Vol. 194, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1993 Pages 246-252

HUMAN FETAL-TO-ADULT GLOBIN GENE SWITCHING IN TRANSGENIC MICE: PERSISTENT EXPRESSION OF THE Gγ- GLOBIN GENE IN THE JAPANESE HPFH

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Received May 27, 1993

SUMMARY: We linked a 3.3 kb fragment containing the human γ-globin gene together with a 5.5 kb fragment containing the human β-globin gene. This construct was introduced into the germ line of mice. Analysis of the resulting transgenic mice showed that expression of the human γ- and β-globin genes were regulated as that of the mouse embryonic and adult globin genes and human γ-to-β globin switching was reconstructed in the mice. By replacing the γ-globin gene in the construct with modified counterparts, this transgenic mouse system enables us to analyze cis-acting elements essential for erythroid-specific and developmental stage-specific expression of the test gene under the condition in which human β-globin gene expression is regulated. In this system expression of the Gγ-globin gene bearing the point mutation found in a Japanese patient of hereditary persistence of fetal hemoglobin (HPFH) (1) persisted at a equivalent level to β-globin expression in fetal and adult mice.

Distinct members of the human globin gene families are expressed in different tissues and at different times during development (2,3). This phenomenon provides us with an interesting model to investigate the mechanisms underlying the switches in gene expression at the molecular level. Using erythroid cell lines, considerable progress has been made in our understanding of cis-acting elements necessary for the transcriptional regulation of globin genes (4-6). However, the cell systems simulate in vivo erythropoiesis only in part. They, therefor have certain disadvantages, in particular, for studying the switch from embryonic to fetal to adult globin gene expression. A more powerful approach to the study of switching mechanisms is the development of transgenic mouse systems in which expression of the introduced human globin genes are regulated as their endogenous counterparts. Constructs containing the human γ- or β-globin gene and the immediate flanking sequences are expressed in a tissue- and developmental stage-specific manner (7,8), however their expression levels are affected by their integrated positions. The constructs containing the β-globin gene locus control region (LCR) confer erythroid specific and position independent high level expression in transgenic mice, but in some situations, override correct developmental control of the human γ- or β-globin gene (9-12). We generated the construct in which the human γ- and β-globin genes were

0006-291X/93 $4.00

juxtaposed and oriented in the same directions. Transgenic mice containing this construct mimicked the human γ-to-β globin switch. By replacing the γ-globin gene in the construct with modified counterparts, this transgenic mouse system enables us to examine expression of the test gene placed in the chromosome, where the integrated β-globin gene is expressed in a tissue-specific and stage-specific manner without the LCR. To evaluate usefulness of this system we analyzed expression of the Gγ-globin gene which has the point mutation associated with the Japanese type of HPFH (hereditary persistence of fetal hemoglobin) (1).

MATERIALS AND METHODS

Production of transgenic mice: The construction of pAγ/β was described previously (13). pGγ^{-114}/β was created by replacing a 3.3 kb *Hin*dIII fragment of the Aγ-globin gene in pAγ/β with a 3.3 kb fragment containing the entire Gγ-globin gene associated with the C-T substitution at nucleotide position -114. This Gγ-globin fragment was the product of polymerase chain reaction using Japanese HPFH patient's genomic DNA. Two oligonucleotide primers with the sequence 5'-GGTCTTTTAGCCGCCTAACA-3' and 5'-TTTCTTCGTTGAGCCCCTTC-3' were used for amplification. A 8.9 kb *Cla*I-*Sal*I fragments (Fig. 1) of the pAγ/β and pGγ^{-114}/β were purified by agarose gel electrophoresis from vector sequences. After further purification by banding in cesium chloride and dialysis, the DNA was microinjected into male pronuclei F2 hybrid eggs obtained by mating C57BL/6J X DBA/2 parents as described (14,15). Transgenic mice were identified by Southern blot analysis (16) of the DNA obtained from tail biopsies (17). The transgene copy numbers were determined by dot blot hybridization using human genomic DNA as a standard.

Analysis of mRNA in transgenic mice tissues: Total RNA was prepared using the guanidine thiocyanate procedure (18) from 10.5-day yolk sac, 13.5 and 16.5-day fetal liver, and adult spleen (about two months old). The levels of β-, γ-, β_{maj}-, and βh1-globin mRNAs were determined using quantitative ribonuclease protection assay (19) and intensities of protected bands were quantitated with Fuji Bio Image analyzer BAS2000 (Fuji Film Co, Japan). The following globin-specific probes were used: (i) for human β-globin mRNA, pBHβ2, a 655 bp *Acc*I fragment linearized with *Pst*I to give a 143 bp protected fragment; (ii) for human γ-globin mRNA, pBHγ2, a 342 bp *Ava*II fragment linearized with *Pst*I to give a 145 bp protected fragment; (iii) for mouse β_{maj}-globin mRNA, pBMβ_{maj}, a 441 bp *Hin*cII-*Bam*HI fragment linearized with *Ava*II to give a 189 bp protected fragment; (iv) for mouse βh1-globin mRNA, pBMβh1, a 423 bp *Xba*I-*Bam*HI fragment linearized with *Xba*I to give a 143 bp protected fragment.

RESULTS

We linked the 3.3 kb fragment containing the entire human γ-globin gene (fetal type) to the 5.5 kb β-globin gene (adult type) containing fragment. This construct is illustrated in Fig. 1. Five lines bearing various numbers of the Aγ/β-globin gene construct were obtained (Table 1) (13). Southern blot analysis showed that the injected fragments appeared to be arranged in the head-to-tail configuration in all lines (data not shown). Expression of the introduced β-globin gene in adult spleen of transgenic mice was measured by ribonuclease protection assay. Two lines, 3-4 and 11-5 expressed detectable amounts of human β-globin mRNA (Table 1). Further analysis for these two lines revealed that human β-globin mRNA was not detected in liver, brain and skeletal muscle of the adult mice. The expression of the β-globin gene was limited to the erythroid tissues.

To analyze the expression pattern of the introduced human globin genes throughout development, we prepared total RNA from 10.5-day yolk sac, 13.5 and 16.5-day fetal liver, and adult spleen of the

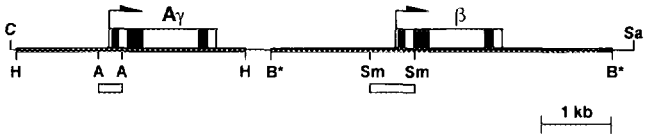

Fig. 1. The Aγ/β-globin gene construct introduced into mice. The human Aγ-globin *Hin*dIII fragment and the human β-globin *Bgl*II fragment (thick bar) were juxtaposed and oriented in the same direction. Thin bars show the sequences derived from plasmid (pBR322). Arrows indicate the transcription start sites. The coding regions of each globin gene are shown by solid boxes. Open boxes under the diagram indicate the locations of the probes used for ribonuclease protection assay. Abbreviations are: A, *Acc*I; B*, *Bgl*II (these sites are lost); C, *Cla*I; H, *Hin*dIII; Sa, *Sal*I; Sm, *Sma*I.

lines 3-4 and 11-5. The relative abundance of the human and mouse globin mRNAs were analyzed by ribonuclease protection assay using an appropriate amount of total RNA samples. Fig. 2 illustrates a representative result obtained from line 3-4. Total RNA of 5 μg (for β) or 10 μg (for γ) extracted at four developing stages was hybridized to labeled RNA probes. Although intensity of the protected bands could not be compared directly between different developmental stages because of different RNA sources, a significant increase of β-globin expression was observed between 13.5 and 16.5-days. On the other hand, a striking change was not observed in γ-globin gene expression. Table 2 shows relative expression levels of human and mouse globin mRNAs represented as a percentage of the level of mouse β_{maj}-globin mRNA expressed at adult spleen. In 10.5-day yolk sac of line 3-4, the β-globin expression level is higher than that of the γ-globin gene. As described previously (8,20), the βh1-globin gene (mouse embryonic type) is predominantly expressed, but the β_{maj}-globin gene (mouse adult type) is barely expressed in 10.5-day yolk sac of the mouse. It is likely that the presence of β-globin mRNA in embryos is due to contamination by maternal erythroid tissues because β_{maj}-globin mRNA was detected in 10.5-day yolk sac (Table 2). Contamination by maternal RNA seems to be

Table 1. Copy number of transgenic mice and relative expression of the human β- to mouse β_{maj}- globin gene in adult mouse spleen

Lines	Copy number[a]	human β/mouse β_{maj} (%)[b]
Aγ/β 3-4	80-150	34.5
11-5	33-42	6.9
5-3	6-13	0
4-5	50	0
8-2	3	0
$G\gamma^{114}/\beta$	5-10	1.7

[a] Copies of the transgene per haploid genome.
[b] Human β-globin expression is represented as a percentage of the level of endogenous β_{maj}-globin RNA expressed in adult mouse spleen.

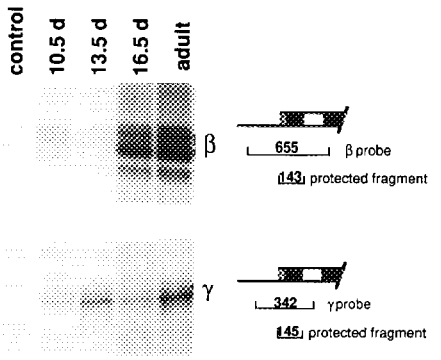

Fig. 2. RNase protection analysis against human γ- and β-globin mRNAs in transgenic mice (line 3-4) during development. Five μg or 10 μg total RNA prepared from erythroid tissues at indicated days of gestation was hybridized to human β- or γ-globin specific RNA probes and RNase resistant fragments were analyzed by electrophoresis on a 6% polyacrylamide sequence gel. RNase protected fragments are indicated at the right. Negative control consisted of 20μg tRNA. Probes are diagramed at the right.

more significant in line 3-4 because exogenous β-globin expression in adult of line 3-4 is much higher (34.5 % of endogenous β_{maj}) than that in line 11-5. Since the amounts of β- and γ-globin mRNA derived from maternal tissues in embryonic samples should correlate with the relative level of mouse β_{maj}-globin mRNA in 10.5-day yolk sac, the amounts of contaminated messages during embryo preparation of line 3-4 appear to be 1.33 and 0.05 as β- and γ-globin messages, respectively. After

Table 2. Relative abundance of human and mouse globin mRNAs per microgram total RNA prepared from transgenic mice

Lines	mRNA	10.5 day	13.5 day	16.5 day	Adult
Aγ/β 3-4	human β	0.42 (0)	0.95*	13.3	34.5
	human γ	0.10 (0.05)	0.41	0.28	1.19
	mouse β_{maj}	3.87	63.6	168.2	100
	mouse βh1	9.75	0.83	0	0
Aγ/β 11-5	human β	0	1.11*	2.42	6.50
	human γ	0.05	0.09	0.07	0.24
	mouse β_{maj}	2.05*	155.3*	219.9	100
	mouse βh1	3.80	0.06*	0*	0*
$G\gamma^{-114}/\beta$	human β	0	7.65*	15.4	1.7
	human γ	0.24	9.68	8.29	1.15
	mouse β_{maj}	1.17	258.8	351.2	100
	mouse βh1	9.85*	0.24*	0*	0*

Human and mouse globin expression levels in 1 μg total RNA prepared at indicated time points are represented as a percentage of the mouse β_{maj}-globin mRNA level in 1 μg adult mice spleen. Expression values shown are averages from duplicated or triplicated analyses except those marked by asterisks. Compensated values for maternal RNA contamination are in parentheses.

compensation for contamination by maternal RNAs, relative expression levels of β- and γ-globin mRNAs turn to be 0 and 0.05, respectively in 10.5-day yolk sac of line 3-4 (Table 2 parenthesized). Fig. 3 shows the ratio of the γ-globin mRNA expression to the total exogenous mRNA (γ/β+γ) at four developmental stages of transgenic mice. The switch from γ- to β-globin expression is observed around 13.5 day of gestation of transgenic mice.

To test whether the point mutation found in the Japanese non-deletion HPFH (1) is the cause of the raised γ-globin levels in the adult stage and is not just a linked polymorphism, we generated the $G\gamma^{-114}/\beta$-globin construct in which the Aγ-globin gene was replaced with the Gγ-globin gene containing the cytosine to thymine substitution at nucleotide position -114. Functional significance of this base change was recently supported by another line of evidence that the same substitution was found at position -114 of the Aγ-globin gene of patients with the Georgia type of HPFH (21). Expression levels of the γ- and β-globin mRNAs throughout mouse development were analyzed by RNase protection assay (Table 2). Fig. 3 shows the γ/β+γ ratio during development. The expression level of the γ-globin gene was not reduced in fetal and adult stages and remained equivalent to that of the β-globin gene. It is concluded that the C-T substitution in the distal CCAAT box of the Gγ-globin promoter overcomes the suppression in fetal and adult mice.

DISCUSSION

We developed the transgenic mouse system using the Aγ/β-globin construct schematically shown in Fig. 1 in which the 3.3 kb *Hin*dIII fragment containing the entire human Aγ-globin gene and the 5.5 kb *Bgl*II fragment containing the entire human β-globin gene were juxtaposed in the same orientation. In this system the human β-globin gene was regulated as the adult mouse globin gene and the human γ-globin gene was regulated as the mouse embryonic globin gene as reported previously (7,8). The human γ-to-β switch was observed around 13.5 day in gestation of transgenic mice.

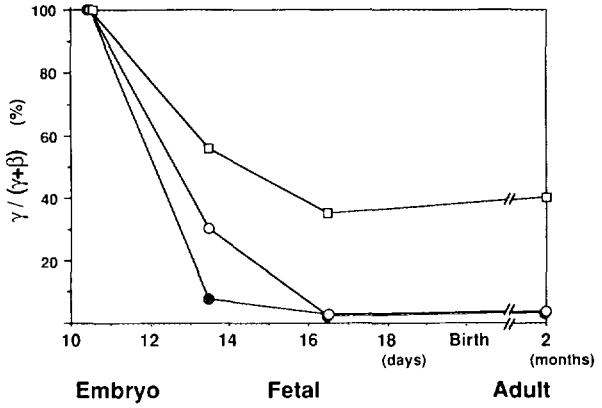

Fig. 3. Relative change in γ- and β-globin gene expression during ontogeny of transgenic mice. The relative γ-globin mRNA levels were plotted as a fraction of total exogenous human globin (γ+β) mRNA. Filled circle, Aγ/β(3-4); open circle, Aγ/β(11-5); open square, $G\gamma^{-114}/\beta$(3-4).

Because expression of introduced genes depends on the effect of the integration loci, it is difficult to analyze the effect of modification on the introduced gene construct. To overcome position-dependent expression of the globin gene in transgenic mice, the globin gene constructs with the locus control region (LCR) were used in some studies (9-12,22-25). Although the LCR conferred high-level position independent erythroid-specific expression, the γ- or β-globin gene attached individually to the LCR was expressed throughout mouse development (9-11,24). The LCR-linked gene with heterologous promoters such as that of the thymidine kinase gene, was also expressed in an erythroid-specific manner (25,26), indicating that the effect is not promoter specific. These observations suggest that each globin gene should escape the effect of the LCR at specific stages of development to be expressed in a developmentally regulated manner. Recently it was revealed that the relative order of the globin genes and distances from the LCR are essential for temporally-specific interaction between the LCR and the globin genes (12,27). Therefore, the globin gene constructs, whose expression were strictly regulated in transgenic mice, were mostly large (10,11,23,28) and its manipulations were laborious. In addition, it is difficult to obtain adult lines that pass on the transgenes because of severe globin chain imbalance due to overexpression of the exogenous gene driven by the LCR (10,12). The construct used in this study contain two genes. The one gene can be considered as a reference for the another and both genes are regulated individually without the LCR through mouse development. This transgenic mouse system enables us to analyze the expression of the test gene by regarding the expression of the β-globin gene linked to it as the erythroid-specific reference gene.

In this system the Gγ-globin gene associated with the C-T substitution position -114 represented persistence of γ-globin expression at a equivalent level in fetal and adult mice (Fig.3). It was previously shown that the Aγ-globin gene associated with the Greek form HPFH point mutation (G-A substitution at position -117) also overrides the repression in adult mice (28). These one base substitutions probably abolish the binding of transcription factors, which could be involved in globin switching (1,28,29).

These results indicate that this transgenic mouse system is useful for functional analysis of cis-acting elements in the erythroid-specific gene through development.

ACKNOWLEDGMENTS

We are grateful to Drs. Toru and Akiko Iwaki for technical advice and assistance. This work was supported in part by grants from the Ministry of Education, Science and Culture in Japan.

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