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High Levels of Human γ-Globin Gene Expression in Adult Mice Carrying a Transgene of Deletion-Type Hereditary Persistence of Fetal Hemoglobin

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Persistent expression of the γ-globin genes in adults with deletion types of hereditary persistence of fetal hemoglobin (HPFH) is thought to be mediated by enhancer-like effects of DNA sequences at the 3′ breakpoints of the deletions. A transgenic mouse model of deletion-type HPFH was generated by using a DNA fragment containing both human γ-globin genes and HPFH-2 breakpoint DNA sequences linked to the core sequences of the locus control region (LCR) of the human β-globin gene cluster. Analysis of γ-globin expression in six HPFH transgenic lines demonstrated persistence of γ-globin mRNA and peptides in erythrocytes of adult HPFH transgenic mice. Analysis of the hemoglobin phenotype of adult HPFH transgenic animals by isoelectric focusing showed the presence of hybrid mouse α_2-human γ_2 tetramers as well as human γ_4 homotetramers (hemoglobin Bart's). In contrast, correct developmental regulation of the γ-globin genes with essentially absent γ-globin gene expression in adult erythroid cells was observed in two control non-HPFH transgenic lines, consistent with autonomous silencing of normal human γ-globin expression in adult transgenic mice. Interestingly, marked preferential overexpression of the LCR-distal $^{A}\gamma$-globin gene but not of the LCR-proximal $^{G}\gamma$-globin gene was observed at all developmental stages in erythroid cells of HPFH-2 transgenic mice. These findings were also associated with the formation of a DNase I-hypersensitive site in the HPFH-2 breakpoint DNA of transgenic murine erythroid cells, as occurs in normal human erythroid cells in vivo. These results indicate that breakpoint DNA sequences in deletion-type HPFH-2 can modify the developmentally regulated expression of the γ-globin genes.

The human β-like globin gene cluster is located on the short arm of chromosome 11 and contains five linked functional globin genes and an upstream locus control region (LCR). The tissue-specific expression of the embryonic, fetal, and adult globin genes is developmentally regulated with sequential activation and silencing of individual genes during ontogeny, characterized by two switches in the pattern of β-like globin gene expression during development. The first switch is observed early in development as the major site of hematopoiesis shifts from the embryonic yolk sac to the fetal liver and is characterized by the high-level expression of the fetal $^{G}\gamma$- and $^{A}\gamma$-globin genes that replaces the expression of the embryonic ε-globin gene. A second switch, from γ- to δ- and β-globin gene expression, occurs late in gestation as well as in the perinatal period and results in high-level expression of the adult β-globin genes in the erythroid cells from the bone marrow, replacing the expression of the fetal γ genes following birth (6, 9, 39, 44). The exact mechanisms that regulate globin gene switching are as yet incompletely understood. However, developmental regulation of this multigene locus appears to be mediated by complex developmental stage- and tissue-specific interactions between *cis*- and *trans*-acting regulatory elements within the gene cluster.

Genetic disorders that alter hemoglobin (Hb) switching have provided naturally occurring molecular models for the study of the regulation of globin gene transcription and the mechanisms of Hb switching during development. Naturally occurring deletions within the β-globin cluster that interfere with fetal-to-adult Hb switching result in two related but discrete clinical syndromes, δβ-thalassemia and hereditary persistence of fetal Hb (HPFH), which are characterized by inappropriate, persistent expression of the fetal γ-globin genes in adult life. Individuals heterozygous for deletion-type HPFH have normal erythrocyte (RBC) parameters, but their Hb consists of 20 to 30% Hb F which is distributed in a pancellular fashion among the RBCs. In contrast, individuals with heterozygous δβ-thalassemia have smaller-than-normal RBCs and lower Hb F levels (10 to 15%), distributed in a heterocellular fashion (5, 34, 39). One hypothesis to explain persistent γ-globin gene expression in adult life in these conditions is that the deletions remove sequences downstream of the γ-globin genes which are necessary for the silencing of the genes in adult life (25). Another mechanism for persistent high levels of Hb F expression could involve the introduction into the vicinity of the γ-globin genes of DNA sequences from the breakpoint regions that can influence γ-globin gene expression following birth through enhancer-like effects (1, 18, 35, 42). These two mechanisms are not mutually exclusive, however, and a different balance of these and possibly other effects could play a role in the different phenotypes of persistent γ-globin gene expression observed in adults with HPFH and δβ-thalassemia.

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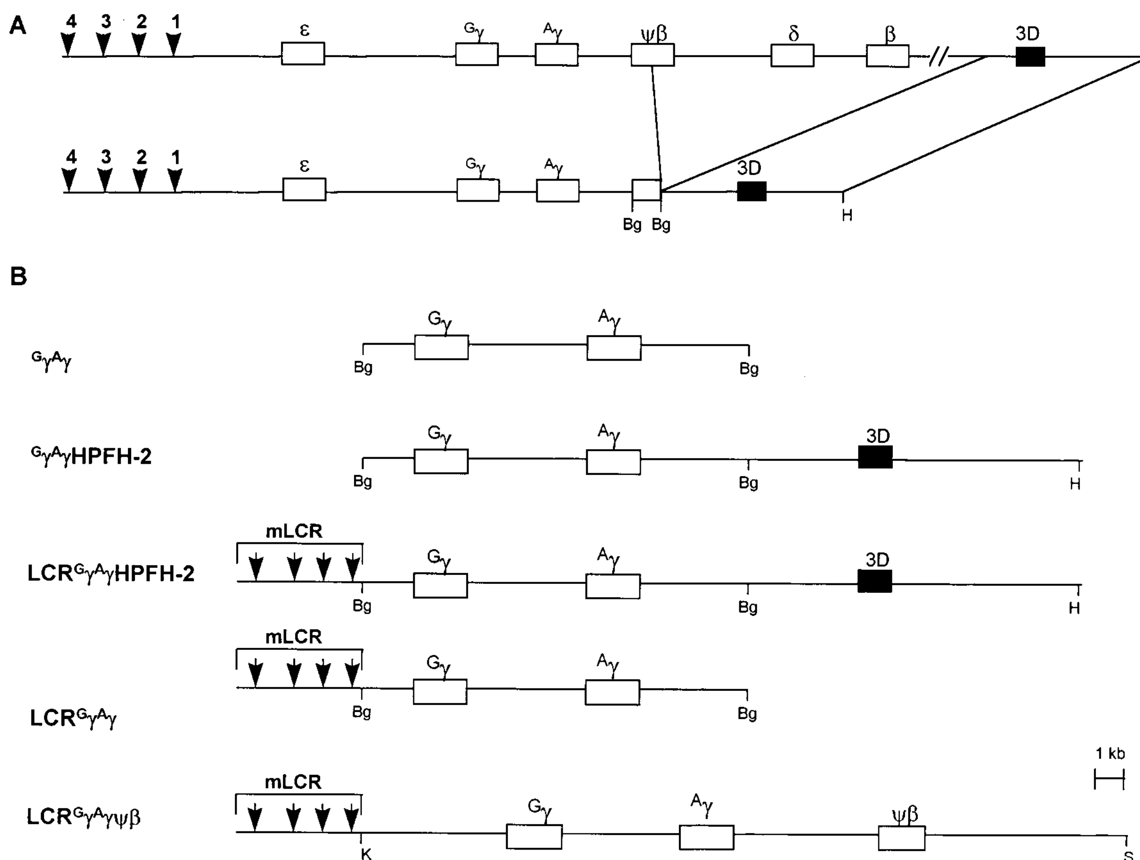


FIG. 1. (A) Diagrams of the normal and mutant (HPFH-2) human β-globin loci. The arrowheads indicate the DNase I-HSSs in the LCR. (B) DNA fragments that were used to generate transgenic mice. See Materials and Methods for a detailed description of the DNA fragments used in microinjection. mLCR, mini-LCR cassette; Bg, *Bgl*II; H, *Hind*III; K, *Kpn*I; S, *Sal*I.

A large body of experimental results has been generated by the analysis of a large number of different transgenic mice carrying a variety of fragments from the human β-globin gene cluster. In the absence of the LCR, individual human γ-globin transgenes with short segments of flanking DNA have been shown to be expressed during mouse development in yolk sac-derived but not adult erythroid cells (7, 26). These early results suggested that DNA sequences necessary to mediate developmental stage-specific expression of the γ-globin genes are located within or near the genes themselves. However, later experiments by Enver et al. (16) and Behringer et al. (3) showed that when linked with the LCR, γ-globin transgenes in the absence of linked β-globin genes were expressed in both embryonic and adult RBCs and that silencing of γ genes in adult erythroid cells was achieved by linking the LCR to the intact γδβ region. These results suggested a competitive model for regulation of the γ- and β-globin genes in transgenic mice (12, 15, 16, 23, 24). Subsequent experiments by Dillon and Grosveld (11) showed that γ-globin genes linked to the LCR are in fact silenced autonomously in adult erythroid tissues in the absence of an adjacent competing β-globin gene. Experiments with transgenic mice carrying a 70-kb transgene containing the entire human β-globin locus, including its LCR, in their normal structural configuration, as well as analyses of mice carrying the total β-globin gene cluster and LCR in large yeast artificial chromosome (YAC) transgenes, showed that the human γ-globin genes are correctly regulated during development and are expressed during the embryonic and early fetal stages of murine erythropoiesis (20, 32, 41). The phenotype of mice carrying large transgenes of the β-globin gene cluster but with deletions of both the δ- and β-globin genes demonstrated autonomous silencing of the γ-globin genes in adult transgenic mice, supporting the possible role of 3′ breakpoint DNA sequences in the generation of δβ-thalassemia and deletion-type HPFH phenotypes (33, 41).

To investigate the molecular mechanisms of γ-globin gene overexpression in adults with deletion-type HPFH, we have studied a model for the two most common forms of deletion-type $^{G}\gamma^{A}\gamma$ pancellular HPFH, HPFH-1 and HPFH-2. These disorders are associated with extensive deletions of the β-globin cluster involving approximately 105 kb of DNA (10) that includes both the δ- and β-globin genes (Fig. 1A). The purpose of this study was to test the functional significance of the breakpoint DNA sequences in deletion-type HPFH and to determine whether the presence of the β-cluster LCR is required for generation of the HPFH phenotype in transgenic mice carrying a 25-kb human DNA fragment mimicking the structure of the β cluster in HPFH-2. We generated two transgenic lines carrying the HPFH-2 transgene without the LCR and six transgenic lines with the HPFH-2 transgene with the LCR. Analysis of the phenotypes of these transgenic lines indicates that adult mice carrying the HPFH-2 transgene without the LCR displayed a delay in the silencing of γ-gene expression during fetal development but did not have persistent γ-gene expression in adult erythroid cells. On the other hand, analysis of the HPFH-2 transgenic lines with the LCR revealed

that human γ-globin mRNA and peptide chains were persistently expressed at significant levels and incorporated into mouse α_2-human γ_2 hybrid Hb tetramers. This persistent γ-gene expression was associated with formation of a DNase I-hypersensitive site (HSS) within HPFH-2 3′ breakpoint DNA region 3D in erythroid tissues of adult mice with the LCR/HPFH-2 transgene. In contrast, correct developmental regulation of γ-globin gene expression was observed in animals from two different control non-HPFH transgenic lines carrying LCR$^G\gamma^A\gamma$ and LCR$^G\gamma^A\gamma\psi\beta$ transgenes in which there was appropriate silencing of the γ-globin transgenes in adult erythroid cells.

MATERIALS AND METHODS

DNA fragments. The following human DNA fragments shown in Fig. 1B were prepared for injection into mouse oocytes. (i) $^G\gamma^A\gamma$ is a 13-kb *Bgl*II fragment containing both γ-globin genes and downstream sequences up to approximately the 5′ HPFH-2 breakpoint site. (ii) $^G\gamma^A\gamma$HPFH-2 is a 25-kb transgene containing 12 kb of HPFH-2 3′ breakpoint DNA sequences linked to $^G\gamma^A\gamma$. (iii) LCR$^G\gamma^A\gamma$HPFH-2 is an ~30-kb transgene containing a 4.7-kb LCR cassette linked to the 25-kb fragment. (iv) LCR$^G\gamma^A\gamma$ is an ~18-kb transgene with the LCR cassette linked to the 13-kb *Bgl*II genomic DNA fragment. (v) LCR$^G\gamma^A\gamma\psi\beta$ is an ~32-kb transgene containing the LCR cassette, both γ-globin genes, and 14 kb of downstream sequences ending 5′ to the δ-globin gene. The 13-kb *Bgl*II genomic DNA fragment containing both the $^G\gamma$ and $^A\gamma$ genes (GenBank Humhbb coordinates: 32820 to 45698) was purified from a cosmid clone containing the β^S-globin cluster described previously (8) and subcloned into the *Bam*HI site of cosmid vector pWE15, which contained a modified polylinker. To construct the LCR$^G\gamma^A\gamma$HPFH-2 transgene, a 12-kb *Bgl*II-*Hin*dIII genomic DNA fragment from a λ phage clone (λB-1) containing the normal DNA overlapping the 3′ breakpoint of HPFH-1 and HPFH-2 (42) was subcloned downstream of the 13-kb *Bgl*II genomic fragment containing the γ-globin genes to yield a 25-kb DNA fragment whose structure is virtually identical to the structure of HPFH-2 DNA.

An LCR cassette was constructed by ligating 5′ HSS1 and HSS2 isolated as a 1.4-kb *Pst*I-*Hin*dIII fragment from the μLAR plasmid (19) to a 1.2-kb *Xmn*I-*Hin*dIII fragment containing HSS3 and a 2.0-kb *Bam*HI-*Xba*I fragment containing HSS4 (GenBank Humhbb coordinates: HSS1, 13062 to 13769; HSS2, 8486 to 9218; HSS3, 3975 to 5172; HSS4, 308 to 2352). This 4.7-kb LCR cassette was subcloned in the genomic orientation upstream of the γ-globin genes in the $^G\gamma^A\gamma$HPFH-2 DNA fragment to yield the LCR$^G\gamma^A\gamma$HPFH-2 transgene. The first control transgene, LCR$^G\gamma^A\gamma$, contained the LCR cassette, which was subcloned in the genomic orientation upstream of the 13-kb *Bgl*II genomic fragment containing the γ-globin genes up to the HPFH-2 5′ breakpoint. The second control transgene, LCR$^G\gamma^A\gamma\psi\beta$, was obtained by inserting the LCR cassette, in the genomic orientation, upstream of a 27-kb *Kpn*I-*Sal*I genomic fragment (GenBank Humhbb coordinates: 27250 to 54726) containing the $^G\gamma$- and $^A\gamma$-globin genes and downstream sequences including the ψβ pseudogene up to the δ-globin gene.

Transgenic mouse lines. The DNA fragments were prepared for microinjection as described previously (40). A solution containing 10 ng of DNA per μl was injected into the pronuclei of (C57BL/6 × SJL)F_2 zygotes and transferred to pseudopregnant females. Transgenic mice were identified as described by Starck et al. (40), and transgenic founder mice were bred with nontransgenic B6/SJL mates to establish transgenic lines. Studies of transgene expression during development were performed with each transgenic line by mating hemizygous transgenic males with nontransgenic B6/SJL females to obtain embryos from timed pregnancies at 11.5, 13.5, and 16.5 days postcoitum. The morning on which the mating plug was observed was designated day 0.5. The copy numbers of the transgenes were determined by digestion of 10 μg of genomic DNA from the tail skin of progeny of two or three different animals from each line. Digestion with *Eco*RI, *Bam*HI, or *Sac*I was carried out and followed by 0.8% agarose gel electrophoresis and Southern blotting as described by Sambrook et al. (36). Several different probes from the transgenes were hybridized to the blots, and the hybridization signals were quantitated with a PhosphorImager (Molecular Dynamics). The measurements were made several times, and averages were taken. Copy numbers were determined by comparing the signals from the human transgene to signals from mouse GATA-1 as described by Starck et al. (40).

RNA analysis. Blood samples were obtained from mouse embryos and adult mice as described previously (40), and total RNA was isolated by using TRIzol reagent (Gibco-BRL) according to the manufacturer's protocol. Human and murine globin mRNAs were analyzed by quantitative RNase protection assays. The human γ-globin probe was a 403-bp *Nae*I-*Bam*HI fragment (IVS1 deleted) in pGEM4 (Promega) containing 5′ upstream sequences and exons 1 and 2 of the γ-globin genes. The antisense probe was synthesized by transcription with Sp6 polymerase to identify a 350-bp protected fragment. For the differential detection of $^G\gamma$- and $^A\gamma$-globin transcripts, the $^G\gamma$ probe was transcribed with T7 polymerase from pBluescript (Stratagene) containing a 721-bp *Hin*cII-*Hin*dIII $^G\gamma$-globin fragment and the $^A\gamma$ probe was transcribed with Sp6 polymerase from pBluescript containing a 980-bp *Pvu*II $^A\gamma$-globin fragment, both of which contained the third exon including the 3′ untranslated regions of the $^G\gamma$- and $^A\gamma$-globin genes, respectively, where four consecutive base differences are present between the $^G\gamma$ and $^A\gamma$ sequences beginning 3 bases 3′ to the termination codon. Both probes give a 215-bp protected fragment with the respective $^G\gamma$ or $^A\gamma$ transcript. Different riboprobes were used for the differential detection of $^G\gamma$- and $^A\gamma$-globin transcripts in the experiments with the mouse lines carrying the transgenes without the LCR. These probes and the riboprobes for detection of murine α-globin and ζ-globin mRNAs and quantitative mRNA analysis methods used for the mouse lines carrying the transgenes without the LCR have been described previously (40). For the mRNA analysis of samples from transgenic mouse lines with the LCR, three different probes (human γ-globin and mouse α- and ζ-globins) of 1.5×10^6 cpm total were simultaneously hybridized to 500 ng of total RNA from 11.5-day RBCs and 100 to 200 ng of RNA from RBCs of 13.5- and 16.5-day fetuses as well as newborn and adult mice. Samples were digested with RNases A (2.5 U/ml) and T_1 (100 U/ml). Conditions of probe excess were confirmed in separate experiments. The protected fragments were detected by autoradiography after electrophoresis in 8% polyacrylamide–8-mol/liter urea gels. Quantitation of human and murine mRNAs was performed with a PhosphorImager.

Isoelectric focusing (IEF). Hb samples were separated on IEF by using a Resolve-Hb kit (Isolab, Akron, Ohio). Hb concentrations were adjusted to between 2 and 4.5 g% by using the supplied sample preparation solution containing 0.05% KCN. Gels were run until sharp bands formed, usually 2 h. To determine the percentage of each band on the IEF gel, the gel was fixed with 10% trichloroacetic acid solution; soaked in distilled water for 15 min; stained with the JB-2 Staining System (Isolab) containing *o*-dianisidine, a heme-specific stain; and scanned at 520 nm with a model 710 densitometer (Corning, Medfield, Mass.). To identify bands, the band was removed from the unfixed gel, eluted with distilled water, spun to remove gel, and analyzed by high-performance liquid chromatography (HPLC).

HPLC. The globin chain composition of mouse RBCs was determined by HPLC using a denaturing solvent that separates the globin chains and a Vydac large-pore (300-Å) C_4 column (4.6 by 250 mm; Separations Group, Hesperia, Calif.) with a modified acetonitrile-H_2O-trifluoroacetic acid (TFA) gradient similar to that used by Schroeder et al. (37) for separating human globin chains. Two buffers were used: A (0.18% TFA in 36% acetonitrile) and B (0.18% TFA in 46% acetonitrile). Starting with 35% B, the amount of buffer B was increased by 0.67%/min until all of the globin chains were eluted.

Determination of oxygen affinity of hemoglobin (P_{50}). Blood samples were collected from the mouse tail and placed directly into heparinized mouse saline (330 mosM). The samples were washed three times with 10 mM HEPES buffer containing 5 mM KCl, 5 mM glucose, and enough NaCl to adjust the osmolarity to 330 mosM. The buffer pH was 7.4 at 37°C. O_2 equilibrium curves were obtained by running the samples at 37°C on a Hem-O-Scan O_2 Dissociation Analyzer (Aminoco, Silver Spring, Md.) at hematocrits between 10 and 20%.

DNase I hypersensitivity assays. Adult mice carrying the LCR$^G\gamma^A\gamma$HPFH-2 transgene were made anemic by three injections of 0.2 ml of 0.4% acetyl phenylhydrazine per 25 g of body weight and sacrificed 5 days after the start of the injections to harvest the spleen. The spleen was placed in a 10-ml syringe and passed through an 18-gauge hypodermic needle several times and resuspended in RSB buffer (10 mM Tris · HCl [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$). Intact nuclei were isolated as described elsewhere (43), and aliquots were subjected to DNase I digestion at various concentrations. The genomic DNA was purified by standard procedures (36). Appropriate restriction endonucleases were used to digest 15 μg of genomic DNA, and the digest was fractionated in 1% agarose gels. Southern blots were performed as described previously (36), and hybridization was carried out with a probe from the breakpoint region known from previous experiments not to contain repetitive DNA sequences.

RESULTS

For studies of globin mRNA expression during development, F_1 and F_2 progeny of the founders were utilized and RBCs for mRNA analysis from multiple transgenic animals from each line were obtained at desired stages of development. For a given developmental stage in a transgenic line, RNA samples from multiple fetuses and adult animals were analyzed to minimize experimental error. Only adult animals 5 weeks old or older (range, 5 weeks to 2 years) were utilized in these studies to prevent any bias resulting from analyzing adult animals shortly after birth for γ-globin gene expression.

HPFH-2 3′ breakpoint DNA sequences modify the developmental regulation of the γ-globin genes in transgenic mice in the absence of the LCR. Two transgenic mouse lines carrying the $^G\gamma^A\gamma$ transgene (lines 310 and 829) and two lines with the $^G\gamma^A\gamma$HPFH-2 transgene (lines 63 and 332) were established.

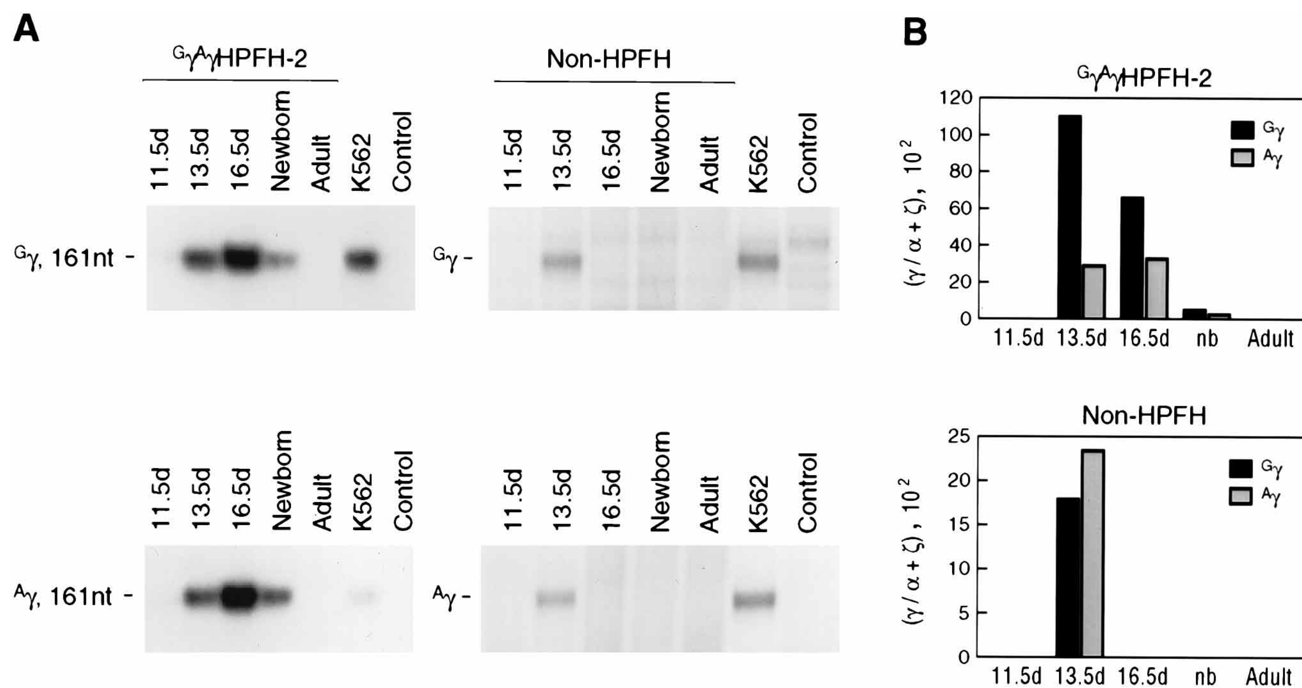


FIG. 2. (A) Human γ-globin mRNA expression in RBCs of transgenic mice carrying the $^{G}\gamma^{A}\gamma$HPFH-2 transgene (line 332) and control (non-HPFH) $^{G}\gamma^{A}\gamma$ transgene (line 310) without the LCR. Ten micrograms of total RNA isolated from mouse RBCs at each indicated developmental stage was hybridized to $^{G}\gamma$- or $^{A}\gamma$-globin riboprobes in RNase protection assays as described by Starck et al. (40). The expected position of the specific 161-nucleotide (nt) $^{G}\gamma$ or $^{A}\gamma$ protected RNA fragment is indicated. Total RNA from human K562 cells and nontransgenic mouse blood RNA (Control) served as positive and negative controls, respectively. (B) Quantitative representation of the ratios of human $^{G}\gamma$ or $^{A}\gamma$ mRNA to murine α plus ζ mRNAs at each developmental stage. Results of HPFH-2 line 332 are shown on the top, and results of non-HPFH line 310 are shown on the bottom. Expression of the murine α- plus ζ-globin mRNAs was quantitated in separate experiments using probes with different specific activities and 100 ng of total RNA in each hybridization (data not shown). Therefore, vertical-axis values should be regarded not as absolute values but as arbitrary units of the ratios of human to murine mRNAs. nb, newborn.

Total RNA isolated from blood cells of mouse embryos at day 11.5, from 13.5- and 16.5-day fetuses, and from newborn pups and adult animals was analyzed for the presence of human mRNAs. Autoradiographs of representative RNase protection assays of lines 332 (HPFH-2) and 310 (non-HPFH) are shown in Fig. 2A. Expression of $^{G}\gamma$- and $^{A}\gamma$-globin mRNAs was detected at day 13.5 in both HPFH-2 (line 332) and non-HPFH (line 310) lines. After day 13.5, however, the pattern of γ-globin mRNA expression during development differed significantly in HPFH-2 and non-HPFH mice. At day 16.5, levels of the two γ mRNAs decreased markedly in mice carrying the $^{G}\gamma^{A}\gamma$ transgene (Fig. 2A). In contrast, γ mRNA expression is detected in 16.5-day fetuses as well as newborn mice carrying the $^{G}\gamma^{A}\gamma$HPFH-2 transgene (Fig. 2A). Quantitative analysis of these results is illustrated in Fig. 2B. The ratios of $^{G}\gamma$- and $^{A}\gamma$-globin mRNAs relative to murine ζ plus α mRNAs for each stage of development were determined. Because the level of expression of the murine globin genes is markedly greater than that of the human transgenes in the absence of the LCR, human and murine globin mRNAs were analyzed on different gels with different amounts of total cellular RNA as described by Starck et al. (40). Therefore, the quantitative data illustrated in Fig. 2B represent not absolute values but relative arbitrary units of the ratio between human and murine globin gene expression at each stage of development. Similar results were obtained with lines 63 and 829 (data not shown). Thus, both $^{G}\gamma$- and $^{A}\gamma$-globin mRNAs were detected in RBCs of 16.5-day fetuses as well as newborn pups in the two HPFH-2 mouse lines, in contrast to the non-HPFH control mice carrying the $^{G}\gamma^{A}\gamma$ transgene, where levels of γ-globin mRNAs decreased markedly by fetal day 16.5 and were not detectable in RBCs of newborn pups. Although no γ-globin gene expression was observed in RBCs of adult animals carrying the $^{G}\gamma^{A}\gamma$HPFH-2 transgene without the LCR, these preliminary results indicated that DNA sequences located 3′ to the breakpoint of the HPFH-2 deletion could modify the developmental expression of the γ-globin genes in transgenic mice and may be involved in the generation of the HPFH phenotype.

The γ-globin genes are persistently expressed in erythroid cells of adult transgenic mice carrying the LCR$^{G}\gamma^{A}\gamma$HPFH-2 transgene. Because our initial experiments with transgenic mice carrying the $^{G}\gamma^{A}\gamma$HPFH-2 transgene without LCR showed γ-globin gene expression later during development than control γ transgenes but not in adult erythroid cells, we next wished to examine the effect of the LCR on γ-globin gene expression and asked whether the LCR is required to reproduce the full HPFH phenotype in transgenic mice. We analyzed, by quantitative RNase protection assays, six transgenic lines carrying the 30-kb LCR$^{G}\gamma^{A}\gamma$HPFH-2 transgene for expression of human γ- and endogenous mouse α- and ζ-globin mRNAs during mouse development. An autoradiograph of a representative RNase protection assay of line 3012 is shown in Fig. 3. Following PhosphorImager quantitation, human γ-globin mRNA levels were expressed as a percentage of mouse α- and ζ-globin mRNA levels and then corrected for the copy number of the transgenes as determined by Southern blotting. The results of γ-globin gene expression in the six transgenic mouse lines carrying the LCR$^{G}\gamma^{A}\gamma$HPFH-2 transgene are shown in Table 1. During the fetal stage of development, the highest level of human γ-globin gene mRNA per transgene copy ranged from 7.3% ± 1.2% to 29% ± 6% of the total mouse α- plus ζ-globin mRNA level. In erythroid cells of all

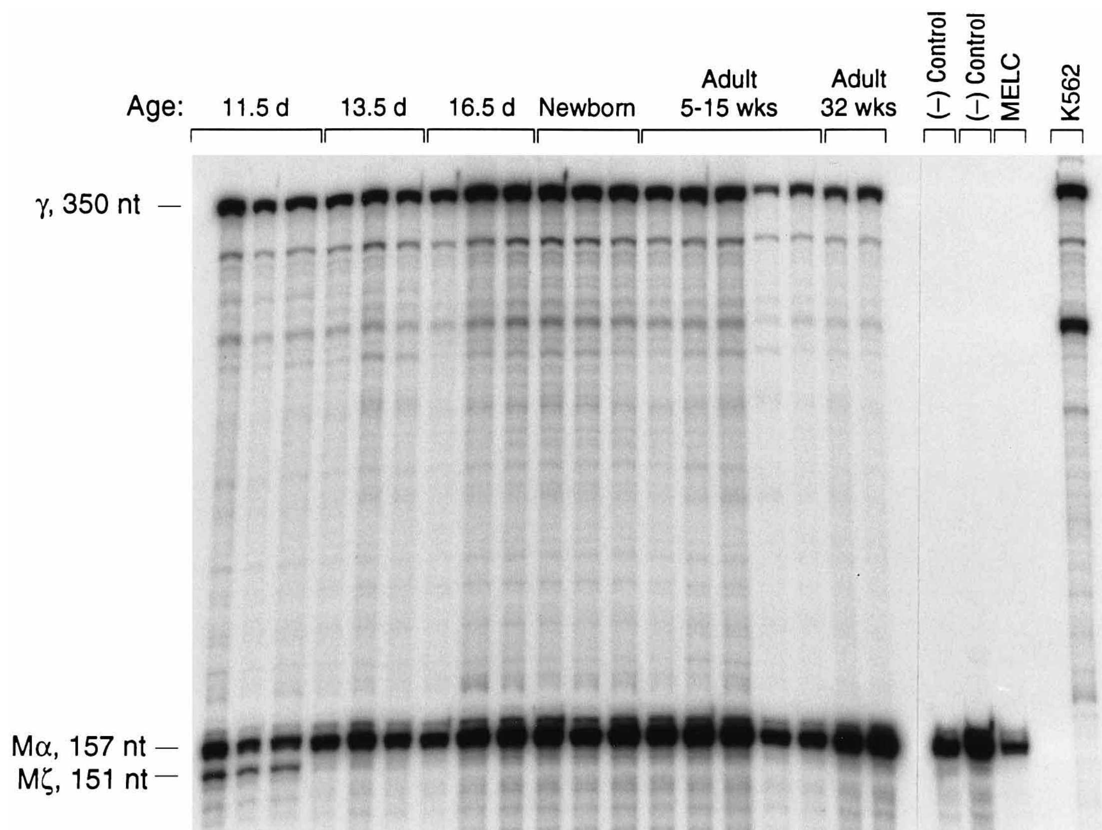


FIG. 3. Human γ-globin mRNA expression in HPFH-2 line 3012 carrying the LCR$^{G}\gamma^{A}\gamma$HPFH-2 transgene. Total RNA, isolated from blood of multiple individual animals at each developmental stage as indicated at the top of the figure, was hybridized simultaneously to human γ-, mouse α-, and mouse ζ-globin riboprobes (see Materials and Methods for the specific riboprobes and RNA quantities used for hybridization). The expected positions of the specific 350-nucleotide (nt) (human γ), 157-nt (mouse α), and 151-nt (mouse ζ) protected RNA fragments are indicated. The positive control samples were total RNA isolated from mouse erythroleukemia cells (MELC) and human K562 cells. The negative (−) control samples were RNA extracted from blood of 13.5-day fetal and adult nontransgenic mice.

adult mice carrying the LCR$^{G}\gamma^{A}\gamma$HPFH-2 transgene, persistent expression of γ-globin mRNA was present, ranging from 3.4% ± 0.2% to 8% ± 3.5% per transgene copy of endogenous mouse α-globin mRNA. Thus, as illustrated in Fig. 4, in adult animals, the expression of the γ-globin genes declined to only about one-third the level of maximal expression during fetal development, with an average percentage of adult γ mRNA level relative to the highest fetal γ mRNA level of 34% ± 11% (range, 21 to 48%). The decline in the level of γ-globin mRNA during development occurred predominantly prior to birth, with only a relatively minor decline after the newborn period, i.e., during the first 5 weeks following birth. The γ-globin mRNA levels remained quite stable up to 2 years of age in all adult animals tested (data not shown). Treatment with phenylhydrazine did not have an effect on γ-globin mRNA levels (data not shown). In all transgenic lines except line 3035, γ-globin gene expression was maximal at fetal day 11.5. Line 3035, which has 20 copies of the transgene, showed the highest γ-globin mRNA levels in 16.5-day fetuses in repeated experiments (Table 1). The results of γ-globin gene expression in six different transgenic lines carrying the LCR$^{G}\gamma^{A}\gamma$HPFH-2 transgene lead us to conclude that HPFH-2 breakpoint DNA sequences can modify the developmental pattern of γ-globin gene expression, resulting in significant levels of γ-globin mRNA in adult RBCs. Since our experiments using the 25-kb $^{G}\gamma^{A}\gamma$-HPFH transgene without the LCR did not reveal persistent γ-globin gene expression in adult erythroid cells, we further conclude that the LCR is required for generation of the HPFH phenotype in transgenic mice.

Distribution of γ-globin chains in RBCs was examined by staining of peripheral blood with monoclonal anti-γ-chain antibodies. Figure 5 shows representative slides. The distribution of γ-globin chains in adult RBCs was not pancellular, but overall a high percentage of the cells, ranging from 38 to 86%, stained positive.

The γ-globin genes are silenced in RBCs of adult transgenic mice carrying the control LCR$^{G}\gamma^{A}\gamma$ and LCR$^{G}\gamma^{A}\gamma\psi\beta$ transgenes. To serve as controls for the experiments carried out with the HPFH transgenic mice, one transgenic line with the 18-kb LCR$^{G}\gamma^{A}\gamma$ transgene and one line with the 32-kb LCR$^{G}\gamma^{A}\gamma\psi\beta$ transgene were generated. We asked whether γ-globin gene silencing could occur in the absence of a linked β-globin gene or HPFH-2 breakpoint DNA sequences in transgenic mice. Studies of gene expression during development by RNase protection assays were carried out as described above. A representative autoradiograph of an RNase protection gel for the transgenic line carrying the LCR$^{G}\gamma^{A}\gamma\psi\beta$ transgene is shown in Fig. 6. Table 2 summarizes the results of γ-globin gene expression during development for each control line. In the line carrying the control LCR$^{G}\gamma^{A}\gamma$ transgene, the highest γ-globin mRNA level during the fetal stage of development was 31% ± 2.7% (per transgene copy) of mouse α- plus ζ-globin mRNA, and the level declined significantly to 0.4% ± 0.2% in adult animals. Similarly, in the line carrying the LCR$^{G}\gamma^{A}\gamma\psi\beta$ trans-

TABLE 1. Globin gene expression in HPFH-2 transgenic mouse lines with the LCR

Line (copy no.)	Developmental age	No. of animals	% (mean ± SD)		Adult γ mRNA/peak fetal γ mRNA (%)
			Human γ mRNA/(mouse α + ζ mRNA)[a]	Human γ mRNA/mouse α mRNA/copy	
3012 (4)	11.5 days	3	86 ± 6.5	22 ± 1.6	
	13.5 days	3	61 ± 6.3	15 ± 1.5	
	16.5 days	3	65 ± 6.3	16 ± 1.6	
	Newborn	3	49 ± 6.1	12 ± 1.5	
	Adult (5–15 wk)	6	31 ± 6.0	7.8 ± 1.5	36
3017 (2)	11.5 days	3	58 ± 11	29 ± 6.0	
	13.5 days	2	49 ± 13	25 ± 6.5	
	16.5 days	3	30 ± 3.2	15 ± 1.6	
	Newborn	3	15 ± 3.4	8 ± 1.7	
	Adult (6–36 wk)	6	12 ± 5.0	6 ± 2.5	21
3021 (9)	11.5 days	3	79 ± 24	9 ± 2.6	
	13.5 days	3	63 ± 22	7 ± 2.4	
	16.5 days	3	58 ± 1.6	6.4 ± 0.2	
	Newborn	3	65 ± 20	7.2 ± 2.2	
	Adult (5–22 wk)	8	38 ± 11	4.2 ± 1.2	48
3025 (4)	11.5 days	3	94 ± 14	24 ± 3.5	
	13.5 days	3	58 ± 6.6	15 ± 1.6	
	16.5 days	2	46 ± 4.1	12 ± 1.0	
	Newborn	3	39 ± 5.5	9.8 ± 1.4	
	Adult (6–21 wk)	6	32 ± 14	8 ± 3.5	34
3028 (4)	11.5 days	3	65 ± 7.0	16 ± 1.7	
	13.5 days	3	58 ± 13	14 ± 3.2	
	16.5 days	3	38 ± 3.2	9 ± 0.8	
	Newborn	2	27 ± 2.1	6.8 ± 0.5	
	Adult (5–6 wk)	4	15 ± 3.9	3.8 ± 0.9	24
3035 (20)	11.5 days	3	95 ± 7.5	4.8 ± 0.4	
	13.5 days	2	132 ± 2.9	6.6 ± 0.1	
	16.5 days	3	145 ± 23.8	7.3 ± 1.2	
	Newborn	3	159 ± 14	8 ± 0.7	
	Adult (5–40 wk)	7	67 ± 4.3	3.4 ± 0.2	46

[a] When only two animals were available, results of multiple quantitations from separate experiments were used to calculate means and standard deviations.

gene, the γ-globin mRNA level in RBCs of 13.5-day fetuses was 23% ± 2.9% (per transgene copy) of endogenous α- plus ζ-globin mRNA, compared to 0.3% ± 0.1% in RBCs of adult animals. Thus, γ-globin gene expression in the adult stage of development from both control transgenic lines declined 70-fold, to 1.3% of maximal fetal-stage expression, compared to an only 3-fold decline, to 34% ± 11%, in the animals carrying the LCR$^G\gamma^A\gamma$HPFH-2 transgene (Fig. 4). Staining of adult RBCs of transgenic mice with anti-γ-chain antibodies showed 1% positive cells in the line carrying the LCR$^G\gamma^A\gamma\psi\beta$ transgene (Fig. 5B) and 0.12% positive cells in the LCR$^G\gamma^A\gamma$ line (data not shown). Although only two LCR-containing control lines were available for study, we conclude that γ-globin transgene silencing in adult mice can occur in the absence of competition from the downstream δ- and β-globin genes and that sequences required for this silencing effect reside within the LCR$^G\gamma^A\gamma$ and LCR$^G\gamma^A\gamma\psi\beta$ transgenes used in these experiments. Therefore, our results were similar to those obtained by Dillon and Grosveld (11) using LCR-γ transgenes of somewhat different structure.

IEF of Hb shows the presence of mouse α_2-human γ_2 globin chain tetramers ($\alpha_2^M\gamma_2$) and γ_4 homotetramers. The Hb phenotype of transgenic mouse RBCs was analyzed by IEF of peripheral blood of adult animals carrying the LCR$^G\gamma^A\gamma$HPFH-2 transgene, and the results were compared to those obtained from RBCs of nontransgenic animals and the control transgenic lines carrying the LCR$^G\gamma^A\gamma$ and LCR$^G\gamma^A\gamma\psi\beta$ transgenes. The adult animals were not treated with phenylhydrazine prior to phlebotomy. IEF of blood samples from mice carrying the LCR$^G\gamma^A\gamma$HPFH-2 transgene shows the presence of $\alpha_2^M\gamma_2$ tetramers (Fig. 7). Thus, human γ-globin chains are effectively incorporated into Hb tetramers, forming hybrids with mouse α-globin chains. Interestingly, the formation of γ-globin homotetramers (Hb Bart's) is also noted running as a faster band in IEF. This band was present in the blood of both a higher-copy-number transgenic animal (line 3035, 20 copies) as well as a relatively lower-copy-number transgenic mouse (line 3012, 4 copies). None of the animals carrying the control LCR$^G\gamma^A\gamma\psi\beta$ and LCR$^G\gamma^A\gamma$ transgenes had any detectable $\alpha_2^M\gamma_2$ hybrids or γ_4 homotetramers in their RBCs (Fig. 7).

Hematologic data of adult mice carrying the LCR$^G\gamma^A\gamma$ HPFH-2 transgene. The hematologic parameters of adult HPFH-2 transgenic mice were examined for changes which could be associated with the presence of high levels of human γ-globin chains in RBCs. Peripheral blood was obtained, and measurements of hematocrit (Hct), Hb, mean corpuscular volume, mean corpuscular Hb concentration, reticulocyte count, and P_{50} were performed. The values obtained from the LCR$^G\gamma^A\gamma$HPFH-2 line (line 3035) were compared to values

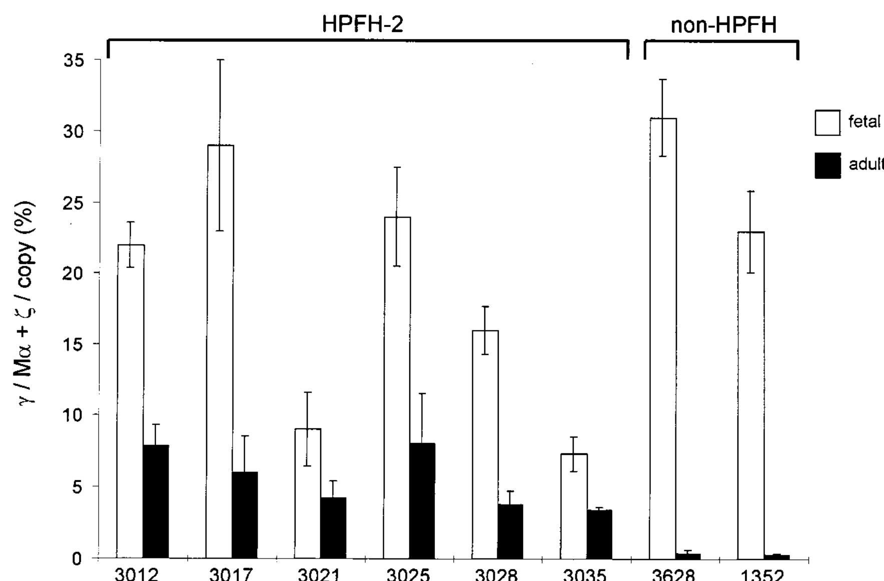
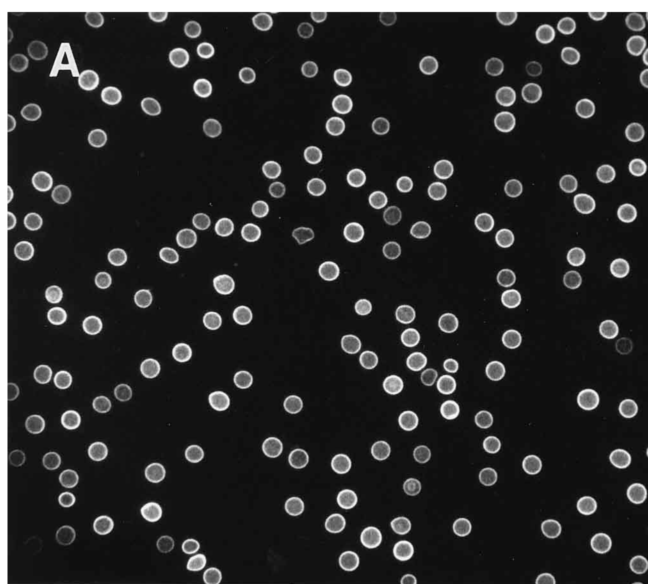


FIG. 4. Quantitative analysis of γ-globin mRNA expression per transgene copy. Peak levels of expression in 11.5- or 13.5-day fetuses are compared to those in adult mice (Tables 1 and 2). Results of six lines carrying the LCR$^G\gamma^A\gamma$HPFH-2 transgene are shown on the left. Results of non-HPFH lines 3628 and 1352, carrying the LCR$^G\gamma^A\gamma$ and LCR$^G\gamma^A\gamma\psi\beta$ transgenes, respectively, are shown on the right.

from nontransgenic animals as well as the non-HPFH control transgenic lines. The Hct and Hb of HPFH-2 mice (45.4% ± 1.3% and 15.1 g/dl, respectively) were not different from the Hct and Hb of nontransgenic mice (45.6% ± 1.6% and 15.6 g/dl, respectively) or the Hct and Hb of non-HPFH transgenic mice carrying the LCR$^G\gamma^A\gamma$ transgene (45.3% ± 1.7% and 15.7 g/dl, respectively). Similarly, no significant differences in the mean corpuscular volume, mean corpuscular Hb concentration, or reticulocyte counts were observed (data not shown). The P_{50}, on the other hand, was significantly lower (34.6 ± 1.8 mm Hg) in the transgenic mice carrying the LCR$^G\gamma^A\gamma$HPFH-2 transgene than in nontransgenic controls (42.4 ± 0.4 mm Hg), as well as in transgenic mice carrying the LCR$^G\gamma^A\gamma$ transgene (43.7 ± 1.2 mm Hg) and LCR$^G\gamma^A\gamma\psi\beta$ transgene (40.8 ± 2.3 mm Hg). This finding suggests that the presence of significant amounts of γ_4 homotetramers (Hb Bart's), which have a very high oxygen affinity, and possibly the presence of hybrid $\alpha_2{}^M$-γ_2 are associated with shift of the oxyhemoglobin dissociation curve to the left.

$^A\gamma$-Globin mRNA is overexpressed compared to $^G\gamma$-globin mRNA at all developmental stages in erythroid cells of mice carrying the LCR$^G\gamma^A\gamma$HPFH-2 transgene but not in mice with the control transgenes. The differential expression of $^G\gamma$- and $^A\gamma$-globin mRNAs was examined because human heterozygotes with deletion-type HPFH-2 preferentially express $^A\gamma$-globin chains ($^A\gamma/^G\gamma$ ratio, ~70:30), as shown by Kutlar et al.



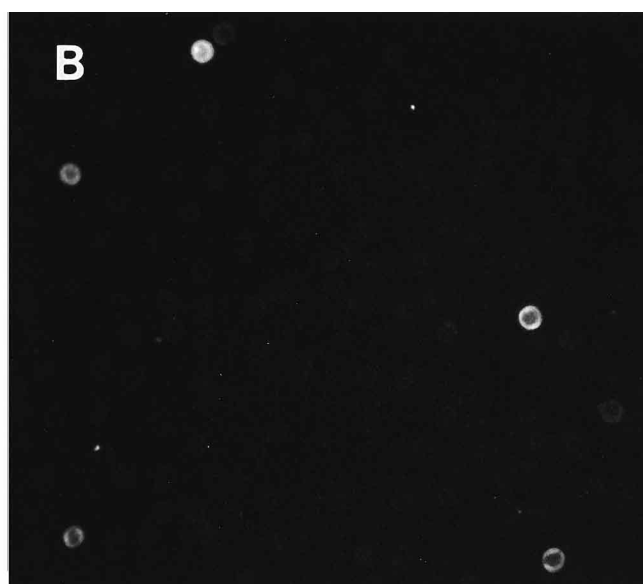


FIG. 5. F-cell staining of peripheral blood using monoclonal anti-γ-chain antibodies was performed as described previously (30). (A) Line 3035, with the LCR$^G\gamma^A\gamma$HPFH-2 transgene; (B) line 1352, with the LCR$^G\gamma^A\gamma\psi\beta$ transgene.

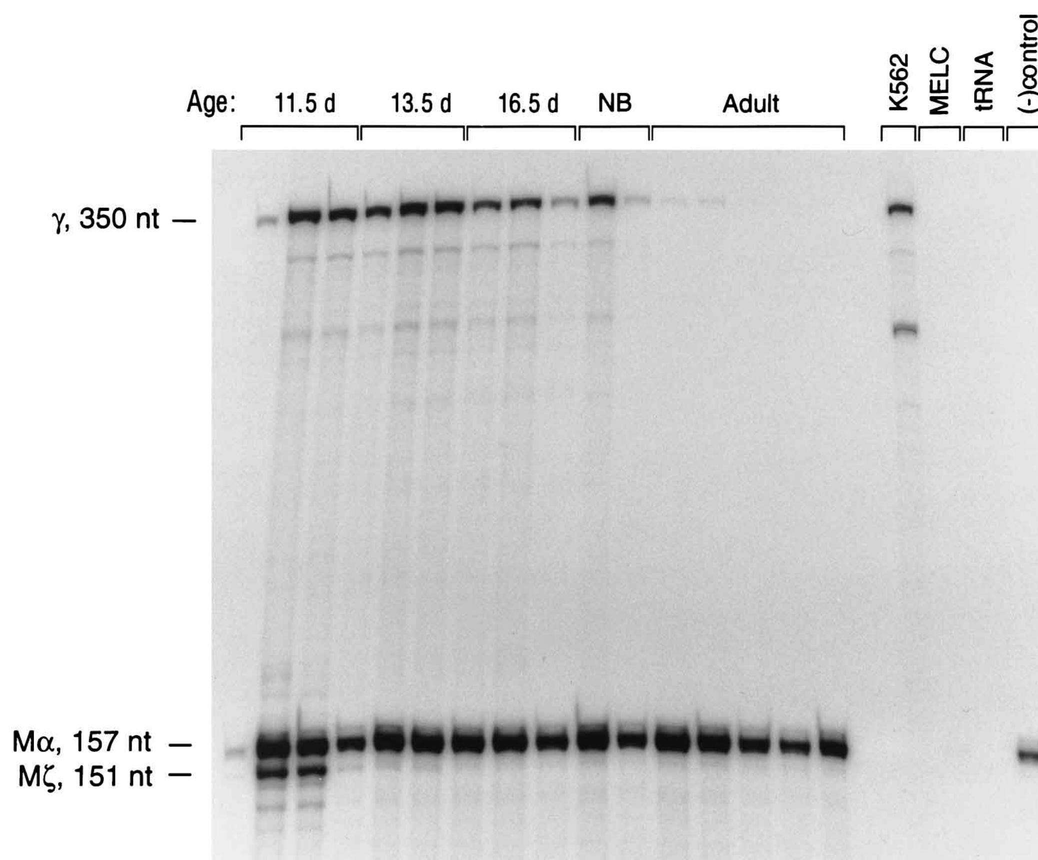


FIG. 6. Human γ-globin mRNA expression in RBCs of transgenic mice carrying the non-HPFH LCR$^{G}\gamma^{A}\gamma\psi\beta$ transgene. RNase protection assay of line 1352 is shown. RNase protection assays were performed as described in the legend to Fig. 2. The positions of the protected fragments are indicated. MELC, mouse erythroleukemia cells.

(27). Riboprobes from the 3′ ends of the $^{G}\gamma$- and $^{A}\gamma$-globin genes were chosen because a 4-bp mismatch in the 3′ untranslated region of the mRNAs permits differentiation of transcripts specific for the individual genes in RNase protection assays. Figure 8 shows the results of RNase protection assays utilizing riboprobes to differentiate $^{A}\gamma$- and $^{G}\gamma$-globin transcripts. As expected, preferential expression of $^{A}\gamma$-globin mRNA was observed at all stages of mouse development in RBCs of transgenic mice (Fig. 9A) in all HPFH-2 transgenic lines. However, at peak levels of γ-gene expression during fetal development, the average $^{A}\gamma/^{G}\gamma$ ratio was ~90:10, and in adult HPFH-2 transgenic mice the average $^{A}\gamma/^{G}\gamma$ ratio was ~95:5 (range, 92% to 98% $^{A}\gamma$ in four different mouse lines). Therefore, the $^{A}\gamma/^{G}\gamma$ mRNA ratio observed in adult HPFH-2 transgenic mice is somewhat higher than the $^{A}\gamma$-globin/$^{G}\gamma$-globin chain ratio observed in adult humans with HPFH-2. Although low levels of $^{G}\gamma$-globin mRNAs were observed in RBCs of 11.5- and 13.5-day fetuses (4 to 8% of mouse α plus ζ mRNAs), the expression declined markedly in 16.5-day fetuses and was less than 0.5% of mouse α mRNA in adult animals (Fig.

TABLE 2. Globin gene expression in non-HPFH transgenic mouse lines with LCR

Line (copy no.)	Developmental age	No. of animals	% (mean ± SD)		Adult γ mRNA/peak fetal γ mRNA (%)
			Human γ mRNA/(mouse α + ζ mRNA)[a]	Human γ mRNA/mouse α mRNA/copy	
LCR$^{G}\gamma^{A}\gamma$, 3628 (1)	11.5 days	2	31 ± 2.7	31 ± 2.7	
	13.5 days	3	13 ± 1.5	13 ± 1.5	
	16.5 days	2	1.5 ± 0.3	1.5 ± 0.3	
	Adult (5–6 wk)	4	0.4 ± 0.2	0.4 ± 0.2	1.3
LCR$^{G}\gamma^{A}\gamma\psi\beta$, 1352 (2)	11.5 days	6	41 ± 9.0	20 ± 4.5	
	13.5 days	4	45 ± 5.8	23 ± 2.9	
	16.5 days	6	17 ± 4.9	8.8 ± 2.0	
	Newborn	5	6.9 ± 1.2	3.5 ± 0.5	
	Adult (5–11 wk)	5	0.5 ± 0.3	0.3 ± 0.1	1.3

[a] See Table 1, footnote *a*.

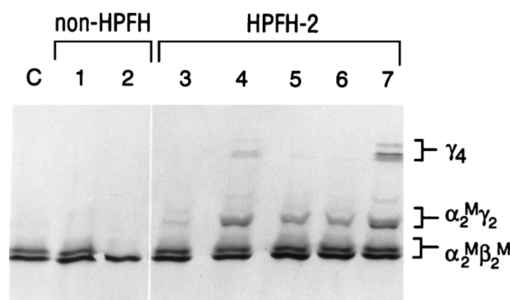


FIG. 7. IEF of Hb. Peripheral blood lysates obtained from adult transgenic mice were analyzed. Lane C, sample from a nontransgenic mouse; lane 1, sample from a mouse carrying the LCR$^G\gamma^A\gamma\psi\beta$ transgene (line 1352); lane 2, sample from a mouse carrying the LCR$^G\gamma^A\gamma$ transgene (line 3628); lanes 3 to 7, samples from mice carrying the LCR$^G\gamma^A\gamma$HPFH-2 transgene (lines 3017, 3021, 3025, 3028, and 3035, respectively). The positions of $\alpha_2{}^M\beta_2{}^M$, $\alpha_2{}^M\gamma_2$, and γ_4 are indicated. The two different murine α-globin isoforms result in the presence of double bands visualized at the position of the endogenous $\alpha_2{}^M\beta_2{}^M$ tetramers as well as hybrid $\alpha_2{}^M\gamma_2$ tetramers.

9A). In contrast, embryonic and fetal erythroid cells of the non-HPFH control line carrying the LCR$^G\gamma^A\gamma\psi\beta$ transgene expressed the LCR-proximal $^G\gamma$-globin gene at a level approximately three- to sevenfold higher than that of the distal $^A\gamma$-globin gene (Fig. 9B), which is consistent with the results of previous studies that examined the role of gene order in the β-globin locus (13, 24, 31). Similarly, in mice carrying the second non-HPFH control transgene (LCR$^G\gamma^A\gamma$), predominant expression of the LCR-proximal $^G\gamma$-globin gene was observed (data not shown).

Although the same parent clone of human genomic DNA was used to construct all transgenes, we wanted to rule out the presence of a mutation in the promoters of the γ-globin genes within the LCR$^G\gamma^A\gamma$HPFH-2 transgene which could result in exaggerated predominance of $^A\gamma$-globin mRNA expression. With transgenic HPFH-2 mouse DNA as the template, the promoter regions of the $^G\gamma$- and $^A\gamma$-globin transgenes were amplified by PCR, subcloned, and sequenced. No mutations were detected in the promoter regions of the $^G\gamma$- or $^A\gamma$-globin gene of the HPFH-2 transgenic mice (data not shown).

HPLC confirms the exaggerated preferential expression of $^A\gamma$-globin chains in adult mice carrying the LCR$^G\gamma^A\gamma$HPFH-2 transgene. HPLC was performed to analyze the globin chain composition in RBCs of adult transgenic animals. Peripheral blood was obtained from animals, untreated with phenylhydrazine, from the LCR$^G\gamma^A\gamma$HPFH-2 transgenic lines as well as the control LCR$^G\gamma^A\gamma\psi\beta$ and LCR$^G\gamma^A\gamma$ transgenic lines. Figure 10 illustrates a representative HPLC analysis of a peripheral blood RBC lysate of an adult transgenic animal carrying the LCR$^G\gamma^A\gamma$HPFH-2 transgene. Significant amounts of human γ-globin chains were detected in RBCs of mice from all HPFH-2 lines (data not shown). Essentially, all of the γ chains were $^A\gamma$-globin, whereas no $^G\gamma$-globin chains detectable, again demonstrating significant overexpression of the $^A\gamma$ gene compared to the $^G\gamma$ gene at the protein level, similar to what was observed at the mRNA level. In the peripheral blood samples of an adult mouse carrying the control LCR$^G\gamma^A\gamma\psi\beta$ and LCR$^G\gamma^A\gamma$ transgenes, no γ-globin chains were detectable by HPLC (data not shown).

Minor position effects are observed on γ-globin gene expression in mice carrying the LCR$^G\gamma^A\gamma$HPFH-2 transgene. We investigated the effect of integration site on the level of γ-transgene expression in the six HPFH-2 transgenic lines. Position effects on the expression of various LCR–γ-globin transgenes have been reported previously by other investigators and can significantly alter the developmental pattern of expression of the γ-globin genes in transgenic mice (11, 28, 29, 38). The copy numbers of the transgenes were calculated by analyzing genomic DNA from multiple individual transgenic animals from each of the six HPFH-2 transgenic lines (see Materials and Methods). In the HPFH-2 lines, there appeared to be some variability in the level of γ-globin mRNA expression per transgene copy at various developmental stages, suggesting the presence of integration site-dependent position effects on γ-transgene expression. A 4-fold variation in levels of γ-globin gene mRNA expression was observed between fetuses of different HPFH-2 lines, whereas only a 2.3-fold difference was present between adult HPFH-2 transgenic mice of different lines. These differences reflect the presence of only minor position effects, particularly in the adult stage of development, where only a 2.3-fold difference in transgene copy expression was shown. Figure 11 illustrates the relationship between the total level of γ-globin mRNA levels in adult HPFH-2 mice and the transgene copy number. The correlation between transgene copy number and total level of γ-globin mRNA expression argues against the possibility that integration site position effects resulted in persistent expression of the γ-globin gene in adult RBCs. A similar analysis could not be carried out for the lines carrying the control transgenes, since only one line carrying the LCR$^G\gamma^A\gamma$ transgene and one line carrying the LCR$^G\gamma^A\gamma\psi\beta$ were established.

A DNase I-HSS is formed in the 3′ HPFH breakpoint DNA region in erythroid tissue of adult transgenic animals carrying the LCR$^G\gamma^A\gamma$HPFH-2 transgene. Demonstration of an erythroid cell-specific DNase I-HSS in the breakpoint DNA sequences of HPFH-1 and 2 in normal fetal and adult nucleated erythroid cells by Elder et al. (14) suggested that alterations of chromatin structure flanking the γ-globin genes contribute to abnormal regulation of this expression in deletion-type HPFH. In transgenic mice carrying the $^G\gamma^A\gamma$HPFH-2 transgene without the LCR, a DNase I-HSS was not formed within the 3′ breakpoint DNA sequences (data not shown). In order to demonstrate whether a DNase I-HSS is formed in erythroid tissue of adult transgenic mice carrying the LCR$^G\gamma^A\gamma$HPFH-2 transgene, nuclei were isolated from spleen cells of anemic mice and treated with increasing concentrations of DNase I. Figure 12 illustrates a Southern blot of *Xba*I-digested genomic DNA which was hybridized to ^{32}P-labelled 0.7-kb *Eco*RI-*Xba*I HPFH-2 from the HPFH-2 breakpoint region. A 3-kb subband is visualized in the lanes containing DNase I but not in the lane without prior DNase I digestion. This site maps within the same region previously shown by Elder et al. (14) to contain an erythroid cell-specific DNase I-HSS.

DISCUSSION

In this study, we have analyzed the phenotypes of transgenic mice carrying a DNA fragment virtually identical to the DNA structure in deletion-type HPFH-2, with and without the LCR. In the absence of the LCR, a delay in γ-globin gene silencing was observed, but γ-globin mRNA expression was not present in erythroid cells of adult transgenic mice. In studies similar to our experiments with the $^G\gamma^A\gamma$HPFH-2 transgene without the LCR, Anagnou et al. (1) have shown that sequences located 3′ to the breakpoint of the HPFH-3 deletion can modify the developmental regulation of a linked $^A\gamma$-globin gene in transgenic mice in the absence of the LCR. In these studies, $^A\gamma$-globin mRNA was found in both 11.5-day embryonic and 16-day fetal liver erythroid cells but was not observed in RBCs of adult animals. These results are consistent with our findings that, in

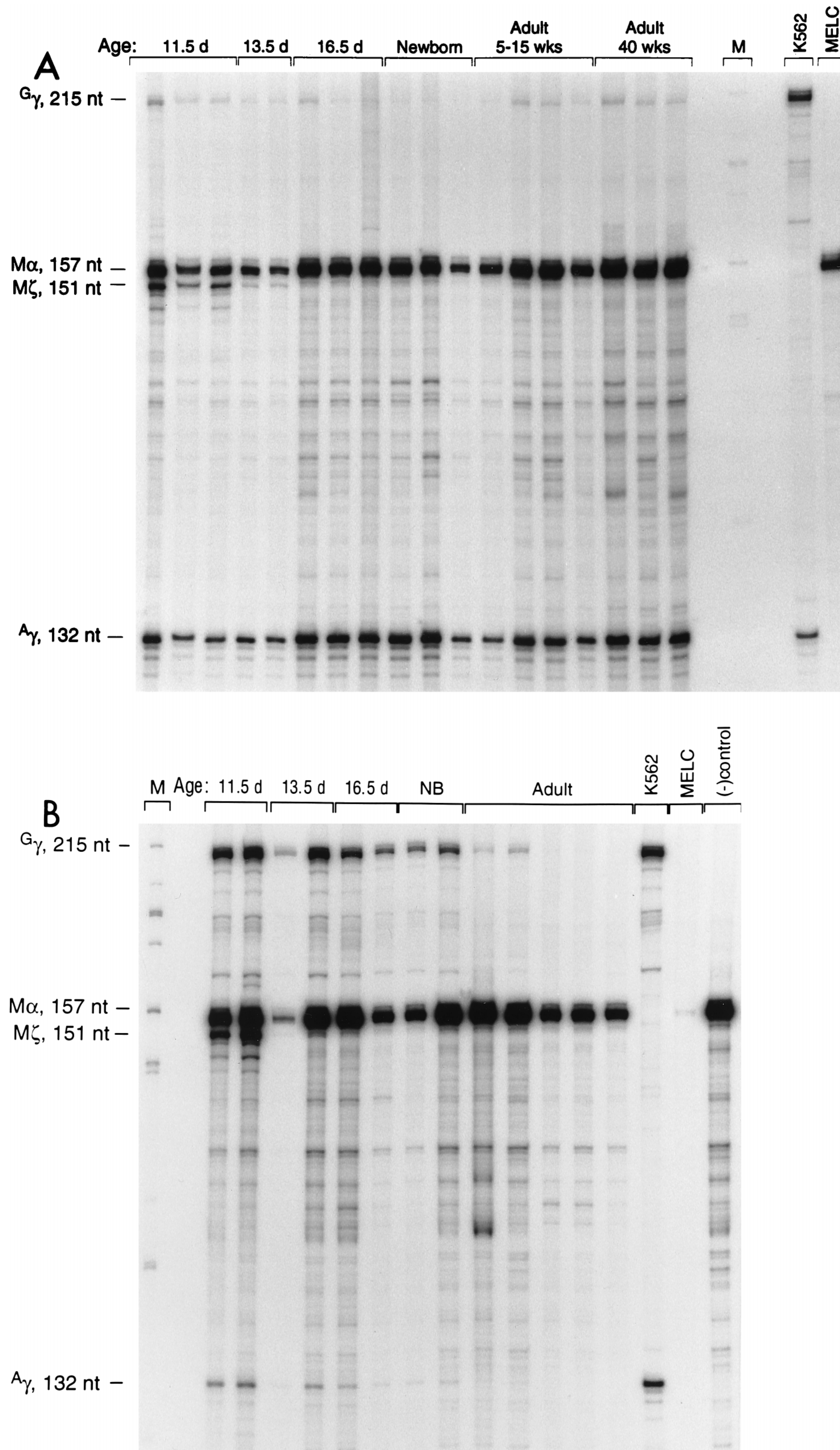


FIG. 8. Human $^{G}\gamma$- and $^{A}\gamma$-globin mRNA expression in RBCs of transgenic mice. (A) RNase protection assay using a $^{G}\gamma$-globin riboprobe of representative line 3035, carrying the LCR$^{G}\gamma^{A}\gamma$HPFH-2 transgene. The brackets on the top indicate the developmental age. The positions of the protected fragments are indicated. The size marker (lane M) was *Msp*I-digested pBR322 DNA. RNA samples from K562 human erythroleukemia cells and mouse erythroleukemia cells (MELC) served as controls. (B) RNase protection assay using a $^{G}\gamma$-globin riboprobe of line 1352 carrying the LCR$^{G}\gamma^{A}\gamma\psi\beta$ transgene. Developmental age is indicated on the top. NB, newborn. The negative (−) control sample was RNA extracted from blood of a nontransgenic mouse.

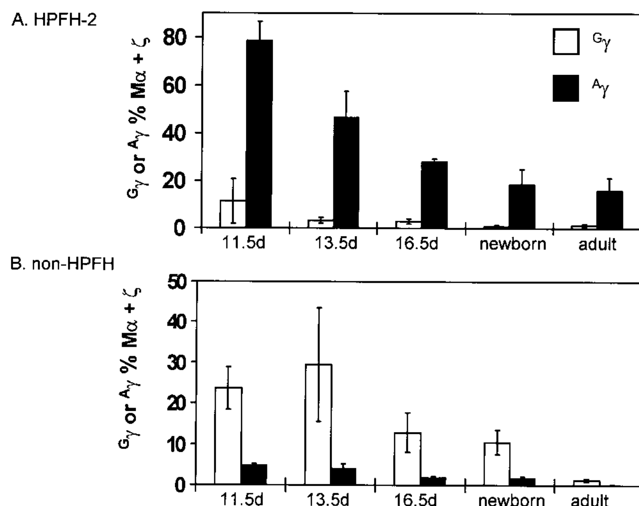


FIG. 9. Differential expression of $^{G}\gamma$- and $^{A}\gamma$-globin mRNAs in transgenic mice. (A) Results of quantitative analysis of $^{G}\gamma$- and $^{A}\gamma$-globin mRNA expression during development in representative line 3025, carrying the LCR$^{G}\gamma^{A}\gamma$ HPFH-2 transgene; (B) results of quantitative analysis of $^{G}\gamma$- and $^{A}\gamma$-globin mRNA expression in the non-HPFH line carrying the LCR$^{G}\gamma^{A}\gamma\psi\beta$ transgene (line 1352). The developmental time points are indicated below each set of two bars.

the absence of the LCR, the presence of HPFH-2 3′ breakpoint DNA sequences is not sufficient to result in generation of a full HPFH phenotype with persistent expression of the γ-globin genes in adult erythroid cells. On the other hand, experimental data obtained from mice carrying the $^{G}\gamma^{A}\gamma$HPFH-2 transgene linked to the LCR (LCR$^{G}\gamma^{A}\gamma$HPFH-2) show persistent human γ-globin mRNA and peptide chain expression in adult erythroid cells. Although transgenic mouse models for the nondeletion form $^{A}\gamma$HPFH (−117G→A) demonstrating persistent human $^{A}\gamma$-globin mRNA expression in adult erythroid cells have been previously described (4, 33), we report the generation of a deletion-type HPFH transgenic mouse model and provide experimental data suggesting that interactions between LCR sequences, the regulatory elements of the γ-globin genes, and 3′ HPFH-2 breakpoint DNA sequences are required for persistent γ-globin gene expression in adult HPFH-2 transgenic mice.

One hypothesis to explain persistent adult γ-globin gene expression in deletion-type HPFH has proposed that the sequences normally located downstream of the γ-globin genes but deleted in HPFH are required for the silencing of the γ-globin genes in adults (25). The observation in transgenic mice of inappropriate embryonic expression of the human β-globin gene when it was linked to the LCR and restoration of developmental regulation in the presence of a competing LCR-proximal γ-globin gene led to the competitive model for globin switching where differential competition exists between the promoters of the γ- and β-globin genes for the activating or stimulating effects of the LCR. The competition model for globin gene switching is supported by in vivo genetic evidence from individuals with nondeletion-type HPFH resulting from point mutations in γ-globin gene promoters, where persistent adult expression of the affected γ-globin gene is associated with downregulation of the β-globin gene from the same chromosome (21, 34). However, small deletions that abolish β-globin gene transcription result in only less than 5% γ-globin gene expression, whereas the large deletions in deletion-type HPFH syndromes result in high levels of γ-globin expression, ranging from 10 to 30% (34). Transgenic mouse studies have suggested that polarity in the locus and stage-specific elements flanking the genes result in nonreciprocal competition in the locus (4, 12, 23, 24). Data from transient transfection experiments that showed enhancer-like activity in HPFH-2 breakpoint DNA sequences (18) and the demonstration of an erythroid cell-specific DNase I-HSS (14) suggest that changes in the chromatin structure flanking the γ-globin genes interfere with promoter-mediated silencing of the γ genes rather than the lack of silencing being due to a lack of competition as a result of the deletion of sequences downstream of the $^{A}\gamma$-globin gene, including the β-globin gene.

Previous studies with transgenic mice to address the mechanisms of γ-globin gene silencing have reported variable results. Deregulation of the γ genes by the LCR had been suggested by earlier studies of Enver et al. (16) and Behringer et al. (3), who found that γ-globin gene expression continued in the adult stages of mouse development. Interestingly, the data from our LCR$^{G}\gamma^{A}\gamma\psi\beta$ control line differ from those of Behr-

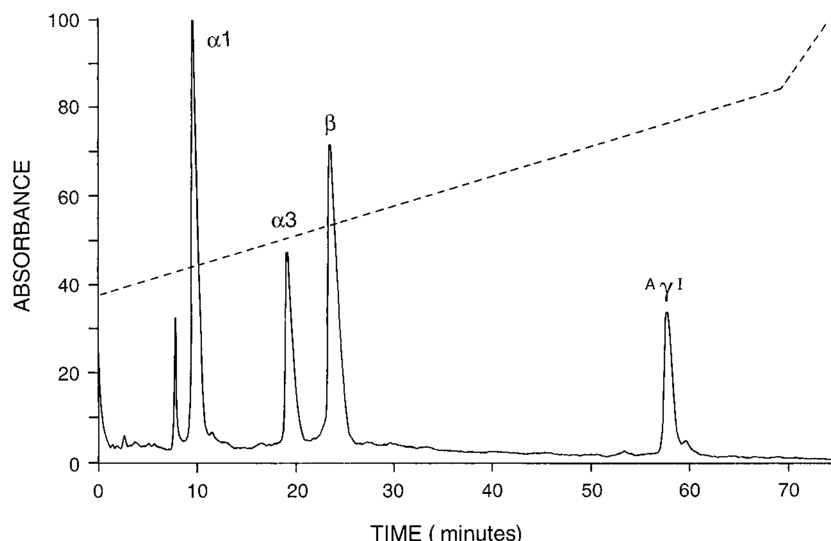


FIG. 10. HPLC analysis of blood lysate of an HPFH-2 transgenic mouse (line 3035). Peak $^{A}\gamma^{I}$ corresponds to human γ-globin chains.

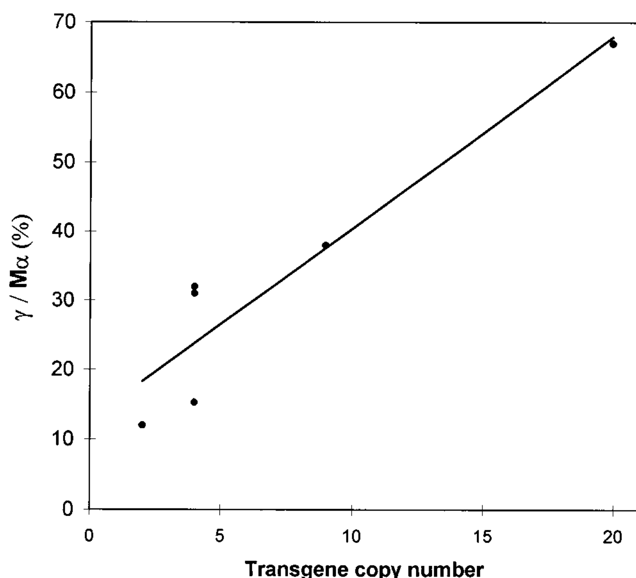


FIG. 11. Correlation between total γ-globin mRNA levels and transgene copy numbers. Results of γ-globin mRNA expression in adult mice from the six different lines carrying the LCR$^{G}\gamma^{A}\gamma$HPFH-2 transgene are shown ($r = 0.93$).

inger et al. (3), who also used a *Kpn*I-*Sal*I γ-globin gene construct which is identical to the $^{G}\gamma^{A}\gamma\psi\beta$ genomic γ-globin gene fragment used to construct the LCR$^{G}\gamma^{A}\gamma\psi\beta$ transgene in our experiments. In the experiments by Behringer et al. (3), the 27-kb $^{G}\gamma^{A}\gamma\psi\beta$ fragment was coinjected with a 22-kb LCR transgene, whereas in our experiments a single 32-kb fragment was microinjected. Persistent γ-globin gene expression in erythroid cells of adult mice was reported in two transgenic lines by Behringer et al. (3); however, it is possible that coinjection of the fragments resulted in structures which influenced γ-gene expression, such as the introduction of sequences between the LCR and γγψβ junctions which were not characterized in this study. Our results obtained from the analysis of γ-globin gene expression in the non-HPFH control transgenic lines carrying the LCR$^{G}\gamma^{A}\gamma$ and the LCR$^{G}\gamma^{A}\gamma\psi\beta$ transgenes suggest that γ-globin gene silencing in adult transgenic mice can occur in the absence of competition from linked δ- and β-globin genes. These results are consistent with those of Dillon and Grosveld (11), who found autonomous silencing of the $^{A}\gamma$ gene within a mini-LCRγ construct without the presence of an adjacent β-globin gene. Similar results were reported by Peterson et al. (33) in their analysis of β-YAC transgenic mice containing the human β-globin gene cluster but lacking the δ- and β-globin genes which demonstrated silencing of the γ-globin genes in adult transgenic mice despite the absence of a linked β-globin gene. Some of the variability in the results of the earlier experiments has been attributed in part to differences in the specific DNA fragments used in different laboratories, the copy number of the transgenes, and the sensitivity of the γ-globin genes to position effects resulting from the surrounding chromatin at the integration site of the transgene (11, 38).

Mild integration site-dependent position effects were observed in the lines carrying the LCR$^{G}\gamma^{A}\gamma$HPFH-2 transgene. Fourfold variability in γ-globin gene expression was observed among fetuses carrying the same transgene, whereas in adults, γ-globin gene expression showed only a 2.3-fold variation. The persistent γ-globin gene expression in erythroid cells of newborn and adult animals cannot be explained only on the basis of integration site-dependent position effects, since a similar developmental pattern of γ-globin gene regulation was observed in all six transgenic lines carrying the same LCR$^{G}\gamma^{A}\gamma$HPFH-2 transgene, i.e., none of the six lines with this transgene exhibited correct developmental regulation of the γ-globin genes. Importantly, a correlation between the total level of γ-globin mRNA expression in adult erythroid cells and transgene copy numbers was observed. Lastly, the LCR$^{G}\gamma^{A}\gamma$ control transgene which does not include HPFH-2 breakpoint DNA sequences resulted in correct developmental regulation of the γ-globin genes. A similar result was obtained with the transgenic mice carrying the LCR$^{G}\gamma^{A}\gamma\psi\beta$ transgene.

Interestingly, at the levels of accumulation of both the mRNA and globin chains, exaggerated preferential expression of $^{A}\gamma$-globin over $^{G}\gamma$-globin was observed at all developmental stages in the transgenic lines carrying the LCR$^{G}\gamma^{A}\gamma$HPFH-2 transgene. In contrast, transcripts from the LCR-proximal $^{G}\gamma$-globin gene were about three- to sevenfold higher than those of the more distal $^{A}\gamma$-globin gene in both non-HPFH control lines. This latter finding is consistent with results of studies with transgenic mice that examined the role of gene order on the level of expression of individual genes in the β-globin locus and found that an LCR-proximal gene is expressed at a higher level than a structurally similar gene located more distally (13, 24, 31). Preferential $^{A}\gamma$-globin expression in erythroid cells of adult HPFH-2 transgenic mice is consistent with the predominant expression of $^{A}\gamma$-globin chains (67.7%) over $^{G}\gamma$-globin chains (32.3%) in erythroid cells of human heterozygotes for deletion-type HPFH-2, as shown by Kutlar et al. (27). Interestingly, the same study showed that HPFH-1 heterozygotes expressed almost equal amounts of $^{G}\gamma$- and $^{A}\gamma$-globin chains

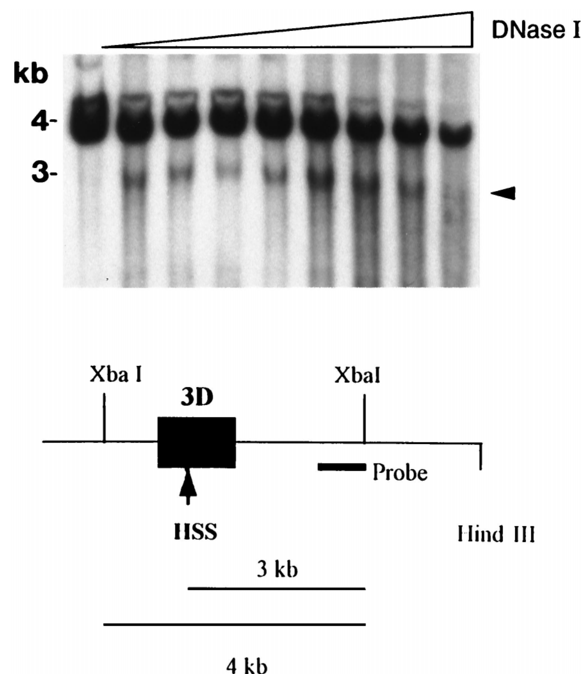


FIG. 12. Mapping of DNase I-HSS within HPFH-2 3′ breakpoint region 3D in erythroid cells from the spleen of an anemic mouse carrying the LCR$^{G}\gamma^{A}\gamma$HPFH-2 transgene. An autoradiograph of Southern blotting of *Xba*I-digested genomic DNA is shown at the top. The first lane on the left contains the control sample without prior DNase I digestion. Lanes contain increasing concentrations of DNase I is from left to right as indicated. The 3-kb subband generated by the HSS is indicated by the arrowhead. A diagram of the HPFH-2 3′ breakpoint region is shown on the bottom. The position of the probe is displayed below the restriction map as a solid horizontal bar. The arrow indicates the position of the HSS.

(27). The HPFH-1 deletion is the same size as that of HPFH-2 but is shifted in the 3′ direction (relative to the HPFH-2 deletion) by approximately 6 kb at both breakpoints (42). Bakioglu et al. (2) proposed that the difference in $^{G}\gamma/^{A}\gamma$ ratios between HPFH-1 and HPFH-2 is due to the presence, in the HPFH-1 $^{G}\gamma$-gene promoter, of the −158C→T base change that is associated with preferential overexpression of the $^{G}\gamma$ gene over the $^{A}\gamma$ gene (22) and therefore would result in a $^{G}\gamma/^{A}\gamma$ ratio different from that in HPFH-2, the $^{G}\gamma$-gene promoter of which does not contain the base change. The γ-globin transgene used in our studies does not contain the $^{G}\gamma$ −158C→T change. In transgenic mice carrying the 25-kb $^{G}\gamma^{A}\gamma$HPFH-2 transgene without the LCR, similar levels of $^{G}\gamma$- and $^{A}\gamma$-globin mRNA levels were observed. An important consideration with regard to the LCR$^{G}\gamma^{A}\gamma$HPFH-2 transgene is the fact that the LCR, which is normally separated from the γ-globin genes by approximately 20 kb in vivo, is in much closer proximity to the γ genes and to the HPFH-2 breakpoint DNA sequences in the transgenic mice. Thus, it is possible that in the mouse model, the in vivo interactions between the LCR, *cis*-acting regulatory elements of the $^{A}\gamma$-globin gene, and breakpoint DNA sequences favoring $^{A}\gamma$- over $^{G}\gamma$-globin expression are exaggerated due to the structure of the transgene.

The analyses of the cellular distribution of human γ-globin chains in the blood of adult HPFH-2 mice using anti-γ-globin monoclonal antibodies demonstrate that expression is heterocellular (39 to 86% positive cells). Blood from mouse lines with higher copy numbers and higher γ/mouse α ratios had higher percentages of positive cells. In human heterozygotes with HPFH-2, however, there is relatively uniform (pancellular) distribution of Hb F in RBCs (5, 6). The molecular genetic determinants of this pancellular Hb F distribution in HPFH syndromes is unknown, although it has been noted in previous studies that the percentage of positive cells correlates with the level of Hb F. In the two transgenic mouse models for nondeletion HPFH (pancellular Greek $^{A}\gamma$), one of which was present in a β-YAC transgene, adult expression of human γ-globin mRNAs was observed; however, the cellular distribution patterns of γ-globin chains were not reported (4, 33). The HPFH-2 transgenic animals analyzed in our studies were not chimeric mice. It is possible that the lack of strictly pancellular γ-globin chain distribution in the mouse model (in contrast to human heterozygotes) is related to the absence in the transgene of DNA sequences from the normal β locus that are required for pancellular expression. Another factor that may play a role in the lack of strictly pancellular expression in HPFH-2 mice is the fact that, in contrast to the in vivo situation in humans, the HPFH-2 transgenes are randomly integrated and not present within the normal chromosomal context of the β-globin gene cluster.

This transgenic mouse model for deletion-type HPFH-2 suggests that alterations in the chromatin structure flanking the γ-globin genes in deletion-type HPFH-2 are associated with persistent expression of the γ-globin genes. The DNA sequence in the vicinity of the DNase I-HSS in 3′ HPFH-2 breakpoint region 3D (Fig. 1A) contains putative binding sites for erythroid transcription factors such as the GATA family of transcription factors and CACCC-binding proteins. It is tempting to speculate that the presence of an erythroid cell-specific DNase I-HSS (14), the enhancer-like activity of the 3′ breakpoint DNA in transient transfection assays (18), and the persistent γ-globin gene expression in adult RBCs in HPFH-2 transgenic mice are all related to the binding of these transcription factors to the breakpoint DNA. Taken together, our results support the hypothesis that breakpoint region DNA sequences can modify the developmental regulation of the γ-globin genes in deletion-type HPFH-2 and that LCR sequences are required for generation of the HPFH phenotype in transgenic mice. The expression of high levels of human γ-globin chains in RBCs of adult HPFH-2 transgenic mice provides the opportunity to study the modifying effects of γ chains on the phenotype of one of the recently described transgenic mouse models for sickle cell disease (17).

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