MOLECULAR AND CELLULAR BIOLOGY, Sept. 1994, p. 6087–6096
0270-7306/94/$04.00+0

Vol. 14, No. 9

Position Independence and Proper Developmental Control of γ-Globin Gene Expression Require both a 5′ Locus Control Region and a Downstream Sequence Element

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Received 11 April 1994/Returned for modification 9 May 1994/Accepted 23 June 1994

We have analyzed the expression of human γ-globin genes during development in F_2 progeny of transgenic mice carrying two types of constructs. In the first type, γ-globin genes were linked individually to large (~4-kb) sequence fragments spanning locus control region (LCR) hypersensitive site 2 (HS2) or HS3. These LCR fragments contained not only the core HS elements but also extensive evolutionarily conserved flanking sequences. The second type of construct contained tandem γ- and β-globin genes linked to identical HS2 or HS3 fragments. We show that γ-globin expression in transgenic mice carrying HS2γ or HS3γ constructs is highly sensitive to position effects and that such effects override the *cis* regulatory elements present in these constructs to produce markedly different developmental patterns of γ-globin expression in lines carrying the same transgene. In contrast, γ-globin expression in both HS2γβ and HS3γβ mice is sheltered from position effects and the developmental patterns of γ-globin expression in lines carrying the same transgene are identical and display stage-specific regulation. The results suggest that *cis* regulatory sequences required for proper developmental control of fetal globin expression in the presence of an LCR element reside downstream from the γ genes.

The β-like globin genes in humans are organized into a locus on chromosome 11 that contains coordinately expressed embryonic, fetal, and adult globin genes and an upstream locus control region (LCR) (see Fig. 1) (49). Within the LCR, regulatory sequences necessary for high-level globin gene expression in cell and transgenic assays have been found to coincide with the presence of focal alterations in chromatin structure that are hypersensitive (HS) to cleavage by nucleases such as DNase I (22, 24, 25, 30, 56, 57). These HS elements have been studied both collectively and individually for their effects on globin gene expression and its regulation during development with both erythroleukemia cell (11, 23, 38, 51, 59) and transgenic systems (3, 18, 30, 51). In particular, the regulatory elements coinciding with LCR HS sites 2 (HS2) (6, 11, 12, 15, 23, 26, 27, 35, 37, 38, 45, 52, 58) and 3 (HS3) (23, 26, 27, 41, 42) have demonstrated the greatest capacity for stimulating expression from linked γ- or β-globin genes. Moreover, it appears that the developmental regulation of γ- and β-globin expression under the control of the entire LCR (28, 40, 50) requires complex interactions between DNase HS elements that, taken individually, support different patterns of globin gene expression (27, 29).

A number of models have been proposed to explain the role of the LCR in modulating developmental globin gene switching; these models have in common the assumption that the γ- and β-globin genes engage one another in competitive interaction for enhancer sequences located within the LCR (3, 16, 18, 29, 31, 47, 54). These models were based in part on experiments demonstrating mutually exclusive expression of primitive and definitive globin species in the chicken (9). A competitive basis for human globin switching is compatible with the increases in adult-stage γ-globin expression that accompany the removal or inactivation of the downstream β gene in several naturally occurring deletion mutants, the δ-β-thalassemias and the hereditary persistence of fetal hemoglobin (5). Recently, such models have received support from transgenic experiments demonstrating that a human γ-globin gene situated between an upstream LCR element and a 3′ β gene is expressed at the expense of β-globin during earlier developmental stages (3, 18, 27, 31, 50). However, extensive developmental data that address the competition hypothesis quantitatively have thus far been lacking.

An important concept that has emerged from studies of the regulation of human transgenes in transgenic animals is that the heterologous chromatin environment present at a given site of integration may exert a dominant influence on transgene expression. Such site-of-integration or position effects manifest as marked variability in transgene expression between transgenic lines carrying identical constructs when expression is corrected for the number of integrated copies. With respect to the globin genes, significant variability in per-copy expression between different lines of transgenic mice characterizes the activity of individual human γ- and β-globin transgenes (2, 7, 8, 34, 39, 55) or that of a cosmid vector transgene containing both genes in their natural local environment (27). By contrast, transgenes comprising a β-globin gene linked either to the full-length LCR, to a mini-LCR construct, or to individual LCR HS sites exhibit small coefficients of variation (CV = σ/μ) in per-copy β-globin expression between lines carrying the same construct. A small CV entails that β-globin expression from such constructs be proportionate to transgene copy number (copy number dependent) (26, 27, 30, 41, 51, 53). These results have been interpreted to signify that expression proceeds independently of negative or positive influences on transcription stemming from the character of chromatin surrounding the integration site (21). In LCR element-β gene constructs, copy-proportional expression is observed both during the fetal stage, when β-globin expression is low, and in the

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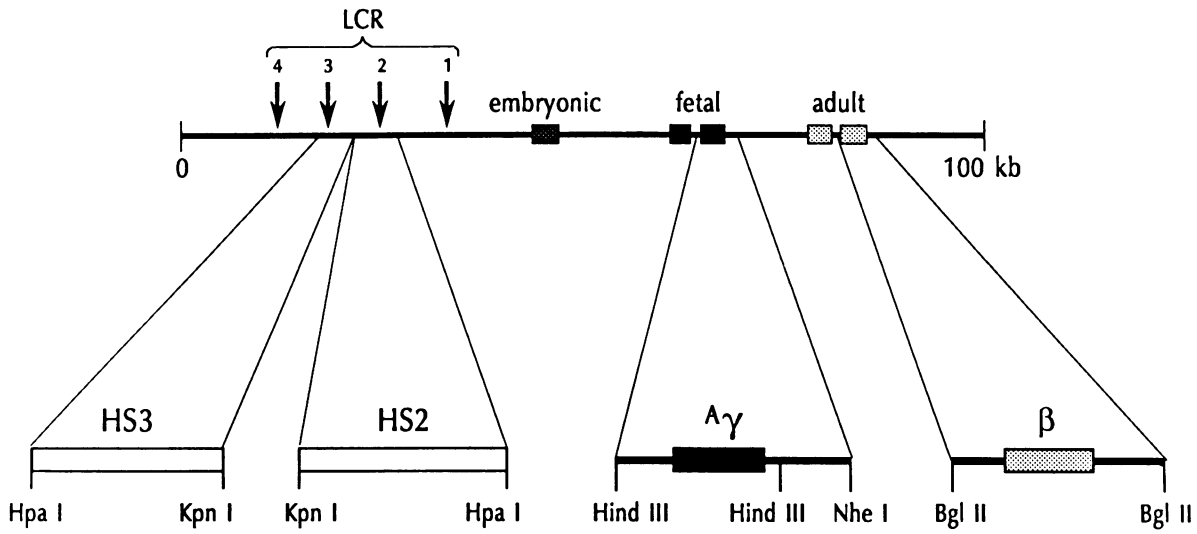

FIG. 1. The β-globin locus and details of HS2, HS3, and γ- and β-globin gene sequence fragments. Fragments of 4.2 kb (*Kpn*I-*Hpa*I) and 4.4 kb (*Hpa*I-*Kpn*I) from the β-globin LCR spanning HS2 and HS3, respectively, were fused independently with the *Hin*dIII fragment spanning the ^{A}γ-globin gene to create the HS2γ and HS3γ constructs. HS2γβ and HS3γβ constructs contained HS sequences linked to a *Hin*dIII-*Nhe*I ^{A}γ fragment and a *Bgl*II β-globin fragment.

adult stage, when it has risen considerably, indicating that the determinants of integration site-independent β-globin expression function independently of changes in the *trans* environment. This suggests either that position-independent transgene expression is entirely a consequence of linkage to an LCR element or that *cis*-acting sequences within or flanking the β-globin gene interact with an upstream regulatory element to mediate expression that is sheltered from position effects.

On the basis of chromatin studies and functional data garnered from experiments with transgenic mouse and erythroleukemia cell systems, a number of properties have been ascribed to the LCR. These include a capacity for erythroid tissue-specific activation of both homologous and heterologous linked genes (31, 59); control over the replication timing of the globin locus (19, 20); and the propagation of structural changes to the surrounding chromatin, resulting in increases in generalized DNase sensitivity far removed from the LCR HS sites themselves (20, 22, 24). Taken together, these observations have been cited in support of a model of LCR operation which attributes the ability to override influences from the surrounding chromatin environment (position effects) to a dominant regulatory function intrinsic to its constituent HS site elements (14, 54). Recent studies, however, indicate that the ability to effect opening of the surrounding chromatin, with consequent dampening of position effects on the expression of an approximated gene, is not an inherent property of LCRs (44, 48). These studies support the notion that position-independent transgene expression requires the interaction of an LCR with *cis* elements within or immediately flanking a linked gene.

We have investigated the control of human fetal globin genes during development by LCR HS2 and HS3 with two types of constructs, those that contained HS2 or HS3 linked independently to a lone ^{A}γ-globin gene and constructs that contained the same regulatory elements linked independently to tandem γ- and β-globin genes. Analysis of per-copy, γ-globin expression in F_2 progeny of transgenic mice carrying HS2γ or HS3γ transgenes showed that γ-globin expression was highly sensitive to site-of-integration or position effects at all developmental stages. Markedly varied developmental patterns were observed among lines transgenic for the same HSγ construct. These data indicate that the surrounding chromatin environment may exert a dominant influence on the expression profile of a developmentally regulated gene. By contrast, analysis of γ-globin expression in F_2 progeny of transgenic mice carrying an HS2γβ or HS3γβ construct revealed that the γ-globin transgenes in these constructs were sheltered from position effects throughout development. The developmental patterns of γ-globin expression in these mice showed stage-specific regulation and were nearly identical in different transgenic lines carrying the same construct. In mice carrying an HS2γβ or HS3γβ construct, γ-globin expression is sheltered from position effects at all developmental stages, indicating that position-independent expression does not depend on either the absolute level or the relative level of γ- or β-globin gene transcriptional activity. This suggests that the sheltering of γ-globin expression from position effects is a transcription-independent effect of *cis* sequences contained in the HSγβ constructs but missing from the HSγ constructs. It therefore appears that the interactions between a fetal globin gene promoter and an upstream LCR that result in proper developmental control of fetal globin expression require the presence of additional regulatory sequences downstream from the γ genes.

MATERIALS AND METHODS

Recombinant constructs (Fig. 1). (i) HS2^{A}γ^{m}. A 4.2-kb *Kpn*I-*Hpa*I fragment (GenBank Humhbb coordinates 7764 to 11976) containing HS2 was cloned into pBluescript SK (Stratagene) and subsequently released by a *Cla*I-*Kpn*I double digest. The resulting fragment was ligated into a similarly digested plasmid containing a 3.3-kb *Hin*dIII fragment spanning a marked human ^{A}γ-globin gene (15). The HS2^{A}γ^{m} fragment was released from the base vector by double digestion with *Asp*718I and *Not*I.

(ii) HS3^{A}γ^{m}. A 4.4-kb *Hpa*I-*Kpn*I fragment (coordinates 3375 to 7764) containing HS3 was cloned into the modified plasmid pUC18. This sequence and part of the flanking

ampicillin resistance gene were subsequently released by digestion with *Sca*I-*Kpn*I. The liberated fragment was conjoined with a *Sca*I-*Kpn*I fragment containing the distal portion of the ampicillin resistance gene along with the marked human $^{A}\gamma$ gene ($^{A}\gamma^{m}$), and drug-resistant colonies containing properly ligated constructs were isolated. The HS3$^{A}\gamma^{m}$ fragment used in transgenic studies was released by digestion with *Not*I-*Nsi*I.

(iii) HS2$^{A}\gamma^{m}\beta^{m}$ and HS3$^{A}\gamma^{m}\beta^{m}$. Assembly of the HS2γβ and HS3γβ constructs was facilitated by the construction of a cassette containing the human $^{A}\gamma$- and β-globin genes. A novel *Nru*I site was introduced into the polylinker of the pSP73 vector (Promega) containing a marked β-globin gene (coordinates 60630 to 65480) (16). A *Hin*dIII-*Nhe*I human $^{A}\gamma^{m}$ fragment (coordinates 38085 to 43202) was then cloned upstream of the β^{m} gene in the same orientation. The resulting 10-kb γβ cassette was linked to either a 4.2-kb HS2 fragment (coordinates 7764 to 11976) or a 4.4-kb HS3 fragment (coordinates 3375 to 7764), preserving genomic orientation. HSγβ inserts were then released by *Nru*I-*Kpn*I (HS2γβ) or *Nru*I-*Not*I (HS3γβ) double digestions for use in transgenesis.

Transgenic mice. Fragments utilized in microinjection were purified by electrophoresis over 0.8% agarose and recovered on NA45 paper (Schleicher and Schuell). Eluted DNA was purified through Centrex microfilters (Schleicher and Schuell), ethanol precipitated, washed, and resuspended in Tris-EDTA buffer. Transgenic lines carrying HS2γ, HS3γ, HS2γβ, and HS3γβ fragments (individually) were established at the transgenic facility of the University of Washington by microinjection of male pronuclei of fertilized eggs, with subsequent transfer to pseudopregnant females. Transgenic founder (F_0) animals were identified by Southern blot with probes directed to γ- and β-globin gene sequences (see below). Founders were bred with nontransgenic mice, and the resulting F_1 progeny were screened for correct integration and the presence of mosaicism in the founders. F_1 mice were subsequently bred with nontransgenic lines to produce the F_2 animals used in developmental studies.

Copy number determination. DNA from carcasses of F_2 progeny in each line was isolated by standard procedures (46). Either two or three samples were obtained from each of three animals from each line. Individual samples were then digested with a different restriction enzyme. For lines carrying HS2γ and HS2γβ transgenes, *Sac*I and *Eco*RI and/or *Pst*I were used; for analysis of HS3γ and HS3γβ lines, *Spe*I and *Eco*RI and/or *Pst*I were used. Five milligrams of DNA from each enzyme reaction performed on samples from a given line was loaded onto a separate gel, and DNA fragments were resolved by electrophoresis over 0.8% agarose. Southern blots were performed with a 1.6-kb *Eco*RI-*Bam*HI probe spanning the 5′ portion of the human $^{A}\gamma$-globin gene and a 0.7-kb *Bam*HI-*Eco*RI probe fragment derived from the large intron of the β-globin gene. Signals were quantitated on a PhosphorImager, and means were computed from data sets that included multiple samples (two or three) from three individual animals from each line. Copy numbers were then determined by comparing the signals from a given transgenic line with those of human genomic (i.e., two-copy) DNA (Promega); the use of human sequences as a standard has the benefit of controlling for the specific activities of the probes. In cases in which the computed value was not an integer, the copy number was rounded to the nearest integer in standard fashion (i.e., $\geq x.5$, round up; $<x.5$, round down).

To ensure the accuracy of quantitation in cases in which samples from a given line provided a signal that was significantly stronger than that of human genomic DNA (i.e., higher-copy-number lines), samples were reexamined following serial dilution with mouse DNA (total DNA remained at 5 mg). The lack of activity of probes with mouse DNA was confirmed in separate experiments, and the stringent wash conditions under which hybridizations were performed effectively eliminated nonspecific interactions.

TABLE 1. Expression and copy number data for HS2γ lines A to C

Line (F_0)	No. of copies	Type of sample	No. of fetuses/ animals	(γ/α)/copy[a]	Ratio of γ-globin[b]
A (4896)	4	d10 YS and blood	6	6.4 ± 2.2	
		d12 liver	2	2.1 ± 0.28	
		Adult blood	6	0.08 ± 0.03	80
B (4901)	16	d10 YS and blood	6	10.9 ± 0.41	
		d14 liver	4	26.6 ± 3.2	
		Adult blood	3	3.5 ± 0.12	3.1
C (4890)	1	d9.5 YS and blood	6	15.9 ± 3.2	
		d14 liver	3	2.3 ± 2.7	
		Adult blood	5	1.4 ± 0.27	11

[a] Data are means and standard deviations derived from a data set that included multiple determinations by RNase protection on samples from each fetus or animal.

[b] Obtained by dividing the peak fetal (d12 to d15) levels by the adult expression levels.

RNA isolation and RNase protection assay. Total RNA was isolated from transgenic tissues by the methods of Karlinsey et al. (33) and Chomczynski and Sacchi (10). Human and murine globin RNAs were quantitated by RNase protection assay and resolved over 6% acrylamide gels. RNA probes were synthesized with either bacteriophage SP6 or T7 RNA polymerases. Probe pSP6Mζ, utilized to detect the mouse ζ transcript, has been described previously (1). pT7Mα, directed against murine α-globin mRNA, was derived from pSP6Mα through the replacement of the SP6 promoter with T7 promoters (1). Probe pT7β^{m} (16) was used to identify a 206-bp protected fragment within the second exon of the human β-globin gene. A 530-bp *Nco*I-*Pvu*II fragment (coordinates 39290 to 39820) encompassing the 5′ region of the $^{A}\gamma$ gene was cloned in pSP73 to generate the plasmid pT7$^{A}\gamma$(170), which was subsequently used to identify a 170-bp protected fragment originating from $^{A}\gamma$ exon 2. The RNA quantities used in protections were as follows: day 9 (d9) yolk sac (YS), 1,000 ng; d10 YS, 1,000 ng; d11 YS, 1,500 ng; d12 fetal blood, 80 ng; d12 fetal liver (f/l), 500 ng; d13 f/l, 500 ng; d14 f/l, 500 ng; d15 f/l, 200 ng; d16 f/l, 400 ng; adult (>3 weeks) blood, 50 ng. All RNase protection signals were quantitated on a PhosphorImager.

RESULTS

Analysis of γ-globin gene expression in HS2γ and HS3γ mice. We established three lines with correctly integrated HS2γ transgenes containing 4, 16, and 1 copies (lines A, B, C, respectively [Table 1]) and four lines containing 3, 10, 35, and 6 copies (lines D, E, F, and G, respectively [Table 2]) of correctly integrated HS3γ transgenes. Studies of γ gene expression during development were performed. Pregnant females were sacrificed at intervals corresponding to embryonic (up to d11), fetal (d12 to d16), and adult (>3 weeks) developmental stages. RNA was isolated from the YS, f/l, or blood of individual animals; RNAse protection was performed on each sample as described above; and the resulting signals were quantitated on a PhosporImager (Fig. 2) (Tables 1 and 2). To minimize experimental error, samples from individual animals were quantitated independently. In addition, samples from the

TABLE 2. Expression and copy number data for HS3γ lines D to G

Line (F_0)	No. of copies	Type of sample	No. of fetuses/ animals	γ/α copy[a]	Ratio of γ-globin[b]
D (9773)	3	d11 blood and YS	6	8.4 ± 2.0	
		d15 liver	3	13.3 ± 0.99	
		Adult blood	6	4.7 ± 0.55	2.8
E (4341)	10	d10 blood and YS	6	24.2 ± 4.4	
		d16 liver	4	18.4 ± 3.7	
		Adult blood	4	0.89 ± 0.17	27.2
F (7089)	35	d10 blood and YS	6	4.3 ± 0.82	
		d15 liver	2	3.4 ± 0.46	
		Adult blood	3	2.9 ± 0.61	1.5
G (9776)	6	d9 YS	2	18.1 ± 4.7	
		d13 liver	3	20.2 ± 2.3	
		Adult blood	2	18.6 ± 0.97	1.1

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

same animal were subjected to multiple determinations by RNase protection to ensure the accuracy of results. In total, human γ-globin expression and murine α- and ζ-globin expression levels in YS, f/l, and adult blood samples from 88 different F_2 progeny carrying HS2γ or HS3γ transgenes were quantitated.

The developmental patterns of γ-globin expression in three lines of HS2γ and four lines of HS3γ transgenic mice are shown in Fig. 3. All seven HSγ lines displayed moderate to high levels of γ-globin expression per transgene copy in the embryo. γ gene expression in primitive erythroid cells ranged from 6.4% ± 2.2% to 15.9% ± 3.2% (mean, 11.1% ± 4.8%) of mouse α- plus ζ-globin expression per copy in HS2γ lines A to C and from 4.3% ± 0.8% to 24.2% ± 4.4% (mean, 13.8% ± 9.1%) of mouse α- plus ζ-globin expression per copy in lines D to G carrying the HS3γ transgene (Table 1) (Fig. 3). γ-Globin expression in HS2γ lines A and C was downregulated in the fetal stage to 2.1% ± 0.3% (line A) and 2.3% ± 2.7% (line C) of mouse α- plus ζ-globin expression, reflecting seven- and threefold reductions, respectively. Expression in these lines

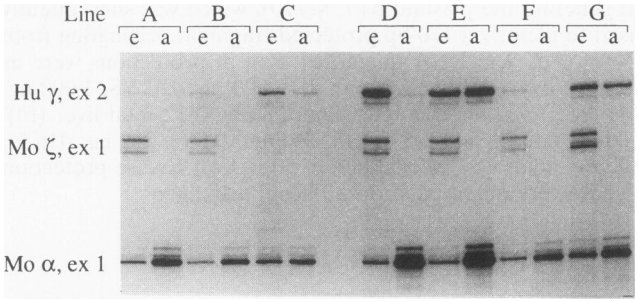

FIG. 2. mRNA analysis of F_2 progeny of transgenic mice carrying an HS2γ or HS3γ transgene. Expression of human γ-globin and murine α- and ζ-globin was analyzed by RNase protection. Protected fragments and sizes are as follows: human ^{A}γ-globin (Hu γ), 170 bp; mouse ζ-globin (Mo ζ), 151 bp; mouse α-globin (Mo α), 128 bp. (For the RNA quantities used in protection assays, see Materials and Methods; copy numbers for transgenic lines A to G are indicated in Table 1.) Data represent a single determination of an individual embryonic YS (e) or adult blood sample (a) from each line. At each developmental time point, multiple individual animals were analyzed in this manner; moreover, at least two independent determinations were performed on samples from each animal. In total, human γ-like globin expression and murine α-like globin expression were quantitated by PhosphorImager analysis of RNase protections on YS, f/l, and adult blood samples from 88 different F_2 progeny carrying HS2γ or HS3γ transgenes. ex, exon.

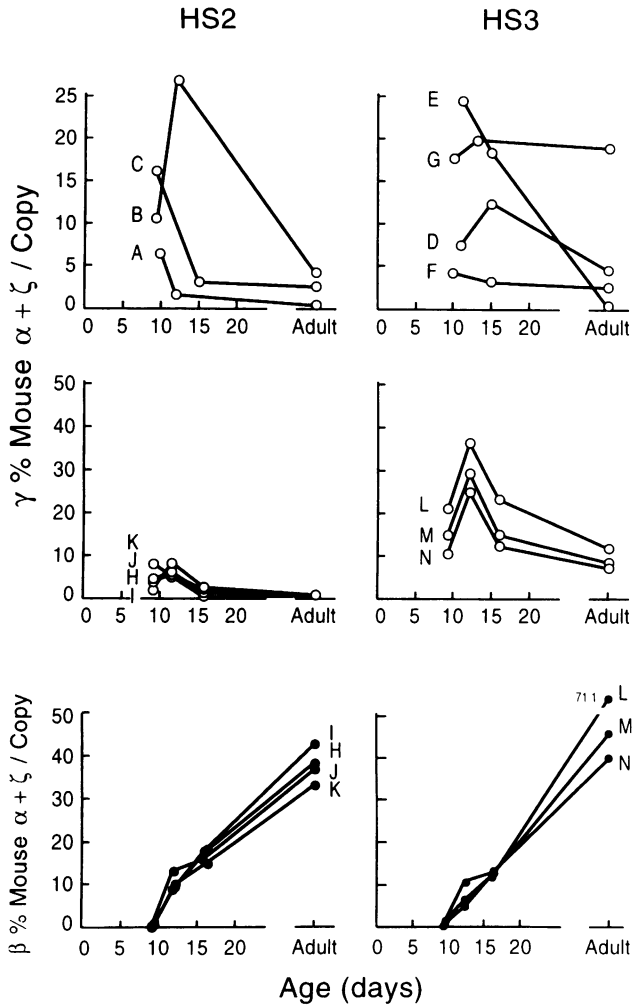

FIG. 3. Developmental patterns of human γ- and β-globin expression in F_2 progeny from HS2γ, HS3γ, HS2γβ, and HS3γβ transgenic mice. Human γ-globin expression per copy (open circles) and β-globin expression per copy (solid circles) are plotted as a percentage of mouse α-locus output (*y* axis). Gestational age is indicated on the *x* axis. The top pair of graphs shows the developmental pattern of γ-globin expression in HS2γ lines A to C and HS3γ lines D to G. Significant CVs at all developmental stages underlie the markedly different developmental patterns shown in these figures, indicating that γ-globin transgenes are highly sensitive to site-of-integration effects throughout development in HS2γ and HS3γ mice. The middle pair of graphs shows γ-globin expression in HS2γβ lines H to K and HS3γβ lines L to N. The bottom pair of graphs shows β-globin expression in HS2γβ lines H to K and HS3γβ lines L to N. The developmental patterns of both γ- and β-globin expression in all four HS2γβ lines and in all three HS3γβ lines are nearly identical. Small CVs at d9, d12, d16, and adult time points indicate a lack of position effects on the expression of either human globin gene throughout development. The variable patterns of γ-globin expression evident in the HSγ lines compared with the consistency characteristic of the HSγβ lines suggests important differences between the two types of constructs with respect to site-of-integration effects on γ-globin expression.

TABLE 3. Expression and copy number data for HS2γβ lines H to K

Line (F_0)	No. of copies	Type of sample	No. of fetuses/animals	(γ/α)/copy[a]	(β/α)/copy[a]	γ/(γ + β) (%)
H (3136)	17	d9 YS	3	4.0 ± 0.17	0.04 ± 0.01	99.0
		d12 blood	3	7.6 ± 0.17	2.0 ± 0.04	79.2
		d12 liver	3	5.3 ± 0.39	9.9 ± 0.56	34.9
		d16 liver	2	2.1 ± 0.05	18.0 ± 0.87	10.3
		Adult blood	3	0.48 ± 0.09	38.4 ± 3.8	1.2
I (3116)	19	d9.5 YS	3	2.6 ± 0.04	0.04 ± 0.01	98.3
		d12 blood	3	7.8 ± 0.41	2.0 ± 0.13	79.6
		d12 liver	3	7.7 ± 1.1	9.1 ± 0.95	45.9
		d16 liver	4	2.3 ± 0.17	18.3 ± 1.6	11.1
		Adult blood	3	0.93 ± 0.37	42.9 ± 4.3	2.1
J (3122)	7	d9 YS	3	5.5 ± 0.14	0.17 ± 0.03	97.0
		d12 blood	1	10.8 ± 0.13	4.4 ± 0.53	71.1
		d12 liver	1	3.9 ± 0.70	13.7 ± 2.6	22.1
		d16 liver	2	1.6 ± 0.09	16.1 ± 1.3	9.1
		Adult blood	4	0.73 ± 0.11	36.5 ± 2.6	2.0
K (3155)	2	d9 YS	3	7.9 ± 0.65	0.08 ± 0.01	99.0
		d12 blood	4	12.2 ± 2.0	2.9 ± 0.55	80.8
		d12 liver	4	3.5 ± 0.40	10.4 ± 2.9	25.0
		d16 liver	3	0.9 ± 0.03	15.2 ± 0.6	5.6
		Adult blood	2	0.6 ± 0.05	33.5 ± 3.2	1.8

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

declined further in the adult stage to 1.4% ± 0.3% (line A) and 0.08% ± 0.03% (line C) of mouse α-globin. By contrast, γ-globin expression in line B increased substantially in the fetal stage to 26.6% ± 3.2% of mouse α- plus ζ-globin expression per copy before declining sharply to a low level (3.5% ± 0.1% of mouse α-globin) in the adult.

γ-Globin expression in HS3γ line D was upregulated 1.5-fold in the fetal stage (to 13.3% ± 1.0% of mouse α- plus ζ-globin expression) and declined to 4.7% ± 0.55% of mouse α-globin in the adult. In HS3γ lines F and G, adult-stage γ-globin expression (2.9% ± 0.6% and 18.6% ± 0.97% of mouse α-globin, respectively) did not differ significantly from corresponding embryonic- (4.3% ± 0.8% [line F] and 18.1% ± 4.7% [line G] of mouse α- plus ζ-globin expression) and fetal-stage values (3.4% ± 0.5% [line F] and 20.2% ± 2.3% [line G] of mouse α- plus ζ-globin expression). The developmental profile of γ gene activity in line E diverged markedly from those of the other HS3γ lines. γ-Globin expression in line E declined from a peak of 24.2% ± 4.4% of mouse α- plus ζ-globin expression in the YS to 18.4% ± 3.7% in the f/l before dropping precipitously to a nearly undetectable value (0.89% ± 0.17% of mouse α-globin) in the adult stage. As such, the developmental patterns of γ-globin expression differed markedly among transgenic lines carrying the same HS2γ or HS3γ construct.

γ-Globin expression in HS2γ and HS3γ mice is sensitive to position effects. Evaluation of position effects on transgene expression involves comparisons in expression per copy between transgenic lines carrying the same construct and therefore requires accurate determination of the number of integrated transgene copies. To calculate transgene copy numbers, we employed a multiply redundant protocol that minimized several potential sources of experimental error (see Materials and Methods). The data in Tables 1 and 2 evince pronounced variability in per-copy, γ-globin expression in HS2γ and HS3γ mice throughout ontogeny. For example, fetal-stage HS2γ mice show 12-fold variation in per-copy, γ-globin expression (2.1% ± 0.28% to 26.6% ± 3.2% of mouse α- plus ζ-globin expression). HS3γ mice exhibit 6-fold variation in per-copy expression during the fetal stage and >30-fold variability in the adult stage. Such variability in copy-corrected transgene expression is suggestive of pronounced site-of-integration effects on expression.

A more rigorous statistical measure of variability employed in the analysis of position effects (41, 53) is the CV between lines in per-copy transgene expression. This statistic expresses the standard deviation in per-copy expression as a percentage of the mean (σ/μ). To assess the impact of the site of transgene integration on γ-globin expression in each construct, we computed the CV in per-copy, γ-globin expression at different developmental stages. The CV is significant (>0.5 or 1 standard deviation of >50% of mean per-copy expression) for fetal- and adult-stage HS2γ mice and for HS3γ mice at embryonic, fetal, and adult stages of development. A significant CV indicates that γ-globin expression in mice transgenic for these constructs is highly sensitive to position effects (13, 41, 53). Consistent with these observations were the markedly varied developmental patterns of γ-globin expression seen in HS2γ and HS3γ mice (Fig. 3).

Analysis of γ-globin expression in HS2γβ and HS3γβ mice. Four lines carrying correctly integrated HS2γβ constructs (lines H to K; Table 3) and three lines carrying correctly integrated copies of the HS3γβ construct (lines L to N; Table 4) were obtained for developmental studies. Developmental studies were performed on F_2 progeny from these lines. RNA was isolated from the YSs, livers, and blood of individual fetuses and from the blood of adult mice. RNase protection assays were performed on these samples, and the levels of human γ- and β-globin mRNA were quantitated on a PhosphorImager (Fig. 4). By following the protocol used with the HS2γ and HS3γ mice, samples from individual fetuses were quantitated independently to reduce experimental error, and multiple determinations by RNase protection were performed on samples from the same fetus to ensure the reliability of

TABLE 4. Expression and copy number data for HS3γβ lines L to N

Line (F_0)	No. of copies	Type of sample	No. of fetuses/animals	(γ/α)/copy[a]	(β/α)/copy[a]	γ/(γ + β) (%)
L (3044)	4	d9 YS	3	21.3 ± 1.6	0.07 ± 0.03	99.6
		d12 blood	3	24.6 ± 3.9	1.3 ± 0.38	95.1
		d12 liver	3	34.9 ± 5.0	5.4 ± 1.2	86.7
		d16 liver	3	23.6 ± 3.5	11.7 ± 2.3	66.8
		Adult blood	4	10.7 ± 1.5	71.1 ± 7.0	13.1
M (3307)	5	d9 YS	4	15.5 ± 1.1	0.07 ± 0.01	99.5
		d12 blood	2	18.6 ± 0.4	0.72 ± 0.10	96.3
		d12 liver	2	27.9 ± 0.22	4.5 ± 0.24	86.0
		d16 liver	3	14.5 ± 0.52	10.9 ± 0.72	57.2
		Adult blood	3	8.0 ± 0.96	45.9 ± 3.4	14.9
N (3100)	6	d9.5 YS	3	10.0 ± 0.41	0.11 ± 0.01	99.0
		d12 blood	3	17.1 ± 1.5	3.2 ± 0.83	84.2
		d12 liver	3	22.6 ± 0.35	10.1 ± 0.72	69.0
		d16 liver	3	11.3 ± 0.73	12.3 ± 1.6	47.8
		Adult blood	3	6.6 ± 0.11	39.4 ± 1.9	14.4

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

measurements. In total, d9 YSs, d12 fetal livers and blood, d16 fetal livers, and adult blood samples from 102 individual animals from the HS2γβ and the HS3γβ lines (Fig. 4) were analyzed in this manner. From these data, the means and standard deviations of γ- and β-globin expression levels in each line were computed at different developmental stages (Tables 3 and 4).

γ-Globin expression at all stages of development was highly consistent in all lines carrying an HS2γβ transgene. γ-Globin was expressed in lines H to K at a moderate level in the embryo (mean, 5.0% ± 2.3% of mouse α- plus ζ-globin expression at d9), remained roughly constant in the fetal stage (at d12, 5.1% ± 1.9% of mouse α- plus ζ-globin expression), and declined during the progression to the adult stage to a low level (1.73% ± 0.62% of mouse α- plus ζ-globin expression [d16] and 0.69% ± 0.19% of mouse α-globin [adult blood]). The expression of β-globin in HS2γβ mice also exhibited high degree of consistency between lines throughout ontogeny. β-Globin expression in lines H to K was nearly undetectable in the embryo and rose steadily with the progression of development, reaching a mean peak of 37.8% ± 3.9% of mouse α-globin in the adult. Qualitatively correct developmental regulation of both γ- and β-globin expression levels was therefore observed. Consistency in the patterns of globin gene regulation among the HS2γβ lines is evident when the data from Table 3 are represented graphically (Fig. 3).

In all lines containing an HS3γβ transgene, higher levels of γ-globin expression per copy relative to murine α-like genes were observed in the embryo (d9 mean, 15.6% ± 5.7% of mouse α- plus ζ-globin expression). Mean γ-globin expression rose to a mean peak of 28.5% ± 6.2% of mouse α- plus ζ-globin expression at d12. By d16, γ-globin mRNA had declined to a level approximately equal to that found in the embryo (16.5% ± 6.4% of mouse α- plus ζ-globin expression) and fell further to a mean of 8.4% ± 2.1% of mouse α-globin in the adult. β-Globin expression in the HS3γβ lines rose throughout development, from barely detectable levels in the embryo (0.08% ± 0.02% of mouse α- plus ζ-globin expression) to a high level in the adult (mean, 52.1% ± 16.7% of mouse α-globin).

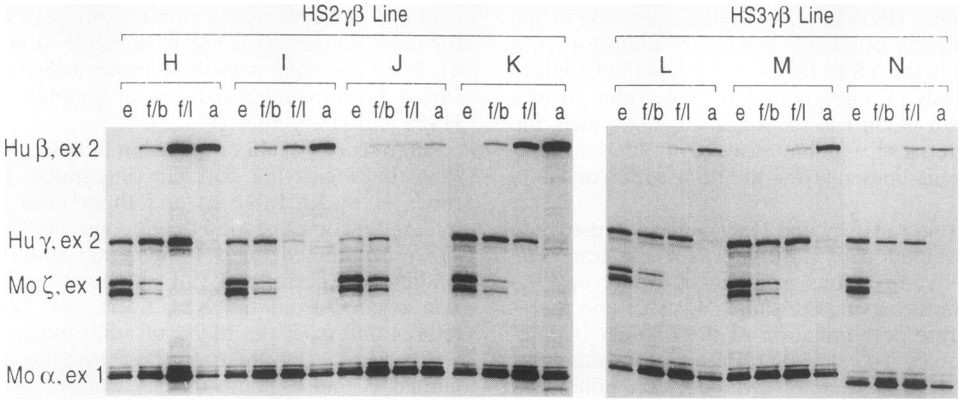

FIG. 4. mRNA analysis of F_2 progeny of transgenic mice carrying an HS2γβ or HS3γβ transgene. Data represent a single determination on an individual embryonic d9 YS (e), d12 fetal blood (f/b), and f/l or adult blood (a) sample from each line. Human γ- and β-globin expression and murine α- and ζ-globin expression levels were analyzed by RNase protection with PhosphorImager quantitation of the resulting signals. Protected fragments and sizes are as follows: human β-globin (Hu β), 205 bp; human ^{A}γ-globin (Hu γ), 170 bp; mouse ζ-globin (Mo ζ), 151 bp; mouse α-globin (Mo α), 128 bp. ex, exon.

Both quantitative and qualitative differences in the pattern of γ-globin expression were apparent with respect to the linked regulatory element. The magnitude of quantitative changes in γ-globin expression during development (i.e., greater range between the peak and minimum developmental values recorded) was significantly higher in the presence of HS3 than of HS2 (~20% versus ~5% of mouse α- plus ζ-globin expression, respectively). In addition, γ-globin expression under the control of HS3 exhibited the rising-and-falling pattern of developmental expression expected from a fetal gene, in spite of the fact that the endogenous murine erythropoiesis appears to lack a definitive fetal stage. β-Globin genes both in HS2γβ mice and in HS3γβ mice were correctly regulated. However, while *cis*-linked γ-globin genes in HS2γβ mice were silenced in the adult stage, γ-globin transgenes in HS3γβ mice underwent downregulation after d12 but failed to exhibit appropriate developmental silencing (Fig. 3).

γ-Globin expression in HS2γβ and HS3γβ mice is insulated from position effects. To study position effects on γ-globin expression in lines transgenic for an HS2γβ or HS3γβ construct, the CV in per-copy expression between lines (see above) was computed at all developmental time points. Small CVs (<0.5) in γ-globin expression between lines carrying the same transgene are present throughout development. Small CVs in β-globin expression are likewise present. In contrast to that in HS2γ and HS3γ mice, γ-globin expression in HS2γβ and HS3γβ lines is sheltered from position effects at all stages of development.

DISCUSSION

In the presence of an LCR, *cis* sequences 3′ to the ^{A}γ gene may provide sheltering from position effects. We employed two types of constructs to examine how two LCR elements (HS2 and HS3) that have demonstrated significant activity with the adult β gene interact with a human fetal γ-globin gene during development in transgenic mice. The first set of constructs contained HS2 or HS3 linked independently to a lone ^{A}γ-globin gene while the second set of constructs contained the same regulatory elements linked independently to tandem γ- and β-globin genes. Figure 3 contains a comparison of γ-globin expression in seven lines carrying HS2γ or HS3γ transgenes with expression in seven lines carrying HS2γβ or HS3γβ transgenes. This comparison highlights the significant differences between the two types of constructs with respect to site-of-integration effects on γ-globin expression and their impact on its developmental profile (Fig. 3). In HS2γ and HS3γ constructs (Fig. 3), γ-globin expression is sensitive to position effects throughout development, which results in different developmental patterns in different lines carrying the same transgene. In HS2γβ and HS3γβ constructs, γ-globin expression is sheltered from position effects and all lines carrying the same transgene display the same pattern of developmental control.

During the genesis of transgenic mice, DNA carrying the transgene integrates into the mouse genome at one or more apparently random locations, with each integration site containing single or multiple transgene copies, usually organized in a tandem head-to-tail array. By breeding transgenic founder animals with nontransgenic mice, lines that harbor a single locus of integration can be established. Integration position effects represent the impact of heterologous chromatin environments on transgene expression. Such effects can be studied by computing expression per transgene copy in a given line at a particular developmental time point and comparing this value with per-copy expression at an analogous time point in other lines carrying identical transgenes. The presence of significant variability in per-copy expression between lines carrying the same transgene indicates that expression is highly influenced by the site of genomic integration. A statistical measure of variability that has been applied to the study of position effects (41, 53) is the CV. This statistic expresses the standard deviation in per-copy expression between lines as a percentage of the mean (σ/μ). A significant CV, namely, when the standard deviation exceeds 50% of the mean (CV, >0.5), is indicative of pronounced position effects on transgene expression. By contrast, a small CV, reflecting a lack of significant variability in per-copy expression between lines transgenic for the same construct, is observed when transgene expression is independent of integration position. When several transgenic lines carrying a wide range of different copy numbers are analyzed, small CVs in per-copy expression translate into transgene expression that is positively correlated with the number of integrated copies (i.e., it is copy number dependent).

Our results show that γ-globin expression in mice carrying HSγβ constructs is sheltered from position effects throughout development. As such, γ-globin expression is position independent, irrespective of the absolute level or the relative level of γ- or β-globin gene activity at a particular developmental stage. This observation also holds for β-globin expression, indicating that the mechanism underlying insulation from position effects does not depend on transgene expression. Differences between the HSγ and HSγβ constructs may, therefore, be ascribed to a transcription-independent effect of *cis* sequences contained in HSγβ constructs but missing from HSγ constructs. Since identical 4.2-kb (HS2) and 4.4-kb (HS3) LCR fragments were used in the genesis of both the HSγ and HSγβ constructs, the difference between the two construct types with respect to the sheltering of γ-globin expression from position effects should be due to the presence of a *cis* element in the additional 3′ sequences (including the β-globin gene) contained in HSγβ constructs but absent from HSγ constructs. Taken together, the data suggest that sequences external to the promoter regions of the fetal globin genes are required for appropriate interaction with elements in the LCR. One possibility is that a *cis* element within or flanking the β-globin gene interacts with the linked HS element to form a stable regulatory domain in which the normally position-sensitive γ gene is sequestered. Previous studies have demonstrated that a 4.6-kb *Bgl*II fragment containing the β-globin gene supplies sufficient sequence information for appropriate interaction with HS2 or HS3 that results in expression insulated from position effects. An alternative hypothesis is that matrix/scaffold attachment regions such as that found in the β-globin large intron (32) provide the basis for positionally stable expression of both γ- and β-globin genes in the presence of LCR sequences. The γ-globin fragment contained in the HS2γ and HS3γ constructs was the standard well-studied 3.3-kb *Hin*dIII fragment (3, 4, 17, 18, 36, 48). In HSγβ constructs, a slightly larger γ-globin fragment that contained a putative 3′ regulatory element originally identified in transient-transfection assays (4, 43) and recently reported to contain a matrix/scaffold attachment region (11a) was used.

The fetal pattern of γ gene expression may be determined by HS3. Direct comparisons between the patterns of human transgene expression in different transgenic lines in this study were made possible by the standardization of human γ- and β-globin expression to output from the murine α-globin locus. Murine α-locus output remains stable on a per-copy basis throughout development (60), and the calculation of γ- and β-globin expression levels as a percentage of mouse α-like

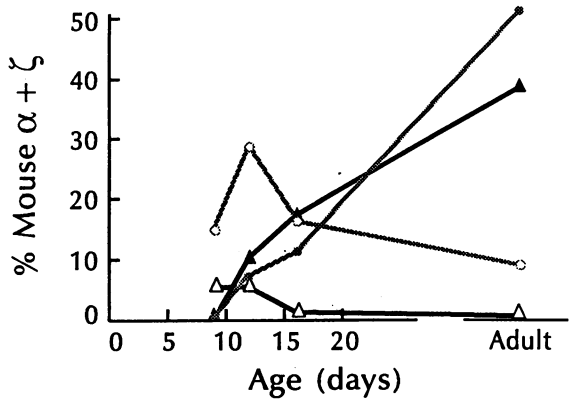

FIG. 5. Mean γ- and β-globin expression levels in HS2γβ and HS3γβ lines. Mean d9, d12, d16, and adult blood γ- (circles) and β-globin (triangles) expression levels from HS2γβ (black lines) lines H to K and HS3γβ (gray lines) lines L to N is plotted as a percentage of murine α-locus output. Data show significant differences between HS2 and HS3 constructs with respect to the patterns of γ-globin expression. By contrast, similar patterns of β-globin expression are seen with both constructs. Note that γ-globin expression rises and falls with a peak in the d12 liver and thus exhibits the expression profile expected of a fetal gene. In contrast, with HS2γβ mice, downregulation of γ-globin expression after d12 does not culminate in adult-stage silencing.

globin has the advantage of showing the absolute expression profile of each transgene during development (Fig. 3 and 5). Mean γ- and β-globin expression levels at different developmental stages for HS2γβ and HS3γβ transgenics are plotted on the same axes in Fig. 5. The patterns of β-globin expression observed with the two HSγβ constructs are quite similar. Differences between the HS2 and HS3 constructs with respect to the pattern of γ-globin regulation during development, as well as the magnitude of expression at different developmental stages, are readily apparent. The results presented herein confirm those reported previously by Fraser et al., who observed qualitative differences between HS2 and HS3 with respect to the patterns of γ-globin expression they engender when they are linked to constructs containing juxtaposed γ- and β-globin genes (27). The wider range of developmental time points sampled in this study exposes two significant features of γ-globin expression under the control of these LCR HS elements that were not evident previously. Data from the HSγ and HSγβ constructs show that both HS2 and HS3 are capable of driving moderate to high levels of γ-globin expression in the f/l (Tables 3 and 4) (Fig. 2 and 4) and thus that differences in the activity of these two regulatory elements during development cannot be accounted for satisfactorily by positing the existence of f/l-specific *trans*-acting factors. The developmental profile of γ-globin expression under the control of HS3 exhibits the rising, peaking (at d12), and falling pattern of expression expected of a genuine fetal-stage-specific gene (Fig. 5). Compared with the embryonic and fetal patterns seen in HS2γβ mice, the fetal expression profile of γ-globin in HS3γβ mice suggests that the developmental pattern of fetal globin expression in humans may be due primarily to the influence of HS3.

The lack of a discrete fetal stage of erythropoiesis in the mouse that is defined by the expression of a specific fetal murine β-like globin gene has been cited in support of the hypothesis that the fetal-stage predominance of γ-globin expression in humans is the result of fetal recruitment of an embryonic gene via evolutionary changes in the complement of *trans*-acting factors (50). An alternative hypothesis is that stage-predominant expression of a given β-like globin gene is due primarily to the activity of different *cis* regulatory elements in the LCR during discrete stages of development (16). The latter hypothesis is compatible with the observation that γ-globin transgenes lacking linked LCR sequences are expressed (sometimes at high levels) only in YS cells of the embryonic erythropoiesis (7), whereas linkage to an LCR element expands the developmental spectrum to include expression in fetal and adult tissues (17, 48).

Position effects can influence the pattern of developmental expression. The extension of the analysis of site-of-integration effects on transgene expression to include a range of developmental time points permits an evaluation of position effects present during a particular stage of development as well as an evaluation of the impact of such effects on the overall pattern of transgene expression during ontogeny. A priori, several possible outcomes of the effects of different chromosomal environments on the developmental expression profile of a position-sensitive transgene can be postulated. One possibility is that the position effect present in each transgenic line establishes a baseline upon which developmentally controlled increases or decreases in transgene expression are superimposed. In this scenario, large CVs between lines would be manifest at all developmental time points because of the different expression baselines for each line. However, the developmental patterns of transgene expression, i.e., the absolute changes in expression over successive developmental intervals that dictate the morphology of the transgene's developmental expression curve, would be qualitatively similar among all lines.

An alternative possibility for the impact of the locus of integration on the developmental expression pattern of a susceptible transgene is that the integration position effect gives rise to large CVs at all developmental time points, but the nature of the position effect changes during development (presumably because of developmentally coordinated alterations in the character of chromatin surrounding the integration site). Yet another possibility is that the response of the gene to a constant position effect changes in a developmentally coordinated fashion (presumably mediated by changes in the *trans* factors acting at the level of the transgene). These two alternatives are not mutually exclusive and would be expected to manifest different developmental patterns of transgene expression in lines carrying the same transgene. The markedly varied developmental profiles of γ-globin expression observed in HS2γ and HS3γ mice (Fig. 3) are compatible with these last alternatives.

By contrast, transgenes that are sheltered from site-of-integration effects throughout ontogeny exhibit small CVs in per-copy expression at all developmental time points. Since variability is minimized, the developmental expression patterns of such transgenes are of necessity highly consistent between lines. Such consistency characterizes the profiles of γ- and β-globin expression levels in HS2γβ and HS3γβ mice (Fig. 3). The uniformity of developmental patterns among HSγβ mice contrasts sharply with the variable patterns seen in HSγ mice (Fig. 3).

Irrespective of the underlying mechanism, our results demonstrate that different chromosomal environments can exercise a dominant influence over the pattern of transgene expression during ontogeny. They suggest further that 3′ *cis* sequences as well as those in the promoter region are essential for mediating the LCR element and γ-globin interactions that determine correct and consistent patterns of fetal globin expression during development. Further analyses of the contribution of

intragenic and 3′ flanking sequence elements to LCR-facilitated globin gene expression are clearly required for understanding the mechanism of LCR function, and such analyses should provide important insights into the control of globin gene regulation.

ACKNOWLEDGMENTS

We thank Betty Josephson for expert technical assistance, Mary Eng for the production of transgenic mouse lines, and Margo Gibson for performing PhosphorImager analysis. We are grateful to George Stamatoyannopoulos for extensive discussions and a critical reading of the manuscript.

This work was supported by grants HL 20899 and DK 45365 from the National Institutes of Health.

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