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Mammalian β-globin loci contain multiple β-like genes that are expressed at different times during development. The murine β-globin locus contains two genes expressed during the embryo stage, Ey and βh1, and two genes expressed at both the fetal and postnatal stages, β-major and β-minor. Studies of transgenic human β-like globin loci in mice have suggested that expression of one gene at the locus will suppress expression of other genes at the locus. To test this hypothesis we produced mouse lines with deletions of either the Ey or βh1 promoter in the endogenous murine β-globin locus. Promoter deletion eliminated expression of the mutant gene but did not affect expression of the remaining embryonic gene or the fetal/adult β-globin genes on the mutant allele. These results demonstrate a lack of competitive effects between individual mouse embryonic β-globin gene promoters and other genes in the locus. The implication of these findings for models of β-globin gene expression are discussed.

In mammals there are multiple β-globin genes, expressed at different stages of development, that encode β-like globins which combine with α-like globins to form hemoglobin. The developmentally regulated pattern of β-globin gene expression is known as β-globin switching (reviewed in ref. 1). During embryonic development, primitive erythrocytes produced in the yolk sac blood islands express ε-globin in humans and predominantly Ey and βh1 globins in mice. Later during development, the embryonic genes are silenced, and definitive erythrocytes, produced in the fetal liver, express Gγ and Aγ β-like globins in humans or β-major and β-minor in a 60/40 ratio in mice. Close to the time of birth, the site of erythropoiesis switches to the bone marrow, and a second globin switch occurs in humans, with both γ genes being silenced and the β gene becoming the predominant β-like globin expressed throughout postnatal human life. In mice the definitive erythrocytes produced in the bone marrow express the β-major and β-minor genes as during fetal liver erythropoiesis but with a changed ratio of 80/20. In both human and mouse loci, all genes are expressed from the same strand and are arranged in their order of expression during development (Fig. 1*A*). β-Globin gene switching is of interest both as a model for complex stage- and tissue-specific gene expression and as a potential target for gene therapy, because activation of the silenced human γ-globin genes postnatally could greatly ameliorate the common genetic disorders of sickle cell disease and β-thalassemia.

Regulation of the β-globin locus has been studied extensively in transgenic mice carrying portions of the human locus and in mice in which the endogenous mouse locus is modified by homologous recombination. Additional insights into globin gene regulation have been derived from studies of the many human β-globin locus mutations found in human populations that coexist with *Plasmodium falciparum*, the red-cell parasite that causes malaria (reviewed in ref. 2). However, understanding of the mechanisms of globin gene switching remains incomplete. One consensus is that β-globin gene activation involves both gene-proximal and -distal regulatory elements (reviewed in ref. 1). The most prominent distal regulatory element is the locus control region (LCR). The murine β-globin LCR spans 5–22 kb 5′ of the Ey gene and has five tissue-specific but not stage-specific DNase I hypersensitive sites (HSs) and a variety of less prominent HSs located between the major HSs. The human β-globin locus has a similar LCR. Although it is clear from studies in which the LCR is deleted from the murine locus that normal levels of expression require the LCR (3, 4), the mechanism by which the LCR (or any other enhancer) acts to increase expression is unknown (reviewed in ref. 5) despite considerable information concerning proteins bound there. There are several plausible models but few experiments differentiate between them (reviewed in ref. 6; ref. 7). One model assumes that the LCR forms a large protein–DNA complex that physically interacts with the promoter of the gene it is activating, with the intervening DNA looped out (looping) (8, 9). An alternative is that activators bound to the LCR either proceed down the chromatin fiber until they reach and bind to a promoter (tracking) or form a chain of proteins that reaches and activates the promoter (linking). Another possible mechanism is that the LCR does not interact with promoters either directly or indirectly but rather directs the locus to a subnuclear compartment in which high levels of transcription can be achieved.

When linked individually to the LCR in transgenic mice, the stage specificity of the embryonic (ε) and fetal (Gγ and Aγ) human β-like globin genes is similar to that from large transgenes that contain the whole locus in its normal arrangement (10–13). Thus, the developmental-specific expression of these genes seems to be regulated by proximal elements. However, when the β gene is linked directly to the LCR in transgenes, it is deregulated and expressed throughout development (14–17). Proper regulation is largely restored when a γ gene is interposed between the β gene and the LCR (16, 17). These and other experiments involving rearrangements of β-globin genes in transgenic human loci in mice (18–20) have suggested that expression of one β-globin gene interferes with expression of other genes within the locus. Furthermore, these experiments have suggested that the appropriate developmental pattern of β-globin gene expression depends on the arrangement of the genes within the locus. In general, genes located more proximal to the LCR are activated to higher levels and at earlier developmental stages than those located more distally; full activation of the distal genes seems to require the autonomous silencing of the proximal genes.

The apparent role of proximity to the LCR in β-globin gene switching has led to the prevalent hypothesis of an active competition between β-globin gene promoters for physical in-

Abbreviations: LCR, locus control region; HS, DNase hypersensitive site; D, diffuse β-globin haplotype; S, single β-globin haplotype.

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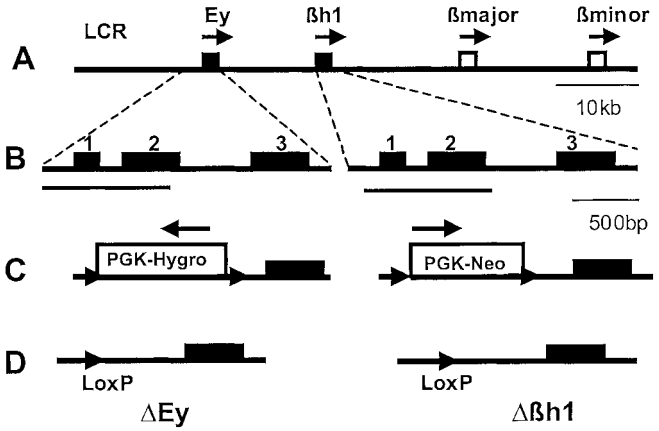

Fig. 1. Strategies for the murine β-globin embryonic gene promoter deletions. (*A*) Schematic map of the D haplotype murine β-globin locus. Ey and βh1 are solid boxes, and β-major and β-minor are open boxes. (*B*) Enlarged map of Ey and βh1 genes with exons numbered. The underlined regions are the deletions. (*C*) Promoter substitution allele after homologous recombination. The Ey promoter region is replaced by the PGK-Hygro gene flanked by loxP sites. The βh1 promoter region is replaced by the PGK-Neo gene flanked by loxP sites. loxP sites are denoted by arrowheads. The horizontal arrows above the selectable marker denote the transcription direction of the selectable marker. (*D*) Promoter deletion alleles after selectable markers are removed by Cre recombinase. The Cre-mediated reaction deletes the selectable marker and leaves a 34-bp loxP site at the locus.

teraction with the LCR, which presumably provides an activity that is otherwise rate-limiting for transcription (reviewed in ref. 7). A more proximal β-globin gene would be favored for interaction with the LCR because of the higher probability, in a random walk model, of an LCR-bound protein complex contacting it. When the proximal gene is silenced, the LCR is free to interact with the distal gene. The finding that a γ gene driven by the spectrin promoter did not suppress β in the embryo when it was interposed between the LCR and β in a transgene suggests that such potential competition may be promoter-specific (21).

The role of the LCR in β-globin gene switching is developmentally neutral. Deletion of the LCR at the murine locus (3, 4) or its omission in human transgenes (22) results in a severe decrease in expression levels of all the genes but does not affect the developmental pattern of gene expression. Thus, all the necessary information for developmental-specific β-globin gene expression is encoded within gene-proximal elements, and the LCR seems to act by increasing transcription levels at all developmental stages. Although the results of some studies of deletions of individual LCR elements in transgenic human β-globin loci have led to the suggestion of stage-specific roles for these elements (23), this conclusion is not true for the murine locus (24–26) and is unclear for human locus transgenes in general. Deletions of different HSs from human transgenes have parallel effects on developmental regulation, with the greatest decreases in expression occurring at the embryonic and fetal stages (9, 27–29). These results suggest that any given HS deletion is not associated with a defect in switching but that there is a general difference in sensitivity of the human locus to LCR mutations at different developmental stages.

To date, models of developmental regulation of β-globin gene expression have been derived mostly from studies of transgenic human loci. It is not clear yet that all effects seen in studies of transgenes apply to the endogenous human or mouse loci. To further test models of β-globin gene switching and LCR–gene interactions, we have examined the effects of promoter deletions in the mouse β-globin locus. The looping model predicts that when two genes are expressed at the same stage of development, deletion of either gene promoter will increase expression of the remaining gene, because a competitor for direct physical interaction is removed (reviewed in ref. 7). A tracking/linking model predicts that the gene that is closer to the LCR would likely interfere with transmission of the signal to the downstream gene, and therefore deletion of the proximal gene might increase expression of more distal genes, whereas deletion of a distal gene promoter would not be expected to affect expression of more proximal genes (6). If the LCR directs subnuclear localization of the locus, deletion of one promoter would not affect expression of the remaining genes. The models are not mutually exclusive, and more complex models are also possible.

The murine β-globin locus is a good system to test gene interaction effects, because there are two genes expressed in yolk sac-derived primitive cells and two genes expressed in definitive cells from fetal liver or bone marrow. Gene interactions therefore can be examined between normally expressed genes at either stage. We report here the effects of deletion of the promoter of either the murine Ey or βh1 genes.

Materials and Methods

Constructs. The targeting vector for Ey promoter deletion (pEy-prd-Hygro) consists of a 2.6-kb *Xba*I fragment located between Ey and HS1 as the 5′ homologous arm, a loxP site-flanked selectable marker (phosphoglycerate kinase I gene promoter driving the hygromycin resistance gene), and a 3.7-kb *Bam*HI fragment spanning Ey intron 2 and exon 3 as the 3′ homologous arm. The targeting vector for βh1 promoter deletion (pβh1prd-Neo) contains a 3.4-kb *Bam*HI–*Hin*cII fragment between Ey and the βh1promoter as the 5′ homologous arm, a loxP-flanked selectable marker (phosphoglycerate kinase I gene promoter driving a neomycin resistance gene), and a 2.6-kb *Hin*dIII fragment spanning the βh1 intron 2 and exon 3 and part of the intergenic region between βh1 and β-major as the 3′ homologous arm. Homologous fragments were isolated from a 129/Sv genomic library (Stratagene) and cloned into parent vector pGEM-3Z with negative selection marker DT-A. Homologous recombination replaced the Ey promoter from −673 to +442 and the βh1 promoter from +11,010 to +12,208 (relative to the cap site of Ey gene as +1) with the selectable marker.

Generation of Embryonic Gene Promoter Substitution and Deletion Mice. Twenty micrograms of targeting vector was linearized with *Sgr*AI and electroporated into R1 embryonic stem cells for each targeting construct. Embryonic stem cell clones were selected with hygromycin (150 μg/ml) for Ey promoter targeting and G418 medium (180 μg/ml) for βh1 promoter targeting. Hygromycin- and G418-resistant clones were confirmed for correct targeting events by Southern blot hybridization with probes differentiating the WT and targeted allele. Targeting frequency was 15% for ΔEyhygro and 10% for Δβh1neo. Multiple targeted embryonic stem cell clones were used to inject day-3.5 blastocysts from C57BL/6 mice to generate chimeric mice. Chimeras were backcrossed with C57BL/6 mice to identify germ line-transmitting founders. Mice were maintained in a mixed C57BL/6/129/BALB/c background. These promoter-replacement transgenic founders were bred with cytomegalovirus-Cre transgenic mice (30) (The Jackson Laboratory) to remove the selectable marker and obtain promoter deletion mouse lines. All the mutations were constructed on the diffuse haplotype. For assays the mutant/diffuse β-globin haplotype (D) males were mated with C57BL/6 [single β-globin haplotype (S)/S] females to get mutant D/S and WT D/S littermate offspring and for timed matings to get day-10.5 and day-15.5 embryos.

Steady-State mRNA Quantitation. Total RNAs were isolated with RNA-Bee (Tel-Test, Friendswood, TX) from day-10.5 yolk sac, day-15.5 fetal liver, and adult peripheral blood using manufac-

turer instructions and were used for reverse transcription with hexamer random primers. The method has been described in detail (24). The primers used recognize Ey, βh1, or β-major and β-minor from both the D and S haplotypes of the murine β-globin locus. Amplified cDNA from each allele can be differentiated by restriction-site polymorphisms between the diffuse and single alleles in the amplified regions. Therefore the WT single allele is the control for expression from the diffuse allele with or without a mutation, and expression is presented as the D/S ratio of the mutant divided by the D/S ratio of WT controls. Quantitation is achieved by amplifying in the presence of ^{32}P-labeled cytosine, running the reaction on a polyacrylamide gel, and measuring the radioactivity in each band with a PhosphorImager (Molecular Dynamics). Assays used at least three mice of each genotype for each analysis. Data are presented as the mutant D/S ratio divided by the WT D/S ratio. Error bars are the standard deviation between multiple individual mice of a given genotype.

HPLC Assay of β-Like Globin Gene Expression. To measure levels of β-major and β-minor protein in bone marrow-derived erythrocytes, peripheral blood was collected from adult mice. The cells were rinsed with mouse saline (170 mM NaCl) three times and subjected to hemolysis and HPLC assay as described (31). Assays used at least five mice of each genotype for each analysis, and the data are presented similarly to the mRNA analysis.

Results

Production of Ey and βh1 Gene Promoter Deletion Mice. To test the hypothesis that expression of one embryonic globin gene interferes with expression of the other globin genes through a mechanism involving transcriptional interference, we used homologous recombination to replace the promoter region of either Ey or βh1 with a selectable marker. The promoter regions and part of the coding region were deleted rather than the entire gene to minimize structural perturbation of the locus, particularly the distances from gene promoters to the LCR.

The strategy for these deletions involved using homologous recombination in embryonic stem cells to replace the promoters with selectable markers and then deletion of the selectable markers by using Cre recombinase, as diagramed in Fig. 1, to avoid any potential influence of the selectable marker on regulation of the locus. Both targeting vectors were designed to replace a 1.1-kb region from ≈700 bp 5′ to the transcription start site to near the 3′ end of exon 2. Drug-resistant clones were analyzed, and targeted clones were identified by Southern blot (Fig. 2). Additional Southern analyses with different restriction digests and probes were used to confirm the integrity of the homologous-recombination ends (data not shown). Mice derived from the replacement with a selectable marker (denoted as ΔEyhygro and Δβh1neo) were bred with cytomegalovirus-Cre transgenic mice (The Jackson Laboratory), which resulted in deletion of the selectable marker in the F_1 progeny with nearly 100% efficiency. These derived strains, denoted as ΔEy and ΔβH1, were verified by Southern analyses as shown in Fig. 2.

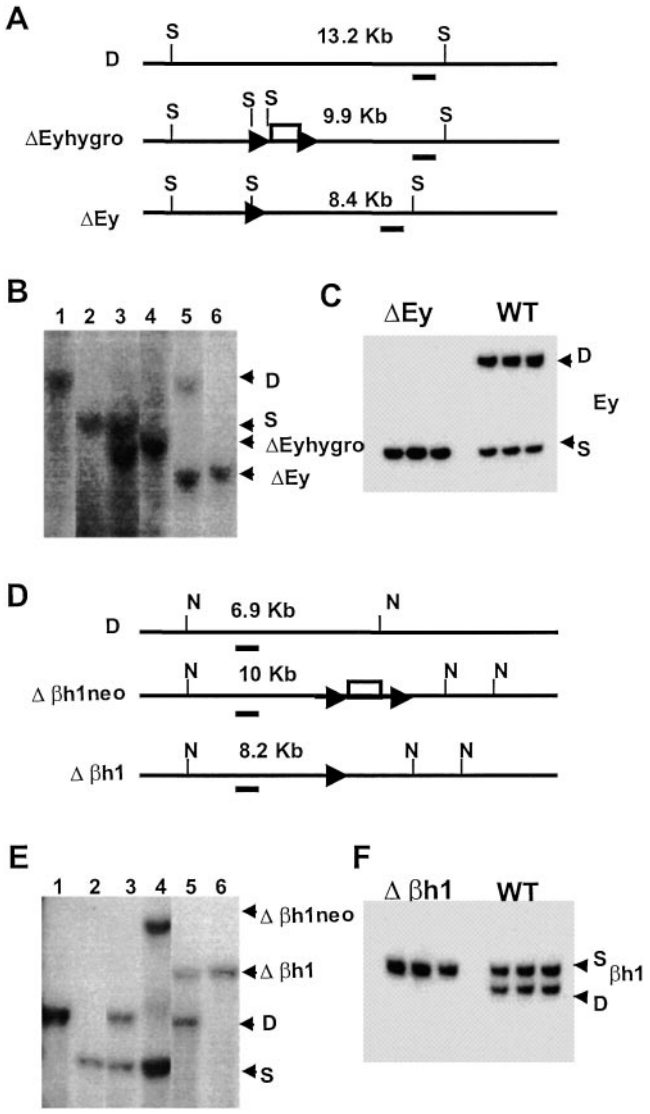

Fig. 2. Verification of deletion of Ey and βh1 promoters and associated loss of expression. (*A*) Southern blot strategy to differentiate WT diffuse, ΔEyhygro, and ΔEy mice. The open box is the PGK-hygro-selectable marker. Sites for the restriction enzyme *Sca*I are marked by S. (*B*) ΔEy Southern blot. Lane 1, D/D; lane 2, S/S; lane 3, ΔEyhygro/S; lane 4, ΔEyhygro/ΔEyhygro; lane 5, ΔEy/D; lane 6, ΔEy/ΔEy. (*C*) Representative RT-PCR assay of yolk-sac Ey expression in WT D/S mice (WT) and ΔEy/S (ΔEy) mice. Expression from D and S alleles is labeled. (*D*) Southern blot strategy to differentiate WT diffuse, Δβh1neo, and Δβh1 mice. The open box is the PGK-neo-selectable marker. Sites for the restriction enzyme *Nsi*I are marked by N. (*E*) Δβh1 Southern blot. Lane 1, D/D; lane 2, S/S; lane 3, D/S; lane 4, Δβh1neo/S; lane 5, Δβh1/D; lane 6, Δβh1/Δβh1. (*F*) Representative assay of yolk-sac βh1 expression in WT D/S mice (WT) and Δβh1/S (Δβh1) mice. Expression from D and S alleles is labeled.

Deletion of Either the Ey or βh1 Promoter Has No Effect on Expression of the Remaining Embryonic Gene. Loss of expression of a β-like globin gene will contribute to thalassemia and associated perturbation of erythropoiesis. To minimize such effects expression is analyzed in heterozygous animals where expression from the mutant allele is compared with expression from the WT allele of a different haplotype in the same animal. The mutations described here are on the D haplotype, whereas the control allele is the WT S haplotype. The effects of mutation are reflected by the ratio of gene expression between the mutant D allele and the S allele, which are compared with the WT D/S ratio (24). cDNA from the D allele is distinguished from cDNA from the S allele by a combined RT-PCR/restriction fragment length polymorphism assay. The system amplifies messages from either allele with equal efficiency and uses restriction fragment length polymorphisms in the amplified region to differentiate the products of each allele.

As expected, the ΔEy promoter deletion eliminates expression of Ey (Fig. 2*C*). An assay of βh1 mRNA from the embryonic yolk sac shows no change in βh1 expression compared with littermate WT D/S mice (Fig. 3).

Similar studies were performed on Δβh1 mice. Using the RT-PCR assay, we found that deletion of the βh1 promoter also completely eliminated βh1 expression from the mutated allele

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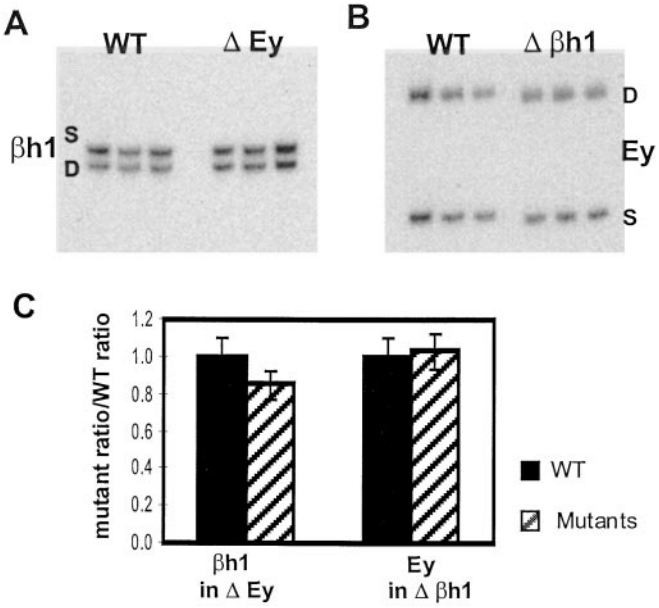

Fig. 3. Deletion of either Ey gene promoter or βh1 promoter does not change mRNA expression of the remaining embryonic gene in the yolk sac. (*A*) Representative assay of βh1 expression in yolk sac of WT and ΔEy mice heterozygous for the D and S alleles. (*B*) Representative assay of Ey expression in yolk sac of WT and Δβh1 mice heterozygous for the D and S alleles. (*C*) Graph of composite data for mRNA expression of βh1 in ΔEy mice and Ey in Δβh1 mice. Shown is the mutant D/S ratio divided by the WT D/S ratio. WT values are the WT D/S ratio divided by itself to normalize all data to 1.0. Error bars are the standard deviation of the mean for multiple individual mice before normalization.

(Fig. 2*F*). The deletion of the βh1 promoter did not perturb expression of Ey in the yolk sac (Fig. 3).

Deletion of Either the Ey or βh1 Promoter Has No Effect on β-Major and β-Minor Expression at Any Developmental Stage. To assay the potential effect on expression of the adult β-globin genes, β-major and β-minor mRNA levels were quantified by an RT-PCR/restriction fragment length polymorphism assay that is similar to the assay used to quantify either Ey or βh1 (24). Although β-major and β-minor are expressed predominantly in definitive cells, with a sufficiently sensitive assay their expression can be detected in primitive cells. Based on the amount of amplification required to get a clear signal, levels of β-major and β-minor are ≈15- to 20-fold lower in yolk sac than in fetal liver. As shown in Fig. 4, the expression of β-major and β-minor from the D allele of either mutant or WT mice was similar to expression of the cognate genes from the single allele in D/S heterozygous mice. This clearly shows that deletion of either one of the promoters has not increased expression of β-major and β-minor in the yolk sac.

Expression of β-major and β-minor was also assayed in definitive erythrocyte precursor cells from fetal liver and reticulocytes from peripheral blood. As shown in Fig. 4, there was no change in mRNA levels of β-major and β-minor in either the ΔEy or Δβh1 mice. This was confirmed by HPLC analysis of globin proteins in mature erythrocytes from peripheral blood (Fig. 4*F*).

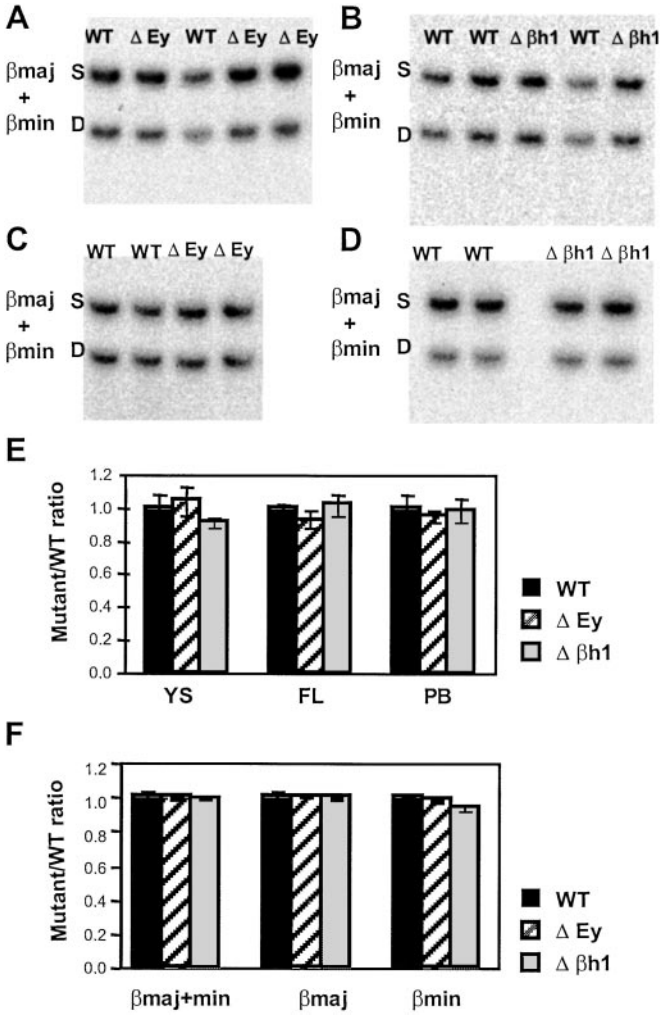

Fig. 4. Expression of β-major (βmaj) and β-minor (βmin) does not change at any developmental stage in ΔEy or Δβh1 mice. (*A*) Representative assay of β-major plus β-minor expression in yolk sac from ΔEy mice. (*B*) Representative assay of β-major plus β-minor expression in yolk sac from Δβh1 mice. (*C*) Representative assay of β-major plus β-minor expression in peripheral blood from ΔEy mice. (*D*) Representative assay of β-major plus β-minor expression in peripheral blood from Δβh1 mice. (*E*) Graph of composite data for mRNA expression of β-major plus β-minor in yolk sac, fetal liver, and peripheral blood from ΔEy and Δβh1 mice. Shown is the mutant D/S ratio divided by the WT D/S ratio. WT values are the WT ratio divided by itself to normalize all data to 1.0. Error bars are the standard deviation of the mean for multiple individual mice before normalization. (*F*) Graph of composite data for protein assay by HPLC from peripheral blood of ΔEy and Δβh1 mice. Normalization and data presentation are done as described for *B*.

Discussion

Implications for Models of LCR Mechanisms. Deletion of the Ey or βh1 gene promoters eliminated expression of the mutated gene but had no effect on expression of the remaining genes in the locus. This shows that there is no transcriptional interference between the embryonic genes, and that embryonic β-globin gene expression is not limited by competition for LCR activity. Although this observation does not prove or disprove any models of LCR function fully, it does show that any LCR–promoter interaction that does occur is not rate-limiting for murine embryonic gene expression.

The expectation of competition has been implicit in the looping model, which is based on the apparent intergenic competition revealed by studies of human transgenes and chicken globin genes (8). Most recently, analysis of nascent mRNA transcripts by nuclear RNA-fluorescence *in situ* hybridization was used to suggest that active transcription alternates between the Ey and βh1 globin genes in primitive erythrocytes (32), which if true would represent a strong indication of

competition between the two genes, perhaps for the activating function of the LCR. However, based on the results reported here, the looping model must be modified, at least in regard to the murine embryonic β-like globin genes. It is possible that for these embryonic genes there is no physical interaction with the LCR, and the looping model does not describe how the murine embryonic genes interact with the LCR. Conversely, such a physical interaction could occur simultaneously with both embryonic genes, and therefore there is no competition despite a requirement for the interaction. Perhaps the physical interaction does occur with one promoter at a time but is not rate-limiting, but this possibility would still undermine basic aspects of the model substantially. Finally, it is formally possible that the physical interaction occurs and is rate-limiting but involves regions of the genes other than the promoters or first exon and intron, which were deleted.

These results do not support the expectation from the tracking/linking models that removal of a proximal gene would increase expression of distal genes, because βh1 gene expression did not increase after deletion of the Ey promoter. Again, however, more complex permutations of such models can accommodate the data; for example, the active Ey promoter simply may not interrupt the procession of proteins tracking or linking along the chromatin fiber and thus would not influence expression of the βh1 gene. The subnuclear localization model of LCR function, which does not contain an implicit expectation of transcriptional interference, is fully compatible with this result.

The experiments reported here show that the embryonic murine genes are not in competition with each other, which raises the question of whether the data from human transgenes that support competition have any relevance to the murine locus. There are data from a spontaneous mouse mutant, thal-1, showing that transcriptional interference does occur between the murine fetal/adult genes. A 50% increase in β-minor transcription is observed in the thal-1 mouse, which has a spontaneous 3-kb deletion of the β-major gene and its promoter (33). Combined with the data presented here, the thal-1 result implies that the regulation of the fetal/adult genes is fundamentally different from the regulation of the embryonic genes. Such differences could be due to gene-proximal effects such as differences between the embryonic and fetal/adult gene promoters. Alternatively, because the embryonic genes are expressed in primitive erythrocytes and the fetal/adult genes are expressed predominantly in definitive erythrocytes, the different regulation of these genes could represent a fundamental difference between these cell types. Finally, the greater size of the deletion in the thal-1 mouse could encompass elements in addition to the promoter that might interact with the LCR.

The currently favored model for human β-globin gene switching, as applied to the mouse genes, proposes that expression of the embryonic genes is required for suppression of the fetal/adult genes in primitive cells. Once again this is thought to involve competition for physical interaction with the LCR (reviewed in ref. 1). Deletion of Ey or βh1 had no effect on the fetal/adult genes in the embryo, fetus, or adult. This shows that deletion of one of the two embryonic genes is not sufficient to up-regulate the fetal/adult genes in the embryo and implies that the fetal/adult genes are not in competition with the embryonic genes. However, it is possible that the remaining embryonic gene is sufficient to compete with and suppress the fetal/adult genes despite the fact that the expression of the remaining embryonic gene did not increase. Until a mouse line is produced with both embryonic genes deleted from the same allele, this question regarding the murine locus will remain open.

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