The Human γ-Globin TATA and CACCC Elements Have Key, Distinct Roles in Suppressing β-Globin Gene Expression in Embryonic/Fetal Development†

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The competition model of globin gene regulation states that the γ-globin gene precludes expression of the β-globin gene in early development by competing for the enhancing activity of the locus control region. The γ-globin gene with a -161 promoter is sufficient for suppressing β-globin gene expression, and the γ-globin TATA and CACCC elements are necessary for this effect. In this work, stable transfection and transgenic mouse assays have been performed with constructs containing HS3 and HS2 from the locus control region, the γ-globin gene with promoter mutation(s), and the β-globin gene. The data indicate that the γ-globin TATA and CACCC elements together have at least an additive effect on the β/γ-globin mRNA ratio in early erythroid cells, suggesting that the elements work coordinately to suppress β-globin gene expression. The TATA and CACCC are the major γ-globin promoter elements responsible for this effect. Transgenic mouse experiments indicate that the γ-globin TATA element plays a role in γ-globin expression and β-globin suppression in the embryo and fetus; in contrast, the CACCC element has a stage-specific effect in the fetus. The results suggest that, as is true for the erythroid Kuppel-like factor (EKLF) and the β-globin promoter CACCC, a protein(s) binds to the γ-globin CACCC element to coordinate stage-specific gene expression.

The major human β-like globin genes are the embryonic ε-globin, the two fetal γ-globin, and the adult β-globin genes (see Fig. 1A). The β-globin locus also includes the locus control region (LCR), which encompasses five DNase I super-hypersensitive sites (HS1-HS5) located between ~6 and 21 kb 5′ of the ε-globin gene (1, 2). The LCR has strong enhancer activity (3, 4) and can initiate and maintain erythroid-specific open chromatin structure to allow for the activation of gene expression (1, 5). A naturally occurring deletion of this region results in γβ-thalassemia even in the presence of the intact genes (6), and LCR sequences must be included in transgenic mouse constructs to activate human globin gene transcription to a level similar to that of the endogenous mouse globin genes (4). Recent experiments suggest that, at least in the mouse, the LCR is required for high levels of β-like globin expression, but other elements are sufficient to establish the open chromatin structure of the locus (7, 8). Evidence also supports a tracking model in which intergenic transcripts within the β-globin locus play a role in remodeling specific chromatin domains to facilitate regulation of the temporal expression of the β-like globin genes (9).

It is well established that suppression of β-globin gene expression in early development depends on the presence of the γ-globin gene (10–13). Transgenic mice with a construct including LCR sequences, the intact β-globin gene, and a partially deleted γ-globin gene without its promoter, first exon, and first intron inappropriately express the human β-globin gene in the embryo (14). In studies with a construct containing the β-globin gene and a γ-globin gene with the β-spectrin promoter, the β-globin gene is also expressed in the embryo (15). Together these experiments support a specific role for the β-globin promoter in β-globin gene suppression. Competition with the γ-globin gene for the enhancing activity of the LCR, therefore, appears to be at least one mechanism by which β-globin expression is inhibited in early development. Further support for this competition model comes from experiments in which in situ hybridization of nascent globin transcripts demonstrate that only one β-like globin gene is generally transcribed at a time at each β-globin locus, suggesting that the genes are competing for a shared enhancer (16, 17).

The mechanism by which this gene competition might occur is not known. Protein-protein interactions between factors binding to the LCR and to the γ-globin promoter may occur by DNA looping and result in the competitive inhibition of the β-globin gene (18, 19). Later in development, interactions between the β-globin promoter and the LCR would be favored. Evidence for DNA looping comes from studies of the long range activity of the chick βε enhancer on the β-globin gene (20). An alternative to DNA looping or perhaps a complementary mechanism for gene activation by the LCR is linking (21, 22). The linking model postulates that protein complexes bound to the chromatin between the LCR and a particular globin gene provide a connection between them. In early development, the link may reach from the LCR to the γ-globin gene, but later it connects with the β-globin gene. Other mechanisms may also be involved, including transcriptional interference of LCR activation of the β-globin gene by the intervening γ-globin gene (11, 14) and/or the presence of insulators between or within the genes of the locus (21, 23). It is clear that the mechanism(s) for the coordinate regulation of the β-globin genes must involve stage-specific factors.
In previous work, we have found that a γ-globin gene with 161 bp of promoter sequence 5′ of the transcription start site is sufficient to suppress early β-globin gene expression in tissue culture assays. Using transgenic mice, we have also shown that the −136 to +56 region of the γ-globin promoter is necessary for embryonic inhibition of β-globin gene expression. The γ-globin promoter beginning around position −145 contains a CACCC element, two CCAAT boxes, the stage-selector element, and a TATA box, all of which have been investigated for their roles in developmental regulation (Fig. 1A). Simultaneous mutations of the CCAAT boxes in transgenic mice revealed that these elements play a negligible role in γ-globin expression and β-globin suppression (24). Using stable transfection assays of constructs containing HS3, HS2, and the γ- and β-globin genes in human erythroleukemia (HEL) and K562 cells, we have shown that the γ-globin CACCC and TATA elements, but not the stage-selector element, are important in suppressing β-globin expression in erythroid cells with an early developmental program (25).

The purpose of this work is to further characterize the γ-globin gene promoter elements necessary for suppression of β-globin gene expression in erythroid cells at various stages in development. We show that the γ-globin TATA and CACCC elements play key, distinct roles in predicting early β-globin expression and coordinate to regulate the expression of the γ- and β-globin genes at different stages in transgenic mice. The TATA element plays a constitutive role in γ-globin gene expression, and the CACCC element is essential for expression of the γ-globin gene in the fetus. Both elements are required for optimal γ-globin expression and β-globin silencing in early development. Therefore, the γ-globin TATA and CACCC elements are likely to establish early β-globin silencing by binding factors that favor the expression of the γ-globin gene and preclude the expression of the β-globin gene.

**Experimental Procedures**

Preparation of the Constructs for Stable Transfection Assays and Transgenic Mice—The constructs used in the stable transfection assays and in generating transgenic mice are shown in Fig. 1B and are as follows: HS3HS2, HS3HS2-CACCCβ, HS3HS2-TATAβ, HS3HS2-TATA/CCAC/CCACβ (HS3HS2-TATA/CCACβ), and HS3HS2-TATA/TATA/CCACβ. These constructs contain a 1.9-kb HS HindIII fragment (GenBank™ HUMHBB 13017 coordinates 3266–5172) and a 1.9-kb HS2 KpnI-PvuII fragment (GenBank™ HUMHBB U01317 coordinates 7764–9653) from the LCR region of the human β-globin locus. The β-globin gene is a 4.5-kb Apa1-EcoRV fragment (−1250 to +3291 relative to the transcription start site) containing the 3′ enhancer region and is derived from the cosmid clone FC14 (12, 26). The constructs contain a HindIII fragment (−1350 to +1061) with the human γ-globin gene. The HS3HS2-Δβs/β construct has a deletion of 192 bp from the γ-globin promoter (−136 to +56), including some of the promoter, the transcription start site, and the 5′ untranslated region. The γ- and β-globin genes in the constructs (except for the γ-globin gene in HS3HS2-Δβs/β) are marked by a 4-bp insertion at +50 to distinguish their mRNAs from the endogenous globin mRNAs in the tissue culture assays. The constructs and the mutations in the γ-globin promoter were previously described (13, 25). The mutations in the γ-globin promoter are a 4-bp substitution in the TATA element and/or a 5-bp substitution in the CACCC element.

**Stable Transfection Studies—** HEL (ATCC CCL 137), human erythroleukemia cells, were grown, selected, and transfected, and RNA isolation and primer extension analysis were performed as previously described (25). Eleven or 12 pools of HEL cells were transfected per construct. The mRNA from the transfected γ- and β-globin genes was quantitated with a Molecular Dynamics PhosphorImager and normalized to and expressed as a percentage of the endogenous γ-globin mRNA. For cells with the HS3HS2-TATAβ and HS3HS2-TATAβ constructs, which have the mutation in the γ-globin TATA box, the transfected γ-globin mRNA was larger than the expected size, as indicated in the results. The statistical analyses were done using the nonparametric rank sum test,

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**Fig. 1.** A, the human β-globin locus and regulatory elements in the γ-globin promoter. The arrows represent the five DNase I super-hypersensitive (HS) sites in the LCR. Below the locus is a diagram indicating the locations of the CACCC, the two CCAAT, the stage-selector element, and the TATA elements downstream of −161 in the γ-globin promoter. The CACCC and/or TATA elements in the γ-globin promoter have been mutated in the constructs described below. B, the DNA constructs tested in stable transfection assays and transgenic mice. All of the constructs contain a 1.9-kb fragment containing HS3 from the LCR, a 1.9-kb fragment containing HS2, a 3.3-kb human γ-globin gene, and a 4.5-kb human β-globin gene. The HS3HS2-ββ construct has the wild-type γ-globin gene, and the other constructs contain the indicated mutations in the γ-globin promoter. HS3HS2-TATAβ has base substitutions in the TATA box at −30, HS3HS2-γ-CACCCβ has base substitutions in the CACCC element at −140, and HS3HS2-T/C/β has both the TATA and the CACCC mutations. HS3HS2-Δβsβ has a deletion from −136 to +56 in the γ-globin promoter.
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The amounts of transfected γ- and β-globin mRNA expression are as a percentage of endogenous γ-globin mRNA. Data are the mean of at least two primer extension assays for each pool of transfected cells, and the mean values are the average for the 12 transfections per construct; S.D. is the standard deviation. The β-γ-globin mRNA ratio indicates the net effect of the γ-globin promoter mutations on the competition between the γ- and β-globin genes within each pool. The rank sum test was used (α < 0.01).

| Construct     | Pool No. | %β | %γ | β/γ ratio | Construct     | Pool No. | %β | %γ | β/γ ratio |
|---------------|----------|----|----|-----------|---------------|----------|----|----|-----------|
| HS3HS2γβ      | 1        | 2.5| 67.8| 0.04      | HS3HS2γCACCCβ| 1        | 5.6| 99.8| 0.06      |
|               | 2        | 3.0| 109.0| 0.03      |               | 2        | 8.0| 75.2| 0.11      |
|               | 3        | 3.1| 57.6| 0.05      |               | 3        | 5.4| 95.6| 0.06      |
|               | 4        | 2.2| 37.1| 0.06      |               | 4        | 8.2| 96.5| 0.09      |
|               | 5        | 3.2| 65.5| 0.05      |               | 5        | 4.7| 54.5| 0.09      |
|               | 6        | 3.0| 77.4| 0.04      |               | 6        | 7.6| 103.1| 0.07     |
|               | 7        | 2.3| 62.3| 0.04      |               | 7        | 7.5| 84.1| 0.09      |
|               | 8        | 2.3| 57.6| 0.04      |               | 8        | 4.9| 81.4| 0.06      |
|               | 9        | 2.3| 62.7| 0.04      |               | 9        | 6.3| 85.4| 0.08      |
|               | 10       | 3.2| 43.0| 0.07      |               | 10       | 11.9| 173.5| 0.07     |
|               | 11       | 2.5| 49.7| 0.05      |               | 11       | 7.1| 92.0| 0.08      |
|               | 12       | 3.0| 62.8| 0.05      |               | 12       | 7.2| 92.0| 0.08      |
| Mean ± S.D.  | 2.7 ± 0.4| 62.7 ± 18.2| 0.05 ± 0.01| Mean ± S.D.  | 7.0 ± 1.9| 94.5 ± 28.1| 0.08 ± 0.02^a |
| HS3HS2γTATAβ | 1        | 3.5| 23.1| 0.15      | HS3HS2γTCβ    | 1        | 7.7| 19.4| 0.39      |
|               | 2        | 6.5| 32.2| 0.20      |               | 2        | 8.5| 18.3| 0.46      |
|               | 3        | 6.7| 40.0| 0.16      |               | 3        | 8.9| 19.7| 0.44      |
|               | 4        | 3.9| 28.0| 0.14      |               | 4        | 10.2| 24.2| 0.40      |
|               | 5        | 5.1| 25.8| 0.19      |               | 5        | 10.9| 25.2| 0.54      |
|               | 6        | 3.0| 17.1| 0.17      |               | 6        | 10.4| 18.3| 0.54      |
|               | 7        | 5.8| 30.1| 0.19      |               | 7        | 11.6| 23.1| 0.48      |
|               | 8        | 11.1| 54.1| 0.20      |               | 8        | 11.1| 18.6| 0.58      |
|               | 9        | 4.1| 40.9| 0.10      |               | 9        | 8.5| 18.2| 0.46      |
|               | 10       | 6.5| 32.4| 0.20      |               | 10       | 8.7| 17.9| 0.47      |
|               | 11       | 3.1| 15.4| 0.20      |               | 11       | 12.0| 25.0| 0.46      |
|               | 12       | 3.6| 18.7| 0.19      |               | 12       | 9.4| 25.5| 0.38      |
| Mean ± S.D.  | 5.2 ± 2.3  | 29.8 ± 11.2  | 0.18 ± 0.03^a | Mean ± S.D.  | 9.8 ± 1.4b | 20.9 ± 3.0  | 0.46 ± 0.06^b |

^a Mean values for the HS3HS2γTATAβ and HS3HS2γCACCCβ constructs are significantly different from those of the wild-type HS3HS2γβ construct.

^b Values for the HS3HS2γTCβ construct are significantly different from those of the HS3HS2γβ, HS3HS2γTATAβ, and HS3HS2γCACCCβ constructs.

**Fig. 2.** Graph of the mean β/γ-globin mRNA ratios for transfections of the HS3HS2γβ, HS3HS2γCACCCβ, HS3HS2γTATAβ, and HS3HS2γTCβ constructs. The bar graphs represent the mean of 12 individual HEL cell transfection assays with each of the four constructs. The S.D. are indicated by the lines above the bars. Cells with the HS3HS2γTCβ construct, with both the TATA and CACCC mutations, have at least an additive β/γ-globin mRNA ratio compared with those with the HS3HS2γCACCCβ and HS3HS2γTATAβ constructs. The asterisks draw attention to data that is significantly different from that obtained with the HS3HS2γβ construct. The double asterisks indicate that the data obtained with the HS3HS2γTCβ construct is significantly different from all three of the other constructs.

and all findings were judged to be significant at an α-level of 0.01.

**Generation of Transgenic Mice**—The cesium chloride-purified HS3HS2γβ, HS3HS2γTATAβ, HS3HS2γCACCCβ, and HS3HS2γTCβ constructs were linearized and cleaved away from their vectors by restriction enzyme digestion followed by gel purification and electrophoresis. Further purification of the DNA for microinjection was performed using the Schleicher and Schuell Elutip column and the QIAGEN QIAexII gel extraction kit following the protocols provided. Transgenic mice were generated by microinjection of the DNA constructs into C57BL/6 × SJL F2 hybrid mouse eggs at the NIH transgenic facility at the University of Alabama, Birmingham. The F0 transgenic mice were identified by polymerase chain reaction amplification of two 5′ regions of the human β-globin gene using DNA from tail biopsies. The first set of primers amplifies a product from position −1158 to −761 (5′-CCCCAGCTGACCTCATAAATGC-3′ and 5′-GAGGGGAAAAGGTCTTCTACTTGG-3′) and the second set amplifies −1232 through −905 (5′-CCCTACGCTGACCTCATAAATGC-3′ and 5′-GAGGGGAAAAGGTCTTCTACTTGG-3′). The integrity of the inserted DNA construct was confirmed, and the number of copies inserted per cell was determined for each mouse line using diagnostic restriction endonuclease digestions and Southern blotting. The Southern blots indicated that two of the F0 mice had multiple sites of integration of the transgene, and mice from lines 2–9 HS3HS2γβ and 4–6 HS3HS2γTCβ were each bred to obtain two separate lines containing single integration sites (designated 2–9A, 2–9B, 4–6A, and 4–6B). Subsequent identification of transgenic embryos and fetuses in established lines was made by polymerase chain reaction amplification of the same 5′ regions in the human β-globin gene discussed above using DNA prepared from placentas. All of the 10.5-days-post-coitum (dpc) embryos and 14.5-dpc fetuses used in this study were of either the F1 or F2 generation. The adult animals tested in primer extension analyses were of the F1 generation to avoid the possibility that F0 animals are mosaic.
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| Construct | Transfection | %β mRNA | %γ mRNA | β/γ mRNA ratio |
|-----------|--------------|---------|---------|----------------|
| HS3HS2γβ  |              | 1.0     | 46.2    | 0.02           |
| 1         | 1.0          | 50.6    | 0.02    |
| 2         | 1.7          | 48.6    | 0.03    |
| 3         | 1.6          | 50.2    | 0.03    |
| 4         | 1.8          | 48.7    | 0.04    |
| 5         | 0.9          | 14.0    | 0.06    |
| 6         | 1.1          | 10.7    | 0.10    |
| 7         | 1.1          | 14.9    | 0.07    |
| 8         | 1.2          | 20.8    | 0.06    |
| Mean ± S.D. | 1.2 ± 0.3   | 33.4 ± 18.1 | 0.05 ± 0.03 |

| Construct | Transfection | %β mRNA | %γ mRNA | β/γ mRNA ratio |
|-----------|--------------|---------|---------|----------------|
| HS3HS2γT/Cβ |              | 11.6    | 17.9    | 0.65           |
| 1         | 11.6         | 17.9    | 0.65    |
| 2         | 13.9         | 13.3    | 0.92    |
| 3         | 7.8          | 20.3    | 0.38    |
| 4         | 6.6          | 12.2    | 0.54    |
| 5         | 5.1          | 12.7    | 0.40    |
| 6         | 4.6          | 12.7    | 0.36    |
| 7         | 14.6         | 14.0    | 1.05    |
| 8         | 8.8          | 11.9    | 0.74    |
| 9         | 10.6         | 9.2     | 0.50    |
| 10        | 4.6          | 9.3     | 0.50    |
| Mean ± S.D. | 7.3 ± 3.4   | 13.5 ± 3.3 | 0.54 ± 0.21 |

RNA Analysis for Transgenic Mice—RNA was prepared from normal and transgenic mice from 10.5-dpc yolk sacs and 14.5-dpc fetal livers, the tissues that contain erythropoietic cells at those stages of development. For the vast majority of time points and lines, the data were obtained from two embryos or fetuses, and the mean is reported. The oligonucleotides used for primer extension analysis of the human γ- and β-globin and the mouse ε-, βH1-, and β-globin mRNAs and their product sizes have been described (12). Human β-like globin mRNA amounts were normalized to and expressed as a percentage of the mouse β-like globin mRNA(s) and have not been corrected to assign a value for expression per gene copy, because expression per gene copy and gene copy number are inversely proportional to each other in cell lines with the HS3HS2γβ construct (12, 27). The copy numbers per cell in each line are listed in Table III for reference.

RESULTS

The TATA and CACCC Elements Are the Major Elements in the γ-Globin Promoter Involved in Competitive Inhibition—We have previously shown that the γ-globin TATA and CACCC elements, but not the stage-selector element, are important in competitive inhibition of β-globin gene expression using constructs containing HS3 and HS2 from the LCR in stable transfections of HEL cells (25), which express the ε- and γ- but not the β-globin genes. To determine whether the TATA and CACCC elements act together in β-globin suppression and to investigate whether these are the two major γ-globin promoter elements involved, we have tested additional constructs in stable transfection assays in HEL cells (Fig. 1B). The constructs in these experiments contain a normal γ-globin gene (HS3HS2γβ), a γ-globin CACCC mutation (HS3HS2γCACCC-β), a γ-globin TATA mutation (HS3HS2γTATAβ), both a CACCC and a TATA mutation (HS3HS2γT/Cβ), or a γ-globin promoter deletion from position −136 to +56 (HS3HS2ΔTBS). The TATA and CACCC mutations are 4- or 5-base substitutions that eliminate the consensus DNA binding sites for transcription factors. HS3 and HS2 from the LCR were included in the constructs because these elements provide most of the enhancer activity for the LCR (28), and they allow for correct embryonic/fetal expression of the γ- and β-globin genes (25).

Transfections of the HS3HS2γβ, HS3HS2γCACCCβ, HS3HS2γTATAβ, and HS3HS2γT/Cβ constructs were performed in HEL cells, and RNA was isolated and analyzed by primer extension analysis (Table I). There was a statistically significant increase in β-globin gene expression in cells with each of the mutant constructs compared with HS3HS2γβ, as indicated by the asterisks in Table I. A mutation of the TATA or CACCC element resulted in 5.2- or 7.0% β-globin mRNA, representing a 2- or 2.6-fold increase in β-globin gene expression, respectively, compared with HS3HS2γβ (2.7% β-globin). When both elements were mutated, there was an approximately a 4-fold increase in β-globin gene expression (9.8% β-globin). Mutations in the TATA element resulted in the use of alternative upstream transcription start sites for the γ-globin gene, as previously described (25). These upstream start sites are clustered in two regions and are also observed in cells with the HS3HS2γT/Cβ construct (see Fig. 3). The effects of the mutations on γ-globin gene expression were also determined. There was no decrease in γ-globin expression in cells with the HS3HS2γCACCCβ construct. There was 2- or 3-fold less γ-globin mRNA in cells with the HS3HS2γTATAβ or HS3HS2γT/Cβ constructs, respectively, than in cells with HS3HS2γβ. Transcription of both the γ- and β-globin genes was affected more by the double mutation than by either mutation alone.

The β/γ ratios for each pool of cells transfected with the HS3HS2γβ, HS3HS2γCACCCβ, and HS3HS2γTATAβ, and HS3HS2γT/Cβ constructs are a comparison of the relative expression of the β- and γ-globin genes in each pool and are not dependent upon the position of integration or the number of copies of the construct per cell (Table I, Fig. 2). This is therefore an effective measure of the competition between the γ- and β-globin genes. There was approximately a 4-fold increase in the ratio with the TATA mutation and a 2-fold increase with the CACCC mutation, but when both elements were mutated, the β/γ ratio was 10-fold higher than in cells with the wild-type HS3HS2γβ construct. The ratio for the cells with the HS3HS2γT/Cβ construct is statistically significantly higher than with the TATA or CACCC mutation alone. The data indicate that the TATA and CACCC elements have at least an additive effect on the competition between the γ- and β-globin genes.

We have previously shown that β-globin gene expression is suppressed similarly in the presence of a γ-globin gene with a −161 and a −1350 promoter (13), suggesting that elements downstream of position −161 are sufficient for competitive inhibition. To test whether TATA and CACCC are the major elements in the γ-globin promoter responsible for β-globin suppression, the HS3HS2γβ, HS3HS2γT/Cβ, and HS3HS2ΔTBS constructs were stably transfected into 11 pools of HEL cells. These three constructs were transfected simultaneously for a direct comparison. The results for the cells with the
HS3HS2γI/Cβ constructs were similar to the previous experiment in that there was ~2.5-fold less γ-globin mRNA than those with the wild-type construct (13.5% compared with 33.4%) (Table II, Fig. 3). Consistent with previous results, the mean β/γ ratio of pools of cells containing the HS3HS2γI/Cβ construct (0.54) was 10-fold higher compared with those with the wild-type HS3HS2γβ construct (0.05). β-Globin mRNA was expressed at means of 7.3 and 10.6% in cells with the HS3HS2γI/Cβ construct and in cells with the HS3HS2γatsβ construct, respectively, compared with 1.2% β-globin mRNA in those with the HS3HS2γβ construct. These differences are statistically significant from the cells with HS3HS2γβ construct. The difference in the percentage of β-globin mRNA between the HS3HS2γI/Cβ and the HS3HS2γatsβ constructs, however, is not statistically significant at α ≤ 0.01. The Δts deletion removed the TATA element and all other DNA elements between positions −136 and +56; the CACCC is preserved in this construct. Because β-globin expression is similar with the HS3HS2γatsβ construct and with the construct that has both a TATA and a CACC substitution mutation, the CACCC element has as great an effect on β-globin suppression as does any combination of other DNA elements in the −161 γ-globin promoter. Therefore, the TATA and CACCC elements are the major DNA elements involved in β-globin suppression. Further evidence supporting the crucial role of the CACCC element comes from the transgenic mouse studies described below.

The γ-Globin TATA and CACCC Elements Have Coordinate Yet Distinct Roles in Human γ- to β-Globin Switching in Development—To study the roles of the human γ-globin TATA and CACCC elements in γ-globin expression and in suppression of β-globin gene expression in an in vivo developmental system, transgenic mice with the HS3HS2γβ, HS3HS2γCACCCβ, HS3HS2γTATAβ, HS3HS2γI/Cβ constructs, Mouse lines 2–9A, 2–9B, and 4–1 contain the HS3HS2γβ construct, lines 2–4 and 3–6 have HS3HS2γCACCCβ, lines 1–6 and 7–3 have HS3HS2γTATAβ, and lines 4–6A, 4–6B, 6–1, and 4–7 contain HS3HS2γI/Cβ constructs. The amounts of human γ- and β-globin mRNA were measured in the 10.5-dpc embryonic yolk sac, in the 14.5-dpc fetal liver, and in adult blood from mice in each of the lines. The products from primer extension of the human RNAs are labeled as follows. γ is the endogenous γ-globin mRNA, γ(−9) and γ(−33) are the mRNAs that start upstream of the normal start site due to the TATA mutation, and β is the human β-globin mRNA. Mouse β-like globin mRNA (mε and mβh1 in 10.5 dpc, mβm in 14.5 dpc and adult) was measured to normalize the human mRNAs between samples.
The amounts of human γ- and β-globin mRNA were measured at 10.5 dpc, 14.5 dpc, and in adult mice for each of the mouse lines. Data are the mean of two primer extension assays, usually performed on two different animals for each time point. The human γ- and β-globin mRNA is expressed as a percentage of the endogenous mouse β-globin mRNA present at the same time point. The β- to γ-globin mRNA ratio indicates the net effect of the γ-globin promoter mutations on the competition between the γ- and β-globin genes. The number of copies of the construct per cell in each mouse line is indicated; however, the % mRNA is not expressed per gene copy (see “Results”). To determine the amount of expression per transgene copy, divide %mRNA by the copy number and multiply by four, for the four mouse β-like globin genes per cell. d, day.

| Transgene Line | % γ-globin mRNA | % β-globin mRNA | β/γ globin mRNA ratio | Copy No. |
|----------------|-----------------|-----------------|-----------------------|---------|
|                | 10.5 d | 14.5 d | Adult | 10.5 d | 14.5 d | Adult | 10.5 d | 14.5 d | Adult |
| HS3HS2γβ       | 2–9A   | 38.9  | 54.4  | 10.9  | 1.6   | 3.3   | 1.7   | 0.04  | 0.06  | 0.15  | 2     |
|                | 2–9B   | 27.5  | 21.1  | 4.6   | 0.7   | 1.0   | 0.8   | 0.03  | 0.05  | 0.18  | 2     |
|                | 4–1    | 6.6   | 4.8   | 0.4   | 0.2   | 0.5   | 0.3   | 0.02  | 0.10  | 0.65  | 2     |
| HS3HS2γCACCCβ  | 2–4    | 22.8  | 0.3   | 0.1   | 1.0   | 4.4   | 5.3   | 0.04  | 14.00 | 57.27 | 7     |
|                | 3–6    | 39.6  | 0.5   | 0.1   | 3.8   | 6.3   | 1.5   | 0.10  | 12.14 | 15.00 | 7     |
| HS3HS2γTATAβ   | 1–6    | 1.6   | 0.9   | 0.1   | 0.7   | 1.1   | 0.3   | 0.47  | 1.33  | 3.24  | 3     |
|                | 7–3    | 4.0   | 9.2   | 0.3   | 1.5   | 8.6   | 2.1   | 0.38  | 0.93  | 7.08  | 19    |
| HS3HS2γTCβ     | 4–6A   | 5.7   | 0.1   | 0.1   | 8.7   | 6.1   | 5.7   | 1.53  | 60.61 | 57.33 | 4     |
|                | 4–6B   | 10.4  | 0.2   | 0.1   | 19.3  | 5.5   | 24.3  | 1.85  | 23.67 | 243.23 | 6    |
|                | 4–7    | 1.0   | 0.1   | 0.1   | 4.6   | 2.1   | 2.4   | 4.37  | 30.74 | 23.63 | 5     |
|                | 6–1    | 0.9   | 0.1   | 0.1   | 8.6   | 6.4   | 3.8   | 9.74  | 64.35 | 37.90 | 6     |

DISCUSSION

According to the competition model, transcription of the γ-globin gene early in development precludes the expression of the β-globin gene because the γ-globin promoter directly or indirectly engages the LCR, limiting interactions between the LCR and the β-globin gene (18, 19). We had previously shown that elements downstream of −161, including the γ-globin TATA and CACCC elements, are important for suppression of the β-globin gene in embryonic/fetal erythroid cells. In this work, we confirm these results in an in vivo developmental system, demonstrate that the γ-globin TATA and CACCC are the major regulators of embryonic β-globin silencing, show that these elements function coordinately, and establish that the CACCC element has stage-specific effects in γ- to β-globin switching. The results indicate that the γ-globin TATA element is required both for normal levels of embryonic transcription and for initiation of transcription at the appropriate site.

Data from transgenic mice and transfected cells indicate that both the TATA and CACCC elements are required in order for the γ-globin gene to most effectively inhibit β-globin gene expression in early development. Experiments in transfected HEL cells show that the TATA and CACCC elements are the
major γ-globin promoter elements responsible for β-globin suppression in early development. In previous work, we studied an HS2γΔtsβ construct in transgenic mice (13). In three lines of HS2γΔtsβ mice, the percentage of γ-globin expression compared with mouse β-like globin at 10.5 dpc were 6, 21, and 21%. In 5 lines of HS2γβ mice, there was between 0.0 and 1.4% γ-globin expression at 10.5 dpc. The average % γ-globin expression at 10.5 dpc is 10.3 for the lines with HS3HS2γCACCCβ and HS3HS2γTATAβ constructs.

In comparing the expression of constructs with mutations in the two elements to those with the single mutations, there is at least an additive effect on increasing the γ/β globin mRNA ratio in HEL cells, and this effect is even more evident in an in vivo developmental system in embryonic and fetal transgenic mice. This suggests that the proteins binding to the γ-globin TATA and CACCC elements can work independently but coordinate to establish a greater effect on the regulation of globin gene developmental switching.

The data indicate that the γ-globin TATA and CACCC elements each have different roles. Transgenic mouse experiments show that the γ-globin TATA element plays a role in γ-globin expression and β-globin suppression in the embryo and fetus; in contrast, the CACCC element has a stage-specific effect in the fetus. Ryan et al. (30) studied the effects of a mutation in the γ-globin CACCC element on the human β/γ-globin mRNA ratio in the yolk sac of transgenic mice. Their results differed from the data presented here in that they observed an increased β/γ globin mRNA ratio in the yolk sac, whereas in our experiments the ratio was not increased at 10.5 days but was increased at 14.5 days. However, qualitative inspection of their data shows that the β/γ ratio is much higher at 14.5 than at 10.5 dpc, and that their CACCC mutation appears to affect γ-globin transcription at 14.5 and not 10.5 dpc, which is consistent with the stage-specific effects described here. Most importantly, in the current work, the CACCC and TATA mutations are compared, demonstrating that both are important for γ-globin gene silencing but that the CACCC element is unique in that it has a stage-specific effect. Ryan et al. (30) show that the γ-globin GATA-1 and Oct-1 binding elements, not involved in β-globin suppression, is consistent with our previous data indicating that the elements necessary for this effect are downstream of −161 (13).

The fact that the γ-globin CACCC element has a stage-specific effect is consistent with the role of the β-globin CACCC element. Targeted gene knock-outs in mice indicate that the
erythroid Krüppel-like factor exerts its effects through the proximal CACCC element in the β-globin promoter, and it is responsible for positively regulating this gene in the adult (31–33). A lack of the erythroid Krüppel-like factor does not reduce γ-globin gene expression (31), suggesting that a different protein binding to the γ-globin CACCC element has a significant role in stage-specific expression. In the future, it will be of interest to identify the transcription factor(s) that binds to the γ-globin CACCC element to elicit its stage-specific effects. Two fetal erythroid kruppel-like factors (FKLF-1 and FKLF-2) have been identified and are candidates that may have this function (34, 35). FKLF-1 and FKLF-2 increase transcription of a γ-globin promoter-reporter gene fusion construct in transient transfection assays, and they require the CACCC element for this activity. DNA binding assays have not been performed to demonstrate that either FKLF-1 or FKLF-2 binds the γ-globin CACCC element. The factors do not appear to be specific for the γ-globin gene because they increase ε- and β-globin gene transcription as well. Further work will need to be done to determine whether one of these factors or some other factor(s) is responsible for the stage-specific activity of the γ-globin CACCC element.

In adult mice with the HS3HS2β construct, the γ-globin gene is not silenced to the same extent as it is in the native locus in humans. This has previously been observed with other constructs (10, 12, 36). There are several possible explanations for this finding. It is possible that an element necessary for adult silencing is absent from the HS3HS2β construct. The shorter than normal distance between the LCR elements and the γ-globin gene in this construct may reduce silencing. It is also possible that HS3 and HS2, in the absence of HS1 and/or HS4, are not sufficient to correctly down-regulate the γ-globin gene in the adult.

The competition model for the developmental regulation of the human γ- and β-globin genes is consistent with a mechanism of interactions between the genes and the LCR via a looping model, a linking model, or both. The finding that the γ-globin CACCC and TATA elements are both necessary for competition does not distinguish whether looping and/or linking occurs, but it does add some structure to the model for globin gene switching. If looping is a major mechanism in establishing the enhancer activity of the LCR, then factors binding to the γ-globin CACCC and TATA elements are required to establish protein-protein interactions with factors binding to the LCR, thereby forming an LCR-γ-globin gene loop. If linking is the major mechanism, then when the γ-globin CACCC and TATA elements are occupied, this inhibits the ability of protein complexes to establish a complete connection along the chromatin between the LCR and the β-globin gene. For example, binding of factors to the γ-globin CACCC and TATA elements could change the chromatin conformation so that the linking complexes could not bind to the DNA between the γ- and the β-globin genes. Alternatively, since the binding of CACCC and TATA factors facilitates transcription of the γ-globin gene, transcription may in turn diminish looping or linking between the LCR and the β-globin gene. Whether looping, linking, or some other mechanism is involved, β-like globin gene regulation must depend on the presence of stage-specific factors, such as CACCC-binding proteins, to establish whether LCR-γ-globin or LCR-β-globin gene interactions are favored. These data demonstrate that the γ-globin TATA and CACCC elements are essential for coordinate regulation of the γ- and β-globin genes in development.

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