**Analysis of the Human \( \xi \)-Globin Gene Promoter in Transgenic Mice**

By Daniel E. Sabath, Elizabeth A. Spangler, Edward M. Rubin, and George Stamatoyannopoulos

\( \xi \)-Globin is the embryonic form of the \( \alpha \) chain of hemoglobin. Transgenic mice generated with \( \xi \)-globin constructs containing the \( \xi \)-globin gene, 557 bp of 5' flanking sequence, and 2-kb of 3' flanking sequence linked to the \( \beta \)-globin locus control region hypersensitive site 2 (HS2) expressed human \( \xi \)-globin only in embryonic yolk sac erythroid tissue, and not in definitive erythroid tissue in the fetal liver or in adult peripheral blood. To determine what sequences in the 5' flanking region of the \( \xi \)-globin gene might be important for developmental specificity, a series of 5' deletion constructs of the \( \xi \)-globin gene were made and used to generate transgenic mice. The 5' ends of these constructs were located 417, 207, and 128 bp 5' to the \( \xi \)-globin transcriptional start site, and HS2 was included to increase the level of erythroid-specific expression. In all lines of mice tested, human \( \xi \)-globin was expressed only in embryonic tissue, and not in fetal livers or in adult peripheral blood. Expression was independent of copy number and appeared to be dependent on the site of transgene insertion. These data suggest that the proximal 128 bp of the \( \xi \)-globin promoter is sufficient to properly regulate \( \xi \)-globin expression during development.

**MATERIALS AND METHODS**

**Constructs.** The constructs used to generate transgenic mice in these experiments (Fig 1) were derived from HS2-\( \xi \)6 here referred to as HS2(\( \xi \))557. This construct contains HS2 of the \( \beta \)-globin LCR placed 5' to a 4.8-kb EcoRI fragment containing the human \( \xi \)-globin gene, 557 bp of 5' flanking sequence, and approximately 2 kb of 3' flanking sequence in the vector pUC19. To generate deletion constructs, a 662-bp EcoRI-Bgl II fragment from the 5' end of the human \( \xi \) genomic clone was subcloned into pUC19 so as to preserve the Bgl II site. This plasmid was cut with Rsa I, Hinfl, or Bgl I to generate 5' ends at 417, 207, and 128 bp 5' to the \( \xi \)-globin transcription start site, respectively. The ends were made blunt, 

**Kpn I** linkers were added, and the resulting Kpn I-Bgl II fragments were subcloned into Kpn I-Bgl II cut pUC19 containing the 4.8-kb \( \xi \)-globin genomic EcoRI fragment. Finally, the deleted \( \xi \) genomic structures were isolated as Kpn I-EcoRI fragments and subcloned into phS2(\( \xi \))557 in place of the full-length \( \xi \) DNA, which was removed by digestion with Kpn I and EcoRI. The structures of all three constructs were verified by sequencing around the newly generated Kpn I site at the 5' end of the \( \xi \) sequence and around the internal Bgl II site.

**Transgenic mice.** The HS2-\( \xi \) constructs were separated from plasmid sequences by HindIII-EcoRI digestion and agarose gel electrophoresis. The DNA was eluted from the gel and purified as de...
Transgenic mice were generated as described except that a 662-bp EcoRI-Bgl II fragment from the 5' end of the \( \zeta \)-globin genomic clone was used as a probe to identify transgenic progeny.

**Copy number determination.** Genomic DNA was prepared from the tails of F1 progeny or from day-14 F1 embryo carcasses. Five micrograms of DNA was digested with BamHI, separated by agarose gel electrophoresis, and blotted; blots were probed with a mixture of radiolabeled \( \zeta \)5' DNA probe and a 600-bp BamHI fragment of murine \( \zeta \) cDNA (generously provided by R. Perlmutter, University of Washington). Probes were labeled to a specific activity of \( 1 \times 10^6 \) cpm/\( \mu \)g using a random primer method (Prime-It Kit; Stratagene, La Jolla, CA). The blots were washed at 60°C in 0.2X SSC (1X SSC is 0.15 mol/L sodium chloride, 15 mol/L sodium citrate, pH 7.0)/0.1% sodium dodecyl sulfate (SDS), and the signals were quantitated using a Molecular Dynamics Phosphor Imager equipped with ImageQuant software. Undigested probe was electrophoresed and probed with a radiolabeled DNA fragment from the tails of F1 progeny or from day-14 F1 embryo carcasses.

**RNA probes.** Plasmids pSP6M[151] and pSP6Mao143 were used as DNA templates to generate antisense RNA probes for the detection of murine \( \zeta \) and \( \alpha \) RNA, respectively. Radiolabeled RNA was generated by in vitro transcription with SP6 polymerase using EcoRI-cut pSP6M[151] and HindIII-cut pSP6Mao143 with an in vitro transcription kit (Ambion, Austin, TX) according to the method of the manufacturer. For the detection of human \( \zeta \) RNA, a probe (pT7H[207]) was constructed by ligating human \( \zeta \) genomic DNA from the Psi I site at -84 to the Bgl II site at +105 to human \( \zeta \) cDNA from the Bgl II site at position 47 to the Sna I site at position 149 of the cDNA sequence. Template DNA was generated by digestion with BamHI, and radiolabeled antisense RNA was transcribed in vitro by T7 RNA polymerase. All RNA probes were isolated by spin column chromatography through Select D-RF columns (5 Prime → 3 Prime, Boulder, CO).

**RNAase protection.** Five micrograms of total RNA was combined with \( 10^6 \) cpm of the murine \( \zeta \), murine \( \alpha \), and human \( \zeta \) RNA probes; ethanol-precipitated; dried; and dissolved in \( 20 \mu \)L of hybridization buffer (80% formamide, 40 mmol/L piperazine-\( N,N \)-bis[2-ethanesulfonic acid] [PIPES], pH 6.4, 400 mmol/L sodium acetate, 1 mmol/L EDTA). Each probe was combined with \( 2 \mu \)g of yeast tRNA in duplicate as controls. The mixtures were heated to 95°C for 3 minutes and then incubated overnight at 47°C. Two hundred microliters of a mixture of 0.5 U/mL RNAase A and 10 U/mL RNAase T1 was added (except for one yeast tRNA tube for each probe), digestion was performed for 30 minutes at 37°C, and the reaction was stopped by the addition of 300 \( \mu \)L of a guanidinium isothiocyanate-isopropanol mixture (from the RPA II kit; Ambion), which simultaneously precipitates the RNA. The precipitated RNA was dissolved in \( 8 \mu \)L of loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromphenol blue, 2 mmol/L EDTA) and separated by electrophoresis on a 0.4-mm 5% acrylamide gel containing 50% urea and 1X TBE (90 mmol/L Tris, 90 mmol/L boric acid, 2 mmol/L EDTA). The human \( \zeta \), mouse \( \zeta \), and mouse \( \alpha \) mRNAs protect fragments of 207, 151, and 134 bases of human \( \zeta \), murine \( \alpha \), and human \( \zeta \) RNAs, respectively.

**Isolation of nucleic acids.** DNA was isolated by digesting tissue overnight with proteinase K in 0.5% SDS, 0.1 mol/L sodium chloride, 50 mmol/L tri(hydroxymethyl)aminomethane (Tris), pH 7.5, 4 mmol/L (ethylenedinitri1o)tetraacetic acid (EDTA) at 50°C. The mixture was made 1.2 mol/L with potassium acetate (pH 5), extracted once with an equal volume of chloroform:isoamyl alcohol (24:1 vol/vol), and DNA was precipitated by the addition of 2 vol of ethanol. DNA was spooled out, rinsed with 70% ethanol (vol/vol), dried briefly, and dissolved in 10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0. DNA was quantitated by fluorimetry. DNA was isolated by the acid phenol method and quantitated by spectrophotometry.
RESULTS

Constructs and transgenic mice. Three constructs were made containing HS2 and various deletions of the \( \beta \)-globin 5' flanking region (Fig 1), and these constructs were used to generate lines of transgenic mice. The deletions used were based on examination of the sequence of the \( \beta \)-globin 5' flanking region and footprinting data, because functional studies had been performed on the proximal \( \beta \) promoter, but not on the distal 5' flanking region. The 5' ends of the constructs were located 417, 207, and 128 bp 5' to the \( \beta \)-globin transcription start site. The HS2(\( \beta \)–417) construct removes a potential Sp1 site at -479. The HS2(\( \beta \)–207) construct removes a GATA-1 site at -230 as well as other less well-characterized sites shown by DNAase footprinting. Finally, the HS2(\( \beta \)–128) construct was designed to contain a minimal \( \beta \) globin promoter, with binding sites for Sp1, GATA-1, and other uncharacterized DNA binding activities. Two lines were established containing the HS2(\( \beta \)–417) construct, two lines with the HS2(\( \beta \)–207) construct, and four lines with the HS2(\( \beta \)–128) construct. The transgene copy numbers of each line were determined and are shown in Table 1. All transgenes were judged to be intact based on genomic DNA blot analysis, which showed both the integrity of the \( \beta \)-globin gene, as judged by the presence of 2.202-, 1.992-, and 1.913-bp BamHI fragments for the HS2(\( \beta \)–557) transgenic mice, and the HS2(\( \beta \)–207) and HS2(\( \beta \)–128) constructs, respectively, as well as the presence of tandemly repeated transgene copies, as judged by the presence of Sac I fragments of about 5.6 kb (data not shown). All founders were bred to produce \( F_1 \) transgenic offspring to avoid mosaicism.

There is no silencer 5' to the minimal \( \beta \)-globin promoter. Timed pregnancies were established by breeding \( F_1 \) transgenic animals with nontransgenic animals. Embryos were removed at day 9 or day 14 of gestation. Transgenic day-9 embryos were identified by DNA blot analysis of placental DNA. Transgenic day-14 embryos were identified by analysis of carcass DNA. Whole transgenic day-9 embryos were pooled for each line, whereas transgenic yolk sacs, livers, brains, and peripheral blood were pooled separately for day-14 embryos. RNA was isolated from each of these tissues and subjected to quantitative RNAase protection analysis using RNA probes specific for mouse \( \beta \), mouse \( \alpha \), and human \( \gamma \) RNAs. In addition, RNA isolated from the peripheral blood of adult \( F_1 \) animals was examined for the presence of human \( \gamma \) RNA and mouse \( \alpha \) RNA.

We initially examined embryonic tissues from mice containing the HS2(\( \beta \)–557) construct. As shown originally, human \( \gamma \) mRNA was easily detected in day-9 transgenic embryos using an RNAase protection assay (Fig 2, lane 1). Quantitative analysis showed that the steady-state level of human \( \gamma \) mRNA was 21.5% the level of mouse \( \gamma \) mRNA (Table 1). When day-14 tissues were examined, only trace amounts of human \( \gamma \) mRNA were detected in the fetal liver, yolk sac, and brain (Fig 2, lanes 2, 3, and 5). Relatively large amounts of human and mouse \( \gamma \) mRNA were present in day-14 embryonic peripheral blood (Fig 2, lane 4), as expected, because at day-14 primitive erythroblasts are still circulating. It is likely that the signals observed in the liver, yolk sac, and brain were due to contamination with peripheral blood. No human or mouse \( \gamma \) mRNA was detectable in the peripheral blood of adult transgenic animals containing the HS2(\( \beta \)–557) construct (Fig 2, lane 6).

Similar results were obtained with the two lines of animals containing the HS2(\( \beta \)–417) construct. Low but detectable levels of human \( \gamma \) mRNA were detected in the fetal liver, yolk sac, and brain (Fig 2, lanes 2, 3, and 5). Human \( \gamma \) mRNA was 21.5% the level of mouse \( \gamma \) mRNA, whereas transgenic yolk sacs, brain, and peripheral blood showed a 10-fold increase over mouse \( \gamma \) mRNA in the same tissues (Table 1). The HS2(\( \beta \)–207) construct was designed to contain a minimal \( \beta \) globin promoter, with binding sites for Sp1, GATA-1, and other uncharacterized DNA binding activities. Two lines were established containing the HS2(\( \beta \)–417) construct, two lines with the HS2(\( \beta \)–207) construct, and four lines with the HS2(\( \beta \)–128) construct. The transgene copy numbers of each line were determined and are shown in Table 1. All transgenes were judged to be intact based on genomic DNA blot analysis, which showed both the integrity of the \( \beta \)-globin gene, as judged by the presence of 2.202-, 1.992-, and 1.913-bp BamHI fragments for the HS2(\( \beta \)–557) transgenic mice, and the HS2(\( \beta \)–207) and HS2(\( \beta \)–128) constructs, respectively, as well as the presence of tandemly repeated transgene copies, as judged by the presence of Sac I fragments of about 5.6 kb (data not shown). All founders were bred to produce \( F_1 \) transgenic offspring to avoid mosaicism.

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able amounts of human ζ mRNA were detected in day-9 transgenic embryos (Fig 3, lanes 1 and 7). Quantitative analysis showed that the level of human ζ mRNA was 2.6% and 2.8% the level of mouse ζ mRNA in lines 6 and 13, respectively (Table 1). Human ζ mRNA was not detected in the peripheral blood of day-14 transgenic embryos from line HS2(−417)−6 (Fig 3, lane 4), whereas human ζ mRNA was detectable in the peripheral blood of day-14 embryos of line HS2(−417)−13 (Fig 3, lane 10). However, no human ζ mRNA was detectable in the yolk sac, liver, or brain of day-14 embryos from either line (Fig 3, lanes 2, 3, 5, 8, 9, and 11). As with animals containing the HS2(−557) construct, human ζ mRNA was undetectable in the peripheral blood of adult transgenic animals containing the HS2(−417) construct (Fig 3, lanes 6 and 12).

Lines 3 and 7 of mice containing the HS2(−207) construct expressed human ζ mRNA at 5.2% and 3.2% the level of mouse ζ mRNA in day-9 embryos, respectively (Table 1 and Fig 4, lanes 1 and 7). Human ζ mRNA could not be detected in transgenic day-14 embryos of HS2(−417)−3 mice (Fig 4, lanes 2 through 5); low levels of human ζ mRNA could be detected only in the peripheral blood of transgenic day-14 embryos of HS2(−417)−7 mice (Fig 4, lanes 8 through 11). As with the other mice examined, no human ζ mRNA was detectable in the peripheral blood of adult transgenic mice (Fig 4, lanes 6 and 12).

Four lines of mice with the HS2(−128) construct were obtained for analysis. Line 14 containing this construct was the only one of nine lines described in this study in which human ζ mRNA was detectable in day-9 transgenic embryos, as well as in tissues from transgenic day-14 embryos and adults (Fig 5, lanes 1 through 6). When quantitated, the level of human ζ mRNA was less than 1% the level of mouse ζ mRNA (Table 1). The remaining lines expressed human ζ mRNA in a pattern similar to that of those mice containing the other constructs. Human ζ mRNA was detected in day-9 transgenic embryos from lines 16, 17, and 19 at 10.1%, 3.5%, and 5.6% the level of mouse ζ mRNA, respectively (Table 1 and Fig 5, lanes 7, 13, and 19). These three lines of mice had easily detectable human ζ mRNA in the peripheral blood of transgenic embryos (Fig 5, lanes 10, 16, and 22). Only trace amounts of human mRNA were detectable in the fetal livers and brains of day-14 embryos (Fig 5, lanes 9, 11, 15, 17, 21, and 23). Line 19 had a considerable amount of human ζ mRNA in the yolk sac of transgenic day-14 embryos (Fig 5, lane 20), whereas no such expression was observed in the yolk sacs of transgenic day-14 embryos from lines 16 or 17 (Fig 5, lanes 8 and 14). Relatively large amounts of mouse ζ mRNA were also present in the line 19 day-14 yolk sacs, suggesting that this particular RNA sample contained more peripheral blood contamination than the others. Again, adult transgenic mice containing the HS2(−128) constructs did not express the human gene in their peripheral blood (Fig 5, lanes 12, 18, and 24).

Expression of human ζ is copy number independent. The constructs used in this study contained HS2 of the β-globin LCR. This element has been shown to confer high level expression of human β-globin, 19-25 γ-globin, 25 and α-globin.26 In addition, copy number dependence has been observed in some systems.21,23,27-31 To determine whether human ζ-globin can be regulated by HS2 in a similar manner, the copy number of each line of mice was determined by comparing the intensity of the human ζ signal to that of an endogenous single-copy mouse gene, lek, on genomic DNA blots hybridized simultaneously to probes for human ζ-globin and murine lek, the results of which are shown in Table 1. Copy number ranged from 2 copies in three of the HS2(−128) lines to 16 copies in the HS2(−207)−7 line. When corrected for copy number, the ratio of human ζ mRNA to mouse ζ mRNA in mice in which human ζ mRNA was detectable ranged from 0.4% per gene copy in HS2(−207)−7 mice to 10.1% per gene copy in HS2(−128)−16 mice. No relationship is apparent between copy number and level of human ζ expression, and no con-
that HS-40 could be more effective at increasing

genic mice were made, an enhancer element from the

struct expressed human

bin locus was described (HS-40).32*33 Although it is possible

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site of insertion of the transgene in the mouse genome, be-

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eral blood of any of the adult mice examined. A similar

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construct.

At least two lines of mice were obtained containing each

site were generated and linked to HS2 of the @-globin LCR.

Three

promoter deletion constructs with

ends located

417, 207, and 128 bp 5’ to the \( \zeta \)-globin transcription start

site were generated and linked to HS2 of the \( \beta \)-globin LCR.

At least two lines of mice were obtained containing each

construct.

With one exception, human \( \zeta \) mRNA was detected in
day-9 embryos and day-14 peripheral blood of all lines of
human \( \zeta \) transgenic mice, regardless of the construct they
contained. No human \( \zeta \) mRNA was detected in the periph-
eral blood of any of the adult mice examined. A similar
pattern of expression was observed for mouse \( \zeta \) mRNA,
showing that expression of human \( \zeta \) mRNA was under
proper developmental control. The only exception was in
line 14 containing the HS2\( (-128) \) construct, in which the
level of human \( \zeta \) mRNA was less than 1% the level of mouse
\( \zeta \) mRNA. The reason for this is unclear, as DNA blotting
suggested that the transgene was intact in this line. Presum-
ably, the lack of detectable human \( \zeta \) mRNA was due to the
site of insertion of the transgene in the mouse genome, be-
cause mice with identical copy numbers of the same con-
struct expressed human \( \zeta \) mRNA.

Subsequent to the time that these constructs and transgen-
ic mice were made, an enhancer element from the \( \alpha \)-glo-
bin locus was described (HS-40).32,33 Although it is possible
that HS-40 could be more effective at increasing \( \xi \) expres-
sion than HS2, this is unlikely for two reasons. First, HS2
and HS-40 are quite similar in structure, containing multi-
ple binding sites for the \emph{trans}-acting factors GATA-1 and
NF-E2.22,33-37 Second, HS2 and HS-40 are essentially inter-
changeable when used to drive expression of \( \alpha \) or \( \zeta \) pro-
moters in transient transfection experiments (unpublished
data). It is more likely that the low expression observed is a
property of embryonic globin genes, because low and vari-
able expression of both \( \alpha \) and \( \zeta \)-globin has been observed
when linked to HS2.7,8

As shown in Table 1, no relationship between transgene

copy number and the level of expression was observed \((r^2 =
.244)\). Copy number dependence, along with integration site

independence, are features associated with locus control
regions. HS2, alone or in association with other hypersensi-
tive sites from the \( \beta \)-globin LCR, clearly is able to increase
the level of expression of globin genes in transgenic mice or
eythroid cell lines, but the issue of copy number depend-
ence is less clear. Although copy number-dependent, integra-
tion site-independent expression of globin genes coupled
to HS2 or larger portions of the LCR has been observed in
some experiments,21,23,27-31 other experiments show no
relationship between copy number and level of globin ex-
pression,7,8,19,20,25,38 and there are even cases in which there
is an inverse relationship between copy number and globin
expression.22,24 An inverse relationship between copy

umber and expression per gene copy is also seen in trans-
genic mice containing the human \( \alpha \)-globin gene linked to the
upstream \( \alpha \)-globin enhancer HS-40.39 In all these ex-
periments, there is no apparent correlation between the globin
genes or enhancer constructs used, and thus the copy num-
ber issue remains unresolved.

One purpose of the experiments described in this study
was to determine if any sequences are present in the 5’ flank-
ing region of the \( \zeta \)-globin gene that are responsible for the
lack of \( \xi \)-globin expression during fetal development and
adult life, as are found in the \( \epsilon \)-globin 5’ flanking region.34 If

-FIG 5. RNAase protection of RNA from HS2\( (-128) \) transgenic mice. Lanes 1 through 6 contain RNA from line HS2\( (-128) \) - 14.
Lanes 7 through 12 contain RNA from line HS2\( (-128) \) - 16. Lanes 13 through 18 contain RNA from line HS2\( (-128) \) - 17. Lanes 19
through 24 contain RNA from line HS2\( (-128) \) - 19. Five micrograms of total RNA from day-9 embryos (lanes 1, 7, 13, and 19), day-14
fetal yolk sacs (lanes 2, 8, 14, and 20), day-14 fetal livers (lanes 3, 9, 15, and 21), day-14 fetal peripheral blood (lanes 4, 10, 16, and 22),
day-14 fetal brain (lanes 5, 11, 17, and 23), and adult peripheral blood (lanes 6, 12, 18, and 24) was analyzed as in Fig 2.
a developmental silencer had been deleted in any of the constructs, human $\gamma$ expression should have been observed in the fetal liver, but no $\gamma$ expression was observed in fetal or adult mice. Because no developmental silencer for $\gamma$-globin can be detected between $-557$ and $-128$, developmental regulation of expression must be mediated by sequences in the 128 bp proximal to the transcription start site, within the gene, or in the 2 kb of 3' flanking sequence. Because transgenic mice containing the bacterial $\beta$-galactosidase gene linked to the $\gamma$-globin promoter express $\beta$-galactosidase only in primitive erythroid cells, it would appear that the 128 bp of 5' flanking sequence proximal to the $\gamma$-globin transcription start site are sufficient to regulate the expression of $\gamma$-globin during development, because the $\beta$-galactosidase constructs lack $\gamma$-globin gene sequences as well as 3' flanking sequences. Future efforts will be directed toward characterizing the factors that interact with this region of the $\gamma$ promoter.

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