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Dissection of the enhancer activity of β-globin 5′ DNase I-hypersensitive site 2 in transgenic mice

(locus control region/nuclear factor erythroid 2/activator protein 1)

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ABSTRACT **The β-globin locus control region (LCR) consists of four erythroid-specific DNase I-hypersensitive sites, which are necessary for high-level expression of the β-like globin genes in erythroid tissues. One of these sites, designated 5′HS-2, functions as an erythroid-specific enhancer element in transfection and transgenic mouse experiments. Recent transfection experiments and studies of DNA–protein interactions have localized the 5′HS-2 enhancer to 18 nucleotides that contain a binding site for both the erythroid-specific factor nuclear factor erythroid 2 (NFE-2) and for activator protein 1 (AP-1). To define the sequences necessary for *in vivo* enhancer activity, several deletion mutants of 5′HS-2 were linked to the human β-globin gene and their activity was tested in transgenic mice. Three upstream fragments of 5′HS-2 [341, 374, and 412 base pairs (bp)], each of which contained the NFE-2/AP-1 sequences, resulted in β-globin expression at levels equivalent to or higher than those observed with the entire 732-bp 5′HS-2 fragment. In contrast, a 358-bp downstream portion of 5′HS-2, which lacked the NFE-2/AP-1 sequences, resulted in β-globin expression at the low levels seen with the β-globin gene alone. Removal of the NFE-2/AP-1 sequences by a 67-bp internal deletion resulted in similar low levels of β-globin expression. A 100-bp 5′ fragment that contained the NFE-2/AP-1 sequences resulted in β-globin expression that was higher than the β-globin gene alone but lower than the entire 5′HS-2 fragment or the three larger upstream fragments. These studies demonstrate that the NFE-2/AP-1 sequences are essential for enhancer activity of 5′HS-2 but that other sequences are required for full activity *in vivo*.**

The β-globin gene cluster spans 70 kilobases (kb) of DNA and consists of five functional genes, which are expressed in a developmentally regulated fashion in erythroid tissues (see Fig. 1) (1). The high level expression of these genes is dependent on regulatory sequences localized by four erythroid-specific DNase I-hypersensitive sites (designated 5′HS1 to 5′HS4), which together comprise the β-globin locus control region (LCR) (2, 3). The importance of this region was suggested by the discovery that deletions that completely or partially removed the LCR result in inactivation of the normal β-globin gene in cis (4–6). Subsequent experiments in which LCR sequences were linked to human β-globin genes and used to make transgenic mice demonstrated that these sequences are necessary for high-level, position-independent globin gene expression (7).

The activities of the individual hypersensitive sites have been examined in transfection and transgenic experiments. Only 5′HS-2 can function as a classical enhancer in both transfection and transgenic mouse studies (8–10). Transient transfection analysis of deletion and point mutation constructs derived from 5′HS-2 indicates that an 18-nucleotide portion is essential for enhancer activity (11, 12). These 18 nucleotides contain a tandem repeat of the consensus recognition site for the activator protein 1 (AP-1) family of DNA binding proteins (13). In addition, the upstream repeat is also a binding site for the erythroid-specific factor nuclear factor erythroid 2 (NFE-2) (14). Both *in vitro* and *in vivo* studies of DNA–protein interactions at these sequence motifs suggest that a specific pattern of binding is present in samples from erythroid cells, particularly when those cells have been induced to increase globin gene expression (11, 12, 15, 16). Other sequence motifs within 5′HS-2 that display unique patterns of protein binding in erythroid-derived samples are four binding sites for the erythroid-specific factor GATA-1 (17) and four copies of a GGTGG sequence motif. The functional importance of these latter sites in 5′HS-2 remains unclear.

In this study, we have attempted to identify the sequences within 5′HS-2 that are necessary for full enhancer activity *in vivo*. We have linked various portions of 5′HS-2 to β-globin gene fragments and examined their activity in transgenic mice. Our findings indicate that while the NFE-2/AP-1 motif is required for enhancer activity in transgenic mice, additional sequence elements are necessary for full enhancer function.

MATERIALS AND METHODS

Construction of 5′HS-2 β-Globin Gene Fragments. The 5-kb *Bgl* II β-globin gene fragment was used in all constructs. Fig. 1 shows the portion of 5′HS-2 that was joined upstream of the β-globin gene by blunt-end ligation in each construct. The 732-base-pair (bp) *Hin*dIII/*Bgl* II 5′HS-2 fragment (GenBank coordinates 8486–9218), which has been shown to function as an erythroid-specific enhancer in transfection and transgenic studies, was joined to the β-globin gene to produce 5′HS2-(H-Bg)β. The 358-bp *Xba* I/*Bgl* II downstream portion (residues 8860–9218) and 374-bp *Hin*dIII/*Xba* I upstream portion (residues 8486–8860) of the 732-bp fragment were linked to the β-globin gene to produce 5′HS-2(X-Bg)β and 5′HS-2(H-X)β, respectively. The 341-bp *Bal* I/*Sna*BI fragment (residues 8568–8909) and 412-bp *Bal* I/*Ssp* I fragment (residues 8568–8980) were used to construct 5′HS-2(B-Sn)β and 5′HS-2(B-Ss)β, respectively. A reverse polymerase chain reaction was used to introduce a 67-bp internal deletion (residues 8648–8715) in the 732-bp 5′HS-2 fragment and this fragment was used to construct 5′HS-2(H-BgΔ)β (11). Oligonucleotides bearing artificial restriction sites were used to amplify

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Abbreviations: AP-1, activator protein 1; NFE-2, nuclear erythroid factor 2; LCR, locus control region.

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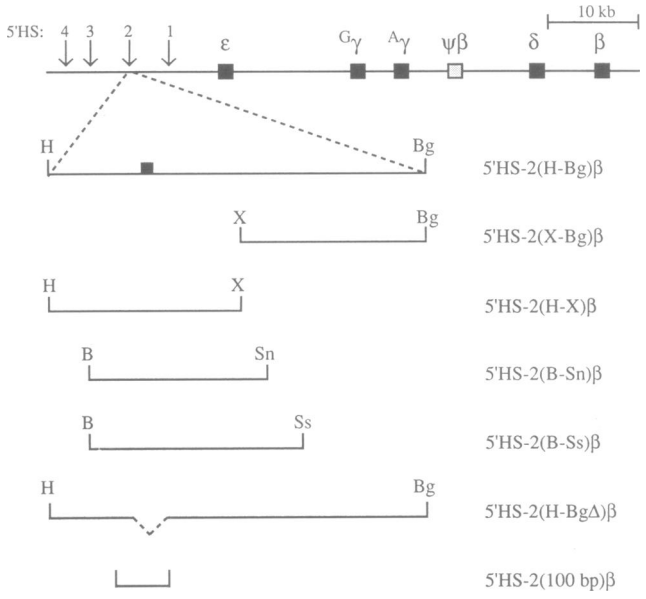

FIG. 1. 5'HS-2 β constructs analyzed in transgenic mice. The human β-globin cluster is shown at the top with genes represented by boxes and locations of the DNase I-hypersensitive sites indicated by arrows. The 732-bp *Hin*dIII/*Bgl* II 5'HS-2 fragment is shown immediately below with the NFE-2/AP-1 binding site shown as a box. Six portions of this 5'HS-2 fragment that were linked to the β-globin gene and assayed in transgenic mice are shown below. H, *Hin*dIII; Bg, *Bgl* II; X, *Xba* I; B, *Bal* I; Sn, *Sna*BI; Ss, *Ssp* I.

a 100-bp portion of 5'HS-2 (residues 8619–8719), which was cloned and used to construct 5'HS-2(100 bp)β. The orientation and sequence of the 5'HS-2 fragments were confirmed by restriction and sequence analysis.

Production and Analysis of Transgenic Mice. Each construct was purified from vector sequences and injected into fertilized mouse eggs; the eggs were transferred to pseudopregnant recipients (10). Day 16 fetuses were sacrificed and DNA was prepared from the hindquarters of each fetus. Southern analysis was performed to identify transgenic mice, to ensure that the injected construct was intact, and to determine gene copy number. DNA samples were digested with *Nco* I, which cuts once in each construct, and filters were probed with the 0.9-kb *Bam*HI/*Eco*RI human β-globin gene probe to identify transgenic mice. Digestion with additional restriction enzymes and hybridization with a 5'HS-2 probe were performed to ensure that the injected construct was intact in each transgenic sample. DNA was also prepared from liver and brain of some fetuses to identify mosaic transgenic mice. Mice that were mosaics or that contained rearranged copies of the injected construct were excluded from this analysis. Southern filters were scanned on a PhosphoImager (Molecular Dynamics, Sunnyvale, CA) and gene copy number was determined by comparison with human DNA standards. Quantitation of gene copy number in each transgenic mouse was performed on at least two filters.

Total RNA was prepared from fetal liver as described by Chomczynski and Sacchi (18) with the addition of a proteinase K digestion step followed by phenol extraction and ethanol precipitation. The RNase protection assay was used to compare human β-globin mRNA to mouse α-globin mRNA in each sample with antisense RNA probes derived from the 3' portion of each gene (19). Samples (0.5 μg) of total liver RNA from each transgenic mouse were hybridized with 2×10^5 cpm of each probe. RNA samples from mice that expressed human β-globin RNA at very high levels were also tested with smaller amounts of total liver RNA to ensure conditions of probe excess. Hybridized samples were digested with RNase A and T1 and separated by electrophoresis in polyacrylamide/urea gels. The gels were scanned on a PhosphoImager to allow quantitation of human β-globin and mouse α-globin mRNA. At least two separate RNA gels were analyzed for each transgenic mouse. Since [^{32}P]UTP was used to synthesize each RNA probe, the relative quantity of human β-globin mRNA signal and mouse α-globin mRNA signal counted was corrected for the relative number of uridine residues in the respective protected probe fragments. Expression of human β-globin mRNA was calculated both as a percentage of endogenous mouse α-globin mRNA {[(human β-globin mRNA)/(mouse α-globin mRNA)] × 100} and as percentage expression per gene copy ({[(human β-globin mRNA)/(human β-globin gene copy)]/[(mouse α-globin mRNA)/(mouse α-globin gene copy)]} × 100).

RESULTS

Production and Analysis of 5'HS-2 Transgenic Mice. We and others have previously demonstrated that 5'HS-2 can markedly enhance expression of a linked human β-globin gene in transgenic mice (9–11). To define the sequences within 5'HS-2 that are responsible for full enhancer activity, it was first necessary to produce control transgenic mice bearing the β-globin gene alone and the β-globin gene joined to the 732-bp *Hin*dIII/*Bgl* II 5'HS-2 fragment. Two transgenic mice were obtained with the β-globin gene alone (Fig. 2*A*). Neither of these mice expressed human β-globin mRNA (Table 1). Seven transgenic mice were produced with the 5'HS-2(H-Bg)β construct (Fig. 2*B*). These mice expressed human β-globin mRNA at high levels with a mean expression per gene copy of 18.5% (range, 8.7–40%).

Six different portions of the 732-bp 5'HS-2 fragment were linked to the human β-globin gene and these constructs were used to make transgenic mice. Five transgenic mice were obtained with the β-globin gene joined to the 358-bp *Xba* I/*Bgl* II downstream portion of 5'HS-2 (Fig. 3*A*). This fragment does not contain the NFE-2/AP-1 sequences that are essential for enhancer activity in transfection assays. These mice expressed human β-globin mRNA at low levels with a mean expression per gene copy of 0.2% (range, 0.0–0.4%).

Three upstream portions of 5'HS-2, each of which contained the NFE-2/AP-1 sequences, were also tested. Six transgenic mice bearing the 374-bp *Hin*dIII/*Xba* I portion of 5'HS-2 were obtained (Fig. 3*B*). The mean expression per gene copy of these mice was 30.8% (range, 5.5–70%). Six transgenic mice were also produced bearing the 341-bp *Bal* I/*Sna*BI 5'HS-2 fragment (Fig. 3*C*). These mice expressed

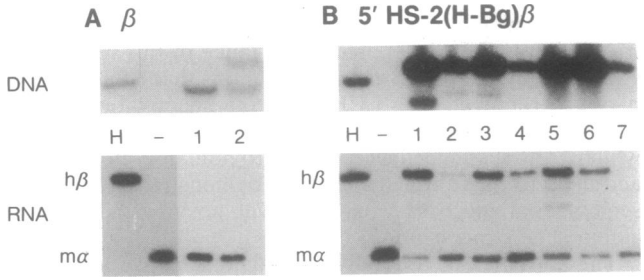

FIG. 2. Southern and RNase protection analysis of samples from β and 5'HS-2(H-Bg)β transgenic mice. Above are human (lanes H), nontransgenic mouse (lanes −), and transgenic mouse (numbered lanes) DNA samples that were digested with *Nco* I and hybridized with a human β-globin gene probe. Below are the corresponding RNA samples that were subjected to RNase protection analysis using antisense mouse α-globin and human β-globin RNA probes. The bands resulting from probe protection with authentic human β-globin (hβ) and mouse α-globin (mα) mRNA are indicated.

Table 1. Human β-globin gene copy number and expression

Transgene	Gene copy number	% mouse α-globin mRNA*		% expression per gene copy†
β				
1	4	0.0		0.0
2	1	0.0		0.0
5'HS-2(H-Bg)β				
1	40	343.6		34.4
2	4	8.7		8.7
3	30	82.3		11.0
4	2	20.0		40.0
5	34	133.1		15.7
6	36	96.0		10.7
7	2	4.5		9.0
			Mean	18.5
5'HS-2(X-Bg)β				
1	15	1.5		0.4
2	3	0.0		0.0
3	5	0.0		0.0
4	1	0.1		0.4
5	13	0.3		0.1
			Mean	0.2
5'HS-2(H-X)β				
1	4	22.0		22.0
2	14	162.2		46.3
3	1	17.5		70.0
4	4	5.5		5.5
5	4	19.6		19.6
6	5	27.1		21.7
			Mean	30.8
5'HS-2(B-Sn)β				
1	16	71.0		17.8
2	54	194.7		14.4
3	2	5.2		10.5
4	3	106.1		141.5
5	1	34.3		137.2
6	2	112.0		224.0
			Mean	90.9
5'HS-2(B-Ss)β				
1	1	8.6		34.4
2	8	30.9		15.4
3	4	25.8		25.8
4	35	112.4		12.8
5	10	84.2		33.7
6	36	131.2		14.6
			Mean	22.8
5'HS-2(H-BgΔ)β				
1	6	0.0		0.0
2	7	7.1		4.1
3	1	0.0		0.0
4	8	0.0		0.0
			Mean	1.0
5'HS-2 (100 bp)β				
1	4	2.8		2.8
2	8	7.4		3.7
3	2	1.1		2.1
4	1	0.6		2.2
5	30	19.6		2.6
			Mean	2.7

*[(Human β-globin mRNA)/(mouse α-globin mRNA)] × 100.
†{[(Human β-globin mRNA)/(human β gene)] ÷ [(mouse α-globin mRNA)/(mouse α gene)]} × 100.

human β-globin mRNA at very high levels, with a mean expression per gene copy of 90.9% (range, 10.5–224%). The extremely high level of expression observed in three of these animals suggests that they may be mosaics. Unfortunately, DNA samples from liver or other organs were not obtained from these mice. Six transgenic mice were obtained bearing the 412-bp *Bal* I/*Ssp* I portion of 5'HS-2 (Fig. 3*D*). These mice also expressed human β-globin mRNA at high levels, with a mean expression per gene copy of 22.8% (range, 12.8–34.4%). Thus, these three 5'HS-2 fragments retained enhancer activity that was equivalent to or greater than that of the 732-bp fragment.

To determine whether the NFE-2/AP-1 sequences were essential for enhancer activity, a 67-bp internal deletion that removed these sequences was introduced into the 732-bp 5'HS-2 fragment. Four transgenic mice were produced with this construct (Fig. 3*E*). The mean expression per gene copy of these mice was 1.0% (range, 0–4.1%). Thus, deletion of a portion of 5'HS-2, which includes the NFE-2/AP-1 motif, resulted in loss of enhancer activity.

A 100-bp portion of 5'HS-2, which contained the NFE-2/AP-1 sequences, was also tested and five transgenic mice bearing this construct were obtained (Fig. 3*F*). The mean level of expression per gene copy in these mice was 2.7% (range, 2.1–3.7%). Thus, the NFE-2/AP-1 motif along with the nearby flanking sequence retains some enhancer activity but at a much lower level than the entire 732-bp fragment.

DISCUSSION

The β-globin LCR consists of four erythroid-specific DNase I-hypersensitive sites that are necessary for the high-level, tissue-specific expression of the β-globin-like genes (3). A similar LCR has recently been described in the α-globin gene cluster (20). The function of the LCR includes both activation of the chromatin of the globin gene cluster to allow gene expression in erythroid tissue and marked enhancement of globin gene expression. While the ability to increase expression of a linked β-globin gene in transgenic mice is present in 5'HS-2, 5'HS-3, and 5'HS-4 (21), only 5'HS-2 contains enhancer activity in transient transfection assays. Examination of deletion and point mutation constructs derived from 5'HS-2 has shown that the sequences essential for this activity reside in an 18-nucleotide portion that contains a binding site for an erythroid-specific factor, NFE-2, and a tandem repeat of the AP-1 motif. The NFE-2 binding motif was first identified in the promoter of the erythroid-specific gene encoding porphobilinogen deaminase (14). Binding sites for this factor have subsequently been identified in the regulatory elements of the globin genes and other genes expressed in erythroid tissues. The AP-1 motif binds the jun/fos family of DNA binding proteins, which participate in regulation of a wide array of genes in many tissues. Studies of DNA–protein interactions and examination of functional activity after specific mutagenesis suggest that binding of NFE-2 is the important activating event in 5'HS-2 (22, 23).

We have previously shown that 5'HS-2 can function as an erythroid-specific enhancer element when linked in either orientation upstream of the human β-globin gene in transgenic mice (10). It has become clear, however, that the levels of β-globin gene expression we observed in both β-globin and 5'HS-2 β-globin transgenic mice were higher than those reported by others. At least two factors contributed to this overestimation of expression. First, excessive amounts of total liver RNA (5–10 μg) were used in the RNA probe hybridizations and thus conditions of probe excess were not achieved. In the current studies, we have found that 0.5-μg total liver RNA samples allow accurate quantitation. Second, the reported mRNA percentages were not corrected for the >2-fold greater number of (radiolabeled) uridine residues in the human β-globin protected probe fragment relative to that of the mouse α-globin protected fragment. Correcting for these two factors resulted in levels of expression that are comparable to those reported by other investigators (9, 21).

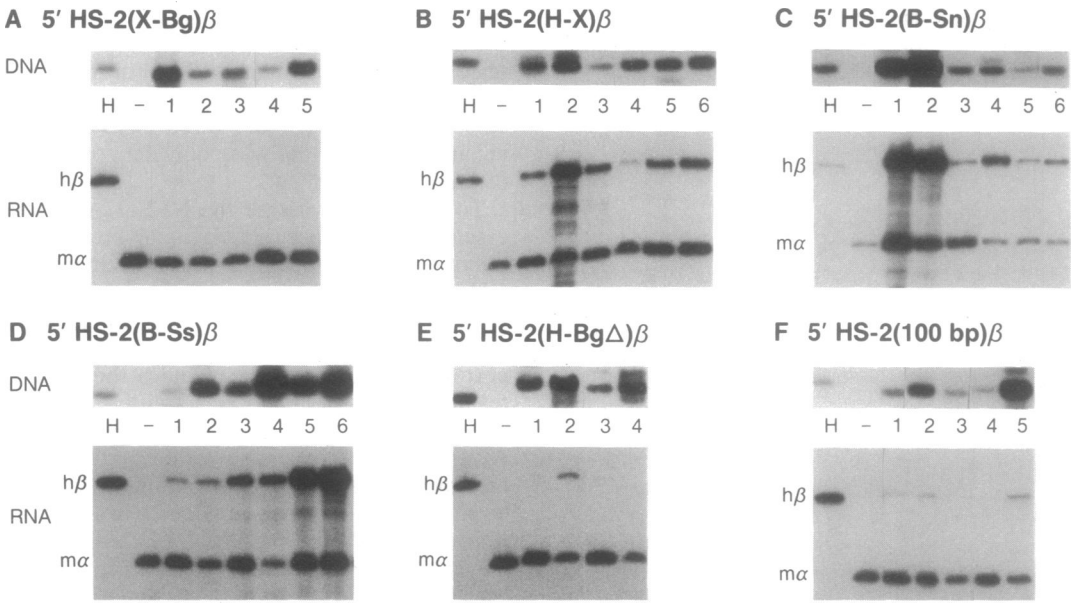

FIG. 3. Southern and RNase protection analysis of samples from transgenic mice bearing portions of 5′HS-2 linked to the human β-globin gene. The fragment tested in each group of transgenic mice is shown in Fig. 1. Lanes representing human (H), nontransgenic mice (−), and transgenic mice (numbered) are indicated. Southern analysis with a human β-globin gene probe is shown above. RNase protection assay, using antisense mouse α-globin (mα) and human β-globin (hβ) RNA probes, is shown below.

To determine whether the NFE-2/AP-1 motif was necessary and sufficient for full enhancer activity, we tested the ability of several 5′HS-2 fragments to activate human β-globin gene expression in transgenic mice. Two constructs that lacked the NFE-2/AP-1 sequences, 5′HS-2(X-Bg)β and 5′HS-2(H-BgΔ)β, resulted in low levels of β-globin gene expression (mean percentage expression per gene copy of 0.2% and 1.0%, respectively). These levels are equivalent to those observed in transgenic mice bearing the β-globin gene alone and indicate that these fragments lack enhancer activity. In contrast, three 5′HS-2 constructs that contained the NFE-2/AP-1 sequences, 5′HS-2(H-X)β, 5′HS-2(B-Sn)β, and 5′HS-2(B-Ss)β, resulted in high expression levels (30.8%, 90.9%, and 22.8%, respectively). Three 5′HS-2(B-Sn)β transgenic mice expressed at very high levels, suggesting that they may be mosaics. If these three mice are omitted, expression levels are equivalent to those observed with the entire 732-bp fragment used in 5′HS-2(H-Bg)β (18.5%). A construct bearing the NFE-2/AP-1 sequences in a 100-bp fragment, 5′HS-2 (100 bp), resulted in mean expression levels (2.7%) significantly lower than the larger 5′HS-2 fragments bearing the NFE-2/AP-1 motifs but significantly higher than the constructs that lack these sequences. Thus, this study indicates that the NFE-2/AP-1 sequences are necessary for enhancer activity but that additional sequences within 5′HS-2 are necessary for full activity. These findings are in agreement with the transgenic studies of Talbot and Grosveld (24) and Caterina *et al.* (25), which also localized the 5′HS-2 enhancer to the upstream portion of the *Hin*dIII/*Bgl* II fragment and demonstrated marked reduction in enhancer activity upon deletion or mutation of the NFE-2/AP-1 motifs.

Recent studies of 5′HS-2 using the *in vivo* footprint technique have allowed examination of DNA–protein interactions within erythroid and nonerythroid cells both with and without agents that induce globin gene expression (15, 16). These studies have shown erythroid-specific and in some cases inducible binding patterns at the NFE-2/AP-1 motifs, at GGTGG sequences both upstream and downstream of those motifs, and at several binding sites for the erythroid-specific binding protein GATA-1. Thus, these sequences are candidate sites for the interactions that are necessary for full enhancer activity of 5′HS-2. Moi *et al.* (26) have recently reported the results of transient transfection experiments using 5′HS-2 fragments bearing point mutations in sequence motifs other than NFE-2/AP-1. These studies suggest that the enhancer activity of 5′HS-2 may be both positively and negatively modulated by sequence motifs within 400 bp of the NFE-2/AP-1 motifs.

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