Functional Properties of the β-Globin Locus Control Region in K562 Erythroleukemia Cells

By Anne M. Moon and Timothy J. Ley

In this report, we compare the function of the human β-globin locus control region (LCR) in three K562 erythroleukemia cell assays, including (1) a transient transfection assay for "classical" enhancer activity, (2) a colony assay that detects "productive integration events," and (3) an assay that detects the ability of LCR fragments to confer hemin inducibility on linked, stably integrated γ-globin promoters. Various LCR fragments were inserted into an expression vector consisting of an S-γ-globin promoter driving the neomycin phosphotransferase gene (γ-neo). Using these vectors, we determined that a 2.5-kb DNA fragment containing LCR sites I through IV (previously named μ locus activation region [μLAR]) had activity in all three assays; of the individual LCR sites, only site II was highly active in all three assays. One region within site II, consisting of tandem AP-1/NF-E2 consensus elements, had ~10% as much colony assay activity as the entire μLAR.

However, this region did not have detectable activity in a transient enhancer assay in uninduced K562 cells, nor was it capable of conferring hemin inducibility on linked γ-globin promoters in stably transfected cells. Finally, we tested the ability of the μLAR to activate promoters (β-globin and cathepsin G) that are not normally expressed in K562 cells. β-neo was minimally activated by the μLAR in transient transfection experiments. The μLAR increased the number of stably transfected colonies produced by β-neo, but the absolute number of β-neo colonies, with or without the μLAR, was approximately 10% to 20% that of γ-neo. In contrast, a minimal cathepsin G promoter was activated by the μLAR in K562 cells. Our results suggest that LCR functions are dependent in part on the environments and the promoters with which the LCR is tested.

© 1991 by The American Society of Hematology.
complex rearrangements and deletions of the linked human β-globin gene. Ryan et al.\textsuperscript{1} and Curtin et al.\textsuperscript{2} have demonstrated that site II is highly active in transgenic mice; the studies of Ryan et al.\textsuperscript{1} showed site II to be about half as active as all four LCR sites together, but those of Curtin et al.\textsuperscript{2} suggested that site II alone was necessary and sufficient for full LCR activity.

In this report, we extend these studies and directly compare the individual LCR elements in three K562 cell assays that test LCR functions with or without stable integration into the genome. Only LCR site II is active in all three assays. Furthermore, the LCR appears to interact differently with different promoters in the K562 cell environment. These studies suggest mechanisms by which the LCR may function in erythroid environments.

**MATERIALS AND METHODS**

**Expression vectors.** The γ-neo and β-neo expression vectors were constructed as previously described.\textsuperscript{2,3} They contain either the γ-β-globin promoter (−299 to +36) or the β-globin promoter (−375 to +46) driving the gene for neomycin phosphotransferase (neo). The transcription units were isolated from the expression vector as a BamHI fragment (see Fig 1).

LCR-containing plasmids were derived from the “pSP65 μLAR” plasmid\textsuperscript{1} by first cleaving that plasmid at a unique EcoRI site, filling-in the ends with Klenow polymerase, and incorporating BamHI linkers to generate the plasmid “ppLAR Bam.” The γ-neo or β-neo transcription units, derived as BamHI fragments from their respective expression vectors, were then ligated into the BamHI site of pUC9 or pSP65, and plasmids with γ-neo in both orientations relative to the μLAR sequences were isolated. When we refer to LCR fragments derived from the “μLAR” plasmids, we have retained the “LAR” nomenclature to indicate their source.

The I/II half-LAR was constructed by isolating the 1.4-kb HindIII fragment from the μLAR plasmid that contains DNAse I HS I and II. This fragment was subcloned into the HindIII site of pUC9 to generate pUC9 LAR I/II. The γ-neo transcription unit was then ligated into the BamHI site in both orientations to generate the γ-neo LAR I/II plasmids.

The III/IV half-LAR was generated by isolating the 1.1-kb HindIII-EcoRI fragment containing DNAse I HS III and IV, filling-in the ends with Klenow polymerase, and ligation the blunt-ended fragment into the HindIII site of pUC9. The γ-neo transcription unit was then inserted into the BamHI site of this plasmid in both orientations to generate the γ-neo LAR III/IV plasmids.

Individual LCR sites were isolated in pUC9 as follows.

**Site I.** Site II was deleted from pUC9 LAR I/II as a Pst I-Nco I fragment, and the remaining 3.4-kb fragment containing site I in pUC9 was blunt-ended with S1 nuclease, “polished” with Klenow polymerase, and religated to itself. The γ-neo transcription unit was inserted as a BamHI fragment.

**Site II.** The 0.68-kb Pst I-Nco I fragment containing site II (isolated during the construction of pLAR I) was blunt-ended with S1 nuclease, polished with Klenow polymerase, and inserted into the HindIII site of pUC9. The γ-neo transcription unit was inserted as a BamHI fragment in both orientations.

**Site III.** Site IV was deleted from pUC9 LAR III/IV as an Apa I-EcoRI fragment, and the remaining 3.2-kb fragment was blunt-ended with S1 nuclease, polished with Klenow polymerase, and religated to itself. The γ-neo transcription unit was subsequently inserted as a BamHI fragment.

**Site IV.** A 0.52-kb Apa I-EcoRI fragment containing site IV (generated during the construction of pUC9 LAR III) was isolated, and the ends of this fragment were made blunt with S1 nuclease and polished with Klenow polymerase. This fragment was ligated into HindIII-cleaved pUC9, and the γ-neo transcription unit was inserted as a BamHI fragment in both orientations.

The mutant (m)γ-neo expression vector contains the HPFH γ-β-globin promoter (−299 to +36) containing a C → G mutation at position −202.\textsuperscript{4,5} The mγ-neo transcription unit was released from the expression vector as a BamHI fragment and was subcloned into the BamHI site of pUC9 LAR Bam in both orientations.

A 3.0-kb genomic EcoRI fragment containing the human cathespin G gene (with 76 bp of 5' flanking sequence)\textsuperscript{7} was either inserted into the EcoRI site of pUC9 or the EcoRI site of pSP65, pUC9, and pSP65, pUC9.

**Tissue culture.** K562 cells were maintained in modified Iscove's Modified Eagle's Medium (IMEM) containing 5% fetal calf serum (FCS; Gibco, Grand Island, NY), 5% Control Process Serum Replacement-4 (Sigma Chemical Company, St Louis, MO), and 10 μg/mL gentamicin at 37°C in 5% CO\textsubscript{2} with 100% humidity.

**Electroporation.** K562 cells were transiently transfected by electroporation as previously described,\textsuperscript{11} with BTX-T-300 electroporator settings of 800 μF and 300 V. Cells were harvested 22 to 24 hours post-electroporation for RNA preparation and analysis. For the colony assay, cells were electroporated at 200 V and 600 microfarads, placed in nonselective complete media for 24 hours, and then were plated in complete media with 1 mg/mL G418 in 0.3% agarose. Visible colonies were quantitated 2 to 3 weeks after plating.

**RNA preparation.** Transiently transfected cells were quantitatively harvested, resuspended in 1 mL of 1X Hebs buffer,\textsuperscript{12} and transferred to an Eppendorf tube. The cells were then pelleted and resuspended in 50 μL of 1X Hebs. Total cellular RNA was

---

**Fig 1.** Diagram of expression vectors. DNA fragments containing either the γ-β-globin promoter (−299 to +36) or the β-globin promoter (−375 to +46) were inserted into the HindIII site of pUC or pSP65 so that the 3' end of the promoter was juxtaposed next to the HindIII site of the polylinker. These promoters (designated as a thin line) were linked with a DNA fragment containing the neomycin phosphotransferase (neo) gene with SV40 splice/poladenylation signals (III). The wavy line indicates pUC9 or pSP65 sequences. DNA fragments containing various test sequences were inserted upstream of the γ-neo or β-neo transcription units at a unique EcoRI site, or downstream of the transcription unit at a unique Sal I site located between the BamHI and HindIII sites. The genomic orientations of the inserted fragments were used to describe subsequent plasmids, as shown. Orientation A is upstream and tandem to the γ-neo transcription unit, B is upstream opposed, C is downstream tandem, and D is downstream opposed.
prepared from these samples by the guanidinium/acid-phenol extraction technique, as previously described.\textsuperscript{23,25} Probes were end-labeled for S1 nuclease protection assays as previously described.\textsuperscript{25} Probe-specific activities were \( \sim 1.0 \times 10^7 \) cpm/\( \mu g \) DNA.

\textit{S1 nuclease protection assay.} For transient expression analyses, all of the total cellular RNA derived from each transfection point (approximately 30 to 40 \( \mu g \)) was lyophilized with 50 to 100,000 cpm (5 to 10 ng) of probe DNA and analyzed exactly as described.\textsuperscript{23} All hybridizations were performed in probe excess.

\section*{RESULTS}

\textit{Vector design.} The wild-type \( \gamma \)-neo and \( \beta \)-neo expression vectors contain the human \( \gamma \)-globin promoter or the \( \beta \)-globin promoter driving the neomycin phosphotransferase (neo) gene as previously described\textsuperscript{23,27,29} (Fig 1). The transient transfection control plasmid consisted of the Rous Sarcoma Virus long terminal repeat driving the neo gene (RSV-neo).\textsuperscript{23} A unique \( BgI \) II site in the neo sequence of the \( \gamma \)-neo or \( \beta \)-neo plasmids was end-labeled to prepare probes that would detect correctly initiated transcripts derived from \( \gamma \)-neo or \( \beta \)-neo (see Fig 2B). Because \( \gamma \)-neo/\( \beta \)-neo and RSV-neo transcripts diverge at the 5' end of the neo sequence, correctly initiated \( \gamma \)-neo transcripts protect a \( \gamma \)-neo probe fragment of 375 nt, \( \beta \)-neo transcripts protect a \( \beta \)-neo probe fragment of 385 nt, and RSV-neo mRNA protects \( \gamma \)-neo or \( \beta \)-neo probe fragments of 325 nt. These

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{RNA and DNA analyses of pools of stably transfected K562 cells. (A) A Southern blot analysis was performed as previously described\textsuperscript{28} using DNA derived from various pools of stably transformed K562 cells. Ten micrograms of total cellular DNA (derived from pools of clones containing each of the indicated plasmids) was digested to completion with \textit{BamHI} and \textit{BglI}. DNA was then electrophoresed on a 1% agarose gel, blotted to Gene Screen Plus (New England Nuclear, Boston, MA), and hybridized with a 0.335-kb \textit{AluI} fragment extending from position –299 to position +36 of the \( \gamma \)-globin gene (black box below \( \gamma \)-gene). This fragment hybridizes with a 4.53-kb fragment containing the 5' end of the \( \gamma \)-gene (see diagram below) and a 2.3-kb fragment containing the 5' end of the \( \beta \)-gene. This probe also hybridizes with the \( \gamma \)-neo transcription unit, which does not contain any \( BgI \) I sites. The sizes of simultaneously run size markers, in kilobases, are shown on the left. Nontransfected K562 cells ("K562," lane 1) contain only the 4.5- and 2.3-kb endogenous \( \gamma \)-globin fragments. All of the pooled colonies containing \( \gamma \)-neo transcription units contain an additional \( \gamma \)-neo band at 2.7 kb. Note that plasmids containing pLAR in either the B or C orientation (lanes 3 and 4) have amplified numbers of \( \gamma \)-neo transcription units. (B) RNA analyses from the pools of K562 clones are displayed. K562 cells containing the indicated transcription units were treated with nothing (–) or with 20 \( \mu M \) hemin (+) for 5 days, and total cellular RNA was harvested. Ten micrograms of RNA was hybridized with an end-labeled \( \gamma \)-neo probe (shown at the bottom) and an end-labeled endogenous \( \gamma \)-globin probe as previously described.\textsuperscript{23,25} The correctly initiated \( \gamma \)-neo mRNA protects a probe fragment of 375 nt from S1 nuclease digestion, as indicated on the right side of the figure. Correctly spliced endogenous \( \gamma \)-globin mRNA protects a 209 nt endogenous \( \gamma \)-globin exon 2 probe fragment from S1 digestion.\textsuperscript{29} Nontransfected K562 cells ("K562," lanes 2 and 3) do not contain \( \gamma \)-neo mRNA. Endogenous \( \gamma \)-globin mRNA levels are induced in all pools (lanes 3, 5, 7, and 9), but levels of \( \gamma \)-neo mRNA are increased only in pools containing the pLAR linked to the \( \gamma \)-neo transcription unit (lanes 4 and 5 v lanes 6 and 7 or 8 and 9). The marker lane contains radiolabeled fragments from Ha\textit{III}-digested phage \( \phi X174 \). Sizes are in nucleotides.}
\end{figure}
vectors allowed us to quantitate levels of correctly initiated γ-neo or β-neo mRNA while controlling for transfection efficiency in transient assays. The same vectors were also used in the stable transfection assays described below. To test the effects of various fragments on γ-neo or β-neo transcription, DNA was subcloned into unique sites upstream or downstream of the γ-neo or β-neo transcription units as shown in Fig 1. “A” indicates that the test fragment is upstream and tandem to γ-neo or β-neo, “B” is upstream opposed, “C” is downstream tandem, and “D” is downstream opposed.

The $^\Delta$γ-globin promoter is enhanced by the LCR. We linked the $\mu$LAR fragment described by Forrester et al to the γ-neo transcription unit to establish our transient expression assay. The $^\Delta$γ-globin promoter is enhanced by the $\mu$LAR (see Figs 3 through 5); this effect is independent of the orientation of $\mu$LAR sequences relative to the transcription unit. While enhancement was detectable over a range of transfection voltages, the fold increase varied with different conditions. We chose to perform all experiments with transfection parameters that optimized detection of γ-neo mRNA derived from the nonenhanced promoter (800 µF and 250 to 300 V); we also demonstrated that the enhancer effect was independent of the quantity of DNA transfected, and determined that our assay was in the linear range using γ-neo DNA inputs from 5 to 30 µg (data not shown). We performed all experiments with 10 µg of the γ-neo construct or molecular equivalents of larger constructions.

The $\mu$LAR increases the number of G418-resistant colonies formed by γ-neo in stably transfected K562 cells. Table 1 contains data from a series of “colony assays” similar to those previously described. K562 cells were stably transfected and then plated on semi-solid selective media; the number of G418-resistant colonies per 10⁵ transfected cells was quantitated 2 to 3 weeks posttransfection. The number of colonies detected with γ-neo alone is approximately the same as that described by Acuto et al and by Rutherford and Nienhuis. Note that the whole $\mu$LAR markedly and consistently increases the number of colonies formed when linked in either orientation with the γ-neo transcription unit.

Mechanism of the colony assay effect. Figure 2A displays an autoradiogram of a Southern blot performed with genomic DNA derived from large pools of stably transfected K562 cell clones. A single probe derived from the $^\Delta$γ promoter region (black box) detects both the endogenous γ-globin loci and the integrated γ-neo transcription units (see diagram at bottom of the figure). The ratio of γ-neo copies to endogenous $^\Delta$γ copies is 0.4:1 for γ-neo (lane 2), 4.2:1 for γ-neo/$\mu$LAR B (lane 3), and 5.2:1 for γ-neo/$\mu$LAR C (lane 4). Amplified numbers of γ-neo/$\mu$LAR genomic copies were consistently found in several pools of clones. When the γ-neo mRNA levels shown in Fig 2B (lanes 4, 6, and 8, see below) are quantitated and corrected for γ-neo copy number, γ-neo is actually expressed at a 10-fold higher level per gene copy than γ-neo/$\mu$LAR B or C. These results indicate that the increase in colony number directed by the $\mu$LAR (Table 1) is not due to increased γ-neo mRNA levels in the γ-neo/$\mu$LAR clones; rather, the $\mu$LAR seems to increase the probability that individual γ-neo genomic integration events will yield the threshold levels of neomycin phosphotransferase activity required for cellular survival in 1 mg/ml G418.

Figure 2B shows the results of an S1 nuclease protection analysis of total RNA prepared from pools of stably transfected K562 cells with or without hemin induction. The amount of γ-neo mRNA is essentially the same in all of these uninduced pools, regardless of whether the $\mu$LAR is present (compare lane 4 with lanes 6 and 8). In lanes 5, 7, and 9, note that hemin treatment of these pooled clones causes increased amounts of endogenous γ-globin mRNA to accumulate. γ-neo alone is not inducible with hemin (lane 4 v 5), consistent with previous reports. However, when the $\mu$LAR sequences are added in either orientation, γ-neo becomes hemin inducible (compare lane 6 v 7, and lane 8 v 9; also see Figs 4 and 6); similarly, Donovan-Peluso et al have shown that the 3' β-globin enhancer can confer hemin inducibility on γ-neo in stably transfected K562 cells.

Enhancer and colony-forming activities of the $\mu$LAR in K562 cells are predominantly contained within LCR site II. We analyzed the function of individual LCR sites in both the transient and stable expression assays to determine the locations of LCR functions. Initially, we divided the $\mu$LAR into two halves, containing either sites I and II or sites III and IV. Figure 3A shows that only the I/II half-LAR has enhancer function in transiently transfected K562 cells. When we tested sites I and II individually, only site II has enhancer activity. Figure 3B shows that sites III and IV together or individually have no detectable enhancer activity in uninduced K562 cells.

Table 1 contains the results of a series of colony assays comparing the entire $\mu$LAR with the half-LCR or single-site-containing plasmids. Site I is as active in this assay as the entire $\mu$LAR. Additionally, site I has a small but consistent effect in the colony assay; the effects of sites I and II are not synergistic. Sites III and IV alone or in combination are inactive. We also tested pools of K562 cell clones for the hemin inducibility of γ-neo; like Sorrentino et al, we discovered that only site II conferred this property on linked γ-globin promoters (data not shown).

LCR site II contains a highly conserved region that has detectable activity only in the colony assay. Previous experiments by several groups have suggested that the enhancer region of site II is contained within a small region that contains tandem AP-1/NF-E2 consensus binding sites. Figure 7 shows an homology comparison of this region among the human, mouse, and goat species. The high level of conservation across species (greater than 90% over 60 bp) suggests that this area is critical for some aspect of LCR function. Figure 7 also depicts the sequences of oligonucleotides and mutations in this region tested by various groups (see legend, Fig 7 for details). For our studies, we chose to test the function of an oligonucleotide (Fig 7, line E) containing the AP-1/NF-E2/triple repeat motif (but excluding an adjacent SV40 enhancer core-like sequence) linked to the γ-neo transcription unit. Figure 4A shows the results of an S1 protection assay of total RNA
Fig 3. Transient transfection analyses of individual LAR sites in uninduced K562 cells. Ten micrograms of the \( \gamma \)-neo plasmid, or the molar equivalents of \( \gamma \)-neo plasmids with inserts, were cotransfected with 2 \( \mu \)g of RSV-neo into uninduced K562 cells as described in Materials and Methods. (A) Analysis of the whole \( \mu \)LAR versus sites I + II versus sites I or II individually are displayed. Note that sites I + II or site II alone have activity in this classical enhancer assay. (B) The results with the \( \mu \)LAR versus sites III + IV or sites III and IV alone are displayed. These sites have little or no effect in this assay.
Fig 4. Analysis of an oligomer derived from LCR site II in transiently and stably transfected K562 cells. The oligomer shown in Fig 7, line E, was inserted into the γ-neo expression plasmid in the orientations shown and transfected transiently into K562 cells using the parameters described in Materials and Methods. Enhancement of γ-neo by the μLAR is demonstrated in (A), lanes 1 through 3, and by site II alone in lanes 4 through 6. Plasmids containing the AP-1 sites alone are shown in lanes 7 through 9, whom no definite effect on γ-neo mRNA levels can be detected. (B) Pools of stably transfected K562 cells containing γ-neo or γ-neo with the indicated test fragments were treated with nothing or with 20 μmol/L hemin for 5 days, and total cellular RNA was isolated. Ten micrograms of RNA from each pool was analyzed with γ-neo and the γ-exon 2 probes in an S1 nuclease protection assay, as described in the legend of Fig 2. Lanes 1 through 5 contain analyses with pools that were untreated. Levels of γ-neo mRNA are essentially the same in all pools. Lanes 6 through 10 contain analyses of RNAs derived from cells treated for 5 days with hemin. Addition of the μLAR sequences permits the γ-neo transcription unit to become hemin inducible; addition of the AP-1/NF-E2 oligomer had a minimal effect on γ-neo inducibility.
Fig 5. Transient transfection analyses of β-globin and cathepsin G promoters in K562 cells. (A) The results of transient transfections of γ-neo, β-neo, or HPFH (my-neo) plasmids cotransfected with 2 μg of RSV-neo into K562 cells. The first lane demonstrates a transfection with 10 μg of RSV-neo alone; no γ-neo signal is present. Lanes 2 through 4 contain an analysis with 10 μg of transfected γ-neo or the molar equivalent of γ-neo with the μLAR. In lanes 5 through 7, a similar experiment is performed with the β-neo plasmid with or without μLAR sequences. Note that only a trace amount of β-neo is present in lanes 6 and 7, even though the cells were efficiently transfected, as demonstrated by the presence of RSV-neo bands. The specific activity of the β-neo probe was essentially equivalent to that of γ-neo, because the intensity of the RSV-neo protected band is the same for the γ-neo and β-neo probes. In lanes 8 through 10, analysis of a mutated (my)-globin promoter is shown; the myneo promoter contains a mutation at position -202 (C → G) associated with HPFH. This panel is identical to Fig 4 of an invited paper by M.J. Ulrich, A.M. Moon, T.J. Ley: Function of transfected globin promoters and the globin locus activator in K562 erythroleukaemia cells. Ann NY Acad Sci 612:207, 1990. Reprinted with permission. (B) An analysis of cathepsin G transcription in transiently transfected K562 cells is shown. Two micrograms of RSV-neo was cotransfected with a plasmid containing a 3.0-kb cathepsin G genomic fragment with or without pLAR sequences. Correctly processed cathepsin G exon 5 mRNA (present in 10 μg of U937 cell RNA, lane 5) protects a cathepsin G probe fragment of 139 nt, as previously described. No cathepsin G mRNA is detected in cells transfected with RSV-neo alone (lane 1) or RSV-neo and pUC9-cathepsin G without added LAR sequences (lane 2). Marker lanes contain Hae III-digested φX174 DNA.

prepared from uninduced K562 cells transiently transfected with γ-neo plasmids containing this oligomer. Note that the level of correctly initiated γ-neo mRNA is not detectably increased when the oligomer is linked with the γ-neo transcription unit. Table 2 contains the results of colony assays performed with the same plasmids; this region of site II has a reproducible effect in this assay (approximately 10% of that measured with the whole site II fragment). We also created a G → C mutation (underlined in Fig 7, line E) in the AP-1/NF-E2/triple repeat motif. This mutation abolishes the activity of this oligomer in the colony assay (Table 2). Figure 4B shows the results of an S1 protection assay performed with total RNA derived from pools of stably transfected K562 cells treated with or without hemin for 5 days. The AP-1/NF-E2/triple repeat region of site II is not sufficient to render the linked γ-globin promoter hemin inducible in K562 cells (compare lanes 7 and 8 with lanes 9 and 10).

The μLAR interacts differently with two promoters that are not normally active in K562 cells. Neither the endogenous β-globin genes nor transfected β-globin promoters or genes are expressed in K562 cells. Berg et al have
reported that deletion of the β-globin promoter to position -233 results in increased activity of this promoter in induced K562 cells, suggesting that the β-globin promoter is repressed. We tested the function of a β-globin promoter extending from position -375 to position +46 driving neo (β-neo) in the transient expression assay or the colony assay. The whole μLAR fragment was added to the β-neo plasmid to determine whether LCR sequences could enhance β-neo.

The results of transient expression analyses with β-neo are shown in Fig 5A. In lanes 1 through 3, the effect of the μLAR on γ-neo function is again demonstrated. The results with β-neo are shown in lanes 4 through 6; a very faint β-neo signal can be detected only in the lanes containing μLAR sequences (lanes 5 and 6). To determine whether the β-neo plasmids functioned properly in an adult erythroid environment, both γ-neo/μLAR and β-neo/μLAR containing plasmids were stably transfected into mouse erythroleukemia cells; both γ-neo and β-neo were shown to function at approximately equivalent levels (data not shown).

We have previously shown that wild-type γ-neo and γ(=-202 C→G) hereditary persistence of fetal hemoglobin (HPFH) γ-neo plasmids function at equivalent levels in transiently transfected K562 cells.23 We wished to determine whether
MOON AND LEY

Fig 7. Sequence analysis of a highly conserved region within LCR site II. The first three lines indicate sequence from the human LCR site II (positions -10896 -10825), from the mouse β-globin LCR site II (positions -9784 -9713), and from the goat LCR site II. Asterisks identify nucleotides that differ from the human LCR site II sequence. "AP-1" indicates the locations of the consensus AP-1 sites. The NF-E2 consensus site(s) contain an additional two base pairs upstream from the consensus AP-1 site. TR indicates the presence of a sequence that is identical to a small region within the SV40 enhancer. Lines A, B, C, and D demonstrate sequences used by other investigators to study the function of this region. Line A contains the sequence of a scrambled mutation (within the context of the whole site II) that abolished site II function in K562 cells. Line B contains a sequence of an oligomer used by Ney et al. and Sorrentino et al. in studies of site II function in K562 cells. Line C contains the sequence of an oligomer used by Talbot et al. in MEL cells and transgenic mice. Line D contains the wild-type oligomer used by Moi and Kan. 

Table 2. Activity of the AP-1/NF-E2 Region of LAR Site II in the K562 Cell Colony Assay

<table>
<thead>
<tr>
<th>Transfected Plasmid</th>
<th>Average Colonies per 10⁶ Cells</th>
<th>Fold Increase Relative to γ-neo</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt;1</td>
<td>---</td>
</tr>
<tr>
<td>γ-neo</td>
<td>11 ± 4</td>
<td>8</td>
</tr>
<tr>
<td>γ-neo/LAR II B</td>
<td>1,480 ± 240</td>
<td>2</td>
</tr>
<tr>
<td>γ-neo/LAR II C</td>
<td>1,470 ± 600</td>
<td>2</td>
</tr>
<tr>
<td>γ-neo/AP-1 A</td>
<td>108 ± 48</td>
<td>8</td>
</tr>
<tr>
<td>γ-neo/AP-1 D</td>
<td>139 ± 85</td>
<td>8</td>
</tr>
<tr>
<td>γ-neo/γ-AP-1 C (mutant)</td>
<td>11 ± 8</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 3. Activity of γ-neo versus β-neo in the K562 Cell Colony Assay

<table>
<thead>
<tr>
<th>Transfected Plasmid</th>
<th>Average Colonies per 10⁶ Cells</th>
<th>Fold Increase Relative to γ-neo or β-neo</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt;1</td>
<td>---</td>
</tr>
<tr>
<td>γ-neo</td>
<td>20 ± 2</td>
<td>2</td>
</tr>
<tr>
<td>γ-neo/μLAR B</td>
<td>2,000 ± 141</td>
<td>2</td>
</tr>
<tr>
<td>γ-neo/μLAR C</td>
<td>1,350 ± 210</td>
<td>2</td>
</tr>
<tr>
<td>β-neo</td>
<td>4 ± 1</td>
<td>2</td>
</tr>
<tr>
<td>β-neo/μLAR B</td>
<td>360 ± 78</td>
<td>2</td>
</tr>
<tr>
<td>β-neo/μLAR C</td>
<td>305 ± 64</td>
<td>2</td>
</tr>
</tbody>
</table>

Experiments were performed as described in Table 1. γ-neo/AP-1 plasmids contain the oligomer shown in Fig 7, lane E. The mutant plasmid contains a G → C substitution at the position underlined in Fig 7, line E.

The mutant HPFH γ-globin promoter (expressed in fetal and adult red cell precursors) interacted with the μLAR in the same way as the wild-type γ-globin promoter. The result of this experiment is shown in Fig 5 lanes 7 through 9; μLAR sequences clearly act as a transient enhancer of the my-globin promoter. The function of the wild-type and HPFH promoters is essentially identical in K562 cells.

Table 3 shows the results of K562 colony assays performed with γ-neo or β-neo plasmids with or without attached μLAR sequences. The "basal" level of β-neo activity in the colony assay was considerably lower than that of γ-neo, similar to previous reports. However, μLAR sequences clearly increased the total number of β-neo colonies, and the fold increase in colonies with β-neo versus β-neo/μLAR was similar to that observed with γ-neo versus γ-neo/μLAR. The absolute number of colonies obtained with β-neo/μLAR was only about 10% to 20% that obtained with γ-neo/μLAR containing plasmids. Even so, β-neo/μLAR mRNA levels were approximately the same as γ-neo/μLAR, and β-neo/μLAR was as inducible as γ-neo/μLAR in pooled K562 clones (data not shown).

The cathepsin G gene encodes a neutral serine protease normally found in the azurophil granules of polymorphonuclear granulocytes; this gene is expressed only at the promyelocyte stage of myeloid maturation. Previous experiments have shown that this gene is not expressed at detectable levels in K562 erythroleukemia cells. Therefore, we wished to determine whether the μLAR could activate expression of another gene that is not normally expressed in K562 cells. We inserted a 3.0-kb EcoRI fragment containing the genomic cathepsin G gene into a μLAR-containing plasmid. The cathepsin G fragment used included 76 bp of 5' flanking sequence and approximately 300 bp of 3' flanking sequence. When this gene was transiently transfected into K562 erythroleukemia cells, as shown in Fig 5B, correctly spliced cathepsin G mRNA molecules containing exon 5 were easily detected only when the μLAR sequences were added in the "B" orientation. A longer exposure of the gel shown in this film showed that the C orientation of the μLAR also enhanced cathepsin G activity in K562 cells.
expression to a limited extent. The B orientation juxtaposed the cathepsin G promoter with site IV of the P-LAR plasmid, while the C orientation increased the distance between the cathepsin G promoter and the P-LAR sequences.

To determine whether the P-LAR could confer hemin inducibility on the minimal cathepsin G promoter, we stably cotransfected P-neo and the cathepsin G plasmids into K562 cells. Cotransfection of the cathepsin G/P-LAR plasmids increased P-neo colony numbers by 15- to 20-fold, implying that the P-neo and cathepsin G/P-LAR plasmids integrated into the same genomic sites. No effect on P-neo colony number was observed when the non-P-LAR-containing cathepsin G plasmid was cotransfected (data not shown). Pools of clones were obtained and treated for 5 days with hemin. RNA was harvested and cohybridized with three probes: P-neo, P-exon 2, and a genomic cathepsin G probe that detects correctly spliced cathepsin G exon 5 mRNA. Figure 6 shows that no cathepsin G mRNA is detected in K562 cells unless cells contain transfected cathepsin G/P-LAR sequences (compare lanes 1 through 8 with 9 through 12). When these pools are treated for 5 days with hemin, levels of cathepsin G mRNA increase substantially (compare lanes 9 and 11 with lanes 10 and 12), paralleling the hemin inducibility of P-neo and the endogenous P-globin genes.

DISCUSSION

We have analyzed the function of the human LCR in three K562 cell assays. A 2.5-kb fragment containing LCR sites I through IV increases the level of correctly initiated P-neo mRNA in transiently transfected K562 cells, increases the number of G418-resistant K562 cell clones obtained with P-neo, and renders linked promoters hemin inducible. Of the individual LCR sites, site II alone has activity in all three assays; site I had a small effect only in the colony assay. An oligomer containing the AP-1/NF-E2/triple repeat region of site II had ~10% of the activity of the entire site II region in the colony assay, but did not contain detectable enhancer or inducibility functions. Sites III and IV had no activity in any of these assays. The P-LAR enhanced an HPFH P-globin promoter and a nonerythroid, cathepsin G promoter in transiently transfected K562 cells, but minimally enhanced the activity of the human P-globin promoter. However, the P-LAR did increase the number of K562 cell colonies formed by P-neo-containing vectors, and conferred hemin inducibility on a linked P-globin gene promoter.

We have demonstrated that the increase in the number of stable colonies resulting from the linkage of the P-LAR with P-neo is not due to increased P-globin promoter activity; the level of P-neo mRNA in uninduced pools of stably transfected clones is the same whether or not LCR sequences are present. So, while a threshold level of P-neo mRNA is required for colony formation, there is no evidence that the LCR elevates P-neo mRNA levels above this threshold. Although the productive events detected with P-neo alone may represent integration into "enhanced" sites, these integration sites do not contain all LCR functions, because P-neo alone is not hemin inducible unless the LCR is linked in cis.

Our results are consistent with earlier experiments performed by Moreau et al.66 and by Weintraub,68 who studied the effects of the SV40 enhancer in single transiently transfected cells. Both groups found that the SV40 enhancer increased the percentage of cells that expressed the transfected promoter; the enhancer did not increase the level of promoter activity in the transfected cells. Similar results have recently been reported by Fiering et al.,69 who showed that stably integrated promoters containing NF-AT binding sites were either "on" or "off" in individual Jurkat cells. Activation of the inducible promoter was dependent upon a threshold level of NF-AT protein in the cell; once that threshold was reached, the promoter "fired" at a high level of activity. Therefore, promoters seem to be on or off in a given cell at a given time; enhancers increase the probability that they will be on. These data suggest a model for how the LCR can increase numbers of colonies without affecting the level of P-neo mRNA within the colonies. The LCR may act to increase the probability that a transcription complex will form on the P-globin promoter; the P-globin promoter would then function at a level that is "preset" by sequences within the promoter. P-neo mRNA levels would therefore be the same whether or not LCR sequences were linked; the number of productive events would be influenced by the LCR, which may act to isolate the P-globin promoter from negative regulatory sequences that may lie nearby.

The dramatic effect of site II in the colony assay may reflect a unique chromatin organizing property of the LCR. Experiments in erythroleukemia cells and in transgenic mice suggest that the LCR contains DNA sequences that allow linked genes to be efficiently organized into transcriptionally competent domains that are minimally influenced by flanking DNA.69-72 The colony assay may detect this function by allowing us to measure single, productive integration events that result in the formation of a G418-resistant colony. We have shown that the AP-1/NF-E2/triple repeat region of site II has a fraction of the activity of site II in the colony assay, but that it has no detectable enhancer or hemin-inducibility functions. Further studies will be required to determine whether other regions of site II contain enhancer or inducer activities in K562 cells.

The function of individual LCR sites in the K562 environment is different from that observed in MEL cells or transgenic mice. Sites III and IV have no activity in any K562 cell assay (this report);52 Sorrentino et al.51 and we have shown that sites III and IV alone or together are unable to render a linked P-globin promoter hemin inducible in stably transfected K562 cells. However, Forrester et al.51 demonstrated that sites III + IV were approximately half as active as the entire P-LAR in MEL cells. Collis et al.77 and Novak et al.80 also found that sites II, III, and IV had activity in MEL cells, and Fraser et al.19 described activity of each of these sites in transgenic mice. Some authors have suggested that LCR sites III and IV may exert their effects only when integrated into chromatin, and that they would therefore not be expected function in transient expression.
systems. We and others\textsuperscript{8,12} have tested each site in transient and stable expression systems; the cumulative evidence from these studies suggests that the LCR sites may function differently because of factors that differ between the K562 and MEL environments. An alternative interpretation (that nonetheless implies developmental specificity) can be proposed if one considers that most experiments in K562 cells have been performed with the \( \gamma \)-globin promoter or gene, but that most MEL cell experiments have been performed with the \( \beta \)-globin gene. The LCR sites may, therefore, interact in different ways with different promoter elements, as suggested by Antoniou and Grosveld,\textsuperscript{4} or may interact with specific intragenic sequences.\textsuperscript{7} Regardless, these observations support the idea that the LCR may contain regions that function differently in different erythroid environments.

Previous studies by Ney et al,\textsuperscript{10,11} Moi and Kan,\textsuperscript{11} Sorrentino et al,\textsuperscript{12} and Talbot et al\textsuperscript{14} have directed attention to a region of site II containing two tandem AP-1/NF-E2 sites. Studies in transiently transfected K562 cells have used \( \gamma \)-globin promoters driving a luciferase reporter\textsuperscript{10,12}; Ney et al\textsuperscript{10} have shown that a scrambling mutation of 31 bp (see Fig 7, line A) containing the AP-1/NF-E2 region abolished the activity of a wild-type site II fragment. Moi and Kan\textsuperscript{11} and Ney et al\textsuperscript{10,12} independently showed that mutations in either of the AP-1 sites or the NF-E2 sites significantly reduced the function of site II in induced K562 cells. However, a single copy of the oligomer containing the AP-1/NF-E2 region (Fig 7, line D) contained only \( \sim 25\% \) of the inducible enhancer function of site II.\textsuperscript{11} Sorrentino et al\textsuperscript{12} first demonstrated the hemin-inducibility function of site II in stably transfected K562 cells; inducibility was abolished if the AP-1/NF-E2 region was scrambled. However, an oligomer (Fig 7, line B) containing this region was not able to direct full hemin-inducible expression unless it was linked with sites III and IV.\textsuperscript{12} Talbot et al\textsuperscript{14} studied the same region in stably transfected MEL cells and in transgenic mice. They found that deletion of the AP-1/NF-E2 region from site II abolished the function of this site in MEL cells. However, one to six copies of a 56-bp fragment containing this region (see Fig 7, line C) were insufficient to confer full inducibility on linked \( \gamma \)-globin genes in MEL cells, or to activate linked \( \beta \)-globin genes in transgenic mice. Collectively, these results suggest that the AP-1/NF-E2 region of site II is necessary but not sufficient for full activity of site II in erythroleukemia cells or in transgenic mice.

We have recently cloned and sequenced the murine homologue of LCR site II,\textsuperscript{12} and have observed a very high degree of conservation in the region containing the AP-1/NF-E2 sites (see Fig 7). We also noticed that a triple repeat motif lies “over” the AP-1/NF-E2 sites, and that an adjacent conserved region has homology with the SV40 enhancer.\textsuperscript{12} We designed an oligonucleotide to test the AP-1/NF-E2/triple repeat motif, and excluded the adjacent region with SV40 enhancer homology (Fig 7, line E). This oligomer had no detectable enhancer function, nor did it render the linked \( \gamma \)-globin promoter hemin inducible in stably transfected K562 cells (see Fig 4). However, it did reproduce a fraction of site II function in the colony assay (see Table 2). The significance of this low level activity was confirmed by a point mutation that changed both the triple repeat and the AP-1/NF-E2 motifs, eliminating the colony assay activity. Our results support the notion that this region is necessary but not sufficient for at least one site II function; we do not yet understand why our oligomer does not confer hemin inducibility on the \( \gamma \)-globin promoter, as did the oligomers of Ney et al,\textsuperscript{10} Moi and Kan,\textsuperscript{11} and Sorrentino et al\textsuperscript{12}; subtle differences between the actual sequences used (see Fig 7) or the assay systems may be important. Regardless, our studies also suggest that the AP-1/NF-E2 region does not contain all of the information necessary for complete site II function. Several other regions upstream from the \( \beta \)-globin gene clusters are highly conserved between mouse and human;\textsuperscript{2} these conserved regions will help to direct the search for other elements that contribute to LCR function.

Studies by Behringer et al\textsuperscript{46} and by Enver et al\textsuperscript{55,56} suggested that LCR sequences linked to individual globin genes can disrupt normal developmental regulation. In contrast, our experiments with the \( \beta \)-globin promoter indicate that the developmental repression of the \( \beta \)-promoter in the K562 fetal erythroid environment is not completely overturned by the \( \mu \)-LAR, a result that is similar to that described by van Assendelft et al;\textsuperscript{12} these authors demonstrated that stably integrated \( \beta \)-globin genes linked with the LCR were expressed at \( \sim 10\% \) of the level of the endogenous \( \gamma \)-globin genes in K562 cells. In our studies, the \( \beta \)-globin promoter is expressed at undetectable levels in transiently transfected K562 cells with or without the LCR; the absolute number of colonies produced by \( \beta \)-neo/\( \mu \)-LAR is 10\% to 20\% that of \( \gamma \)-neo/\( \mu \)-LAR. These studies confirm and extend the original studies of Acuto et al\textsuperscript{17} and of Rutherford and Nienhuis,\textsuperscript{5,28} who showed that the number of stable colonies formed with \( \gamma \)-neo was greater than \( \beta \)-neo in K562 cells. However, in the \( \beta \)-neo/\( \mu \)-LAR colonies the \( \beta \)-globin promoter is actively expressed, and it is inducible with hemin. Perhaps this result mimics the deregulation of the human \( \beta \)-globin gene by the LCR in transgenic mice, where the \( \beta \)-gene is inappropriately expressed in embryonic yolk-sac-derived erythroblasts.\textsuperscript{5} In contrast, the cathepsin G promoter is expressed at very high levels in transiently or stably transfected K562 cells if it is linked with the \( \mu \)-LAR. We doubt that this minimal none erythroid promoter fortuitously contains the positively regulated sites required for high level promoter function in a fetal erythroid environment. Therefore, our results support the notions that the LCR sequences themselves carry at least some of the information necessary for the hemin-inducibility response,\textsuperscript{45} and that the \( \beta \)-globin promoter is actively repressed in the K562 cell environment.

Experiments performed by a number of groups suggest that the LCR may have multiple functional properties in vivo and in vitro. Assays that can distinguish between these properties will be crucial for the separation and study of LCR functions. For example, if we can define the specific DNA elements that organize chromatin or confer hemin
inducibility, we can study these elements to determine how they exert their effects in vivo. The data presented here suggest that the colony assay may allow us to detect the chromatin organizing properties of the LCR; our goal is to determine whether the DNA sequences containing this function can be separated from those with enhancer and/or hemin-inducibility functions.

REFERENCES

31. Mignotte V, Wall L, deBoer E, Grosveld F, Romeo PH: Two
tissue-specific factors bind the erythroid promoter of the human
32. Li Q, Powers PA, Smithies O: Nucleotide sequence of 16
kilobase pairs of DNA 5' to the human e-globin gene. J Biol Chem
260:14901, 1985
33. Moon AM, Ley TJ: Conservation of the primary structure,
organization, and function of the human and mouse β-globin locus
activating regions. Proc Natl Acad Sci USA 87:7693, 1990
34. Li Q, Zhou B, Powers P, Enver T, Stamatoyannopoulos G:
β-globin locus activation regions: Conservation of organization,
structure and function. Proc Natl Acad Sci USA 87:8207, 1990
35. Dean A, Ley TJ, Humphries RK, Fordis M, Schechter AN:
Inducible transcription of five globin genes in K562 human leukemia
cells. Proc Natl Acad Sci USA 80:5515, 1983
36. Donovan-Peluso M, Young K, Dobkin C, Bank A: Erythro-
leukemia (K562) cells contain a functional β-globin gene. Mol Cell
Biol 4:2553, 1984
37. Fordis CM, Anagnou NP, Dean A, Nienhuis AW, Schechter AN:
A β-globin gene, inactive in the K562 leukemic cell, functions
normally in a heterologous expression system. Proc Natl Acad Sci
USA 81:4485, 1984
38. Young K, Donovan-Peluso M, Cubbon R, Bank A: Trans-
acting regulation of β-globin gene expression in erythroleukemia
expression of human globin genes introduced in K562 cells. EMBO J
4:927, 1985
40. Fordis CM, Nelson N, McCormick M, Padmanabhan R,
Howard B, Schechter AN: The 5'-flanking sequences of human
globin genes contribute to tissue-specific expression. Biochem
Biophys Res Commun 134:128, 1986
41. Lin HJ, Anagnou NP, Rutherford TR, Shimada T, Nienhuis
AW: Activation of the human β-globin promoter in K562 cells by
DNA sequences 5' to the fetal γ- or embryonic ζ-globin genes. J
Clin Invest 80:374, 1987
42. Berg PE, Williams DM, Qian R-L, Cohen RB, Cao S-X,
Mittelman M, Schechter AN: A common protein binds to two
silencers 5' to the human β-globin gene. Nucleic Acids Res
17:8833, 1989
43. Hanson RD, Connolly NL, Burnett D, Campbell EJ, Senior
RM, Ley TJ: Developmental regulation of the human cathepsin G
P: The SV40 72 base pair repeat has a striking effect on gene
expression both in SV40 and other chimeric recombinants. Nucleic
45. Weintraub H: Formation of stable transcription complexes
as assayed by analysis of individual templates. Proc Natl Acad Sci
USA 85:5819, 1988
46. Fiering S, Northrop JP, Nolan GP, Mattila PS, Crabtree GR,
Herzenberg LA: Single cells assay of a transcriptional factor reveals a
threshold in transcription activated by signals emanating from the T-cell antigen receptor. Genes Develop 4:1823, 1990
47. Antoniou M, Grosfeld F: β-globin dominant control region
interacts differently with distal and proximal promoter elements.
Genes Develop 4:1007, 1990
48. Behringer RR, Ryan TM, Palmiter RD, Brinster RL, Townes
TM: Human γ- to β-globin gene switching in transgenic mice.
Genes Develop 4:380, 1990
49. Enver T, Ebens AJ, Forrester WC, Stamatoyannopoulos G:
The human β-globin locus activation region alters the developmen-
tal fate of a human fetal globin gene in transgenic mice. Proc Natl
Acad Sci USA 86:7033, 1989
50. Enver T, Raich N, Ebens AJ, Papayannopoulos T, Costan-
tini F, Stamatoyannopoulos G: Developmental regulation of hu-
man fetal-to-adult globin gene switching in transgenic mice. Nature
344:309, 1990
51. van Assendelft GB, Hanscombe O, Grosfeld F, Greaves
DR: The β-globin dominant control region activates homologous
and heterologous promoters in a tissue-specific manner. Cell
56:969, 1989
Functional properties of the beta-globin locus control region in K562 erythroleukemia cells

AM Moon and TJ Ley