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Regulation of embryonic/fetal globin genes by nuclear hormone receptors: a novel perspective on hemoglobin switching

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The CCAAT box is one of the conserved motifs found in globin promoters. It binds the CP1 protein. We noticed that the CCAAT-box region of embryonic/fetal, but not adult, globin promoters also contains one or two direct repeats of a short motif analogous to DR-1 binding sites for non-steroid nuclear hormone receptors. We show that a complex previously named NF-E3 binds to these repeats. In transgenic mice, destruction of the CCAAT motif within the human ε-globin promoter leads to substantial reduction in ε expression in embryonic erythroid cells, indicating that CP1 activates ε expression; in contrast, destruction of the DR-1 elements yields striking ε expression in definitive erythropoiesis, indicating that the NF-E3 complex acts as a developmental repressor of the ε gene. We also show that NF-E3 is immunologically related to COUP-TF orphan nuclear receptors. One of these, COUP-TF II, is expressed in embryonic/fetal erythroid cell lines, murine yolk sac, intra-embryonic splanchnopleura and fetal liver. In addition, the structure and abundance of NF-E3/COUP-TF complexes vary during fetal liver development. These results elucidate the structure as well as the role of NF-E3 in globin gene expression and provide evidence that nuclear hormone receptors are involved in the control of globin gene switching.
Keywords: COUP-TF/erythropoiesis/globin genes/nuclear hormone receptors

Introduction

Human β-like globin genes are organized as a cluster of five genes (ε, ^{G}γ, ^{A}γ, δ and β), in the order of their temporal expression. The ε gene is expressed in the embryonic yolk sac, the γ genes in the fetal liver, and the δ and β genes are activated from late gestation onward. Transcriptional activity of all these genes is controlled by their proximal regulatory regions and by a dominant element, the Locus Control Region (LCR), located 6–20 kb 5′ from the ε gene (Grosveld *et al*., 1987). The molecular mechanisms leading to globin gene switching are poorly understood, but are thought to be caused by subtle changes in transcription factor expression (Enver and Greaves, 1991; Crossley and Orkin, 1993; Dillon and Grosveld, 1993). The organization of β-like globin genes in the mouse is different, with two embryonic β-like genes, εY and βh1, and two adult genes, β major and β minor. Expression of the εY and βh1 genes is restricted to primitive erythroblasts; these originate mostly from the yolk sac, but it has been shown that precursors derived from the intra-embryonic splanchnopleura also express εY and βh1 (Cumano *et al*., 1996). Expression of βmaj and βmin starts only after the onset of definitive erythropoiesis, around day 11 post coitum (11 d.p.c.). Although mice do not possess fetal globin genes, a human γ-globin gene linked to a LCR element is expressed at high levels in fetal liver and switched off around 16 d.p.c. (Dillon and Grosveld, 1991), suggesting that murine erythroblasts undergo similar changes in transcription factor expression to human erythroblasts during development.

Hereditary persistence of fetal hemoglobin (HPFH) is an inherited human condition resulting in continued expression of γ-globin in adult life (Poncz *et al*., 1989). HPFHs have been associated either with deletions in the β-globin locus or with mutations in the ^{G}γ- or ^{A}γ-globin promoter. These mutations can be found in a few distinct places, including the –200, –175 and –110 regions. Mutations in the –200 region have been shown to alter the binding affinity of Sp1 and other nuclear factors (Ronchi *et al*., 1989; Fischer and Nowock, 1990; Jane *et al*., 1993), and also the ability of this region to form a triple helix structure *in vitro* (Bacolla *et al*., 1995; Pissard *et al*., 1996). Mutations at the –175 nucleotide increase the binding affinity of GATA-1 (Martin *et al*., 1989; Nicolis *et al*., 1989). Finally, several mutations in the distal CCAAT-box region of the γ-globin promoters (–114, –117 or deletion of –114 to –102) have been shown to disrupt or enhance the binding of several transcription factors (Superti-Furga *et al*., 1988; Mantovani *et al*., 1989; Fucharoen *et al*., 1990; Berry *et al*., 1992). The –117 mutation in the ^{A}γ-globin promoter, which is associated with Greek HPFH (Collins *et al*., 1985; Gelinas *et al*., 1985), is sufficient to produce a strong HPFH phenotype in transgenic mice (Berry *et al*., 1992; Peterson *et al*., 1998). Several transcription factors can bind this region, including CP1/NF-Y, the CCAAT displacement protein (CDP), GATA-1 and a poorly characterized protein called NF-E3 (Superti-Furga *et al*., 1988; Mantovani *et al*., 1989; Ronchi *et al*., 1995). Further dissection of the CCAAT-box region in transgenic mice failed to correlate γ-globin expression with the binding or loss of one particular transcription factor (Ronchi *et al*., 1996).

In transgenic mice, a human ε-globin gene linked to the LCR is expressed in primitive erythroid cells only, and its pattern of expression resembles that of the endogenous murine εY gene (Raich *et al*., 1990; Enver and Greaves, 1991). Regulatory sequences mediating extinction of

ε expression in definitive erythroid cells have been mapped to four regions: around –3000 relative to the cap site (J.Li *et al*., 1998), between –2000 and –460 (Q.Li *et al*., 1998), between –460 and –180 (Raich *et al*., 1992, 1995; Peters *et al*., 1993; J.Li *et al*., 1998; Q.Li *et al*., 1998) and in the proximal promoter (Q.Li *et al*., 1998). As in the case of the γ-globin promoters, deletions or point mutations in these negative elements yield detectable ε-globin transcription in adult cells. In the –180 to –460 silencer element, it has been shown that disruption of either a GATA-1 or a YY1 binding site was sufficient for derepression in adult transgenic mice (Raich *et al*., 1995). The role of GATA-1 as a repressor of ε-globin transcription was later confirmed as its overexpression in transgenic mice results in a specific decrease of a human ε-globin transgene (Li *et al*., 1997).

Since their proximal promoter contributes to the extinction of both embryonic (ε) and fetal (γ) globin genes at the adult stage, we focused our attention on this regulatory element. We noticed that the CCAAT-box region of ε/γ globin promoters actually contains one or two direct repeats of a short motif that is analogous to binding sites for non-steroid nuclear hormone receptors. This region is bound by several protein complexes in erythroid nuclear extracts, including CP1 and NF-E3. The NF-E3 complex appears to recognize specifically the direct repeats. Experiments in transgenic mice carrying mutated ε promoter constructs in which either the binding of CP1 or the binding of NF-E3 has been abolished show that CP1 acts as an ε gene activator while NF-E3 acts as an ε gene repressor. We show that the NF-E3 complex is recognized by antibodies directed against COUP-TF orphan nuclear receptors. One of these, COUP-TF II (also called ARP-1), is expressed in the murine yolk sac and para-aortic splanchnopleura, as well as in the fetal liver. We also present evidence that the structure of NF-E3/COUP-TF complexes varies during fetal liver development. In addition to elucidating the role of NF-E3, these data provide the first *in vivo* and *in vitro* evidence that nuclear hormone receptors play a role in the regulation of globin genes during the course of development.

Results

The CCAAT-box regions of embryonic and fetal globin genes contain direct repeats

As the distal CCAAT-box region of the γ-globin promoter is important for correct stage-specific expression, we examined its sequence and noticed that it contains an approximate direct repeat, TGACCAATAGCCT. This is reminiscent of the binding sites for non-steroid nuclear hormone receptors (Glass, 1994). Nuclear receptors of this subgroup, which includes the receptors for retinoic acid, vitamin D_3 and thyroid hormone, as well as the so-called orphan receptors, bind direct repeats (DR) of an AGGTCA (TGACCT on the complementary strand) consensus sequence. The spacing of these two half-sites (DR-n), contributes to the binding specificity of each receptor (Mangelsdorf and Evans, 1995). Using this nomenclature, the direct repeat found in the γ promoter can be described as a DR-1 element (Figure 1). We examined further the human globin promoters and found that the CCAAT-box regions of the ε and ζ genes also contain a DR-1-type sequence; interestingly, the corresponding regions of the adult α and β promoters diverge from the nuclear receptor site consensus (Figure 1). DR-1 elements can also be found in the promoters of the murine εY and βh1 genes (Figure 1).

One of the complexes that bind the CCAAT region recognizes the DR-1 motif

DR-1 elements are considered as specific for retinoic acid receptors (made of either RXR–RAR or RXR–RXR dimers), peroxisome proliferator activated receptors (made of RXR–PPAR heterodimers) and various orphan receptors (Glass, 1994). In order to test the ability of the globin motifs to bind nuclear receptors, we performed gel retardation assays with oligonucleotides containing the ε CCAAT/DR-1 and γ CCAAT/DR-1 regions, as well as known retinoic acid receptor binding sites, RARE 1 (a DR-2 element) and RARE 2 (a DR-1 element) (Figure 1; Durand *et al*., 1992). In these and all other experiments, oligos RARE 1 and RARE 2 behaved identically and were therefore used interchangeably. We used nuclear extracts from two human erythroleukemia cell lines, HEL (Martin and Papayannopoulou, 1982) and K562 (Lozzio and Lozzio, 1975), which express detectable levels of embryonic and fetal, but not adult globin genes (Villeval *et al*., 1985).

The upper complex formed on the γ CCAAT/DR and ε CCAAT/DR probes in HEL extracts (marked with an asterisk in Figure 2) was previously identified as the CCAAT-binding protein CP1/NF-Y (Ronchi *et al*., 1995). The lower complex binding on the γ CCAAT/DR probe is NF-E6 (Berry *et al*., 1992). The intermediate complex was previously described and named NF-E3 (Mantovani *et al*., 1989; Ronchi *et al*., 1995). This protein does not bind the β-globin CCAAT box (our data not shown; deBoer *et al*., 1988; Delvoye *et al*., 1993). The NF-E3 complex is efficiently competed by an excess of cold γ CCAAT/DR or ε CCAAT/DR, but also by RARE 1 and RARE 2 (Figure 2A, left and middle panels). The RARE 1 probe binds only one complex in HEL extracts, which is displaced by ε CCAAT/DR and, with lower efficiency, by γ CCAAT/DR (Figure 2A, right panel). We conclude that NF-E3 is indeed able to bind consensus DR-1 and DR-2 motifs. Previous determination of the NF-E3 binding site by methylation interference showed a repeated pattern of interaction with DNA (Ronchi *et al*., 1996), which is actually identical to that of RAR-RXR or COUP-TF complexes (Cooney *et al*., 1992; Carter *et al*., 1994). In K562 extracts, similar results were obtained, except that the putative receptor complexes appeared as a doublet (Figure 2B). Finally, no complex containing both CP1 and NF-E3 is formed, which indicates that their binding is mutually exclusive.

From these results, we conclude that the NF-E3 factor recognizes a DR-1-type nuclear receptor binding site in the ε- and γ-globin CCAAT-box regions. Relative affinities of the different sequences are in the order RARE 2 > ε CCAAT/DR > γ CCAAT/DR (Figure 2A, right panel).

The ε promoter contains a second DR-1 element

Immediately upstream from the CCAAT region in the human ε promoter lies a second DR-1 element (Figure 1). Gel retardation experiments with oligonucleotides span-

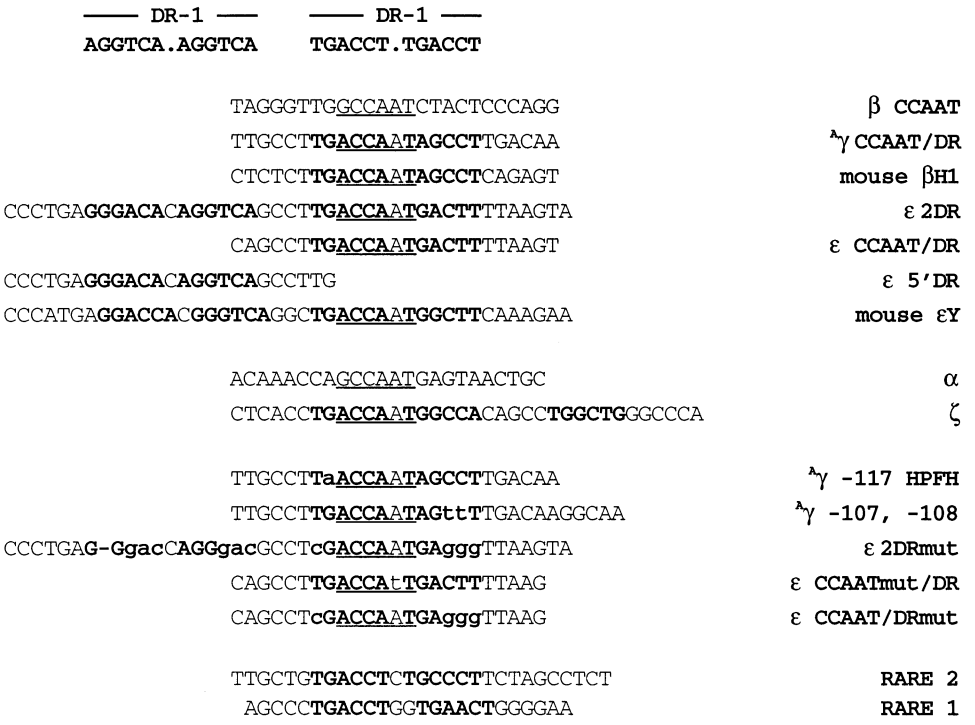

Fig. 1. Embryonic and fetal globin genes contain nuclear receptor binding sites in their CCAAT box region. Sequence alignment of globin CCAAT boxes ('coding' strand): β, $^{A}\gamma$ and ε are human β-like globin genes, α and ζ are human α-like genes, and βh1 and εY are murine β-like globin genes. With the exception of βh1-, εY- and α-globin, all these sequences correspond to oligonucleotides used in this study. CCAAT motifs are underlined and DR motifs are in bold type. The distal CCAAT box from the $^{A}\gamma$ gene is shown. Oligo ε 2DR contains the two DR-1 motifs from the ε promoter (see text). Sequences of mutant oligonucleotides used for bandshift and/or mutagenesis are shown in the third block; mutations are in lower case. The –117 HPFH oligo contains the G to A mutation from Greek HPFH patients. Oligo $^{A}\gamma$ –107, –108 mut is an experimental mutant (Ronchi *et al*., 1996). Other mutants are described in the text. The RARE 2 (DR-1) and RARE 1 (DR-2) oligos are high-affinity binding sites for retinoic acid receptors (Durand *et al*., 1992).

ning either this second DR-1 (oligo 5′ DR) or both DR-1s and the CCAAT motif (oligo 2DR) show that the upstream element is also able to bind NF-E3 in K562 extracts (Figure 2B, right panel). This second NF-E3 binding site, which can also be found in the murine εY promoter (Figure 1) had not been noticed in previous studies.

Mutations in the γ-globin CCAAT-box affect binding of several nuclear proteins

The guanine to adenine mutation at –117 in the $^{A}\gamma$-globin promoter (Figure 1, –117 HPFH), which causes the Greek form of hereditary persistence of fetal hemoglobin, results in loss of NF-E3 binding (Figure 2A and C, left panel; Berry *et al*., 1992; Ronchi *et al*., 1996). Therefore, the NF-E3 complex seems to be important for appropriate expression of the γ-globin gene. However, another experimental mutation on nucleotides –107 and –108 (Figure 1), which destroys the NF-E3 binding site, does not yield a HPFH phenotype in transgenic mice (Ronchi *et al*., 1996). Although the main effect of this mutation is indeed abolition of NF-E3 binding, we have found that it also causes an increase in CP1 affinity and a decrease in NF-E6 affinity and allows weak binding of a complex we identified as GATA-1 (Figure 2C, left panel; data not shown). We conclude that the complicated pattern of nuclear factor binding on the γ-globin CCAAT box makes it an imperfect model to study the function of NF-E3.

Specific mutations in the ε promoter abrogate binding of either CP1 or NF-E3

The ε-globin CCAAT box, on the other hand, binds only CP1 and NF-E3 in HEL and K562 cells (Figure 2); it also has higher affinity for NF-E3 than the γ-globin CCAAT box (Ronchi *et al*., 1995). Finally, the second DR-1 element upstream from the CCAAT box is able to bind NF-E3 only (Figure 2B). These data suggest that the ε-globin promoter may be more suitable for further analysis of NF-E3 function.

In order to test this hypothesis, we made point mutations in the ε-globin promoter that were able to disrupt binding of CP1 and NF-E3 independently. This was facilitated by the knowledge of sequence requirements for nuclear receptor binding to DNA (Glass, 1994). We synthesized mutant oligonucleotides ε CCAATmut/DR and ε 2DRmut (Figure 1). Oligonucleotide ε CCAATmut/DR contains an A to T mutation in the middle of the 3′ DR-1; this abrogates binding of CP1 (Figure 2C, right panel and 2A, middle panel). Oligonucleotide ε 2DRmut contains mutations in both DR-1 elements which prevents nuclear receptor binding (Figure 2C, right panel and 2A, middle and right panels). Importantly, each mutation preserves the *in vitro* binding affinity of the other factor and does not lead to binding of novel nuclear proteins (Figure 2C, right panel). We conclude that it is possible to introduce mutations into the ε CCAAT region that only disrupt binding of one transcription factor.

The CCAAT box mediates activation of the ε promoter

To test the role of the CCAAT motif on ε gene expression *in vivo*, we introduced the CCAATmut mutation into a vector containing a 3.1 kb μLCR cassette linked with an

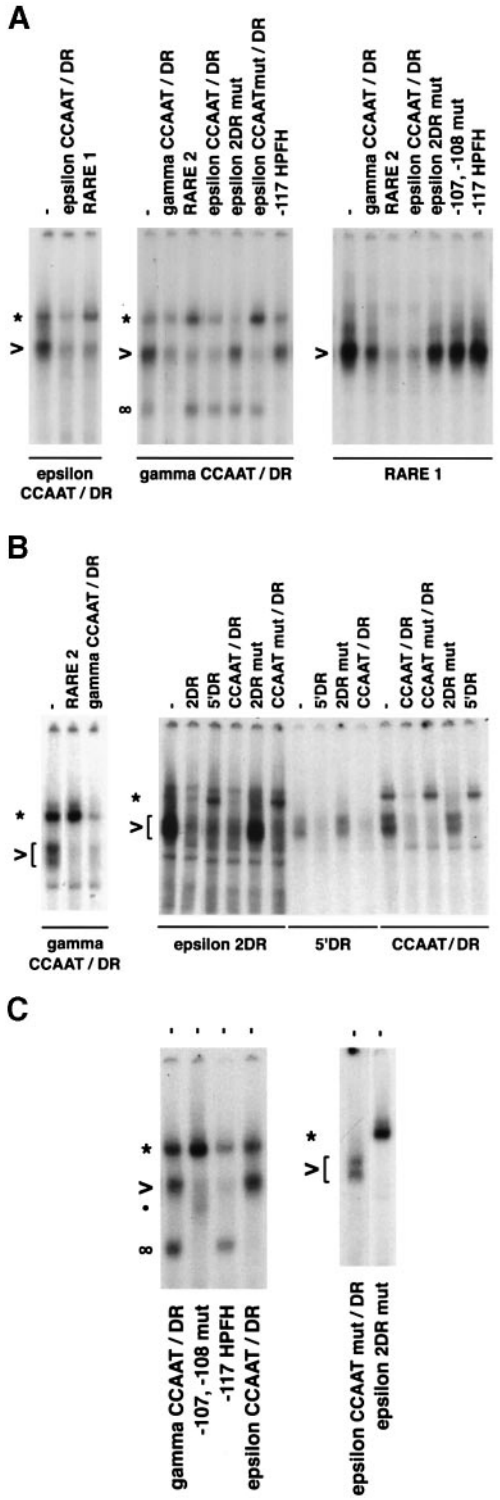

Fig. 2. Binding of nuclear proteins to globin CCAAT-box regions. In this and following figures, only the top half of the gel retardation image is shown; probe is always in excess. The oligonucleotide probe is indicated under each gel or lane, and when added, competitors are indicated above individual lanes; –, no competitor. Complexes are indicated with the following symbols (see text): *, CP1; >, NF-E3; ∞, NF-E6 and ●, GATA-1. (**A**) Gel retardation assays in HEL extracts. (**B**) Gel retardation assays in K562 extracts. Note that the NF-E3 complex appears as a doublet. (**C**) Comparison of gel retardation profiles of wild-type and mutant γ and ε CCAAT-box regions. Left panel, HEL extract; right panel, K562 extract.

ε globin gene with 2 kb of 5′ flanking sequence (Li *et al*., 1998b). This mutated construct cannot bind CP1 and is called μLCRε (mCCAAT). This construct was used to produce transgenic mice and analyze ε gene expression during development.

Human as well as mouse ε gene expression in transgenic mice is confined in primitive erythroblasts. The day 10 yolk sac and peripheral blood of these animals are composed exclusively of primitive erythroblasts. At 12 d.p.c., the contribution of cells of fetal liver origin in the periphery is still minimal so that the yolk sac and the peripheral blood of 12 d.p.c. transgenic embryos are composed predominantly of embryonic erythroblasts. The fetal liver of 12 d.p.c. transgenic embryos is composed of definitive erythroblasts and few contaminating embryonic erythroblasts. At day 16, the fetal liver consists only of definitive erythroblasts and there are predominantly definitive red cells in the blood. Therefore, in our studies, we used the 10 and 12 d.p.c. yolk sac and peripheral blood for the analysis of globin expression in embryonic erythropoiesis, and the 12 d.p.c. fetal liver, 16 d.p.c. fetal liver, 16 d.p.c. blood and adult transgenic blood to analyze globin gene expression in definitive erythropoiesis.

The developmental expression of five μLCRε (mCCAAT) lines is shown in Tables I and II. All lines had substantially lower ε gene expression in the embryonic cells compared to the μLCRε controls with wild-type ε promoter. In the day 10 yolk sac and blood, expression was decreased by 71 and 75%, respectively, and in the day 12 blood and yolk sac by 46 and 43%, respectively. As in the controls, ε expression was barely detectable in cells of definitive erythropoiesis. These data indicate that the mutation that prevents CP1 binding at the ε CCAAT box prevents activation of ε gene expression.

The DR-1 elements are involved in repression of the ε-globin gene in definitive erythroblasts

To investigate the role of the DR-1 elements on ε gene expression, the 2DRmut mutation was also introduced into the μLCRε construct, yielding μLCRε (mDR). This construct, which cannot bind NF-E3, was used to produce transgenic mice. Five lines with 1–10 copies of the transgene were analyzed. Of these, one line displayed striking position effects and was excluded from the evaluation of our results (data not shown). Findings in the remaining four lines are shown in Tables I and II.

Epsilon expression in the 10 and 12 d.p.c. embryonic cells of the μLCRε (mDR) mice was ~20–90% higher compared with control mice, raising the possibility that the binding of NF-E3 on the DR-1 sites may repress ε gene transcription. This possibility was demonstrated with the analysis of ε expression in the definitive erythropoiesis of the μLCRε (mDR) mice. In the day 12 fetal liver, ε expression in control mice is ~1–1.5% of murine α and is due to contamination of the fetal liver by embryonic erythroblasts; in contrast, ε expression in the 12 day fetal liver of the μLCRε (mDR) mice was increased 14-fold. Epsilon expression is barely detectable in the 16 d.p.c. hematopoietic tissues of the controls, but in the μLCRε (mDR) transgenic mice it ranged from 8.8 to 16.7% of murine α. In the adult μLCRε (mDR) mice, ε expression was 15- to 40-fold higher than in controls. These results demonstrate that the destruction of the

Table I. Human ε mRNA levels in the embryonic erythropoiesis of transgenic mice with mCCAAT and mDR promoter mutations

			ε mRNA % of murine α + ζ per copy			
			Day 10		Day 12	
Line	Construct	Copy number	Yolk sac	Blood	Yolk sac	Blood
A	mCCAAT	12	6.3 ± 2.8	6.8 ± 2.6	8.3 ± 1.2	9.8 ± 3.3
B		12	3.1 ± 0.38	2.4 ± 0.08	7.4 ± 1.4	9.4 ± 1.7
C		2	5.7 ± 1.7	1.7 ± 0.45	6.8 ± 0.5	5.5 ± 0.6
D		6	5.2 ± 0.5	3.7 ± 0.3	8.2 ± 0.6	7.5 ± 0.02
E		10	5.9 ± 1.6	4.8 ± 1.3	11.8 ± 1.2	9.8 ± 1.1
Mean			**5.2 ± 1.3**	**3.9 ± 2.0**	**8.5 ± 1.9**	**8.4 ± 1.9**
F	mDR	4	17.5 ± 3.0	13.0 ± 0.81	24.8 ± 3.8	23.6 ± 1.6
G		1	27.0 ± 5.0	27.3 ± 6.4	36.7 ± 3.8	31.9 ± 2.8
H		10	19.1 ± 1.8	16.2 ± 0.47	29.5 ± 1.1	22.4 ± 2.0
I		7	22.3 ± 0.99	18.8 ± 0.95	29.4 ± 2.6	28.7 ± 2.2
Mean			**21.5 ± 4.2**	**18.8 ± 6.1**	**30.1 ± 4.9**	**26.7 ± 4.4**
J	μLCRε	3	18.2 ± 1.2	15.5 ± 1.4	15.8 ± 3.0	19.6 ± 1.5

Table II. Human ε mRNA levels in the definitive erythropoiesis of transgenic mice with mCCAAT and mDR promoter mutations

			ε mRNA% of murine α per copy			
			Day 12	Day 16		Adult
Line	Construct	Copy number	Liver	Liver	Blood	Blood
A	mCCAAT	12	2.3 ± 0.53	0.41 ± 0.09	0.39 ± 0.06	0.08 ± 0.05
B		12	1.7 ± 1.1	0.03 ± 0.05	0.07 ± 0.11	0.03 ± 0.01
C		2	1.3 ± 0.12	0.05	0.09	0.08
D		6	1.4 ± 0.3	0.07 ± 0.05	0.10 ± 0.02	0.07 ± 0.02
E		10	1.4 ± 0.3	0.28	0.24	0.11 ± 0.03
Mean			**1.6 ± 0.4**	**0.17 ± 0.17**	**0.18 ± 0.14**	**0.07 ± 0.03**
F	mDR	4	17.8 ± 1.1	8.8 ± 1.1	9.5 ± 2.1	7.2 ± 0.24
G		1	16.3 ± 1.0	10.0 ± 1.8	9.4 ± 1.2	2.4 ± 0.8
H		10	19.1 ± 1.0	12.4 ± 2.1	13.9 ± 1.8	4.4 ± 1.2
I		7	17.9 ± 1.0	14.4 ± 2.6	16.7 ± 1.7	5.9 ± 0.58
Mean			**17.8 ± 1.1**	**11.4 ± 2.5**	**12.4 ± 3.6**	**5.0 ± 2.1**
J	μLCRε	3	1.3 ± 0.26	0.05 ± 0.07	0.18 ± 0.10	0.18 ± 0.07

NF-E3 binding sites in the ε promoter prevents repression of the ε gene in definitive erythropoiesis.

The ε and γ DR-1 elements bind a member of the COUP-TF subfamily

In order to determine the nature of the NF-E3 complex, we included various antibodies in our gel shift assays. The NF-E3 complex was not disturbed by antibodies directed against RXR (α, β, γ), RARα, RARγ or orphan receptor HNF4; however, it was entirely supershifted by anti-COUP-TF antibodies (Figure 3A; data not shown). This was observed both with HEL (Figure 3A, left and right panels) and K562 (Figure 3A, middle panel) extracts. Among the COUP-TF subfamily, the antibodies we used are able to recognize COUP-TF I and COUP-TF II, but not COUPβ. They may, however, be able to recognize unknown COUP-related proteins (Wang *et al*., 1989; Jonk *et al*., 1994). We examined the expression of members of the COUP-TF subfamily in HEL cells by reverse transcription-polymerase chain reaction (RT–PCR) analysis. This showed that COUP-TF II, but neither COUP-TF I nor COUPβ, is highly expressed in this cell line (Figure 3B). In order to ensure that the NF-E3 complex contains COUP-TF II, we transfected an expression vector for COUP-TF II (kindly donated by Dr M.-J.Tsai) into COS cells, and used a protein extract from these cells in a gel retardation assay. Figure 3C shows that electrophoretic mobility of the complex obtained on either RARE 2 or ε CCAAT/DR oligo was similar in transfected COS extracts and HEL extracts. We also isolated two full-length COUP-TF II cDNAs by screening a HEL cDNA library (data not shown).

COUP-TF II is expressed in ε and γ erythroid tissues

We then analyzed expression of COUP-TF I and COUP-TF II during development. In the mouse, a first generation of hematopoietic precursors appears in the yolk sac, beginning at 7.5 d.p.c., which gives rise to primitive erythroid cells. Slightly later, an independent generation of hematopoietic precursors occurs in the intra-embryonic splanchnopleura (SP; Cumano *et al*., 1996). These precursors are most probably responsible for the establishment of definitive hematopoiesis. Following their emergence in the SP, hematopoietic precursors are present in the para-aortic splanchnopleura (PSP; Godin *et al*., 1995) and then in the aorta-gonad-mesonephros (AGM) region (Muller *et al*., 1994; Medvinsky and Dzierzak, 1996). These intra-

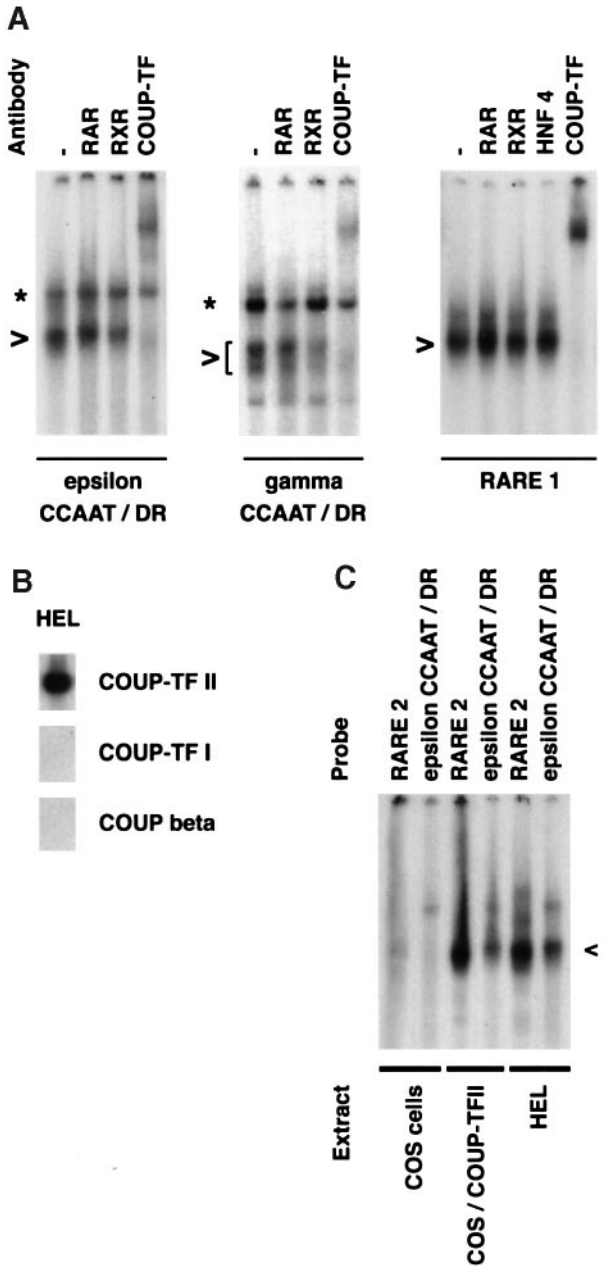

Fig. 3. (**A**) The NF-E3 complex is recognized by anti-COUP-TF antibodies. Antibodies specific for RAR, RXR or COUP proteins (see text) were included in gel retardation assays. Left and right panels, HEL extract; middle panel, K562 extract. (**B**) RT–PCR analysis of the expression of COUP-TF II, COUP-TF I and COUP β in HEL RNA. (**C**) Gel retardation assay with oligos RARE 2 and ε CCAAT/DR in extracts from untransfected COS cells, COS cells transfected with a COUP-TF II expression vector, and HEL cells. The major retarded complex is shown (<). The minor upper band seen with the ε probe corresponds to CCAAT-binding proteins.

embryonic precursors are responsible for colonization of the fetal liver, together with yolk sac-derived cells. From 10.5 d.p.c., the expansion and differentiation of definitive hematopoietic precursors is carried out in the fetal liver, then progressively shifts to the bone marrow, which becomes the major hematopoietic site at the end of gestation. COUP-TF II mRNA was readily detected in the earliest two sites of murine embryonic hematopoiesis, the yolk sac and intra-embryonic splanchnopleura. A weak signal could be detected at 7.5 d.p.c. in the splanchnopleura, which increased between 7.5 and 9.5 d.p.c. in both splanchnopleura and yolk sac (Figure 4A). As circulation is established at around 8.5 d.p.c., both sites contain primitive and definitive progenitors at 9.5 d.p.c. (Cumano *et al*., 1996). Therefore, it is not possible to determine whether COUP-TF II expression stems from primitive or definitive cells. Embryoid bodies (EB) derived from murine embryonic stem cells at day 5 (Mitjavila *et al*., 1998) also co-express εY-globin and COUP-TF II (Figure 4A). As we were interested in globin gene switching, we also performed a detailed analysis of εY-globin, β major-globin, COUP-TF I and COUP-TF II during fetal liver development. In this organ, expression of εY is contributed by circulating primitive erythroblasts, whereas βmaj expression comes from fetal liver definitive erythroblasts. Figure 4 shows that COUP-TF II expression rises around 11 d.p.c., when εY is already highly expressed, peaks at 12 d.p.c., then decreases in parallel with εY from 13 d.p.c. COUP-TF II mRNA is almost undetectable in purified (human) adult hematopoietic progenitors ($CD34^+CD38^-$ and $CD34^+CD38^+$), erythroblasts or megakaryocytes (Figure 4A).

The structure, affinity and abundance of nuclear receptor complexes vary during development

We performed gel retardation experiments with the high-affinity RARE 2 probe and the medium-affinity ε CCAAT/DR probe in nuclear extracts from HEL cells, murine erythroleukemia (MEL) cells (which express adult globins; see Materials and methods), as well as from day 12.5 and day 14.5 mouse fetal liver (FL 12.5 and FL 14.5; Figure 5). Nuclear receptor complexes formed on the RARE 2 (Figure 5A) and ε CCAAT/DR (Figure 5B) oligos were comparable in HEL and FL 12.5 extracts. The complexes found in fetal liver were again displaced by anti-COUP-TF antibodies. A different picture emerged from the analysis of FL 14.5 and MEL extracts. In both cases, nuclear receptor complexes were much less abundant and had increased mobility (Figure 5A, last two panels); most, but not all, were displaced by anti-COUP-TF antibodies, and the supershifted band also displayed a change in mobility. A middle complex can be seen with FL 14.5 extract, but not MEL extract and may actually originate from non-hematopoietic cells present in day 14.5 fetal liver. It also appears that the ε CCAAT/DR and γ CCAAT/DR oligos do not displace the complexes formed on RARE 2 as efficiently in FL 14.5 as in FL 12.5 (Figure 5A). When ε CCAAT/DR is used as a probe, it forms very weak nuclear receptor complexes compared to RARE 2 in FL 14.5 extract (Figure 5). Although our present data do not allow us to conclude on the exact structure of COUP-TF complexes, it is clear that profound alterations in the nature, abundance and affinity of these complexes occur during development.

Discussion

The CCAAT-box region of ε/γ globin genes contains DR-1 elements

In nuclear extracts of erythroid cells and tissues, the ε and γ CCAAT-box regions are recognized by proteins with the same sequence specificity as non-steroid nuclear

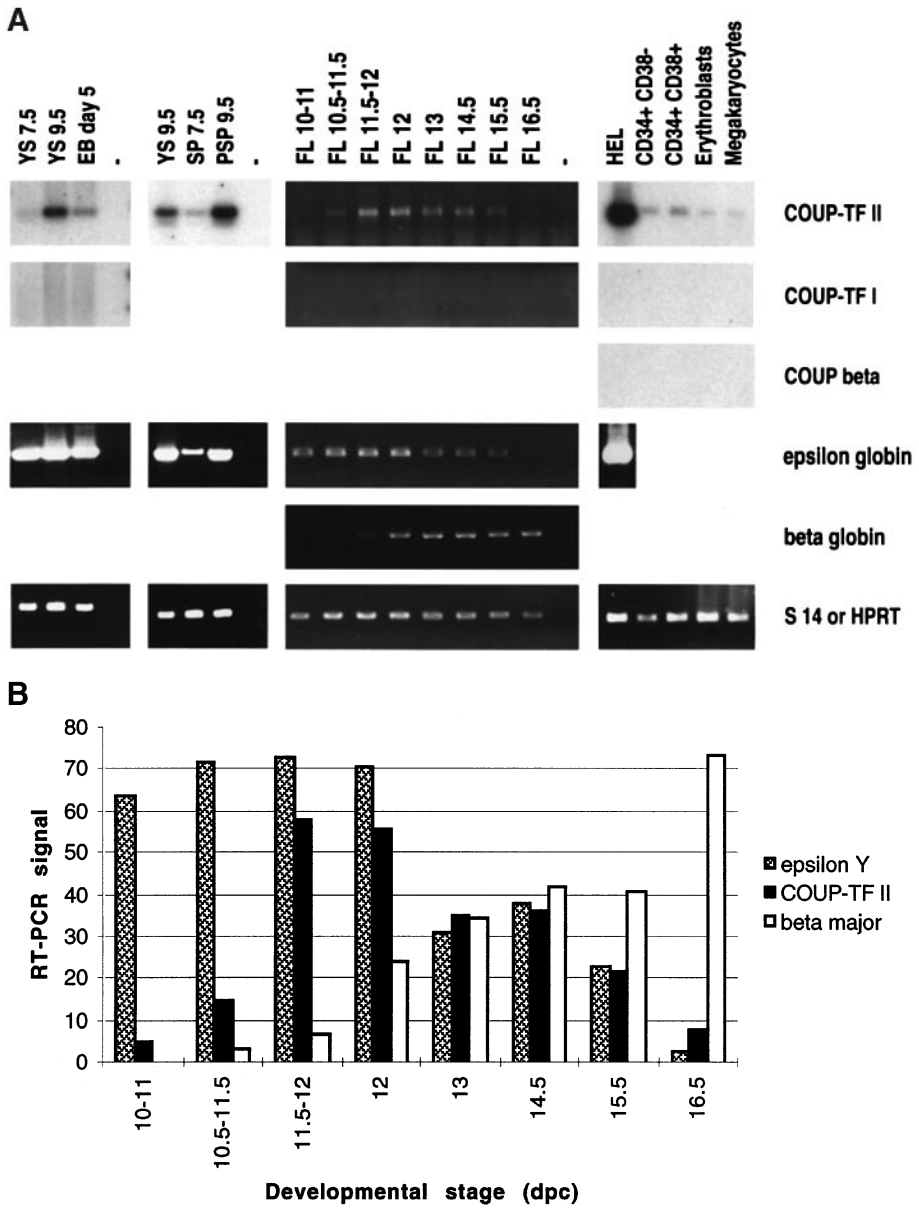

Fig. 4. (**A**) RT-PCR analysis of the expression of COUP-TF II, COUP-TF I, COUP β, ε (for HEL) or εY (for murine tissues) globin, β major-globin and, as controls, S14 ribosomal RNA (for human hematopoietic cells, last set of RNAs) or hypoxanthine phosphoribosyl transferase (HPRT, for murine tissues). See text and Materials and methods for details. YS, embryonic yolk sac; EB, embryoid bodies from embryonic stem cells; SP, intra-embryonic splanchnopleura; PSP, para-aortic splanchnopleura; FL, fetal liver; for murine embryonic tissues; numbers indicate d.p.c. (**B**) Diagram showing the amount of PCR product for εY-globin, COUP-TF II and βmaj-globin at different stages of fetal liver development. The amount of HPRT PCR product was set as a constant.

hormone receptors. The embryonic α-like promoter ζ also contains a DR-1 motif in its CCAAT-box region (Figure 1), which forms a similar complex with low affinity (data not shown). On the other hand, the β-globin CCAAT-box region is unable to form this complex (our data not shown; deBoer *et al*., 1988; Delvoye *et al*., 1993). We have shown that the specific destruction of the DR-1 elements in the ε-globin promoter is sufficient to create a persistence of ε expression at the adult stage. This result is reminiscent of the effects of the –117 mutation in the ^{A}γ-globin promoter, although the complex pattern of protein binding on the γ promoter made its analysis difficult (Ronchi *et al*., 1996). Due to the higher affinity of NF-E3 for the ε CCAAT region, multiple point mutations were necessary to destroy the DR-1 elements in this promoter. Therefore, although ε and γ have very different expression profiles during human development, some of the mechanisms that ensure their repression at the adult stage are shared by these two genes.

Nuclear hormone receptors are transcriptional switches

Ligand-dependent non-steroid nuclear hormone receptors can bind DNA irrespective of the availability of ligand. In the absence of ligand, they recruit co-repressors such as N-CoR, SMRT or SUN-CoR (Chen and Evans, 1995; Hörlein *et al*., 1995; Zamir *et al*., 1996) and histone deacetylases such as mRPD3/HDAC1 (Heinzel *et al*., 1997; Nagy *et al*., 1997). In the presence of ligand, they undergo conformational changes (Moras and Gronemeyer,

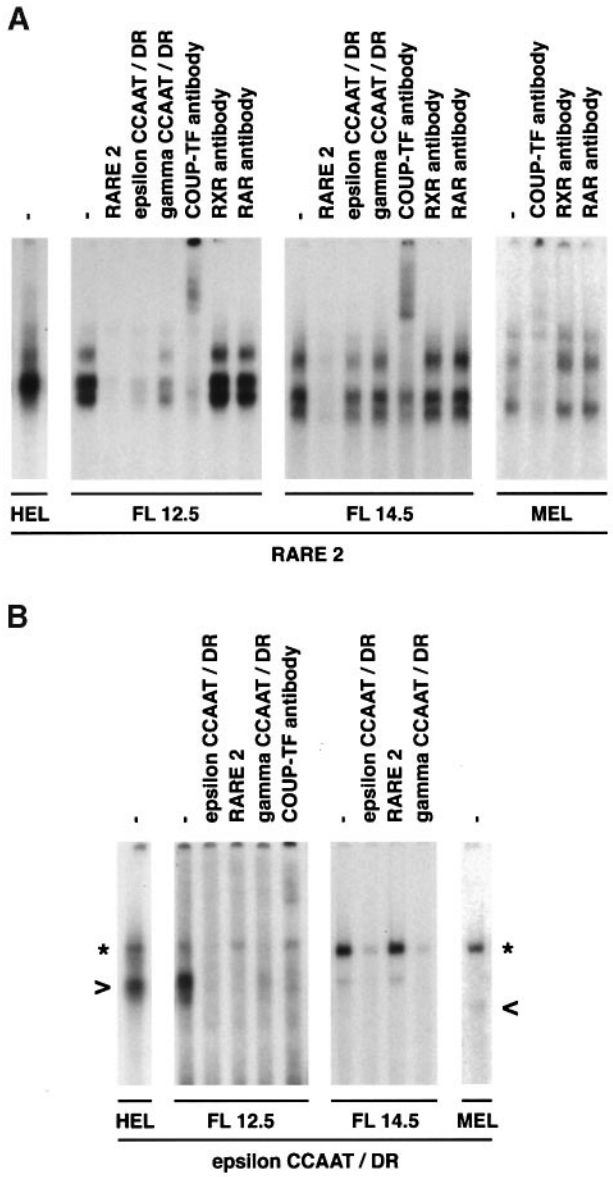

Fig. 5. Gel retardation assays in extracts from HEL cells, 12.5 and 14.5 d.p.c. fetal liver, and MEL cells. Competitors and antibodies, when added, are indicated above individual lanes. (**A**) RARE 2 probe, (**B**) ε CCAAT/DR probe.

1998) and recruit co-activators such as Trip1 (Lee *et al*., 1995), TIF1 (Le Douarin *et al*., 1995), TRAPs (Fondell *et al*., 1996), CBP/p300 (Kamei *et al*., 1996) and ACTR (Chen *et al*., 1997). These, in turn, interact with histone acetyl-transferases (HAT) or exert HAT activity by themselves (Ogryzko *et al*., 1996). These properties allow nuclear receptors to control cellular decisions. For instance, the thyroid hormone receptor (RXR-TR) is involved in avian erythroid differentiation (for a review see Gandrillon *et al*., 1995; Beug *et al*., 1996). In the absence of thyroid hormone, overexpression of TR orients avian erythroid progenitors toward proliferation and blocks differentiation; in the presence of physiological concentrations of hormone, overexpression of TR accelerates differentiation, activates target genes and induces a decrease in histone deacetylase activity (Bauer *et al*., 1998).

Orphan nuclear receptors, on the other hand, have no known ligand. Some behave as activators, such as HNF-4 (Sladek, 1994) and others, like COUP-TF proteins, are often considered as constitutive repressors (for a review see Tsai and Tsai, 1997). COUP-TF I has been shown to interact with co-repressors SMRT and N-CoR (Shibata *et al*., 1997). COUP-TF II is also able to interact with a co-repressor, RIP13Δ1 (Bailey *et al*., 1997). However, COUP-TF proteins have been shown to act as transcriptional activators in a few cell types. They can activate the transferrin, fatty acid-binding protein, mouse mammary tumor virus, vHNF-1 and ornithine transcarbamylase promoters (for a review, see Tsai and Tsai, 1997). Therefore, it is possible that these orphan receptors can also function as transcriptional switches.

What is the role of COUP-TF II in erythroid cells?

Our results show that COUP-TF II is highly expressed in K562 and HEL cells, together with ε- and γ-globin, which argues against a constitutive repression by COUP-TF II. In support of this, we have characterized DR-1 elements in another hematopoietic promoter, on which COUP-TF II acts as a weak activator in HEL cells (A.Filipe and V.Mignotte, unpublished). COUP-TF II is also expressed in the yolk sac and para-aortic splanchnopleura at 9.5 d.p.c. when blood contains only primitive erythroblasts. It is not clear, however, whether non-erythroid cell types contribute to COUP-TF II expression in these sites.

The murine fetal liver contains both primitive erythroblasts (that contain εY- and βh1-globin) and definitive erythroblasts (that express βmaj- and βmin-globin). Although the switch between embryonic and adult globin gene transcription occurs in a narrow window of time around 12 d.p.c., primitive erythroblasts can still be detected at 16.5 d.p.c. (Fraser *et al*., 1998). Therefore, RT–PCR experiments indicate the quantity of globin mRNA present at each stage, but not the transcriptional activity of the corresponding genes. They also do not indicate whether COUP-TF II is expressed in primitive cells, definitive cells or both. Interestingly however, COUP-TF II mRNA is detectable in 10.5 day fetal liver and its level increases 10-fold between 10.5 and 12 d.p.c., then decreases again, in parallel with εY mRNA (Figure 4B). Therefore, the peak in COUP-TF II expression coincides with the embryonic-to-adult switch. Such an expression profile suggests that COUP-TF II may trigger repression of embryonic globin genes, because of a threshold effect and/or because of recruitment of co-repressors and histone deacetylases. The observed alteration of COUP-TF-related complex mobility between 12.5 and 14.5 d.p.c. suggests that COUP-TF II undergoes a conformational change or that it is replaced by an unknown member of the COUP-TF subfamily in late development. Additional experiments will be necessary to answer these questions.

It has been clearly shown that the 5′ end of both the ε and γ genes contain numerous sequences that are necessary for repression at the adult stage. Most of these regions seem to differ between these genes, which is coherent with the fact that they are expressed with different profiles. However, destruction of one region or motif can be sufficient to allow a high level of expression at the adult stage, suggesting that several non-redundant mechanisms are involved. It is therefore possible that

COUP-TF II induces an initial (and transient) chromatin condensation which would be followed by the action of other repressors at other sites. COUP-TF II-deficient embryos die at 10 d.p.c. (M.-J.Tsai, personal communication), which precludes the analysis of their erythroid development. Again, additional experiments that include overexpression of COUP-TF II in adult erythropoiesis or *in vitro* differentiation of COUP-TF II-deficient ES cells are needed to understand the exact contribution of COUP-TF II to hematopoietic development.

Materials and methods

Cell lines

HEL cells express erythroid and megakaryocytic markers (Villeval *et al*., 1985; Deveaux *et al*., 1997). Our clone of this cell line, 5J20 (provided by Dr William Vainchenker) expresses relatively high levels of both γ- and ε-globin. K562 cells derive from acute erythroblastic leukemia in a chronic myeloid leukemia patient (Lozzio and Lozzio, 1975); they express erythroid markers including embryonic and fetal globins, but neither adult globins nor megakaryocytic markers (Villeval *et al*., 1985; Deveaux *et al*., 1996). MEL cells derive from Friend viral complex-induced leukemia; they express adult, but not embryonic or fetal globins (Friend *et al*., 1971).

Oligonucleotides

The oligos used for gel retardation and/or mutagenesis are shown in Figure 1. The following were used for RT–PCR (sense and antisense). Human ε-globin: 5′ CAG GAT CCA GCA CAC ATT ATC ACA AAC 3′ and 5′ ACC CTT CAT TCC CAT GCA TTG AGA A 3′; mouse εY: ATG GCA AGA AGG TGC TGA CTG CTT and TGT GCA GAG AGG AGG CAT AGC GGA; mouse β major-globin: CTG ACA GAT GCT CTC TTG GG and CAC AAC CCC AGA AAC AGA CA; human COUP-TF II: CCC AGC CAG CAC GCC AGC CC and CAC CGC TTC CCG TCT CAT GC; mouse COUP-TF II: CCC GCC CAG CAC GCC GGC CCA and TAC AGC TTC CCG TCT CAT GCC C; human COUP-TF I: CAG CAG CAG GCG GGC TCG GG and GGT GGT GCT GGT CGA TGG GA; mouse COUP-TF I: CCC GGA GCG CCC GCC ACC CCC and TCG CTG AAC CGC TTC CCG CCT C; and human COUP β: GAC ACG AAC GGC GTG GAC AA and ACT TCT TGA GAC GGC AGT AC.

Transgenic mice

Transgenic mice carrying the pμLCRε mDR and pμLCRε mCCAAT constructs were produced as described previously (Li and Stamatoyannopoulos, 1994). Founder animals were identified by slot blotting with a HS3 probe. F_1 progeny were obtained by breeding founder animals with non-transgenic mice and were screened for correct integration and to exclude the presence of mosaicism in the founders. To study the developmental pattern of human ε gene expression, staged pregnancies were interrupted on days 10, 12 and 16 of development. Samples from blood and yolk sac were collected on day 10 embryos; blood, yolk sac and liver were collected on day 16 fetuses.

ε mRNA quantitation

Total RNA was isolated from transgenic tissues as described (Chomczynski and Sacchi, 1987). The ε mRNA level was measured by the quantitative RNase protection assay described previously (Li and Stamatoyannopoulos, 1994). Briefly, riboprobe for ε mRNA was labeled by transcribing the linearized plasmid pT7ε(188) using T7 RNA polymerase. The ε probe protects a 188 nucleotide fragment in exon 2 of ε mRNA. The mouse α and ζ riboprobes were used in RNase protection assays as internal globin mRNA controls. mRNA levels were determined in all transgenic siblings of each litter. RNA samples from different tissues were analyzed at least twice in order to reduce experimental error in mRNA quantitations. Human ε, and mouse α and ζ signals were quantitated with a PhosphorImager (Molecular Dynamics). Levels of human mRNA per transgene copy were expressed as percentages of mouse α-like mRNA levels per copy, taking into account that the mouse possesses four copies of the α-globin gene and two copies of the ζ-globin gene. In the adult, murine α mRNA per copy was calculated by dividing the levels of murine α mRNA by four.

Copy number determination

Copy number determination was accomplished by the multiply redundant protocol described previously to reduce experimental errors. Multiple DNA samples were obtained from each of at least three animals from each transgenic line. These samples were digested with restriction enzyme *Eco*RI and resolved by electrophoresis over a 1% agarose gel. Southern blots were hybridized with a radiation-labeled ε probe (0.6 kb *Bam*HI fragment). The signals were quantitated on a PhosphorImager. Copy numbers were calculated by determining the relative intensity of signals from a given transgenic line compared with the signals obtained from diploid human genomic DNA.

Other methods were carried out as described previously: dignam nuclear extract preparation (Wall *et al*., 1988), gel retardation assays (deBoer *et al*., 1988) and supershifts (Rochette-Egly *et al*., 1994). Specific mutations in the ε-globin promoter were introduced via recombinant PCR (Deveaux *et al*., 1997). All mutagenized fragments were entirely sequenced.

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