Inactivation of the human β -globin gene by targeted insertion into the β -globin locus control region

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The human β -globin locus control region (LCR) is a complex regulatory element that controls the erythroid-specific expression of all *cis*-linked globin genes. The LCR is composed of five DNase I hypersensitive sites (HS) spanning 16 kb and located >50 kb upstream of the β -globin gene on chromosome 11. Constructs containing all or some of these HS have been shown to produce high-level erythroid-specific expression of linked genes in transgenic mice and transfected cells. In all transgenic and transfection experiments reported to date, however, the spatial relationships between the LCR and globin genes have been disrupted. We have used homologous recombination (HR) as an approach to gain insights into the potential interactions between the LCR and globin genes in their native locations. A hygromycin B resistance (*hygro*^R) gene was inserted into the human β -globin LCR on chromosome 11 in a mouse/human hybrid erythroid cell line that expresses the human β -globin gene after the induction of differentiation. As a consequence of this targeted insertion, the β -globin gene is transcriptionally inactive and not inducible. In contrast, the *hygro*^R gene within the LCR is inducible, whereas randomly integrated *hygro*^R genes are not inducible in these cells. The chromatin structure of the targeted locus is also altered. A new DNase I HS is present in the enhancer/promoter of the *hygro*^R gene inserted into the LCR, whereas a HS normally present in the LCR 3' to the insertion is lost and the β -globin gene promoter HS is not detectable. These results are consistent with the promoter/enhancer competition model for LCR function and globin gene switching.

[Key Words: Homologous recombination; LCR; promoter/enhancer competition; human β -globin gene; human/MEL hybrid]

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The human β -globin locus is composed of five linked functional β -like globin genes that span ~50 kb on chromosome 11. These genes are arranged in the same transcriptional orientation and in the order in which they are expressed developmentally. The human β -globin locus control region (LCR), located 6–22 kb upstream of the ϵ -globin gene on chromosome 11, is a recently recognized regulatory unit that controls the replication timing, chromatin structure, and transcription of the β -globin domain (for review, see Epner et al. 1991). The LCR has been operationally defined by five DNase I hypersensitive sites (5'HS1–5'HS5), four of which are erythroid specific, developmentally stable (Tuan et al. 1985; Forrester et al. 1986), and present prior to transcriptional activation of the globin genes (Forrester et al. 1987). The contribution of 5' ϵ elements to expression of all *cis*-linked globin genes was surmised originally from the phenotype of naturally occurring deletions of this region, which result in the failure to activate the linked globin

genes (Curtin et al. 1985; Van der Ploeg et al. 1986; Driscoll et al. 1989). Detailed analysis of the Hispanic ($\gamma\delta\beta$)⁰ thalassemia, in which a 35-kb region 5' of 5'HS1 of the LCR (located ~50 kb upstream of the β -globin gene) is deleted, has not only implicated the LCR with transcriptional regulation but also with the establishment of the erythroid-specific DNase I-sensitive chromatin domain and timing of DNA replication of the locus. In this disorder, no transcription of the β -globin gene on the deletion chromosome is observed, and the locus is DNase I resistant and replicated late in S phase (Driscoll et al. 1989; Forrester et al. 1990). This is in contrast to the normal allele in which the β -globin gene is expressed and the locus is DNase I sensitive and replicated early in S phase (Epner et al. 1988; Forrester 1990). Thus, in addition to the classical enhancer effects revealed in transfection and transgenic assays (see below), the LCR also influences the chromatin structure and replication timing of the locus.

Functional assays have shown that various LCR elements can overcome the position effects exhibited by constructs integrated randomly into chromosomal loca-

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tions. Initially, a 38-kb minilocus containing the LCR was shown to alleviate position effects in transgenic mice: A construct containing the LCR linked to the β -globin gene demonstrated copy number-dependent expression and expression levels equivalent to the endogenous mouse β -globin genes (Grosveld et al. 1987). Similar results were also observed in subsequent transgenic studies, in which subsets of the LCR were linked to the β -globin gene (Curtin et al. 1989; Ryan et al. 1989; Talbot et al. 1989; Enver et al. 1990). In combination with β -globin promoter sequences, the LCR also confers high-level, erythroid-specific expression in transfected cell lines (Blom van Assendelft et al. 1989; Forrester et al. 1989). Although, as in transgenic mice, the strongest effects are seen when all of the HS are linked together, some individual sites can also augment transcription (Forrester et al. 1989; Tuan et al. 1989; Collis et al. 1990). These effects vary, depending on whether transient or stable assays are employed. Transfection studies performed in MEL cells showed that 5'HS2, 5'HS3, and 5'HS4 all elevate expression of linked β -globin genes (Forrester et al. 1989; Collis et al. 1990) when stably integrated into the chromosome. However, only 5'HS2 shows enhancer activity in transient assays in murine erythroid leukemia (MEL) and K562 cells (Tuan et al. 1989; Moon and Ley 1991). Elements analogous to the human β -globin LCR have also been identified in the chicken (Reitman et al. 1990), goat (Li et al. 1990), and mouse (Moon and Ley 1990) β -globin and human α -globin loci (Higgs et al. 1990).

Recent transgenic studies also suggest that the human LCR may be involved in stage-specific globin gene switching (Behringer et al. 1990; Enver et al. 1990; Hanscombe et al. 1991) in a manner analogous to the enhancer competition model described for chicken β -like globin gene switching (Choi and Engel 1988). The essence of this and derivative models (Evans et al. 1990; Orkin 1990; Townes and Behringer 1990; Stamatoyannopoulos 1991) is that the stage-specific expression of any globin gene is dependent on the presence of positive factors that allow a high-affinity interaction between that gene and the enhancer within the LCR, and/or the presence of stage-specific autonomous silencing of the other globin genes.

An important limitation in interpreting the analyses of LCR function in transgenic and transfection assays is that the spatial relationships between the LCR and *cis*-linked globin genes have been disrupted in all such experiments. Thus, as an approach to gain further insights into the potential interactions among the LCR and globin genes in their native locations, we have performed homologous recombination (HR) into the human β -globin LCR. Here, we report that insertion of a hygromycin B-resistance (*hygro*^R) gene driven by the Friend viral long terminal repeat (LTR) enhancer/promoter between 5'HS1 and 5'HS2 in the β -globin LCR abolishes expression of the human β -globin gene located >50 kb downstream of the insertion site. The inserted *hygro*^R gene located within the LCR shows inducible expression, whereas randomly integrated *hygro*^R genes are not

inducible. Chromatin analyses reveal a new DNase I HS in the Friend virus promoter/enhancer, whereas 5'HS1 in the LCR region and the β -globin promoter HS are eliminated. These results present evidence that the LCR influences the inducible expression of the downstream β -globin gene from its natural position. We speculate that the inactivation of the β -globin gene may involve competition between the Friend viral LTR and the β -globin gene for elements of the LCR in a manner analogous to that proposed by Choi and Engel (1988).

Results

Isolation of homologous recombinants

The positive-negative selection strategy described by Capecchi and colleagues (Mansour et al. 1988; Capecchi 1989) was used to construct a replacement-type vector (Fig. 1) designed to insert a selectable marker gene into the human β -globin LCR by a double crossover event. This vector contains 1 kb of 5' homology and 8 kb of 3' homology, including 5'HS1 and part of the ϵ -globin gene. The positive-selection *hygro*^R marker gene is inserted at a unique *Hpa*I site ~7.5 kb upstream of the ϵ -globin gene, and the negative-selection marker gene, herpes simplex virus (HSV) thymidine kinase (*tk*), is placed outside the 3' homologous sequences (for details of construction, see Materials and methods). The Friend virus enhancer/promoter, which is active in many cell types and shows high-level expression in erythroid cells (Cowie and Myers 1988), was used to drive both selectable marker genes. Correctly targeted integration of this vector by HR will result only in the insertion of the *hygro*^R gene between 5'HS1 and 5'HS2, with no alterations in LCR sequences *per se*.

The linearized vector was electroporated into a human lymphoid/MEL hybrid cell line (N-MEL) containing a single normal human chromosome 11. As described previously (Forrester et al. 1987; 1990), these cells are adult-stage erythroid cells in which only the adult β -globin gene is transcribed and the entire human β -globin gene domain spanning >100 kb is DNase I sensitive and early replicating. After selection with hygromycin B and gancyclovir, pools of cells resistant to both drugs were screened by polymerase chain reaction (PCR) and Southern blot analysis (Kim and Smithies 1988). Two primers were used for PCR amplification: Primer 99 is located outside and ~100 bp upstream of the targeting vector, and primer H1 is derived from sequences in the *hygro*^R gene and is specific to the vector DNA (Fig. 1). If a targeting event juxtaposes these two primers, specific PCR amplification should result in a diagnostic 1.5-kb band that hybridizes to a 1.0-kb 5' ϵ *Eco*RI-*Hpa*I probe. Sib selections from the pool that demonstrated the strongest specific hybridization signal were performed serially, with ~100, ~10, and ~1 cell per pool in 24-, 48-, and 96-well plates, respectively. During the sib selection, the enriched pools were diluted into 96-well plates so that only 30–50% of the wells received a cell. After growth to adequate density, samples were removed and analyzed

Figure 1. Homologous recombination into the human β -globin LCR. (A) Schematic map of the human β -globin locus showing the positions of the HS and the arrangement of β -globin-coding genes. (B) Enlarged region of A to show the targeting locus. (C) The targeting construct aligned with a chromosomal copy of this region. The hygro^R gene is inserted into the unique *HpaI* site between 5'HS1 and 5'HS2, and the HSV tk gene is juxtaposed 3' of the ϵ -globin second intron region (see Materials and methods). (D) Homologous recombination between genomic DNA (B) and the targeting vector (C). In A, solid and open rectangles represent the functional and nonfunctional globin genes, respectively. In B, C, and D the solid and open boxes represent exons and introns in the ϵ -globin gene, respectively. Large arrows denote developmentally stable, erythroid-specific HS; small arrows represent HS within the promoters of the fetal and adult β -genes. The asterisks (*) above the arrows indicate the subsets of HS that are found in the parent MEL/human hybrid cells. PCR primers are indicated with identifying numbers. The probe for the PCR–Southern analysis is also indicated by the horizontal bar. Restriction sites are as follows: (B) *Bam*HI; (F) *Fsp*I; (H) *Hpa*I; (RI) *Eco*RI.

Figure 2 shows a Southern blot analysis of DNA from parental cells and the eight presumptive targeted clones cut with *Bgl*III and probed with junction and *hygro*^R probes. If a correct targeting event has occurred, both probes should detect a 4.4-kb band in the recombinants, whereas the junction probe, but not the *hygro*^R probe, should detect a 7.7-kb band in parental DNA. In seven of eight clones the predicted 4.4-kb band was detected by the 5' junction probe (probe 1, Fig. 2A). No band, however, was observed in clone 1, whereas two clones (2 and 6) had additional ~9-kb bands, suggesting that these clones are not pure or contain multiple rearrangements. In contrast, in three of five clones showing the single expected band hybridizing to the junction probe, the single predicted 4.4-kb band was detected by the *hygro*^R probe, whereas the other two clones showed additional bands (Fig. 2B). Thus, this analysis suggests that three targeted clones (HR9, HR20, and HR39) contain a single insertion at the desired location, whereas others contain additional nonhomologous insertions or are not homogeneous clones. The homogeneity, correct targeting, and integrity of the β -globin locus of clones HR9, HR20, and HR39 were confirmed by Southern analyses using several additional restriction enzymes and probes (data not shown).

Gene expression

Total cellular RNA was prepared from the three single-copy targeted clones before and after hexamethylene bisacetamide (HMBA)-induced differentiation, and globin RNA content was determined by an RNase protection assay. Surprisingly, human β -globin gene expression is not detectable in the three targeted clones, whereas mouse α - and β -globin (not shown) are expressed at high levels in both HMBA-induced parental and recombinant cells (Fig. 3A). In addition, the human γ -globin gene, which is not expressed in normal human/MEL hybrids, is not expressed in our targeted clones (data not shown). However, the *hygro*^R gene inserted into the LCR region by homologous recombination is expressed and induced to high levels during the *in vitro* differentiation of the homologous recombinant clones. The induction of *hygro*^R is observed independent of maintenance of the targeted clones in selective media. Although the *hygro*^R gene shows high-level HMBA-dependent inducible expression in the three homologous recombinant clones (Fig. 3A), the basal expression of this gene is not inducible during the *in vitro* differentiation of clones containing nonhomologous integrants (Fig. 3B). These results suggest that the inducible effect of the LCR remains functional in the targeted β -globin gene locus, but the inserted and LCR-proximal *hygro*^R gene may replace the downstream β -globin gene as the recipient of this effect (see Discussion).

It should be noted, however, that individual subclones of parental hybrid cells that do not contain the homologously inserted *hygro^R* gene display substantial variation in human β -globin gene expression, as assayed by RNase protection or by immunofluorescence with an allele-spe-

Figure 2. Southern blot hybridization of genomic DNA from putative homologous recombinants. After electroporation and positive-negative double selection, PCR positive cells were sib selected and genomic DNA from eight putative clones was cleaved with *Bgl*II (lanes 1–8). Control DNA from untransfected parent cells (lane P) was also included in Southern blot hybridization. Southern blots were hybridized with a 0.3-kb 5' *Eco*RI–*Eco*RV junction probe (probe 1 in C) (A) and a 1.0-kb *Nco*I–*Bgl*II fragment from the *hygro*^R gene (probe 2 in C) (B). (C) Relevant restriction maps of parent (N-MEL) and homologous recombinant (HR-MEL). The HR-MEL restriction map shows the insertion of the Friend virus enhancer/promoter (Fep), *hygro*^R gene, and SV40 poly(A) addition and termination sites [poly(A)] between 5'HS1 and 5'HS2. The junction probe detects a 7.7-kb band in control N-MEL DNA and a 4.4-kb band in HR-MEL DNA. The *hygro*^R probe does not hybridize to control DNA and detects the same 4.4-kb band in HR-MEL DNA. Restriction enzyme sites are as follows: (Bg) *Bgl*II; (H) *Hpa*I; (X) *Xba*I.

cific human β -globin antibody (Shesely et al. 1991 and data not shown). Thus, it is possible that the absence of human β -globin expression in the targeted clones originated in a variant cell within the original parent cell clonal population. This type of variability in expression, known as extinction, has been reported to be attributable to random de novo methylation of genomic DNA, which occurs continually during cell culture (e.g., Antequera et al. 1990), and is reversible by 5-azacytidine (Comper and Palmiter 1981; Groudine et al. 1981; Jones et al. 1982; Ley et al. 1982). As shown in Figure 3C, 5-azacytidine treatment of uninduced parent hybrid cells results in increased levels of human β -globin expression both before and after HMBA-induced differentiation (Fig. 3C). In addition, we have observed significant increases in human β -globin gene expression in all 5-azacytidine treated subclones of the parental cells (data not shown). In contrast, treatment of the targeted clones with 5-azacytidine has no effect on human β -globin gene expres-

sion before or after HMBA-induced differentiation. Therefore, it seems likely that the lack of human β -globin gene expression in targeted clones is not the result of a methylation event in a subset of cells from the original population but is due to the targeted insertion of the *hygro*^R gene.

Chromatin structure

We have demonstrated previously that the chromatin structure of the human β -globin domain is faithfully reproduced in MEL cell hybrids containing a chromosome 11 derived from either human lymphoid cells or fibroblasts (Forrester et al. 1987; 1990). To determine whether the failure to activate β -globin gene expression in our targeted clones is related to the formation of the developmentally stable and/or gene-specific HS in the globin gene domain, we analyzed the chromatin structure of the β -globin gene locus in the HR clones.

Figure 3. Analysis of globin gene expression. (A) RNase protection assays of HR. Total cellular RNA was prepared from uninduced (–) and HMBA induced (+) parental MEL/human hybrid cells (129⁰) or three clones of HR (HR9, HR20, and HR39, which correspond to clones 3, 5, and 8 in Fig. 2, respectively) cultured in the presence (+) or absence (–) of selection (see text). RNase protection analysis was performed by an established method (see Materials and methods) with mouse α -globin ($m\alpha$), human β -globin ($h\beta$), and $hygro^R$ gene ($hygro^R$) probes. The locations of protected fragments corresponding to each gene are indicated. Bands marked by asterisks (*) indicate residual undigested probes. (M) pBR322 *Msp*I-cut DNA molecular weight markers. (B) RNase protection assays of three independent nonhomologous recombinant clones. Clone B represents a clone that does not contain human chromosome 11. Bands labeled $m\beta 1$ and $m\beta 2$ correspond to mouse β -globin exons 1 and 2, respectively. (C) RNase protection assays of 5-azacytidine-treated parent and HR cells. Cells were treated with 5-azacytidine for 20 hr at a final concentration of 3 μ M, and then the cells were allowed to recover in culture for 1 week.

Nuclei from targeted clone HR9 (HR-MEL), from a subclone of the parental hybrid in which the human β -globin is expressed (N-MEL), and a hybrid in which human β -globin expression is extinguished (X-MEL) were treated with DNase I, and Southern blot analyses were performed on the purified DNAs. As shown in Figure 4A, hybridization of *Bam*HI-restricted samples with end-labeled probe A (1.7-kb 5' ϵ *Bgl*II–*Eco*RV fragment) reveals the presence of 5'HS1 and 5'HS2 in the high expressing and extinguished cells. Similar analysis of the HR clone (HR-MEL) reveals the expected sub-band indicative of 5'HS2, but the 5'HS1 sub-band signal is reduced significantly and a new HS is detected in the inserted Friend virus enhancer/promoter region. The presence of this novel HS and the absence of 5'HS1 in the targeted clone were confirmed by hybridization of *Bam*HI-digested samples with probe B (1.3-kb 5' ϵ *Hind*III–*Bam*HI fragment), which labels the other end of the restriction fragment (Fig. 4B). Hybridization of *Eco*RI-digested samples with probe C (0.8-kb 5' ϵ *Nco*I–*Bgl*II fragment) reveals the expected 10.2-kb parental fragment and sub-bands corresponding to 5'HS2, 5'HS3, and 5'HS4 in the control and homologous recombinant cells (Fig. 4C), as well as in the extinguished cells (not shown). Additional analyses revealed that 5'HS5 (21.5-kb 5' to ϵ) and 3'HS1 (21.8-kb 3' to β) were formed in both control and targeted

clones (not shown). Thus, the insertion of our targeting vector into the LCR does not result in a global change in chromatin structure of the β -globin locus. Rather, within the LCR, the detectable changes are the loss of 5'HS1 and the formation of a new promoter/enhancer-based HS in the inserted Friend virus LTR. Analyses of the other homologous recombinant clones (HR20 and HR39) revealed similar changes in the chromatin structure of the LCR (data not shown).

We also analyzed the human β -globin gene promoter HS, which is associated with the expression of this gene in normal adult erythroid cells and MEL/human hybrids (Groudine et al. 1983; Forrester et al. 1987). A Southern blot of *Eco*RI-digested samples from control, targeted, and extinguished cells was hybridized with a 0.6-kb *Hinc*II fragment located 5' to the β globin gene. As shown in Figure 5, the HS in the promoter region is formed as expected in N-MEL chromatin but is not detected in HR-MEL or in X-MEL chromatin. Previously reported minor HS located upstream of the promoter HS (Groudine et al. 1983) are detectable in these hybrid cells and are more prominent in HR-MEL and X-MEL owing to the absence of the promoter HS in these cells (Fig. 5).

Thus, our analysis of the chromatin structure of these cell lines reveals that the HS associated with expression of the human β -globin gene is absent in cell lines in

Homologous recombination into the human β -globin LCR

Figure 4. Chromatin structure in the LCR. After digestion of nuclei with DNase I at increasing concentrations (indicated by direction of the arrow above each sample), the DNA was purified, digested with *Bam*HI, and blot-hybridized to a 1.7-kb 5' ϵ *Bgl*II–*Eco*RV fragment (probe A in D) (A) or a 1.3-kb 5' ϵ *Hind*III–*Bam*HI fragment (probe B in D) (B). Both probes detect a 15.0-kb *Bam*HI fragment in parent MEL/human hybrid clones that show high (N-MEL) or no (X-MEL) expression of the human β -globin gene after HMBA induction, and a 17.5-kb *Bam*HI fragment in the HR9 clone (HR-MEL). In A, sub-bands of 5.5 and 10.0 kb represent 5'HS1 and 5'HS2, respectively, and are detected in both N-MEL and X-MEL. In HR-MEL, 9.5- and 12.5-kb sub-bands correspond to the new HS in the Friend virus LTR and 5'HS2, respectively. In B, a 5.0-kb sub-band corresponding to 5'HS2 is detected in all samples, whereas a 9.5-kb sub-band corresponding to 5'HS1 is seen in both N-MEL and X-MEL and a 8.5-kb sub-band representing a new HS in the Friend virus LTR appears in HR-MEL. Sub-bands corresponding to each HS are indicated by arrows at right labeled HS. (M) Molecular weight standards of *Hind*III λ and *Hae*III-digested ϕ X 174 DNAs. (C) *Eco*RI-digested DNA samples were blot hybridized with 0.8-kb 5' ϵ *Nco*I–*Bgl*II fragment (probe C in D) to detect 5'HS2, 5'HS3, and 5'HS4. This probe detects a 10.2-kb parent *Eco*RI band in all samples and sub-bands of 1.6, 5.7, and 9.0 kb representing 5'HS2, 5'HS3, and 5'HS4, respectively. (D) Simplified maps of N-MEL and HR-MEL. HS are depicted by solid arrows; stippled arrows represent the loss of an HS. The *Eco*RI (RI) and *Bam*HI (B) restriction sites are also indicated. Probes used in A, B, and C are shown below the map.

which the gene is inactive. However, all LCR HS remain intact in control cells and cells in which the gene is inactivated by extinction, whereas the insertion of hygro^R gene into the LCR between 5'HS1 and 5'HS2 results in formation of a new HS in the inserted promoter/enhancer region and significant reduction of the normal 5'HS1 located 3' of the inserted sequences. In combination with the expression data presented in Figure 3, the chromatin structure results suggest that the inactivation of the human β -globin gene ~50 kb downstream of the targeted region may be the result of interaction of LCR elements with the proximal Friend virus enhancer/pro-

motor, preventing the normal interaction between the LCR and the β -globin gene promoter (or proximal control elements).

Discussion

We have used the positive-negative selection homologous recombination strategy to introduce a Friend virus LTR-driven hygro^R gene into the human β -globin LCR in human/MEL hybrid cells containing one copy of human chromosome 11. Our results (summarized in Fig. 6) show that β -globin gene expression and chromatin structure of

the locus are affected by insertion of the Friend virus LTR-driven *hygro*^R gene into the β -globin LCR between 5'HS1 and 5'HS2. As shown by RNase protection assays (Fig. 3), the correctly targeted selection marker is induced to high-level expression when the cells are differentiated, whereas the human β -globin gene is no longer expressed either before or after induction. *Hygro*^R genes integrated randomly outside the β -globin locus are not inducible. Chromatin structure studies (Figs. 4 and 5) show that the promoter-based HS in the β -globin gene is eliminated in the targeted clones, and a new enhancer/promoter-based HS is formed in the Friend virus LTR, which drives transcription from the inserted *hygro*^R gene. LCR 5'HS1, which is displaced 2.7 kb downstream by insertion of the *hygro*^R gene, is greatly reduced or eliminated, whereas other HS in the β -globin locus (5'HS2–5'HS5 and 3'HS1) are maintained. The abrogation of human β -globin gene expression in our homologous recombinants may be attributable to the establishment of a new promoter/enhancer competition (Choi and Engel 1988), such that the *hygro*^R gene replaces the β -globin gene as a recipient of the downstream effects of the LCR. This possibility is supported by the induction of high-level expression of the *hygro*^R gene following HMBA treatment of the three targeted clones and the low-level constitutive expression of the *hygro*^R gene in clones containing nonhomologous integrants (Fig. 3).

Support for the promoter/enhancer competition model (Choi and Engel 1988) is derived from recent transgenic experiments addressing the influence of the LCR on human β -like globin gene expression. The human β -globin genes are arranged in the order in which they are expressed developmentally: Expression switches from embryonic (ϵ) in the yolk sac, to fetal (γ) during intrauterine life, and to adult (β) after birth. Transgenic experiments have shown that in the absence of the LCR, the ϵ - and γ -globin genes are silenced autonomously during later developmental stages, when the

β -globin gene is expressed (Cao et al. 1989; Raich et al. 1990; Dillon and Grosveld 1991). This silencing mechanism can not be overcome completely by the presence of the LCR. In contrast, the β -globin gene is expressed

equally well at all developmental stages when linked to the LCR. In transgenic constructs containing linked γ - and β -globin genes, the level of stage-specific expression of each gene has been shown to be affected by the positions of these genes relative to the LCR (Blom van Asseveldt et al. 1978; Behringer et al. 1990; Enver et al. 1990; Lindenbaum and Grosfeld 1990; Hanscombe et al. 1991). In combination, these results suggest that the outcome of competition between genes for activation by the LCR is determined both by stage-specific elements immediately flanking the genes, as proposed by Choi and Engel (1988), and by their relative distances from the LCR, with the more proximal gene having an advantage (Hanscombe et al. 1991). In this view, the stage-specific and autonomous turning off of the ϵ - and γ -globin genes would be the rate-limiting steps in the subsequent LCR-dependent transcriptional activation of the β -globin gene during development. Thus, promoter competition for LCR elements appears to be the principal mechanism for β -globin gene switching in humans and chickens, as proposed originally by Choi and Engel (1988). In this context, it is not surprising that a competition between the Friend enhancer/promoter *hygro*^R cassette and the β -globin gene may also occur, given the preferential activity of the Friend LTR in erythroid cells (Cowie and Myers 1988) and its proximity to the LCR. In essence, the inactivation of the β -globin gene in our homologous recombinants may be through a mechanism similar to that operating in development, when the active ϵ -globin gene out-competes the β -globin gene for the effects of the LCR.

Others have reported the insertion of positive selectable marker genes into the human β -globin locus in MEL/human hybrids by HR. In one case (Smithies et al. 1985), two transcription units, an SV40 enhancer/promoter-driven neomycin resistance (*neo*^R) gene and an additional truncated β -globin gene, were inserted at a site 2.3 kb upstream from the cap site of the human β -globin gene. Expression of the endogenous β -globin gene was reduced, and the *neo*^R gene displayed inducible expression (Nandi et al. 1988). The observed decrease in β -globin expression in this recombinant is somewhat difficult to interpret, given the introduction of two additional genes into this locus. However, in the second case (Sheseley et al. 1991), insertion of a single polyoma enhancer/tk promoter-driven *neo*^R gene ~600 bp upstream of the β -globin gene cap site did not significantly affect expression of the downstream β -globin gene. Although this result and our results seem contradictory, they can be reconciled by considering the model described above, in which competition between genes for the LCR is influenced by their relative distances from the LCR and/or the differential affinity of stage-specific elements for the LCR. In this context the proximity of the Friend virus promoter/enhancer to the LCR in our targeted clones could result in its successful competition over the distantly located β -globin gene promoter for LCR-conferred inducible expression. In contrast, given the similar locations of the *neo*^R and β -globin genes ~50 kb downstream from the LCR, less effective competition for the LCR

would occur. Sequences within the Friend virus enhancer/promoter may also be stronger competitors for the LCR than the sequences within the polyoma cassette in the MEL cell background.

It is also possible that the insertion of the *hygro*^R gene between 5'HS1 and 5'HS2 may disrupt critical spacing requirements for LCR activity, including the formation of 5'HS1. We believe, however, that several observations make this a less likely explanation for the inactivation of the downstream β -globin gene than the promoter/enhancer competition model. Stable transfection assays in erythroid cells in which the β -globin gene is linked to fragments of the LCR have revealed that in combination with other HS sequences, 5'HS1 can augment globin gene expression by a maximum of only twofold (Collis et al. 1990). Moreover, in contrast to 5'HS2, 5'HS3, or 5'HS4, sequences within 5'HS1 do not confer elevated globin gene expression autonomously. Furthermore, the retention of sequences comprising 5'HS1 is not sufficient to form an active β -globin chromatin domain or activate β -globin gene expression in the Hispanic ($\gamma\delta\beta$)⁰ thalassemia, a de novo 35-kb deletion that removes all LCR elements 5' of 5'HS1 (Driscoll et al. 1989; Forrester et al. 1990). It has also been reported that β -globin gene expression is not altered by a naturally occurring 3.03-kb deletion upstream of the ϵ -globin gene, including sequences comprising 5'HS1 (Kulozik et al. 1991). In addition, the *hygro*^R genes introduced into the LCR in our homologous recombinants demonstrate inducible expression, in the absence of 5'HS1. Thus, 5'HS1 is neither necessary nor sufficient for the erythroid specific expression of genes under LCR control.

Regardless of the mechanism of β -globin inactivation in our targeted recombinants, our results emphasize the need to remove the positive selectable marker gene following HR, if a positive selection HR strategy is to be useful in the analysis of regulatory regions. Recently, several in/out strategies to delete sequences inserted by HR have been described (Reid et al. 1990; Dale and Ow 1991; Hasty et al. 1991; O'Gorman et al. 1991; Valancius and Smithies 1991). We are currently investigating the feasibility of these methods for use in the functional analysis of the human β -globin LCR by HR.

Materials and methods

Targeting construct preparation

The targeting construct (p5' ϵ Hygro^Rtk) is an 18-kb pUC19-based plasmid that contains a 9-kb 5' ϵ *Eco*RI–*Bam*HI DNA fragment, with the *hygro*^R gene (1.5-kb *Sac*I–*Bgl*II fragment; Santerre et al. 1984) inserted at a unique *Hpa*I site and the HSV tk gene (2.5-kb *Bgl*II–*Bam*HI fragment of pHSV 106; McKnight and Gavis 1980) inserted at the terminal *Bam*HI site (see Fig. 1). Both genes are driven by a 0.5-kb *Kpn*I–*Pst*I fragment that contains the Friend virus LTR enhancer/promoter sequences (Clark et al. 1985) to allow high-level constitutive expression in the hybrid erythroleukemia cells. The *hygro*^R gene contains a tandem duplicated 0.85-kb *Bgl*II–*Bam*HI fragment with the SV40 poly(A) addition signal sequence and small t antigen intronic splicing sequence.

Cell culture

The parent cells for homologous recombination (N-MEL-129°), which contain a copy of normal human chromosome 11, are one of the MEL/human hybrid cell lines described previously (N-MEL; Forrester et al. 1990). Cells were maintained in nonselective Dulbecco's modified Eagle medium (DMEM) with 10% (vol/vol) heat-inactivated bovine calf serum and periodically enriched for the presence of human chromosome 11 by panning with a monoclonal antibody as described (Yagi et al. 1987; Forrester et al. 1990).

For electroporation, 1×10^7 cells were resuspended in 0.6 ml of DMEM without serum, and 5 μ g of *FspI*-linearized targeting vector was added. After incubation at room temperature for 10–15 min, electroporation was performed at 350 μ F capacitance and 250 V by use of a BTX device. After 10–15 min at room temperature, cells were diluted into warm growth medium and plated in either 150-mm petri dishes or 48-well plates. Hygromycin B (0.6–0.8 mg/ml; Calbiochem) was added the next day for 2 weeks, and gancyclovir (10 μ M; Syntex) was then added for an additional week. After both positive and negative selections, drug-resistant cells were maintained in 0.5 mg/ml of hygromycin B-containing media. Several sib selections were performed in microtiter plates to isolate pure populations of recombinant cells.

Induction of erythroid differentiation of cells was performed by the addition of 5 mM HMBA (Sigma) to the culture medium for 3 days. 5-Azacytidine (Sigma) treatment was accomplished by addition to a final concentration of 3 μ M to the culture medium for 20 hr, and surviving cells were allowed to recover by cultivation in normal medium for 1 week.

PCR

PCR reactions were performed using standard conditions in a total reaction volume of 50 μ l. Other ingredients such as glycerol (10%) or tetramethylammonium chloride (TMAC, 50 μ M) were added to PCR reactions when crude cell lysates were used. Thirty-five cycles were performed with denaturation for 1 min at 92°C, annealing for 30 sec at 63°C, and extension for 2.5 min at 72°C. A portion of each sample (15 μ l) was analyzed by electrophoresis in 1% agarose gels. Putative homologous recombinants were identified by the PCR product size of ethidium bromide-stained bands in the gel or after Southern blotting and probing with a random-primed 5' ϵ 1.0-kb *HpaI*–*EcoRI* fragment. Primer sequences are as follows: PCR 1 (vector, 3' homology region), 5'-CAG GGT GAA AGG GTA TCG CTA C-3'; PCR 99 (genomic, 5' junction), 5'-GCA CTC CAG CCT CCA GAG CG-3'; PCR H1 (vector, *hygro*^R gene), 5'-ATC TCA TTG CCC CCG GAC GA-3'; PCR 8 (vector, 5' homology region), 5'-CCC ATC AAA TTC CCT GAC CG-3'.

Chromatin structure and genomic Southern blotting

Isolation of nuclei, DNase I digestions, purification and restriction enzyme digestion of genomic DNA, and Southern blotting were as described (Forrester et al. 1990). Probes for hybridization were labeled by the nick translation or random priming methods. The junction probe (0.3-kb *EcoRI*–*EcoRV* probe used in Fig. 3) was made by a PCR-based primer extension method (Bednarczuk et al. 1991).

RNAse protection analysis

Total cellular RNA from cultured and HMBA-induced cells was prepared by the RNazol-based quick method (Kim et al. 1990)

as recommended by the manufacturer (Cinna/Biotech), and the purified RNA was resuspended in water according to the total cell number per volume (RNA from 1×10^7 cells/50 μ l of water).

RNAse protection assays were performed as originally described (Zinn et al. 1983; Kim et al. 1990). Antisense probe RNAs were prepared in vitro with SP6, T7, or T3 RNA polymerase (Promega) using standard methods (Zinn et al. 1983; Melton et al. 1984; Kim et al. 1990). As controls, mouse α - and/or β -globin gene probes were also included in the reaction containing 1 μ l of purified cellular RNA. The antisense RNA probes are as follows: human β -globin, pSP64h β ³⁵¹ as described in Charnay et al. (1984); *hygro*^R, pVZhygro^R containing a 295-bp *HindIII*–*EcoRI* fragment of the *hygro*^R gene; mouse α -globin, pSP64m α containing a *BamHI*–*PstI* fragment of the mouse genomic α -globin gene; mouse β -globin, pGEMm β containing a 0.8-kb *HindIII*–*BamHI* fragment of the genomic mouse β -globin gene (two protected bands corresponding to exon 1 and exon 2).

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