

THE ROLE OF P22 NF-E4 IN HUMAN GLOBIN GENE SWITCHING

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SUMMARY

The human stage selector protein (SSP), a complex containing the ubiquitous transcription factor CP2, and the erythroid-specific factor p22 NF-E4, facilitates the interaction of the γ -globin genes with the LCR in fetal erythroid cells. Enforced expression of p22 NF-E4 in K562 cells and human cord blood progenitors increases fetal globin gene expression, and in progenitors, reduces β -globin expression. To examine the role of NF-E4 in an *in vivo* model of hemoglobin switching, we enforced the expression of p22 NF-E4 in transgenic mice carrying the human β -globin locus YAC. Although murine erythropoiesis and globin gene expression is unaffected in these mice, the expression profile of the human globin genes is altered. All three transgenic lines displayed an increased γ : β -globin ratio in d12.5-d14.5 fetal liver, resulting in a delay in the fetal/adult switch. At d12.5, this is primarily due to a reduction of β -gene expression, whereas at d14.5, the increased γ : β ratio is due to enhanced γ -gene expression. Despite this, the switch in globin subtype is fully completed in the adult bone marrow. These findings indicate that p22 NF-E4 is capable of influencing human globin gene expression *in vivo*, but is incapable of overriding the intrinsic mechanisms governing γ -gene silencing in this context.

INTRODUCTION

Expression of the human β -like globin genes (ϵ , γ^G , γ^A , δ , β) is co-ordinately regulated throughout human development under the influence of both local and distal transcriptional control elements. The high level of gene activation observed at each developmental stage is dependent on the presence of key regulatory sequences 6-20 kb upstream of the ϵ -gene (1-4). These sequences, characterised by the presence of five DNaseI hypersensitivity sites (5'HS1-5), are known as the Locus Control Region (LCR) (5-7). The HSs act cooperatively as a holocomplex which focuses the LCR to a single globin gene at any given time point during ontogeny (8). Recent data suggests that the primary role of the LCR is to enhance the transition from transcription initiation to elongation (9). However, the LCR does not provide the temporal specificity of globin gene expression, as transgenic mice lacking this region still exhibit appropriate developmental switching (10). Similarly, mice lacking the endogenous LCR retain the normal developmental profile of globin gene expression, albeit at markedly reduced levels (11). From these studies it is evident that the sequences conferring stage-specific expression reside in the regions immediately flanking the globin genes.

Several diverse regulatory mechanisms govern the highly restricted pattern of globin gene expression. These include: the gene order within the locus (12, 13); competition between globin gene promoters for the enhancer sequences of the LCR (14-16); the transcription factor milieu at different developmental stages (17-19); and tissue and stage-specific chromatin remodelling (20-22). Studies in transgenic mice have emphasised the importance of gene order and promoter competition. Transgenic lines carrying a β -gene in a more proximal position or lines in which an isolated β -gene is linked to the LCR show promiscuous adult globin expression in yolk sac and fetal liver (12, 14, 16). Correct temporal regulation of β -gene expression is restored in lines in

which the ϵ - and/or γ -genes are linked more proximally in cis. In contrast, the ϵ - and γ -globin genes are silenced autonomously in transgenic mice, requiring no linked globin gene for correct stage-specific expression (23, 24).

The concept of promoter competition for single enhancer elements was first proposed in the chicken globin system (15). The preferential expression of the β -globin gene in this system was mediated by a stage selector element (SSE) in proximal β -promoter. We defined analogous elements in the human γ -globin promoters that provide these promoters with a competitive advantage over the β -promoter in fetal erythroid cells (25). The human SSE lies immediately adjacent to the γ -TATA box and binds a fetal/erythroid transcription factor complex known as the stage selector protein (SSP) (17). The SSP consists of a complex between the ubiquitous transcription factor CP2, an erythroid-specific partner p22 NF-E4 (17, 26).

The in vivo role of developmental transcription factors in human globin gene competition is best characterised in studies of EKLF^{-/-} mice transgenic for the human β -globin locus (18, 19). These mice exhibit a significant increase in the number of transcriptionally active γ -genes in the fetal liver in parallel to the dramatic reduction in β -globin expression. Despite this, the majority of the cells transcribing the γ -genes have only one active locus suggesting that silencing of the other locus had occurred independently of promoter competition (19).

The SSP is a candidate factor for mediating the preferential expression of the γ -genes in the fetal liver. Deletion of the SSE in mice transgenic for the human β -globin locus, results in an increase in the $\beta/\gamma+\beta$ ratio in d12-d16 fetal liver. This effect is lost when these lines are crossed with EKLF \pm mice indicating that a balance of fetal and adult stage-specific factors governs competition between the promoters for the LCR (27). To further examine the importance of this balance, we have now generated transgenic mice in which the expression of NF-E4 has been

enforced in the context of the β YAC. These animals display an increase in the γ/β ratio in d12.5-d14.5 fetal liver, but still complete the γ/β switch in adult bone marrow.

EXPERIMENTAL PROCEDURES

Production and screening of transgenic mice

The NF-E4 transgenic construct was generated with a 600 bp *EcoRI* fragment containing the coding region of the p22NF-E4 cDNA, 3' end tagged with the HA epitope sequence. The internal ATG codon at position 101 was mutated using PCR and the final cDNA was sequenced and cloned into the *EcoRI* site of pCAGGS. The transgenic insert was prepared by cutting the plasmid with *Hind III/Pvu I* and purified from the gel for microinjection. 75 μ l of the plasmid at 2 μ g/ml was injected into a fertilised egg of C57BL/6 mice and implanted into C57BL/6 recipient mice as described (28). Tails were clipped at weaning and genomic DNA extracted for genotyping. Animals were screened by Southern blotting of *PstI*-digested DNA using the 0.3kb *PstI/EcoRI* p22 NF-E4 fragment as a probe. Founder animals were crossed to C57BL/6 mice to establish transgenic lines. Pups or embryos were tested for presence of the transgene by Southern blot analysis or PCR analysis of tail or embryo genomic DNA. PCR was performed with NF-E4 sense primer 5'- ACC CGG GAG GGG CTC CGG TCT T - 3' and NF-E4 antisense primer 5' - CCC TTG GCT CAG ATG AAG CGA TGG TAG T - 3';

Total cellular RNA was extracted from mouse tissues, including bone marrow, spleen, heart and kidney using TRIZOL reagent (Gibco BRL, Life Technologies, Inc., Grand Island, N.Y.). The expression of NF-E4 was analyzed using Northern blotting, primer extension and RPA. To detect the expression of transgene at protein level, spleen cell lysates were immunoprecipitated using anti-HA monoclonal antibody and blotted with antisera against NF-E4. Detection of the

immunoprecipitated proteins was achieved using the ECL detection system according to the manufacturers instructions (Amersham Pharmacia).

Analysis of globin production in transgenic mice

Transgenic mice carrying a β YAC have been described previously (29). Genotyping of β YAC transgenic mice was carried out by PCR performed with γ -globin sense primer 5'- AAG CTC CTA GTC CAG ACG CCA-3' and γ -globin antisense primer 5'-GGC CAC TCC AGT CAC CAT CTT-3'. To study the effects of NF-E4 on globin gene expression, staged pregnancies were interrupted at days 10.5, 12.5, 14.5, and 16.5 of development. Samples from blood and yolk sac from embryos at day 10.5 and liver from fetuses at days 12.5, 14.5, and 16.5 were collected. Total cellular RNA was extracted from 10.5-day embryo yolk sac, 12.5, 14.5 and 16.5-day fetal livers, adult bone marrow and spleen using TRIZOL reagent. Primer extensions and RPA were performed as described (30). Primers used were human γ - 5' - CCAGCATCTTCCACATTCACC - 3'; human β - 5' - AGACGGCAATGACGGGACACC - 3'; mouse α - 5' - CAGGCAGCCTTGATGTTGCTT - 3'; mouse β maj - 5' - TGATGTCTGTTTCTGGGGTTGTG - 3'; mouse β h1 - 5' - ATAGCTGCCTTCTTCCTCAGCT - 3'. Probes used were as described previously (31).

Extract preparation and Western analysis

Nuclear extracts were prepared by the method of Dignam as previously described (32). For Western analysis, nuclear extract or fetal liver whole cell lysate were resolved by SDS-PAGE using a 12% gel. After transfer to PVDF, the samples were immunoblotted with specific antiserum and developed with ECL.

Semisolid culture of fetal liver CFCs

Cultures were performed as described by Metcalf (33) and enumerated *in situ* using a dissection microscope after staining for hemoglobinized erythroid colonies with 2, 7-diaminofluorene.

RESULTS**Generation of transgenic lines expressing human NF-E4**

To examine the role of NF-E4 in hemoglobin switching in an *in vivo* model, we initially generated transgenic mouse lines in which the expression of NF-E4 was driven off the β -spectrin promoter to confine expression to the erythroid compartment at all developmental stages (34). Unfortunately, despite deriving numerous transgenic lines, we were unable to identify a single line in which a significant increase in NF-E4 expression was observed (data not shown). We therefore turned to the pCAGGS system (containing the chicken beta-actin promoter and cytomegalovirus enhancer, beta-actin intron and rabbit globin polyadenylation signal) that has been widely used in the generation of transgenic lines. These regulatory elements drive high-level expression of linked genes in virtually all tissues throughout development (35). The NF-E4 cDNA initiates at a non-AUG codon and contains a single methionine at codon 101 that is the predicted initiation codon for a shorter 14 kD isoform, observed in K562 cells, fetal liver and bone marrow (26). We inserted the NF-E4 cDNA tagged at the 3' end with the hemagglutinin epitope (HA), and containing a mutation of methionine at codon 101 into our vector construct (Fig. 1A). Five independent lines were generated using this construct, and NF-E4 RNA expression was observed in a variety of organs in four of these lines (#4, 26, 33 and 34) (Fig. 1B). Expression of NF-E4 was noted to be low in the bone marrow and spleen of line 26. To confirm expression of the transgene at protein level, whole cell lysates from splenocytes were obtained from all lines and immunoprecipitated with anti-HA antisera and blotted with anti-NF-E4 antisera

(Fig. 1C). In accordance with our RNA data, p22 NF-E4-HA protein expression was readily detected in lines 4, 33 and 34. No expression was detected in line 5 and low levels were detected in line 26, and so these lines were not studied further. To examine the expression of the transgene during erythroid development, we obtained RNA from yolk sac (E10.5), fetal liver (E12.5-E16.5) and adult bone marrow from line 33 and control wild-type littermates and performed RNase protection using an NF-E4 probe (Fig. 1D). Abundant message was present at all stages of erythroid development in the transgenic mice. As expected, no signal was detected in the wild-type control mice, as no murine homologue of NF-E4 has been identified.

Enforced expression of p22 NF-E4 does not affect murine erythropoiesis or globin gene expression

All NF-E4 transgenic mice appeared healthy and were fertile, indicating that enforced expression of p22 NF-E4 outside its normal tissue range had no apparent adverse effects. To ensure that expression of the transgene did perturb erythropoiesis, we initially examined the full blood counts of NF-E4 transgenics and their littermate controls. The hematocrit and reticulocyte counts were identical in the two groups (Fig. 2A), and all other parameters, including the red cell indices were also identical (data not shown). To extend these studies, we examined the immunophenotypes of erythroid cells from E13.5 fetal livers from NF-E4 transgenics and littermate controls, co-staining for Ter-119 and CD71 (Fig. 2B). This combination of cell surface markers can be used to discriminate several stages of erythroid maturation (36). The relative and absolute numbers of each erythroid phenotype was indistinguishable between the two groups. To further examine erythropoietic activity in these animals we performed colony forming assays on fetal liver cells from NF-E4 transgenic and control E13.5 embryos (Fig. 2C). The frequency and behaviour of BFU-E colonies was identical in the two groups, as were myeloid CFCs (G-CFC, GM-CFC and

M-CFC). These results indicate that hematopoiesis was unaffected by the presence of the NF-E4 transgene, and in particular, the mice exhibited no evidence of a stress erythropoiesis response. We next examined the profiles of murine globin gene expression in the transgenic mice and wild-type control littermates (Fig. 2D). We observed a small increase in β h1 expression in d12.5 fetal liver in line 33, but no other change in the level or timing of globin gene expression was observed in the transgenic lines compared to controls. This finding indicated that enforced expression of p22 NF-E4 did not significantly influence murine erythroid maturation or development.

Enforced expression of p22 NF-E4 delays the human γ/β switch in transgenic mice

To determine the effects of enforced expression of p22 NF-E4 on human globin gene expression we crossed our transgenic lines (lines 4, 33 and 34) with mice homozygous for the β -globin locus on a yeast artificial chromosome (β YAC) (29) (a kind gift of Dr K. Gaensler). Yolk sac (E10.5), fetal liver (E12.5-E16.5) and adult bone marrow RNA were obtained and analysed by RNase protection using probes specific for the human γ -, and β -globin genes. A murine α -globin probe served as the loading control (Fig. 3A-C). In the d10.5 yolk sac, in which the γ -genes are highly expressed and the β -gene is silent, increased levels of p22 NF-E4 had no additional effect on γ -gene expression. Although there was some minor variability between the lines, at d12.5-d14.5 when the γ - and β -genes are actively competing for the LCR, increased levels of NF-E4 resulted in an increase in the γ/β ratio in all three NF-E4+/ β YAC+ transgenic lines (line 33 - 3A; line 4 - 3B; and line 34 - 3C and summarised in Fig. 4A) compared to the NF-E4-/ β YAC+ littermate controls. The increased γ/β ratio was due to different mechanisms at the respective time points (Fig. 4B and C). At d12.5, the altered ratio was predominantly due to a decrease in the level of β -gene expression as evidenced by the decrease β/α ratio in fetal liver (Fig. 4C, panel 1). This was most marked in lines 4 and 33, where expression of the β -gene at d12.5 was almost completely

absent. Line 34 also displayed a reduction in β -gene expression at this time point, although this line also demonstrated increased γ -gene expression at d12.5. In the d14.5 fetal liver, the increased γ/β ratio was predominantly due to a significant increase in γ -gene expression, as evidenced by the increased γ/α ratio at this time point (Fig. 4B, panel 2). Again, this was most marked in lines 4 and 33, but also present in line 34. Overall, we observed an approximate 6-fold increase in the γ/β ratio at d12.5 and a 4-fold increase at d14.5 (Fig. 4A). These reciprocal changes suggest an NF-E4-induced shift in the competitive balance between the fetal and adult globin genes to favour the fetal genes. Despite this, the γ/β -switch was completed in the adult bone marrow of all NF-E4+/ β YAC+ mice.

The effect of EKLF dosage on fetal globin expression in the NF-E4+/ β YAC+ mice

The erythroid transcription factor, EKLF has been shown to be a critical determinant in the activation of the β -globin gene and to play a key role in the competition between the γ - and β -genes in the fetal liver (18, 19, 37, 38). To determine the effect of EKLF dosage on human globin gene expression in the context of enforced expression of NF-E4, we crossed the NF-E4 transgenic mice with EKLF^{+/-} mice (a kind gift of Dr S. Orkin) to obtain EKLF^{+/-}/NF-E4+ mice. These were subsequently crossed with the homozygous β YAC mice and human globin gene expression examined in the d12.5 and d16.5 fetal liver (Fig. 5). Both the presence of the NF-E4 transgene, and a dose reduction of EKLF increased the level of fetal globin expression at d12.5 in isolation. The combination of the two resulted a small additional increase in γ -gene expression at this time point. In contrast, loss of one EKLF allele at d16.5 had a more marked effect on γ -gene expression than the NF-E4 transgene, and no additive effect was observed in the context of altered expression of both transcription factors.

DISCUSSION

The SSE has been shown in human cell lines and in transgenic mice to contribute to the preferential expression of the γ -genes when in competition with the β -genes for the enhancer sequences of the LCR (25, 27). Enforced expression of p22 NF-E4, the erythroid-specific component of the SSP, augments fetal globin expression in the γ -globin producing cell line, K562 (26). In human cord blood progenitors, in which both γ - and β -genes are expressed, enforced expression of p22 NF-E4 has modest effects on γ -gene output, but significantly reduces β -gene expression (26). We now demonstrate that enforced expression of p22 NF-E4 in mice transgenic for the human β -globin locus has similar effects to those observed in cord blood, shifting the balance between the γ - and β -globin genes to favour the fetal gene. In the d10.5 yolk sac, in which the γ -genes are highly expressed and the β -gene is silent, p22 NF-E4 appears to have no effect on γ -gene expression. Similarly, at d12.5, when γ -gene expression still predominates, the change in the γ/β ratio was primarily due to a reduction in β -gene expression. In contrast, at d14.5, when γ -gene silencing has commenced the increased γ/β ratio is predominantly due to elevated levels of γ -gene expression. This suggests that when the two genes are actively competing for the LCR, increased levels of NF-E4 swing the balance further in favour of the fetal genes by maintaining repression of the β -gene. Once the intrinsic processes to silence γ -gene and activate β -gene expression commence, the elevated levels of p22 NF-E4 serve to maintain the active state of the fetal gene rather than repress the adult globin gene. These effects are observed in the absence of any alteration in murine erythropoiesis or globin gene expression, highlighting the specificity of NF-E4 in human fetal globin regulation. Our findings suggest that the predominant role of p22 NF-E4 is to direct the LCR to the γ -promoter in the setting of globin gene competition, and to maintain γ -gene expression in the setting of intrinsic silencing.

The data obtained from mice carrying a human β -globin mini-locus with a deletion of the SSE support our conclusions (27). These animals display no reduction in γ -gene expression prior to the onset of gene competition, but then display a significant increase in the $\beta/\gamma+\beta$ ratio in the fetal liver from d12 to d16. These findings also indicate that this region is not a typical promoter element but functions to provide an advantage for the γ -promoter in the setting of gene competition. This is reflected by the reduced number of transcriptional events detected by primary transcript FISH occurring at the γ -gene versus the β -gene in mice carrying the SSE deletion (27). We are currently examining whether enforced expression of p22 NF-E4 also increases the number of active loci using this technique.

Our studies provide further insight into the balance that exists between p22 NF-E4 and EKLF in the control of globin gene switching. A reduction in EKLF levels in the context of normal gene competition results in a shift in the balance toward the γ -promoter with prolonged and increased levels of γ -gene expression (18, 19). Similarly, loss of the SSE shifts the balance away from the γ -promoter towards the adult β -gene (27). When EKLF is reduced in this context, the competition again becomes even and the timing of switching is restored to normal (27). The increase in the γ/β ratio observed in our transgenic lines implicates p22 NF-E4 in human hemoglobin switching. The change in this ratio is due to both a reduction in β -gene expression and increased levels of γ -gene expression. Our observation that enforced expression of p22 NF-E4 in the setting of reduced levels of EKLF confers no significant additional advantage on the γ -promoter suggests that the two developmental factors, NF-E4 and EKLF play equally important roles in determining the balance between the γ - and β -globin genes at the time of switching. The concept of a dynamic balance of transcription factors regulating developmental expression of the globin genes is not novel. Studies of the γ - to β -globin switch in the fetal livers of mice transgenic for the β -globin locus YAC by primary transcript in situ hybridisation reveal that the LCR flip-flops back and

forward between the two genes as the transcription factor milieu changes to favour the adult gene (8). More recent data indicates that this occurs through a physical approximation between the LCR and the individual promoters (39, 40). Presumably, both EKLF and NF-E4 play essential roles in this direct interaction. The mechanism by which this is achieved will provide a cornerstone in our understanding of switching.

Finally, the completion of the γ/β switch in the adult bone marrow of the NF-E4⁺/EKLF^{+/-} mice indicates that p22 NF-E4 and EKLF, although able to influence gene competition, are incapable of completely overriding the globin developmental program. Despite persistently high levels of NF-E4 expression in d16.5 fetal liver in the setting of reduced EKLF dosage, γ -gene expression is virtually silenced at this time point. These findings support the models of competitive silencing of the β -globin gene and autonomous regulation of the γ -genes and indicate that altered levels p22 NF-E4 expression are not responsible for fetal globin gene silencing.

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FIGURE LEGENDS

Fig. 1. Generation of p22 NF-E4 transgenic mice. (A) Schematic representation of transgenic construct. The regulatory elements in pCAGGS are shown. The 3' end of the full-length NF-E4 cDNA was fused in frame to a hemagglutinin epitope tag. The ATG at codon 101 was mutated by PCR to a TTG codon. (B) NF-E4 expression in tissues of transgenic lines. 10 µg of total RNA from spleen, bone marrow, kidney and heart of one wild-type and the five NF-E4 transgenic lines (#4, 5, 26, 33 and 34) was analysed by Northern blot with human NF-E4 and mouse GAPDH probes. Protected products are indicated to the right of each panel. (C) Expression of NF-E4 protein in transgenic mice. Nonidet P-40 lysates from splenocytes of wild-type (lane 1) and transgenic lines (lanes 2-6) were immunoprecipitated with anti-HA antisera and the precipitate resolved by SDS-PAGE on a 12% gel and transferred to PVDF. The membrane was immunoblotted with anti-NF-E4 polyclonal Ab and developed with ECL. The positions of p22 NF-E4-HA, immunoglobulin heavy and light chains and the molecular weight size standards (in kilodaltons) are indicated. (D) NF-E4 expression during erythroid development. 10 µg of total RNA from d10.5 yolk sac, 12.5, 14.5, 16.5 fetal liver and adult bone marrow (Ad) from wild-type and transgenic line 33 was analysed by RNase protection with NF-E4 and mouse β -actin probes. Protected products are indicated to the right of each panel.

Fig. 2. Enforced expression of p22 NF-E4 does not alter murine erythropoiesis or globin gene expression. (A) Hematocrit (%) and reticulocyte counts (%) in 6 week old NF-E4 transgenics and controls. Values are shown as a mean \pm SD for $n=4$ animals. (B) Ter119/CD71 analysis of E13.5 fetal livers from NF-E4 transgenics ($n=8$) and controls ($n=11$). Single cell suspensions of fetal liver cells (10,000) were immunostained with Ter119 and CD71 antibodies. Live cells were analysed by flow cytometry and erythrocytes were excluded by gating out cells with low forward

scatter. (C) Numbers of progenitor cells present in samples of 25,000 fetal liver cells from NF-E4 transgenics ($n=11$) and controls ($n=11$) in response to cytokines. Cells were plated in methycellulose in the presence of SCF, IL3 and Erythropoietin and grown for 7 days prior to counting. (D) Total RNA was extracted from d10.5 yolk sac, 12.5, 14.5, 16.5 fetal liver and adult bone marrow (Ad) from wild-type and transgenic lines 4 and 34, and analysed by primer extension and RNase protection respectively. Probes for murine β h1, β maj- and α -globin are described in materials and methods. Bands corresponding to the products of the murine globin genes are indicated to the left of each panel.

Fig. 3. Enforced expression of p22 NF-E4 delays the γ/β switch. Expression of human γ - and β -genes in NF-E4 transgenic mice. Total RNA from d10.5 yolk sac, 12.5, 14.5, 16.5 fetal liver and adult bone marrow (Ad) from wild-type and transgenic mice were analysed by primer extension. The results of all three lines 33 (A), 4 (B) and 34 (C) are shown. Probes for the human γ - and β - and mouse α -globin genes are described in the materials and methods section and the migration of the respective products are indicated on the left. All mice were hemizygous for the NF-E4 transgene and the β YAC.

Fig. 4. Effect of the NF-E4 transgene on human γ - and β -globin synthesis. Bar diagrams of results from the three transgenic lines studied. The results are given as: (A) the ratio of γ - to β -RNA expressed in fetal liver at d12.5 (panel 1), d14.5 (panel 2), and d16.5 (panel 3) in β YAC (grey columns) and NF-E4/ β YAC littermates (black columns); (B) the ratio of human γ - to mouse α - RNA expressed in fetal liver at d12.5 (panel 1) and d14.5 (panel 2), in β YAC (grey columns) and NF-E4/ β YAC littermates (black columns); (C) the ratio of human β - to mouse α -

RNA expressed in fetal liver at d12.5 (panel 1) and d14.5 (panel 2), in β YAC (grey columns) and NF-E4/ β YAC littermates (black columns). Note the change in axes units at each time point.

Fig. 5. Effect of EKLF dosage in NF-E4 transgenic mice. Primer extension analysis of total RNA from d12.5 and d16.5 fetal liver from NF-E4/ β YAC transgenic mice in either the EKLF +/+ or EKLF +/- background, as indicated. The probes are as described above and the migration of the products is shown to the left.

Fig 1

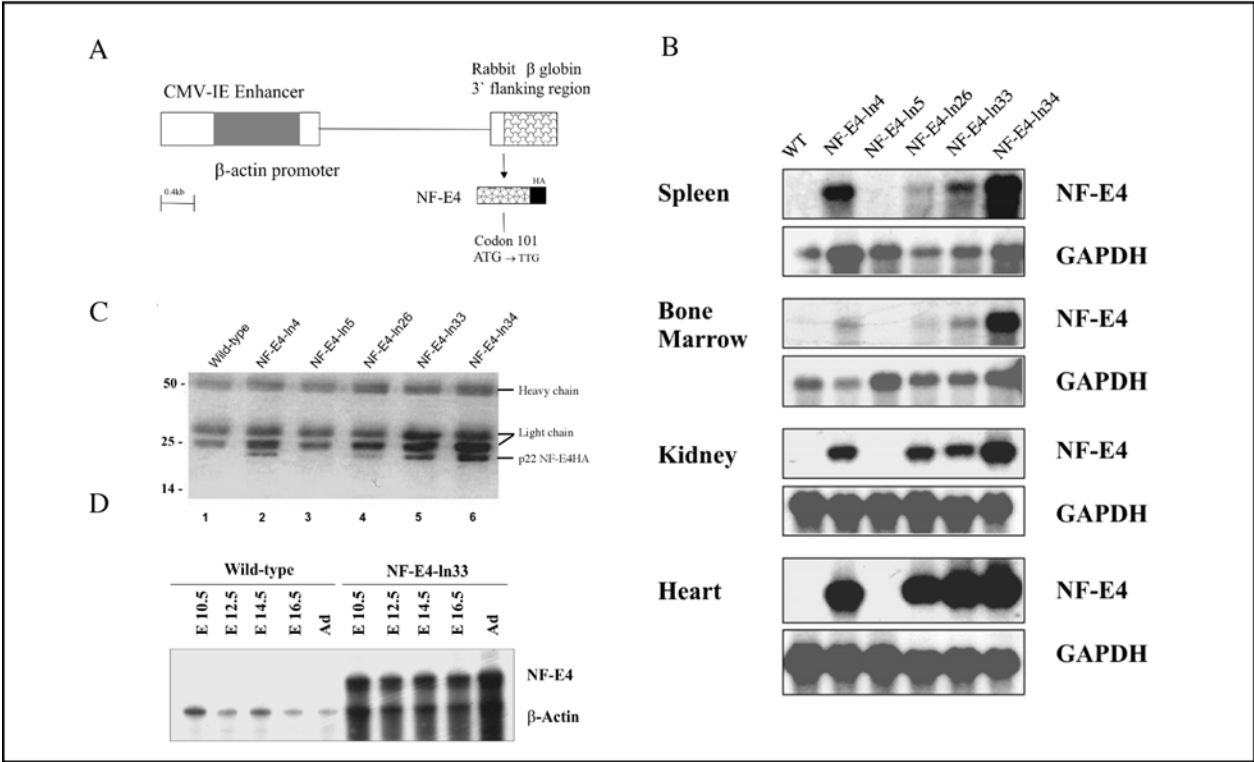


Fig 2

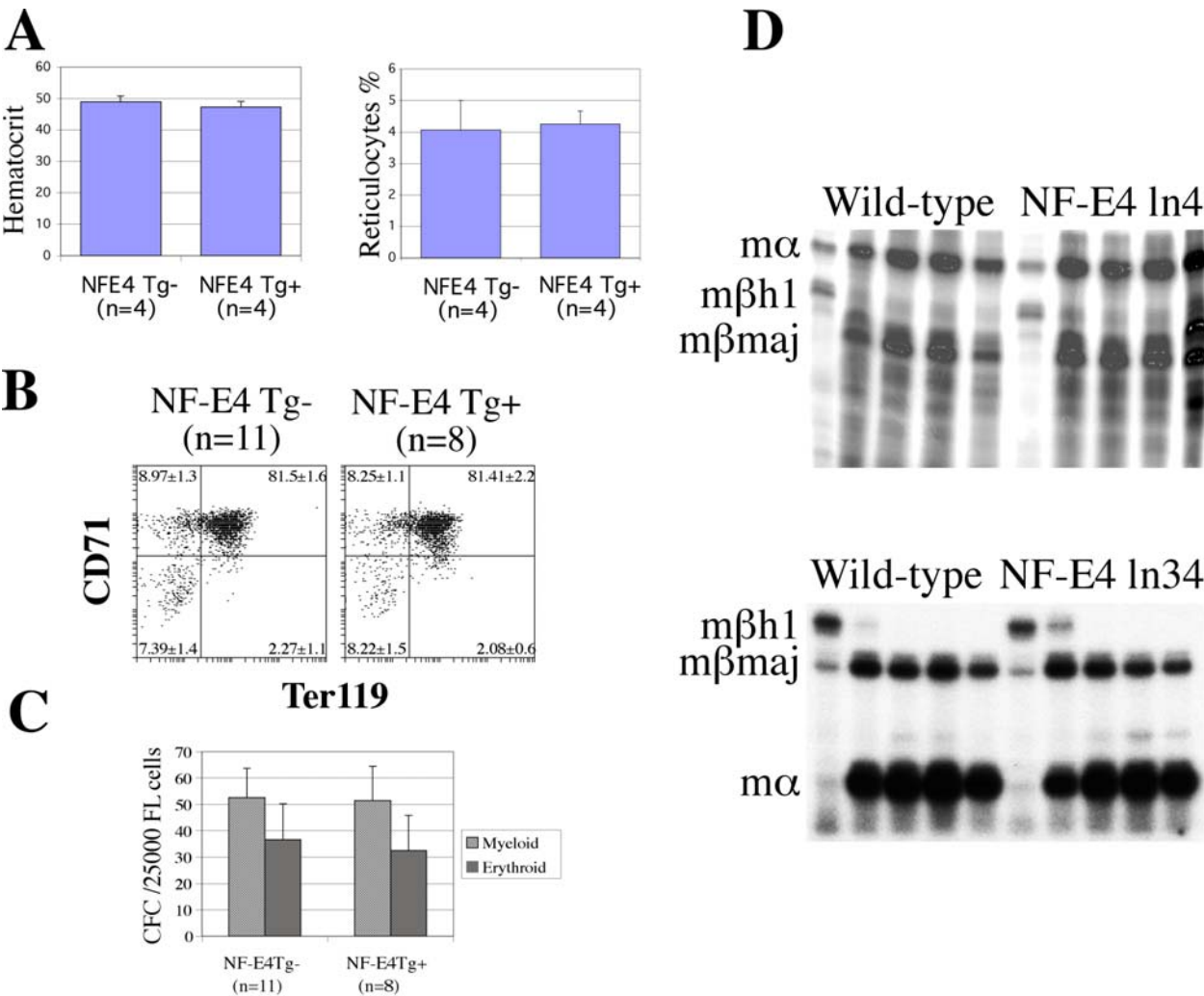


Fig 3

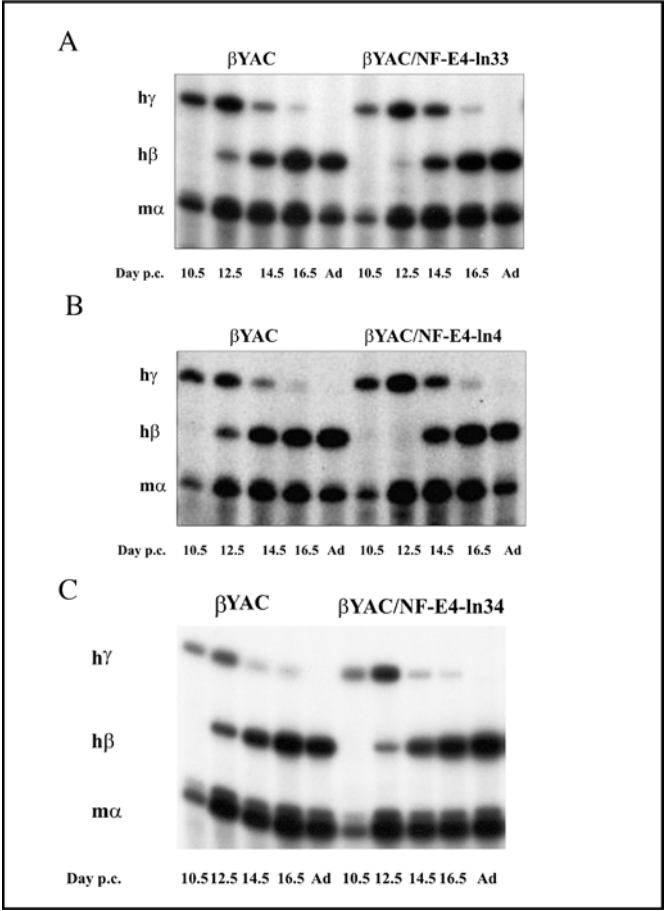


Fig 4

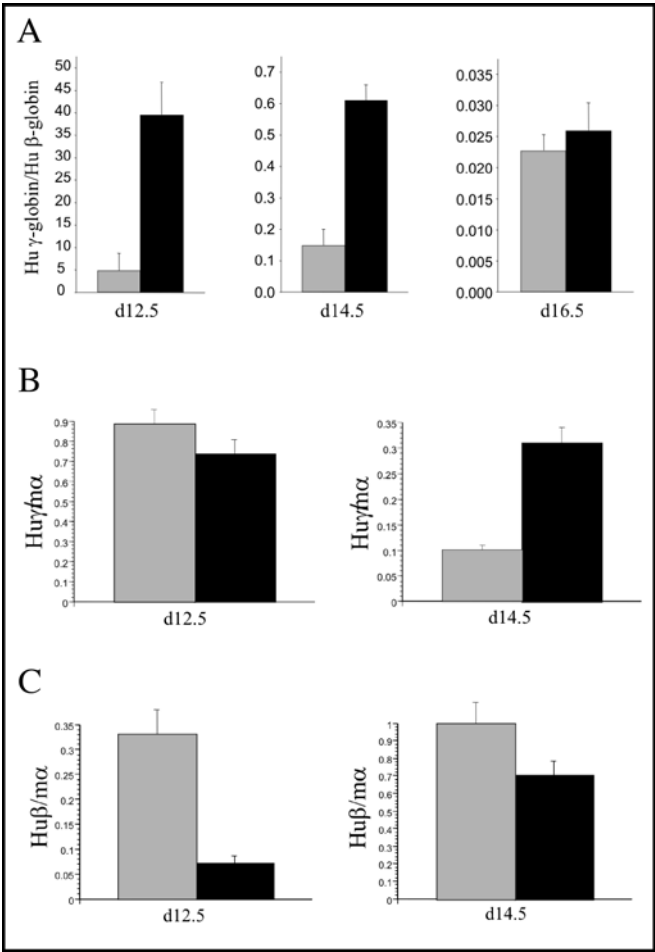
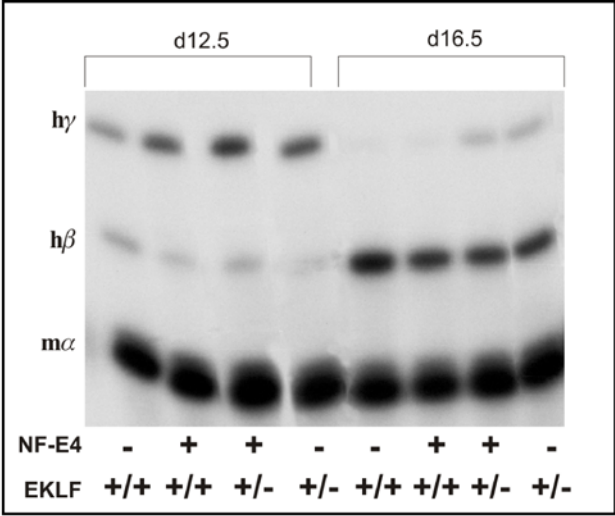


Fig 5



The role of P22 NF-E4 in human globin gene switching

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