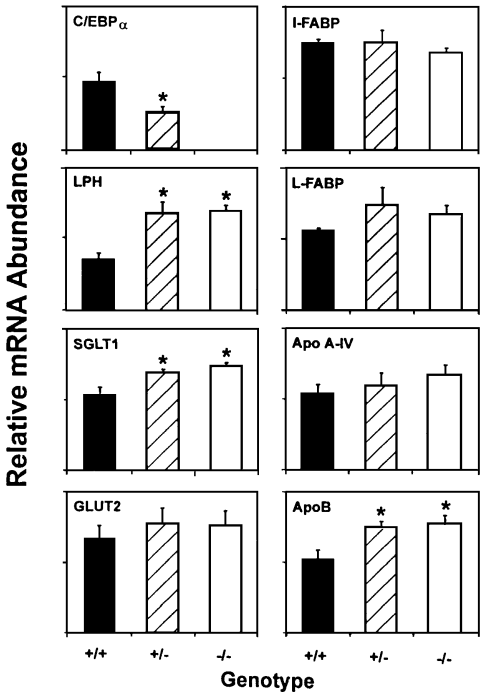

Figure 3 Relative abundance of eight intestinal mRNAs from the three C/EBPα genotypes

Data generated by phosphorimaging of Northern blots shown in Figure 2 are expressed as a ratio of the hybridization signal for the mRNA of interest to that of the constitutive marker EF-1α. Because the phosphorimager yields arbitrary units, the scale on each ordinate is an arbitrary one. Bars show means ± S.E.M. for three or four mice of each genotype: ■, +/+; ▨, +/−; □, −/−. The effect of genotype on the relative abundance of each mRNA was assessed by a one-way ANOVA. When significance was found by the ANOVA ($P < 0.05$), *post-hoc* Fisher LSD tests were performed to compare both the null (−/−) animals and the heterozygotes (+/−) with the wild-types (+/+). Bars marked with an asterisk (*) denote null or heterozygote groups with relative mRNA abundance significantly different from those of the wild-type group ($P < 0.05$).

to increase prenatally in rodents: I-FABP, L-FABP, apo A-IV and apoB. All of these probes detected single transcripts with sizes as expected from the literature. The mRNAs for I-FABP, L-FABP and apo A-IV showed no systematic variation with genotype, whereas for apoB mRNA there was a trend towards lower levels in the wild-type animals. The last panel of Figure 2 shows the same blot probed with EF-1α, a housekeeping gene that has previously been used as a constitutive marker in the intestine [28].

The Northern blots shown in Figure 2 were quantified by phosphorimaging. To correct for possible loading inequalities and for differences in the total mRNA pool as compared with the rRNA pool, the signal for each mRNA of interest was expressed as a ratio to that for EF-1α in the same sample. The results are shown in Figure 3. The pattern for intestinal C/EBPα mRNA is very similar to that previously reported for liver [10], with levels in heterozygotes being significantly reduced compared with those in wild-type animals. Despite the total absence of C/EBPα mRNA in the −/− animals, none of the seven other mRNAs examined showed reduced abundance in these intestines. On the contrary, for LPH, SGLT1 and apoB, the mRNA abundance was significantly higher in the null animals than in the wild-types. For each of these mRNAs, the abundance in heterozygotes was also significantly higher than in wild-type animals. For all other mRNAs studied there was no significant effect of genotype on relative abundance.

Relative abundance of C/EBPβ and C/EBPδ mRNAs in small intestine of C/EBPα-null mice and their littermates

In view of the absence of detectable phenotype in intestines of animals completely lacking C/EBPα, it was of interest to assess whether there was a compensatory up-regulation of other C/EBP isoforms. To this end, the set of mRNAs used in Figures 2 and 3 were probed for C/EBPβ and C/EBPδ mRNA. As can be seen from the Northern blot in Figure 4(A), both mRNAs were detectable in mice of all three genotypes. The abundance of C/EBPβ mRNA appeared to be relatively constant in all animals studied. In contrast, C/EBPδ mRNA showed large animal-to-animal variations in both the +/+ and +/− animals. As a consequence, there was a trend toward increased expression in the null animals in two of the groups but not in the third. The quantitative data are shown in Figure 4(B). Statistical analysis showed that there was no significant effect of genotype on the relative abundance of either C/EBPβ or C/EBPδ mRNA.

Developmental patterns of C/EBP isoforms in small intestine of wild-type mice

Although C/EBPα-null mice displayed no consistent compensatory increase in the expression of the β and δ mRNAs, the fact that these other isoforms were clearly present in the newborn intestine raises the possibility that they may substitute for C/EBPα in regulating intestinal gene expression in the late fetal period. To assess this possibility further, the normal developmental patterns of expression of C/EBPβ and C/EBPδ were studied by Northern blotting of RNA from intestines of wild-type fetal mice collected during the last 4 days of gestation. For the purpose of comparison, the blots were probed with C/EBPα also. The Northern blots (Figure 5A) show that the mRNAs for all three isoforms were detectable at the earliest age studied (E15.5) and all increased toward term. The quantitative data derived from the blots are shown in Figure 5(B). The relative abundance of each isoform was found to increase significantly with gestational age: $P = 0.016$, $P = 0.008$ and $P = 0.004$ for α, β and δ respectively). The pattern for C/EBPα is consistent with the original report in mouse intestine [7]. Although the literature contains no information on expression of C/EBPβ and C/EPBδ mRNAs in fetal mouse intestine, the developmental patterns shown in Figure 5 are qualitatively similar to those recently reported in fetal rat intestine [13]. The latter study showed only single samples on Northern blots with no quantification.

DISCUSSION

In this study we have used mice with a deletion of the gene for C/EBPα in order to assess directly the role of this transcription factor in the structural and functional maturation of the small intestine. These mice display normal rates of fetal growth and are physically indistinguishable from their wild-type and heterozygote littermates at birth. However, if left untreated, the C/EBPα-null mice rapidly become lethargic and die within 8 h. Their most striking phenotype is the failure to accumulate both glycogen in the liver and lipid in the adipose tissue. The lack of hepatic glycogen has been ascribed to an ablation of the prenatal rise of the mRNA for glycogen synthase, whereas the basis for the lack of triacylglycerol in adipose tissue has not yet been

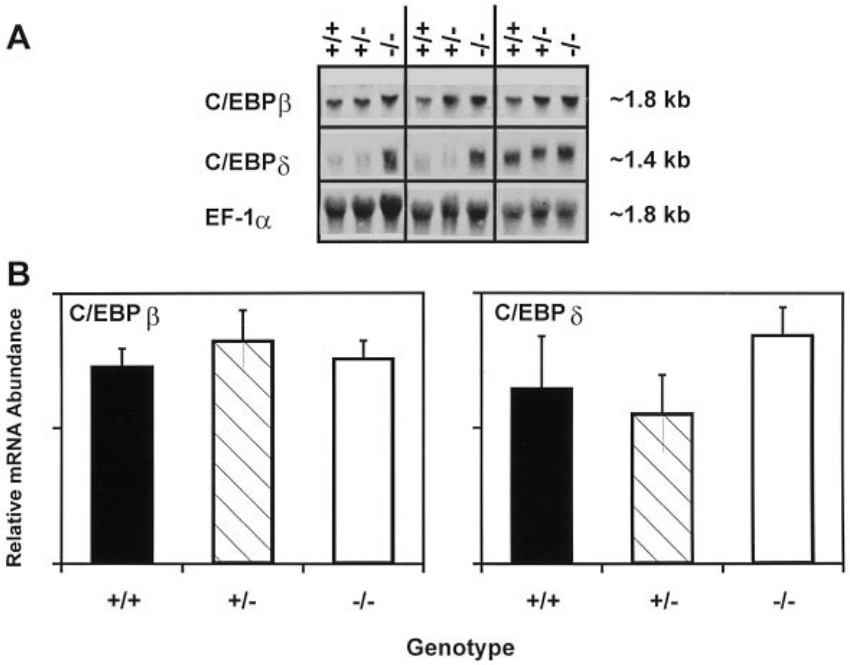

Figure 4 Expression of C/EBPβ and C/EBPδ mRNAs in intestines of newborn mice from the three C/EBPα genotypes

(**A**) Northern blots with 20 μg of total RNA per lane (same samples as in Figure 2) were probed for C/EBPβ, C/EBPδ and EF-1α. Approximate sizes are shown on the right. (**B**) Relative abundance of C/EBPβ and C/EBPδ mRNAs expressed as in Figure 3. Bars show means $\pm$ S.E.M. for three mice of each genotype: ■, +/+; ▨, +/−; □, −/−. One-way ANOVA of these data showed no significant effect of genotype on either C/EBPβ mRNA ($P > 0.74$) or C/EBPδ mRNA ($P > 0.45$).

determined [1,10,11]. These findings in the liver and adipose tissue constitute the first proof of an *in vivo* role for C/EBPα in biochemical maturation. In the same manner, in the current studies these mice have allowed a definitive test of the hypothesis [13] that C/EBPα plays a causal role in both morphogenesis and differentiation of the intestinal epithelium.

Our assessment of the structural development of the small intestine showed no differences between C/EBPα-null mice and their wild-type and heterozygote littermates. As expected for neonatal mice, the villi were well developed and the crypts somewhat less so [14,32]. All three villus cell lineages (enterocytes, goblet cells and enteroendocrine cells) were present and displayed normal morphology and frequency. In the crypts, neither the C/EBPα-null mice nor their heterozygote and wild-type littermates displayed eosinophilic staining typical of Paneth cells. This is in agreement with the report by Bry et al. [31] that this lineage matures postnatally in the mouse, with staining for specific markers appearing around days 7–10. Preservation of intestinal structure in the C/EBPα-null mice is consistent with the findings in liver and adipose tissue that indicate that tissues develop normally and acquire dysmorphologies secondarily to the lack of glycogen granules and lipid droplets respectively (G. J. Darlington, unpublished work).

Although normal overall morphology in C/EBPα-null animals was predictable, the lack of an effect of genotype on proliferation of the intestinal epithelium was unexpected. *In vitro* studies with a variety of cell lines [2,5,9,36] including a rat intestinal epithelial cell line [9] have demonstrated that elevation of expression of C/EBPα is associated with growth arrest. Conversely, studies in transgenic mice have shown that, if villus enterocytes are engineered to re-enter the cell cycle, their expression of C/EBPα is silenced [12]. Moreover, previous *in vivo* studies with C/EBPα-deficient mice have shown evidence of hyperproliferation in both liver and lungs [11]. In contrast, the quantitative data shown in Table 1 indicate that intestines of the C/EBPα-deficient mice have no expansion of the proliferative compartment. Thus it appears that, in the intact intestine, in contrast with other tissues, C/EBPα has no essential role in cessation of cell division. These findings reiterate the need for caution in the extrapolation of regulatory mechanisms from one tissue to another as well as from the *in vitro* setting to the intact animal.

To assess the effect of C/EBPα deficiency on functional differentiation of the intestinal epithelium, we first studied mRNAs that encode proteins involved in digestion and absorption of milk carbohydrate. LPH is the enzyme responsible for luminal hydrolysis of lactose; SGLT1 actively transports both glucose and galactose monosaccharides into the cell [37]; and GLUT2 allows facilitated diffusion of glucose from the basolateral side of the epithelium into the circulatory system [38]. Of these three genes, LPH is known to have prenatal onset of expression as well as C/EBPα sites in its promoter [15,39]. For SGLT1 and GLUT2 there are no published mRNA data for the prenatal intestine; however, both are present at birth in rats [40], consistent with the fact that carbohydrate absorption is fully functional at this time [14,41]. The promoter for SGLT1 has been found to have five putative C/EBP sites (M. G. Martín and E. M. Wright, personal communication) and that for GLUT2 has two such sites [42]. Nevertheless, as can be seen in Figures 2 and 3, mRNA levels for these three genes were not reduced in the small intestine of neonatal C/EBPα-null mice as compared with their heterozygote and wild-type littermates. In the case of LPH we also assessed the expression of the protein by assaying for lactase activity (results not shown). There was no significant difference among the three genotypes. Although functional assays have not been performed for SGLT1 and GLUT2, the presence of normal levels of their mRNAs suggests that all components of

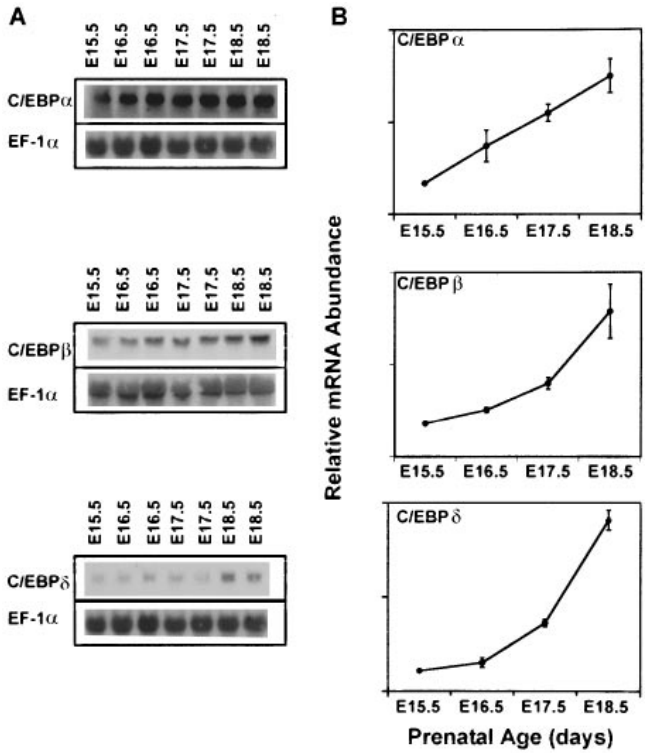

Figure 5 Levels of C/EBPα, C/EBPβ and C/EBPδ mRNA in intestines of wild-type mice during late fetal development

(**A**) RNA was prepared from pooled intestines of mice during the last 4 days of gestation as described in the Materials and methods section. Northern blots with 10 μg of total RNA per lane were hybridized with probes for C/EBPα, C/EBPβ, C/EBPδ and the constitutive marker EF-1α. (**B**) Relative abundance of C/EBPα, C/EPBβ and C/EPBδ mRNA was expressed as in Figure 3 and plotted against gestational age. Values are given as means $\pm$ range for two pools of RNA from four to eight fetuses at E16.5–E18.5 and for a single pool at E15.5. Lack of error bar indicates that the range was smaller than the symbol. The statistical significance of the effect of age on relative abundance of each mRNA was assessed by regression analysis.

lactase digestion and absorption are intact in the C/EBPα-null mice.

Four mRNAs encoding proteins associated with lipid absorption were studied: I-FABP, L-FABP, apo A-IV and apoB. Of these, the two FABPs are believed to play a role in the transport of fatty acids across the enterocyte, whereas the two apolipoproteins are involved with export and circulation [14]. All four mRNAs are known to have prenatal onset of expression in both rats and mice [14]. Moreover, three of the four (namely I-FABP, L-FABP and apoB) have been reported to have C/EBP sites in their promoters [16–18]. In the case of I-FABP, the putative C/EBP site identified by sequence analysis has been confirmed by DNase footprint analysis with recombinant C/EBPα [17]. A functional role for this site in I-FABP has also been indicated by the fact that its deletion (in transgenic animals) results in more than a sixfold reduction in the expression of the reporter gene [17]. Despite these correlations and predictions, neither I-FABP mRNA nor those for L-FABP, apo A-IV and apoB were reduced in the C/EBPα-deficient mice. The expression of this group of mRNAs in the mutant mice is consistent with the original histological observations that reported lipid globules in both enterocytes and lacteals [10]. Quantitative assessment of functional lipid absorption remains a challenge with these tiny animals.

For the seven genes studied (LPH, SGLT1, GLUT2, I-FABP, L-FABP, apo A–IV and apoB), our findings indicate that C/EBPα is not essential for their developmental onset of expression, despite the presence of putative C/EBP sites in six of their promoters and a coincident prenatal surge of C/EBPα expression in the intestine. This situation is reminiscent of several mRNAs in adipose tissue (namely stearoyl-CoA desaturase, 422/aP2 and GLUT4) which are unaffected in C/EBPα-deficient mice and yet known to have C/EBP response elements [1]. Since other isoforms of C/EBP recognize the same DNA-binding sequence [24], one possible explanation for these apparent anomalies is that there is functional redundancy among various C/EBP isoforms in the developmental regulation of these genes in intestine and adipose tissue. Although we found no compensatory up-regulation of C/EBPβ and C/EBPδ mRNAs in the intestines of C/EBPα-deficient mice, both were readily detectable and thus may have been present in sufficient amounts to provide adequate levels of the respective protein isoforms. As there were no published data on the patterns of expression of C/EBPβ or C/EBPδ during development of the mouse intestine, we assessed them in wild-type animals. Both mRNAs were detectable from day E15.5 onward and displayed increased relative abundance toward term in parallel with C/EBPα mRNA. Thus it would appear that either C/EBPβ or C/EBPδ could substitute for C/EBPα in regulating the late fetal onset of expression of various genes in the intestine. It can be argued that duplication of control mechanisms such as this may have evolved to ensure the timely expression of critical genes under severe selective pressure [43]. An alternative explanation for the lack of effect of C/EBPα deficiency on the expression of LPH, SGLT1, GLUT2, I-FABP, L-FABP and apoB is that the putative C/EBP sites in their promoters are not in fact functional sites. If this is the case, the role of the prenatal surge of expression of all three C/EBP isoforms remains to be determined.

For three of the mRNAs examined (namely LPH, SGLT1 and apoB), there was actually a significant increase in relative abundance in both the C/EBPα-null animals and the heterozygotes as compared with wild-type littermates. This suggests that there may have been a compensatory up-regulation of other transcription factors. Alternatively, C/EBPα may normally exert a negative effect on transcription of LPH, SGLT1 and apoB, and thus either the complete absence of C/EBPα (as in the null animals) or reduced levels (as in the heterozygotes) leads to increased expression of these three genes. Although C/EBPα is most commonly viewed as a transcriptional activator, there is precedent for its having repressive effects [44].

Overall, the studies in this paper show that lack of C/EBPα has no obvious effects on either the structural development or the size of the proliferative compartment of the neonatal intestinal epithelium, nor does such lack interfere with the late fetal phase of functional differentiation of the intestinal epithelium. Thus, although the temporal correlation of the onset of C/EBPα expression with this critical period of morphogenesis and biochemical maturation of the intestine made causality an attractive hypothesis [13], direct investigation has shown no essential role for C/EBPα in these phenomena. Given the widespread tendency to build models based on spatial and temporal patterns of expression of transcription factors such as C/EBPα, these findings in the developing intestine illustrate the importance of *in vivo* studies in which causality is investigated directly by perturbation of the normal pattern of expression of the factor in question.

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