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Genetics

Synergism between hypersensitive sites confers long-range gene activation by the β-globin locus control region

(transcription/erythroid/chromatin/chromosomal domain)

EMERY H. BRESNICK* AND LINA TZE

University of Wisconsin Medical School, Department of Pharmacology, 387 Medical Science, 1300 University Avenue, Madison, WI 53706

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ABSTRACT **The human β-globin locus control region (LCR) consists of four erythroid-specific DNaseI hypersensitive sites (HSs) at the 5′ end of the β-globin cluster. The LCR functions over a long distance on chromosome 11 to regulate transcription and replication of the β-globin genes. To determine whether the HSs function independently or as an integrated unit, we analyzed the requirements for long-range transcriptional activation. If the HSs function independently, individual HSs would be expected to have long-range activity. In contrast, if long-range activity requires multiple HSs, individual HSs would have a limited functional distance. HS2, HS3, and a miniLCR containing multiple HSs, were separated from a γ-globin promoter by fragments of phage λ DNA. After stable transfection into K562 cells, HS2 had strong enhancer activity, but only when positioned close to the promoter. HS3 also had strong enhancer activity, although it was weaker than HS2 and more sensitive to the spacer DNA. The miniLCR had the strongest enhancer activity and functioned even at a distance of 7.3 kb. A model is proposed in which synergistic interactions between HSs confer long-range activation by creating a *stable* LCR nucleoprotein structure, which is competent for recruiting chromatin-modifying enzymes. These enzymes would mediate the well-characterized activity of the LCR to modulate chromatin structure.**

Regulation of transcription in higher eukaryotes often requires genetic elements that function over long distances on chromosomes. These enhancers or locus control regions (LCR) are characterized by clusters of transcription factor binding sites, many of which are also found on promoters (1). One mechanism to explain action-at-a-distance is the recruitment of basal transcription machinery through protein–protein interactions with factors bound to distal sites (2). The intervening DNA would form a loop, reducing the constraints for the protein–protein interactions (3). An alternative model assumes that the distal nucleoprotein complex modulates the chromatin structure of the promoter, and this structural transition is crucial for assembly of a preinitiation complex (4). Both mechanisms may be intertwined and work together (5).

A poorly understood issue relevant to the models described above is what parameters determine the active distance of a genetic regulatory element. The looping model assumes that the ability of the intervening DNA to bend and the strength of the protein–protein interactions are important. Parameters controlling the distance affected by a chromatin disruption mechanism are less clear. A localized chromatin disruption involving nucleosome disruption (6–8), histone modifications (9, 10), and/or changes in higher-order chromatin folding (11–13), could be propagated along a chromosome. We have been using the β-globin LCR to study requirements for long-range activation.

The human β-globin LCR consists of four erythroid-specific hypersensitive sites (HSs) at the 5′ end of the β-globin gene cluster on chromosome 11 (14, 15). The LCR controls the chromatin structure, transcription, and replication of the β-globin domain (16, 17). Molecular genetic studies have shown that the LCR confers copy-number-dependent and position-independent expression of globin transgenes in mice (16). In addition, a naturally occurring chromosomal translocation in Hispanic thalassemia removes part of the LCR, resulting in inactivation of the β-globin genes (17).

Inclusive and *exclusive* models have been proposed for how the LCR regulates the β-globin genes. *Inclusive* models assume that the activation property of the LCR can be shared by multiple promoters (18). In contrast, *exclusive* models assume a mutually exclusive interaction of the LCR with promoters. Competition between promoters for the LCR has been observed in various systems and has been hypothesized to be crucial for developmental regulation of the β-globin genes (19–22).

Three recent results support an *inclusive* mechanism. First, the LCR can generate HSs on Gγ and Aγ promoters on the same chromosome (18). Second, hybrid cells containing one copy of human chromosome 11 can express multiple globin genes within the same cell (23). Third, primary RNA transcripts from the embryonic, fetal, and adult β-globin genes were colocalized in single cells, containing one copy of a β-globin locus transgene (24). Based on the dynamic synthesis of the transcripts, it was postulated that the LCR engaged in transient looping with globin promoters. Transient looping would allow a LCR to be shared by multiple genes. At any given time, however, the LCR would interact with only a single promoter.

An unresolved issue is whether the four HSs function as a unit or independently. Deletion of HS2 or HS3 by homologous recombination in mice only has a small inhibitory effect on transcription of the β-globin genes (25, 26), suggesting considerable redundancy, if the HSs function together. If the HSs function independently, one would predict that each HS could activate transcription over long distances on a chromosome. In contrast, if the HSs function as a unit, the enhancer activity of each HS may be restricted to short distances. To distinguish between these possibilities, we have investigated the requirements for long-range activation using a stable transfection assay in K562 cells.

MATERIALS AND METHODS

Cell Culture. The human erythroleukemia cell line K562 (27) was propagated in Iscove's modified Eagle's medium (IMEM) (Biofluids, Rockville, MD), containing 10% fetal calf serum (FCS) (GIBCO/BRL), 2 mM glutamine, and gentamycin (25 μg/ml). Cells were grown in a humidified incubator at 37°C, in the presence of 5% CO_2. Hygromycin (0.2 mg/ml) was included in

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Abbreviations: IMEM, Iscove's modified Eagle's medium; HS, hypersensitive site; LCR, locus control region; YAC, yeast artificial chromosome.

medium for selection of stably transfected pools or clones of K562 cells.

Plasmid Construction. A *Sma*I–*Hin*dIII fragment of the human γ-globin promoter (−260 to +35) was subcloned into the *Sma*I and *Hin*dIII sites of pGL3basic vector (Promega) to yield pγluc. The *Kpn*I (7768)–*Bgl*II (9218) human HS2 fragment was modified with a *Kpn*I linker and subcloned into the *Kpn*I site of pGL3basic vector (Promega) to yield pHS2luc. A *Sma*I–*Hin*dIII fragment of the human γ-globin promoter (−260 to +35) was subcloned into the *Sma*I and *Hin*dIII sites of pHS2luc to yield pHS2γluc. *Mlu*I fragments of phage λ DNA were subcloned into the *Mlu*I site of pHS2γluc to yield pHS2(2.2)γluc, pHS2(3.4)γluc, pHS2(5.1)γluc, and pHS2(7.3)γluc. The 1.0-, 1.2-, 2.2-, 2.4-, and 5.1-kb phage λ inserts used in this study were derived from phage λ DNA by cleavage with *Mlu*I at the following positions: 1.0, 19996–20952; 1.2, 20952–22220; 2.2, 17791–19996; 2.4, 15372–17791; 5.1, 458-5548. The pHS2(3.4)γluc plasmid contains 2.4- and 1.0-kb phage λ inserts. The pHS2(7.3)γluc plasmid contains 5.1- and 2.2-kb phage λ inserts.

The pγluc plasmid was cleaved with *Sac*I, and an oligonucleotide containing an *Eco*RI site was cloned into the *Sac*I site to generate pGL3RIγluc. The *Hin*dIII (3266)–*Hin*dIII (5172) human HS3 fragment was subcloned into the *Hin*dIII site of plasmid Bluescript SK$^+$ (Stratagene) to yield pBHS3. The HS3 fragment was excised as an *Eco*RI–*Kpn*I fragment and subcloned into *Eco*RI–*Kpn*I sites of pGL3RIγluc to yield pHS3γluc. *Mlu*I fragments of phage λ DNA were subcloned into the *Mlu*I site of pHS3γluc to yield pHS3(2.2)γluc, pHS3(3.4)γluc, pHS3(5.1)γluc, and pHS3(8.5)γluc. The pHS3(3.4)γluc plasmid contains 2.4- and 1.0-kb phage λ inserts. The pHS3(8.5)γluc plasmid contains 5.1-, 2.2-, and 1.2-kb phage λ inserts.

The *Eco*RI–*Sal*I miniLCR fragment (28) was subcloned into plasmid Bluescript SK$^+$ to yield pminiLCR. The miniLCR fragment was excised as an *Eco*RI–*Kpn*I fragment and subcloned into *Eco*RI–*Kpn*I sites of pGL3RIγluc to yield pminiLCRγluc. This fragment contains the conserved core regions of HS1–HS4 and natural flanking sequences (coordinates: HS1, 10946–15180; HS2, 7764–9218; HS3, 4277–5122; HS4, 951-2199). *Mlu*I fragments of phage λ DNA were subcloned into the *Mlu*I site of pminiLCRγluc to yield pminiLCR(2.2)γluc, pminiLCR(2.4)γluc, pminiLCR(5.1)γluc, and pminiLCR(7.3)γluc. The pminiLCR-(7.3)γluc plasmid contains 5.1- and 2.2-kb phage λ inserts. The integrity of the plasmids was confirmed by restriction enzyme digestion analysis and partial DNA sequencing.

Transfections. For stable transfection assays, linearized test plasmids (3 μg) and a linearized selection plasmid, containing the thymidine kinase promoter driving a hygromycin resistance gene (0.3 μg), were cotransfected into K562 cells (1×10^7) as described (29). Test and selection plasmids were linearized at unique *Not*I and *Sal*I sites, respectively, in the vector. K562 cells were washed with ice-cold TBS (25 mM Tricine, pH 7.4/140 mM NaCl/5 mM KCl/0.5 mM $MgCl_2$/0.7 mM $CaCl_2$) and resuspended in TBS at a concentration of 1×10^7 cells per ml. DNA was electroporated into cells using a Bio-Rad Gene Pulser II apparatus at 260 V and 950 μF. After 48 h of growth in IMEM medium containing 10% FCS, stably transfected pools of cells were selected with hygromycin B (0.2 mg/ml) for approximately 4 weeks. For isolation of clonal cell lines, cells were grown for 48 h in IMEM medium containing 10% FCS, incubated with hygromycin for 1 week, and then subjected to limiting dilution. After selecting for hygromycin-resistant pools or clones, cells were propagated in IMEM medium containing 10% FCS and 0.1 mg/ml of hygromycin.

Luciferase Assay. Cells (12 ml of a near-confluent culture, 4.8×10^6-7.2×10^6 cells) were isolated by centrifugation at $240 \times g$ for 6 min at 4°C and washed by resuspension in ice-cold PBS (2 ml) and recentrifugation. The cells were lysed in reporter lysis buffer (100 μl) (Promega) for 15 min at 23°C, and the supernatant was isolated after centrifugation for 2 min at $18,700 \times g$. The luciferase activity generated by the supernatant in 30 s was determined with a Berthold Lumat LB9501 luminometer (Nashua, NH). Protein concentrations were estimated by the Bradford assay using γ globulin as a standard, and luciferase values were normalized by protein concentration.

Southern Blot Analysis. Genomic DNA was purified from clonal cell lines and analyzed by Southern blot analysis as described (30). DNA (15 μg) was digested to completion with *Hin*dIII and *Xba*I. After resolving on a 1.2% agarose gel, DNA was transferred to a Magnagraph nylon membrane and cross-linked to the filter with a Stratalinker (Stratagene). A random-primed luciferase DNA fragment [6×10^7 cpm/10 ml Quick-hyb (Stratagene) hybridization buffer] was used as a probe. The relative copy number of the integrated constructs was determined by analysis of the blot with a PhosphorImager (Molecular Dynamics). The lowest copy number was designated an arbitrary value of one.

RESULTS AND DISCUSSION

Individual Hypersensitive Sites Strongly Activate Transcription only When Positioned Close to a Promoter. The HS2 and HS3 subregions of the LCR can strongly activate transcription of adjacent promoters in stable transfection assays and transgenic mice (31–36). HS4 also has moderate enhancer activity (37), whereas no significant enhancer activity has been observed with HS1. To differentiate between models of LCR function where individual HSs function independently or as an integrated unit, a stable transfection assay was used in human K562 erythroleukemia cells. Constructs were prepared in which a γ-globin promoter was linked to a luciferase gene, in the absence or presence of HS2, HS3, and the miniLCR. The miniLCR contains sequences spanning HS1–HS4, but lacks sequences between the core sites of the intact LCR (28). To define the distance constraints for transcriptional activation by individual HSs, we systematically separated HS2, HS3, and the miniLCR from the γ-globin promoter, using variable-length fragments of phage λ DNA. The constructs were electroporated into K562 cells, and pools of stably transfected cells were isolated and assayed for luciferase activity.

As shown in Fig. 1*A*, the integrated γ-globin promoter had very weak activity. HS2 strongly activated the promoter when positioned near the promoter. Insertion of a 2.2-kb fragment of phage λ DNA between HS2 and the promoter had a small inhibitory effect on transcription. In contrast, larger fragments resulted in almost complete inactivation of the promoter. Thus, in contrast to the endogenous LCR in its native chromosomal environment, HS2 can only strongly activate a promoter over short distances on a chromosome in a stable transfection assay.

The distance constraints for the transcriptional activation property of HS3 were analyzed in a similar manner to that described above for HS2. The luciferase activity induced by HS3 was approximately 5-fold lower than with HS2 (Fig. 1*B*). In contrast to the HS2 construct, insertion of a 2.2-kb fragment of phage λ DNA between HS3 and the promoter almost completely inactivated the promoter. Restriction enzyme digestion analysis indicated that the orientation of the 2.2-kb phage fragments in the HS2(2.2λ)γluc and HS3(2.2λ)γluc vectors was identical, ruling out a trivial explanation for the differential inhibitory effect. The larger phage λ DNA fragments also strongly inhibited transcription. Thus, HS3 exhibited greater sensitivity than HS2 to the phage λ DNA fragments.

Because the intact LCR can function over long distances in its normal chromosomal context, it was critical to determine whether long-range activation could be achieved with the stable transfection assay. Thus, a series of constructs were prepared in which the miniLCR was separated from a γ-globin promoter by phage λ DNA fragments as described above. The overall activity decreased as a function of increasing distance between the promoter and the miniLCR (Fig. 1*C*). However, strong transcriptional activity was retained at the longest distance of 7.3 kb.

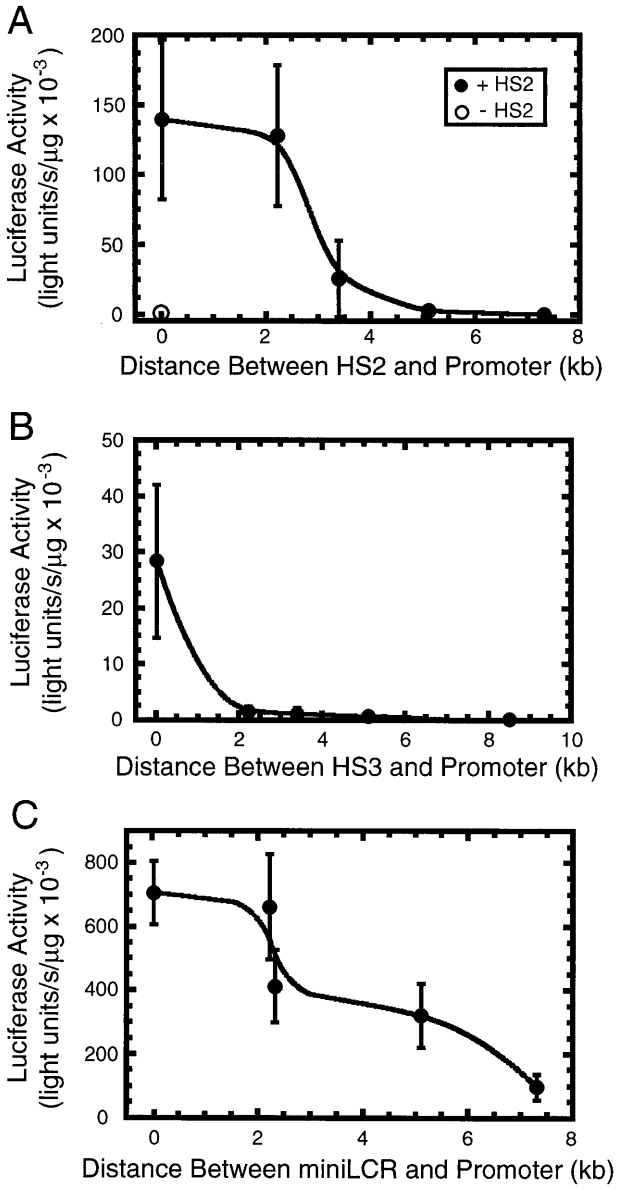

FIG. 1. Distance constraints for the transcriptional activation property of HS2, HS3, and the miniLCR. The three activating elements (*A*) HS2, (*B*) HS3, and (*C*) miniLCR, were subcloned upstream of a γ-globin promoter linked to a luciferase gene. Variable-length phage λ DNA fragments were subcloned between the activating element and promoter. The test constructs and a selection construct were linearized and co-transfected into K562 cells. Stably transfected pools of cells were isolated and assayed for luciferase activity as a measure of γ-globin promoter activity (mean ± SEM). The number of pools analyzed for the various constructs was: HS2γluc, 7; HS2(2.2)γluc, 7; HS2(3.4)γluc, 6; HS2(5.1)γluc, 7; HS2(7.3)γluc, 4; HS3γluc, 7; HS3(2.2)γluc, 8; HS3(3.4)γluc, 8; HS3(5.1)γluc, 5; HS3(8.5)γluc, 7; miniLCRγluc, 16; miniLCR(2.2)γluc, 12; miniLCR(2.3)γluc, 12; miniLCR(5.1)γluc, 12; miniLCR(7.3)γluc, 11. (*A*) The activity of a construct containing only the γ-globin promoter fused to the luciferase gene (pγluc) are shown by ○, whereas in *A–C* the activity of constructs containing activating elements are shown by ●. The luciferase activity of pγluc was 0.43 ± 0.49 light units per s/μg × 10^{-3} (n = 3).

The strength of the three activating elements can be ranked in the following descending order: miniLCR > HS2 > HS3. Previous observations have shown that HS4 has moderate enhancer activity (37) and HS1 lacks enhancer activity. Thus, the high level of activation achieved with the miniLCR may result from synergistic interactions between the HSs. Synergistic effects of LCR HSs on transcriptional activation have been observed previously when the regulatory elements were positioned close to an ε-globin promoter (38, 39). In addition, synergism of transcriptional activation properties of HS3 and HS4 was observed with a β-globin locus yeast artificial chromosome (YAC) in transgenic mice (40). A distinct question, however, is whether synergism allows the HSs to function over long distances on a chromosome. Since both HS2 and HS3 differed from the miniLCR in that they had very low activity at the longest distance tested, this is consistent with a requirement for synergism between HSs to generate the long-range activation function.

Fig. 2 shows a replot of the data of Fig. 1 to illustrate the strong synergistic transcriptional activation when regulatory elements are separated from the promoter by the 5.1-kb phage λ DNA fragment. When the regulatory elements are positioned next to the promoter (23-, 25-, and 25-bp away for HS2, HS3, and miniLCR, respectively), the activity of the pminiLCRγluc construct is 4.2-fold higher than the sum of the activities of the pHS2γluc and pHS3γluc constructs (Fig. 1). In contrast, when the regulatory elements are separated from the promoter by 5.1 kb, the activity of the pminiLCRγluc construct is 94-fold higher than the sum of the activities of the pHS2γluc and pHS3γluc constructs (Fig. 2).

Studies were performed with clonal cell lines to confirm the results described above, which used pools of stably transfected cells. Fig. 3*A* shows the luciferase activity of clonal lines containing pHS2(2.2λ)γluc and pHS2(7.3λ)γluc constructs. The luciferase activity was normalized to the relative copy number of the integrated template, determined by Southern blot analysis (Fig. 3*B*). Twelve of the 15 clonal lines containing pHS2(2.2λ)γluc exhibited high luciferase activity, whereas all 8 of the lines containing pHS2(7.3λ)γluc had very low activity. The average activity was 9,361 ± 3,001 and 89.0 ± 30.9 light units per s/μg per relative copy for pHS2(2.2λ)γluc and pHS2(7.3λ)γluc constructs, respectively (mean ± SEM, n = 15 and 8, respectively). The differential activity of the two constructs in clonal cell lines (105-fold) is consistent with the differential activity observed with pools of stably transfected cells (329-fold).

One could argue that the clonal lines are blocked at distinct stages of erythroid differentiation and, thus, variations in γ-globin promoter activity would be expected. To address this issue, five clones containing each construct were induced with 20 μM of hemin for 70 h. Hemin alters the differentiation state of K562 cells, resulting in higher levels of embryonic and fetal globin (27, 41). Constitutively "induced" clones would be expected to synthesize higher levels of globin than clones blocked at an earlier stage. Hemin could have a preferential stimulatory effect on clones blocked at an early stage. However, hemin treatment had less than a 2-fold effect on luciferase activity of all clones tested, inconsistent with this idea (data not shown).

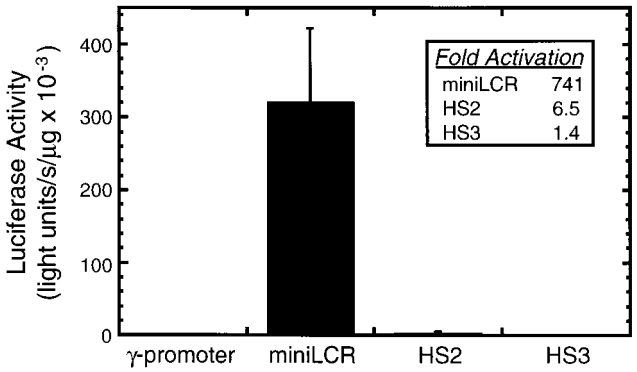

FIG. 2. Synergistic transcriptional activation by multiple hypersensitive sites. The luciferase values from Fig. 1, representing the activity of constructs containing a 5.1-kb phage λ insert between the activating element and promoter, are expressed in the graph. (*Inset*) The -fold activation of the test construct relative to the control plasmid containing the γ-globin promoter linked to the luciferase gene.

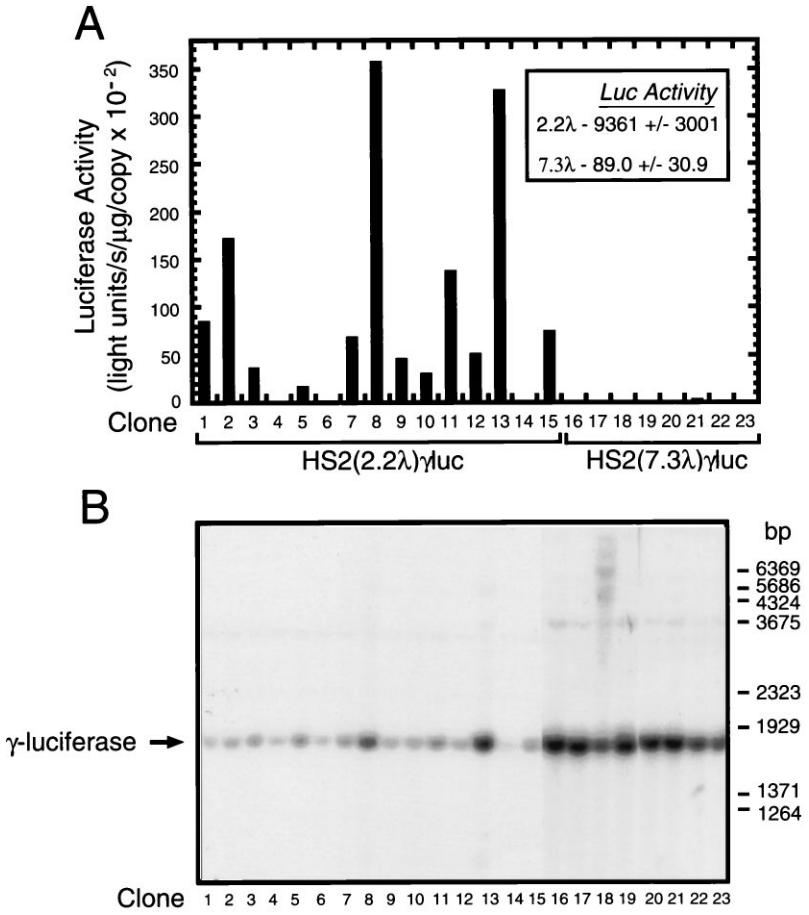

FIG. 3. Low luciferase activity of cells containing pHS2(7.3λ)γluc is unrelated to template copy number. (*A*) The pHS2(2.2λ)γluc and pHS2(7.3λ)γluc constructs were linearized and cotransfected into K562 cells. Stably transfected clonal cell lines were isolated and assayed for luciferase activity as a measure of γ-globin promoter activity. (*Inset*) Mean luciferase activity of each set of clones [mean ± SEM, $n = 15$ and 8 for pHS2(2.2λ)γluc and pHS2(7.3λ)γluc constructs, respectively]. (*B*) Southern blot analysis of integrated templates. Genomic DNA was purified from the clonal cell lines of *A* and digested to completion with *Hin*dIII and *Xba*I to excise a fragment containing the γ-globin promoter linked to the luciferase gene. Genomic DNA (15 µg) was analyzed by Southern blot analysis with a luciferase probe.

The low activity of integrated constructs containing large phage λ fragments could be explained by increasing the distance between the activating element and the promoter beyond a critical length. On the other hand, low activity could result from an effect of the phage DNA to induce heterochromatinization (42). Although we are not aware of a precedent for this phenomenon, we tested this hypothesis by comparing the activity of constructs containing phage λ DNA between the activator and promoter with constructs containing phage λ DNA upstream of the activator. If the phage λ DNA is a nucleation site for heterochromatinization, one would expect regulatory sequences of the activating element and surrounding sequences to become occluded by condensed chromatin, regardless of the position of the phage λ DNA in the vector. Thus, strong inhibition would be observed with both constructs. In contrast, if inhibition is due to separation of the activator from the promoter, the construct containing the phage λ DNA upstream of the activator should be minimally effected. This argument is equivocal, however, for constructs integrated as a tandem array. Bidirectional activation could potentially occur with tandemly arranged copies. Thus, even if inhibition resulted from altering the spacing between the activating element and the promoter, some inhibition would be observed with the phage DNA upstream of HS2, due to inhibition of activation of the upstream promoter. Of course, the probability of bidirectional activation for an integrated construct is unknown, because the functional distance of the regulatory elements is unclear.

A construct was prepared with the 5.1-kb fragment of phage λ DNA placed upstream of HS2, p(5.1λ)HS2γluc (Fig. 4*A*). The activities of this construct and the pHS2(5.1λ)γluc construct were compared in stably transfected pools of K562 cells (Fig. 4*B*). As observed in Figs. 1–3, strong inhibition resulted from placement of the phage DNA between HS2 and the promoter. In contrast, no inhibition was observed with the p(5.1λ)HS2γluc construct. This result is consistent with the interpretation that inhibition by long phage λ DNA fragments results from increasing the distance between the activating element and the promoter, rather than a general repressive function, such as inducing heterochromatin formation. This result also argues against the following trivial mechanism of inhibition. The phage DNA fragments could direct the integration of the constructs into regions of condensed chromatin, resulting in repression. Based on the requirement for placement of phage λ DNA between HS2 and the promoter, it is unlikely that the mechanism of inhibition is similar to the mechanism of silencing of globin constructs flanked by retroviral long terminal repeats (43).

Molecular Basis of Synergism Between Hypersensitive Sites. We have shown that regulatory complexes competent to strongly activate transcription of proximal promoters are incapable of functioning over long distances on a chromosome. In contrast, a cluster of complexes, the miniLCR, is competent for long-range activation. If the HSs normally function independently, one would expect that they could function over long distances. Since the long-range activation property requires multiple HSs, it is likely that the HSs normally function in a coordinated manner, rather than as independent regulatory units. Although the results favor a mechanism involving interacting HSs, we have not addressed whether all four HSs must interact. It is conceivable that only a

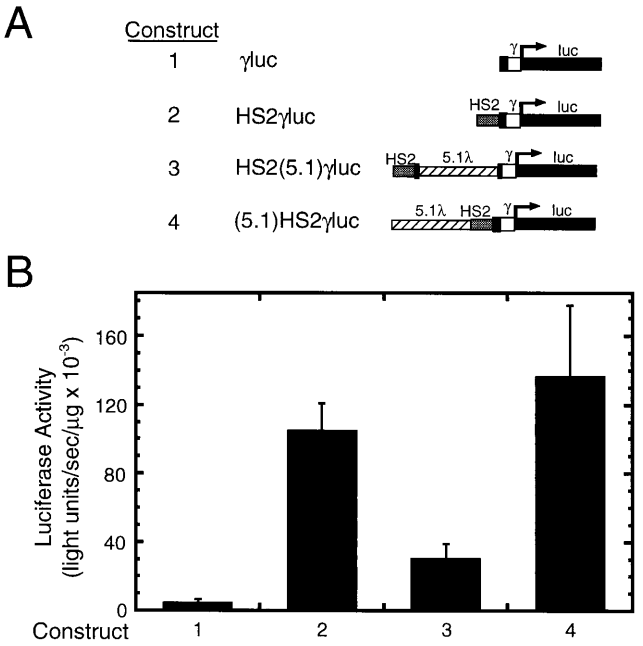

FIG. 4. Inhibition of transcriptional activation by placement of the 5.1-kb phage DNA fragment between HS2 and the promoter. (*A*) Structure of test constructs. (*B*) Test and selection constructs were linearized and cotransfected into K562 cells. Stably transfected pools of cells were isolated and assayed for luciferase activity as a measure of γ-globin promoter activity (mean ± SEM, n = 11, 9, and 9, and 9 for constructs 1–4, respectively).

subset of the HSs interact to confer long-range activation. It should be noted, however, that the cotransfected selectable marker could potentially influence the chromatin structure at the integration site. Thus, the requirements for long-range activation in this system may differ from the homologous chromosomal environment. A holo-LCR complex involving interactions between all four HSs has been proposed (44).

The magnitude of enhancer activity of the three activating elements correlated with their functional distance on a chromosome. This is consistent with the hypothesis that strong enhancer activity is required for long-range activation. It has been suggested that enhancer activity and the activity of the LCR to confer copy number-dependent and position-independent expression are separable properties. Talbot and Grosveld (45) showed that the enhancer activity of HS2 was greatly decreased upon mutation of the tandem NF-E2 sites, but despite this, the mutated HS2 retained the ability to confer copy number-dependent gene expression when positioned close to a promoter. Our results suggest that weak activating elements have a restricted functional distance and therefore lack long-range activity characteristic of LCRs. In addition, based on considerable variation in expression per copy commonly observed (Fig. 3), these results suggest that individual HSs lack the defining properties of the intact LCR. The variation implies that HS2 is not competent to overcome position effects and confer copy number-dependent expression in this system.

The results described herein are inconsistent with the proposal that HS3 has a unique domain-opening property (44), since HS3 was very sensitive to the phage λ DNA fragments. Based on a domain-opening function, HS3 would be expected to increase the accessibility of the γ-globin promoter to the basal transcription machinery and strongly activate transcription. The recent disruption of HS3 by homologous recombination (26), which only had a modest effect on the β-globin locus, also argues against a domain-opening activity of HS3. We suggest that efficient domain opening requires a group of HSs, rather than individual sites. This is not to say that a single HS could not influence chromatin structure over short distances. The recent report showing that HS deletions in β-globin locus transgenes resulted in loss of the position-independent function of the LCR (46) is consistent with our observations that the intact LCR is required for long-range activation in a *heterologous chromosomal environment*.

Various combinations of transactivators have been shown to exert synergistic effects on transcription. An obvious question is what is the specificity of HSs to generate synergism? Does the synergism require HS1, which lacks enhancer activity, or are HS2, HS3, and HS4 sufficient? A more fundamental question is whether synergism is determined by the number and strength of transcription factors associated with the complex. Transcriptional synergism can occur in a manner independent of the number of acidic activation domains bound to a promoter (47). It was suggested that cooperative DNA binding or changes in chromatin structure mediate synergism. *In vitro* transcription and transient transfection studies have provided evidence that recruitment of TFIIB to the preinitiation complex and an unidentified post-TFIIB recruitment step can be targets for synergism (48). In addition, because transcription factors can bind cooperatively to nucleosomal templates (49), synergism can also be explained by the assembly of stable complexes on a chromosome.

Synergism of LCR HSs may be related to the ability of the transacting factors to form a stable nucleoprotein complex on the LCR. The probability of stable complex formation would be significantly greater when multiple HSs are clustered within a chromosomal region. This may be analogous to the observation that multiple recognition sites for factors within a HS increases the probability of HS formation (50). A stable complex would confer a quantal activity, whereas an unstable complex would lack activity. Thus, differences in luciferase activity between cell populations may reflect an all-or-none phenomenon, in which the number of cells containing an active γ-globin promoter varies, rather than the promoter activity within each cell. All-or-none transcriptional responses may be quite common and have been observed in multiple systems (51–55).

Does the Intervening DNA Between an Activator and Promoter Modulate the Efficiency of Transmission of the Activation Signal? The strong inhibitory property of the phage λ DNA fragments in the stable transfection assay may not be representative of all DNA fragments. DNA fragments that differ in base composition and physical characteristics could have different efficacies *vis-a-vis* inhibition of promoter activation. DNA sequences from the endogenous β-globin locus may have evolved to allow efficient propagation of the activation function over a long distance on chromosome 11. The studies with phage λ fragments may facilitate the development of regulatory elements which, when associated with transgenes, function efficiently in various chromosomal environments. The observation that individual HSs can have a limited active distance in synthetic constructs after stable transfection also has implications for studies on the developmental specificity of HSs. In constructs containing a HS linked to two genes, the HS would have a strong preference for the proximal promoter.

LCR Function in Homologous Versus Heterologous Chromosomal Environments. Our results need to be reconciled with recent homologous recombination experiments that studied LCR function in the normal chromosomal environment of the β-globin locus (25, 26). These seminal studies showed that disruption of HS2 or HS3 by homologous recombination only has subtle effects on globin gene expression. One explanation may be that in a homologous chromosomal environment sufficient redundancy exists within the LCR to maintain an active locus, even when single HS elements are deleted.

In contrast to the endogenous β-globin locus, disruption of HS3 and HS4 in transgenic YACs, containing the human β-globin cluster, has a strong inhibitory effect on globin transcription in mice (40). Similarly, disruption of individual HSs from an intact LCR in a β-globin locus YAC construct, containing the complete β-globin locus, resulted in loss-of-position-independent globin

gene expression (46). However, a related study failed to observe significant inhibitory effects upon deletion of HS3 and HS4 from comparable YACs (56). One explanation for these disparate results is suggested by the experiments of Milot *et al.* (46). Constructs containing incomplete LCRs may be subject to chromosomal position effects. Nevertheless, it remains unclear how the HS requirements for LCR function compare between YACs and the endogenous mouse chromosome. Since all four HSs are not required for regulation of the β-globin locus in a homologous chromosomal environment, additional sequences within the endogenous locus may modulate the stability and efficacy of the endogenous LCR.

Mechanism of Long-Range Gene Activation by LCRs. Once the LCR complex has assembled on a chromosome, how does it regulate gene transcription? The activity of the LCR to generate an active domain may involve protein–protein interactions with promoter-associated components, chromatin disruption, or a combination of the two. The *inclusive* model of LCR function (30) assumes that the LCR is shared by multiple promoters within a domain. A second assumption is that the LCR is crucial for generation of an active domain but not for globin gene switching during development. One explanation for the molecular basis of sharing is that chromatin disruption increases the accessibility of all promoters and replication origins within the domain. The generation of an active domain would be a necessary prerequisite for the subsequent developmental regulation, conferred by local regulatory elements, such as promoters, enhancers, and silencers. Evidence that the LCR is not required for globin gene switching (57) is consistent with the *inclusive* model.

Although it is easy to envision how the cooperative binding of transcription factors to nucleosomes could disrupt histone–DNA contacts and thus facilitate the binding of transacting factors, the mechanism whereby the chromatin structure of an entire domain is affected is unclear. It seems reasonable to invoke the involvement of known chromatin modifying enzymes (58), such as histone acetyltransferases (59, 60) and the nucleosome remodeling complex, Swi/Snf (61, 62). What could be the basis for targeting these regulatory components to the β-globin cluster? The mechanism may be analogous to a recent report of a yeast transcription factor recruiting a histone acetyltransferase to a specific chromosomal site through protein–protein interactions (59). Furthermore, several transcription factors (63–65) require a common cofactor, CREB binding protein (66), for transcriptional activation. CREB binding protein has recently been shown to be a histone acetyltransferase (67). CREB binding protein binds to another histone acetyltransferase, P/CAF (60), suggesting a similar recruitment mechanism to that described above. Local changes in histone acetylation and/or nucleosome positioning could provide an initial signal that is propagated along the chromosome in a regulated fashion.

In addition to the chromatin disruption mechanism described above, it is certainly possible that regulatory complexes of the LCR engage in transient looping with promoter-associated factors (24, 46, 68). However, based on considerations of the complex architecture of the four HSs, each harboring multiple recognition sites for transacting factors, it is difficult to conceptualize the nature of the protein–protein interface that would mediate these looping interactions.

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1. Felsenfeld, G., Bresnick, E. H., Chung, J. & Reitman, M. (1995) *Molecular Biology of Hemoglobin Switching* (Intercept, Andover, MA).
2. Ptashne, M. (1986) *Nature (London)* **322,** 697–701.
3. Schleif, R. (1992) *Annu. Rev. Biochem.* **61,** 199–223.
4. Hager, G. L., Archer, T. K., Fragoso, G., Bresnick, E. H., Tsukagoshi, Y., John, S. & Smith, C. L. (1993) *Cold Spring Harbor Symp. Quant. Biol.* **58,** 63–71.
5. Archer, T. K., Lefebvre, P., Wolford, R. G. & Hager, G. L. (1992) *Science* **255,** 1573–1576.
6. Richard-Foy, H. & Hager, G. L. (1987) *EMBO J.* **6,** 2321–2328.
7. Bresnick, E. H., Bustin, M., Marsaud, V., Richard-Foy, H. & Hager, G. L. (1992) *Nucleic Acids Res.* **20,** 273–278.
8. Fascher, K. D., Schmitz, J. & Horz, W. (1993) *J. Mol. Biol.* **231,** 658–667.
9. Wolffe, A. P. & Pruss, D. (1996) *Cell* **84,** 817–819.
10. Brownell, J. E. & Allis, C. D. (1996) *Curr. Opin. Genet. Dev.* **6,** 176–184.
11. Felsenfeld, G. & McGhee, J. D. (1986) *Cell* **44,** 375–377.
12. Hansen, J. C. & Wolffe, A. P. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2339–2343.
13. Schwarz, P. M. & Hansen, J. C. (1994) *J. Biol. Chem.* **269,** 16284–16289.
14. Forrester, W. C., Thompson, C., Elder, J. T. & Groudine, M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 1359–1363.
15. Tuan, D. & London, I. M. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 2718–2722.
16. Grosveld, F., van Assendelft, G. B., Greaves, D. R. & Kollias, G. (1987) *Cell* **51,** 975–985.
17. Forrester, W. C., Epner, E., Driscoll, M. C., Enver, T., Brice, M., Papayannopoulou, T. & Groudine, M. (1990) *Genes Dev.* **4,** 1637–1649.
18. Bresnick, E. H. & Felsenfeld, G. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1314–1317.
19. Enver, T., Raich, N., Ebens, A. J., Papayannopoulou, T., Costantini, F. & Stamatoyannopoulos, G. (1990) *Nature (London)* **344,** 309–313.
20. Behringer, R. R., Ryan, T. M., Palmiter, R. D., Brinster, R. L. & Townes, T. M. (1990) *Genes Dev.* **4,** 380–389.
21. Lloyd, J. A., Krakowsky, J. M., Crable, S. C. & Lingrel, J. B. (1992) *Mol. Cell. Biol.* **12,** 1561–1567.
22. Jane, S. M., Ney, P. A., Vanin, E. F., Gumucio, D. L. & Nienhuis, A. W. (1992) *EMBO J.* **11,** 2961–2969.
23. Furukawa, T., Zitnik, G., Leppig, K., Papayannopoulou, T. & Stamatoyannopoulos, G. (1994) *Blood* **83,** 1412–1419.
24. Wijgerde, M., Grosveld, F. & Fraser, P. (1995) *Nature (London)* **377,** 209–213.
25. Fiering, S., Epner, E., Robinson, K., Zhuang, Y., Telling, A., Hu, M., Martin, D., Enver, T., Ley, T. J. & Groudine, M. (1995) *Genes Dev.* **9,** 2203–2213.
26. Hug, B. A., Wesselschmidt, R. L., Fiering, S., Bender, M. A., Epner, E., Groudine, M. & Ley, T. J. (1996) *Mol. Cell. Biol.* **16,** 2906–2912.
27. Dean, A., Erard, F., Schneider, A. P. & Schechter, A. N. (1981) *Science* **212,** 459–461.
28. Forrester, W. C., Novak, U., Gelinas, R. & Groudine, M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 5439–5443.
29. Lam, L. & Bresnick, E. H. (1996) *J. Biol. Chem.* **271,** 32421–32429.
30. Bresnick, E. H., John, S. & Hager, G. L. (1991) *Biochemistry* **30,** 3490–3497.
31. Forrester, W. C., Novak, U., Gelinas, R. & Groudine, M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 5439–5443.
32. Curtin, P. T., Liu, D. P., Liu, W., Chang, J. C. & Kan, Y. W. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 7082–7086.
33. Philipsen, S., Talbot, D., Fraser, P. & Grosveld, F. (1990) *EMBO J.* **9,** 2159–2167.
34. Talbot, D., Philipsen, S., Fraser, P. & Grosveld, F. (1990) *EMBO J.* **9,** 2169–2177.
35. Hardison, R., Xu, J., Jackson, J., Mansberger, J., Selifonova, O., Grotch, B., Biesecker, J., Petrykowska, H. & Miller, W. (1993) *Nucleic Acids Res.* **21,** 1265–1272.
36. Moon, A. M. & Ley, T. J. (1991) *Blood* **77,** 2272–2284.
37. Pruzina, S., Hanscombe, O., Whyatt, D., Grosveld, F. & Philipsen, S. (1991) *Nucleic Acids Res.* **19,** 1413–1419.
38. Jackson, J. D., Miller, W. & Hardison, R. C. (1996) *Nucleic Acids Res.* **24,** 4327–4335.
39. Jackson, J. D., Petrykowska, H., Philipsen, S., Miller, W. & Hardison, R. (1996) *J. Biol. Chem.* **271,** 11871–11878.
40. Bungert, J., Dave, U., Lim, K. C., Lieuw, K. H., Shavit, J. A., Liu, Q. & Engel, J. D. (1995) *Genes Dev.* **9,** 3083–3096.
41. Dean, A., Ley, T. J., Humphries, R. K., Fordis, M. & Schechter, A. N. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 5515–5519.
42. Shaffer, C. D., Wallrath, L. L. & Elgin, S. C. (1993) *Trends Genet.* **9,** 35–37.
43. McCune, S. L. & Townes, T. M. (1994) *Nucleic Acids Res.* **22,** 4477–4481.
44. Ellis, J., Tan-Un, K. C., Harper, A., Michalovich, D., Yannoutsos, N., Philipsen, S. & Grosveld, F. (1996) *EMBO J.* **15,** 562–568.
45. Talbot, D. & Grosveld, F. (1991) *EMBO J.* **10,** 1391–1398.
46. Milot, E., Strouboulis, J., Trimborn, T., Wijgerde, M., De Boer, E., Langeveld, A., Tan-Un, K., Vergeer, W., Yannoutsos, N., Grosveld, F. & Fraser, P. (1996) *Cell* **87,** 105–114.
47. Oliviero, S. & Struhl, K. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 224–228.
48. Chi, T., Lieberman, P., Ellwood, K. & Carey, M. (1995) *Nature (London)* **377,** 254–257.
49. Adams, C. & Workman, J. L. (1995) *Mol. Cell. Biol.* **15,** 1405–1421.
50. Boyes, J. & Felsenfeld, G. (1996) *EMBO J.* **15,** 2496–2507.
51. Weintraub, H. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5819–5823.
52. Ko, M. S., Nakauchi, H. & Takahashi, N. (1990) *EMBO J.* **9,** 2835–2842.
53. Fiering, S., Northrop, J. P., Nolan, G. P., Mattila, P. S., Crabtree, G. R. & Herzenberg, L. A. (1990) *Genes Dev.* **4,** 1823–1834.
54. Hug, B. A., Moon, A. M. & Ley, T. J. (1992) *Nucleic Acids Res.* **20,** 5771–5778.
55. Walters, M. C., Fiering, S., Eidemiller, J., Magis, W., Groudine, M. & Martin, D. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7125–7129.
56. Peterson, K. R., Clegg, C. H., Navas, P. H., Norton, E. J., Kimbrough, T. G. & Stamatoyannopoulos, G. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 6605–6609.
57. Starck, J., Sarkar, R., Romana, M., Bhargava, A., Scarpa, A. L., Tanaka, M., Chamberlain, J. W., Weissman, S. M. & Forget, B. G. (1994) *Blood* **84,** 1656–1665.
58. Kingston, R. E., Bunker, C. A. & Imbalzano, A. N. (1996) *Genes Dev.* **10,** 905–920.
59. Brownell, J. E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D. G., Roth, S. Y. & Allis, C. D. (1996) *Cell* **84,** 843–851.
60. Yang, X. J., Ogryzko, V. V., Nishikawa, J., Howard, B. H. & Nakatani, Y. (1996) *Nature (London)* **382,** 319–324.
61. Cairns, B. R., Kim, Y. J., Sayre, M. H., Laurent, B. C. & Kornberg, R. D. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1950–1954.
62. Peterson, C. L., Dingwall, A. & Scott, M. P. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2905–2908.
63. Arias, J., Alberts, A. S., Brindle, P., Claret, F. X., Smeal, T., Karin, M., Feramisco, J. & Montminy, M. (1994) *Nature (London)* **370,** 226–229.
64. Chakravarti, D., LaMorte, V. J., Nelson, M. C., Nakajima, T., Schulman, I. G., Juguilon, H., Montminy, M. & Evans, R. M. (1996) *Nature (London)* **383,** 99–103.
65. Dai, P., Akimaru, H., Tanaka, Y., Hou, D. X., Yasukawa, T., Kanei-Ishii, C., Takahashi, T. & Ishii, S. (1996) *Genes Dev.* **10,** 528–540.
66. Chrivia, J. C., Kwok, R. P., Lamb, N., Hagiwara, M., Montminy, M. R. & Goodman, R. H. (1993) *Nature (London)* **365,** 855–859.
67. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H. & Nakatani, Y. (1996) *Cell* **87,** 953–959.
68. Hanscombe, O., Whyatt, D., Fraser, P., Yannoutsos, N., Greaves, D., Dillon, N. & Grosveld, F. (1991) *Genes Dev.* **5,** 1387–1394.