RAPID COMMUNICATION

Demonstration of a Human $\epsilon$-Globin Gene Silencer With Studies in Transgenic Mice

By Natacha Raich, Thalia Papayannopoulou, George Stamatoyannopoulos, and Tariq Enver

The human $\epsilon$-globin gene displays normal developmental regulation in transgenic mice; it is expressed only in embryonic and in definitive erythroid cells. We show here that deletion of a negative element located between $-182$ and $-467$ bp upstream of the $\epsilon$-globin gene cap site results in continuation of $\epsilon$ gene expression in the definitive erythroid blasts of the fetal liver and in the red blood cells of adult transgenic mice. These data provide direct in vivo evidence that cis acting silencing elements are involved in the developmental control of the $\epsilon$-globin gene.

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MATERIALS AND METHODS

Production of $\mu\text{LCR}\Delta\epsilon$ transgenic mice. $\mu\text{LCR}\Delta\epsilon$ was constructed using standard procedures and verified by DNA sequencing. Transgenic mice were produced using the Not I-Sal I $\mu\text{LCR}\Delta\epsilon$ insert, purified using NA45 paper (Shleicher and Schuell, Keene, NH). Developmentally staged embryos were produced as described.18 Nucleic acid analysis. RNA and DNA were prepared as described.19 Fidelity of transgene integration was assessed by DNA restriction (BamHI, Asp700, and SpeI) and Southern blotting. One hundred nanograms of RNA was used in each RNase protection analysis, performed as described20 using globin-specific probes. Quantitations of RNase protection analyses were achieved by phosphoimaging analysis (Molecular Dynamics, Sunnyvale, CA). The human $\epsilon$-probe is derived from a marked gene,21 making it unsuitable for $5'$ end analysis. $5'$ end analysis was therefore performed by S1-mapping using a purified, $5'$ end-labeled, oligonucleotide-primed, single-stranded probe, extending from positions $+142$ to $+276$ on the $\epsilon$-globin gene.

Immunocytochemistry. Cyto centrifuge-prepared cellular smears were made using single cell suspensions of fetal liver or peripheral blood cells. Cellular smears were fixed in methanol at room temperature before washing in phosphate-buffered saline and distilled water. Fixed smears were stained at $37^\circ$C with, first, an antihuman $\epsilon$-globin-specific monoclonal antibody and, second, a fluoroscein-conjugated antimouse IgG polyclonal antibody as previously described.5

RESULTS

Analysis of founders. To test if the upstream negative element plays a role in silencing the $\epsilon$-globin gene during development, we deleted the sequence contained in the interval $-182$ to $-467$ (with respect to the canonical $\epsilon$ cap site) from the $\epsilon$-globin gene. After linking this mutated $\epsilon$-globin gene containing fragment to an LCR cassette,5 we examined the stage specificity of the resultant construct, $\mu\text{LCR}\Delta\epsilon$ (Fig 1A), in transgenic animals. DNA prepared

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on the other hand, is composed of nonnucleated definitive erythrocytes released from the fetal liver together with nucleated primitive erythroid cells persisting from yolk sac-derived erythropoiesis. These latter cells are more prevalent in day 13 blood than in day 15 blood. All six µLCRΔε transgenic embryos expressed the ε-globin gene in both blood (Fig 1B) and liver (Fig 1C) samples. In contrast, control µLCRε transgenic embryos at the same developmental stage express human ε-globin messenger RNA (mRNA) only in the blood and not in the liver (Fig 2).12

Cellular smears of day 13 embryonic blood and fetal liver were stained with a monoclonal antibody specific for the human ε-globin chain. Both the embryonic erythrocytes in the embryonic blood and the definitive erythroblasts of the fetal liver reacted positively with anti-ε antibody (Fig 3), indicating that the µLCRΔε transgenics express ε-globin in the cells of primitive as well as definitive erythropoiesis.

To see if µLCRΔε expression was restricted to the fetal liver stage of murine definitive erythropoiesis, or persisted in the bone marrow stage of definitive erythropoiesis, we generated adult µLCRΔε transgenic mice. These adult mice expressed the µLCRΔε transgene as judged both by RNase protection (Fig 1D) and immunofluorescence analysis (Fig 3). Expression of the human ε-globin gene in the definitive erythrocytes of control adult µLCRε transgenic mice could not be detected even when a 10-fold excess of RNA was used.12

Previous work has shown that the ε-globin gene has multiple transcription initiation sites located upstream of the major cap site.10 Using an S1 mapping probe covering this region, we tested whether the pattern of transcriptional initiation was altered by the deletion of the negative element (Fig 1E). ε-Globin transcription was found to be initiated at the major cap site in all erythroid tissues of the day 13, day 15, and adult µLCRΔε transgenic mice.

Analysis of lines. Quantitative comparison of transgene expression in different founders can sometimes be complicated both by the site (position effect) and tissue distribution (mosaicism) of transgene integration. We therefore analyzed the level of ε-globin gene expression in the developmentally staged progeny of lines of µLCRΔε transgenic mice. Two independent lines of mice, µLCRΔε1 (Fig 4B) and µLCRΔε2 (Fig 4C), were used.

When normalized to mouse ε expression (the evolutionary mouse homolog of the human ε-globin gene) and corrected for transgene gene copy number, the level of human ε-expression in the day 10 to 11 embryonic blood of both µLCRΔε1 (38%) (Fig 4B) and µLCRΔε2 (34%) (Fig 4C) is similar to the level seen in control µLCRε embryonic blood (34%) (Fig 4A) at the same developmental stage. These results suggest that the ~182 to ~467 region is not critical for the LCR-dependent transcription of the ε-globin gene in embryonic erythrocytes.

Human ε expression during development was quantitated by normalization relative to total mouse α (α + ζ) globin expression (Fig 4 and Table 1). The human ε-globin gene is clearly expressed in definitive erythrocytes of both µLCRΔε1 and µLCRΔε2 lines at a time when mouse embryonic genes (ε' and ζ) are quiescent. However, its level

Fig 1. Analysis of µLCRΔε transgenic founders. (A) µLCRΔε construct. The BamHI to Afl II region was deleted from a 3.7-kb EcoRI ε-globin gene fragment,14 which was then linked to a 2.5-kb µLCR cassette. Open boxes denote 5' hypersensitive sites identified by their native location in kilobases relative to the tglobin gene. Parentheses sequence motifs with homology to negative elements found in the tissues analysed are indicated. Controls are: 16, day 10 pLCRt through 6 and 16, 0.5 RNase protection analyses of erythroid tissues (blood and liver) from and adult µLCR transgenic embryo; 17, the e-globin expressing human erythroleukemic cell line HEL-R. The RNA amount used in the analyses is: lanes 1 through 6 and 16, 0.5 μg; lanes 7 through 15 and 17, 1 μg.

from adult mice as well as from embryos was analyzed by Southern blotting to identify animals with correctly integrated transgenes (data not shown). RNA was prepared from the erythroid tissues of the transgenic animals and analyzed by RNase protection.

Figure 1 shows an RNase protection analysis of human ε-globin gene expression in the blood (Fig 1B) and liver (Fig 1C) of µLCRΔε transgenic embryos killed at either day 13 or day 15 of development. At this stage of mouse development, the fetal liver is the primary site of definitive erythropoiesis. The peripheral blood of the mouse embryo,
Fig 2. Analysis of μLCRε transgenic mice. (A) μLCRε construct. The construct is identical to μLCRAc except that the BamHI-Afl II fragment spanning the silencer has not been deleted (see legend to Fig 1A for explanation of symbols). (B, C, and D) RNase protection analyses of erythroid tissues (blood and liver) from a d14 μLCRε transgenic founder and a nontransgenic littermate (control). The probes used and the size of their respective protection products in nucleotides are indicated. RNA (0.5 μg) was used in each analysis.

Embryonic Blood

Fetal Liver

Adult Blood

Fig 3. Cellular distribution of ε-globin chain production in μLCRAc transgenic mice. The figure shows typical results from the immunofluorescence analysis of cellular smears prepared from embryonic blood, fetal liver, and adult blood, using an antibody specific for human ε-globin chain.

Fig 4. RNA analysis of ε-globin expression in the peripheral blood of developmentally staged progeny of μLCRAc transgenic mouse lines. (A, B, and C) RNase protection analyses using the probes indicated to the left of the figure. One hundred nanograms of RNA was used in each RNase protection analysis. The size of the protected fragments is indicated in nucleotides on the left. (A) RNA from day 10 transgenic offspring of a two-copy control μLCRε transgenic mouse. (B) Analysis of RNA from the staged transgenic offspring of the four-copy μLCRAε1 transgenic line. (C) Similar analysis of the two-copy μLCRAε2 line and its progeny. (D) An S1 nuclease mapping analysis of ε-transcriptional initiation in the blood (0.5 μg RNA) and liver (1 μg RNA) in developing μLCRε1 transgenic mice. The results indicate that ε-transcription initiates from the major cap site in all developmental stages of μLCRε transgenic mice. RNA from HEL-R cells (1 μg), day 10 μLCRε embryo blood (0.5 μg), and yeast tRNA (25 μg) serve as controls. The relative migration of molecular weight markers in nucleotides is indicated on the left.
Transgenic mice having a pLCRα construct (i.e., a construct in which the silencer is present) totally lack β-gene expression in the fetal liver and adult stages of erythropoiesis. However, the deletion of the silencer in the pLCRα transgenic mice suggests that the bulk of β-gene expression is normalized and corrected for transgene copy number, allowing for the diploid nature of the endogenous mouse α-globin locus. Transgenic mice having a μLCRα construct (i.e., a construct in which the silencer is present) totally lack ε-gene expression in the fetal liver and adult stages of erythropoiesis.13

of expression in definitive cells is considerably lower than that in embryonic cells (Table 1).

DISCUSSION

Our results clearly show that the deletion of the silencer region from the ε-globin gene causes its developmentally aberrant expression in definitive erythroid cells. However, our quantitative analysis of the relative levels of human ε expression in embryonic versus definitive erythroid cells of μLCRα transgenic mice suggests that the bulk of ε-globin gene developmental regulation is independent of this element. Taken together, these results support a multistep model for the developmental regulation of the ε-globin gene. In such a model, silencing of the ε-globin gene in definitive cells is achieved in part by the definitive stage-specific silencer, but is further dependent on either (1) additional negative regulatory elements specific to the definitive stages of erythropoiesis, or (2) the absence of embryonic stage-specific transactivators necessary for high-level ε-globin gene transcription.

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REFERENCES


Table 1. Quantitation of Human ε-Globin Gene Expression in Developing μLCRα Transgenic Animals

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>Gene Expression (%)</th>
<th>Mouse α + ε</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRα1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 10</td>
<td>29.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 14</td>
<td>12.0</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>Day 17</td>
<td>1.8</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCRαε2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 11</td>
<td>14.0</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>Day 14</td>
<td>3.7</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Day 19</td>
<td>0.6</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
</tbody>
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Human ε expression is normalized to total mouse α-globin production (Mouse α + ε) and corrected for transgene copy number, allowing for the diploid nature of the endogenous mouse α-globin locus. Transgenic mice having a μLCRα construct (i.e., a construct in which the silencer is present) totally lack ε-gene expression in the fetal liver and adult stages of erythropoiesis.13
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