

# Erythroid overexpression of C/EBP $\gamma$ in transgenic mice affects $\gamma$ -globin expression and fetal liver erythropoiesis

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**The CCAAT boxes of the  $\beta$ -like globin genes interact with three proteins: NF-Y, GATA-1 and NFE-6. We demonstrate that NFE-6 contains C/EBP $\gamma$ , and address its role in globin gene regulation by erythroid overexpression of C/EBP $\gamma$ , and a dominant-negative form C/EBP $\gamma\Delta B$ , in mice. Elevated levels of C/EBP $\gamma$ , but not C/EBP $\gamma\Delta B$ , increase expression of the (fetal)  $\gamma$ -globin relative to the (adult)  $\beta$ -globin gene. Interestingly, fetal liver erythropoiesis is ablated when the C/EBP $\gamma$  and C/EBP $\gamma\Delta B$  levels are further increased in homozygous transgenics. We suggest that targeted expression of dominant-negative leucine zipper proteins is a generally applicable approach to ablate specific tissues in mice.**

**Keywords:** C/EBP $\gamma$ /dominant-negative proteins/erythropoiesis/globin gene switching/locus control region

## Introduction

Many mRNA coding genes contain functionally important CCAAT boxes in their promoter (Mantovani, 1998). Our interest in this sequence motif stems from the study of the human  $\beta$ -globin gene cluster. CCAAT consensus sequences are present in the minimal promoters of the human  $\beta$ -like globin genes (deBoer *et al.*, 1988; Liberati *et al.*, 1998) between a CACC box and a TATA box motif, all of which are necessary for efficient transcription (deBoer *et al.*, 1988; Antoniou *et al.*, 1995). The relevance of the TATA box and the CACC box has been deduced from patients with mutations in these motifs (Thein, 1993). In addition, genetic ablation studies in mice have shown that EKLf, a factor binding to the CACC box, is absolutely required for  $\beta$ -globin expression (Wijgerde *et al.*, 1996).

The  $\beta$ -globin CCAAT box binds three major complexes in nuclear extracts from murine erythroleukaemia (MEL) cells, a tissue-culture model for adult erythropoiesis. These complexes have been designated NF-Y (previously called CP1), GATA-1 and NFE-6 (Berry *et al.*, 1992). NFE-6 displays a similar mobility on the  $\beta$ -CCAAT box to a factor denoted DSFr (Delvoye *et al.*, 1993), which was shown through expression cloning and antibody studies to contain C/EBP $\gamma$  (Wall *et al.*, 1996). C/EBP $\gamma$  is a member

of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors, a class of bZIP proteins characterized by a basic domain followed by a leucine zipper. The leucine zipper serves as a dimerization motif that is necessary for DNA binding by the basic domain. Each member can form homodimers as well as heterodimers with C/EBP family members. In addition, they can form dimers with several other leucine zipper proteins such as jun and fos, raising the possibility that C/EBP proteins exert their biological function by switching partners during differentiation. The C/EBP family consists of six members, designated C/EBP $\alpha$  to C/EBP $\zeta$ . The genes encoding C/EBP $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\zeta$  have been disrupted by homologous recombination, thus elucidating some of the functions of their gene products. C/EBP $\alpha$  is necessary for the establishment and maintenance of energy homeostasis in neonates (Wang *et al.*, 1995). C/EBP $\zeta$  is involved in the induction of apoptosis under conditions associated with impaired function of the endoplasmic reticulum (Zinszner *et al.*, 1998). C/EBP $\alpha$  and C/EBP $\delta$  have a synergistic role in terminal adipocyte differentiation (Tanaka *et al.*, 1997). C/EBP $\beta$  has a critical role in ovarian follicle development (Pall *et al.*, 1997; Sterneck *et al.*, 1997) and in the immune system (Screpanti *et al.*, 1995; Tanaka *et al.*, 1997). C/EBP $\gamma$  null mice show a high mortality rate within 48 h after birth. The analysis of lymphoid cells of these mice has demonstrated a critical role for C/EBP $\gamma$  in the functional maturation of NK cells (Kaisho *et al.*, 1999).

C/EBP proteins are expressed in the fetal liver at the onset of definitive haematopoiesis. Therefore, these proteins are likely to have a role in the differentiation and lineage commitment of the developing haematopoietic system (Scott *et al.*, 1992; Zhang *et al.*, 1997; Nerlov *et al.*, 1998).

In this study, we have explored the relationship between NFE-6 and C/EBP $\gamma$ , and we have addressed the role of bZIP proteins in globin gene expression and erythropoiesis. C/EBP $\gamma$  was first cloned from B cells as a factor that binds to the Ig heavy chain promoter and enhancer. Unlike other C/EBP proteins, C/EBP $\gamma$  is ubiquitously expressed (Roman *et al.*, 1990). The cDNA predicts a small polypeptide of 16.4 kDa lacking the consensus N-terminus activation domain experimentally defined in C/EBP $\alpha$  (Friedman *et al.*, 1989) and C/EBP $\beta$ . Thus, C/EBP $\gamma$  might function as LIP, a truncated version of C/EBP $\beta$  that operates as a modulator of a series of activators (Descombes and Schibler, 1991; Cooper *et al.*, 1995).

The CCAAT boxes of the  $\beta$ -like globin genes all conform to the C/EBP consensus, which is dyad symmetric, consisting of abutted half-sites bearing the CCAAT sequence (Osada *et al.*, 1996). A role for the CCAAT boxes in globin gene expression is best demonstrated by mutations in the duplicated CCAAT box of the A $\gamma$ -globin (Berry *et al.*, 1992; Ronchi *et al.*, 1996). Such mutations

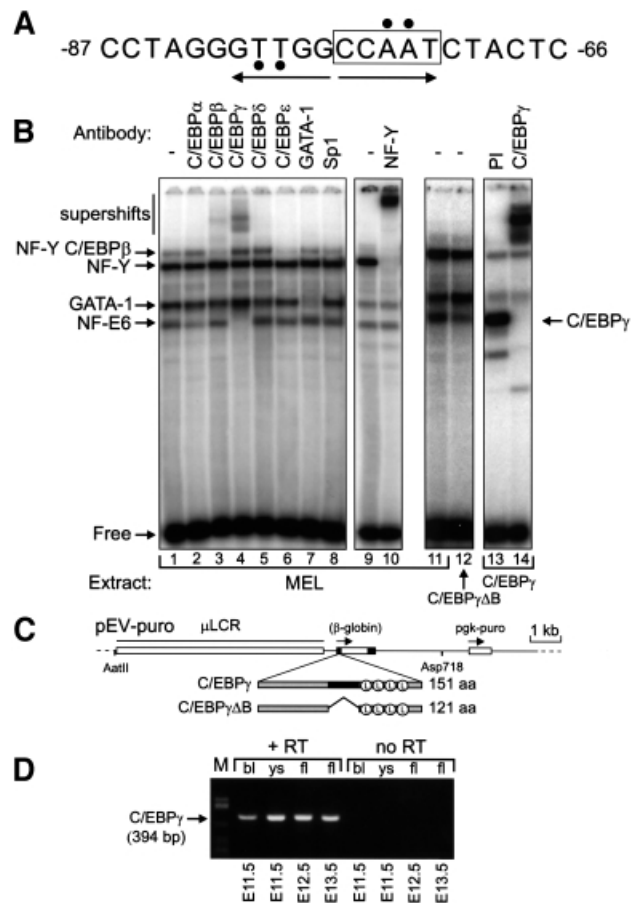
can result in the extemporary expression of fetal  $\gamma$ -globin in adult life, a condition known as hereditary persistence of fetal haemoglobin (HPFH) and hence hint that factors binding to the CCAAT boxes have a role in  $\gamma$ -globin silencing. To investigate the potential function of C/EBP $\gamma$  in globin gene expression and erythroid differentiation, we have generated transgenic mice expressing C/EBP $\gamma$  under the control of  $\beta$ -globin regulatory sequences. We also produced mice expressing a dominant-negative version of C/EBP $\gamma$  that lacks the DNA binding domain but retains the leucine zipper (C/EBP $\gamma$  $\Delta$ B). Such a protein interferes with DNA binding and activity of bZIP transcription factors (e.g. Moitra *et al.*, 1998). We found that moderate overexpression of C/EBP $\gamma$ , but not C/EBP $\gamma$  $\Delta$ B, results in increased  $\gamma$ -globin gene expression in transgenic mice carrying the human  $\beta$ -globin locus. Interestingly, higher overexpression of both proteins causes a block in definitive erythropoiesis leading to a severe anaemia and fetal lethality.

## Results

### C/EBP $\gamma$ is an integral part of NFE-6

The CCAAT box of the human  $\beta$ -globin gene spans from position -62 to -87 of the start site (Figure 1A). At least three activities bind these sequences in MEL cell extracts. Two of these have been identified as NF-Y and GATA-1; the third was called NFE-6 (Berry *et al.*, 1992). A complex migrating with similar mobility to NFE-6 was shown by another group to contain C/EBP $\gamma$  (Wall *et al.*, 1996). To characterize NFE-6 further we analysed its contact points on the human  $\beta$ -globin CCAAT box by deperunation analysis (Wall *et al.*, 1988). The contact points of NFE-6 (Figure 1A) are in agreement with the consensus binding site of C/EBP proteins (Osada *et al.*, 1996). We therefore determined which members of this family are contained in NFE-6. For this purpose, we performed bandshifts on the  $\beta$ -globin CCAAT box with antibodies raised against C/EBP family members. NFE-6 is supershifted by antibodies against C/EBP $\gamma$  (Figure 1B, lane 4) and not by any of the other sera used. In addition, the reduced intensity of the slowest migrating complex in lane 3 correlates with the C/EBP $\beta$  supershift. The NF-Y antibodies also remove this shift (Figure 1B, lane 10) and we conclude that this activity is composed of both NF-Y and C/EBP $\beta$ , in agreement with Wall *et al.* (1996).

To confirm further that NFE-6 contains C/EBP $\gamma$ , we cloned the cDNA by RT-PCR and expressed two versions of C/EBP $\gamma$  under the control of the  $\beta$ -globin promoter and locus control region (LCR) in MEL cells (Needham *et al.*, 1992). The first construct, pEV-C/EBP $\gamma$ , contains the 0.54 kb open reading frame of C/EBP $\gamma$ . The second construct, pEV-C/EBP $\gamma$  $\Delta$ B, is a dominant-negative version of the former, in which we deleted the DNA sequences coding for amino acids Met58–Glu89. This removes the basic DNA binding domain but leaves the leucine zipper dimerization domain intact (Figure 1C). We then analysed nuclear extracts of transfected populations in bandshifts with the human  $\beta$ -globin CCAAT box oligonucleotide. This shows that C/EBP $\gamma$  $\Delta$ B causes a reduction in the intensity of the NFE-6 band (Figure 1B, lanes 11 and 12). Furthermore, C/EBP $\gamma$  protein displays the same mobility as NFE-6 (Figure 1B, lanes 13 and 14).



**Fig. 1.** Proteins interacting with the CCAAT box of the human  $\beta$ -globin gene. (A) Sequence of the CCAAT box with the contact sites of NFE-6 indicated by black circles. The imperfect dyad symmetric C/EBP binding site is shown by arrows. (B) Bandshift analysis of proteins binding to the  $\beta$ -globin CCAAT box. An oligonucleotide probe covering the CCAAT box was incubated with 4  $\mu$ g of MEL cell nuclear extract plus the indicated antibodies; PI, pre-immune serum. Lanes 12–14 contain extracts from MEL cells overexpressing the indicated EBPs. (C) Constructs used to express C/EBP $\gamma$  and C/EBP $\gamma$  $\Delta$ B. Restriction sites used to isolate the microinjection fragments are indicated. (D) RT-PCR analysis of C/EBP $\gamma$  expression in embryonic and fetal haematopoietic tissues. Two micrograms of RNA from the indicated tissues (bl, blood; ys, yolk sac, fl, fetal liver) were used for RT-PCR (30 cycles). No RT, reverse transcriptase was omitted to exclude amplification of contaminating DNA.

These data are in agreement with the notion that C/EBP $\gamma$  is an integral part of NFE-6. Although it appears that NFE-6 is a homodimer of C/EBP $\gamma$ , our results do not formally exclude the possibility that NFE-6 is a heterodimer of C/EBP $\gamma$  and another polypeptide.

### Overexpression of C/EBP $\gamma$ in transgenic mice

It has been suggested that C/EBP $\gamma$  could be involved in the regulation of the  $\beta$ -globin gene in MEL cells (Wall *et al.*, 1996). However, we did not observe a change in the expression of the mouse adult globin genes upon MEL cell differentiation when C/EBP $\gamma$  or C/EBP $\gamma$  $\Delta$ B were overexpressed (data not shown). The outcome of this experiment suggests either that C/EBP $\gamma$  may not be directly involved in the transcription of the globin genes or that the role of this factor is important at a stage during the differentiation of erythroid cells other than that represented by MEL cells. C/EBP $\gamma$  is ubiquitously

**Table I.** EBP transgenic mice generated for this study

Founder	Copy number	Remarks	$\gamma$ -globin expression
<b>PEV-C/EBP<math>\gamma</math></b>			
7	(10)	no transmission	n.a.
8 <sup>a</sup>	6	homozygous alive	$\gamma$ up in het. and hom.
13	4	transmission	n.d.
14	(42)	no transmission	n.a.
19	26	homozygous lethal	$\gamma$ up in het.
21 <sup>a</sup>	20	homozygous lethal	$\gamma$ up in het.
22	(22)	no transmission	n.a.
28	12	homozygous alive	n.d.
<b>pEV-C/EBP<math>\gamma</math><math>\Delta</math>B</b>			
1 <sup>a</sup>	22	homozygous lethal	$\gamma$ normal in het.
2	(16)	no transmission	n.a.
10	(20)	no transmission	n.a.
12	6	homozygous alive	$\gamma$ normal in het. and hom.
19	5	homozygous alive	$\gamma$ normal in het. and hom.
21	(20)	no transmission	n.a.
22 <sup>a</sup>	40	heterozygous lethal	n.d.
14	(< 1)	no transmission	n.a.

n.a., not applicable; n.d., not determined; het., heterozygous; hom., homozygous for the EBP transgene. Expression of  $\gamma$ -globin refers to  $\gamma$  mRNA levels in E12.5 fetal liver in crosses with mice carrying the human  $\beta$ -globin locus (line 72; Strouboulis *et al.*, 1992). There was no significant difference in the upregulation of  $\gamma$ -globin between the different C/EBP $\gamma$  transgenics.

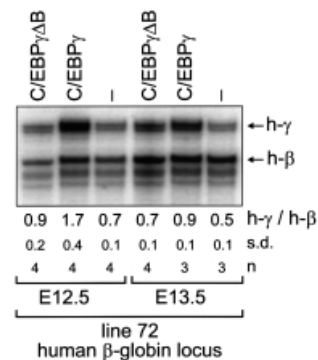
<sup>a</sup>Data obtained with these lines are shown in the figures.

expressed in adult tissues and in most tissues of fetuses at 14 days of gestation (E14) (Cooper *et al.*, 1995). Because we are interested in the function of C/EBP $\gamma$  in erythropoiesis, we extended these studies to the erythroid organs of E11.5–E13.5 mouse fetuses through an RT-PCR analysis of C/EBP $\gamma$  expression. C/EBP $\gamma$  mRNA is detected in E11.5 yolk sac and E12.5/E13.5 fetal liver (Figure 1D) demonstrating that C/EBP $\gamma$  is already expressed before the onset of adult erythropoiesis. Thus, C/EBP $\gamma$  may have a role in erythroid cells of embryonic and fetal origin, and in globin gene switching. To investigate this, we generated transgenic mice with the pEV-C/EBP $\gamma$  and pEV-C/EBP $\gamma$  $\Delta$ B constructs (Figure 1C). These expression vectors are active in embryonic, fetal and adult erythropoiesis. Several founders were generated and bred to obtain germline transmission (Table I). Transgenic F<sub>1</sub> pups reached adulthood with no obvious phenotype. Collectively, these mice will be referred to as EBP transgenics in this paper.

### Expression of C/EBP $\gamma$ , but not C/EBP $\gamma$ $\Delta$ B, results in upregulation of $\gamma$ -globin expression

The EBP transgenics were bred with mice harbouring the 70 kb human  $\beta$ -globin locus (line 72; Strouboulis *et al.*, 1992), in order to analyse the effects of transgene-derived C/EBP $\gamma$  and C/EBP $\gamma$  $\Delta$ B on the regulation of the human  $\beta$ -like globin genes. This is particularly useful to study the switch from fetal ( $\gamma$ -) to adult ( $\beta$ -) globin expression, which is absent in the endogenous mouse  $\beta$ -globin locus.

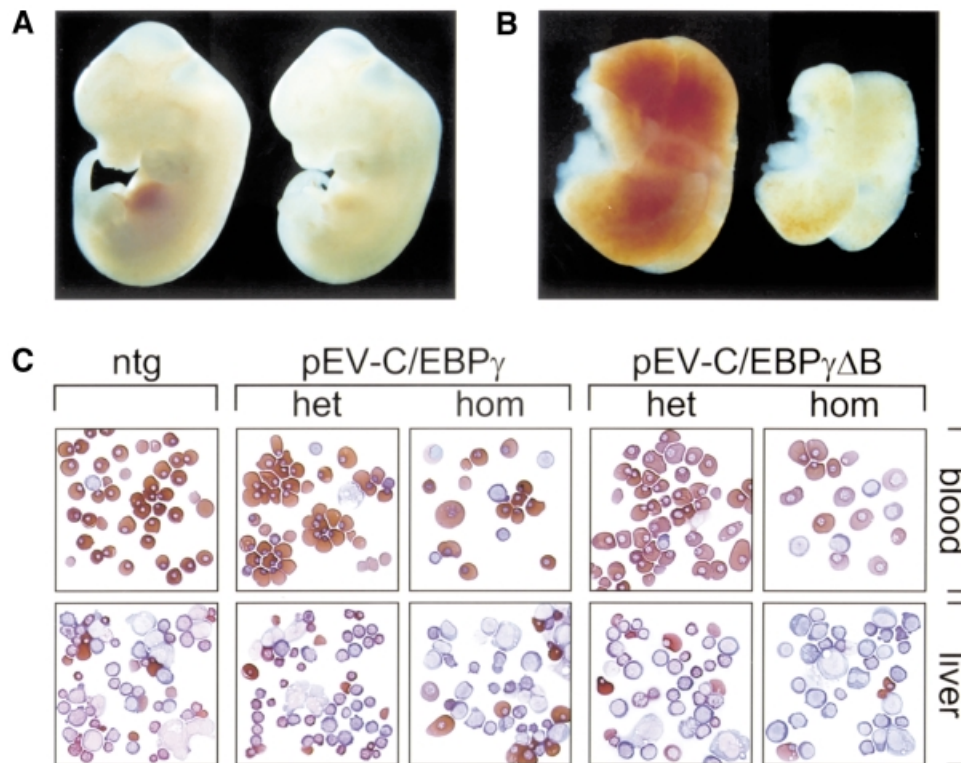
Fetal liver globin mRNA levels of compound transgenic fetuses dissected at E12.5 and E13.5 were assayed by S1 nuclease protection (Figure 2). For each transgene combination, several fetuses from independent litters were analysed. The RNA samples were also assayed for expression of the murine  $\beta$ H1 and  $\epsilon\gamma$  genes to assess the contribution of primitive erythrocytes derived from the circulation; this was found to be minimal and hence the



**Fig. 2.** Overexpression of C/EBP $\gamma$  affects globin gene switching. Mice harbouring the 70 kb human  $\beta$ -globin locus (line 72) were crossed with C/EBP $\gamma$  $\Delta$ B line 1 and C/EBP $\gamma$  line 21, heterozygous for the EBP transgene. RNA was isolated from E12.5 and E13.5 livers and 1  $\mu$ g was used for quantitative S1 nuclease analysis of human globin gene expression (Wijgerde *et al.*, 1996). Arrows indicate human  $\gamma$ -globin (h- $\gamma$ ) and  $\beta$ -globin (h- $\beta$ ) signals. The average of the h- $\gamma$ /h- $\beta$  signals with standard deviations (s.d.) and the number of fetuses analysed (*n*) are shown below the lanes.

$\gamma$ -globin mRNA measured is derived from fetal liver cells (data not shown).

Interestingly, the  $\gamma$ -globin gene is upregulated in the presence of the pEV-C/EBP $\gamma$  transgene, but not in the presence of the pEV-C/EBP $\gamma$  $\Delta$ B transgene (Figure 2). This is particularly evident in the E12.5 samples when we observe a 2- to 3-fold higher  $\gamma$  to  $\beta$  ratio in the presence of the pEV-C/EBP $\gamma$  transgene (Figure 2). There was no significant difference in the upregulation of  $\gamma$ -globin expression between the different C/EBP $\gamma$  transgenic lines (Table I). In adult animals, the EBP transgenes do not activate  $\gamma$ -globin expression, and these animals have normal numbers of erythrocytes, lymphocytes, granulocytes and platelets in their circulation (data not shown).



**Fig. 3.** Fatal anaemia caused by high level expression of the EBP transgenes. (A) Wild-type (left) and C/EBP $\gamma$  $\Delta$ B-overexpressing line 22 (right) E12.5 littermates. Note the pallor of the transgenic fetus, which has an otherwise normal gross morphological appearance. (B) Fetal livers dissected from the fetuses shown in (A). The transgenic liver (right) is small and pale relative to the control liver (left), indicative of hampered erythropoiesis. (C) Cytospins of peripheral blood and fetal liver single cell suspensions prepared from fetuses with the indicated genotype. ntg, non-transgenic; het, heterozygous; hom, homozygous. Data shown are obtained with C/EBP $\gamma$  $\Delta$ B line 1 and C/EBP $\gamma$  line 21. The slides were stained with a combined histological and neutral benzidine stain.

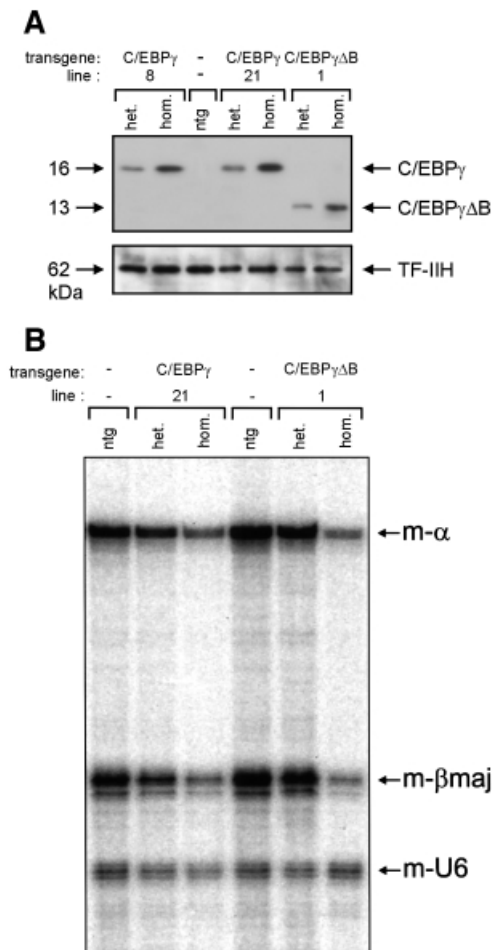
We conclude that overexpression of C/EBP $\gamma$  in the fetal liver results in increased  $\gamma$ -globin expression. This prolongation is dependent on the presence of the basic DNA-binding domain in the C/EBP $\gamma$  protein, suggesting that C/EBP $\gamma$  acts preferentially at the  $\gamma$ -globin CCAAT boxes. These results are in contrast with data obtained from transfection experiments that suggested a function for C/EBP $\gamma$  in transcriptional enhancement through the  $\beta$ -globin CCAAT box (Wall *et al.*, 1996).

#### **High-level expression of the EBP transgenes blocks fetal liver erythropoiesis**

Animals heterozygous for the EBP transgenes are viable and show no morphological abnormalities in blood and fetal liver (see Figure 3). However, one of the pEV-C/EBP $\gamma$  $\Delta$ B founder males gave rise to small litters and the pups analysed were non-transgenic. We extended the analysis of this line to E11.5–E15.5 fetuses and we found normal-sized litters up to E13.5 and re-absorbed fetuses from E14.5 onwards. At E13.5, 30% of the fetuses were severely anaemic. The affected fetuses all carried the transgene and had otherwise developed normally (Figure 3A). The fetal livers of these animals were much smaller and paler than those of non-transgenic littermates (Figure 3B). Since this phenotype could be due to high levels of transgene-derived protein, it might be reproduced in other EBP transgenic lines if the transgene could be expressed at sufficiently high levels. We therefore raised

the expression levels by breeding the EBP lines to homozygosity. Western blot analysis of fetal liver nuclear extracts shows that the levels of transgenic EBP proteins are raised in homozygous transgenics, that the expression levels correlate with the transgene copy number, and that the C/EBP $\gamma$  wild-type and  $\Delta$ B proteins are expressed at comparable levels (Figure 4A). We found that homozygous fetuses of the high copy number lines were anaemic and died at ~E13–E14, confirming that the phenotype observed is due to the overexpression of the EBP proteins, and not a secondary effect of transgene integration. Furthermore, there is a good correlation between transgene copy number and fetal lethality (Table I). Surprisingly, high levels of both C/EBP $\gamma$  and C/EBP $\gamma$  $\Delta$ B proteins cause anaemia and fetal lethality.

The paleness of the fetal livers prompted us to determine by S1 nuclease analysis whether overexpression of the EBP proteins interfered with globin gene expression. We observe a general reduction in the levels of the mouse (Figure 4B) and human (data not shown) globin genes if the fetuses are homozygous for the high copy number EBP transgenes. The ratio between the  $\alpha$ - and  $\beta$ -like genes is comparable to the controls, demonstrating that the block in erythroid differentiation is accompanied by reduced expression of the globin genes from both loci (Figure 4B). Thus, we conclude that the anaemia is not due to chain imbalance caused by a defect in either  $\alpha$ - or  $\beta$ -globin expression, but more likely by an early block in



**Fig. 4.** High levels of the EBP proteins result in reduced expression of the  $\alpha$ - and  $\beta$ -globin genes. **(A)** Ten micrograms of E12.5 fetal liver nuclear extract were used for western blot analysis of EBP protein expression. Results are shown for non-transgenic (ntg), heterozygous (het.) and homozygous (hom.) EBP transgenics of the lines indicated. The polyclonal rabbit antiserum used was raised against the N-terminal part of C/EBP $\gamma$  that is shared by the C/EBP $\gamma$  and C/EBP $\gamma$ ΔB proteins. Note that the levels of endogenous C/EBP $\gamma$ , although present in the RT-PCR analysis (Figure 1D), are too low for detection with this antiserum. A mouse monoclonal antibody recognizing the p62 subunit of TF-IIH was used as a loading control. **(B)** E12.5 livers were dissected from non-transgenic (ntg), heterozygous (het.) and homozygous (hom.) EBP transgenics of the lines indicated and used for RNA isolation. Approximately 1  $\mu$ g of total RNA was used for quantitative S1 nuclease analysis of mouse  $\alpha$ - (m- $\alpha$ ) and  $\beta$ <sub>major</sub>-globin gene expression (m- $\beta$ maj); a probe detecting mouse U6 RNA (m-U6) was used as an internal loading control (Wijgerde *et al.*, 1996).

definitive erythropoiesis resulting in fewer cells transcribing the globin genes.

#### Cytological analysis of fetal livers and blood

Fetal livers of mice expressing high levels of EBP proteins are pale and small (Figure 3B). We therefore analysed the morphology and distribution of the cells in the fetal livers and peripheral blood of wild-type and EBP transgenic fetuses. Cytospins of blood and E12.5 livers were prepared and stained with histological dyes and neutral benzidine (Beug *et al.*, 1982). The pattern found in E12.5 control livers is shown in Figure 3C (ntg). A predominance of small, benzidine-positive, polychromatic

and orthochromatic erythroblasts is observed, followed by fewer basophilic erythroblasts and proerythroblasts. A similar pattern is observed in the livers of heterozygous fetuses (Figure 3C, het). In contrast, liver preparations from homozygous fetuses are composed mainly of small, benzidine-negative blast-like cells. The absence of small, benzidine-positive cells is immediately apparent (Figure 3C, hom). Thus, it appears that the transgenic fetal livers are hypocellular and pale because erythropoiesis is impeded at a stage preceding haemoglobinized polychromatic erythroblasts. The failure of liver erythropoiesis was further confirmed by *in vitro* progenitor assays with pEV-C/EBP $\gamma$  line 21 and pEV-C/EBP $\gamma$ ΔB line 1 fetal livers. E12.5 livers of homozygous fetuses formed virtually no CFU-Es (>15-fold reduction) and a drastically reduced number of BFU-Es (6- to 7-fold reduction). These results indicate that the block in erythropoiesis occurs at the level of relatively early precursor represented by the BFU-E colonies. Since the colony numbers are not significantly different between wild-type and heterozygous fetal livers, we conclude that there is a critical threshold level for the dominant-negative effect of the EBP proteins. Finally, the blood shows a large number of blue nucleated cells distinct from the primitive erythrocytes (compare Figure 3C, ntg and het, with Figure 3C, hom). These abnormal cells are presumably of fetal liver origin, and are probably prematurely released in circulation in response to the severe anaemia.

We conclude that fetal liver erythropoiesis is severely impaired in homozygous EBP transgenics. This defect in erythropoiesis is independent of the presence of the DNA-binding domain of C/EBP $\gamma$ , suggesting that C/EBP $\gamma$  might indeed act as a modulator of the activity of other bZIP proteins that are essential for the proper execution of the erythropoietic program.

## Discussion

### C/EBP $\gamma$ and the developmental regulation of the $\beta$ -globin gene cluster

The work described in this paper was initiated to study the role of the previously described CCAAT box binding protein NFE-6 in the regulation of the human  $\beta$ -globin gene cluster. Several naturally occurring mutations in the promoters of the globin genes denote the involvement of factors binding to these sequences for the proper temporal and topological expression of these genes. For example, mutations in the CACC box of the human  $\beta$ -globin gene or ablation of EKLF, an erythroid restricted factor that binds to this motif, result in impaired expression of the  $\beta$ -globin gene (Thein, 1993; Wijgerde *et al.*, 1996). Intriguingly, no patients with mutations in the  $\beta$ -CCAAT box are known, suggesting that the  $\beta$ -CCAAT box is perhaps not important for  $\beta$  gene expression. The relevance of the globin CCAAT boxes *in vivo* is best demonstrated by naturally occurring mutations clustered around the distal CCAAT box of the fetal  $\gamma$ -globin gene that result in an HPFH phenotype (Poncz *et al.*, 1988). Consequently it would appear that factors binding to these sequences are causative in the proper regulation of the fetal genes and have a role in the switch from  $\gamma$ - to  $\beta$ -globin expression. Reactivation of  $\gamma$ -globin expression in adults is clinically

important because this would be beneficial to large numbers of  $\beta$ -thalassaemia and sickle cell anaemia patients.

We show in this paper that NFE-6 contains C/EBP $\gamma$ , and that it is most likely the same factor as the DSFr activity described by Delvoye *et al.* (1993). It has been postulated that C/EBP $\gamma$  could play a role in the developmental upregulation of the adult  $\beta$ -globin gene (Wall *et al.*, 1996). However, our data in mice overexpressing C/EBP $\gamma$  do not support this model, because we observe an increase, rather than a decrease, in  $\gamma$ -globin expression during the switching period. If C/EBP $\gamma$  were a  $\beta$ -promoter-specific activator,  $\gamma$ -globin transcription should have decreased. For instance, a robust positive correlation between the levels of the  $\beta$ -promoter transcription factor EKLF and  $\beta$  gene expression in the fetal liver has been reported, and higher  $\beta$  levels are accompanied by lower  $\gamma$  levels (Wijgerde *et al.*, 1996). Thus, our data strongly suggest that C/EBP $\gamma$  is not an activator of the  $\beta$ -globin promoter. We can only speculate about the mechanism by which increased C/EBP $\gamma$  levels favour  $\gamma$ -globin expression. One obvious possibility is that it exerts this effect directly through the  $\gamma$ -globin CCAAT boxes. Alternatively, it could affect the developmental progression of fetal liver erythropoiesis, resulting in prolonged maintenance of a transcription factor environment favouring  $\gamma$ -globin expression. In this respect, it is interesting to note that C/EBP proteins are thought to be part of a developmental transcription factor cascade in haematopoiesis (Sieweke and Graf, 1998).

After the fetal liver stage, the  $\gamma$ -globin genes are silenced normally and we do not observe an HPFH phenotype in our EBP transgenic lines. Furthermore, the  $\gamma$ -globin expression levels in adult transgenic mice with the -117 Greek HPFH mutation in the distal  $\gamma$ -globin CCAAT box (Berry *et al.*, 1992) remain unchanged in the presence of the EBP transgenes (data not shown). This is consistent with the notion that C/EBP $\gamma$  is not an activator of the  $\beta$ -globin promoter, because in that case a shift in favour of  $\beta$  expression should occur in the presence of elevated C/EBP $\gamma$  levels. However, the molecular mechanism underlying Greek HPFH remains elusive since neither point mutagenesis of the distal CCAAT box (Ronchi *et al.*, 1996) nor manipulation of one of the factors binding to it (this study) has provided clear-cut insight into the mode of action of the -117 mutation. It appears that either more than one factor is involved, or that the -117 mutation precipitates a secondary effect like localized chromatin remodelling.

#### **High-level expression of the EBP transgenes blocks fetal erythropoiesis**

We have found that overexpression of the EBP transgenes in MEL cells does not interfere with dimethyl sulfoxide-induced MEL cell differentiation. Since MEL cells represent the proerythroblast stage of erythroid development, this suggests that the differentiation programme beyond this stage can be efficiently executed in the presence of high levels of EBP proteins. In sharp contrast, the effects of such high levels on erythropoiesis in mice are dramatic. It results in a developmental block of early erythroid progenitors in the fetal liver. C/EBP family members have distinct pivotal roles in lineage commitment in the haematopoietic system (Cooper *et al.*, 1994;

Screpanti *et al.*, 1995; Zhang *et al.*, 1997; Nerlov *et al.*, 1998; Kaisho *et al.*, 1999). These proteins are known to interact with several other factors in large multiprotein complexes that regulate the tissue-specific expression of several haematopoietic-specific genes (reviewed in Sieweke and Graf, 1998). It has been proposed that C/EBP $\gamma$  is a modulator of the activity of other bZIP proteins (Cooper *et al.*, 1995). Our data are consistent with this proposal, since we observe defective erythropoiesis in mice with high levels of C/EBP $\gamma$  and the dominant-negative version C/EBP $\gamma\Delta B$ . Presumably, provided they are expressed at sufficiently high levels, both EBP proteins interfere with activator functions of bZIP proteins required for fetal liver erythropoiesis.

#### **Tissue-specific expression of dominant-negative leucine zipper proteins**

Inactivation of genes by knockout strategies is a frequently chosen approach to address the function of gene products in whole organisms. In the case of C/EBP family members, much has been learnt about the specific roles of individual family members in the development and physiology of mice (LekstromHimes and Xanthopoulos, 1998). Functional redundancy and early lethality are potential problems of gene knockouts. To some extent, these issues can be tackled by intercrossing knockout lines and by using conditional knockout alleles. However, the number of combinations that can be made is limited for practical reasons. The dominant-negative transgenic approach does not suffer from these limitations. Expression of the transgene can be directed to specific tissues and, unlike toxin genes, leaky expression in other tissues should not adversely affect these tissues because low level expression is unlikely to interfere with the functioning of the cells. Furthermore, dimerization specificity of the leucine zipper can be modulated by *in vitro* mutagenesis (Vinson *et al.*, 1993), and fusion with steroid receptor ligand binding domains would give an additional level of control over the spatio-temporal activity of the dominant-negative protein (Littlewood *et al.*, 1995).

Dominant-negative leucine zippers have been applied successfully in gene expression and differentiation studies of tissue-culture cells (i.e. Olive *et al.*, 1997; Nerlov *et al.*, 1998), and this approach was used to study the function of adipose tissues in mice (Moitra *et al.*, 1998). Expression of a dominant-negative leucine zipper-containing protein under the control of the adipose-specific aP2 enhancer/promoter resulted in the complete ablation of white adipose tissue with dramatic consequences for the physiology and behaviour of the transgenic mice. In our EBP transgenics we have inactivated the erythroid system, but development of the transgenic fetuses is otherwise normal. Thus, we suggest that targeted expression of dominant-negative leucine zipper proteins might be a generally applicable approach to ablate specific cell types and tissues in order to study their role in the development, physiology and behaviour of mice.

## **Materials and methods**

#### **Cloning of C/EBP $\gamma$ and expression constructs**

Total RNA from MEL cells was isolated as described (Antoniu, 1991) and 5  $\mu$ g were reverse transcribed with the  $\gamma$ -r primer

5'-GGCCTGGAAGGAGATCTACT-3'. One-fifth of the first strand product was subjected to 30 cycles of PCR with the  $\gamma$ -f primer 5'-ACGTGCCCAAATGAGCAAAGC-3'. The PCR product was cloned and sequenced. To delete the DNA-binding domain, the 3' end of C/EBP $\gamma$  was amplified with the C/EBP $\gamma$  reverse primer and the  $\gamma$ -1 primer 5'-ATGCCATGGATACACTGCAA-3'. The product was digested with *Nco*I and *Bgl*III, and ligated to the C/EBP $\gamma$  plasmid digested with *Nco*I and *Bgl*III. *Eco*RI-*Bgl*III inserts of C/EBP $\gamma$ - and C/EBP $\gamma$  $\Delta$ B-plasmids were ligated to pEV3-puromycin' (Needham *et al.*, 1992) to generate pEV-C/EBP $\gamma$  and pEV-C/EBP $\gamma$  $\Delta$ B.

#### Cell culture and protein preparation

pEV-C/EBP $\gamma$  and pEV-C/EBP $\gamma$  $\Delta$ B were linearized with *Sca*I and transfected into MEL cells as described (Antoniou, 1991) with the exception that puromycin (1  $\mu$ g/ml) was used for the selection. RNA and nuclear extracts were prepared as described (Wall *et al.*, 1988; Antoniou, 1991).

#### Production of antibodies and gel mobility shift assays

A *Nco*I site was introduced at the first ATG of C/EBP $\gamma$  by PCR. The following primers were used: 5'-CCATGGGCAAGCTGTGCGAGC-CAG-3' and the  $\gamma$ -r primer. The PCR product was cloned and sequenced. The plasmid was digested with *Nco*I and the insert coding for the unique N-terminus of C/EBP $\gamma$  was cloned into the *Nco*I site of pET15 and used for the production of recombinant protein in *Escherichia coli* BL21 (DE3). Gel-purified recombinant protein was used to immunize rabbits.

Gel mobility shift assays were performed as described by Wall *et al.* (1988) using the human  $\beta$ -globin CCAAT box oligonucleotide as probe (deBoer *et al.*, 1988). For supershifts, appropriately diluted antibodies were added to the reactions followed by a further 20 min incubation at room temperature prior to loading of the gel.

#### Generation of transgenic mice and DNA analysis

pEV-C/EBP $\gamma$  and pEV-C/EBP $\gamma$  $\Delta$ B were digested with *Aat*II and *Asp*718. The fragments were isolated and used for microinjection as described (Kollias *et al.*, 1986). Transgenics were screened by Southern blotting of *Eco*RI-*Bam*HI-digested DNA using the 0.54 kb *Eco*RI C/EBP $\gamma$  fragment as probe.

#### RNA analysis

RNA was prepared from yolk sacs, fetal livers and adult blood as described (Strouboulis *et al.*, 1992). RT-PCR was performed with the  $\gamma$ -f and the  $\gamma$ -r2 primer 5'-GGCTGTGCGCATGCTCAAGAAAC-3' (394 bp product) to detect C/EBP $\gamma$  expression. Globin and U6 RNA levels were analysed by S1 nuclease protection (Wijgerde *et al.*, 1996). The S1 signals were quantitated by phosphorimager analysis.

#### Protein analysis

Nuclear protein was isolated from E12.5 liver (Andrews and Faller, 1991) and 10  $\mu$ g were used for western blot analysis with the rabbit anti-C/EBP $\gamma$  polyclonal antibodies. A mouse monoclonal antibody recognizing the 62 kDa subunit of TH1-H was used as a loading control; this antibody was generously provided by Dr E.Citterio (Rotterdam).

#### Colony assays

Colony assays were performed essentially as previously described (Wong *et al.*, 1986). Fetal livers were disaggregated into single cells by passage through a 100  $\mu$ m mesh and plated at a density of  $3 \times 10^5$  cells per ml in methyl cellulose containing 1 U/ml Epo. The appearance of CFU-E and BFU-E colonies was scored after 2 and 14 days, respectively.

#### Cytospin preparations and staining

E12.5 fetuses were dissected from the uterus and bled on a dish to collect fetal blood. Then, the fetal livers were dissected and rinsed several times in cold phosphate-buffered saline. A single cell suspension of the fetal livers was prepared by gentle pipetting with a yellow tip. Aliquots of blood and fetal liver cell suspensions were loaded on a cytofunnel and spun at 400 r.p.m. for 5 min on microscope slides. The preparations were left to air dry and were stained with a combined histological and neutral benzidine stain (Beug *et al.*, 1982).

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