Developmentally regulated and erythroid-specific expression of the human embryonic β-globin gene in transgenic mice

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ABSTRACT

Transgenic mice have proven to be an effective expression system for studying developmental control of the human fetal and adult β-globin genes. In the current work we are interested in developing the transgenic mouse system for the study of the human embryonic β-globin gene, ε. An ε-globin gene construction (HSII,I,ε) containing the human ε-globin gene with 0.2 kb of 3′ flanking sequence and 13.7 kb of extended 5′ flanking region including the erythroid-specific DNase I super-hypersensitive sites HSI and HSII was made. This construction was injected into fertilized mouse ova, and its expression was analyzed in peripheral blood, brain, and liver samples of 13.5 day transgenic fetuses. Fetuses carrying intact copies of the transgene expressed human ε-globin mRNA in their peripheral blood. Levels of expression of human ε-globin mRNA in these transgenic mice ranged from 2% to 26% per gene copy of the endogenous mouse embryonic ε^y-globin mRNA level. Furthermore, the human ε-globin transgene was expressed specifically in peripheral blood but not in brain or in liver which is an adult erythroid tissue at this stage. Thus, the HSII,I,ε transgene was expressed in an erythroid-specific and embryonic stage-specific manner in the transgenic mice. A human ε-globin gene construction that did not contain the distal upstream flanking region which includes the HSI and HSII sites, was not expressed in the embryos of transgenic mice. These data indicate that the human ε-globin gene with 5′ flanking region extending to include DNase I super-hypersensitive sites HSI and HSII is sufficient for the developmentally specific activation of the human ε-globin gene in erythroid tissue of transgenic mice.

INTRODUCTION

The human β-globin gene locus, located on the short arm of chromosome 11, is composed of five functional genes and one pseudogene, in the order of 5′ ε, $^G\gamma$, $^A\gamma$, ψβ, δ, β 3′. During development, the β-type globin genes are expressed in specific erythroid tissues and at specific developmental stages. The ε-globin gene is expressed in yolk sac-derived primitive erythroid cells until about 8 weeks of embryonic development. The main site of erythropoiesis then shifts to the fetal liver where the fetal γ-globin genes are activated in definitive lineage erythroid cells. The γ genes remain fully active until shortly after birth when the adult β-globin gene reaches maximum activity in bone marrow-derived definitive erythroid cells (reviewed in 1,2).

The transgenic mouse expression system has been effectively used to study human β-globin gene switching because mice have a related pattern of developmental switching. During mouse development, primitive nucleated erythrocytes are produced in the yolk sac blood islands between days 8 and 12 of gestation. These cells are released into peripheral blood and persist in the circulation until day 16 of gestation (3). Primitive nucleated erythrocytes produce two embryonic β-type globin chains, y and z (4,5), which are encoded by the ε^y (evolutionarily related to human ε) and the βh1 (related to human γ) genes, respectively (6−8). The level of βh1 expression is highest at days 10 and 11 of gestation and declines afterward, while the expression of the ε^y gene peaks at about day 13 and decreases during further fetal development (8,9). The next site of erythropoiesis is the fetal liver where definitive non-nucleated erythrocytes are produced and then released into peripheral blood beginning on day 12 of gestation and continuing until birth (10). Only the adult β-globin chains, encoded by the adult β-globin genes (related to the human β-globin gene) are produced in these definitive erythrocytes (3−5,11). The spleen and bone marrow begin to produce definitive non-nucleated erythrocytes after day 16 of gestation and continue to be the sites of erythropoiesis after birth. Only adult β-globin chains are synthesized in these cells as well (3−5).

When constructions of the human β- or γ-globin genes that include proximal flanking sequences are introduced into transgenic mice, regulatory elements within these regions are sufficient to confer erythroid and developmentally specific

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expression. The human β gene is expressed with the temporal developmental specificity of the mouse adult β-globin genes and the human γ gene is expressed with the developmental pattern of mouse embryonic βh1, its evolutionary orthologue in mouse (9,12−15). The level of expression was generally low and site-of-integration dependent, however, suggesting that some crucial elements for physiological expression were omitted.

Studies on chromatin structure have revealed the presence of two types of DNase I hypersensitive sites in the human β-globin locus which are closely related to globin gene expression. The first type of hypersensitive site is developmentally specific, and appears near or inside the specific globin gene being expressed (16,17). The second type of hypersensitive site, located in the flanking regions 5′ and 3′ of the β-globin locus, is erythroid-specific and developmentally stable. These sites appear specifically in erythroid cells throughout development, regardless of which β-globin gene is active in that particular cell type. The developmentally stable sites, designated super-hypersensitive (HS), are located 6 kb (HS I), 11 kb (HS II), 15 kb (HS III), 18 kb (HS IV), 21.5 kb (HS V) 5′ of ε-globin and 20 kb (HS VI) 3′ of β-globin (17−20).

Using constructions containing the human β-globin gene with appended flanking DNA containing super-hypersensitive sites HSI-IV (5′) and HSVI (3′), Grosveld and his colleagues first observed erythroid-specific, high-level and position-independent expression of the human β-globin gene in transgenic mouse fetuses and MEL (murine erythroleukemia) cells (20,21). Constructions containing the β-globin gene with all or subsets of only the 5′ super-hypersensitive sites are also expressed erythroid-specifically at a high level in transgenic mice and erythroid cell lines (22−24). In human, these sites (contained in the locus activation region or LAR) may normally potentiate expression for the whole β-globin locus by organizing the locus into an active chromatin domain which facilitates the activation of specific globin genes by developmental stage-specific trans-acting factors or other mechanisms (19,20). Surprisingly, however, the addition of the LAR HS sites to either γ or β resulted in a loss of developmental specificity of expression of the fused gene in transgenic mice (25−27). Proper developmental regulation of these genes was restored when the two genes were linked together in their natural configuration prior to microinjection and co-integration with the LAR. This result led to the suggestion that a competition between γ and β for HS site influence is responsible for normal γ- to β-globin gene switching during development (26,27).

Investigations on the expression of the human ε-globin gene have been carried out in the human erythroid prenatal tissue culture cell line, K562. In this line, inducible, physiological level expression of the human ε-globin gene has been achieved following the transfection of plasmids containing the ε-globin gene with 2 kb or less of 5′ flanking DNA (28−30). Recently a transcriptional silencer in the immediate 5′ flanking region of the human ε-globin gene has been identified in this system, which may play an important role in the regulation of ε-globin gene expression during development (31). In order to further study the regulation of ε-globin gene expression, we have introduced this gene into transgenic mice. This report describes the production of transgenic mouse fetuses containing the ε-globin gene with extended 5′ flanking region which includes super-hypersensitive sites HS I and HS II. These fetuses express the ε-globin transgene in both an erythroid-specific and a developmentally specific manner.

MATERIALS AND METHODS

ε-Globin gene constructions

A 3.7 kb Eco RI fragment containing the human ε-globin gene with 2 kb of 5′ flanking and 0.2 kb of 3′ flanking sequence and an 8-base Xho I linker inserted in the Stu I site between positions +44 and +45 of the 5′ untranslated region was cloned into the Eco RI site of pUC8, forming intermediate pHε3.7. In addition, a 15.4 kb Bam HI fragment extending from 0.2 to 15.6 kb 5′ of the human ε-globin gene was prepared from phage clone 5′ εII (obtained from O. Smithies, 32). This fragment was inserted into the Bam HI site of pUC8, forming p5′HεIIM. The 17.4 kb Sal I-Cla I fragment (containing pUC8 plus sequence from 15.6 to 0.9 kb 5′ of the ε-globin gene) was then prepared from p5′HεIIM and ligated to the 2.6 kb Cla I-Sal I fragment from pHε3.7 (containing the ε-globin gene with 0.9 kb of 5′ flanking and 0.2 kb of 3′ flanking region and a portion of the pUC8 polylinker sequence), forming p5′Hε17.4. This plasmid now contains the linkered ε-globin gene reconstituted with 15.6 kb of natural 5′ flanking sequence.

Preparation of the HSII,I,ε DNA fragment and its microinjection

The 15.5 kb Mlu I-Sal I DNA fragment (named HSII,I,ε; see Fig. 1) from p5′Hε17.4 was separated from vector sequence by electrophoresis on a 0.6% agarose gel, and eluted from the gel by electro-elution. The fragment was cleaned of contaminants using an elu-tip apparatus. After ethanol precipitation, the purified HSII,I,ε DNA fragment was resuspended in sterile microinjection buffer (10 mM Tris-HCl pH 7.5, 0.25 mM EDTA) at a concentration of 2 ng/μl and microinjected into the male pronuclei of F2 hybrid ova from C57BL/6×SJL parents. Injected eggs were immediately transferred to pseudopregnant Swiss Webster foster mothers. The method is as described by Brinster et al. (33). The 3.7 kb Eco RI fragment (Hε3.7) containing the human ε-globin gene (Fig. 1) was prepared from pHε3.7 in a similar way and microinjected using the same procedure.

DNA analysis

DNA isolated from fetal mouse carcasses was analyzed for the presence of intact transgenes by genomic Southern blot hybridization (34). DNA was digested with Rsa I, Xmn I, Kpn I and Bam HI, Kpn I and Nhe I, or Kpn I alone, electrophoresed on 0.7% (or 0.65%) agarose gels, and transferred to nitrocellulose. The filters were then hybridized with one of the following probes, radioactively labeled by the random primer method. The human ε-globin gene probe, the Bgl II-Dra I 0.42 kb fragment from the IVS-2 region of the human ε gene; the HSII region probe, the Hind III 1.9 kb fragment from 9.1 to 11.0 kb 5′ to the human ε gene; or the ε proximal upstream probe, the Eco RI-Bgl II 0.41 kb fragment from 1.6 to 2.0 kb upstream of the ε gene (see Fig. 3E). After hybridization, the filters were exposed to X-ray film at -80°C for 48 hr or longer. Human DNA from K562 cells and diluted plasmid DNA were used as copy number standards.

Preparation of RNA

Blood samples were collected from individual fetuses in 2.5 ml of ice-cold phosphate buffered saline (PBS) containing 10 units/ml of heparin. Blood cells were pelleted by centrifugation at 200 g, 4°C, for 20 minutes, resuspended in 4 ml of ice-cold PBS, and pelleted by centrifugation as above. Then, blood cells were

lysed in 0.5 ml of ice-cold lysis buffer (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.6, 0.5% Triton X-100) to dissociate the plasma membrane, and centrifuged at 200 g, 4°C, for 20 minutes to pellet the nuclei. After centrifugation, the supernatant was collected, combined with one volume of 2× Proteinase K buffer (0.2 M Tris-HCl pH 7.5, 25 mM EDTA, 0.3 M NaCl, 2% SDS), and treated with Proteinase K (final concentration, 200 μg/ml) at 37°C for 30 minutes. The treated solution was then extracted once with an equal volume of phenol/chloroform (1:1) and precipitated with 2.5 volumes of ice-cold ethanol. For liver and brain samples, total nucleic acids were first prepared and then DNase I treated to obtain purified RNA (35,36). To obtain total nucleic acids, frozen liver or brain samples were homogenized in 4 ml of SET Buffer (1% SDS, 5 mM EDTA, 10 mM Tris-HCl pH 7.5) containing 200 μg of proteinase K per ml, incubated at 45°C for 2.5 hr, extracted once with phenol/chloroform, twice with chloroform, and precipitated with 2.5 volumes of ethanol. After resuspension in DNase I buffer (6 mM $MgCl_2$, 10 mM NaCl, 40 mM Tris-HCl pH 7.9), the total nucleic acids sample was digested with DNase I (75 units/ml) for 20 minutes at 37°C, combined with 1 volume of 2× Proteinase K buffer, treated with Proteinase K (100 μg/ml) for 15 minutes at 37°C, extracted once with phenol/chloroform, once with chloroform, and precipitated with 2.5 volumes of ethanol. The purified RNA samples were resuspended in sterile double-distilled water, quantified by UV-spectrophotometry and electrophoresed on 1% agarose gels to determine the integrity of the RNA.

Primer extension

Primer extension analysis was performed essentially as described in Luse et al. (37) with only minor modification. Following primer extension the samples were resuspended in 10 μl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA), treated with RNase A (100 μg/ml) for 30 minutes at 37°C, lyophilized for 1 hr, and dissolved in 4 μl of Maxam-Gilbert loading dye mix (38). The extended primers were then analyzed on 8% denaturing polyacrylamide gels (38) and autoradiography was performed at −80°C with one intensifying screen. Quantifications of mRNA levels were made by densitometric scanning using a Zeineh Soft Laser Scanning Densitometer. For determining the relative expression level per gene copy of human ε-globin mRNA compared to the endogenous mouse ϵ^y mRNA level the following formula was used: (human ε-globin mRNA/mouse ϵ^y mRNA)×(2 endogenous ϵ^y genes/transgene copy #)×100%. The results were corrected for the relative specific activities of the human ε and mouse ϵ^y primers.

The oligonucleotides used in primer extension analyses were as follows: for human ε mRNA, the primer was a 19 nucleotide oligomer (5′-AAAATGCACCATGATGCCA-3′) which is complementary to the natural human ε mRNA sequence from positions +47 to +65, and the Xho I linkered ε mRNA product of HSII,I,ε or Hε3.7 from positions +55 to +73. For mouse β mRNA, the primer was a 19 nucleotide oligomer (5′-ATG-TCTGTTTCTGGGGTTG-3′) which is complementary to the mouse β^s mRNA sequence from positions +33 to +51. For mouse ϵ^y mRNA, the primer was a 20 nucleotide oligomer (5′-GC AAGTCTGGGAGGTTGCTG-3′) which is complementary to the ϵ^y mRNA sequence from positions +29 to +48.

RESULTS

Production and expression analysis of Hε3.7 transgenic mice

Fig. 1 illustrates the human β-globin locus and the two constructions, Hε3.7 and HSII,I,ε, which were injected into fertilized mouse ova. In initial experiments transgenic mice carrying the human ε-globin gene with flanking region sufficient for expression in K562 cells were produced by microinjecting purified human ε 3.7 kb Eco RI fragment (Hε3.7). Transgenic offspring were identified by Southern blot hybridization. The DNA samples were cut with Xmn I and probed with the human ε-globin gene probe (see 'Methods') to detect the 2.7 kb fragment which contains the entire human ε-globin gene including 1.1 kb of upstream flanking sequence. Nine male transgenic mice carrying from 1 to 15 intact copies of the human ε-globin gene were produced (data not shown). These transgenic mice were mated with control C57BL/6×SJL females. Fetuses were isolated

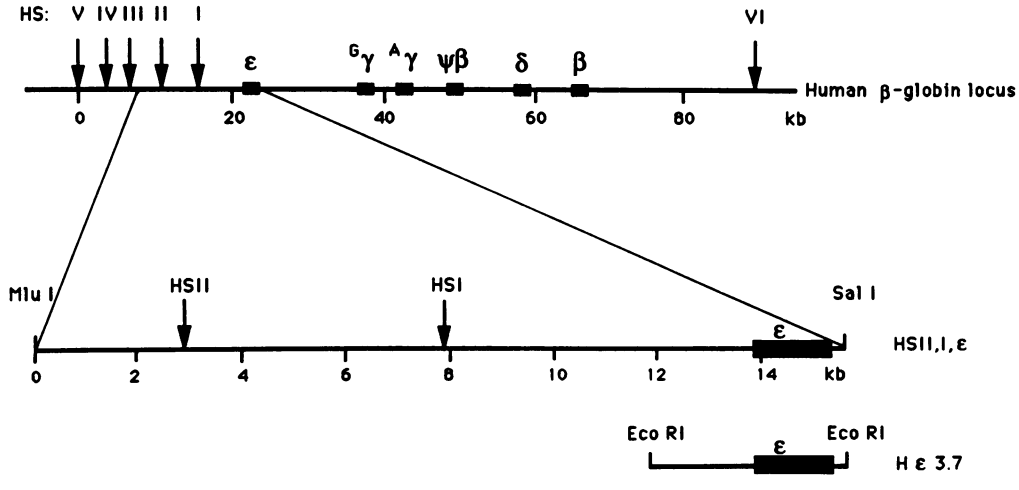

Figure 1. DNA fragments from the human β-globin locus microinjected into fertilized mouse ova. The top line shows the human β-globin locus. The arrows indicate the locations of the erythroid-specific DNase I super-hypersensitive sites. The second and the third lines show the HSII,I,ε and the Hε3.7 constructions, respectively, which were injected into fertilized mouse ova. The connecting lines between the β-globin locus and the HSII,I,ε construction indicate the original position of the HSII,I,ε fragment in the β-globin locus. The HSII,I,ε construction is the 15.5 kb Mlu I-Sal I fragment from p5′Hε17.4; the Hε3.7 construction is the Eco RI fragment from pHε3.7 (see Materials and Methods).

from the uteri of pregnant females at 11.5, 12.5, or 13.5 days of gestation during the expression period of endogenous ϵ^y to collect blood for RNA analysis by primer extension. No human ϵ-globin mRNA was detected in the peripheral blood of these unborn offspring. In several adult founders tested, analysis of blood RNA also indicated no expression of the transgene at this stage. Therefore, the construction containing the ϵ-globin gene with 2 kb of 5′ and 0.2 kb of 3′ proximal flanking region appears to lack some important genetic information which is necessary for the expression of the human ϵ-globin gene in transgenic mice.

Production of HSII,I,ϵ transgenic mice

To generate human HSII,I,ϵ transgenic fetuses, fertilized ova were injected with the purified HSII,I,ϵ fragment (Fig. 1), and transferred into the uteri of foster mothers. At day 13.5 of gestation, fetuses were removed from the uteri. Peripheral blood, liver, and brain samples were collected for later RNA analysis. DNA was isolated from the carcass. Transgenic fetuses were then identified by Southern blot hybridization. The DNA was cut with Rsa I and probed with the ϵ-globin gene probe (the Bgl II-Dra I 0.42 kb probe) to detect the 1.5 kb fragment extending from 69 bp 5′ of the cap site to 6 bp 5′ of the stop codon (see Fig. 2B). The results of the genomic Southern analysis are shown in Fig. 2A. Three fetuses, 6101, 6503, and 6910, were found to carry the ϵ gene portion of the transgene intact in the range of 1 to 6 copies per cell as determined by densitometry (6503, 6101 and 6910 have 1, 2 and 6 copies per cell, respectively).

To examine the intactness of the entire microinjected construction, numerous enzymes and sets of enzymes as well as multiple probes were used in genomic Southern analyses (Fig. 3). In all of these analyses the intensities of the bands generated from the various portions of the transgenes were consistent with

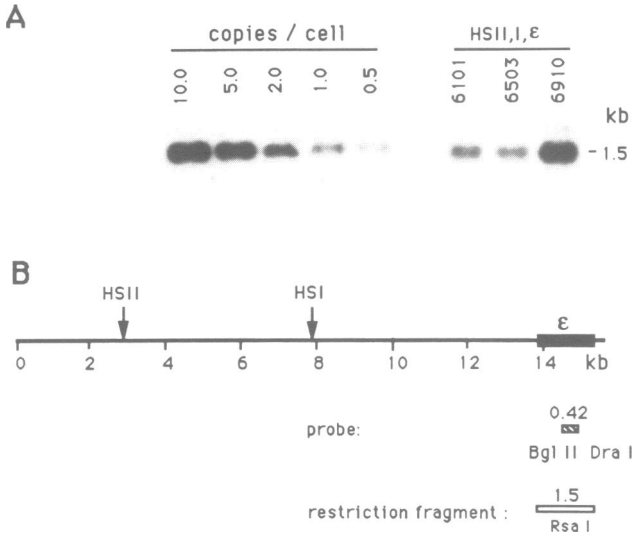

Figure 2. Southern blot analysis of the HSII,I,ϵ transgenic mice. DNA was digested with Rsa I and separated on a 0.7% agarose gel. The DNA was then transferred to a nitrocellulose filter and hybridized with the human ϵ-globin gene probe (the Bgl II-Dra I 0.42 kb probe) as illustrated in B (hatched bar). After washing, autoradiography was performed at −80°C. (A) Lanes 1 to 5 are different dilutions of Rsa I digested p5′Hϵ17.4 plasmid DNA equivalent to 10, 5, 2, 1, and 0.5 copies of the transgene per cell, respectively. Lane 6 is blank. Lanes 7 to 9 are six micrograms of Rsa I digested DNA from mice 6101, 6503, and 6910, respectively. (B) A map of the HSII,I,ϵ construction. The hatched bar below the construction represents the Bgl II-Dra I 0.42 kb probe used in A. The open bar represents the Rsa I 1.5 kb fragment detected by the Bgl II-Dra I probe.

the copy numbers obtained for the ϵ gene portion. To examine the intactness of the upstream region from 11.7 kb to 0.9 kb upstream of the cap site of the ϵ-globin gene, the DNA samples were cut with Kpn I and Nhe I, and probed with the HSII region probe (the Hind III 1.9 kb probe) with the results shown in Fig. 3A. As in the human DNA control, a 10.8 kb band was present in 6101 and 6910 DNA samples, indicating that the upstream DNA in this region is intact in both 6101 and 6910. In contrast, the 6503 DNA sample lacked the 10.8 kb band but had a band of much higher molecular weight, indicating that part of the upstream region of the transgene in 6503 has been rearranged. In a similar experiment using a Kpn I, Bam HI double-digestion which produces an 11.5 kb fragment from 11.7 kb to 0.2 kb upstream of the intact ϵ gene, we also observed that the band of expected size was present in 6101 and 6910 but not in 6503 (data not shown).

To individually examine the intactness of the HSI and HSII regions of the transgenes, the DNA samples were cut with Xmn I and probed with the HSII region probe which detects the Xmn I fragments containing both sites (see Fig. 3E). All of the transgenic fetuses (6101, 6503, and 6910) were found to contain intact HSI and HSII regions as indicated by the presence of 4.3 kb and 3.1 kb bands, respectively (Fig. 3B).

To investigate the proximal upstream region of the transgene in 6503, DNA samples from 6503 and 6101 were cut with Xmn I and probed with the ϵ proximal upstream region probe (the Eco RI-Bgl II 0.41 kb probe, see Fig. 3E). A 4.1 kb fragment extending from 5.2 to 1.1 kb upstream of the ϵ gene is present in 6101 as well as 6503 (Fig. 3C), showing that the ϵ proximal upstream region of the transgene of 6503 is intact.

For the ϵ-globin gene and immediate flanking region, the DNA samples were cut with Xmn I, and probed with the ϵ-globin gene probe (the Bgl II-Dra I 0.42 kb probe, see Fig 3E). As shown in Fig 3D, the 2.7 kb Xmn I fragment containing the entire human ϵ-globin gene with 1.1 kb of 5′ flanking sequence is present in both 6101 and 6910 DNA samples, but not in 6503, indicating that the transgene in mouse 6503 has been rearranged in this region.

Our results indicate that both 6101 and 6910 carry intact copies of the HSII,I,ϵ transgene. The results also show that 6503 has intact upstream region extending from 12.6 kb to 1.1 kb upstream of the ϵ gene (Fig 3B and 3C) and intact ϵ gene extending from 69 bp 5′ of the cap site to 6 bp 5′ of the stop codon (Fig. 2). However, when restriction enzymes such as Nhe I and Bam HI, which cut at sites located between 1.1 kb (Xmn I site) and 69 bp (Rsa I site) upstream of the ϵ gene were used alone or in combination with other enzymes to digest 6503 DNA, the expected fragments were not detected by appropriate probes (Fig. 3A and data not shown). These data indicate that the transgene in 6503 has been rearranged in the region between 1.1 kb and 69 bp upstream of the ϵ gene. Based on this and other data from genomic Southerns (not shown) we conclude that in 6503 the linear HSII,I,ϵ fragment circularized placing the M1u I and Sal I ends together (producing restriction fragments of predictable sizes traversing this junction) and then broke within the region between the Xmn I and Nhe I sites at 1.1 kb and 0.9 kb upstream of ϵ before or during integration into the mouse genome.

To examine whether the transgenes exist as tandem repeats, DNA samples cut with Kpn I, which cuts once within the transgene, were probed with the HSII region probe. A 15.5 kb band is expected if the transgenes are arranged in a head-to-tail tandem repeat and a 31 kb band is expected if head-to-head tandem repeats occurred. The 15.5 kb band is present in both

6101 and 6910 DNA samples but not in 6503, and the 31 kb fragment is not detected in any of the three samples (data not shown). These data indicate that the transgenes in 6101 and 6910 are arranged as head-to-tail tandem repeats, while the transgene in 6503 is integrated into the mouse genome as single copy. This result is consistent with the copy numbers of the transgenes obtained in Fig. 2 and the mechanism for 6503 integration described above.

Expression of the human ε-globin gene in HSII,I,ε transgenic mouse fetuses

At day 13.5 of mouse gestation, there are two populations of erythroid cells existing in peripheral blood, yolk sac-derived primitive nucleated erythroid cells and liver-derived definitive non-nucleated erythroid cells. The yolk sac-derived erythroid cells express the embryonic β-type globin genes, βh1 and ϵ^y, while the liver-derived erythroid cells contain exclusively adult β-globin mRNA expressed from the adult β-globin genes in nucleated precursors in the liver. Since the human ε-globin gene is the evolutionary orthologue of the mouse ϵ^y gene, and is expressed at the same developmental stage *in vivo*, we expected that the human ε-globin gene would have the same tissue and developmental specificity of expression as the mouse ϵ^y gene in these transgenic mouse fetuses. RNA samples purified from peripheral blood of 13.5 day HSII,I,ε transgenic fetuses were analyzed for expression of the transgene by primer extension. As shown in Fig. 4A, correctly initiated human ε-globin mRNA was present in blood samples from all three HSII,I,ε transgenic fetuses. Fetuses 6101 and 6910, that had intact copies of the transgene, expressed human ε-globin mRNA at 2% and 26% of mouse ϵ^y mRNA levels per gene copy, respectively. Transgenic fetus 6503 that contained the one rearranged transgene, on the other hand, expressed human ε-globin mRNA at a very low level, 0.2% of the mouse ϵ^y-globin mRNA level per gene copy.

To study the tissue and developmental specificity of expression of the HSII,I,ε transgene, RNA samples from brain and liver of the 13.5 day transgenic fetuses were also analyzed. No expression of the transgenes occurred in the brains of any of the

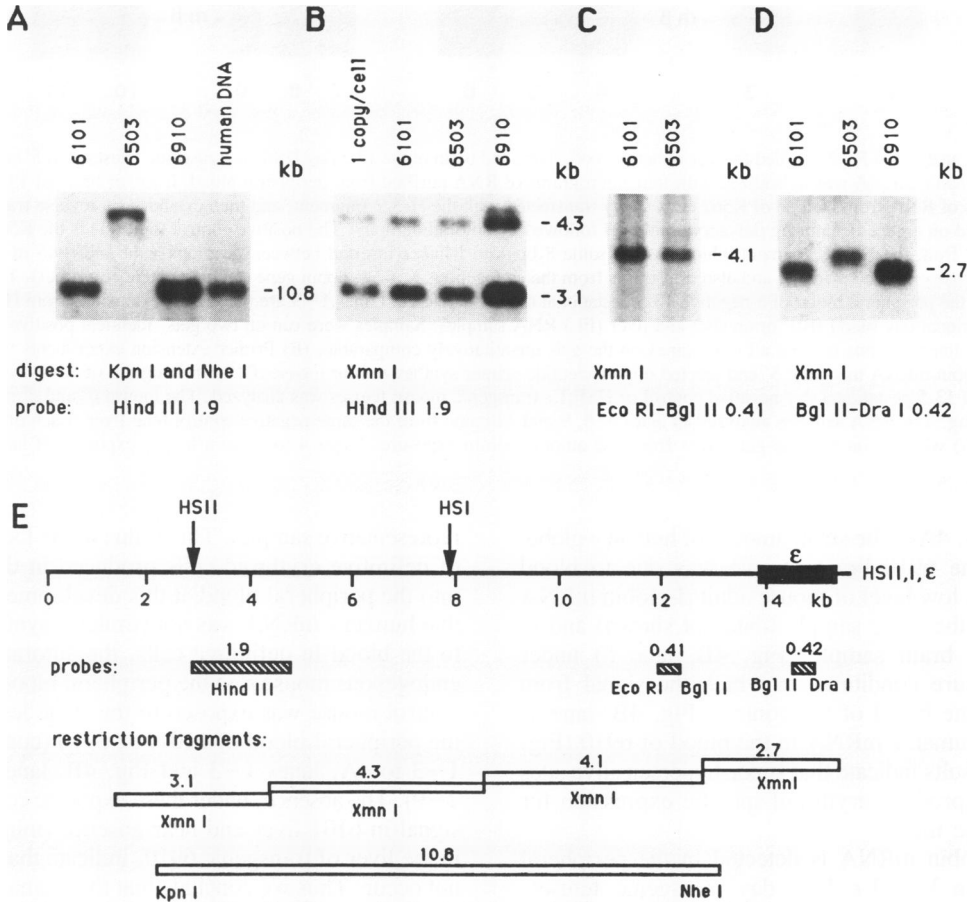

Figure 3. Structural analyses of the transgenes in the HSII,I,ε transgenic mice. DNA was digested with the indicated restriction enzyme(s) and separated on 0.65% agarose gels. The DNA was then transferred to nitrocellulose filters and hybridized with the indicated probes as illustrated in E (hatched bars). After washing, the filters were exposed to film for 48 hr or longer at -80°C. (A) Kpn I and Nhe I digested DNA, Hind III 1.9 kb probe (the HSII region probe). Lanes 1 to 3 are six micrograms of DNA from transgenic mice 6101, 6503, and 6910, respectively. Lane 4 contains 6 micrograms of human DNA. (B) Xmn I digested DNA, Hind III 1.9 kb probe (the HSII region probe). Lane 1 is a dilution of the p5'Hε17.4 plasmid DNA equivalent to 1 copy of transgene per cell. Lanes 2 to 4 are six micrograms of DNA from transgenic mice 6101, 6503, and 6910, respectively. (C) Xmn I digested DNA, Eco RI-Bgl II 0.41 kb probe (the human ε proximal upstream region probe). Lanes 1 and 2 are six micrograms of DNA from transgenic mice 6101 and 6503, respectively. (D) Xmn I digested DNA, Bgl II-Dra I 0.42 kb probe (the human ε gene probe). Lanes 1 to 3 are six micrograms of DNA from transgenic mice 6101, 6503, and 6910, respectively. (E) A map of the HSII,I,ε construction. Below the construction, the hybridization probes for the HSII region (the Hind III 1.9 kb probe), the proximal upstream region (the Eco RI-Bgl II 0.41 kb probe), and the ε-globin gene region (the Bgl II-Dra I 0.42 kb probe) are illustrated as hatched bars. At the bottom, all of the Xmn I fragments, and the only Kpn I-Nhe I fragment resulting from the restriction enzyme digestions of the HSII,I,ε transgene are illustrated as open bars. Sizes are shown in kb.

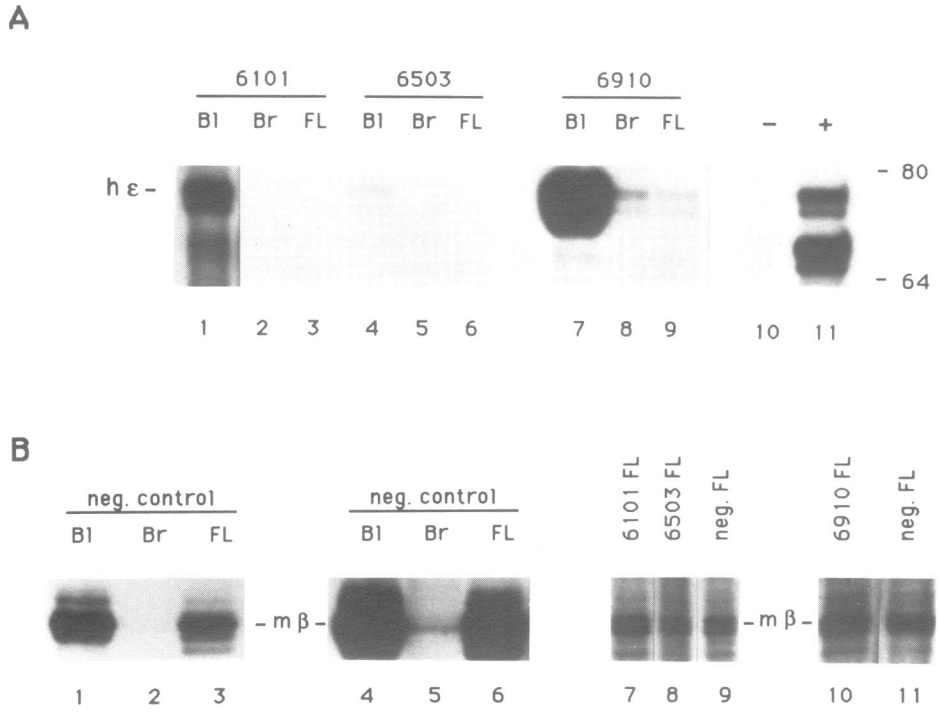

Figure 4. Primer extension analysis of RNA purified from peripheral blood, liver, and brain of the 13.5 day HSII,I,ε transgenic fetuses. (A) 5′ end-labeled oligonucleotide primer specific for the human ε mRNA was hybridized with four micrograms of RNA purified from peripheral blood, liver, or brain of 13.5 gestational day mouse fetuses, or ten micrograms of RNA from a clone of K562 cells stably transfected with the Hε3.7 fragment, and then extended by reverse transcriptase. The extended primers were then analyzed on an 8% denaturing polyacrylamide gel followed by autoradiography. The positive control (lane 11) is the K562 cell transfectant RNA which contains transcripts from the Hε3.7 fragment which has the same 8 bp Xho I linker inserted between positions +44 and +45 of the ε-globin gene as the HSII,I,ε construction (73 base extended primer), and also transcripts from the endogenous K562 ε-globin gene (65 base extended primer). The negative control (lane 10) is RNA purified from the peripheral blood of a negative 13.5 gestational day mouse fetus. Lanes 1−9 are the primer extensions from HSII,I,ε transgenics 6101, 6503 and 6910 13.5 gestational day blood (Bl), brain (Br) and liver (FL) RNA samples. Samples were run on two gels. Identical positive controls on the two gels were exposed to the same intensity thus making all other lanes on the gels quantitatively comparable. (B) Primer extension experiments were conducted as in (A) to assay mouse adult β-globin mRNA using the 5′ end-labeled oligonucleotide primer synthesized for mouse β^s. 0.5 microgram of RNA from peripheral blood (Bl), brain (Br), or liver (FL) of 13.5 gestational day negative control or HSII,I,ε transgenic mouse fetuses was analyzed. The mouse β^s and β^t primer extension products are both 51 nucleotides long. The RNA samples analyzed in lanes 3, 6, 9 and 11 came from the same negative control fetal liver. Each of the four groups of lanes (1−3, 4−6, 7−9, 10−11) was run on a single gel and is from one autoradiogram exposure. Lanes 4 to 6 are a longer exposure of lanes 1 to 3. See 'Results' for experimental details.

transgenic fetuses (Fig. 4A). The small amount of human ε-globin mRNA present in the 6910 brain sample was due to blood contamination since a low level of mouse adult β-globin mRNA was also detected in the same sample (data not shown) and in the negative control brain sample (Fig. 4B, lane 5) under autoradiogram exposure conditions in which the signal from mouse β mRNA in the blood of the control (Fig. 4B, lane 4) approaches that for human ε mRNA in the blood of 6910 (Fig. 4A, lane 7). These results indicate that super-hypersensitive sites HSI and HSII act to produce erythroid-specific expression for human ε in transgenic mice.

While human ε-globin mRNA is detected in the peripheral blood samples of the HSII,I,ε 13.5 day transgenic fetuses, expression of the transgene was not observed in the fetal livers. High levels of endogenous adult β-globin mRNA were detected in all of the 13.5 day fetal liver samples, however, showing that these livers were all erythropoietic at 13.5 days of gestation and expressing approximately the same amount of endogenous adult β-globin mRNA as each other and as the non-transgenic controls (Fig. 4B, lanes 7−11). The HSII,I,ε transgene, therefore, appeared to show embryonic specificity of expression. Endogenous mouse adult β-globin mRNA was present at high levels in both the peripheral blood and the livers of 13.5 day fetuses (see Fig. 4B, lanes 1−3 and 4−6 for non-transgenic representative sample). This is due to the fact that a large number of definitive erythroid cells produced in the liver are released into the peripheral blood at this developmental stage. To verify that human ε mRNA was not similarly synthesized and released to the blood in definitive cells, the autoradiogram signal from endogenous mouse β in the peripheral blood of a non-transgenic control mouse was exposed to the same level as the ε signal in the peripheral blood of 6101, or 6910 (compare Fig. 4B, lanes 1−3 to 4A, lanes 1−3 and Fig. 4B, lanes 4−6 to 4A, lanes 7−9). The absence, under these exposure conditions, of ε mRNA signal in 6101 liver and near absence (minimal contamination) in the liver of transgenic 6910, indicate that this mechanism did not occur. Thus we conclude that this human ε gene construction containing the HSI and HSII sites is expressed with an embryonic pattern in transgenic mice.

DISCUSSION

High level, inducible expression of the human embryonic β-globin gene, ε, was previously observed when plasmids containing this gene with 2 kb of immediate 5′ flanking DNA were transfected into the human embryonic and fetal erythroid cell line K562 (28,29). However, as shown above, a similar construction containing the human ε-globin gene with 2 kb of

5′ and 0.2 kb of 3′ flanking region was not expressed in transgenic mice. These observations suggest that more genetic information is required for expression of the human ε-globin gene in the whole mouse expression system than in the erythroid cell line. When extended 5′ flanking region containing erythroid-specific super-hypersensitive sites HSI and HSII was included to produce the HSII,I,ε construction, the transgenic fetuses carrying intact copies of the transgene expressed human ε-globin mRNA at high although not physiological levels in an erythroid-specific fashion. Our results thus support the notion that the β-globin locus activation region (LAR) is important for the expression of β-type globin genes and may normally activate the whole β-globin locus by organizing it into an active chromatin domain in erythroid cells (19,20). Our data also indicate that only a portion of the locus activation region which contains DNase I super-hypersensitive sites HSII and HSI is sufficient to confer high-level and erythroid-specific expression on the human ε-globin gene in transgenic mice.

The human ε-globin gene introduced as part of the HSII,I,ε construction is expressed with embryonic developmental stage specificity in transgenic mice as expected from orthology to mouse ϵ^y (6). Studies on the expression of the human γ- and β-globin genes indicated that in the case of those genes, developmentally regulated expression that was observed when each gene was introduced into mice with only proximal flanking region was lost when HS sites were included (25−27). This loss occurred whether all LAR hypersensitive sites were included or only sites HSI and HSII, the sites used in the present study. The loss of specificity, characterized by the expression of the globin genes throughout development, was attributed to a dominant activating effect of the LAR HS sites (27). Restoration of developmental specificity in the presence of LAR sequence was achieved for both of these genes by the inclusion of both genes in the same construction in their natural relative positions (26,27). This result led to the suggestion that the fetal to adult switch (26,27) and perhaps all human globin gene switching (27) is controlled by a mutually exclusive competition between individual globin genes for HS sequences. Our results indicate that this is not the case for the human ε-globin gene since developmental specificity is obtained in the LAR construction without the inclusion of an additional globin gene by what can therefore be characterized as a 'non-competitive' mechanism. Thus the HSII,I,ε construction appears to contain sufficient cis-acting elements for high-level, developmentally specific expression of ε and the developmental control elements are not dominated by the erythroid enhancing effects of the HS sites.

The transgene in HSII,I,ε transgenic mouse number 6503 is present in only one copy and is rearranged. Expression of this transgene appears to be developmentally and tissue-specifically regulated, although the very low level of expression does not permit this determination to be made definitively. Previous data (22,23) suggest that HS site-containing transgenes need to be in tandem array, placing HS sites both upstream and downstream of at least one gene copy, in order to express at high levels. The 6503 transgene which has HS sites only downstream (and in reverse orientation) from ε may express at low levels because it does not meet this requirement. The generation of additional single copy transgenic animals with various organizations of HS sites relative to the genes will be required to test this hypothesis.

The mechanism for the switching off of the human ε gene in the adult developmental compartment may involve a transcriptional silencer. Schechter and colleagues (31) have reported that such an element is present in the near 5′ flanking region of human ε based on studies in tissue culture lines. Positively acting elements may also exist for the activation of the ε gene in embryonic development. Numerous positively acting proximal elements for developmental control of the human β-globin gene have been identified and characterized in transgenic mice (39). The transgenic mouse system may now be extremely useful in the identification and study of regulatory elements of the ε gene.

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REFERENCES

1. Collins,F.S. and Weissman,S.M. (1984) In Cohn, W., and Moldave, K. (ed.), Progress in Nucleic Acid Research and Molecular Biology, Academic Press, Orlando, Vol.31, pp.315−462.
2. Karlsson,S. and Nienhuis,A.W. (1985) Ann. Rev. Biochem. **54**, 1071−1108.
3. Craig,M.L. and Russell,E.S. (1964) Devl. Biol. **10**, 191−201.
4. Fantoni,A., Bank.,A. and Marks,P.A. (1967) Science **157**, 1327−1329.
5. Barker,J.E. (1968) Devl. Biol. **18**, 14−29.
6. Hansen,J.N., Konkel,D.A. and Leder,P. (1982) J. Biol. Chem. **257**, 1048−1052.
7. Hill,A., Hardies,S.C., Phillips,S.J., Davis,M.G., Hutchison,C.A.,III and Edgell,M.H. (1984) J. Biol. Chem. **259**, 3739−3747.
8. Farace,M.G., Brown,B.A., Raschellà,G., Alexander,J., Gambari,R., Fantoni,A., Hardies,S.C., Hutchison,C.A.,III and Edgell,M.H. (1984) J. Biol. Chem. **259**, 7123−7128.
9. Chada,K., Magram,J. and Costantini,F. (1986) Nature **319**, 685−689.
10. Rifkind,R.A., Chui,D. and Epler,H. (1969) J. Cell Biol. **40**, 343−365.
11. Kovach,J.S., Marks,P.A., Russell,E.S. and Epler,H. (1967) J. Mol. Biol. **25**, 131−142.
12. Chada,K., Magram,J., Raphael,K., Radice,G., Lacy,E. and Costantini,F. (1985) Nature **314**, 377−380.
13. Magram,J., Chada,K. and Costantini,F. (1985) Nature **315**, 338−340.
14. Townes,T.M., Lingrel,J.B., Chen,H.Y., Brinster,R.L. and Palmiter,R.D. (1985) EMBO J. **4**, 1715−1723.
15. Kollias,G., Wrighton,N., Hurst,J. and Grosveld,F. (1986) Cell **46**, 89−94.
16. Groudine,M., Kohwi-Shigematsu,T., Gelinas,R., Stamatoyannopoulos,G. and Papayannopoulou,T. (1983) Proc. Natl. Acad. Sci. USA **80**, 7551−7555.
17. Tuan,D., Solomon,W., Li,Q. and London,I.M. (1985) Proc. Natl. Acad. Sci. USA **82**, 6384−6388.
18. Forrester,W.C., Thompson,C., Elder,J.T. and Groudine,M. (1986) Proc. Natl. Acad. Sci. USA **83**, 1359−1363.
19. Forrester,W.C., Takegawa,S., Papayannopoulou,T., Stamatoyannopoulos,G. and Groudine,M. (1987) Nucleic Acids Res. **15**, 10159−10177.
20. Grosveld,F., Blom van Assendelft,G., Greaves,D.R. and Kollias,G. (1987) Cell **51**, 975−985.
21. Blom van Assendelft,G., Hanscombe,O., Grosveld,F. and Greaves,D.R. (1989) Cell **56**, 969−977.
22. Ryan,T.M., Behringer,R.R., Martin,N.C., Townes,T.M., Palmiter,R.D. and Brinster,R.L. (1989) Genes & Development **3**, 314−323.
23. Curtin,P.T., Liu,D., Liu,W., Chang,J.C. and Kan, Y.W. (1989) Proc. Natl. Acad. Sci. USA **86**, 7082−7086.
24. Forrester,W.C., Novak,U., Gelinas,R. and Groudine,M. (1989) Proc. Natl. Acad. Sci. USA **86**, 5439−5443.
25. Enver,T., Ebens,A.J., Forrester,W.C. and Stamatoyannopoulos,G. (1989) Proc. Natl. Acad. Sci. USA **86**, 7033−7037.
26. Enver,T., Raich,N., Enbens,A.J., Papayannopoulou,T., Costantini,F. and Stamatoyannopoulos,G. (1990) Nature **344**, 309−313.
27. Behringer,R.R., Ryan,T.M., Palmiter,R.D., Brinster,R.L. and Townes,T.M. (1990) Genes & Development **4**, 380−389.
28. Young,K., Donovan-Peluso,M., Bloom,K., Allan,M., Paul,J. and Bank,A. (1984) Proc. Natl. Acad. Sci. USA **81**, 5315−5319.
29. Allan,M., Montague,P., Grindlay,G.J., Sibbet,G., Donovan-Peluso,M., Bank,A. and Paul,J. (1985) Nucleic Acids Res. **13**, 6125−6136.

30. Kioussis,D., Wilson,F., Khazaie,K. and Grosveld,F. (1985) EMBO J. **4**, 927–931.
31. Cao,S.X., Gutman,P.D., Dave,H.P.G. and Schechter,A.N. (1989) Proc. Natl. Acad. Sci. USA **86**, 5306–5309.
32. Li,Q., Powers,P.A. and Smithies,O. (1985) J. Biol. Chem. **260**, 14901–14910.
33. Brinster,R.L., Chen,H.Y., Trumbauer,M.E., Yagle,M.K. and Palmiter,R.D. (1985) Proc. Natl. Acad. Sci. USA **82**, 4438–4442.
34. Jeffreys,A.J. and Flavell,R.A. (1977) Cell **12**, 429–439.
35. Durnam,D.M. and Palmiter,R.D. (1983) Anal. Biochem. **131**, 385–393.
36. Ryan,T.M., Behringer,R.R., Townes,T.M., Palmiter,R.D. and Brinster,R.L. (1989) Proc. Natl. Acad. Sci. USA **86**, 37–41.
37. Luse,D.S., Haynes,J.R., VanLeeuwen,D., Schon,E.A., Cleary,M.L., Shapiro,S.G., Lingrel,J.B. and Roeder,R.G. (1981) Nucleic Acids Res. **9**, 4339–4354.
38. Maxam,A.M. and Gilbert,W. (1977) Proc. Natl. Acad. Sci. USA **74**, 560–564.
39. Behringer,R.R., Hammer,R.E., Brinster,R.L., Palmiter,R.D. and Townes,T.M. (1987) Proc. Natl. Acad. Sci. USA **84**, 7056–7060.