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An "in–out" strategy using gene targeting and FLP recombinase for the functional dissection of complex DNA regulatory elements: Analysis of the β-globin locus control region

(homologous recombination)

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ABSTRACT **The human β-globin locus control region (LCR) is a complex DNA regulatory element that controls the expression of the cis-linked β-like globin genes located in the 55 kilobases 3′ of the LCR. We have initiated the functional analysis of the LCR by homologous recombination in murine erythroleukemia cell somatic hybrids that carry a single copy of human chromosome 11 on which the β-globin locus is situated. High-level expression of the human β-globin gene normally occurs when these hybrid cells are induced to differentiate. We have reported that the insertion of an expressed selectable marker gene (driven by the Friend virus enhancer/promoter) into the LCR disrupts the LCR-mediated regulation of globin transcription. In these cells, β-globin is no longer expressed when the cells differentiate; instead, expression of the selectable marker gene increases significantly after differentiation. Since present techniques for homologous recombination require the insertion of a selectable marker, further progress in using homologous recombination to analyze the LCR depends on deletion of the selectable marker and demonstration that the locus functions normally after the insertion, expression, and deletion of the selectable marker. Here we show that after precise deletion of the selectable marker by using the FLP recombinase/FRT (FLP recombinase target) system, the locus functions as it did before the homologous recombination event. These studies demonstrate the feasibility of using homologous recombination to analyze the LCR in particular, and other complex cis-regulatory DNA elements in general, in their normal chromosomal context.**

Transcriptional control of eukaryotic genes occurs within an overall context that includes the influences of genomic position, such as chromatin organization and the timing of DNA replication (for review, see refs. 1 and 2). However, most analyses of transcriptional regulatory elements have utilized the transient transfection of reporter gene constructs. In transient assays, the experimental DNA has not integrated into the genome, and therefore the potential influences of an integration site are ignored. Another common approach to studying transcriptional regulatory elements has been to select cells (or transgenic organisms) that have integrated the experimental DNA more or less randomly into the genome. These stable integration assay systems have shown that the integration site has a large influence on the activity of the integrated construct, often rendering assays of stably integrated constructs difficult to interpret.

The development of homologous recombination (HR) in mammalian cell lines and the associated ability to modify ES cells *in vitro* and subsequently derive mice from these lines (reviewed in ref. 3) have opened the possibility of a systematic analysis of cis DNA regulatory elements by making mutations in the endogenous DNA *in situ*. We have begun such an analysis of the human β-globin locus control region (LCR) that is based on introducing mutations into the LCR by homologous recombination in mouse erythroleukemia (MEL) cell hybrids and then assaying the effects of these mutations on the β-globin locus.

The human β-globin locus is composed of five linked functional β-like globin genes. These highly homologous genes are arranged in the same transcriptional orientation and in the order they are expressed during development (5′-ε-G_γ-A_γ-δ-β-3′). The LCR, located 6–22 kilobases (kb) upstream of the ε-globin gene is a regulatory region that controls the timing of replication, chromatin structure, and transcription of the entire 200-kb β-globin domain (for review, see ref. 4). The LCR was first characterized as a series of five DNase I hypersensitive sites (HS) that are present in erythrocyte chromatin regardless of which globin genes are expressed (5, 6). Since its discovery, the LCR has been analyzed extensively by transfection and transgenic studies (refs. 7–11 and others). These studies have revealed that components of the LCR can substantially overcome position effects and confer high-level erythrocyte-specific expression on cis-linked genes; however, the mechanisms underlying the general function of the LCR are not well understood.

We reported previously that the simple insertion of a selectable marker into the β-globin LCR by HR can disrupt the normal functioning of the LCR (12). Here, we report the successful use of the yeast FLP recombinase system (13) to delete the selectable marker that was inserted as part of the homologous recombination selection system. We further show that in multiple independent clones, the LCR functions normally after the deletion of the selectable marker. The implications of these findings are discussed in relation to the analysis of the LCR and other complex genetic regulatory elements in their native chromosomal position.

MATERIALS AND METHODS

Plasmid Constructs. The targeting construct (p5′εFneoF/tk) is similar to the construct (p5′εhygrotk) used previously (12). This construct is a 19-kb pUC19-based vector that contains a 9-kb *Eco*RI/*Bam*HI DNA homology fragment with the bacterial *neo* gene (2.0-kb *Bgl* II/*Bam*HI fragments from pSV2-neo) inserted at a unique *Hpa* I site and the herpes simplex virus (HSV) thymidine kinase (*tk*) gene [2.5-kb *Bgl* II/*Bam*HI fragment of pHSV106 (14)] inserted at the terminal

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Abbreviations: HR, homologous recombination; FRT, FLP recombinase target; LTR, long terminal repeat; F-LTR, Friend virus LTR; MEL, mouse erythroleukemia; h-chr 11, human chromosome 11; LCR, locus control region; β-gal, β-galactosidase; HS, hypersensitive sites; HMBA, *N,N′*-hexamethylenebisacetamide; FACS, fluorescence-activated cell sorting.

*Bam*HI site. Both genes are driven by a 0.4-kb *Eco*RV/*Pst* I fragment that contains the Friend virus long terminal repeat (LTR) enhancer/promoter (F-LTR; ref. 15). To permit the removal of the F-LTR/neo gene by FLP recombinase, 48-base-pair (bp) FLP recombinase target (FRT) oligonucleotides with the same orientation flanked both sides of F-LTR/neo. The chemically synthesized FRT oligonucleotides are FRT-1 (5'-AACGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCGTT) and FRT-2 (the complement of FRT-1).

The pCFIZ plasmid is designed to use an internal ribosome entry site (IRES; ref. 16) to coexpress FLP and β-galactosidase (β-gal; encoded by *lacZ*) from the same message. pCFIZ was constructed by using the pCSIRESβ-gal vector (provided by R. Rupp and D. Turner, Fred Hutchinson Cancer Research Center). pCSIRESβ-gal is constructed in pBluescript and contains in order the promoter/enhancer of the simian cytomegalovirus immediate early region, a polylinker, the IRES, *lacZ*, and the simian virus 40 (SV40) polyadenylylation region. pCFIZ was made by insertion of the FLP gene (*Xba* I/*Xho* I fragment) from pOG44 (17) into the polylinker. The full mRNA contains in order the FLP gene, the IRES sequence, *lacZ*, and finally the SV40 poly(A) sequence. Both the FLP gene and *lacZ* contain translation stop codons near their 3' ends.

Cell Culture. The parent cells for homologous recombination (N-MEL), which contain a single copy of human chromosome 11 (h-chr 11), are one of the MEL–human hybrid cells described previously (18). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) heat-inactivated bovine calf serum.

Following an HR event, the *neo* selectable marker gene is present in the LCR; therefore the h-chr 11 can be maintained by growth of these cells in selective medium (containing G418 at 0.75 mg/ml; Boehringer Mannheim). Before an HR event and following the FLP-mediated removal of the selectable marker, the h-chr 11 does not contain a selectable marker, and the chromosome is lost at a significant rate that varies among individual clones. Analysis of the percentage of cells containing h-chr 11 and selection of cells containing h-chr 11 was performed by utilizing the expression of h-chr 11-specific antigens expressed in these cells. The h-chr 11-specific antibodies used were 53-6 (19) or 4F2 (American Tissue Culture Collection), both of which are highly expressed on MEL cells specifically carrying h-chr 11. The percentage of cells with h-chr 11 was assayed by flow cytometry (Facscan; Becton Dickinson) using fluoresceinated goat anti-mouse antibody as a second step (Jackson ImmunoResearch). Selection was performed either by panning as described (19) or fluorescence-activated cell sorting (FACS; FACS II, Becton Dickinson). For all experiments reported here, 50–98% of the cells carried h-chr 11.

Induction of differentiation was performed by culturing the cells for 3 days in medium containing 5 mM *N,N'*-hexamethylenebisacetamide (HMBA; Sigma). Since high cell density will also induce differentiation, prior to harvest for uninduced RNA, the cells were maintained at <500,000 per ml.

As discussed (12), it is possible that, although observed with multiple independently derived clones, the suppression of β-globin transcription observed in the homologous recombinant clone HR-MEL is due to the *de novo* methylation of the β-globin gene. To address this, all clones were treated with 5-azacytidine prior to collection of RNA. 5-Azacytidine treatment was done by culturing the cells for 20 hr in medium containing 3 μM 5-azacytidine (Sigma). Following this treatment, the cells were allowed to recover for 6 days before induction of erythroid differentiation.

Isolation of Homologous Recombinants. The electroporation of the targeting construct and the selection for HR clones was identical to our previous report (12) with the following exceptions: the vector was linearized with *Sal* I, and selection was performed with G418 at 0.75 mg/ml (GIBCO/BRL). The PCR reactions to identify pools and subsequently clones of HR cells was basically as described (12). However, a *neo* primer, YZ24, was used instead of a primer from the gene for hygromycin resistance; the sequence of YZ24 is as follows: 5'-GTTGTGCCCAGTCATAGCCG.

Isolation of Clones That Had an FLP-Mediated Deletion of the Selectable Marker. The 1140 SF clone with stably integrated FLP was isolated by coelectroporation of HR-MEL with the FLP expression construct pOG44 and pF-LTR/hygro. Electroporation was performed with 10 μg of pOG44 and 1 μg of pF-LTR/hygro. Cells were placed into selective medium (hygromycin B at 0.75 mg/ml; Calbiochem) 24 hr after electroporation. Resistant cells were cloned by limiting dilution, and the clones were analyzed by PCR for deletion of the selectable marker. PCR was performed with primers 1 and 99 as described (12) (these primers are diagrammed in Fig. 1*E*).

Isolation of clones that had an FLP-mediated deletion of the F-LTR/neo gene after transient FLP expression was initiated by either coelectroporating pOG44 with F-LTR/β-gal or electroporating with the pCFIZ construct described above. Forty-eight hours after electroporation, the cells were assayed and sorted for β-gal expression by the viable-cell "FACS-Gal" assay (20, 21). In coelectroporation experiments, 40 μg of pOG44 was used with 2 μg of F-LTR/β-gal or, alternatively, 40 μg of pCFIZ was used. With either system the expression of β-gal was detected in <1% of the cells, and the highest β-gal expressers (about 0.1% of total cells) were cloned by FACS (FACS II; Becton Dickinson). The clones were analyzed by PCR for FLP-mediated deletions of F-LTR/neo. All electroporations of FLP described here were performed on 2 million cells, and all clones studied here were derived from independent electroporations.

Genomic Southern Analysis. Southern analysis was performed as described (18). The probe (1.0-kb *Hpa* I/*Eco*RI fragment as shown in Fig. 1*D*) was labeled with ^{32}P by the random primer method.

RNase Protection Analysis. Total RNA was isolated from 10^7 cells by using RNAzol (Tel-Test, Friendswood, TX) as recommended by the manufacturer. The RNA was quantitated by spectrophotometry, and 3 μg was used for each RNase protection analysis. RNase protection analyses were performed as described (22). Antisense RNA probes were prepared by standard methods (22, 23). The human β-globin probe was prepared from pSP64hβ351 (24) linearized with *Eco*RI. The mouse α-globin probe was prepared from the pSP64mα plasmid (25) linearized with *Hin*dIII. To reduce the signal from mouse α-globin, which is more highly expressed in these cells than human β-globin, the ^{32}P-labeled mouse α-globin probe was mixed in equal molar ratio with unlabeled mouse α-globin probe. The *neo* probe (pT3TKN, linearized with *Eco*RI) was prepared from pTKNeo (unpublished) and contains a 0.6-kb *Pvu* II fragment containing 260 bp of *neo*. The gel shown is representative of results from multiple RNase protection assays done on two independent collections of RNA.

RESULTS

Inactivation of the β-Globin Gene by Targeted Insertion of a Selectable Marker Gene into the LCR. To initiate the work described here, we essentially repeated a previous experiment (12) in which a selectable marker gene was integrated by HR between 5' HS1 and 5' HS2 of the human β-globin LCR (Fig. 1). As before, we used the positive–negative selection strategy (3) to construct a replacement-type vector designed to insert the selectable marker gene into the LCR by a double crossover event (Fig. 1). The genomic homology sequences

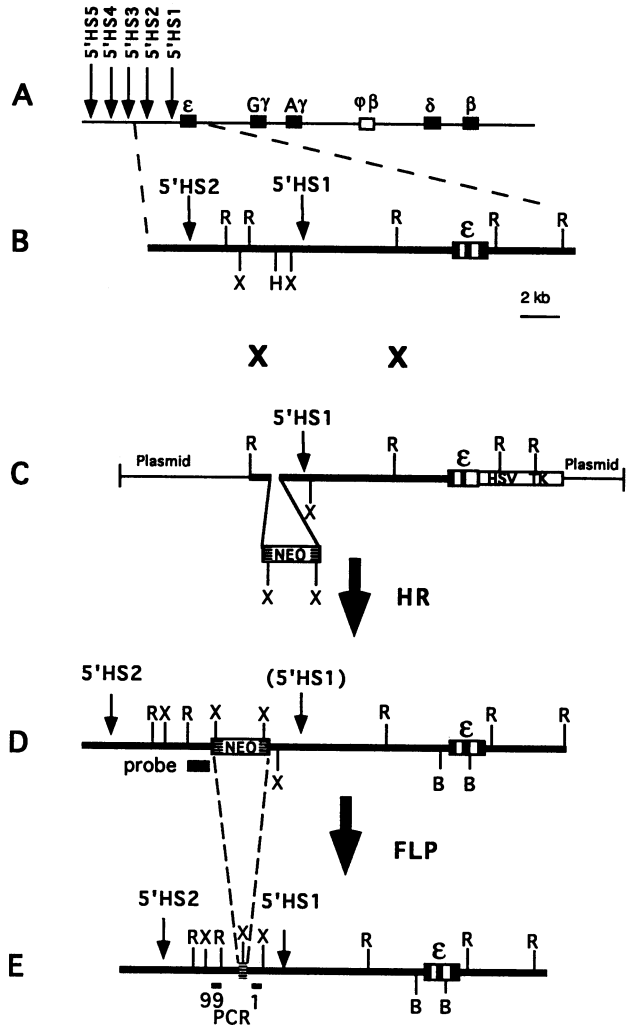

FIG. 1. The β-globin locus and changes made in the LCR by HR and FLP recombination. (*A*) Schematic map of the human β-globin locus showing the positions of the HSs in the LCR and the β-like globin gene coding regions. (*B*) Enlarged region of *A* showing the HR region. (*C*) The HR construct aligned with the chromosomal map of the region. HSV TK, herpes simplex virus thymidine kinase gene; neo, neomycin-resistance gene. (*D*) Product of the HR event between genomic DNA (*B*) and the HR vector (*C*). (*E*) Product of the FLP-mediated deletion of the F-LTR/neo gene. In *A*, solid and open rectangles denote functional and pseudo genes, respectively. In *B*, *C*, *D*, and *E*, the solid and open boxes represent exons and introns of the ε-globin gene, respectively. Labeled arrows denote developmentally stable, erythroid-specific DNase HS sites in *A–E*. The FRTs are horizontally striped boxes in *C*, *D*, and *E*. The probe for the Southern analysis (Fig. 2) is denoted by a labeled solid box in *D*. The positions of PCR primers used to screen for FLP-mediated deletion are denoted in *E*. B, *Bam*HI; H, *Hpa* I; R, *Eco*RI; X, *Xba* I.

are identical to those used previously (12), with 1 kb of 5′ homology and 8 kb of 3′ homology, and the positive and negative selectable marker genes are driven by the same enhancer/promoter, the F-LTR. These experiments utilize the bacterial neomycin-resistance gene as a positive selectable marker, whereas previously we used the hygromycin-resistance gene. The important difference between the construct used previously and the construct used here is that in these experiments, all of the inserted nonhomologous sequences (F-LTR/neo) are flanked by single copies of the 48-bp FRT site (26, 27).

These homologous recombination experiments were performed in a MEL cell somatic hybrid (N-MEL) carrying a single copy of h-chr 11 on which the β-globin locus is situated. MEL cells are transformed erythroid cells that can be induced to differentiate into erythrocytes and, during differentiation, express the adult globin genes. Several clones containing the expected targeted insertion were isolated as described (12), and the Southern analysis of a representative homologous recombinant (HR-MEL) is shown in Fig. 2. Southern analysis with the probe illustrated in Fig. 1*D* shows that the homologous recombination event results in the conversion of the N-MEL 6.5-kb *Eco*RI genomic fragment into a 8.5-kb band (Fig. 2*A*). This 8.5-kb band also hybridized to a *neo* probe (data not shown), further confirming the HR event.

As reported in our previous experiments (12) and shown in Fig. 3 *left*, the simple insertion of a selectable marker gene into the LCR had a marked phenotypic effect on the endogenous β-globin locus. The insertion completely suppressed the expression of the β-globin gene [compare the N-MEL and HR-MEL lanes after (+ HMBA) induction of differentiation]. While β-globin expression is suppressed, expression of the selectable marker gene (*neo*) increased significantly after induction of differentiation (Fig. 3 *center*). Additionally, DNase I sensitivity of 5′ HS1 is greatly reduced (data not shown). These phenotypic results were consistent among multiple independently derived clones and are identical to the results reported previously when using the F-LTR/hygro selectable marker gene (12). One clone, HR-MEL, was selected for subsequent experiments involving the FLP recombinase-mediated deletion of the selectable marker gene.

Reactivation of β-Globin Gene Expression by FLP-Mediated Deletion of the Selectable Marker Gene. The FLP recombinase of *Saccharomyces cerevisiae* catalyzes recombination between two FRTs (13, 17, 26, 27). Depending on the orientation of the FRTs, the FLP-mediated recombination will lead to an inversion or deletion if the sites are linked on the same chromosome or to an insertion or translocation if the FRTs are not linked. Functional FLP can be expressed in mammalian cells, recognize FRT sites integrated in the mammalian genome, and perform a recombination reaction (17). The orientation of the FRT sites in our HR is such that an FLP recombination reaction will delete the intervening sequences and leave a single intact FRT at the HR site. We utilized the FLP system to determine whether precise deletion of the selectable marker gene would result in the reactivation of the β-globin gene.

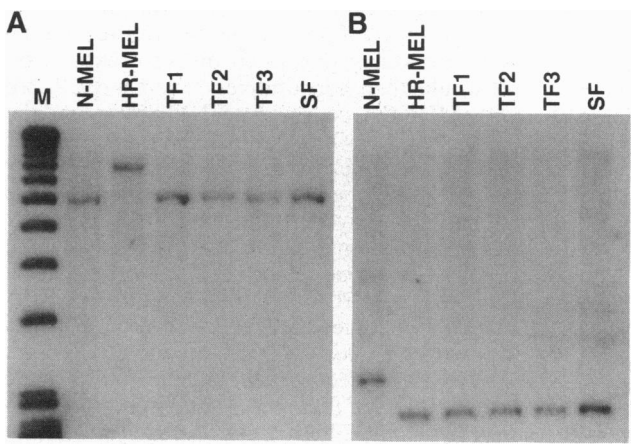

FIG. 2. Genomic Southern blot showing the results of both the original HR and the FLP-mediated deletions of F-LTR/neo. DNA was from clones as labeled: N-MEL, parental MEL hybrid; HR-MEL, homologous recombinant; TF1, TF2, TF3, and SF, clones with FLP-mediated deletions. The probe is as diagrammed in Fig. 1*D*. The marker is the 1-kb ladder (GIBCO/BRL). (*A*) Restriction digestion performed with *Eco*RI. (*B*) Restriction digestion performed with *Xba* I.

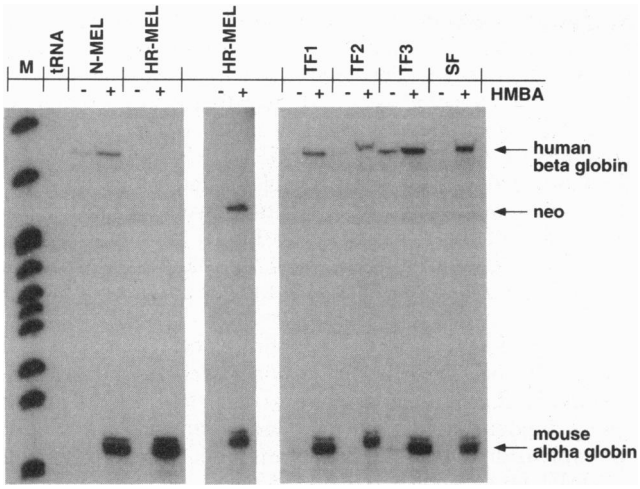

FIG. 3. RNase protection analysis of gene expression. Total cellular RNA was prepared from uninduced (−), and HMBA-induced (+) MEL–human cell hybrids: parental cells, N-MEL; homologous recombinant cells, HR-MEL; clones with FLP-mediated deletions of F-LTR/*neo*, TF1, TF2, TF3, and SF. Lane M contains *Msp* I-cut pBR322 DNA molecular weight markers. (*Left* and *Right*) RNase protection probes were human β-globin and mouse α-globin genes hybridized together with 3 μg of RNA. (Center) RNase protection probes were *neo*, human β-globin, and mouse α-globin genes hybridized together with 3 μg of RNA. The tRNA lane had all three probes hybridized to 20 μg of tRNA. The correct-size protected bands for each probe are as labeled.

Our initial attempts to isolate clones in which the FRT-flanked F-LTR/neo gene had been deleted by an FLP reaction involved transient transfection (via electroporation) of the cells with an FLP expression plasmid [pOG44 (17)] and subsequent PCR analysis for deletion of F-LTR/neo. We screened an estimated 500 clones in pools of 10 clones each, as well as 48 individual clones and obtained no evidence that FLP had deleted F-LTR/neo in any of these clones. This failure is due at least partially to the difficulty in transfecting the MEL cells. Our studies with the F-LTR/β-gal reporter gene have revealed that, in most transfections, only 1–2% of the cells express β-gal (data not shown).

Our further efforts to isolate clones with FLP-mediated deletions of the selectable marker used either stable integration of the FLP expression construct or FACS analysis of cells that had been successfully transiently transfected. To select cells that had stably integrated the FLP expression construct and would then constitutively express FLP, we cotransfected the HR-MEL cells with pF-LTR/hygro and the FLP expression construct pOG44. After selection for hygromycin resistance, 24 clones were screened by PCR for deletion of the F-LTR/neo gene. One such clone (SF) was identified and found to have lost resistance to G418. By Southern blotting, we determined that this clone had indeed deleted the F-LTR/neo gene (Fig. 2*A*; conversion of the 8.5-kb band in lane HR-MEL to a 6.5-kb band in lane SF).

To accomplish the FLP-mediated deletion with transiently expressed FLP, we developed two related systems to enrich for cells that had been transfected successfully. Both systems depend on the expression of β-gal that is either cotransfected or on the same mRNA with FLP [pCFIZ, utilizing an internal ribosome entry site (16)]. The flow cytometer was used to identify and sort the cells with the highest levels of β-gal activity by the viable cell FACS-Gal flow cytometry assay (20, 21). β-Gal-expressing cells were sorted into both clones and multicell pools, allowed to grow, and subsequently screened by PCR. From these experiments, three independent clones with FLP-mediated deletions of the F-LTR/neo gene were obtained (TF1, TF2, TF3). With both β-gal-based enrichment systems, the frequency of cells with the FLP-mediated deletion per total clones analyzed was ≈1 in 30. As with the clone SF, obtained with stable integration of FLP, these three clones all showed the expected 6.5-kb *Eco*RI bands on a Southern blot (Fig. 2*A*) and had lost resistance to G418. In addition, as determined by Southern blotting, these clones have not integrated FLP (data not shown). An FLP recombination reaction leaves a residual FRT site that contains an *Xba* I restriction site. Southern analysis of *Xba* I-cut DNA with the probe diagrammed in Fig. 1*D* shows that all cell lines derived from HR-MEL still have an *Xba* I site at the previous site of an FRT (note the smaller band in the HR-MEL and clones derived from it compared to N-MEL) and therefore still contain an FRT, as expected.

As discussed above, none of the clones with the HR described here or previously (12) expressed human β-globin when induced, even though the mouse α-globin and β-globin genes are well expressed after induction. Thus, as concluded previously, the presence of the selectable marker gene in the LCR disrupts the expression of the downstream β-globin gene, even in the absence of deletion or mutation of specific sequences in the LCR. Obtaining multiple clones with FLP-mediated deletion of the *neo* gene allowed us to determine whether the suppression of β-globin expression due to the insertion of the selectable marker in the LCR could be reversed by removing the selectable marker gene. All four of the clones that underwent the FLP-mediated deletion of F-LTR/neo express normal levels of human β-globin when induced to differentiate (Fig. 3 *Right*). In addition, 5′ HS1 is fully restored in all four of these clones (data not shown).

DISCUSSION

Previous work (12) showed that the simple insertion of a selectable marker (F-LTR/hygro) controlled by the F-LTR suppresses the expression of the β-globin gene 50 kb downstream of the integration site. This suppression of globin transcription is associated with the inducibility of the selectable marker gene when the cells are stimulated to differentiate. In regard to the effects of the insertion on the LCR function, the results described in this communication confirm those reported previously. The minor changes made—replacement of the hygromycin-resistance gene with the neomycin-resistance gene and flanking the F-LTR/neo gene with FRTs—did not affect the resulting phenotype.

Finding that the F-LTR/hygro gene insertion suppresses β-globin transcription led us to redesign our HR strategy to include a system for removing the selectable marker once the HR had been accomplished. Two systems had been shown to function as "in–out" strategies when used in an HR (28, 29), but both systems depend on a spontaneous second recombination event because of a duplication of homologous sequences associated with an "insertion" type of HR strategy. Our long-range goal, to use HR to make specific deletions of the LCR, requires a "replacement" strategy that utilizes a double crossover rather than a single crossover "insertion" HR strategy. Therefore, we chose to work with a recombinase system that could specifically delete sequences flanked by its target sites, even though the use of the available recombinase systems in an HR had not been demonstrated at the time these experiments were performed. A recent report has shown the use of the FLP recombinase system to delete the selectable marker in an HR (30); however, in those experiments, a large deletion in the endogenous sequences was made, and the phenotype reported was not influenced by FLP-mediated removal of the selectable marker.

Most importantly for the potential of HR as a tool to study transcriptional regulation of β-globin, the cells revert to the original phenotype in regard to β-globin expression following the FLP-mediated deletion of the F-LTR/neo gene. Thus, the

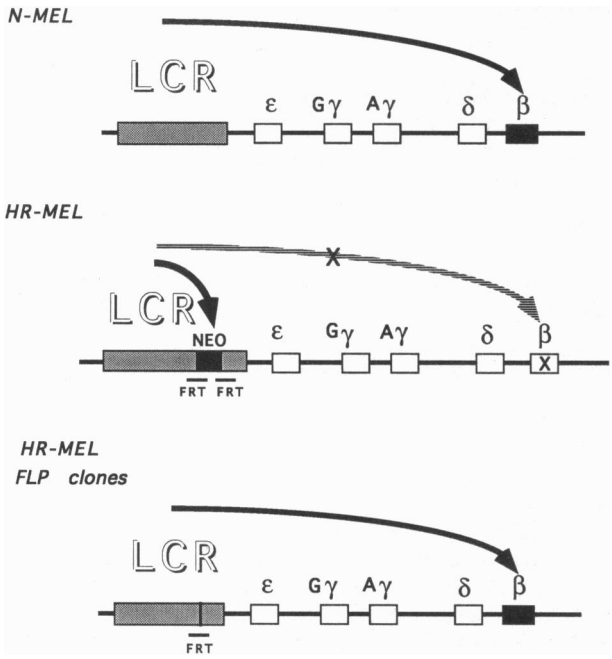

FIG. 4. Model of the competition between the Friend LTR and the β-globin gene promoter for the interaction with the LCR. (*Top*) Diagram of the postulated interaction of the LCR with the β-globin gene. The effect of the LCR on expression of the β-globin gene may be mediated through the effects of one 5′ HS or any combination of 5′ HSs. (*Center*) Diagram of the results of inserting the F-LTR/neo gene. In this situation, LCR-mediated enhanced transcription of the β-globin gene is suppressed by the insertion of the F-LTR/neo gene. (*Bottom*) Diagram of the restored interaction between the β-globin gene and the LCR following FLP-mediated deletion of F-LTR/neo.

postulated interaction between elements of the LCR and the F-LTR/neo gene that suppressed globin expression is reversible when the F-LTR/neo gene is removed. Finally, in regard to the general technique of using the FLP recombinase system to remove the selectable marker used in an HR, the presence of the remaining FRT does not appear to affect the function of the LCR.

One of the prevalent models for how the LCR controls the expression and switching of the β-like globin genes postulates promoter competition between the various β-like genes for interactions with the LCR (31–34). Our previous results (12) showing that the inserted F-LTR/hygro gene suppressed β-globin gene expression can be interpreted as a promoter competition between the F-LTR and β-globin gene for the interaction with the LCR. The results reported here further support the promoter competition model, since removal of the competitor restores normal β-globin transcriptional regulation (model diagrammed in Fig. 4). Clearly, however, these results also could be interpreted to support other models of LCR function.

Since selectable marker genes used previously and in this report were controlled by the F-LTR, we have not addressed the question of what precise elements are able to interact with the LCR to mediate the suppression of β-globin. Recent studies have implied that the LCR-mediated enhancement of β-like globin gene expression requires sites for binding the GATA-1 transcription factor in the promoters of these genes (35, 36). Interestingly, the F-LTR contains two GATA-1 binding sites, raising the possibility that the F-LTR GATA-1 sites may be key elements in the competitive suppression of β-globin expression in our homologous recombinants. This and related questions may be addressed in the future by using FLP recombinase to make further insertions into the single FRT remaining in the LCR.

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