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Dual promoter activation by the human β-globin locus control region

(chromatin/DNase I-hypersensitive site/erythroid gene expression)

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ABSTRACT **The human β-globin locus control region (LCR) is necessary for high-level and position-independent expression of globin genes in erythroid cells. A variety of mechanisms have been proposed for the cis-activation of individual members of the β-globin gene family by the LCR located 10–50 kilobases upstream. It is not known, however, whether a given LCR can activate all developmentally appropriate globin family members on its chromosome or whether, within a given chromosome, the LCR must be committed to activating only a single gene. We have devised an experiment to distinguish between these possibilities. This experiment takes advantage of the fact that if two genes in a cluster are transcriptionally active and their promoters, therefore, are in a conformation hypersensitive to nucleases, restriction enzymes that cleave the promoters will excise the intervening chromatin fragment. The *Apa* I sites on human fetal $^{G}\gamma$- and $^{A}\gamma$-globin gene promoters are accessible to cleavage in nuclei from the human erythroleukemia cell line K562, which expresses these genes, but not in HeLa cells. We find that *Apa* I digestion leads to excision in high yield of the fragment spanning these promoters, showing that a LCR element is capable of sharing its activating function among members of a gene cluster on a single chromosome.**

At the 5′ end of the human β-globin locus are four DNase I hypersensitive sites that regulate the transcriptional activity of individual globin genes within the globin gene cluster (1, 2). This locus control region (LCR) was identified by its ability to confer position independence and copy number dependence on globin transgenes in mice and stably transfected cell lines (3). Subsequent studies showed that the individual DNase I-hypersensitive sites 2 and 3 (HS2 and HS3) (4–8) also have LCR activity. A naturally occurring chromosomal translocation in Hispanic thalassemia patients (9) and gene disruption experiments (10) have provided evidence that the LCR is necessary for formation and/or maintenance of DNase I hypersensitive sites at regulatory regions of the globin locus.

Although the LCR has certain characteristics reminiscent of classical enhancers—e.g., an abundance of transcription factor binding sites (6, 11, 12) and orientation-independent activity (13)—several lines of evidence argue that LCRs and enhancers may operate by distinct mechanisms. First, HS3 lacks enhancer activity in transient transfection assays (14) and, therefore, an element with the properties of a LCR is not also required to have intrinsic enhancer activity. Second, although HS2 has intrinsic enhancer activity (15), deletion of binding sites for the erythroid-specific transcription factor NF-E2 from HS2 decreases enhancer activity by ≈90% without a corresponding loss of the LCR effect (7).

Despite these differences between LCRs and enhancers, many of the same models proposed for enhancer action have also been suggested for LCRs. Thus, the widely invoked looping mechanisms proposed for transcriptional regulation by enhancers (16), in which enhancer- and promoter-bound proteins physically interact, have also been suggested to account for the action of LCRs. The β-globin LCR appears to serve all of the genes in the β-globin cluster; in the looping model, some variation in preference among active promoters might be expected, depending on the relative strength of interaction between promoter- and LCR-bound factors and upon the size of the loop. In the case of the two identical γ-globin promoters (Fig. 1), smaller loop size might favor the promoter closer to the LCR. Such "competitive" models for LCR activity have been proposed (17, 18) and tested (19). When the LCR is coupled to the γ- and β-globin genes and introduced into transgenic mice, both genes are expressed at relative levels that are roughly proportional to the inverse third power of the distance between the LCR and the promoter (19). This has been explained in terms of decreasing probabilities of loop formation.

"Tracking" mechanisms have also been suggested to explain the effects of the LCR (20, 21). In this view, transcription factors or disruptors of DNA and chromatin structure such as helicases would bind to the LCR and migrate to a downstream promoter by progressive movement along the DNA. This model again leads to the prediction that the promoter closest to the LCR should be favored. A third class of models involves action at a distance, through effects on supercoiling, for example. It is difficult to predict how such a mechanism would affect individual promoters.

The presence of the LCR raises another kind of question: In any given cell, does the LCR activate all of the β-globin genes in a cluster compatible with that cell lineage, or only one? One could imagine, for example, that within a chromosome a stable loop forms exclusively between the LCR and a single promoter or that a tracking mechanism can deliver activating factors to only one or the other of the promoters. Whether the choice were made randomly or according to the preferential selection rules described above, the outcome would be that only one of the globin genes would be active on a single chromosome. Alternatively, LCRs might function inclusively in such a way as to activate several genes in the cluster. To distinguish between the *exclusive* and *inclusive* mechanisms, we have devised an assay to ask whether a LCR can establish a transcriptionally competent state on multiple promoters simultaneously. Our results support an inclusive mechanism of LCR action whereby the activation properties of a LCR can be shared by multiple genes on a single chromosome.

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Abbreviations: LCR, locus control region; HS2 and HS3, DNase I-hypersensitive sites 2 and 3.

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MATERIALS AND METHODS

Isolation of Nuclei. The human erythroleukemia cell line K562 was propagated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. K562 cells (1×10^8) were harvested by centrifugation ($600 \times g$) for 5 min at 4°C, washed once by suspension in 20 volumes of ice-cold phosphate-buffered saline, and resuspended in 2 volumes of 10 mM Tris·HCl, pH 7.5/10 mM NaCl/3 mM $MgCl_2$/0.2% Nonidet P-40/10 mM dithiothreitol. After a 5-min incubation at 4°C, nuclei were collected by centrifugation ($1000 \times g$) for 5 min at 4°C. Nuclei were washed by gentle resuspension in 2 volumes of 10 mM Tris·HCl, pH 7.5/10 mM NaCl/3 mM $MgCl_2$/10 mM dithiothreitol and then collected by centrifugation ($600 \times g$) for 5 min at 4°C.

***Apa* I Sensitivity Assay.** Washed nuclei were resuspended in 0.9 ml of digestion buffer (10 mM Tris·HCl, pH 7.9/50 mM NaCl/10 mM $MgCl_2$/1 mM dithiothreitol, divided into 0.2-ml aliquots, and incubated with various amounts of *Apa* I (New England Biolabs, 20 units/μl) for 15 min at 30°C. Reactions were terminated by adding 0.5 ml of 10 mM Tris·HCl, pH 8.0/25 mM EDTA/1% SDS/0.04% proteinase K with rapid vortex mixing. After an incubation for 12 hr at 37°C, genomic DNA was purified by multiple phenol/chloroform extractions. Purified genomic DNA (25 μg) was digested to completion with *Eco*RI and analyzed by Southern blot analysis. To ask whether both γ-globin promoters were hypersensitive on a single chromosome (see Fig. 3), a more dilute nuclear suspension and a longer incubation time were used to increase the efficiency of *Apa* I digestion. Nuclei were isolated from 5×10^7 cells and were suspended in 0.9 ml of digestion buffer. The suspension was divided into 0.2-ml aliquots and the indicated amounts of *Apa* I were added for 20 or 40 min at 30°C. Reactions were terminated, and genomic DNA was purified as described above. Genomic DNA (25 μg) was digested to completion with *Kpn* I and analyzed by Southern blot analysis. The intensity of bands on Southern blots was quantitated with a PhosphorImager (Molecular Dynamics).

RESULTS AND DISCUSSION

Restriction Endonuclease Sensitivity of γ-Globin Promoters in K562 Cells. As the LCR is necessary for formation of DNase I-hypersensitive sites on globin promoters (9, 22), we reasoned that if a LCR can establish hypersensitive sites on two identical promoters in cis, the chromatin fragment spanning the two promoters could be excised from nuclei with restriction enzymes that cleave at the hypersensitive sites. On the other hand, if a LCR can activate only a single promoter in a gene cluster, one would observe cleavage at either of the two promoters in a mutually exclusive manner and, therefore, not excise the intervening chromatin fragment.

The organization of globin genes in the human β-globin locus is shown in Fig. 1. The human $^{G}\gamma$ and $^{A}\gamma$ fetal globin genes represent an ideal system for asking whether a LCR can activate two promoters in a gene cluster. They have identical proximal promoter elements, which are separated by about 5 kilobases and are transcriptionally active in human K562 erythroleukemia cells (23). It is not clear, however, whether both fetal globin genes are transcribed in the same cell. These genes encode fetal globin proteins which differ in only a single amino acid (24) and, therefore, it has not been possible to address this problem by directly assaying for the presence of $^{G}\gamma$ and $^{A}\gamma$ proteins in a single cell. In addition, even if both $^{G}\gamma$ and $^{A}\gamma$ genes are transcribed in the same cell, the $^{G}\gamma$ gene could be transcribed exclusively on one chromosome and the $^{A}\gamma$ gene on the other. As the LCR is required for efficient γ-globin promoter activity, and DNase I-hypersensitive sites are a signature of active promoters, we wished to determine whether the LCR could induce hypersensitive-site formation on both $^{G}\gamma$ and $^{A}\gamma$ promoters in a single gene cluster.

We identified *Apa* I sites on the endogenous γ-globin promoters and asked whether they were sensitive to *Apa* I cleavage when the restriction enzyme was added to intact K562 nuclei. We also asked whether these sites were accessible to nucleolytic cleavage in nuclei from HeLa cells, which do not express globin genes. Nuclei were incubated with various amounts of *Apa* I under conditions in which the nuclei remain intact (25). Genomic DNA was purified, digested with *Eco*RI, and analyzed by Southern blot analysis. Fig. 2*A* shows the predicted two parental DNA fragments (6982 and 2633 bp) and two *Apa* I–*Eco*RI fragments (1626 and 1606 bp) from the *Apa* I/*Eco*RI digests. As shown in Fig. 2*B*, strong cleavage at γ-globin *Apa* I sites was observed with

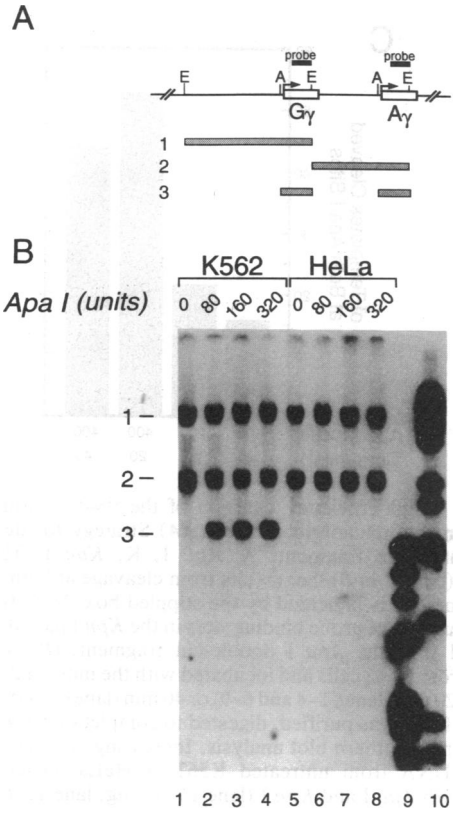

FIG. 2. Sensitivity of γ-globin promoters to *Apa* I cleavage in K562 nuclei. (*A*) Indirect end-labeling strategy for detecting *Apa* I fragments. Nuclei were isolated from K562 cells and incubated with *Apa* I (amounts indicated in *B*) for 15 min at 30°C. Genomic DNA was purified, digested to completion with *Eco*RI, and analyzed by Southern blot analysis. The two *Eco*RI parental fragments (1 and 2), and the *Apa* I–*Eco*RI fragments (3) are indicated by the stippled boxes. A, *Apa* I site; E, *Eco*RI site. (*B*) Southern blot. The two parental fragments (bands 1 and 2) and *Apa* I fragments (band 3) are indicated. Lanes 1–4, K562 DNA; lanes 5–8, HeLa DNA; lane 9, *Hae* III digest of ΦX174 double-stranded phage DNA; lane 10, *Bst*EII digest of λ phage DNA.

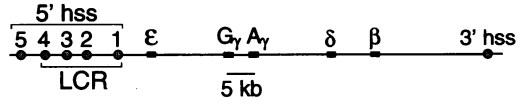

FIG. 1. Organization of the human β-globin locus. The human globin gene cluster consists of one embryonic gene (ε), two fetal genes ($^{G}\gamma$ and $^{A}\gamma$), one minor adult gene (δ), and one major adult gene (β). Four erythroid-specific DNase I-hypersensitive sites at the 5′ end of the locus (hss 1–4) and two ubiquitous hypersensitive sites (hss 5 and 3′ hss) are indicated. The four upstream hypersensitive sites comprise the LCR and regulate the chromatin structure and transcriptional activity of globin genes.

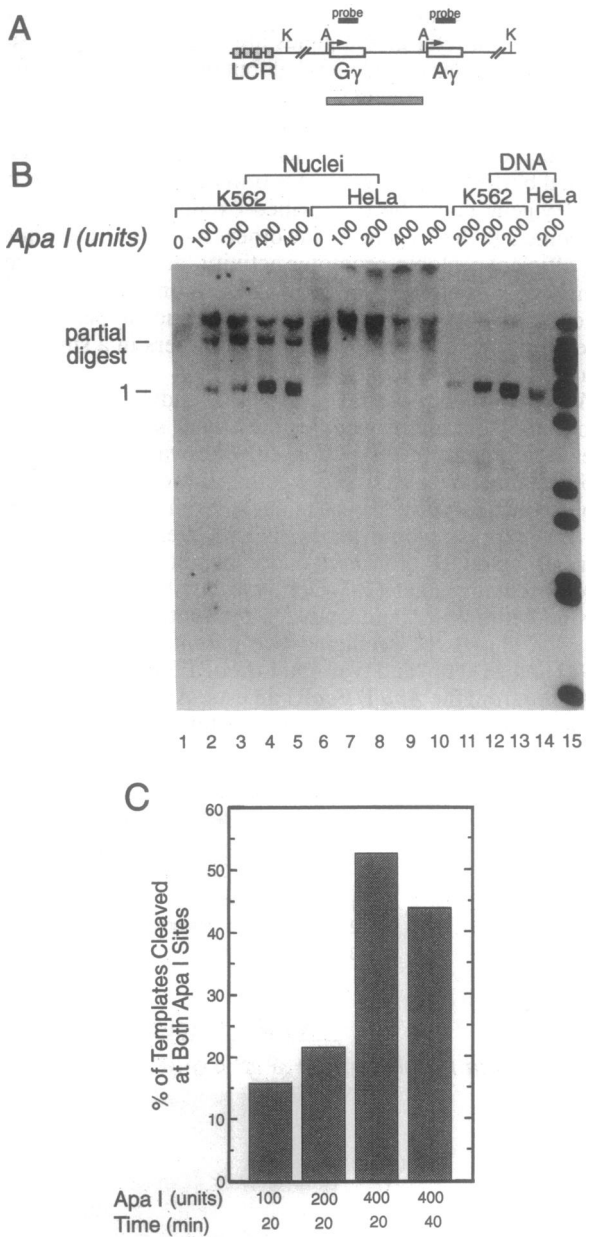

FIG. 3. High-efficiency excision of the $^{G}\gamma$-$^{A}\gamma$ chromatin fragment from the human globin locus. (*A*) Strategy for detecting the $^{G}\gamma$-$^{A}\gamma$ chromatin fragment. A, *Apa* I; K, *Kpn* I. The 4936-bp fragment (band 1 in *B*) that results from cleavage at both *Apa* I sites in the same cell is indicated by the stippled box. Note that there is a 2:1 molar ratio of probe binding sites in the *Kpn* I parental fragment compared with the *Apa* I double-cut fragment. (*B*) Nuclei were isolated from K562 cells and incubated with the indicated amounts of *Apa* I for 20 min (lanes 1–4 and 6–9) or 40 min (lanes 5 and 10) at 30°C. Genomic DNA was purified, digested to completion with *Kpn* I, and analyzed by Southern blot analysis. Increasing amounts of purified genomic DNA from untreated K562 or HeLa nuclei were also cleaved with *Apa* I and *Kpn* I (lane 11, 3.1 μg; lane 12, 6.2 μg; lane 13, 12.5 μg; lane 14, 12.5 μg). Lane 15, *Bst*EII digest of λ phage DNA. (*C*) Quantitation of excision efficiency. The Southern blot was analyzed with a PhosphorImager, and the percentage of total templates cleaved at both $^{G}\gamma$ and $^{A}\gamma$ sites was calculated as a fraction of the corresponding free DNA samples in lanes 11–13.

K562 nuclei, whereas no cleavage was observed with HeLa nuclei. The 1626- and 1606-bp fragments comigrated as a single band (fragment 3). The amounts of both $^{G}\gamma$ and $^{A}\gamma$ parental fragments decreased, demonstrating that both γ-globin promoters were accessible to *Apa* I. Digestion of deproteinized HeLa genomic DNA with *Apa* I resulted in cleavage products identical to those from K562 DNA (see below). The lack of cleavage in HeLa nuclei was not due to the failure of *Apa* I to access HeLa chromatin, as total genomic DNA isolated from *Apa* I-digested HeLa nuclei was digested to approximately the same degree as K562 DNA (data not shown).

Both γ-Globin Promoters Are Hypersensitive on a Single Chromosome. We used the *Apa* I sensitivity assay to ask whether both γ-globin genes were hypersensitive in a single gene cluster. A more dilute suspension of nuclei and longer incubation times were used in these experiments, to maximize the extent of *Apa* I cleavage. Nuclei were digested with *Apa* I, and the purified genomic DNA was then digested with *Kpn* I. Fig. 3*A* shows the fragment expected if both promoters are hypersensitive in the same locus: an *Apa* I–*Apa* I fragment of 4936 bp. In contrast, if only one promoter is hypersensitive in the locus, one would expect only the large parental fragment (39,923 bp) and fragments resulting from partial digestion of globin chromatin. As shown in Fig. 3*B*, the parental and partially digested fragments were detected as well as significant amounts of the 4936-bp fragment. The efficiency of cleavage was quantitated by analyzing the blot with a PhosphorImager (Molecular Dynamics). Approximately 50% of the total templates were cleaved at both *Apa* I sites to generate the 4936-bp fragment (Fig. 3*C*). A similar degree of cleavage was observed in two experiments. No *Apa* I cleavage products were detected in chromatin from HeLa nuclei (Fig. 3*B*, lanes 7–10). The γ-globin promoters in HeLa cells do, in fact, have *Apa* I sites, as cleavage of deproteinized genomic DNA with *Apa* I and *Kpn* I yielded the expected 4936-bp fragment (lane 14).

An Inclusive Mechanism for the Human β-Globin LCR. Fig. 3 shows that in at least half the chromosomes carrying the β-globin genes, both γ-globin promoters are in an active conformation. This is a lower limit because the *Apa* I sites in some nuclei seem to be altogether resistant to cutting (upper band in lane 5 of Fig. 3*B*), perhaps because the nuclei are impermeable to the enzyme or because neither gene is active. This is strong evidence in support of the inclusive mechanism, in which the LCR is capable of acting in cis "simultaneously" on both γ-globin promoters, and appears to eliminate the exclusive model, in which a given LCR must commit itself to the activation of one or the other of these genes.

This behavior is consistent with looping models in which the LCR/promoter interaction leading to promoter hypersensitivity is transient. It would be consistent with tracking models, provided that a successful encounter between the proximal promoter and the advancing activation signal did not derail the latter. It should be kept in mind that we do not know at what point in the cell cycle these critical determinative steps occur. It may be that the hypersensitivity of the promoters is established irreversibly during DNA replication, or it may be that hypersensitivity must be maintained during the life of the cell by continued interactions between LCR and promoters. Whatever the details of the mechanism, our results support models in which a single LCR can be shared among the promoters it is activating.

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