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Multiple elements in human β-globin locus control region 5′ HS 2 are involved in enhancer activity and position-independent, transgene expression

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ABSTRACT

The human β-globin Locus Control Region (LCR) has two important activities. First, the LCR opens a 200 kb chromosomal domain containing the human ε-, γ- and β-globin genes and, secondly, these sequences function as a powerful enhancer of ε-, γ- and β-globin gene expression. Erythroid-specific, DNase I hypersensitive sites (HS) mark sequences that are critical for LCR activity. Previous experiments demonstrated that a 1.9 kb fragment containing the 5′ HS 2 site confers position-independent expression in transgenic mice and enhances human β-globin gene expression 100-fold. Further analysis of this region demonstrates that multiple sequences are required for maximal enhancer activity; deletion of SP1, NF-E2, GATA-1 or USF binding sites significantly decrease β-globin gene expression. In contrast, no single site is required for position-independent transgene expression; all mice with site-specific mutations in 5′ HS 2 express human β-globin mRNA regardless of the site of transgene integration. Apparently, multiple combinations of protein binding sites in 5′ HS 2 are sufficient to prevent chromosomal position effects that inhibit transgene expression.

INTRODUCTION

Four erythroid-specific, DNase I hypersensitive sites are located 11 to 18 kilobases upstream of the ε-globin gene in the human β-globin gene family (Fig. 1). These sequences are critical for high-level expression of human ε-, γ- and β-globin genes and, therefore, have been designated the Locus Control Region (LCR) (1–4). Deletions that remove these LCR sequences result in severe β-thalassemias even in patients that retain an intact copy of the β-globin gene (5–7).

Forrester *et al.* (8) have demonstrated that a deletion of 5′ HS 2-4 in a Hispanic patient with β-thalassemia results in a dramatic change of chromatin configuration and timing of DNA replication. The β-globin locus on the mutant chromosome is resistant to DNase I digestion in erythroid nuclei and is replicated late in S phase of the cell cycle. The β-globin locus on the normal chromosome of this heterozygous patient is sensitive to DNase I digestion in erythroid nuclei and replicates early in S phase.

When the entire LCR is inserted upstream of globin genes and tested for activity in transgenic mice, high-level expression is observed specifically in erythroid cells (9, 10). Expression of the linked globin genes is enhanced 300-fold and, all of the animals that contain intact copies of the transgenes express correctly initiated human globin mRNA.

These data strongly suggest that LCR sequences perform two important functions. First, they 'open' a chromosomal domain that encompasses the entire β-globin locus specifically in erythroid cells. This activity allows them to overcome integration-site, position effects which normally inhibit expression of globin transgenes in 30–70% of the animals. Secondly, they function as a powerful enhancer that stimulates globin gene expression.

We previously demonstrated that a 1.9 kb KpnI–PvuII fragment containing the 5′ HS 2 site (Fig. 1) enhances human β-globin gene expression 100-fold in transgenic mice and also confers position independent expression (10, 11). Several other groups have obtained similar data (12–16). To further define important sequences within this region, 5′ HS 2 β constructs containing deletions of the 1.9 kb fragment were tested for LCR activity in transgenic mice. The results of these experiments demonstrate that multiple protein binding sites are important for maximal enhancer activity. In contrast, no single site is required for position-independent, transgene expression.

METHODS

Construction and analysis of 5′ HS 2 β fragments

Plasmids were constructed by standard procedures (17). Fragment preparation, microinjection, DNA and RNA analysis were as described (18, 19). The limit of detection of β-globin mRNA by the solution hybridization assay (18) is 1.0 pg; this value corresponds to 3 molecules/cell assuming 20 pg of RNA per fetal liver cell and 20 μg of total RNA in the hybridization. Transgene copy numbers for all animals that express human β-globin mRNA are listed in Table 1; transgene copy numbers for non-expressors were in the same range.

Site-specific mutations in 5′ HS 2 (K-P) β were made in the pSELECT system as described by the manufacturer (Promega). The mutagenic oligonucleotides were as follows:

Δ8699-9653: 5′ GGGTGTGTGC/ATCGATGTTCTCAGCC 3′
ΔSP1: 5′ TCAGTGCCCCAC/TTCTGGTTCTGTGTA 3′
ΔH-8641: 5′ CCAGCATCCTCATCTCTGAT/GGCTCAAGCACAGCAATGCT 3′
Δ8641-58: 5′ CCTTCTAAGCAAACCTTCT/GCTGAGTCATGATGAGTC 3′
ΔAP1x2: 5′ CAAGCACAGCAATGC/GCTGAGGCTTAGGGT 3′
8678-82S: 5′ GCTGAGTCATGATGAGTCAttaatAGGCTTAGGGTGTGTGC 3′
NF-E5S: 5′ GATGAGTCATGCTGctaacgAGGGTGTGTGCCCA 3′
8689-98S: 5′ GAGTCATGCTGAGGCTTAGctagctagaCCAGATGTTCTCAGCCTAGAG 3′
ΔGATA1: 5′ GCCTAGAGTGATGAC/GTCCCCAGCAGGATG 3′
ΔUSF: 5′ AAGGAGAAGCTGACCAC/CTCCACCTCAAAC3′
ΔHSC: 5′ AAGAAAATGGATGCC/GTGACATATTCTAGA 3′

The slashes (/) indicate the site of deletion and the small case letters are mutated (scrambled) bases.

RESULTS

Analysis of 5′ HS 2 β-globin constructs in transgenic mice

Figure 1 illustrates the human β-globin locus on chromosome 11 and an expanded view of the 1.9 kb KpnI−PvuII fragment containing 5′ HS 2. Previous experiments demonstrated that this 1.9 kb fragment confers position independent expression in transgenic mice and strongly enhances human β-globin gene expression specifically in erythroid cells. Figure 2A summarizes our previous analysis of this fragment (11) and includes the additional constructs described below. Each 5′ HS 2 fragment was inserted immediately upstream of a 4.1 kb human β-globin gene. The constructs were purified from vector sequences and injected into fertilized mouse eggs. These eggs were transferred into the uteri of pseudopregnant foster mothers and embryos were removed at 16 days of development. Total RNA was extracted from fetal liver and analyzed for correctly initiated human and mouse β-globin mRNA by primer extension (data not shown). Human and mouse β-globin mRNA levels were quantitated by solution hybridization (18). Table 1 lists the results of these experiments and the data are summarized in Figure 2.

Figure 2A demonstrates that all 11 transgenic mice containing intact copies of the 5′ HS 2 (K-P) β construct express the transgene, and the average level of human β-globin mRNA is 41.5% of endogenous mouse β-globin mRNA. When the human β-globin gene without LCR sequences is analyzed in mice only 7 of 23 transgenic animals express human β-globin mRNA and the average level of expression is 0.3% of endogenous mouse β-globin mRNA. Previous experiments also demonstrated that enhancer activity is lost with deletions of both 5′ and 3′ sequences of the 1.9 kb KpnI−PvuII fragment (11); however, all of the transgenic animals that contained the 373 base pair HindIII−XbaI fragment expressed correctly initiated human β-globin mRNA. Constructs that did not contain the HindIII−XbaI fragment [5′ HS 2 (K-H) β, 5′ HS 2 (X-Bg) β and β] were not expressed at high levels and only a fraction of these animals expressed the transgene at all. These results suggested that multiple sequences in the 1.9 kb fragment are involved in enhancer activity and that sequences within the HindIII−XbaI fragment are sufficient to confer position independent expression. Analysis of additional deletion constructs 5′ HS 2 (K-Pp) β and 5′ HS 2 (K-8696) β (Fig. 2) suggested that sequences which confer position independent expression may lie between HindIII and position 8698 (marked by an asterisk). All of the transgenic mice containing these constructs expressed correctly initiated human β-globin mRNA.

No single sequence necessary for position-independent, transgene expression

To further define important sequences in this region a set of 7 constructs containing small deletions within the 217 bp HindIII-8698 fragment were analyzed in transgenic mice. Figure 2B indicates the specific sequences that are deleted or scrambled in these constructs and also compares the human, mouse (20) and goat (21, 22) 5′ HS 2 sequences in this region. We anticipated that mutations which do not inhibit enhancer activity but do affect position-independent, transgene expression would result in high-level expression in only a fraction of the animals. Surprisingly, when all 7 constructs were tested, none of the deletions prevented position-independent expression; all 87 transgenic mice expressed the human β-globin gene. These results suggest that no single sequence in 5′ HS 2 is necessary for position-independent, transgene expression. Apparently, multiple combinations of sites in 5′ HS 2 are sufficient to prevent inhibitory, chromosomal position effects regardless of the site of transgene integration. This conclusion is supported by results from an additional deletion construct, 5′ HS 2 (ΔH-X) β, which is listed at the bottom of Fig. 2A. In this construct the entire HindIII−XbaI fragment was deleted from 5′ HS 2 (K-P) β. Even in the absence of this fragment, all transgenic mice expressed the human β-globin gene. In this case, protein binding sites in the KpnI−HindIII fragment are juxtaposed to sites in the XbaI−PvuII fragment and, presumably, form a novel complex that directs position-independent, transgene expression.

Multiple sequences required for maximal enhancer activity

Although no single site is necessary for position-independence, deletion of individual protein binding sites significantly affect enhancer activity. We and others previously mapped protein binding sites on the HindIII−XbaI fragment by DNase I footprinting (11, 13−15, 23). Three prominent and three weaker footprints were obtained. The SP1, NF-E2, NF-E5, GATA-1, USF and HSC binding sites are indicated in Figure 2B. With the exception of HSC, all of these sites were also detected by *in vivo* footprinting (24, 25) [see Hardison *et al.* (26) for summary]. As we demonstrated previously, deletion of the duplicated NF-E2 sites in the HindIII−XbaI fragment reduces expression of human β-globin mRNA to 2% of endogenous mouse β-globin mRNA. The results in Figure 2A demonstrate that deletion of the SP1, GATA-1 and USF sites also significantly

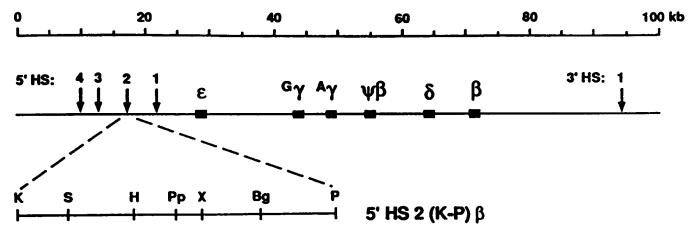

Figure 1. Human β-globin locus. The human β-globin locus on chromosome 11 is illustrated at the top of the figure. Black boxes represent individual globin genes and arrows mark erythroid-specific, DNase I hypersensitive sites that flank the locus. An expanded view of a 1.9 kb KpnI−PvuII fragment containing the 5′ HS 2 sequence is illustrated below the locus. 5′ HS 2 (K-P) β is a construct that contains the 1.9 kb fragment inserted immediately upstream of a human β-globin gene.

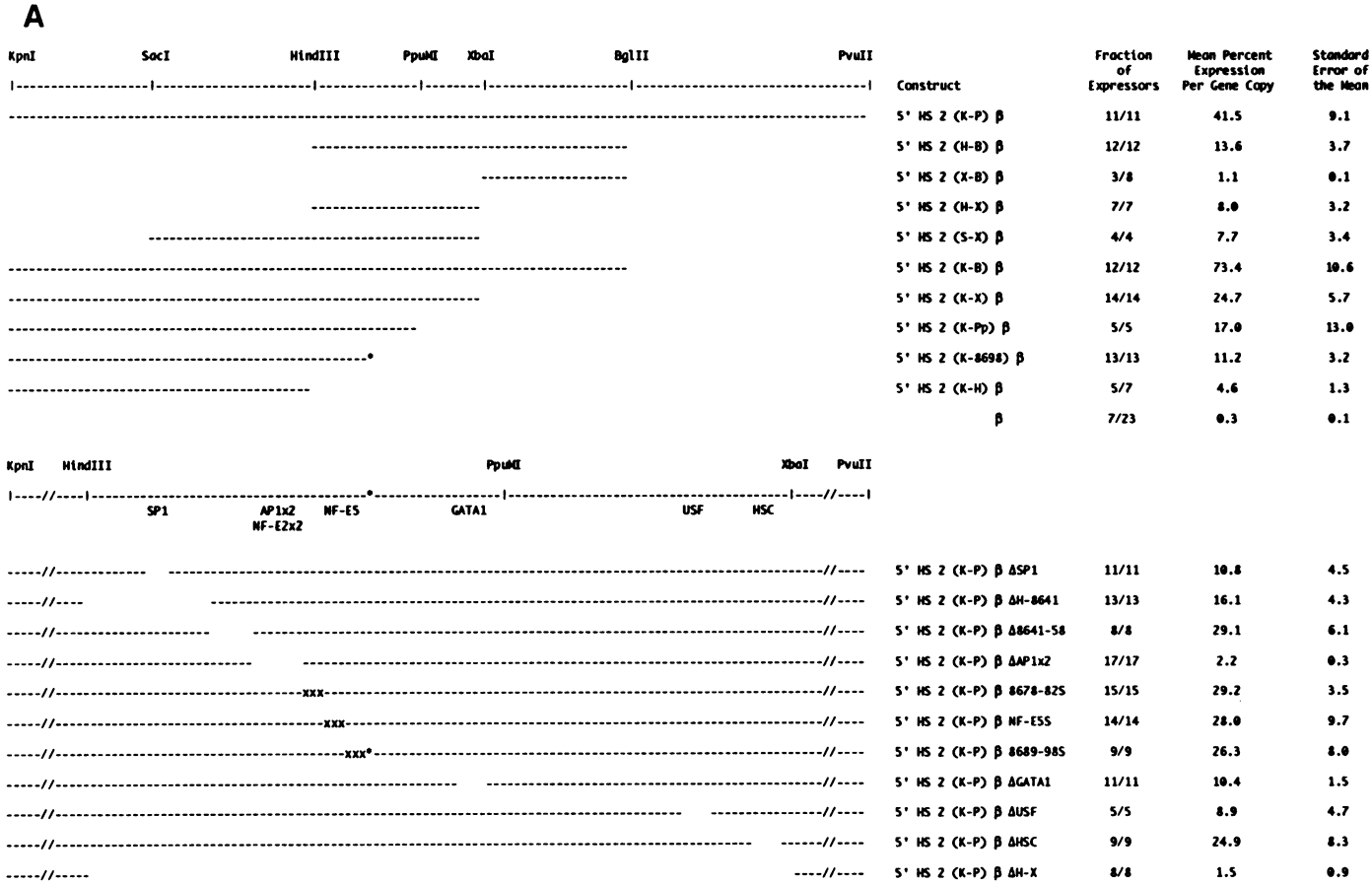

Figure 2. Analysis of 5′ HS 2 β constructs in transgenic mice. Panel **A**. Fragments containing deletions and point mutations in the 5′ HS 2 region were inserted upstream of a 4.1 kb fragment containing the human β-globin gene. 5′ HS 2 β fragments were purified from plasmid sequences and analyzed in transgenic mice as described (10, 11, 18). Restriction sites are as follows: K, KpnI; S, SacI; H, HindIII; Pp, PpuMI; X, XbaI; B, BglII; P, PvuII. Δ refers to sequences or protein binding sites that were deleted. Scramble (S) or xxx refers to sequences or protein binding sites that were replaced with random sequence. Quantitative values of human β-globin and mouse β-globin mRNA were determined by solution hybridization with human β-globin and mouse β-globin specific oligonucleotides as described (10, 18). Data for some of the constructs have been published previously (10, 11) and are included for comparison. The results for ΔUSF include 2 fetuses that were previously designated ΔNF-E (11). The results for 5′ HS 2 (K-B) β and 5′ HS 2 (K-X) β were inadvertently switched in Caterina *et al.* (11) and are listed correctly here. Panel **B**. Sequence homology between the 5′ HS 2 region of the human, mouse, and goat β-globin loci. Sites of protein/DNA interaction are marked and the exact position of deletion and scramble mutants from Panel A are shown.

Table 1.

Construct	Copy Number	Percent Expression	Percent Expression per Gene Copy
5' HS 2 (K-P) β	8	56.0	28.0
	4	63.0	63.0
	5	68.0	54.4
	60	194.0	12.9
	6	21.5	14.4
	10	22.3	8.9
	6	97.1	64.8
	4	46.2	46.2
	22	148.0	26.9
	1	7.5	29.8
	11	295.9	107.6
5' HS 2 (H-B) β	1	0.2	0.8
	2	1.9	3.8
	2	4.1	8.2
	2	4.1	8.2
	3	4.3	5.7
	2	5.2	10.4
	10	16.0	6.4
	5	18.0	14.4
	5	19.0	15.2
	5	44.0	35.2
	20	45.0	9.0
	20	227.0	45.4
5' HS 2 (X-B) β	5	1.4	1.1
	5	1.1	0.9
	10	2.9	1.2
5' HS 2 (H-X) β	0.5	0.7	5.6
	3	1.8	2.4
	1	1.2	4.8
	5	1.2	1.0
	2	5.5	11.0
	3	19.0	25.3
	15	21.0	5.6
5' HS 2 (S-X) β	0.25	0.6	10.3
	0.5	0.4	3.4
	0.5	0.2	1.3
	1	4.0	16.0
5' HS 2 (K-B) β	0.5	7.0	56.0
	0.5	9.0	72.0
	1	11.0	44.0
	1	28.0	112.0
	1	16.0	64.0
	1	24.0	96.0
	4	35.0	35.0
	15	57.0	15.2
	2	72.0	144.0
	3	75.0	100.0
	5	56.0	44.8
	4	98.0	98.0
5' HS 2 (K-X) β	0.5	2.0	16.0
	1	2.0	8.0
	5	4.0	3.2
	1	6.0	24.0
	0.5	8.0	64.0
	1	13.0	52.0
	2	16.0	32.0
	25	110.0	17.6
	50	27.0	2.2
	10	37.0	14.8
	10	46.0	18.4
	3	48.0	64.0
	10	52.0	20.8
	25	54.0	8.6
5' HS 2 (K-Pp) β	1	2.8	11.2
	1	16.9	67.5
	10	10.3	4.1
	10	1.3	0.5
	2	0.9	1.8
5' HS 2 (K-8698) β	0.25	0.4	6.5
	0.5	0.2	1.4
	0.5	4.5	36.2
	7	38.2	21.8
	5	0.1	0.1
	1	5.6	22.3
	1	2.3	9.0
	1	1.4	5.5
	0.5	0.2	1.4
	3	18.6	24.8
	2	6.3	12.6
	2	1.6	3.1
	1	0.1	0.3
5' HS 2 (K-H) β	0.5	0.5	4.0
	1	0.6	2.4
	2	2.0	4.0
	8	6.0	3.0
	5	12.0	9.6
β	8	0.3	0.2
	2	0.2	0.4
	10	0.3	0.1
	30	1.0	0.1
	20	2.0	0.4
	100	10.0	0.4
	150	23.0	0.6
5' HS 2 (K-P) β ΔSP1	1	6.9	27.5
	1	0.8	3.0
	6	12.9	8.6
	1	0.1	0.4
	1	2.2	8.7
	4	16.5	16.5
	2	0.1	0.2
	2	2.5	5.1
	2	23.7	47.4
	1	0.2	0.8
	1	0.1	0.3
5' HS 2 (K-P) β ΔH-8641	1	4.3	17.1
	1	1.4	5.7
	20	49.5	9.9
	1	3.5	14.0
	1	1.9	7.6
	1	0.2	0.8
	2	29.2	58.3
	2	16.8	33.6
	1	3.2	12.7
	2	1.3	2.5
	3	16.4	21.8
	1	2.9	11.7
	15	51.0	13.6
5' HS 2 (K-P) β Δ8641-58	12	40.9	13.6
	5	48.4	38.7
	9	7.8	3.5
	9	104.5	46.5
	1	5.4	21.8
	0.5	2.4	19.2
	1	13.2	53.0
	2	18.2	36.4
5' HS 2 (K-P) β ΔAP1x2	1	0.7	2.8
	2	2.4	4.8
	1	0.8	3.2
	15	8.0	2.1
	15	7.3	2.0
	50	15.3	1.2
	3	1.4	1.9
	27	19.7	2.9
	7	5.5	3.1
	2	0.4	0.7
	36	29.7	3.3
	3	1.8	2.3
	1	0.4	1.7
	0.5	0.1	0.7
	2	1.0	1.9
	3	0.3	0.4
	2	0.9	1.7
5' HS 2 (K-P) β 8678-82S	10	45.0	18.0
	2	11.3	22.6
	1	7.2	28.6
	1	9.1	36.5
	1	3.1	12.4
	0.5	2.5	20.2
	1	8.4	33.5
	0.5	2.8	22.2
	1	11.1	44.4
	4	21.6	21.6
	1	3.4	13.8
	3	31.2	41.5
	2	21.7	43.4
	3	14.6	19.5
	0.5	7.5	60.3
5' HS 2 (K-P) β NF-ESS	20	11.5	2.3
	0.5	1.2	9.4
	20	90.0	18.0
	3	5.4	7.2
	0.5	1.9	15.3
	5	47.8	38.2
	0.5	0.8	6.3
	3	58.7	78.3
	3	55.3	73.7
	1	29.3	117.1
	0.5	0.0	0.4
	0.5	0.1	0.5
	0.5	3.0	23.7
	0.5	0.3	2.2
5' HS 2 (K-P) β 8689-98S	12	111.3	37.1
	1	3.6	14.3
	0.5	4.2	33.8
	2	0.6	1.2
	35	78.3	9.0
	3	14.9	19.9
	1	19.3	77.3
	0.5	0.7	5.4
	20	195.5	39.1
5' HS 2 (K-P) β ΔGATA1	1	1.7	6.8
	1	1.7	6.6
	1	3.5	14.0
	3	10.1	13.5
	15	58.5	15.6
	3	16.3	21.7
	0.5	1.0	7.6
	1	2.1	8.5
	1	1.1	4.5
	1	2.0	8.0
	1	1.9	7.6
5' HS 2 (K-P) β ΔNF-E	3	27.0	36.0
	75	105.0	5.6
	4	0.3	0.3
	3	0.6	0.8
	1	0.5	1.9
5' HS 2 (K-P) β ΔHSC	2	5.1	10.2
	2	17.8	35.7
	6	41.6	27.8
	1	1.0	4.0
	10	46.8	18.7
	8	168.0	84.0
	5	37.1	29.7
	1	1.2	5.0
	1	2.1	8.6
5' HS 2 (K-P) β ΔH-X	10	14.4	5.7
	4	0.4	0.4
	1	0.1	0.4
	1	0.2	0.6
	3	0.2	0.2
	2	0.9	1.8
	100	34.3	1.4
	20	8.3	1.7

decrease enhancer activity; all three deletions reduce β-globin expression to approximately 10% per gene copy. Deletions of the NF-E5 and HSC sites also reduce expression but to a lesser extent (28% and 25%, respectively). These results and the results of deletions upstream and downstream of the HindIII−XbaI fragment (Fig. 2A) demonstrate that the 5′ HS 2 enhancer is extremely complex and that multiple protein binding sites are required for maximal enhancer activity.

The results described above differ from data recently published by Ellis *et al.* (15).who demonstrated that deletion of GATA-1 and USF sites in a 215 bp synthetic 5′ HS 2 did not inhibit expression of a linked human β-globin gene. In addition, Ellis *et al.* (15) showed that mice containing only a single copy of the transgene did not express human β-globin mRNA. In our study all animals with single, intact copies of the transgene expressed correctly initiated human β-globin mRNA and deletion of GATA-1 and USF binding sites decreased expression of the human β-globin gene from approximately 40% per gene copy to 10% per gene copy. The discrepancies in these data probably result from the use of different 5′ HS 2 fragments. Ellis *et al.* (15) used a 215 bp synthetic 5′ HS 2 fragment which has reduced activity in transgenic mice. This 215 bp fragment is 4 fold less active than the wild-type 375 bp HindIII−XbaI fragment and the HindIII−XbaI fragment is approximately 5 fold less active than the 1.9 kb KpnI−PvuII fragment used in our experiments. Our GATA-1 and USF mutations were made in the context of the entire 1.9 kb KpnI−PvuII fragment.

DISCUSSON

We previously suggested that the 5′ HS 2 sequences required for position-independent, transgene expression were located within a 372 bp HindIII−XbaI fragment (11). This conclusion was based on the observation that all constructs which contained these sequences were expressed in transgenic mice regardless of the site of transgene integration. To further define sequences required for position independence, deletions throughout the HindIII−XbaI fragment were analyzed in transgenic mice. Surprisingly, none of the mutations resulted in position-dependent transgene expression; all transgenic mice that contained an intact copy of the transgene expressed human β-globin mRNA. We subsequently deleted the entire HindIII−XbaI sequence from the 1.9 kb KpnI−PvuII fragment and tested this construct in mice. Again, all of the transgenic animals expressed correctly initiated, human β-globin mRNA. Apparently, juxtaposition of protein binding sites in the KpnI−HindIII fragment and sites in the XbaI−PvuII fragment form a novel complex that is capable of directing position-independent transgene expression. At least 10 DNase I footprints are detected in the KpnI−HindIII fragment and 3 footprints are detected in the XbaI−BglII fragment (Caterina and Townes, unpublished). These data suggest that position-independence can be initiated by many different combinations of sequences, and the results are consistent with the observation that 5′ HS 2, 3 and 4 each have LCR activity (27) but share little sequence similarity.

Felsenfeld (28) recently suggested that a primary role of the β-globin LCR is to inhibit nucleosome assembly on globin gene promoters during DNA replication. According to this model, LCR binding proteins form stable interactions with promoter binding factors and keep the promotor in an open conformation specifically in erythroid cells. Several groups have recently shown this type of anti-repression *in vitro* (29−33). Laybourn and Kadonaga (31) demonstrated that a Gal4/VP16 fusion protein acts at a distance to prevent inhibition of target gene expression by histone H1-containing chromatin. Interestingly, the antirepression activity of Gal4/VP16 was characterized by a threshold effect and multiple Gal4 binding sites were required for target gene activation. Although multiple LCR binding proteins may act in a similar manner, this model does not explain the change in chromatin structure throughout the 200 kb globin locus. However, a logical extension of this idea is that LCR binding proteins not only inhibit nucleosome assembly (or at least histone H1-containing chromatin assembly) at promoters but also at several other critical points throughout the β-globin locus; lack of nucleosome assembly at these sites may inhibit the formation of higher order chromatin structures. Alternatively, LCR proteins may prevent histone H1 mediated repression indirectly by binding small proteins such as HMG(I)Y which inhibit highly cooperative H1−H1 interactions (34, 35). Several recent reports suggest that HMG(I)Y and HMG(I)Y-like proteins have a wider role in gene regulation than previously anticipated (36, 37). Regardless of the mechanism involved, it is clear that multiple combinations of 5′ HS 2 binding proteins are capable of erythroid-specific, LCR activity.

In summary, we have demonstrated that several protein binding sites are involved in the enhancer activity of 5′ HS 2. Deletion of SP-1, GATA-1 and USF binding sites in the 1.9 kb KpnI−PvuII fragment containing 5′ HS 2 reduce human β-globin gene expression from 41.5% per gene copy to approximately 10% per gene copy in transgenic mice. As demonstrated previously, deletion of the duplicated NF-E2 sites reduces expression to 2% per gene copy. Although these mutations inhibit enhancer activity, none of them inhibit position-independent transgene expression; all animals express the human β-globin gene regardless of the site of transgene integration. These results demonstrate that no single protein binding site in 5′ HS 2 is necessary for position-independent, transgene expression. Apparently, multiple combinations of protein binding sites in 5′ HS 2 are sufficient to prevent chromosomal position effects that inhibit transgene expression.

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