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Embryonic and fetal β-globin gene repression by the orphan nuclear receptors, TR2 and TR4

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The TR2 and TR4 orphan nuclear receptors comprise the DNA-binding core of direct repeat erythroid definitive, a protein complex that binds to direct repeat elements in the embryonic and fetal β-type globin gene promoters. Silencing of both the embryonic and fetal β-type globin genes is delayed in definitive erythroid cells of *Tr2* and *Tr4* null mutant mice, whereas in transgenic mice that express dominant-negative TR4 (dnTR4), human embryonic ε-globin is activated in primitive and definitive erythroid cells. In contrast, human fetal γ-globin is activated by dnTR4 only in definitive, but not in primitive, erythroid cells, implicating TR2/TR4 as a stage-selective repressor. Forced expression of wild-type TR2 and TR4 leads to precocious repression of ε-globin, but in contrast to induction of γ-globin in definitive erythroid cells. These temporally specific, gene-selective alterations in ε- and γ-globin gene expression by gain and loss of TR2/TR4 function provide the first genetic evidence for a role for these nuclear receptors in sequential, gene-autonomous silencing of the ε- and γ-globin genes during development, and suggest that their differential utilization controls stage-specific repression of the human ε- and γ-globin genes.

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Introduction

The human β-globin locus is composed of ε- (embryonic), Gγ- and Aγ- (fetal), and δ- and β-globin (adult) genes, which are spatially arranged from 5′ to 3′ and developmentally expressed in the same order. The embryonic ε-globin gene is transcribed during the first 8 weeks of human gestation in yolk sac (primitive) erythroid cells. The first switch in β-globin transcription results in the silencing of ε-globin and concomitant activation of fetal γ-globin when definitive erythropoiesis ensues in the fetal liver. Gradually, at around the time of birth, a second switch from γ- to β-globin transcription occurs as the site of hematopoiesis shifts again to the adult bone marrow (Stamatoyannopoulos and Grosveld, 2001).

From genetic analyses of transgenic mice harboring mutated human β-globin loci, two nonexclusive mechanisms for globin gene 'switching' have been postulated: one is regulation by sequences located in the globin promoters (autonomous gene control) (Magram *et al*, 1985; Townes *et al*, 1985; Raich *et al*, 1990; Dillon and Grosveld, 1991), and the other is competition among the globin genes for activation by the locus control region (LCR), an element required for abundant expression of all the globin genes (Grosveld *et al*, 1987; Choi and Engel, 1988; Behringer *et al*, 1990; Enver *et al*, 1990). In a competitive model, the gene closer to LCR should have a higher probability of interaction with the LCR and hence be more abundantly transcribed, unless the gene is autonomously silenced (Hanscombe *et al*, 1991; Tanimoto *et al*, 1999). Autonomous control plays a major role in silencing the human embryonic ε- and fetal γ-globin genes in definitive erythroid cells (Raich *et al*, 1990; Dillon and Grosveld, 1991), whereas competitive control plays a major role in silencing of the adult β-globin gene during the embryonic and fetal stages (Tanimoto *et al*, 1999). However, the molecular basis for the seamless integration of these regulatory phenomena is incompletely understood.

In analyzing possible autonomous silencing mechanisms governing transcriptional regulation of the ε- and γ-globin genes, direct repeat (DR) elements (AGGTCA repeats), consensus binding sites for nonsteroidal nuclear receptors, were identified in the proximal promoters of both genes (Figure 1A). Mutation of the DR sequences in the ε-globin promoter led to ε de-repression in definitive erythroid cells of transgenic mice (Filipe *et al*, 1999; Tanimoto *et al*, 2000). In the hematologic condition known as hereditary persistence of fetal hemoglobin (HPFH), the fetal γ-globin gene is abundantly transcribed in adulthood, with elevated synthesis (up to 30%) of γ-globin in adult erythrocytes (Stamatoyannopoulos and Grosveld, 2001). HPFH mutations include small and large deletions in the locus as well as point mutations in the γ-globin promoters. Of 16 documented naturally occurring HPFH promoter mutations, six are located within DR elements (Huisman *et al*, 1997). Introduction of artificial or naturally occurring mutations into the DR element leads to derepression of γ-globin transcription in transgenic mice (Berry *et al*, 1992; Omori *et al*, 2005). These observations initially suggested a central role for promoter DR elements in both ε- and γ-globin silencing in definitive erythroid cells.

Two nuclear factors, DRED (direct repeat erythroid definitive) and COUP-TFII, can bind to the DR elements in the

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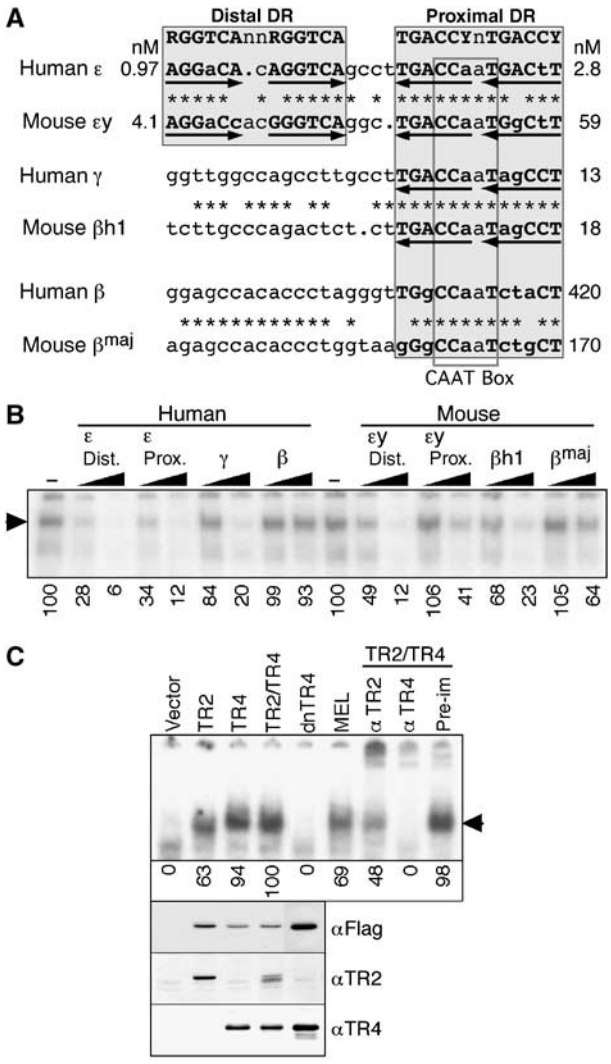

Figure 1 DRED binding to the DR Elements of human and mouse embryonic and fetal β-type globin genes. **(A)** Alignment of the promoter sequences of human and mouse β-type globin gene orthologues. Nucleotides in potential DR elements (horizontal arrows) are shown in bold letters, where as those matching the consensus sequence for nuclear receptor binding are indicated in uppercase. The numbers adjacent to each potential DR element represent the nM K_i determined for that binding site. **(B)** EMSA competitive binding assays using 1.1 nM ^{32}P-labeled ε distal DR probe, and 20 or 200 nM (18- or 180-fold molar excess) unlabeled competitor oligonucleotides. The arrowhead indicates the position of authentic DRED complex. The relative abundance of bound probe is shown at the bottom of each lane (the bound probe with no added competitor set at 100%). −, no competitor. **(C)** A 10 μg portion of pEF-BOS expression vector driving Flag-tagged TR2 or TR4 cDNA (Tanabe *et al*, 2002) was transfected separately or together into 293T cells for nuclear extract preparation and EMSA (top panel) or Western blotting with anti-Flag, anti-TR2, or anti-TR4 antibodies (lower panels). The nuclear extract of TR2/TR4 cotransfectant was preincubated with anti-TR2 or anti-TR4 antibody, or preimmune serum, and then subjected to EMSA (rightmost three lanes). A 10 μg portion of a CMV expression vector driving transcription of putative dnTR4 mutant (Flag-tagged) was also transfected into 293T cells. The arrowhead indicates the mobility of the authentic DRED complex from MEL cell nuclear extract. The relative abundance of bound probe is shown at the bottom of each lane (with TR2/TR4 cotransfection set at 100%).

ε- and γ-globin promoters *in vitro*, and both have been implicated in their repression (Filipe *et al*, 1999; Tanimoto *et al*, 2000). When we purified DRED from nuclear extracts of murine erythroid (MEL) cells, mass spectrometric analysis revealed that DRED is a large macromolecular complex containing the TR2 and TR4 orphan nuclear receptors (Tanabe *et al*, 2002). These data indicated that DRED might play a key role in repressing definitive erythroid ε- and γ-globin transcription.

TR2 and TR4 have diverse biological functions. They form homodimers and heterodimers to bind to AGGTCA DRs separated by a 0–6 nt spacer, and can either activate or repress cellular target genes (Lee *et al*, 2002). TR2 and TR4 share common functions in regulating cellular genes: the CNTF receptor, multiple thyroid hormone-regulated genes as well as genes involved in retinoic acid signaling (Lee *et al*, 2002). Recent gene ablation studies have revealed that mice lacking TR2 are viable and have no overt phenotypes (Shyr *et al*, 2002), whereas TR4 germline mutants display reproductive and neurological deficiencies (Collins *et al*, 2004; Mu *et al*, 2004; Chen *et al*, 2005).

Here, we report biochemical and genetic analyses of TR2 and TR4 and their loss- and gain-of-function effects on the regulation of β-type globin gene transcription. The analyses of *Tr2* and *Tr4* null mutant as well as dominant-negative TR4 (dnTR4) mutant mice showed that TR2/TR4 are stage-selective repressors of the human ε- and γ-globin genes: an ε-globin repressor in both primitive and definitive erythroid cells, but at the same time a definitive stage-specific repressor of the fetal γ-globin genes. Forced TR2/TR4 expression resulted in precocious repression of the human embryonic ε-globin gene, but in contrast, to induction of the γ-globin gene in definitive erythroid cells. This gene-selective repression of the human embryonic and fetal β-type globin genes by TR2 and TR4 provides critical new insight into the molecular basis for gene autonomous, sequential silencing of the ε- and γ-globin genes during embryonic development, and in concert with the competition hypothesis, provides a compelling molecular rationale for how globin gene switching during human development ensues.

Results

DRED differs in affinity for embryonic and fetal globin promoter DR sites

During embryonic development, primitive erythrocytes produced in the murine yolk sac predominantly express the embryonic εy- and βh1-globins. At around 12.5 d.p.c., when definitive erythropoiesis ensues in the murine fetal liver, the εy and βh1 genes are gradually silenced with concomitant activation of the two adult β-globin (β^{major} and β^{minor}) genes whose expression continues after birth as the site of erythropoiesis shifts to the bone marrow (Whitelaw *et al*, 1990). The promoters of the mouse embryonic globin genes contain either two (εy) or one (βh1) DR elements, and are similar to the equivalent regions of their human orthologues, the embryonic ε- and fetal γ-globin genes, respectively (Figure 1A). Neither the mouse nor the human adult β-globin genes contain recognizable DR sites. This suggests that both the mouse and human embryonic or fetal β-type globin genes could be regulated by DRED (Tanabe *et al*, 2002).

To test the hypothesis that TR2/TR4 might negatively regulate embryonic and fetal globin gene transcription, we first asked which of these promoter DR elements could bind to DRED. A ^{32}P-labeled probe from the ε-globin distal

promoter DR element that conforms best to the consensus binding site for nuclear receptors was used in these experiments; an assortment of oligonucleotides corresponding to human and mouse β-type promoter DR elements were used as competitors. From these studies, equilibrium dissociation constants (K_i) representing the affinities of competitor oligonucleotides for DRED were determined.

The K_i value of the human ε distal DR element displayed the highest affinity of all the competitors tested (Figure 1A). The affinity of the γ-globin promoter DR element was equivalent to known functional binding sites for RXR (Medin *et al*, 1994) or HNF-4 (Jiang *et al*, 1997), members of the same subfamily (Laudet, 1997). Not surprisingly, the affinity of the human β-globin promoter was too low to generate functional association. The mouse globin DR-binding sites were of generally lower affinity than their equivalent human promoter counterparts. Typical data for the competitive binding experiments are shown in Figure 1B. These data indicated that the human and mouse embryonic and fetal β-type globin gene promoters have DR elements with differential affinities for DRED in the following order: human ε distal > ε proximal > γ; mouse εy distal > βh1 > εy proximal.

We next compared the affinities of TR2 or TR4 alone and the TR2/TR4 heterodimer to the ε-globin distal DR element by expressing TR2 and TR4 separately or together. After transfection into 293T (kidney) cells, nuclear extracts were prepared and examined for binding to the ε distal DR element by electrophoretic gel mobility shift assay (EMSA) (Figure 1C). Expression of TR2 alone led to a weak signal for a DNA–protein complex that co-migrated with authentic DRED (from MEL cells), whereas expression of TR4 alone yielded a more robust EMSA complex, even though TR2 was slightly more abundant than TR4 (Figure 1C). Coexpression of TR2 and TR4 generated an EMSA complex whose signal intensity was comparable to that of TR4 alone. These data indicate that the affinity of the ε-globin distal DR element for the TR2 homodimer is lower than that for either the TR2/TR4 heterodimer or TR4 homodimer, whose affinities are roughly equivalent.

Effects of Tr2 or Tr4 loss-of-function on embryonic/fetal globin gene transcription

To investigate the *in vivo* roles of TR2 and TR4 in β-type globin gene regulation, we first analyzed the expression of the endogenous globin genes in *Tr2* or *Tr4* null mutant mice. The level of the embryonic εy-, βh1-, and adult β-globin mRNAs in 10.5 d.p.c. yolk sac and 13.5 d.p.c. fetal liver was determined by semiquantitative RT–PCR and normalized to endogenous α-globin mRNA. In the yolk sac, there was no significant difference in the expression of any of the globin genes in the homozygous *Tr2* or *Tr4* mutant embryos or their wild-type littermates (data not shown). In fetal liver definitive erythroid cells, βh1 silencing was significantly delayed in both *Tr2* and *Tr4* mutant fetuses; at the same stage, expression of the mouse embryonic εy and adult β-globin genes was unaffected (Figure 2A). The results indicate a role of TR2 and TR4 in the repression of βh1 in the fetal liver, consistent with the hypothesis that DRED represses both embryonic and fetal DR-regulated β-type globin genes. However, these results do not provide clear evidence regarding a role, if any, for TR2 and TR4 in the regulation of the murine εy gene. As TR2 or TR4 can bind to DR elements as homodimers (Figure 1), they may be functionally redundant in their ability to regulate the embryonic and fetal β-type globin genes. To examine this possibility, we interbred the *Tr2* and *Tr4* mutants to generate compound mutants in which to analyze β-globin gene expression.

Tr2/Tr4 mutant loss-of-function effects on human β-type globin transcription were assessed by breeding to a wild-type human β-globin YAC transgenic line ($Tg^{\beta YAC}$, line 264; Tanimoto *et al*, 2000). The total amount of transgene-derived human β-type globin mRNAs in animals bearing this YAC was only about 10% of mouse endogenous α-globin transcript. ε- and γ-globin silencing was significantly delayed (9- or 3.6-fold increased ε- and γ-globin gene expression in 14.5 d.p.c. fetal livers, respectively) in compound *Tr2/Tr4* homozygous null mutant fetuses as compared to wild type, whereas expression of the human adult β-globin gene was unaffected (Figure 2B). In the fetal livers of the $Tr2^{-/-}$:$Tr4^{-/+}$ or the $Tr2^{-/+}$:$Tr4^{-/-}$ embryos, expression of the ε- and γ-globin genes was induced to levels lying between those of wild-type and compound homozygous null mutant fetuses. The data indicate that TR2 and TR4 play key roles in repression of the ε- and γ-globin genes, and that TR2 and TR4 are genetically partially redundant. Furthermore, the data suggest that TR4 plays a more prominent role than does TR2 *in vivo*, as the $Tr2^{-/+}$:$Tr4^{-/-}$ mutants displayed a more severe phenotype than did the $Tr2^{-/-}$:$Tr4^{-/+}$ mutants.

To determine whether these changes in mRNA accumulation were due to altered transcriptional activity, we quantified primary RNA transcripts recovered from fetal liver samples of human β-type globin genes by RT–PCR using primer sets spanning exon–intron junctions (one primer for an exon sequence, and the other for an adjacent intron sequence). The abundance of β-, γ-, or ε-globin primary transcripts in the 14.5/15.5 d.p.c. fetal livers of wild-type mice was about 0.5, 0.2, or 0.2% of the corresponding mRNA abundance, respectively. In the fetal livers of the $Tr2^{-/-}$:$Tr4^{-/-}$ mutant fetuses, ε and γ primary transcript levels were elevated 2.9- or 2.4-fold, respectively, compared to wild type (Figure 2C). These data show that TR2 and TR4 exert repressive effects on ε- and γ-gene transcription in definitive erythroid cells of the fetal liver, and thus that the effects of TR2/TR4 are transcriptional and are not due to (e.g.) altered longevity of primitive erythroid cells or erythroid cell-specific mRNAs.

Transgenic mice that express wild-type or dominant-negative TR2/TR4

We next wished to address the possible roles of TR2 and TR4 in β-type globin gene regulation after forced transgenic expression of wild-type or mutant receptors. The crystal structure of the DNA-binding domain of RXRα, a member in the same subfamily as TR2 and TR4 (Laudet, 1997), has been resolved, and the amino-acid residues that determine the base and phosphate backbone contacts in complex with the DR element have been identified (Zhao *et al*, 2000) (Figure 3A). Based on that structure, we predicted which residues in TR4 would make contacts with the DR elements, and introduced three amino-acid substitutions for those residues (Lys or Arg to Glu) to generate a potential dnTR4 mutant protein that should be defective in DNA binding, but should retain other (dimerization or co-regulator interaction) activities.

After transfection of dnTR4 into 293T cells, nuclear extracts were examined for binding of the force-expressed

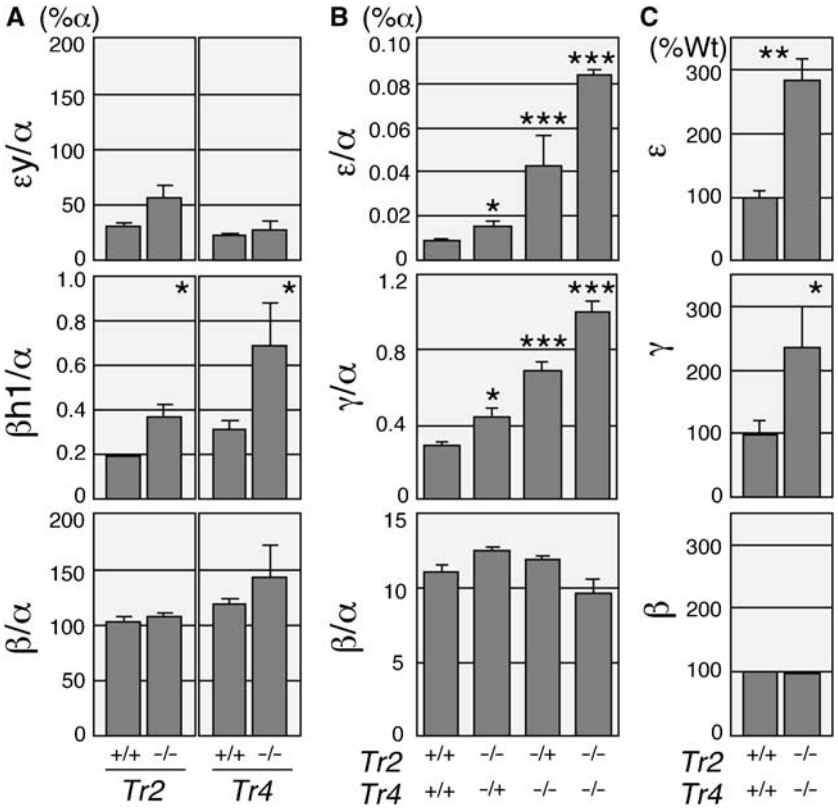

Figure 2 Mouse and human β-type globin gene expression in *Tr2* or *Tr4* null mutant mice. **(A)** The abundance of mRNAs for mouse εy-, βh1-, and adult β-globin normalized to α-globin mRNA abundance in the 13.5 d.p.c. fetal liver of *Tr2* or *Tr4* null mutant fetuses and their wild-type littermates was determined by semiquantitative RT–PCR and graphically depicted with s.e.m. The number of animals of each genotype analyzed was 3–8. **(B)** The abundance of human embryonic ε-, fetal γ-, and adult β-globin mRNA normalized to mouse α-globin mRNA abundance in the 14.5 d.p.c. fetal livers of compound *Tr2/Tr4* null mutant mice bred to a human β-globin YAC transgenic line was determined by semiquantitative RT–PCR and graphically depicted with s.e.m. The number of fetuses of each genotype analyzed was 2–10. **(C)** Abundance of primary RNA transcripts for human ε-, γ-, and β-globin in 14.5 d.p.c. fetal livers of compound *Tr2/Tr4* homozygous null mutant fetuses bearing a wild-type β-globin YAC transgene ($Tr2^{-/-}$:$Tr4^{-/-}$:$Tg^{\beta YAC}$) was determined by RT–PCR and normalized to mouse α-globin mRNA abundance. Relative abundance of primary RNA transcripts normalized to wild-type ($Tr2^{+/+}$:$Tr4^{+/+}$:$Tg^{\beta YAC}$, set at 100%) fetuses is graphically depicted with s.e.m. Two mutant and four wild-type fetuses were analyzed. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ by *t*-test.

receptor to the human ε-distal DR element (Figure 1C). The dnTR4 mutant produced no DNA–protein complex on EMSA, even though the dnTR4 protein is abundantly expressed in the transfected cells (from Western blotting) but is devoid of DNA-binding activity. The dnTR4 mutant was next coexpressed in cells at the same time as the wild-type TR2 or TR4 proteins to ask whether the mutant could, in fact, serve as a dominant-negative receptor (Figure 3B). The DNA-binding activities of TR2 and TR4 were significantly diminished by coexpression of dnTR4 protein. These data showed that dnTR4 could serve as a mutant receptor isoform that could block the intrinsic DNA-binding activity of both TR2 and TR4.

In order to restrict their expression exclusively to hematopoietic cells, the dominant-negative or wild-type TR2 or TR4 cDNAs were cloned into *G1-HRD*, a *Gata1* construct that is sufficient to drive expression of any cDNA exclusively in (primitive and definitive) erythroid cells (Onodera *et al*, 1997). The eGFP gene was also placed under *G1-HRD* control, and by microinjecting the two constructs together into fertilized oocytes, transgenic lines carrying the dnTR4 mutant (Tg^{dnTR4}) as well as wild-type TR2 (Tg^{TR2}) or TR4 (Tg^{TR4}), or both ($Tg^{TR2/TR4}$) were generated (co-integration of eGFP and nuclear receptor transgenes was verified by following multiple generations of breeding in each line).

The abundance and stability of transgene expression was first estimated by eGFP fluorescence, as measured by flow cytometric analysis of adult peripheral blood. Lines that expressed eGFP in >80% of the erythrocytes were selected for further analysis. Expression of the transgenes in the 14.5 d.p.c. fetal liver was determined by real-time quantitative PCR; the level of dnTR4 mRNA was 11- or 9-fold higher than endogenous TR2 or TR4 mRNAs, respectively. In the gain-of-function lines, the level of Tg-derived TR2 mRNA was 1.7- to 7-fold higher than endogenous TR2, whereas Tg-derived TR4 mRNA levels were from 8- to 13-fold greater than those of endogenous TR4 (Figure 3C).

The human ε- and γ-globin genes are activated by dnTR4

We analyzed the effects of transgenic dnTR4 expression on human β-type globin transcription by breeding to a wild-type human β-globin YAC transgenic line (Tanimoto *et al*, 2000) to generate Tg^{dnTR4}:$Tg^{\beta YAC}$ compound transgenic animals. The abundance of human β-type globin mRNAs in the 8.5, 10.5, and 12.5 d.p.c. yolk sacs or 14.5 d.p.c. fetal liver was determined by semiquantitative RT–PCR, normalized to mouse α-globin mRNA (Figure 4A). The data show that the peak of ε-globin mRNA accumulation in the yolk sac was induced 1.5-fold in comparison to $Tg^{\beta YAC}$ littermates upon concomitant

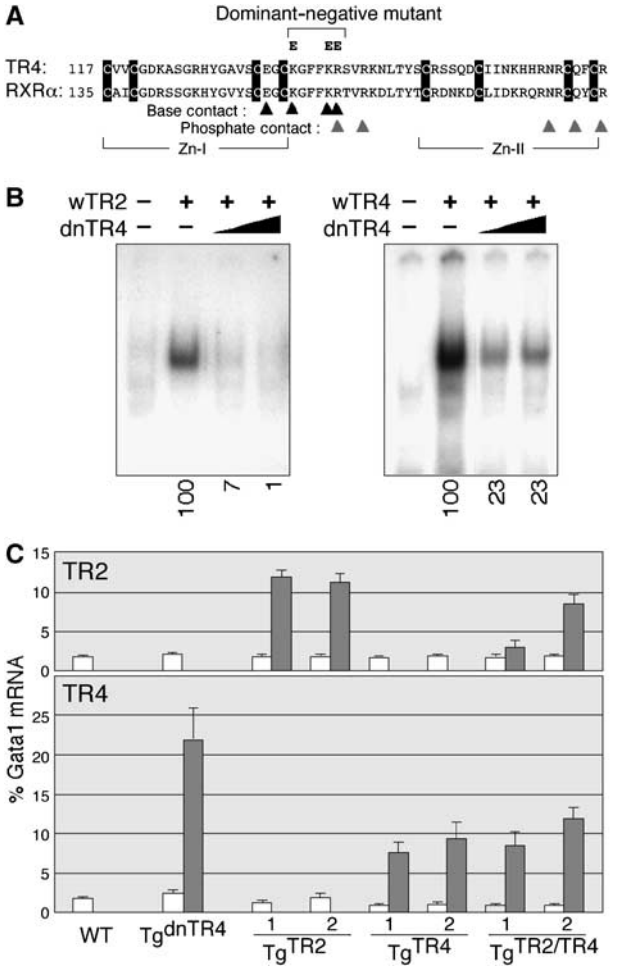

Figure 3 Generation of transgenic mice expressing wild-type and dominant-negative TR2 or TR4. **(A)** Alignment of the amino-acid sequences of the DNA-binding domains of TR4 and RXRα. Mutations introduced to the putative dnTR4 mutant are shown at the top. Residues in RXRα that make base or phosphate contacts are indicated by triangles (Zhao *et al*, 2000). **(B)** A 4 μg portion of a CMV expression vector bearing wild-type TR2 (left panel) or TR4 (right) cDNAs was transfected into 293T cells with or without cotransfection of 4 or 16 μg of a CMV expression vector bearing the putative dnTR4, followed by nuclear extract preparation and EMSA. The relative abundance of DR probe bound to wild-type TR2 or TR4 is indicated at the bottom of each lane (bound probe in the absence of dnTR4 was set at 100%). **(C)** Relative mRNA abundance of the TR2 or TR4 transgenes. The abundance of endogenous (open bars) or transgenic (shaded) TR2 (upper panel) and TR4 (lower) mRNAs in 14.5 d.p.c. fetal livers of transgenic mice expressing dnTR4, or wild-type TR2, TR4 or both was determined by reverse transcription followed by real-time quantitative PCR, and normalized to the abundance of endogenous GATA-1 mRNA (set at 100%). Data represent the averages with s.e.m. of 2–3 fetuses from each transgenic line, or 14 wild-type fetuses.

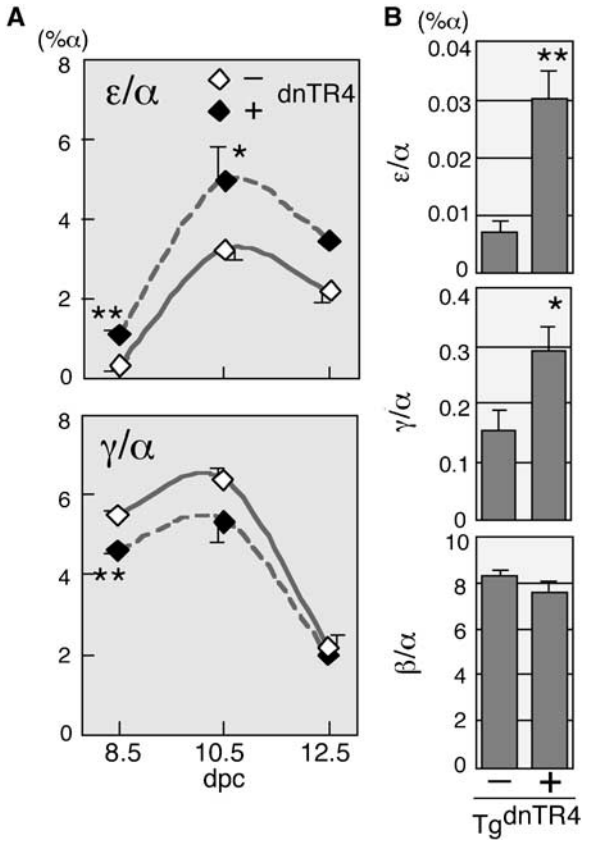

Figure 4 Human β-globin gene transcription in dnTR4 transgenic mice. **(A)** The abundance of the human ε- and γ-globin mRNAs normalized to the mouse endogenous α mRNA abundance in the 8.5–12.5 d.p.c. yolk sac of transgenic mice bearing the wild-type human β-globin YAC, with or without intercrossed Tg^{dnTR4}, was determined by semiquantitative RT–PCR and graphically depicted with s.e.m. One to five embryos of each genotype were examined. **(B)** The abundance of the human ε-, γ-, and β-globin mRNAs normalized to the abundance of α-globin mRNA in 14.5 d.p.c. fetal livers of transgenic mice bearing the wild-type human β-globin YAC, with (+) or without (−) the intercrossed Tg^{dnTR4}, was determined. The number of fetuses of each genotype analyzed was 2–7. $^{*}P<0.05$, $^{**}P<0.01$ by *t*-test.

expression of the dnTR4 mutant, and that ε-globin gene silencing in the fetal liver (Figure 4B) was significantly delayed (4.2-fold increased expression) compared to littermates without Tg^{dnTR4}. In contrast, γ-globin mRNA accumulation was only slightly reduced in comparison to YAC-containing littermates in yolk sac erythroid cells, whereas γ-globin transcription, like ε, was induced 1.9-fold in the fetal liver RNA samples. These data verify the *Tr2* and *Tr4* null mutant mouse analysis, and underscore the conclusion that TR2/TR4 is a repressor of the mouse βh1 gene, as well as the human ε- and γ-globin genes. The data also suggest that TR2/TR4 may act in a stage-selective manner: as an ε-globin repressor in primitive (Figure 4A) and definitive (Figure 4B) erythroid cells, but as a fetal γ-globin repressor only in definitive erythroid cells.

The embryonic εy gene is repressed by forced TR2 and TR4 expression

We next analyzed the consequences of forced transgenic expression of TR2 or TR4 on β-type globin gene transcription. The abundance of mouse endogenous globin mRNAs in the 9.5 d.p.c. yolk sac and 14.5 d.p.c. fetal liver was determined by semiquantitative RT–PCR (Figure 5). In the embryonic yolk sac of the two Tg^{TR4} lines, εy expression was reduced to 70 or 25% in comparison to their wild-type littermates, whereas βh1 mRNA accumulation was unaffected. Unexpectedly, in both Tg^{TR4} lines, expression of the adult β-globin gene was induced by 2.0- or 2.5-fold. In contrast, TR2 forced expression did not cause a change in expression of any of the β-type globin genes (data not shown). The activation of adult β-globin transcription upon forced TR4 transgenic expression

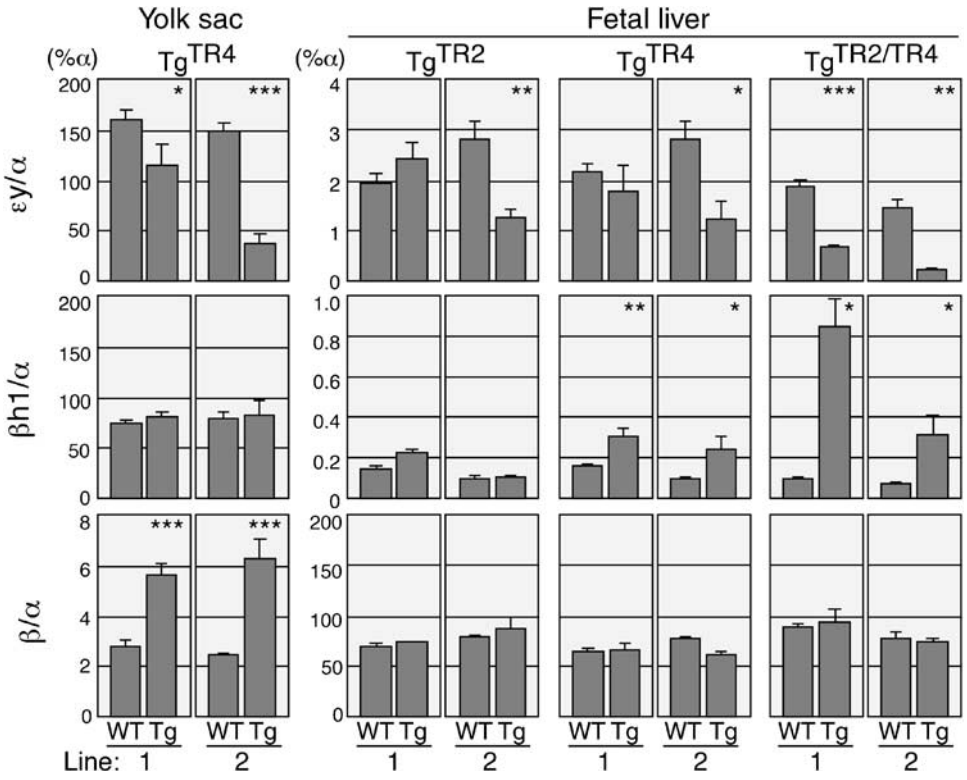

Figure 5 Mouse β-type globin gene expression in transgenic mice forcibly expressing TR2 or TR4. The abundance of mRNAs for mouse embryonic εy-, βh1-, and adult β-globin normalized to the mouse endogenous α mRNA abundance in the 9.5 d.p.c. yolk sacs and 14.5 d.p.c. fetal livers of TR2 or TR4 transgenic fetuses and their wild-type littermates was determined by semiquantitative RT–PCR and graphically depicted with s.e.m. The number of animals of each genotype analyzed was 2–6. $*P<0.05$, $**P<0.01$, $***P<0.001$ by *t*-test.

was unpredicted, but may be due to a secondary consequence of promoter competition by the LCR due to coordinate εy repression.

In the fetal livers of the Tg^{TR2} lines, embryonic εy globin gene expression was repressed to 45% of wild type (line 2), whereas βh1 and adult β transcription was unaffected. TR4 forced expression resulted not only in εy repression to 45% of wild type (line 2), but also in mild induction of βh1 (1.9- or 2.5-fold higher than in wild-type fetal livers). In the $Tg^{TR2/TR4}$ lines, εy gene expression was severely repressed (to 35 or 15% of wild type), but βh1 was even more robustly activated (by 8.8- or 4.3-fold over wild type), whereas again adult β mRNA accumulation was unaffected. The data indicate that both TR2 and TR4 homo- and heterodimers can repress εy transcription. However, the data also show that forcibly expressed wild-type TR2 and TR4 can induce βh1 expression, thereby causing a significant delay in 'fetal' gene silencing in the liver, thus superficially contradicting the loss-of-function data (showing that TR2/TR4 acts as a repressor of the βh1 gene in the fetal liver; see Discussion). The $Tg^{TR2/TR4}$ lines displayed essentially the same, but more robust, phenotypes in the fetal liver than fetuses bearing only TR2 or TR4 transgenes, underscoring the possibility that Tg^{TR2} and Tg^{TR4} function additively or synergistically. Forced expression of TR2 plus TR4 also resulted in induction of βh1 (2.4- and 6.7-fold in the $Tg^{TR2/TR4}$ lines 1 and 2, respectively) in the adult spleen, but did not cause a significant change in εy or adult β-globin mRNA (data not shown).

Finally, we performed a complete time-course analysis of mouse β-type globin mRNA abundance in one of the $Tg^{TR2/TR4}$ lines (line 1; Figure 6). The data show that $Tg^{TR2/TR4}$ forced expression reduced the peak level of εy transcription in the yolk sac, and accelerated εy silencing in the liver. In contrast, βh1 transcription was unchanged in the yolk sac, but induced in the fetal liver, causing a significant delay in βh1 silencing. Adult β-globin transcription was unaffected except in the yolk sac, suggesting that its transient induction was secondary to repression of εy transcription.

Human ε-globin transcription is repressed in TR2/TR4 transgenic mice

We next analyzed the effects of transgenic TR2/TR4 expression on human β-type globin transcription by analysis of $Tg^{TR2/TR4}$:$Tg^{\beta YAC}$ compound transgenic animals (Figure 7A). The abundance of human β-type globin mRNA in the 10.5 d.p.c. yolk sac, 15.5 d.p.c. fetal liver, or adult spleen was determined by semiquantitative RT–PCR and once again normalized to endogenous mouse α-globin mRNA abundance.

In the embryonic yolk sac, forced expression of TR2/TR4 repressed human ε-globin to 28% (line 1) or 3% (line 2) of wild-type ($Tg^{\beta YAC}$ alone) levels, but did not change fetal γ- or adult β-globin (data not shown) transcription at the primitive stage. In the fetal liver, $Tg^{TR2/TR4}$ expression repressed embryonic ε-globin transcription to 54% (line 1) or 10% (line 2) of wild type, and activated γ-globin by 3.9-fold (line 1) or 3.6-fold (line 2), precisely as observed at the same stage in the mouse orthologue, βh1. In the adult spleen, γ-globin expression was also induced 3.9-fold (line 1) or 5.1-fold (line 2), but

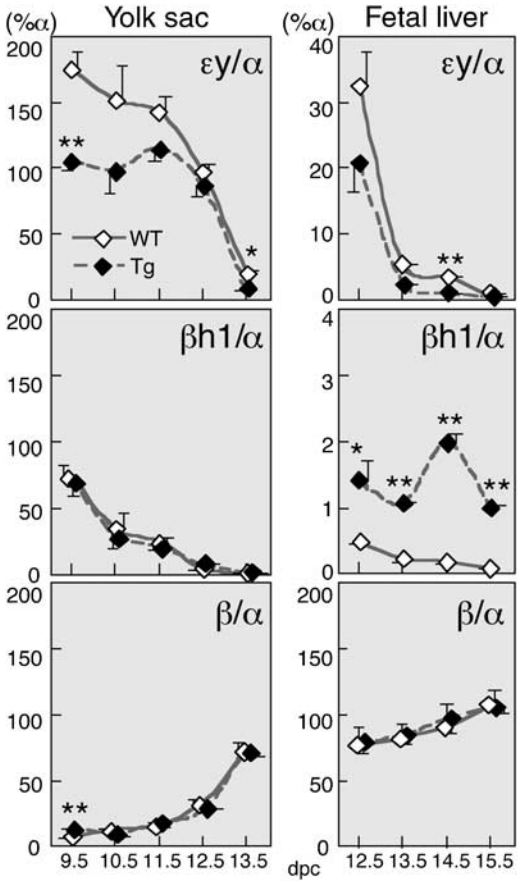

Figure 6 Time course of mouse β-type globin mRNA accumulation in TR2/TR4 transgenic mice. The abundance of mRNAs for mouse embryonic εy-, βh1-, and adult β-globin normalized to mouse α mRNA in the yolk sac and fetal liver of TR2/TR4 transgenic mice (line 1) and their wild-type littermates from 9.5 to 15.5 d.p.c. was determined by semiquantitative RT–PCR and graphically depicted with s.e.m. Note that the scales for the εy- and βh1-mRNA accumulation in the fetal liver are different from the others. Either two or three animals of each genotype were examined. $^{*}P<0.05$, $^{**}P<0.01$ by *t*-test.

neither ε-globin (data not shown) nor β-globin accumulation was affected.

We performed a complete time-course analysis of human β-type globin transcription in the $Tg^{TR2/TR4}$:$Tg^{\beta YAC}$ compound transgenic mice using the $Tg^{TR2/TR4}$ line 1 (Figure 8). The data show that elevated TR2/TR4 expression reduced the peak level of ε-globin transcription in the yolk sac and significantly accelerated ε transcriptional silencing. In contrast, fetal γ-globin expression in the yolk sac was not significantly affected, but was induced 2.2- to 7.3-fold in the fetal liver, causing a delay in silencing of the γ gene in definitive erythroid cells. Adult β-globin mRNA accumulation was unchanged in the yolk sac, but was repressed by 20% in the fetal liver. The consistent repression of human adult β-globin synthesis in the fetal liver was unpredicted, but may be simply a secondary consequence of activation of the γ-globin gene via promoter competition for LCR activity (Choi and Engel, 1988; Carter *et al*, 2002; Tolhuis *et al*, 2002). These data indicate that the transcriptional effects of forced TR2 and TR4 expression are conserved between the mouse and human embryonic and fetal orthologues: between εy and ε, as well as between βh1 and γ.

We quantified the primary RNA transcripts of the human β-type globin genes to analyze the actual transcriptional activity in erythroid cells of the fetal liver and adult spleen. The levels of γ primary transcript of $Tg^{TR2/TR4}$ line 2 animals increased 2.2- and 1.5-fold in the fetal liver or adult spleen, respectively, compared to the wild-type $Tg^{\beta YAC}$ transgene alone, whereas the ε primary transcript level was not significantly altered in the fetal liver (Figure 7B). These data indicated that forcibly expressed TR2 and TR4 induced γ-globin transcription in definitive erythroid cells of the fetal liver and adult spleen. In contrast, the reduction of ε-globin mRNA abundance in the fetal livers of $Tg^{TR2/TR4}$:$Tg^{\beta YAC}$ compound transgenic mice can be largely ascribed to the TR2/TR4 effects on ε-gene transcription in the few residual primitive erythroid cells that remain in the fetal liver.

We next analyzed the effects of $Tg^{TR2/TR4}$ on mutant human β-globin YAC transgenes that bear mutant DR elements in either the ε- or Aγ-globin promoters (Tanimoto *et al*, 2000; Omori *et al*, 2005) in order to determine whether or not the effects were mediated directly through globin promoter DR elements. One transgenic line bears a mutant YAC with 9 nt substitutions in the ε-globin gene promoter that ablates both of its DR elements (Bepsi) (Tanimoto *et al*, 2000). This YAC mutant (Tg^{BEPSI}) was bred to $Tg^{TR2/TR4}$ line 2, and expression of the mutant ε-globin gene in the 10.5 d.p.c. yolk sac was determined. The data show that repression of the ε-globin gene observed in the wild-type YAC is abrogated by the DR site mutations in the Tg^{BEPSI} YAC (Figure 7C). We also examined a different transgenic line: mutDR has a 4 nt substitution in the Aγ-globin gene promoter that specifically abolishes the DR1 element (Omori *et al*, 2005); this mutation induced Aγ-globin gene expression by six-fold compared to the wild-type YAC transgene (line 264). Tg^{mutDR} was bred to $Tg^{TR2/TR4}$ line 2, and expression of the (unmodified) Gγ-globin gene (as the internal control) and the mutant Aγ-globin gene was individually quantified. In contrast to the behavior of the wild-type $Tg^{\beta YAC}$ (Figure 7D), only the Gγ gene (bearing an intact DR element) in the Tg^{mutDR} YAC was activated. The YAC mutant data show that the effects of TR2/TR4 on transcription are direct, and are mediated by the binding of TR2 and TR4 to the DR elements in both the ε- and γ-globin promoters.

Discussion

We showed that the TR2/TR4 heterodimer can bind *in vitro* to the DR elements of both the human and mouse embryonic and fetal β-type globin gene promoters with affinities that are comparable to the known association constants for TR2, TR4 and closely related nuclear receptors. The genetic analysis provides compelling *in vivo* evidence for repression of the human embryonic ε- and fetal γ-globin genes by these receptors. In the transgenic mice bearing the human β-globin YAC as well as a dnTR4 mutant, the human ε-globin gene was activated in both primitive and definitive erythroid cells. In contrast, the human fetal γ-globin gene was only slightly perturbed during the yolk sac stage of erythropoiesis, but was induced at the fetal liver (definitive erythroid) stage. These data suggest that TR2/TR4 comprise a stage-specific, gene-selective repressor of the human embryonic ε- and fetal γ-globin genes, and may provide at least part of the molecular basis for explaining the gene-autonomous, sequential

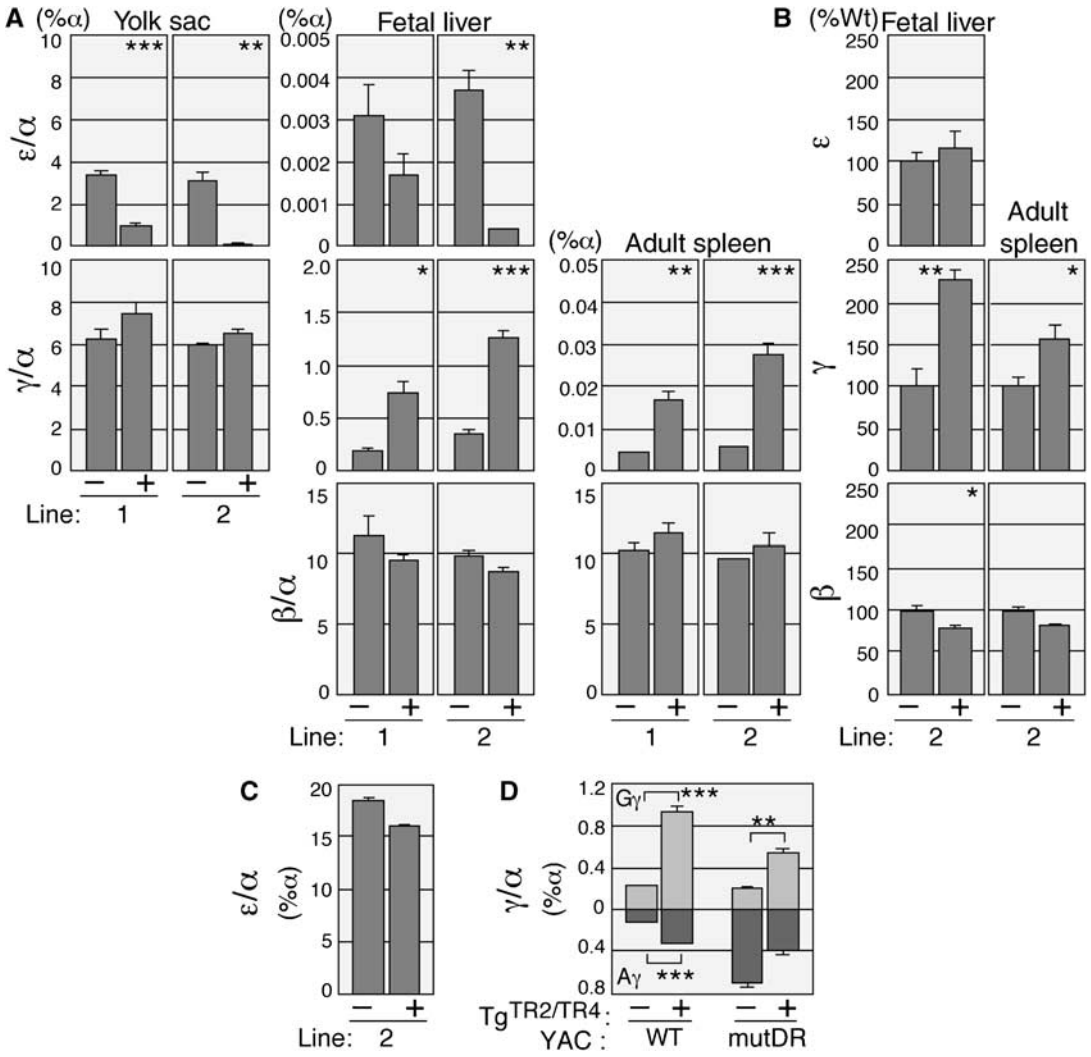

Figure 7 Altered human β-type globin gene transcription in TR2/TR4 transgenic mice. **(A)** The abundance of human ε-, γ-, and β-globin mRNAs normalized to mouse α mRNA in the 10.5 d.p.c. yolk sac, 15.5 d.p.c. fetal liver, or adult spleen of transgenic mice bearing a wild-type human β-globin YAC transgene with (+) or without (−) $Tg^{TR2/TR4}$ was determined by semiquantitative RT–PCR and graphically depicted with s.e.m. Two to five animals of each genotype were examined. **(B)** The abundance of primary RNA transcripts for the same samples as in panel (A) was determined by semiquantitative RT–PCR and normalized to mouse α mRNA abundance. Three animals of each genotype were analyzed. **(C)** The abundance of human embryonic ε-globin mRNA in the 10.5 d.p.c. yolk sac of transgenic mice bearing the DR mutant human β-globin YAC transgene, Bepsi (Tanimoto *et al*, 1999), in the presence (+) or absence (−) of $Tg^{TR2/TR4}$ (line 2) was determined as described in panel (A). Three fetuses of each genotype were examined. **(D)** γ-Globin cDNAs from the 15.5 d.p.c. fetal liver of transgenic mice bearing a wild-type or mutDR (Omori *et al*, 2005) human β-globin YAC transgene either in the presence (+) or absence (−) of $Tg^{TR2/TR4}$ (line 2) were amplified by PCR as in panel (A) and then digested with *Pst*I to determine the Gγ to Aγ molar ratio (Tanimoto *et al*, 1999; Omori *et al*, 2005). The averages with s.e.m. for Gγ- and Aγ-globin mRNAs normalized to mouse endogenous α-globin are graphically depicted. Three fetuses of each genotype were examined. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ by *t*-test.

silencing of those genes during development: that is β-globin gene 'switching'.

Forced erythroid-specific TR2/TR4 expression resulted in precocious repression of human ε-globin transcription, but in contrast, to induction of the γ-globin gene in definitive erythroid cells by a currently unexplained mechanism. The analysis of DR mutant YAC transgenic mice demonstrates that the effects of TR2 and TR4 on the ε- and γ-globin genes are direct. The expression of the DR-mutated Aγ-globin gene was elevated six-fold in the fetal liver compared to the wild-type Aγ gene, whereas γ transcription was elevated 3.6-fold in the compound *Tr2/Tr4* homozygous null mutation. These data indicate that the majority of repressor activity acting through the γ promoter DR element can be attributed to TR2 and TR4, although the involvement of other currently undefined, redundant factors in repression of the γ-globin genes through their DR elements cannot be excluded.

Control of β-type globin gene switching mediated by TR2/TR4

Based on the results presented here, we propose a hypothetical model for how TR2 and TR4 regulate human β-type globin gene transcription (Figure 9). In primitive erythroid cells of the yolk sac, TR2/TR4 binds to the DR elements of the ε-globin gene as a component of the DRED complex, thereby repressing ε-globin transcription. In contrast, TR2/TR4 exerts little or no effect on the γ-globin gene DR sites at this developmental stage; this is consistent with the results of

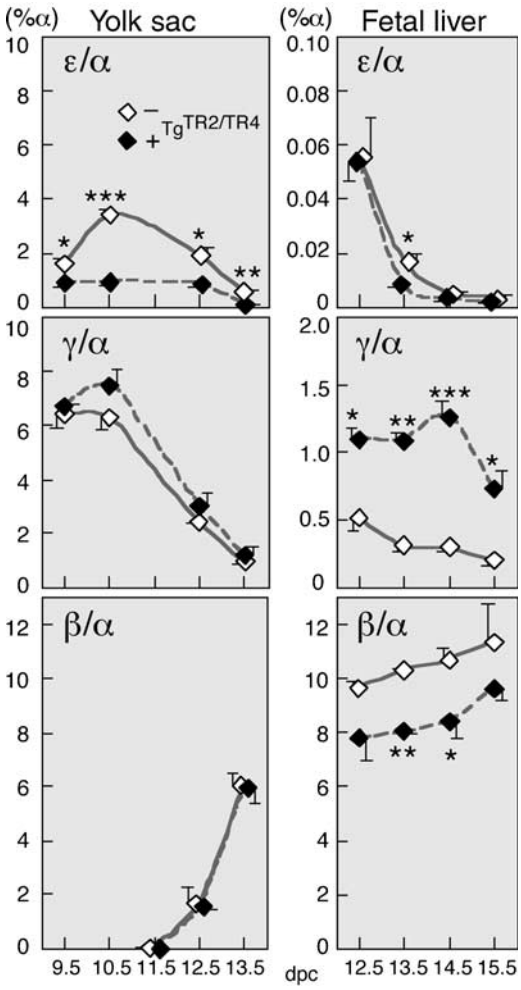

Figure 8 Time course of human β-type globin mRNA accumulation in TR2/TR4 transgenic mice. The abundance of the human ε-, γ-, and β-globin mRNAs normalized to mouse α-globin mRNA in the yolk sac and fetal liver of transgenic mice bearing the wild-type human β-globin YAC, with or without $Tg^{TR2/TR4}$ (line 1), from 9.5 to 15.5 d.p.c. was determined by semiquantitative RT–PCR and graphically depicted with s.e.m. Note that the scales for ε- and γ-globin mRNA accumulation in the fetal liver are different from the others. Two to five animals of each genotype were used. $*P<0.05$, $**P<0.01$, $***P<0.001$ by *t*-test.

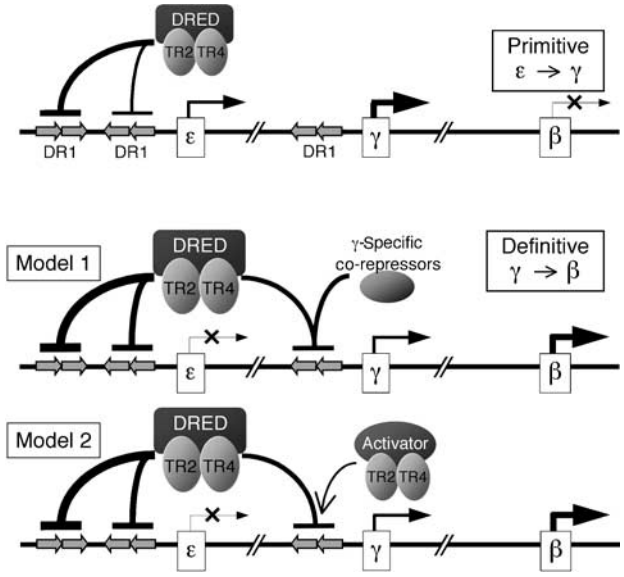

Figure 9 A model for the role of TR2/TR4 in developmental stage-specific silencing of the human ε- and γ-globin genes. In primitive erythroid cells (top), DRED is formed as a complex of TR2/TR4 and other (currently unidentified) co-repressors, and DRED represses ε-globin transcription, but exerts little or no effect on the γ-globin gene in primitive erythroid cells (because of higher affinity for the ε-globin promoter DR sites, and/or because of its activity/abundance at that stage). In definitive erythroid cells (models 1 and 2), the activity of DRED increases, allowing it to gradually repress γ-globin synthesis from the (lower affinity) DR site in the γ-globin promoter. The seemingly contradictory induction of the γ-globin genes in the $Tg^{TR2/TR4}$ transgenic mice may be explained either by a dominant-negative effect of the forcibly expressed wild-type receptors which dilute limiting γ-specific co-repressors for their normal repressor activity against the γ-globin gene (model 1), or by an inherent context-dependent transcriptional activator function (i.e. dual functionality) of the receptors on the γ-globin gene, depending on interaction with specific coactivators that are found only in definitive erythroid cells (model 2).

the dnTR4 mutant analysis, and with the lack of any effect on Tg^{mutDR} transcription in primitive erythroid cells (Omori *et al*, 2005). Such gene-selective repression could result from a greater number of ε promoter-binding sites or to the higher affinity of DRED for the two ε-globin promoter DR sites compared to the single γ-globin promoter DR site.

In definitive erythroid cells of the fetal liver, the DRED complex represses both the ε- and γ-globin genes in a gene-autonomous manner, thus enabling the subsequent γ- to β-globin switch, possibly because the activity or abundance of DRED increases in definitive erythroid cells (we have no evidence to either support or refute this hypothesis). Although ligands for TR2 or TR4 have not been identified, it is also possible that the activity of TR2/TR4 could be mediated by the differential availability of small molecule ligands (if such exist), which would provide a non-cell-autonomous basis for the developmental stage specificity and synchronicity of globin gene switching.

Although autonomous control has been shown to play a major role in the silencing of the human embryonic ε- and fetal γ-globin genes in definitive erythroid cells (Raich *et al*, 1990; Dillon and Grosveld, 1991), roles for the LCR hypersensitive sites 2, 3, and 4 in gene-selective or stage-specific transcriptional activation of the β-type globin genes have been demonstrated (Bungert *et al*, 1995, 1999; Navas *et al*, 1998, 2001). In this context, we would speculate that DRED may repress the ε- and γ-globin genes not only by gene-autonomous mechanisms, but also possibly by modulating the interaction of the globin genes with specific LCR hypersensitive sites.

The precocious repression of the embryonic mouse εy- and human ε-globin genes after forced transgenic expression of TR2/TR4, when considered along with the induction of the ε-globin gene in the compound *Tr2/Tr4* homozygous null fetuses and after breeding to the dnTR4 mutant, provides compelling genetic evidence that TR2/TR4 is a *bona fide* repressor of the mouse and human embryonic globin genes. In contrast, the absence of repression of the murine embryonic βh1- and human fetal γ-globin genes in primitive erythroid cells, and the unexpected induction of those genes in definitive erythroid cells upon TR2 or TR4 forced expression, superficially contradicts the proposed repressor function of TR2 and TR4 for the βh1- and γ-globin genes

deduced from the loss-of-function and dominant-negative analyses. The absence of βh1 or γ repression in primitive erythroid cells could be a secondary consequence of promoter competition for LCR activity due to profound repression of the εy- or ε-globin gene, but the induction of the βh1- or γ-globin gene in definitive erythroid cells cannot be explained by promoter competition, as the βh1- and γ-globin genes are induced even in the adult spleen where the repression of εy- or ε-globin genes is no longer observed, and as the induction of the γ-globin gene in the fetal liver is disproportionately greater than the degree of repression of ε.

We previously showed that DRED is a >500 kDa complex, consisting of TR2 and TR4 as well as other currently unidentified proteins (Tanabe *et al*, 2002). One likely explanation for the induction of the γ- (and βh1-) globin gene may be a dominant-negative effect of forcibly expressed TR2 and TR4, which are DNA-binding subunits of the functional DRED holo-repressor complex. The forcibly expressed orphan receptors could sequester and dilute limiting γ-specific co-repressor(s) in the DRED complex, thereby precluding formation of a functional DRED complex on the γ promoter DR element (model 1 in Figure 9). The lack of γ- (and βh1-) globin gene repression in primitive erythroid cells by the forcibly expressed TR2/TR4 may be also explained by hypothetical γ-specific co-repressor(s), which would also be limited in abundance in the context of forced expression of TR2/TR4. Alternatively, it is possible that TR2/TR4 may bear an inherent context-dependent transcriptional function that is specific to the γ- (βh1-) globin gene, and is dependent on interaction with specific coactivators that are available only in definitive erythroid cells, in keeping with the dual regulatory capacity of most nuclear receptors (model 2 in Figure 9) (Glass and Rosenfeld, 2000). Distinguishing between these two possibilities is an immediate goal.

We finally speculate that the very modest activation of the mouse adult β-globin gene in the yolk sac, and the repression of the human adult β-gene in the fetal liver, upon forced expression of TR2 and TR4 may be ascribed to promoter competition for the LCR due to mouse εy-gene repression, or human γ-globin activation, respectively. However, the possibility that forcibly expressed TR2/TR4 are directly modulating mouse and human adult β-globin gene transcription cannot be formally disproven at this time.

Genetic analyses examining transgenic mice harboring mutated human β-type globin loci have revealed that silencing of the embryonic and fetal β-type globin genes is initiated by gene-autonomous mechanisms (Magram *et al*, 1985; Townes *et al*, 1985; Raich *et al*, 1990; Dillon and Grosveld, 1991), although the relative contributions of autonomous control versus competitive silencing (Tanimoto *et al*, 1999) are unresolved. In analysis of possible *cis*-regulatory elements governing transcription of the ε- and γ-globin genes, multiple silencing elements have been proposed to lie in the proximal or distal regions of those promoters (Raich *et al*, 1992, 1995; Peters *et al*, 1993; Li *et al*, 1998a, b; Tanimoto *et al*, 2000), including those identified through the analysis of the HPFH mutations (Berry *et al*, 1992; Ronchi *et al*, 1995; Li *et al*, 2001; Omori *et al*, 2005). However, identifying the repressors that mediate gene silencing through any of these *cis*-regulatory elements has proven to be remarkably difficult. Nonetheless, some well-known activators (e.g. GATA-1) have been suggested to harbor context- or stage-specific erythroid repressor functions (Li *et al*, 1997).

Taken together, the data presented here represent the first genetic evidence showing that a *trans*-acting factor controls gene-autonomous silencing of the human embryonic and fetal β-type globin genes directly through well-defined *cis*-regulatory elements in their promoters. Considering the presence of many other hypothetical as well as verified negative *cis*-regulatory elements in the proximal promoters of the β-type globin genes, we speculate that multiple *trans*-acting factors, including TR2/TR4, collectively control autonomous embryonic and fetal globin gene silencing. Identifying, and then clarifying the specific roles of each of these transcriptional effectors as well as the co-regulators recruited to these genes via those sequence-specific transcription factors, will be essential for finally elucidating the molecular mechanisms that control β-globin gene switching.

TR2/TR4 as a target for therapeutic intervention in sickle-cell disease

Sickle-cell disease is caused by a missense mutation in the adult β-globin gene and affects millions of people worldwide (Stuart and Nagel, 2004). Based on biochemical and epidemiological evidence, therapeutic agents that increase γ-globin production are widely expected to benefit sickle-cell patients. While the fetal hemoglobin (HbF) level in erythrocytes of normal human adults is <1% of total hemoglobin, HbF concentrations among sickle-cell patients can vary from 0.1 to 30%, and any increment in HbF level was found to increase survival (Steinberg, 2005). A previous clinical study showed that sickle-cell patients with higher (>8.6%) HbF levels experience significantly reduced mortality (Platt *et al*, 1994).

In this study, we found that the abundance of human fetal γ-globin mRNA in fetal liver definitive erythroid cells of compound *Tr2/Tr4* null mutants was 9.5% of human adult β-mRNA abundance, and was nearly four-fold higher than that of genetically matched wild-type (2.5%) embryos. These data, therefore, provide direct evidence that TR2/TR4 may comprise a useful molecular target for possible therapeutic intervention in treating sickle-cell disease: pharmacological inhibition of the repressor activity of TR2/TR4 in definitive erythroid cells is predicted to induce γ-globin synthesis and thereby ameliorate the disease. Identification of natural ligand(s) of TR2 and TR4, if they exist, would facilitate the design of antagonists that would block the repressor function of TR2/TR4. Similarly, identification of co-repressor proteins in the larger DRED complex (Tanabe *et al*, 2002) may also be crucial for developing new therapeutics, as the interfaces between TR2/TR4 and those co-factors would constitute additional targets for drug design that may selectively block effective interactions and therefore diminish DRED repressor function.

Materials and methods

Transgenics

For transient expression in cell culture, the mouse TR2 and TR4 cDNAs were appended to Flag-tags at their amino termini (Tanabe *et al*, 2002), and then cloned into a CMV promoter-driven expression vector pEGFP-N3 (Clontech), replacing the eGFP gene. The cDNA encoding the dnTR4 mutant was generated by PCR-directed mutagenesis, and cloned into the same vector. For

transgenic expression, the Flag-tagged mouse TR2 or TR4 cDNAs, the dnTR4 mutant, and eGFP from the pEGFP-N3 plasmid were ligated to the *Kpn*I–*Not*I fragment from IE3.9int-*LacZ* (*GATA1-HRD*; Onodera *et al*, 1997).

Mice

The *Tr2* and *Tr4* null mutant mice were described previously (Shyr *et al*, 2002; Collins *et al*, 2004). The phenotypes of these mutant mice were confirmed in other independent *Tr2* and *Tr4* null mutant mouse lines (O Tanabe, unpublished). For generation of transgenic mice expressing wild-type TR2 or TR4, or the dnTR4 mutant, the expression DNAs were separated from the plasmid backbones and purified by gel electrophoresis and electroelution. The constructs were injected into fertilized mouse oocytes (CD1; Harlan) that were then transferred to foster dams (Nagy *et al*, 2003). Founder offspring were screened by PCR for the presence of transgenes, and then bred to wild-type CD1 mice; F1 offspring were analyzed for the presence of the transgenes by Southern blots of tail DNA. Transgenic mouse lines bearing the wild-type human β-globin YAC (line 264; Tanimoto *et al*, 1999) and the mutant YAC transgenes Bepsi (line 588; Tanimoto *et al*, 2000) and mutDR (line 74; Omori *et al*, 2005) were described previously.

Semiquantitative RT–PCR

Total RNA from transgenic lines was extracted from the yolk sac, fetal liver, or adult spleen (6-week-old, treated with 1-acetyl-2-phenylhydrazine to induce anemia), and then used as a template for first-strand cDNA synthesis. Details of the RT–PCR assay and primer sequences are described in Supplementary data. To determine the ratio of Gγ to Aγ mRNAs, both cDNAs were coamplified with the common γ primers, and then digested with *Pst*I (Omori *et al*, 2005). The relative abundance (molar ratio) of β-type globin RNAs normalized to mouse α-globin mRNA was calculated according to the following equation:

$$\text{Molar ratio}\,(\%) = \frac{R\beta}{R\alpha} \times \frac{C\alpha}{C\beta} \times \frac{(1+E\alpha)^{N\alpha}}{(1+E\beta)^{N\beta}} \times 100$$

$R\beta$ where $R\alpha$ is the radioactivity of PCR product for β-type or mouse α globin measured by PhosphoImager; $C\beta$, $C\alpha$ number of C nucleotides incorporated by PCR in each amplicon for β-type or α globin; $E\beta$, $E\alpha$ amplification efficiency of a primer set for β-type or α globin; $N\beta$, $N\alpha$ number of PCR cycles for β-type or α globin.

The amplification efficiency for each primer set was experimentally determined for each tissue by plotting radioactivity of PCR products against cycle numbers over a 6–8 cycle range.

Real-time PCR analysis for quantifying TR2, TR4, and GATA-1 cDNA

Real-time PCR analysis was performed with 0.1 μl of 14.5 d.p.c. fetal liver cDNA prepared as described above in a 25 μl reaction using an ABI Prism 7000 and SYBR Green PCR Master Mix (Applied Biosystems). Sequences of the primers are described in Supplementary data. All the primer sets were designed to span introns. The abundance of each cDNA was determined based on its C_t value and an experimentally determined amplification efficiency for each primer set, and then normalized to the abundance of GATA-1 cDNA (as the internal control).

Cell culture and transient transfection

The mouse erythroleukemia cell line MEL and human embryonic kidney cell line 293T were cultured in DME medium (Gibco) with 10% fetal calf serum. For transfection, 2×10^6 293T cells were plated in a 10 cm dish the day before transfection. A total of 20 μg of expression plasmid was transfected into each dish using lipofectamine 2000 (Invitrogen). At 48 h after transfection, cells were harvested and nuclear extracts were prepared.

EMSA

Nuclear extracts preparation, binding reactions, and electrophoresis were performed as described previously (Tanimoto *et al*, 2000). The oligonucleotides used are described in Supplementary data. To determine the affinity of each competitor by EMSA, the dissociation constant ($K_d = 0.56$ nM) for the ^{32}P-labeled ε distal DR probe was initially determined by saturation binding experiments using nuclear extracts from MEL cells. The competitive binding experiments were then performed using 1.1 nM ^{32}P-labeled ε distal DR probe and 70 pM to 2 μM of each competitor to determine the 50% inhibitory concentration (IC_{50}). The equilibrium dissociation constant (K_i) for each competitor was then determined as follows (Cheng and Prusoff, 1973):

$$K_i = \frac{IC_{50}}{1 + \frac{[\text{labeled probe}]}{K_d}}$$

Antibodies

Rabbit antisera against TR2 and TR4 were generated by fusing cDNA fragments (for the amino-terminal regions of mouse TR2 (Leu^{35}–Leu^{100}) and TR4 (Ala^{43}–Tyr^{116})) into the pET-42a plasmid (Novagen), and then expressed as GST-fusion proteins in *Escherichia coli* BL21-CodonPlus (DE3)-RIL (Stratagene). The fusion proteins were affinity-purified with glutathione Sepharose 4B (Amersham), and then used as antigen for the preparation of rabbit antisera (Cocalico Biologicals Inc.). For Western blotting with the anti-Flag mouse monoclonal antibody (Sigma), or the anti-TR2 and -TR4 antisera, horseradish peroxidase-conjugated secondary IgG was used for detection using the ECL system (Amersham).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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References

Behringer RR, Ryan TM, Palmiter RD, Brinster RL, Townes TM (1990) Human γ- to β-globin gene switching in transgenic mice. *Genes Dev* **4:** 380–389

Berry M, Grosveld F, Dillon N (1992) A single point mutation is the cause of the Greek form of hereditary persistence of fetal hemoglobin. *Nature* **358:** 499–502

Bungert J, Dave U, Lim KC, Lieuw KH, Shavit JA, Liu Q, Engel JD (1995) Synergistic regulation of human β-globin gene switching by locus control region elements HS3 and HS4. *Genes Dev* **9:** 3083–3096

Bungert J, Tanimoto K, Patel S, Liu Q, Fear M, Engel JD (1999) Hypersensitive site 2 specifies a unique function within the human β-globin locus control region to stimulate globin gene transcription. *Mol Cell Biol* **19:** 3062–3072

Carter D, Chakalova L, Osborne CS, Dai YF, Fraser P (2002) Long-range chromatin regulatory interactions *in vivo*. *Nat Genet* **32:** 623–626

Chen YT, Collins LL, Uno H, Chang C (2005) Deficits in motor coordination with aberrant cerebellar development in mice lacking testicular orphan nuclear receptor 4. *Mol Cell Biol* **25:** 2722–2732

Cheng Y, Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:** 3099–3108

Choi OR, Engel JD (1988) Developmental regulation of β-globin gene switching. *Cell* **55:** 17–26

Collins LL, Lee YF, Heinlein CA, Liu NC, Chen YT, Shyr CR, Meshul CK, Uno H, Platt KA, Chang C (2004) Growth retardation and

abnormal maternal behavior in mice lacking testicular orphan nuclear receptor 4. *Proc Natl Acad Sci USA* **101:** 15058–15063

Dillon N, Grosveld F (1991) Human γ-globin genes silenced independently of other genes in the β-globin locus. *Nature* **350:** 252–254

Enver T, Raich N, Ebens AJ, Papayannopoulou T, Costantini F, Stamatoyannopoulos G (1990) Developmental regulation of human fetal-to-adult globin gene switching in transgenic mice. *Nature* **344:** 309–313

Filipe A, Li Q, Deveaux S, Godin I, Romeo PH, Stamatoyannopoulos G, Mignotte V (1999) Regulation of embryonic/fetal globin genes by nuclear hormone receptors: a novel perspective on hemoglobin switching. *EMBO J* **18:** 687–697

Glass CK, Rosenfeld MG (2000) The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev* **14:** 121–141

Grosveld F, van Assendelft GB, Greaves DR, Kollias G (1987) Position-independent, high-level expression of the human β-globin gene in transgenic mice. *Cell* **51:** 975–985

Hanscombe O, Whyatt D, Fraser P, Yannoutsos N, Greaves D, Dillon N, Grosveld F (1991) Importance of globin gene order for correct developmental expression. *Genes Dev* **5:** 1387–1394

Huisman THJ, Carver MFH, Baysal E (1997) *A Syllabus of Thalassemia Mutations*. Augusta, GA, USA: The Sickle Cell Anemia Foundation http://globin.cse.psu.edu/globin/html/huisman/thals/IV-a.html

Jiang G, Nepomuceno L, Yang Q, Sladek FM (1997) Serine/threonine phosphorylation of orphan receptor hepatocyte nuclear factor 4. *Arch Biochem Biophys* **340:** 1–9

Laudet V (1997) Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. *J Mol Endocrinol* **19:** 207–226

Lee YF, Lee HJ, Chang C (2002) Recent advances in the TR2 and TR4 orphan receptors of the nuclear receptor superfamily. *J Steroid Biochem Mol Biol* **81:** 291–308

Li J, Noguchi CT, Miller W, Hardison R, Schechter AN (1998a) Multiple regulatory elements in the 5′-flanking sequence of the human ε-globin gene. *J Biol Chem* **273:** 10202–10209

Li Q, Blau CA, Clegg CH, Rohde A, Stamatoyannopoulos G (1998b) Multiple ε-promoter elements participate in the developmental control of ε-globin genes in transgenic mice. *J Biol Chem* **273:** 17361–17367

Li Q, Clegg C, Peterson K, Shaw S, Raich N, Stamatoyannopoulos G (1997) Binary transgenic mouse model for studying the trans control of globin gene switching: evidence that GATA-1 is an *in vivo* repressor of human ε gene expression. *Proc Natl Acad Sci USA* **94:** 2444–2448

Li Q, Duan ZJ, Stamatoyannopoulos G (2001) Analysis of the mechanism of action of non-deletion hereditary persistence of fetal hemoglobin mutants in transgenic mice. *EMBO J* **20:** 157–164

Magram J, Chada K, Costantini F (1985) Developmental regulation of a cloned adult β-globin gene in transgenic mice. *Nature* **315:** 338–340

Medin JA, Minucci S, Driggers PH, Lee IJ, Ozato K (1994) Quantitative increases in DNA binding affinity and positional effects determine 9-*cis* retinoic acid induced activation of the retinoid X receptor β homodimer. *Mol Cell Endocrinol* **105:** 27–35

Mu X, Lee YF, Liu NC, Chen YT, Kim E, Shyr CR, Chang C (2004) Targeted inactivation of testicular nuclear orphan receptor 4 delays and disrupts late meiotic prophase and subsequent meiotic divisions of spermatogenesis. *Mol Cell Biol* **24:** 5887–5899

Nagy A, Gertsennstein M, Vintersten K, Behringer RR (2003) *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press

Navas PA, Peterson KR, Li Q, McArthur M, Stamatoyannopoulos G (2001) The 5′HS4 core element of the human β-globin locus control region is required for high-level globin gene expression in definitive but not in primitive erythropoiesis. *J Mol Biol* **312:** 17–26

Navas PA, Peterson KR, Li Q, Skarpidi E, Rohde A, Shaw SE, Clegg CH, Asano H, Stamatoyannopoulos G (1998) Developmental specificity of the interaction between the locus control region and embryonic or fetal globin genes in transgenic mice with an HS3 core deletion. *Mol Cell Biol* **18:** 4188–4196

Omori A, Tanabe O, Engel JD, Fukamizu A, Tanimoto K (2005) Adult stage γ-globin silencing is mediated by a promoter direct repeat element. *Mol Cell Biol* **25:** 3443–3451

Onodera K, Takahashi S, Nishimura S, Ohta J, Motohashi H, Yomogida K, Hayashi N, Engel JD, Yamamoto M (1997) GATA-1 transcription is controlled by distinct regulatory mechanisms during primitive and definitive erythropoiesis. *Proc Natl Acad Sci USA* **94:** 4487–4492

Peters B, Merezhinskaya N, Diffley JF, Noguchi CT (1993) Protein–DNA interactions in the ε-globin gene silencer. *J Biol Chem* **268:** 3430–3437

Platt OS, Brambilla DJ, Rosse WF, Milner PF, Castro O, Steinberg MH, Klug PP (1994) Mortality in sickle cell disease. Life expectancy and risk factors for early death. *N Engl J Med* **330:** 1639–1644

Raich N, Clegg CH, Grofti J, Romeo PH, Stamatoyannopoulos G (1995) GATA1 and YY1 are developmental repressors of the human ε-globin gene. *EMBO J* **14:** 801–809

Raich N, Enver T, Nakamoto B, Josephson B, Papyannopoulou T, Stamatoyannopoulos G (1990) Autonomous developmental control of human embryonic globin gene switching in transgenic mice. *Science* **250:** 1147–1149

Raich N, Papayannopoulou T, Stamatoyannopoulos G, Enver T (1992) Demonstration of a human ε-globin gene silencer with studies in transgenic mice. *Blood* **79:** 861–864

Ronchi AE, Bottardi S, Mazzucchelli C, Ottolenghi S, Santoro C (1995) Differential binding of the NFE3 and CP1/NFY transcription factors to the human γ- and ε-globin CCAAT boxes. *J Biol Chem* **270:** 21934–21941

Shyr CR, Collins LL, Mu XM, Platt KA, Chang C (2002) Spermatogenesis and testis development are normal in mice lacking testicular orphan nuclear receptor 2. *Mol Cell Biol* **22:** 4661–4666

Stamatoyannopoulos G, Grosveld F (2001) *Hemoglobin Switching*. Philadelphia, PA, USA: WB Saunders

Steinberg MH (2005) Predicting clinical severity in sickle cell anemia. *Br J Haematol* **129:** 465–481

Stuart MJ, Nagel RL (2004) Sickle-cell disease. *Lancet* **364:** 1343–1360

Tanabe O, Katsuoka F, Campbell AD, Song W, Yamamoto M, Tanimoto K, Engel JD (2002) An embryonic/fetal β-type globin gene repressor contains a nuclear receptor TR2/TR4 heterodimer. *EMBO J* **21:** 3434–3442

Tanimoto K, Liu Q, Bungert J, Engel JD (1999) Effects of altered gene order or orientation of the locus control region on human β-globin gene expression in mice. *Nature* **398:** 344–348

Tanimoto K, Liu Q, Grosveld F, Bungert J, Engel JD (2000) Context-dependent EKLF responsiveness defines the developmental specificity of the human ε-globin gene in erythroid cells of YAC transgenic mice. *Genes Dev* **14:** 2778–2794

Tolhuis B, Palstra RJ, Splinter E, Grosveld F, de Laat W (2002) Looping and interaction between hypersensitive sites in the active β-globin locus. *Mol Cell* **10:** 1453–1465

Townes TM, Lingrel JB, Chen HY, Brinster RL, Palmiter RD (1985) Erythroid-specific expression of human β-globin genes in transgenic mice. *EMBO J* **4:** 1715–1723

Whitelaw E, Tsai S-F, Hogben P, Orkin SH (1990) Regulated expression of globin chains and the erythroid transcription factor GATA-1 during erythropoiesis in the developing mouse. *Mol Cell Biol* **10:** 6596–6606

Zhao Q, Chasse SA, Devarakonda S, Sierk ML, Ahvazi B, Rastinejad F (2000) Structural basis of RXR–DNA interactions. *J Mol Biol* **296:** 509–520

