The human β-globin gene domain at chromosomal position 11p15.5 consists of a cluster of globin genes that are expressed only in erythroid cells. The genes are arranged 5′-HBE-HBG2-HBG1-HBD-HBB-3′ from the centromere to the telomere. The genes are temporally expressed in the order of their array along the chromosome, with ε-globin (encoded by HBE) produced embryonically, γ- and δ-globin (encoded by HBG2 and HBG1) produced fetally, and δ- and β-globin (encoded by HBD and HBB) produced in the adult. Controlled expression of the genes occurs through proximal regulators, including the promoters of the individual genes, and distal elements, such as the dominant regulatory region known as the locus control region (LCR) (1–4). The LCR spans at least 17 kilobases of DNA, located 23 to 6 kilobases upstream of the ε-globin gene, and contains 5 DNase I-hypersensitive sites (HS1–5) (5–7). The LCR is defined by its ability to confer high level, tissue-specific expression of linked genes at all sites of integration examined in transgenic mice (8). It is a powerful enhancer (8–11). Analysis of LCR deletions in thalassemic patients (12, 13) and experiments testing LCR function at ectopic sites (e.g. 14, 15) have been interpreted as showing a domain-opening activity, but this has not been seen in directed deletions of the LCR (10, 11). Thus, all the functions of the LCR have not been conclusively defined (16), but it is clear that it is a major cis-regulatory element for the β-like globin gene cluster. DNA fragments containing individual HSs can produce some of the effects of the LCR (17–20), and growing evidence supports the model that multiple HSs work together in a holocomplex at the LCR (9, 15, 21–25). A full set of cis-regulatory sites and the protein(s) that works at them is not yet known either for the function of an individual HS or for its interaction with other sites.

One of the most potent components of the LCR is HS2, which can produce high level, position-independent expression of linked genes in transgenic mice (17, 20, 26) and stably transfected erythroid cell lines, including both human K562 cells and mouse erythropoietin (ME) cells (21, 26, 27). It can also act without stable integration into chromosomes, conferring both strong enhancement (28, 29) and heme-inducibility (30–32) on linked globin genes in transiently transfected K562 cells. The core of HS2 is defined as the smallest region containing position-independent expression of the β-globin gene; it is contained within a 374-base pair HindIII-XbaI fragment (26, 33). Simultaneous alignment of multiple DNA sequences of the β-globin domain from mammals shows several conserved blocks, many of which have been confirmed as protein binding sites needed for the function of HS2 (4, 34). A particularly dense cluster of sites, spaced at 10-base pair intervals within a 100-base pair segment of the HS2 core, is suggestive of a contiguous array of proteins on the same face of the DNA helix. A dimer of MAREs, or Maf-responsive elements (35), is crucial for the enhancement and heme-inducibility by HS2 in K562 cells (30, 31) as well as high level expression in transgenic mice (26, 33, 36). The MAREs are binding sites for AP1, NFE2 (37–39), Nrf1 (40), LCRF1 (41), TCF11 (42), Nrf2 (43), and Bach1 (44). Most of these proteins function as heterodimers with small Maf proteins; hence, the binding site has been named for this common component. The MAREs are not sufficient for full-level enhancement (26, 27, 31, 32), indicating that other proteins are also functioning at HS2. In particular, GATA1 and/or GATA2 (26, 33, 45, 46), basic helix-loop-helix proteins and other E box-binding proteins (32), HS2NFE5 (47), and proteins binding to the highly conserved CAC motif (33) have all been implicated by a combination of mutagenesis, in
vivo footnoting, in vitro binding coupled with antibody studies, and sequence conservation. Additional conserved sites are found throughout and beyond the HS2 core (4), and many of these regions have also been implicated in function of the LCR (33, 48, 49).

To better understand the contribution of the several conserved sequences in the HS2 core to enhancement in erythroid cells, we developed methods using cationic lipids to transiently transfect several erythroid cell lines (50). This has allowed us to test constructs containing promoters from several β-like globin genes in murine MEL cells (which produce β-globin (51)), human K562 cells (which produce ε- and γ-globin (52, 53)), and human erythroleukemia (HEL) cells (which produce mainly γ-globin (54)). The results identify cis-acting sequences within the HS2 core that negatively regulate expression of linked β-like globin genes in erythroid cell lines and in normal human adult erythroid cells. The negative effect is seen both in transiently transfected cells and after integration into stably transformed cells.

**EXPERIMENTAL PROCEDURES**

**Transient Transfection of Erythroid Cells**—The cationic lipid reagent Tfx50 (from Promega) was used to transiently transfect MEL, K562, and HEL cells (50) following the manufacturer’s protocol for suspension cells. Optimal transfection was obtained at a 2:1 ratio (charge to mass) of cationic lipid to DNA. The reagent and DNA remained in the cell culture for 48 h, at which point the cells were harvested. Three different experimental designs were used to examine the level of expression in transient transfections. In the first, each plasmid containing a reporter gene was assayed as a titration of DNA mass from 0.25 μg up to a maximum of 8.0 μg (maintaining the 2:1 charge to mass ratio), with the results reported as luciferase activity in relative light units (RLUs). In the second, plasmids were transfected in triplicate at the single DNA concentration, and sequence conservation. Additional conserved sites are found throughout and beyond the HS2 core (4), and many of these regions have also been implicated in function of the LCR (33, 48, 49).

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FIG. 1. Comparison of HS2 enhancement in transiently transfected K562 and MEL cells. A, diagrams of plasmids illustrate the different promoters used (from the rabbit HBE gene encoding e-globin and the mouse Hbb-b1 gene encoding the β-major globin) as well as the different poly(A) signals from HBE (e) or SV40 (SV). The luciferase cDNA in these reporter gene plasmids is from pGL2Basic (Promega). B–D, panels comparing HS2 enhancement in K562 (left) and MEL cells (right) on the HBE promoter (B) or the Hbb-b1 promoter from −340 (C), or −106 (D) to +26. An increasing mass of DNA was transfected into the cells, and the resulting luciferase activity is plotted. Fold enhancement over the enhancerless construct level of expression is shown in each box.

with and without the HS2 core shows the expected robust enhancement by HS2 for both the HBE and the Hbb-b1 promoters in K562 cells (50- and 30-fold in this experiment, Fig. 1, B and C). However, enhancement in MEL cells is substantially lower, being only 12- and 3-fold for the HBE and Hbb-b1 promoters, respectively (Fig. 1, B and C). Although the measured fold enhancement varies in different experiments, comparisons of transfections done at the same time invariably show a lower level of enhancement in MEL than in K562 cells. Also, as will be shown later, this modest effect of the HS2 core in MEL cells is seen in several different transfection protocols. Furthermore, a similar response from the promoters of rabbit, mouse, or human globin genes to enhancers containing fragments of the human HS2 core suggests that species-specific effects are not evident.

The data in Fig. 1 show that the transfected plasmid templates were active, even in cells in which the endogenous homologous genes are inactive. In particular, the plasmid with an HBE promoter without an enhancer is as active in MEL cells, which do not produce e-globin from the endogenous Hbb-y locus, as in K562 cells, which produce e-globin from the endogenous HBE gene (800 RLU/s in K562 and 500 RLU/s in MEL). However, the response to the HS2 core was considerably lower in transiently transfected MEL cells. Likewise, the plasmid with an Hbb-b1 promoter responded better to the HS2 core enhancer in K562 cells, where the endogenous homolog HBB is inactive, than in MEL cells. The Hbb-b1 promoter used for the data in Fig. 1C extended to nucleotide position −340 (with respect to the cap), and thus contained some sequences implicated in negative regulation of this gene (59, 60). Deletion of these negative elements did not restore enhancement in MEL cells, since the Hbb-b1 promoter extending only to −106 still showed a 6-fold reduction in enhancement in MEL cells compared with K562 cells (Fig. 1D). Thus, these results indicate that the reduced enhancement in MEL cells is not related to the type of globin gene promoter used or the activity of the endogenous homologous genes.

The limited enhancement by the HS2 core in MEL cells is not an artifact of using only parts of the genes. The plasmid c-luc (Fig. 1A) was designed to include almost all of the HBE gene (57, 61). It has the rabbit HBE promoter segment extending from −573 to +85, which includes multiple positive and negative elements (62) that are homologous to those of the human HBE gene (56, 63, 64). Since the luciferase-coding region replaces a segment extending from the 3′ end of exon 1 to almost the 3′ end of exon 2, the reporter gene retains most of the HBE exons, intron 2, and an extensive 3′-flanking region. The difference in enhancement between the two cell lines is seen both for expression plasmids containing the promoter and internal and 3′-flanking regions of HBE) as well as for plasmids containing only the promoter (e.g. from Hbb-b1) but no other parts of the globin gene.

Greater Enhancement by Individual Cis-acting Elements than the HS2 Core in MEL Cells—The HS2 enhancer contains multiple cis-acting sequences, and thus, the reduced enhancement by HS2 in MEL cells could result from less activity from specific positive cis-acting elements, greater activity from negative cis-acting elements, or both. Therefore, we compared the activity of multimers of individual transcription factor binding sites with that of the entire enhancer in both cell lines. Previous observations (26, 27, 31, 32) showed that in K562 cells the tandem MAREs are not sufficient for full enhancement. A similar result is obtained using the cationic lipid as the transfection reagent for K562 cells, assayed at increasing amounts of transfecting DNA (Fig. 2A). A second cis-element of HS2 known to contribute to enhancement in K562 cells is the E box at position 8701 in the sequence file HUMHBB (32). We tested the ability of a multimerized E box sequence, containing five copies, to enhance independently of other elements in HS2 (Fig. 2B). The pentamer of the E box enhanced 3-fold, which is significant (p < 0.05, Table I) but much less than the enhancement by the HS2 core.

In contrast, in MEL cells, the MAREs enhance the HBB promoter more strongly than does the HS2 core (Fig. 2C). Also, the pentamer of the E box stimulates expression of the linked HBB promoter as strongly as the intact core does (Fig. 2D). The enhancements by both the E box multimers and the intact HS2 core are significant (p < 0.01), and they are not significantly different from each other. Thus the reduced enhancement in MEL cells is not exclusively from reduced activity of either of these transcription factor binding sites, since both the MAREs and the E boxes were as active or more active than the intact core. These results suggest that a cis-acting element in HS2 has a strong, negative effect in MEL cells.

Effects of HS2 Cis-elements in MEL Cells—To examine the contributions to enhancement by groups of cis-elements within the HS2 core, we tested the activities of a deletion series from the 5′ and 3′ ends of the HindIII-XbaI HS2 core. Members of each series were linked to the HBE-luciferase reporter gene and transfected into MEL cells. Two different experimental protocols were followed. In the first, the amount of DNA used in the transfection was titrated to find an optimal concentration for enhancement. In the second protocol, the transfections were
FIG. 2. Enhancement from individual cis-acting elements compared with the core of HS2. A and B, in K562 cells expression from e-luc and eHS2 is compared with that from e-luc enhanced by human MARE (hMARE) sequences (A) or a human E box multimer (B) composed of five copies of the 8701 E box sequence. Diagrams of the constructs are shown between panels A and B. The MAREs are represented by two shaded rectangles, and the E boxes are represented by five rectangles with darker shading. Rectangles with lighter shading represent some of the other conserved blocks in the HS2. Results are plotted as luciferase activity as a function of mass of DNA used in the transfection. C and D, in MEL cells, expression from β106 and β106HS2 were compared with that from β106 enhanced by human MARE sequences (C) or a human E box multimer (D) ligated to β106. Diagrams of the constructs are shown between panels C and D.

Repeated in triplicate at the optimal DNA concentration so that the results could be analyzed statistically. As shown in Fig. 3, the terminal deletions reveal negative cis-elements in HS2.

For the triplicate transfections shown in Fig. 3B, the HindIII-XbaI HS2 core enhanced HBE-luciferase expression 6.6-fold (Fig. 3B). Deletion from the 5’ end increased enhancement to 11.7-fold for the BalI-XbaI fragment. This is a significant increase (p < 0.01 for comparison with the intact core; Table II). A similar result was obtained in the DNA titration experiment shown in Fig. 3C. In this case, the enhancement increased 4-fold by deleting the HindIII-BalI fragment (from 2.5-fold for the HindIII-XbaI HS2 core to 10-fold for the BalI-XbaI fragment). Thus cis-acting elements located between HindIII and BalI exerted a negative effect in this assay. Additional deletions into the HS2 core caused a progressive loss of enhancement but no deletion completely removed activity (Fig. 3 and Table II).

Deletions from the 3’ end of the HS2 core revealed a second negative element in MEL cells. For the triplicate transfections shown in Fig. 3B, removal of the 3’ fragment caused an increase in enhancement from 6.6- to 9.5-fold; this increase is significant (p < 0.02; Table II). This result was confirmed by the DNA titration experiment in Fig. 3C in which deletion of the 3’ fragment doubled the level of enhancement, from 3- to 6.8-fold. Similar to the results with the 5’ deletion series, additional deletions from the 3’ end caused a progressive reduction in enhancement (Fig. 3), but again, all fragments retained significant activity (p < 0.001 for each in comparison with e-luciferase, Table II). These data show that truncation of either the 5’ or 3’ end of the core of HS2 partially relieves repression in MEL cells.

The activity of a particular segment of HS2 depends on the context. Deletion of the 5’ region of HindIII to XbaI the 3’ region of 8750 to XbaI shows that both have a negative effect on enhancement when the rest of the HS2 core is present. In contrast, both have a small but significant positive effect when assayed by themselves. This suggests that the negative effect could arise by interference with positive elements within the rest of HS2.

Negative Effect of HindIII-BalI Region on Both HBE and Hbb-b1 Genes in Three Different Erythroid Cell Lines—To examine whether the cis-regulatory sequences at the 5’ end of the HS2 core had a negative effect only on the embryonic e-globin gene promoter in MEL cells, the effect of its deletion was tested on promoters from the e-, γ-, and β-globin genes in K562, HEL, and MEL cell lines (Fig. 4A). In this series of experiments, the test plasmid with the globin gene promoter-driving expression from the firefly luciferase gene was cotransfected with a control plasmid with the globin gene promoter-driving expression from the Renilla luciferase gene expressed from a tk promoter. The results are plotted as a ratio of firefly luciferase activity to Renilla luciferase activity.

As shown in Fig. 4B, removal of the HindIII-BalI fragment doubled the level of enhancement in K562 for both the HBE promoter (from 6-fold in its presence to 13-fold in its absence)
and the Hbb-b1 promoter (from 8- to 15-fold). However, no difference in luciferase expression from the HBG1 promoter was measurable when the HindIII-BalI region was removed. Interestingly, in K562 cells, the 275-base pair BalI-XbaI fragment worked equally well as the 1.4-kilobase KpnI-BglII fragment containing HS2.

HEL cells produce mainly γ-globin and a small amount of ε-globin, but no β-globin, from its endogenous genes (54). Results of transient transfections (Fig. 4B) show that the HindIII-XbaI fragment comprising the HS2 core had almost no effect on either the HBE or Hbb-b1 promoters. However, removal of the HindIII-BalI negative element increased enhancement substantially, to 3.6-fold for HBE and 5.0-fold for Hbb-b1. In contrast to the results with K562 cells, the HBG1 promoter responded more strongly to the BalI-XbaI fragment of HS2 (lacking the negative element) than to the core HindIII-XbaI fragment (3.1- and 2-fold enhancement, respectively, Fig. 4B). This small but significant (p < 0.01) difference indicates that the negative element can decrease enhancement of any of the three promoters tested, although its effect is stronger on HBE and Hbb-b1 than on HBG1.

Results of transfections in MEL cells were similar to those in HEL cells for all three promoters (Fig. 4B). Little to no enhancement of the Hbb-b1 or HBE promoters was observed for plasmids containing the HS2 core (HindIII-XbaI fragment), but both promoters showed substantial enhancement with the BalI-XbaI fragment of HS2 (5.8-fold for HBE and 7.0-fold for Hbb-b1). In contrast, the HBG1 promoter was enhanced by the HS2 core (3.5-fold), but even this increased to 8.8-fold upon deletion of the negative element. Thus the 5′ HindIII-BalI fragment is exerting a negative effect on all three promoters in HEL cells, with its strongest effect on HBE. A striking decline in enhancement is observed in MEL cells for the 5′ HindIII-BalI fragment containing HS2 and the HBG1 promoter (Fig. 4B, plasmid γKB). Transfection of both K562 and HEL cells with this same plasmid DNA showed strong enhancement by the KpnI-BglII fragment. Further investigation would be required to determine whether this corresponds to a difference in species specificity, promoter preference, or stage of differentiation.

Assays described so far test human HS2 on promoters of globin genes from other species, i.e. rabbit and mouse. To test whether the human HS2 core works more effectively with a human HBB promoter, MEL cells were transfected with con-

FIG. 2. 5′ and 3′ deletions of the HS2 core fragment assayed in MEL cells. A, diagrams of a series of progressive deletions from the 5′ end of the HindIII-XbaI fragment and another series of deletions from the 3′ end. These were ligated to euk and assayed by transient transfection. Conserved sites between 8650 and the E box bound by upstream stimulatory factor (E/USF) are indicated by shaded boxes in the map of the HS2 core but are not shown in the terminal regions (HindIII to 8650 and E/USF to XbaI). B, the luciferase activity after transfecting MEL cells at the 2:1 charge:mass ratio with 2 μg of test DNA is plotted for each plasmid. Each sample was transfected in triplicate, assayed for activity, and normalized to the amount of total protein in the sample. The means are plotted, and the error bars show the S.D. The fold increase over no enhancer is shown within or adjacent to the bar for each sample. C, the amount of luciferase expression is plotted as a function of mass of DNA transfected into MEL cells, keeping a 2:1 charge:mass ratio.

**TABLE II**

| Reporter | Enhancer | Mean activity | S.D. | n  | Fold increase | P value<sup>a</sup> | P value<sup>b</sup> |
|----------|----------|---------------|------|----|---------------|-------------------|-------------------|
| ε-luciferase | None     | 1,328         | 292  | 3  | 1             | <0.001            | <0.001            |
|           | HS2      | 8,773         | 566  | 3  | 6.6           | <0.001            | <0.001            |
|           | BX       | 15,581        | 2,482 | 3 | 11.7          | <0.01             | <0.001            |
|           | MX       | 12,124        | 2,881 | 3 | 9.1           | <0.01             | <0.001            |
|           | 8701X    | 6,073         | 513  | 3  | 4.6           | <0.05             | <0.001            |
|           | 8762X    | 3,062         | 822  | 3  | 2.3           | <0.05             | <0.001            |
|           | H8750    | 12,685        | 1,659 | 3 | 9.6           | <0.02             | <0.001            |
|           | H8650    | 10,664        | 2,211 | 3 | 8             | <0.1              | <0.001            |
|           | HCAC     | 4,067         | 371  | 3  | 3.1           | <0.001            | <0.001            |
|           | HB       | 4,139         | 105  | 3  | 3.1           | <0.05             | <0.001            |

<sup>a</sup> The P value is the probability that the observed mean is not different between two sets of values, based on Student’s t test with df = 4. The P value was calculated for the pairwise comparison of each DNA with the previous plasmid in the deletion series as listed (except H8750, which is compared to HS2). 

<sup>b</sup> P value for pairwise comparison with no enhancer.
FIG. 4. Comparison of the effects of deleting the HindIII to BalI fragment of HS2 on three different globin genes in three different cell lines. A, diagram of DNA fragments within or containing HS2, which were ligated to luciferase reporter genes driven by promoters from HBE, HBG1, or Hbb-b1. Conserved blocks in the HS2 core are indicated by the shaded rectangles; the darker-shaded rectangles are the MAREs (labeled E box) at 8701. B, the graphs compare the expression from plasmids containing various combinations of promoter and enhancers after transfection of K562, HEL, or MEL cells. Expression is plotted as the ratio between the activity of firefly luciferase (FF luc) encoded in the test plasmids and Renilla luciferase (R luc) encoded in a cotransfection control. All transfections were in triplicate; the mean is plotted, and the error bars show the S.D. The firefly luciferase in the HBG1 constructs is from pGL3Basic (Promega), which encodes a luciferase enzyme with higher activity and, thus, results in higher RLU output than do the pGL2- derived firefly luciferases in the HBE and Hbb-b1 constructs.

Negative Effect of the Human HindIII to BalI Fragment on Stable Integration of the HBB Promoter—In summary, the data in Figs. 4 and 5 show that the 5' HindIII to BalI fragment of HS2 has a negative effect upon transient transfection of three immortalized erythroid cell lines from two different species (human and mouse), affecting four different globin genes. Hence the effect is not species-specific nor is it unique to one promoter.

Negative Effect of HS2 HindIII to BalI Fragment in Normal Human Adult Erythroid Cells—To test whether the negative effect of the 5' end of HS2 could be seen in normal human cells rather than in continuously growing cell lines, normal hAEC were isolated by culturing peripheral blood by the procedure of Fibach et al. (55). The hAEC were transfected with plasmids containing the β106-luciferase reporter gene with or without DNA fragments containing segments of HS2. The results of the transient expression assays (Fig. 6) show that the HindIII to XbaI HS2 core segment had no effect on the reporter gene, whereas the BalI-XbaI DNA fragment boosted expression over 7-fold. Thus the negative effect seen for the 5' HindIII-BalI fragment is not an artifact of transfecting immortalized cell lines.

Expression after Integration into MEL Cells—The effect of the 5' end of the HS2 core was tested on stably integrated DNA constructs in MEL cells. As shown in Fig. 7A and Table III, in three independently generated pools of stably transfected MEL cells, the BalI-XbaI DNA fragment from HS2 enhanced expression of the β106-luciferase reporter gene much more than did the HindIII to XbaI HS2 core segment. Although the fold enhancement for each HS2-containing construct varied among the three pools, the BalI-XbaI fragment consistently produced a substantially greater enhancement than did the HS2 core fragment. Thus the negative effect of the 5' HindIII-BalI frag-
FIG. 6. Negative effect of the HindIII to BalI region in human adult erythroid cells. A, hAEC cells were transfected in triplicate with \( \beta_{106} \), \( \beta_{106}HS2 \) (HindIII-XbaI), and \( \beta_{BX} \) (BalI-XbaI) then harvested after 2 days, and the amount of luciferase activity was assayed. The means are plotted, and the S.D. shown as error bars. B, diagrams of the plasmid constructs are shown in the lower portion of the figure.

Effects of Induction of MEL Cells—We used these pools of stably transfected MEL cells to test whether induction by HMBA could overcome the negative effect of the HindIII-BalI fragment. As shown in Fig. 7B and Table III, expression of the \( \beta_{106} \)-luciferase reporter gene increased in each of two pools of transfected cells in response to the inducer, HMBA. However, the amount of induction was similar for each construct (almost within a range of 5–10-fold) in the absence or presence of a DNA fragment containing HS2. Thus, for these constructs, the major cis-acting sequence affecting induction appears to be the \( Hbb-b1 \) promoter. In particular, induction by HMBA did not overcome the negative effect of the HindIII-BalI fragment in HS2.

Deletion Series through the HindIII to BalI Region of the HS2 Core—The 5’ negative element was mapped more precisely using additional deletions at a finer resolution. As shown in Fig. 8, the 5’ end of each deletion corresponds to one of several sequences in the HS2 core that are conserved among mammalian globin loci (4). MEL cells were transfected with the series of constructs that contained no enhancer (\( \beta_{106} \)), the HS2 core (\( \beta_{HX} \)), or subfragments of the HS2 core beginning at conserved region 2, 3, or 4 (\( \beta_{CR2}, -3 \), or -4, respectively) or the BalI-XbaI region (\( \beta_{BX} \)). All fragments had the same 3’ end, the SacI site in the polylinker downstream of the HS2 core. The HS2 core conferred no enhancement on the expression of the \( Hbb-b1 \) promoter, but CR2, -3, and -4 constructs all showed a substantial increase of 6.1-, 4.9-, and 5.9-fold (respectively) over the enhancerless construct \( \beta_{106} \). This is similar to the 5.2-fold enhancement obtained with the BalI-XbaI fragment. Thus the sequence responsible for the negative effect on HS2 enhancement is localized to a region of 20 nucleotides at the 5’ end of HS2. Two conserved elements that fall within this area are candidates for the negative cis-regulatory activity, one at the HindIII restriction site and one in a neighboring T-rich motif.

DISCUSSION

Many studies show that HS2 of the \( \beta \)-globin LCR strongly stimulates the expression of linked globin genes in transgenic mice (17, 26, 33, 66), in stably transfected MEL (21, 26) and K562 cells (27, 67), and in transiently transfected K562 cells (29, 30). Multiple cis-acting sequences in HS2 contribute to and modulate the enhancement activity (26, 32, 33, 36, 47, 49, 68). Our studies reported here show that a 20-base pair region within the HindIII to BalI fragment at the 5’ end of the HS2 core exerts a negative effect, whereas the remaining segment of HS2 strongly stimulates expression.

The negative effect was observed in four different types of erythroid cells, including three cell lines (MEL, HEL, and K562) as well as normal hAEC. All of these cells are derived from adults, but HEL and K562 cells are not fully committed to the erythroid lineage and express globin genes that are maximally expressed in fetal and embryonic development. This indicates that the negative effect of the HindIII-BalI fragment can be exerted at many stages of adult erythroid differentiation (e.g. before and after commitment and at various stages of erythroid maturation). Although developmental specificity cannot be definitively addressed with these transfection experiments, we note that a globin gene expressed predominately during primitive erythropoiesis (rabbit \( HBE \)) and one expressed during definitive erythropoiesis (mouse \( Hbb-b1 \)) responded strongly to this negative element. This was seen both in cells expressing the endogenous homolog as well as in cells in which the homolog is silent (e.g. the effect was seen on \( Hbb-b1 \) in MEL cells, where the endogenous gene is expressed, and in HEL and K562 cells, where the homologous gene \( HBB \) is not expressed). Thus the HS2 negative element is active on genes expressed at different stages of development. This is consistent with studies of the human HS2 in transgenic mice; both gain-of-function (19, 20) and loss-of-function (15, 69) experiments show a comparable effect of HS2 at all developmental stages.

Human \( HBG1 \), which is expressed predominately in fetal life, also responded to this negative element in HEL and MEL cells, albeit less dramatically than the other genes tested. Indeed, transfection of a \( HBG1 \) promoter-luciferase reporter in K562 cells showed no effect of the HS2 HindIII-BalI fragment. The absence of an effect in K562 cells and the rather modest effect in MEL cells helps explain why this effect was not reported previously, since the \( HBG1 \) promoter has been commonly used in transfections of these cells (27, 30, 47). Furthermore, the \( HBE \) gene also is frequently used in transfections of K562 cells (28, 56, 64, 65, 70), and its expression is enhanced by the HindIII-XbaI core of HS2, although the enhancement is increased after deletion of the HindIII-BalI negative element. The observation that the HindIII-BalI fragment of HS2 has only a modest, and sometimes no, effect on \( HBG1 \) in the same cells where it has a strong effect on \( HBE \) and \( Hbb-b1 \) shows that this part of HS2 has some promoter specificity.

The HindIII-BalI fragment of HS2 decreases enhancement after stable integration into MEL cell chromosomes as well as in transiently transfected cells. The unintegrated DNA in transiently transfected cells is readily packaged into nucleosomes (71, 72), and thus, the effects of enhancers observed in transiently transfected cells do not result from proteins interacting with naked DNA. However, in these experiments, it is not clear that all templates are in the same chromatin structure. We examined pools of stably transfected MEL cells to test the effects of different segments of the HS2 core on templates integrated into chromosomes, and hence fully packaged into chromatin. Pools were tested so that many different integration sites could be assayed at once. These experiments showed that the negative effect of the HindIII-BalI fragment at the 5’ end of the HS2 core is exerted even when the template is in chromatin.

Changes in the level of enhancement conferred by fragments of the HS2 core support the results of studies where transcription factor binding sites were specifically targeted by point
mutation. For instance, mutations in the 8701 E box reduce the level of expression from the mouse Hbb-b1 gene in stably transfected MEL cells (32). Multimers of this same E box sequence confer a 3-fold increase in the level of expression from the mouse Hbb-b1 promoter in K562 cells (26). Assay of a number of HS2 segments for enhancement of the Hbb-b1 gene in stably transfected MEL cells. A HaeIII-XbaI fragment (of similar end points to the Ball-XbaI fragment discussed here) produced a slightly greater level of enhancement than did the HindIII-XbaI fragment in several pools of clones. Although these differences were not considered significant in the Talbot et al. (26) paper, they are consistent with our demonstration of a negative element at the 5′ end of HS2.

Although our studies utilizing fragments of the LCR in proximity to various globin gene promoters are useful for dissecting regulatory components, they leave open a large number of possibilities for how these components could function during physiological regulation. For instance, the negative function of the HindIII-BalI fragment could be utilized to help keep globin genes silent in nonerythroid cells, to turn them off in erythroid cells at appropriate stages, or to attenuate the activation by HS2 early in erythroid differentiation. The situation is further complicated by the fact that multiple HSs function together in the LCR, and our experiments do not address how this part of HS2 functions in this context. However, it is important to know

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TABLE III

| Cells          | Reporter plasmid | Transfection method | Uninduced | Fold enhancement | Fold induction |
|----------------|-------------------|---------------------|-----------|------------------|---------------|
|                |                   |                     | Mean ± S.D. |                 |               |
|                |                   |                     | Fold enhanced | $P_a$           | $P_{HIXX}$     |
| MEL Stable pool 2 | β106              |                     | 510 ± 44    | 1.0             | NA            |
|                 | βHX               |                     | 1341 ± 203  | 2.6             | <0.01         |
| MEL Stable pool 3 | β106              |                     | 2322 ± 193  | 1.0             | NA            |
|                 | βHX               |                     | 2116 ± 158  | 0.91            | <0.2          |
| hAEC Transient  | β106              |                     | 4362 ± 471  | 1.0             | NA            |
|                 | βHX               |                     | 4175 ± 82   | 0.95            | >0.5          |

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$P_a$ is the probability that the observed mean is not different from that with no enhancer, based on Student’s t test with df, 4.

$P_{HIXX}$ is the probability that observed expression levels from constructs with the HindIII-XbaI enhancer are not different from those with the Ball-XbaI enhancer.
that this segment can play a negative role.

A potential practical application of the identification of negative elements in the LCR HSs may be found in improving vectors for globin gene therapy. Inclusion of LCR HSs in expression constructs, including retroviral vectors, can greatly increase the level of expression of the target globin gene (21, 25, 73, 74), but the set of DNA fragments needed for optimal expression has not yet been defined (4). Our characterization of negative elements within the conventional HS2 and the indication that they also may be operative in transgenic mice (36) raise the possibility that re-engineering LCR constructs to remove all such negative regions could generate an even more potent enhancer of expression.

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A Negative Cis-element Regulates the Level of Enhancement by Hypersensitive Site 2 of the β-Globin Locus Control Region
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