A major positive regulatory element has recently been identified 40 kb upstream from the human \( \alpha \)-globin gene. This regulatory element increases the expression of a linked \( \alpha \)-globin gene in mouse erythroleukemia cells and in transgenic mice. This element has been shown to share many of the structural and functional features of the locus control region (LCR) of the \( \beta \)-globin gene cluster. We have examined the activity of a small fragment from this regulatory domain (\( \alpha \)LCR) in a transient expression system. We show that this element is active as an enhancer in the erythroid environment of K562 cells. It is somewhat less effective as an enhancer in the nonerythroid environment of HeLa cells. This \( \alpha \)LCR fragment does not exhibit promoter specificity because it can activate both the promoter of its endogenous target gene and the heterologous promoter of the SV40 early genes. Although the major activity of this element is mediated by its interaction with the promoter of the \( \alpha \)-globin gene, some increase in activity is seen when structural elements from the 5' end of the \( \alpha \)-globin gene are included with the target promoter. In addition, we show that the enhancing activity of the \( \alpha \)LCR is potentiated by hemin-induction of K562 cells. Whereas phorbol esters that induce megakaryocytic differentiation of K562 cells markedly decrease \( \alpha \)-globin messenger RNA accumulation, they do not seem to have a negative effect on the activity of the \( \alpha \)LCR.

These studies suggest a role for the \( \alpha \)LCR in the basa

activity of the \( \alpha \)-globin gene in erythroid cells and in its increased expression seen with erythroid differentiation. The mechanism of negative regulation of \( \alpha \)-globin gene expression in phorbol-differentiated K562 cells does not appear to be mediated through the action of the \( \alpha \)LCR.

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MATERIALS AND METHODS

Cloning of the \( \alpha \)LCR. The \( \alpha \)LCR fragment used in this study was generated by the polymerase chain reaction (PCR). Two oli-
gonucleotide primers were designed based on the sequence of the 350-bp fragment that was shown by Jarman et al.\textsuperscript{22} to account for the major activity of the α-LCR. The sequence of primer I was 5'-TCT GGA ACC TAT CAG GGA C-3' and that of primer II was 5'-CTC TCA GAT AAA CAG GAG GGG G-3'. A 255-bp fragment was amplified from 0.5 μg of normal human DNA using a GeneAmp kit purchased from Perkin-Elmer (Norwalk, CT). The amplification parameters were as follows: 30 cycles, each consisting of denaturation for 1 minute at 94°C, annealing for 2 minutes at 45°C, and extension for 3 minutes at 72°C. The 255-bp amplification products were eluted from a preparative agarose gel, "blunt-ended" by a DNA polymerase reaction, and cloned in the Sma I cloning site of pBluescript II KS purchased from Stratagene (La Jolla, CA). The DNA sequence of the amplified fragment was determined by the Sanger dideoxy chain termination method\textsuperscript{24} using double-stranded plasmid DNA templates and the universal and reverse primers. The derived sequence differed from the published sequence at 2 nucleotides at position 59 (T for C) and position 106 (A for G).\textsuperscript{22} These differences are unlikely to be a result of infidelity of the Taq polymerase because they were identified in 3 of 3 clones that were sequenced. They are more likely to be silent polymorphisms. One of these PCR-amplified cloned fragments was subcloned in the experimental constructs described below.

**Experimental constructs.** The constructs used to study the activity of the α-LCR are schematically illustrated in Fig 1. The first construct, named PaMSCAT, consists of a fragment containing the α-globin gene promoter from position -575 to position +308 linked to chloramphenicol acetyl-transferase (CAT) coding sequences. This same construct was described earlier in a report from our laboratory.\textsuperscript{23} In
the second set of constructs, named (αLCR)αCAT, the αLCR fragment was cloned upstream of the α/CAT transcription unit in both orientations and downstream in the genomic orientation. In the third construct, named αα'CAT, DNA sequences between position +1 and +308 of the α-globin gene were deleted from the αα'CAT construct. This deletion was generated by exonuclease III/S1 nuclease digestion using the Erase-a-Base kit from Promega (Madison, WI). The endpoint of the deletion was determined by DNA sequence analysis using a custom designed synthetic oligonucleotide primer. The fourth construct, named (αLCR)α20CAT, consists of an SV40 promoter (without an enhancer) linked to CAT coding sequences. This construct was also purchased from Promega. The last construct used in this study was generated by cloning the αLCR in the genomic orientation upstream of the early SV40 promoter of the PSV40-CAT construct described above. This construct was named (αLCR)PSV40-CAT.

Cell culture, transfections, and CAT assays. Two different cell lines were used in this study. The first was an erythroid cell line known as K562 human erythroleukemia cells. These cells were grown in RPMI 1640 supplemented with 5% fetal bovine serum (GIBCO-BRL, Gaithersburg, MD), 5% controlled process serum replacement-4 (Sigma, St Louis, MO), and penicillin-streptomycin. These cells were transfected by electroporation as described by Ulrich and Ley. Cells (1 × 10⁷) were electroporated using 2 µg of the experimental CAT construct and 4 µg of a control Rous Sarcoma Virus (RSV)-β-galactosidase construct. The use of 2 µg of the test constructs instead of the usual 20 µg was necessary to keep the assays in the linear range as a result of the extremely powerful effect of the αLCR. Cells were harvested 24 hours after electroporation and the β-galactosidase activity of their lysates was determined as described. This activity was used to standardize the amount of extracts used in CAT assays that were performed as described by Gorman et al. Each CAT assay was repeated at least three times using three different plasmid DNA preparations with little variation from experiment to experiment. Quantitative data was obtained by cutting out the radioactive spots from thin layer chromatography plates from three different experiments and measuring their radioactivity in a scintillation counter.

The second cell line used in this study was the nonerythroid HeLa cells. These cells were grown in Dulbecco’s Minimal Essential Medium (DMEM) supplemented with 5% fetal bovine serum, 5% controlled process serum replacement-4, and penicillin/streptomycin. Petri dishes containing HeLa cells that were 50% to 70% confluent were transfected by DNA calcium phosphate coprecipitation as described. Twenty micrograms of the experimental construct and 20 µg of the control RSV-β-galactosidase construct were cotransfected in each experiment. Extracts were prepared 48 hours after transfection and used in a CAT assay after standardizing for the β-galactosidase activity.
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Fig 5. Effect of αLCR on SV40 promoter activity in K562 cells. Lane 1 represents CAT activity in K562 cells transfected with PSV-40-CAT and lane 2 represents CAT activity in K562 cells transfected with (αLCR)PSV40-CAT.

as described above. Each experiment was also repeated at least three times in HeLa cells.

In experiments in which CAT activity was compared in induced and uninduced K562 cells, 3 × 10⁶ cells were electroporated using 6 µg of the experimental CAT construct and 12 µg of the RSV-β-galactosidase construct. Immediately after transfection, the cells were split into three Petri dishes. Hemin was added to the first dish to a final concentration of 50 µmol/L, 12-0-tetradecanoylphorbol-13-acetate (TPA) was added to the second dish to a final concentration of 2 × 10⁻⁶ mol/L, and no inducing agent was added to the third dish. Cells were harvested 48 hours later and a CAT assay was performed using equal volumes of each cell extract. This was an appropriate standardization for this experiment since the cells from the three different extracts were electroporated in the same cuvette and thus had the same efficiency of transfection. This avoided the bias that would have resulted from β-galactosidase standardization because TPA was added to activate the RSV LTR promoter of the control β-galactosidase construct in K562 cells (data not shown).

Quantitation of α-globin messenger RNA (mRNA) by S1 analysis. K562 cells were induced by hemin or TPA as described above for 3 days. Total cellular RNA was isolated and S1 analysis was performed as we previously described. 39 The probe used for quantitating α-globin mRNA consisted of a 3' end-labeled 259-bp fragment of the α-globin gene that extends from the Nco I site of the translation initiation codon to the Tag I site in exon 2.

Determination of the site of initiation of transcription from the α-globin promoter. An S1 nuclease assay was used to identify the site of initiation of transcription of the transfected Psα³⁰⁸CAT genes. RNAs isolated from K562 cells transfected with either Psα³⁰⁸CAT or (αLCR)Psα³⁰⁸CAT were hybridized to a 5' end-labeled DNA probe. This probe, which extends from an EcoRI site in the 5' region of the CAT coding sequences to an EcoRI site immediately 5' to the aLCR, was derived from the (αLCR)Psα³⁰⁸CAT construct shown in Fig 2.

RESULTS

The primary aim of the first experiment was to determine whether the αLCR element that was shown earlier to increase the expression of a linked α-globin gene after stable transfer into MEL cells could function as a classical enhancer in a transient expression system. A secondary aim was to determine whether the interaction of the αLCR with the α-globin promoter would be influenced by the presence of a regulatory element in the 5' region of the structural α-globin gene. 25 In a previous study, we showed that this element may be responsible for the enhancer-independent expression of the α-globin gene in heterologous cells. 25 To that end, we cloned the αLCR in a construct that includes this regulatory element downstream from the α-globin promoter (Psα³⁰⁸CAT). The CAT activity in K562 cells transfected with the Psα³⁰⁸CAT construct was compared with CAT activity in cells transfected with three different constructs in which the αLCR was cloned upstream from the α-CAT gene in both orientations or downstream in the genomic orientation. Figure 2 shows very weak CAT activity in K562 cells transfected with the Psα³⁰⁸CAT construct (lane 1). This “apparent” weakness of the α-globin promoter is a result of the use of a very small amount of plasmid DNA and cell extracts in this experiment to keep the CAT assay in the linear range. A much higher CAT activity is seen upon transfection of K562 cells with the (αLCR)Psα³⁰⁸CAT constructs (lanes 2 through 4). Quantitative analysis shows that the activity of the α³⁰⁸CAT gene increases 25- to 33-fold when the αLCR is present on the same plasmid. This experiment shows that the αLCR does indeed function as a classical enhancer in a transient assay in K562 erythroleukemia cells.

We then isolated RNA from K562 cells transfected with these same constructs to analyze the accuracy of initiation of transcription from the α-globin promoter. In other words, we wanted to determine whether the observed increase in CAT activity in the presence of the αLCR is a result of an increase in the level of properly initiated α/CAT mRNA. We mapped the site of initiation of transcription of this mRNA by an S1 analysis assay. Properly initiated mRNA is expected to protect a 383-nt fragment extending from the true α-globin cap site to the EcoRI site within the CAT coding sequences. Figure 3 shows the results of such an S1 mapping experiment in which 50 µg of RNA isolated from K562 cells transfected with Psα³⁰⁸CAT (lane 1) or (αLCR)Psα³⁰⁸CAT (lane 2) was used. The expected 383-nt fragment is seen in both lanes, indicating proper initiation of transcription in the presence or absence of the αLCR. In addition, the level of accumulation of α/CAT mRNA is much higher in the presence of the αLCR, which confirms the findings of the CAT assay in Fig 2.

Because some enhancers function in a tissue-specific manner while others exert their enhancer effect equally well in a variety of cell types, we decided to examine the activity of
this αLCR in nonerythroid cells. In our next experiment, we transfected HeLa cells with the same four constructs used in the experiment illustrated in Fig 2. Here also, the αLCR did increase the activity of the α-globin promoter, as shown in Fig 4. However, the magnitude of the increase in activity was less in HeLa cells (8- to 16-fold) than in K562 cells (25- to 38-fold). These experiments suggest that the αLCR has a relative tissue specificity as demonstrated by its higher level of activity in an erythroid versus a nonerythroid cellular environment. We also investigated the promoter specificity of the αLCR. We compared the effect of the αLCR on an α-globin gene promoter with its effect on the SV40 viral early promoter. When CAT activity was compared in K562 cells transfected with PSV40-CAT (Fig 5, lane 1) and (αLCR)PSV40-CAT (Fig 5, lane 2), a 15-fold increase in the activity of the SV40 promoter is observed in the presence of the αLCR. This experiment demonstrates that the αLCR is also capable of enhancing the activity of a heterologous promoter in the erythroid environment of K562 cells.

It is not clear from the experiments described above whether the enhancing effect of the αLCR on the α-globin promoter depends on the presence of the previously described α-globin regulatory element present within the structural α-globin sequences. To resolve this issue, we deleted those structural sequences from the Pa-αCAT construct. The CAT activity generated by Pa-αCAT and (αLCR)Pa-αCAT was first compared in K562 cells, as shown in Fig 6A. There was a threefold decrease in the activity of the α-promoter as a result of the deletion of the structural α-globin gene sequences. The same constructs were transfected into HeLa cells, in which their CAT activities were found to be essentially identical (Fig 6B). These experiments suggest that the presence of the α-globin structural sequences has a modest positive effect on the activation of the α-globin promoter by the αLCR in erythroid cells in a transient expression assay.

Hemin is an agent that induces erythroid differentiation of K562 cells and increases the expression of the α-globin genes. TPA, on the other hand, induces megakaryocytic differentiation of K562 cells and results in the reduced expression of the γ-globin genes. To our knowledge, the effect of TPA induction on the expression of the α-globin genes in K562 cells has not been described. Figure 7 shows the effect of hemin and TPA induction on the accumulation of α-globin mRNA in the K562 isolate used in this study. In lane 1, 20 μg of mRNA from uninduced K562 cells was used in the S1 assay. The expected 97-nt fragment, which extends from the translation initiation codon to the 3’ end of exon 1, was protected. Lane 2 shows an almost complete absence of α-globin mRNA in 20 μg of RNA from TPA-induced K562 cells, whereas lane 3 shows the expected twofold to threefold increase in α-globin mRNA in hemin-induced K562 cells.

Finally, we studied the effect of induced differentiation of K562 cells on the activity of the αLCR. K562 cells were transfected with either Pa-αCAT or (αLCR)Pa-αCAT, followed by induction of differentiation with hemin or TPA. Figure 8 compares the activity of the αLCR in uninduced (lane 1), hemin-induced (lane 3), and TPA-induced (lane 5) K562 cells. There is an obvious increase in the activity of the αLCR in the presence of hemin. However, it is of interest to note that the αLCR remains very active in the presence of TPA. Table 1 summarizes the results of three such transfections using three different DNA preparations of the same constructs. Hemin induction results in a threefold increase in CAT activity in K562 cells transfected with (αLCR)Pa-αCAT, whereas it has essentially no effect on CAT

Fig 6. Effect of 5’ α-globin structural sequences on the activity of the α-globin promoter in the presence of the αLCR. (A) Lane 1 represents CAT activity in K562 cells transfected with (αLCR)Pa-αCAT and lane 2 represents CAT activity in K562 cells transfected with (αLCR)Pa-αCAT. (B) Lanes 1 and 2 represent CAT activity in HeLa cells transfected with the same constructs used in (A) in the same order.
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Fig 7. Effect of induction on α-globin mRNA accumulation in K562 cells. This autoradiograph represents a quantitative S1 analysis experiment in which RNAs from uninduced cells, TPA-induced cells, and hemin-induced cells were used in lanes 1, 2, and 3, respectively. The arrow denotes the 97-nt protected fragment of the α-globin probe.

activity in Pα-1CAT-transfected cells. This suggests that the increased activity of the native α-globin genes in hemin-induced K562 cells is probably mediated through the αLCR rather than the α-promoter. This is in marked contrast to the findings in TPA-induced K562 cells. In these cells, the αLCR remains quite active (22% conversion) even though the native α-globin gene is almost completely silenced (Fig 7). This suggests that the negative effect of TPA on α-globin gene expression is unlikely to be mediated at the level of the αLCR or the α-promoter.

DISCUSSION

The discovery of the β-globin LCR has provided a very powerful tool for investigating the regulation of genes within the β-globin cluster. This regulatory element has been studied very extensively in transgenic mice and in cultured erythroid cells using transient and stable expression systems. Its subunit structure has been analyzed in great detail. Of the four hypersensitive sites that make up this element, only HS2 has been shown to function as a classical enhancer in a transient expression assay. HS2 has been further dissected and its enhancer activity was shown to localize to an 18-bp region that contains two AP-1 sites that are separated by 10 nt. These AP-1 sites appear to bind the erythroid transcription factor NF-E2 in erythroid cells. A 46-bp fragment of HS2 containing the AP-1 sites was shown to mediate a 10- to 20-fold increase in the expression of a linked γ-globin promoter in hemin-induced cells as compared with uninduced cells.

The α-globin LCR has been discovered much more recently and its role in α-globin gene regulation is just beginning to be appreciated. Jarman et al localized the activity of this element in MEL cells to a single hypersensitive site 40 kb upstream from the β-gene. The 350-bp fragment containing this hypersensitive site was analyzed in detail and was shown to exert a level of activation similar to that of a complete αLCR in MEL cells in a stable expression assay. Whether this abbreviated fragment will be equally effective in transgenic mice remains to be determined. The αLCR shares several of the structural features of HS2 of the βLCR. This element, like βHS2, has paired binding sites for AP-1 in close association with GATA-I sites. However, in the αLCR the two AP-1 sites are inverted and the distance between them is increased.

In this report, we describe our studies of this abbreviated αLCR fragment in a transient expression system. We show that, like the HS2 of the βLCR, the αLCR can function as a classical enhancer in K562 cells. It increases the activity of the α-globin promoter between 25- and 38-fold, depending on its position and orientation (Fig 2). We also show that the increased activity of the α-globin promoter in the presence of the αLCR is associated with an increase in the level of properly initiated α/CAT mRNA (Fig 3). The αLCR fragment used in this study is also capable of activating the α-globin promoter in the nonerythroid HeLa cells between 8- and 16-fold (Fig 4). Therefore, this element by itself is probably not sufficient to impart complete erythroid specificity to α-globin gene expression. It is not surprising that a regulatory element containing 2 sites that could bind the ubiquitous family of AP-1-binding proteins would have some positive enhancer activity in a transient assay in HeLa cells. The higher activity in erythroid cells is probably a result of the effect of the erythroid specific NF-E2 nuclear factor. However, it should be noted that this element exists in a permissive chromatin configuration (ie, hypersensitive domain) in erythroid cells and a nonpermissive configuration in nonerythroid cells in vivo. It may be postulated that the sequences responsible for maintaining this element in a hypersensitive configuration
may contribute to the tissue-specific expression of the α-globin gene. It is not clear whether the stable integration of this abbreviated element in MEL cells would lead to the formation of a hypersensitive domain. However, hypersensitive sites do develop when a cosmid containing this element is stably integrated in the genome of MEL cells.

Tuan et al. have previously shown that the HS2 of the βLCR increases the activity of an SV40 promoter in HeLa cells up to 10-fold. In the same study, the activity of the ε-promoter in HeLa cells was not enhanced by the presence of the βHS2. On the other hand, Moi and Kan observed an 11-fold activation of a γ-promoter by βHS2 in HeLa cells. The reason for these discrepancies in measuring the activity of βHS2 in HeLa cells may be more apparent than real. They may have resulted from the use of different target promoters in the different studies. Alternatively, these apparently big discrepancies may result from minor differences in the very small denominator in these calculations. The denominator in this case is the weak activity of unenhanced β-like globin promoters in HeLa cells.

We have recently described an element within the 5′ region of the structural α-globin gene that may be responsible for the observed enhancer-independent expression of the α-globin gene in heterologous cells. The role of this element in the regulation of α-globin gene expression in vivo is still not clear. We have looked for a possible interaction between this element and the αLCR in a transient expression system. When the enhancing effect of the αLCR is compared using a target promoter that contains this element [(αLCR)PdCAT] versus a target promoter that lacks this element [(αLCR)-PdCAT], a threefold increase in the activity of the α-promoter is seen in the presence of this element in K562 cells (Fig 6A). No effect of this structural α-globin regulatory element is seen in HeLa cells (Fig 6B). These findings suggest that this element may play a small role in the activation of gene in heterologous cells.

Table 1. Effect of Differentiation of K562 Cells on Activity of αLCR

<table>
<thead>
<tr>
<th>% Conversion of 14C-Chloramphenicol</th>
<th>Uninduced</th>
<th>Hemin</th>
<th>TPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PdCAT</td>
<td>0.17 ± 0.09</td>
<td>0.2 ± 0.14</td>
<td>2.3 ± 1.6</td>
</tr>
<tr>
<td>(αLCR)PdCAT</td>
<td>13.6 ± 2.3</td>
<td>41.2 ± 0.8</td>
<td>22.5 ± 9.7</td>
</tr>
</tbody>
</table>

* Means of percent conversion of three different transfections ± standard deviation.

![Figure 8](image-url) Fig 8. Effect of differentiation of K562 cells on the activity of the αLCR. Lane 1 represents the CAT activity of (αLCR)PdCAT in uninduced K562 cells, whereas lanes 3 and 5 represent the activity in the same transfectants after hemin and TPA induction, respectively. Lane 2 represents the CAT activity of PdCAT in uninduced K562 cells, whereas lanes 4 and 6 represent the activity in the same transfectants after hemin and TPA induction, respectively.
the α-globin gene in vivo. Here also, it should be noted that this element does exist in a DNase I hypersensitive domain in erythroid cells, but not in nonerythroid cells. Whether the same sequences responsible for maintaining the αLCR in a DNase I hypersensitive domain are also responsible for the DNase I hypersensitivity of this element is not clear at present.

We also investigated the effect of induced differentiation of K562 cells on the activity of the αLCR. The enhancer activity of the αLCR was potentiated about threefold in the presence of hemin (Fig 8). This is very similar to the increase in the expression of the endogenous α-globin genes in hemin-induced K562 cells. This increase in α-globin gene expression was previously shown to be mediated at the transcriptional level. Thus, it is quite likely that the αLCR may be responsible for the transcriptional activation of the α-globin gene in hemin-induced K562 cells. It is interesting to note that the enhancer activity of βHS2 has previously been shown to be potentiated 10- to 20-fold after hemin induction of K562 cells. This is a higher level of activation than that of the endogenous ε- or γ-globin genes in hemin-induced K562 cells. It is not clear whether these observed differences in the potentiation of the αLCR and βHS2 in response to hemin are genuine differences in the activities of these elements or a result of differences in the constructs and/or differences in the transfected K562 cells.

We finally investigated the effect of TPA-induced megakaryocytic differentiation of K562 cells on the activity of the αLCR. Such induction was previously shown to result in a decrease in the expression of several markers of erythroid differentiation including the accumulation of γ-globin chains and γ-globin mRNA. This decrease in γ-globin mRNA has been shown to be mediated by a combination of transcriptional and posttranscriptional mechanisms. In this study, we demonstrate that the expression of the α-globin gene is also profoundly suppressed in TPA-induced K562 cells (Fig 7). The αLCR is at least as active in TPA-induced cells as in uninduced cells (Table 1). This suggests that the activity of the αLCR in K562 cells is not affected by TPA induction. This, in turn, suggests that the decrease in α-globin gene expression seen with TPA induction of K562 cells is not mediated by the αLCR. Such an effect may either be transcriptionally mediated by a different regulatory element or, alternatively, it may be mediated at a posttranscriptional level.

While this manuscript was in preparation, a new study describing the activity of the αLCR in a transient expression system has been published. Pondel et al. showed an effect of the αLCR on the α-promoter and the SV40 promoter in K562 cells similar to the one we describe in this study. They also demonstrated that the αLCR was less active in HeLa cells than in K562 cells. However, the level of activation they observed in HeLa cells was only twofold to threefold, as compared with the 8- to 16-fold we observed in this study. This may be a result of the use of different αLCR fragments in the two studies. Whereas we used an abbreviated αLCR fragment of 255 bp, Pondel et al. used a much larger 4-kb fragment containing the same hypersensitive site. It is conceivable that the larger fragment may contain elements that contribute to the tissue specificity of the αLCR. Alternatively, these differences may be a result of the small denominator, as described above. It is also interesting to note that they observed weak activity of the αLCR in uninduced MEL cells (threefold enhancement) that increased to 25- to 30-fold enhancement upon hexamethylene bisacetamide (HMBA) induction. These differences in the potentiation of the activity of the αLCR resulting from induction of K562 and MEL cells may reflect inherent differences in the inducibility of these two cell lines. MEL cells accumulate a low level of hemoglobin in the uninduced state that increases 10- to 20-fold after induction. K562 cells, on the other hand, accumulate a very significant amount of hemoglobin in the uninduced state that increases 1.5- to 5-fold after hemin induction. Thus, the observed differences in the degree of potentiation of the αLCR that are seen with induction in these transient assays are commensurate with the differences in the activation of the endogenous genes seen after induction of differentiation of the corresponding cell lines in culture. These observations support the hypothesis that the αLCR may play an important role in the increased expression of the α-globin genes that is associated with the induction of erythroid differentiation of leukemic cells in culture. Its potential role in the developmental regulation of the human α-globin gene in vivo remains to be elucidated.

REFERENCES


The major regulatory element upstream of the alpha-globin gene has classical and inducible enhancer activity

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