A 46 base pair enhancer sequence within the locus activating region is required for induced expression of the gamma-globin gene during erythroid differentiation

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ABSTRACT

The locus activating region (LAR), contained within 30 kb of chromatin flanking the human beta-globin gene cluster, has recently been shown to be essential for high level beta-globin gene expression. To determine the effect of fragments containing LAR sequences on globin gene expression, mRNA from a marked gamma-globin gene linked to LAR fragments was assayed in stably transfected K562 erythroleukemia cells. DNaseI hypersensitive site II (HS II), located 10.9 kb upstream of the epsilon-globin gene, was required for high level gamma-globin gene expression. We also showed that a 46 bp enhancer element within HS II was necessary and sufficient for the increased gamma-globin gene expression observed with hemin induced erythroid maturation of K562 cells. These results localize a distant regulatory element important for activation of globin genes during human erythroid cell maturation.

INTRODUCTION

The human beta-like globin genes are distributed over 40 kb of DNA on the short arm of chromosome 11. Five functional genes contained within this region are regulated in three important ways. The globin genes are expressed in a strictly tissue specific manner so that globin mRNA is found only in erythroid cells. The individual beta-like globin genes are expressed at specific stages of development. Epsilon-globin is produced during embryonic development, ^{G}gamma and ^{A}gamma-globin during fetal development, and delta and beta-globin during the adult stage. A third level of enhanced globin expression coincides with terminal erythroid maturation.

This complex regulation is partly dependent on critical sequences within promoter, enhancer, and intragenic elements that are within or directly flanking the individual globin genes. Interaction of transcription factors with these sequences is one important mechanism of gene regulation. It is clear that these flanking sequences are not sufficient to direct high level expression in gene transfer experiments. When beta-globin constructs containing a full promoter and downstream enhancer sequences are introduced into mice either by creating transgenic animals or by retroviral mediated transfer, expression averages only a few percent that of the endogenous mouse globin genes and is variable among individual animals (1−3). These data suggest that other sequence information is required for high level regulated expression.

Distant sequences located 20 kb upstream and 6 kb downstream of the locus have been shown to be critically important to globin gene expression. These sequences, marked by DNaseI hypersensitivity in isolated nuclei, are located 18.0, 14.7, 10.9, and 6.1 kb 5′ of the epsilon-globin gene transcriptional start site and 21.8 kb 3′ of the beta-globin polyadenylation site. We have used the nomenclature that designates the most upstream site hypersensitive site IV (HS IV) and the others HS III, HS II, HS I, and HS VI, respectively.

The functional importance of these sequences was first suggested by the description of the Dutch gamma-delta-beta thalassemia mutation in which a deletion that removes the upstream sites caused inactivation of the entire chromosomal locus despite the presence of a intact beta-globin gene and its flanking regulatory sequences (4−5). Considerable experimental evidence confirming the importance of these sequences has been obtained using both transgenic mice and tissue culture cells (6−9). Based on this data this large region of chromatin has been called the dominant control region or the locus activating region (LAR) reflecting its apparent critical role in expression of the genes within the beta-globin locus.

The actual sequences within the LAR that are responsible for transcritional activation and the mechanism of action of these sequences are largely unknown. Tuan et. al. first reported that elements within the LAR contain sequences expected for an enhancer and later showed that HS II contains a powerful erythroid specific enhancer (10−11). We have recently localized this enhancer activity to a short sequence containing a tandem repeat for activating protein 1 (AP1) binding sites (12).

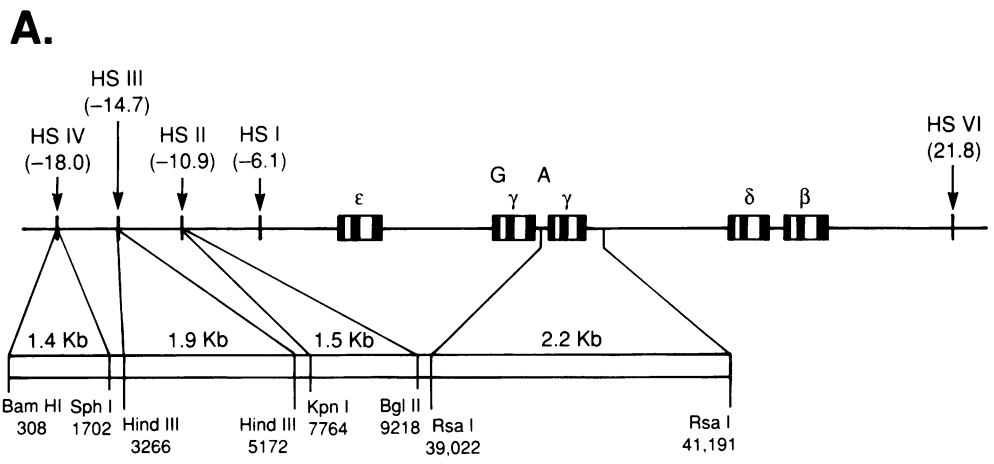
Our goal for the experiments to be described was to develop an assay system to determine the sequences within the LAR that are active in controlling gene expression by stably integrating hypersensitive sites linked to a globin gene into human erythroleukemia (K562) cells. Subfragments containing the individual hypersensitive sites linked to a marked gamma-globin gene were assayed to determine the activity of the individual sites and any synergy between them. We chose to study the three most upstream sites based on data that sites I and VI are not required for full activity (7,9,13−14). Our results indicate that HS II is

necessary for high level gamma-globin expression in stably transfected K562 cell clones and that a 46 bp enhancer within site II is both necessary and sufficient for the increased gamma-globin gene expression seen with induction of erythroid maturation with hemin.

METHODS

DNA constructions

All subfragments were subcloned into pUC007, a pUC based plasmid with a novel polylinker. pUC007 consists of the following polylinker subcloned into the HindIII and EcoRI sites of pUC9: 5′ XhoI, BglII, XbaI, HindIII, SmaI, BamHI, ApaI, SphI, SalI 3′. HS IV was subcloned as a BamHI-SphI fragment into the BamHI and SphI sites of pUC007. HS III was subcloned as a HindIII fragment into the HindIII site of pUC007. HS II was subcloned as a KpnI(blunted)-BglII fragment into the BlgII(blunted) and BamHI sites of pUC007. The ^{A}gamma-globin gene was subcloned as a RsaI fragment into the SmaI site of pUC007 (pUC007-^{A}gamma). The marked ^{A}gamma-globin gene was made by subcloning a synthetic oligonucleotide consisting of wildtype ^{A}gamma-globin sequence from −51 to +50 relative to the mRNA cap site but deleting six bases from +23 to +28. This synthetic fragment was cloned into the AhaII and NaeI sites of the gamma-globin gene in pUC007-^{A}gamma and sequenced. A neomycin resistance transcription unit containing a polyoma



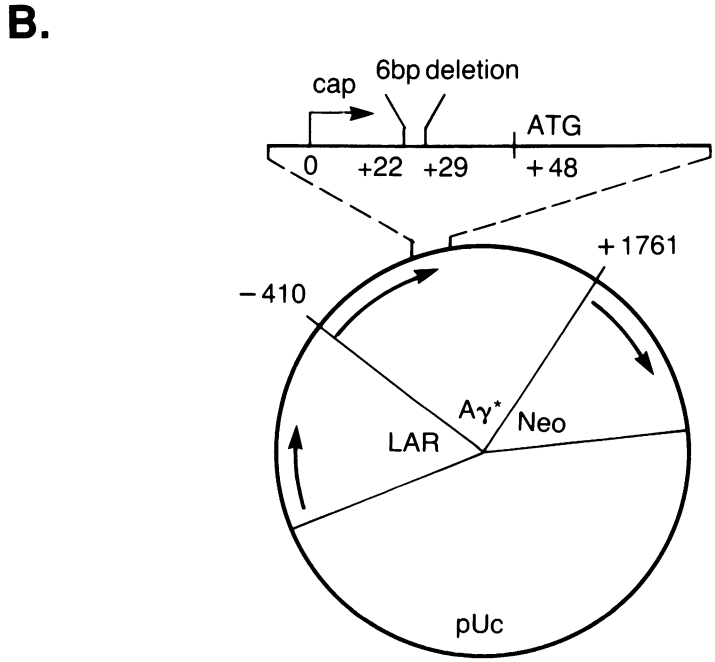


Figure 1. LAR fragments used in plasmid constructions. (A) A schematic diagram of the beta-globin locus showing the relative positions of the major hypersensitive sites and the individual globin genes. Distance in Kb from the epsilon gene (HS IV-I) or the beta gene (HS VI) is given in parenthesis. Cloned fragments are shown with their gene bank coordinates and restriction sites used. Dark bars indicate exons and light bars introns within the individual genes. (B) A general schematic showing plasmid construction. LAR fragments were cloned upstream of a marked ^{A}gamma-gene containing a 6 bp deletion in the non-translated portion of the first exon. Numbers listed show sequence locations relative to the globin transcription start site. A neomycin resisitance gene is cloned downstream of the globin gene.

A enhancer was isolated from the plasmid PMCIPolA (15) as a XhoI-HindIII fragment and subcloned into the XhoI and HindIII sites of pUC007. A 46 bp fragment containing the minimal enhancer was synthesized as an oligonucleotide with a HindIII site on the 5′ end and a BamHI site on the 3′ end. This oligonucleotide was subcloned into the HindIII and BamHI sites of pUC007 and sequenced. The 1.5 Kb enhancer minus version of HS II (HS II-E) was made by replacing 25 bp of the 46 bp enhancer sequence with polylinker (12). This construct disrupts both AP1 sites by replacing wildtype HS II sequence 8664 to 8694 with polylinker while leaving the total size of the fragment unchanged.

Plasmids containing combinations of the fragments described above were all made with the following subcloning strategy: vector plasmids containing the most 5′ fragment(s) were cut with SalI and AatII. Inserts were isolated as XhoI-AatII fragments and cloned into the SalI and AatII sites of the vector. Note that the XhoI and SalI sites used in this cloning step are ablated allowing the same cloning strategy to be applied when isolating vector or insert fragments from the new plasmid.

Tissue culture and cell transfection

Human erythroleukemia K562 cells were grown in Improved Minimal Essential Medium (IMEN, Biofluids) with 10% fetal calf serum and 50 mg/ml gentamicin. Plasmid DNA was linearized with SalI and 7.5 μg of each linear construct was transfected into 1.25×10^7 cells by electroporation (16). A Bio-Rad gene pulser set at 0.2 Kv and 960 μF was used after suspending the cells and DNA in 400 μl HEBS. Transfected cells were grown for 48 hrs in non-selective growth medium, diluted at 2.5×10^3 and 5.0×10^3 cells/ml in medium containing 0.5 mg/ml of G418 (GIBCO), and plated out in Costar 24 well plates. After 12 days individual clones were pooled in 10 mls of growth medium in Costar flasks. Flasks were mixed and split into two equal aliqouts. One half of each split pool was grown in 20 μM hemin (Sigma) and both induced and uninduced aliquots were grown for 3 days in 10 mls of their respective media.

PCR RNA assay

Total cytoplasmic RNA was prepared by NP-40 lysis in the presence of vanadylribonucleoside complex as described previously (17). A first strand cDNA synthesis was performed using the following assay conditions: 0.5−2.0 μg of RNA as a template, 1.33 mM dNTPS, 13.3 units/μl M-MLV-reverse transcriptase (BRL), 1.3 units/μl RNasin (Promega), 6.7 ng/μl oligo$(dT)_{12-18}$, 50mM Tris-HCl (pH8.3), 75mM KCl, 10mM DTT, and 3mM $MgCl_2$ in a 15 μl volume for 30 minutes at 37°C. Samples were then denatured at 95°C for 5 minutes and chilled on ice. 10 μl of each cDNA reaction mixture was amplified by adding the following reagents: 2.5 μl of each 20uM primer, 0.5 μl of TaqI polymerase (Cetus 5.0 units/μl), 5.0 μl of 10× buffer (Cetus), 1.0 μl of ^{32}PdCTP (800uCi/mM), and 28.5 μl dH_2O. The sequence of the upstream primer is 5′ACACTCGCTTCTGGAACG 3′ and that of the downstream primer 5′GCTTGTGATAGTAGCCTTGT 3′. Samples were amplified for 8−10 cycles with denaturation at 94°C for 1.5 mins, reannealing at 56°C for 2.0 mins, and extension at 72°C for 3.0 mins. 6 μl of each reaction mixture was denatured, quick chilled, and electrophoresed on a 8% polyacrylamide 7M urea sequencing gel. The amount of input RNA and the number of amplification cycles were held constant for any given experiment.

T1 RNase protection assay

10 μg of RNA was assayed as previously described (18−19). The probe used was made by cloning a 881 bp BamHI fragment from the wildtype ^{A}gamma-globin gene into the BamHI site of plasmid pTZ18R (Pharmacia). Radiolabelled transcripts were

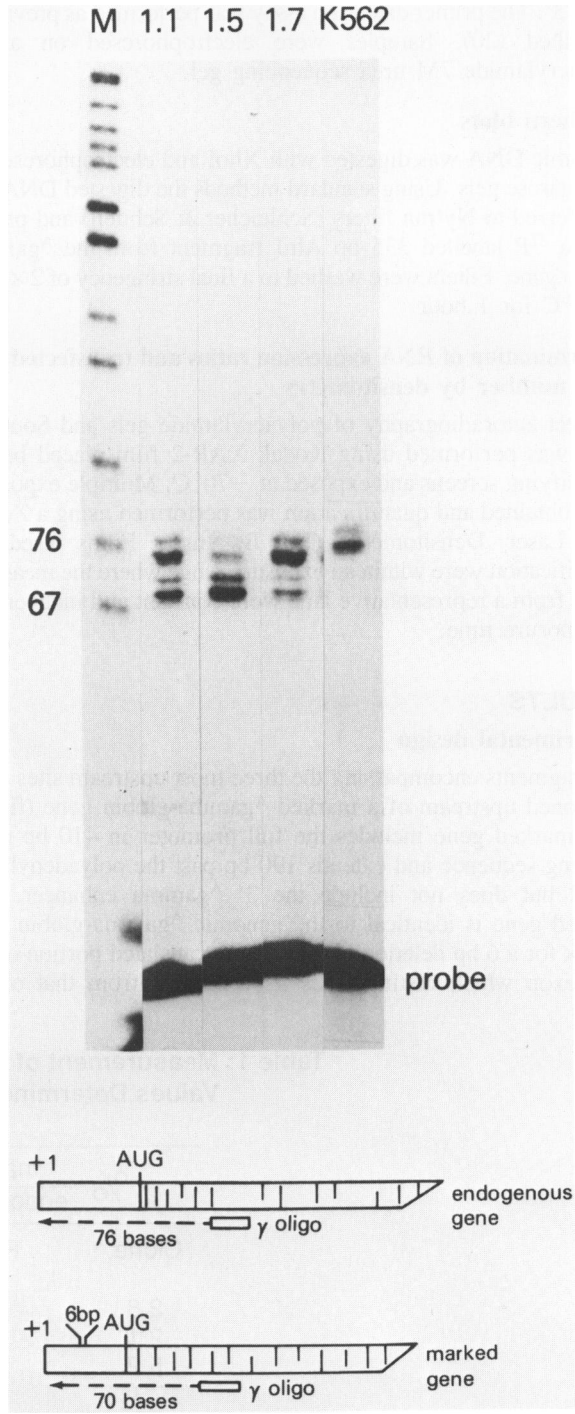


Figure 2. Primer extension analysis of individual K562 cell clones. Clones transfected with constructs containing LAR fragments linked to the marked gamma-globin gene are numbered 1.1, 1.5, and 1.7 and nontransfected K562 cells are shown. Molecular weight marker is shown in the left most lane with 76 bp and 67 bp signals indicated. Free probe is shown at the bottom. The schematic diagram shows that correctly initiated endogenous gamma-globin mRNA gives a 76 bp extension product and that correctly initiated marked gamma-globin mRNA gives a 70 bp extension product.

generated with T7 polymerase. This probe protects a 145 bp fragment of the first exon from the endogenous gamma-globin gene transcript and a 119 bp fragment from the marked transcript.

Primer extension analysis

20 μg of RNA was annealed to a ^{32}P end labelled primer with the following sequence: 5'CCTCCTCTGTGAAATGACCCATGGC 3'. The primer extension assay was performed as previously described (20). Samples were electrophoresed on a 8% polyacrylamide 7M urea sequencing gel.

Southern blots

Genomic DNA was digested with XhoI and electrophoresed on 1% agarose gels. Using standard methods the digested DNA was transferred to Nytran filters (Schleicher & Schuell) and probed with a ^{32}P labelled 335 bp AluI fragment from the ^{A}gamma-globin gene. Filters were washed to a final stringency of 2× SSC at 65°C for 1 hour.

Determination of RNA expression ratios and transfected gene copy number by densitometry

Indirect autoradiography of polyacrylamide gels and Southern blots was performed using Kodak XAR-2 film placed behind intensifying screens and exposed at −70°C. Multiple exposures were obtained and quantification was performed using a Zeineh Soft Laser Densitometer (L.K.B. Inst). Films used for quantification were within an exposure range where the measured ratios from a representative lane were constant and independent of exposure time.

RESULTS

Experimental design

Subfragments encompassing the three most upstream sites were subcloned upstream of a marked ^{A}gamma-globin gene (fig.1). This marked gene includes the full promoter in 410 bp of 5' flanking sequence and extends 190 bp past the polyadenylation signal but does not include the 3' ^{A}gamma enhancer. The marked gene is identical to the genomic ^{A}gamma-globin gene except for a 6 bp deletion in the 5' non-translated portion of the first exon which distinguishes its transcript from that of the endogenous gamma-globin genes in K562 cells. This deletion has previously been shown to be transcriptionally neutral in non-erythroid cells (21). A 1.1 kb transcription unit containing the neomycin resistance gene was cloned 3' to the globin gene. Constructs were linearized, electroporated into K562 cells, selected in G418, and individual clones were isolated. Cytoplasmic RNA and genomic DNA were prepared for analysis. The activity of individual or combined LAR subfragments was defined by the ratio of marked gamma-globin mRNA to endogenous gamma-globin mRNA in stably transfected K562 cells.

Determination of the transfected gene transcription start site

Primer extension analysis was performed to determine if the marked gamma-globin mRNA was correctly initiated (fig 2). Three bands appear clustered around the mRNA cap site for both the marked and endogenous genes. This pattern has been obtained by other investigators using primer extension assays (21−22) or S1 nuclease protections assays (1) and probably represents microheterogeneity in the gamma-globin transcription start site. No upstream transcription start sites were seen for any of the clones analyzed. cDNA derived from stably transfected clones did not amplify with PCR using a primer upstream of the cap site and a second primer on the opposite strand further substantiating that the marked gamma-globin RNA is correctly initiated (data not shown).

Substantial variability in expression of individual clones

Marked gamma-globin RNA from the stably transfected gene was compared to that of endogenous gamma-globin by T1 RNase protection. This ratio was corrected for transfected gene copy number by Southern analysis. 30 clones were analyzed, ten containing HS II, no LAR, or HS IV,III and II. There was substantial variability in expression among clones containing a given construct (data not shown). A construct containing only HS II expressed the marked gene an average of 33% that of an endogenous gene with a standard deviation of 25% and a range from 0−80%. The marked gamma-globin gene within a construct with no LAR expressed an average of 21% that of an endogenous gene with a standard deviation of 32% and a range from 0−108%. A construct containing HS IV, III, and II expressed

Table 1: Measurement of RNA Ratios by PCR Closely Parallels Values Determined by RNase Protection Assays

% $\frac{\text{marked } \gamma\text{-globin RNA}}{\text{endogenous } \gamma\text{-globin RNA}}$		
Clone	RNase protec.	PCR
2.8	0%	3%
2.1	0	5
5.2	10	7
T7B4	26	26
1.7	28	25
5.1	40	33
T7B2	46	41
T7B3	56	68
5.6	95	109
5.1H	162	179
1.5	177	175

the marked gene an average of 53% that of an endogenous gene with a standard deviation of 53% and a range from 7−179%. Comparison showed no statistically significant difference in expression between these constructs. Because of this variability, constructs were then analyzed by pooling clones. For any given LAR construct, 140 to 150 clones were analyzed in 4 to 10 pooled populations each containing 15 to 35 clones.

HS II is required for high level expression of the transfected gamma-globin gene

Cytoplasmic RNA was isolated from each pool. Using PCR amplification of cDNA, a RNA assay was developed that facilitated rapid determinaton of transfected gene to endogenous gene RNA ratios. cDNA made from total cytoplasmic RNA was amplified using a set of primers that anneal to both endogenous gamma-globin and marked gamma-globin cDNA. The 6 bp deletion in the marked gene enabled resolution of marked and endogenous signals on a sequencing gel and measurement of marked to endogenous RNA ratios by scanning densitometry. The ratios correlated well with values determined by T1 RNase protection assays over a wide range of marked gene expression (Table 1). RNA from all pooled populations was analyzed with this PCR assay (fig. 3 and data not shown).

Southern analysis was used to determine average transfected gene copy number for each pool of clones (fig. 4 and data not shown). XhoI sites are located in the 5′ polylinker sequence and

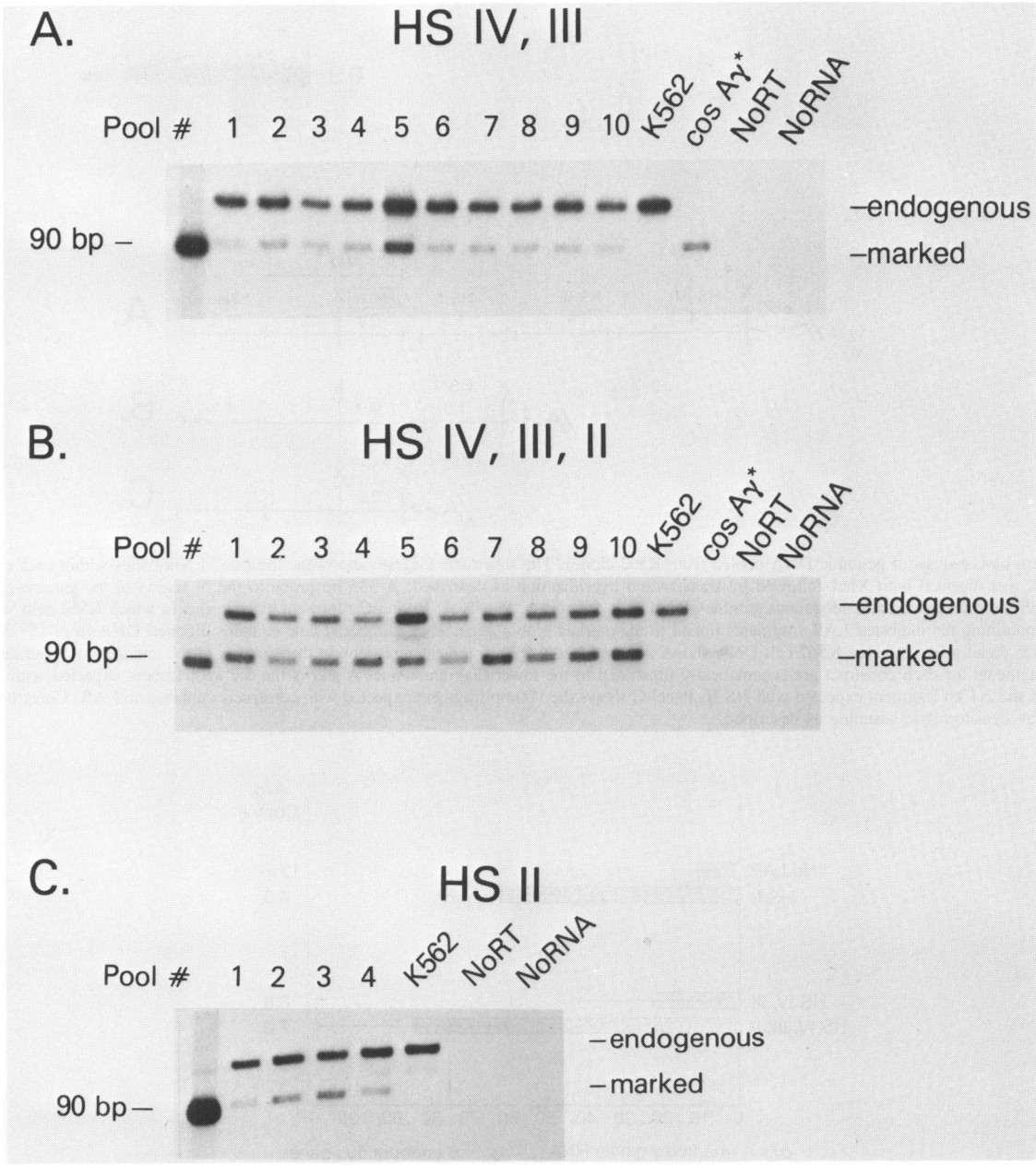


Figure 3. RNA assays by PCR of uninduced pooled K562 cell clones. Amplified cDNA from the endogenous gamma-globin genes migrates at 97bp while that of the marked gamma-globin gene migrates at 90bp. Each lane contains amplified cDNA from 15−35 pooled clones. Controls in the right most lanes are assays of nontransfected K562 cells (K562), Cos cells (which express no endogenous globin) transfected with the marked gamma-globin gene (cos A-gamma*), RNA amplifed without a preceding reverse transcriptase reaction (No RT), and samples with no RNA. Panel A shows clones containing HS IV and III linked to the marked gene. Panel B shows clones containing constructs with sites IV, III, and II. Panel C shows clones containing HS II only.

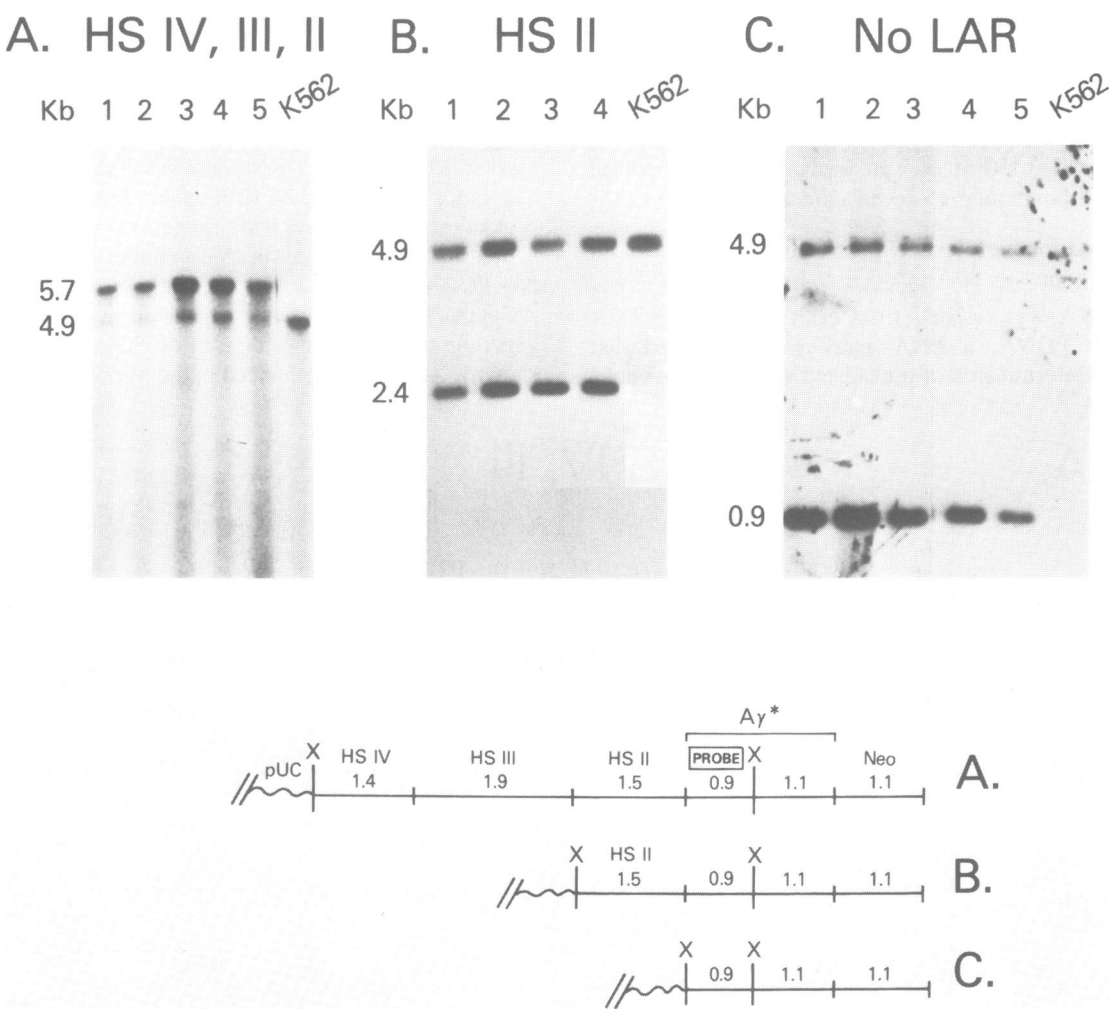


Figure 4. Southern blot analysis of genomic DNA pooled from K562 clones. The schematic diagram shows the location of XhoI sites within each construct. 10 μg of genomic DNA was digested with XhoI followed by transfer and hybridization as described. A 335 bp probe to the 5' region of the gamma-globin gene was used to detect both the transfected and endogenous gamma-globin gene fragments. Panels A, B, and C represent experiments in which K562 cells were transfected with constructs containing the indicated LAR fragments linked to the marked globin gene. Each numbered lane contains digested DNA from 15–35 pooled K562 cell clones. In each panel nontransfected K562 cell DNA shows the predicted 4.9 kb fragment representing three copies of the endogenous ^{A}gamma-globin genes. The predicted fragments for each construct are schematically illustrated in the lower diagram. Panel A shows the 5.7 kb fragment expected with HS IV,III, and II. Panel B shows the 2.4 kb fragment expected with HS II. Panel C shows the 900 bp fragment expected with constructs containg no LAR. Construct copy number was determined by densitometric scanning as described.

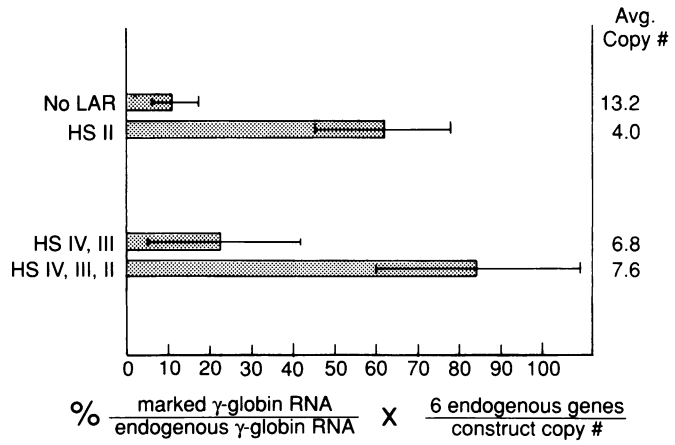


Figure 5. The effect of LAR sequences on gamma-globin gene expression in uninduced K562 cells. Each shaded bar represents the average copy number corrected expression ratio for constructs containing the LAR fragments indicated on the left. For each construct, 150 clones were analyzed in pools as described above. Error bars indicate one standard deviation on each side of the mean. The average transfected gene copy number for each experiment is shown on the right.

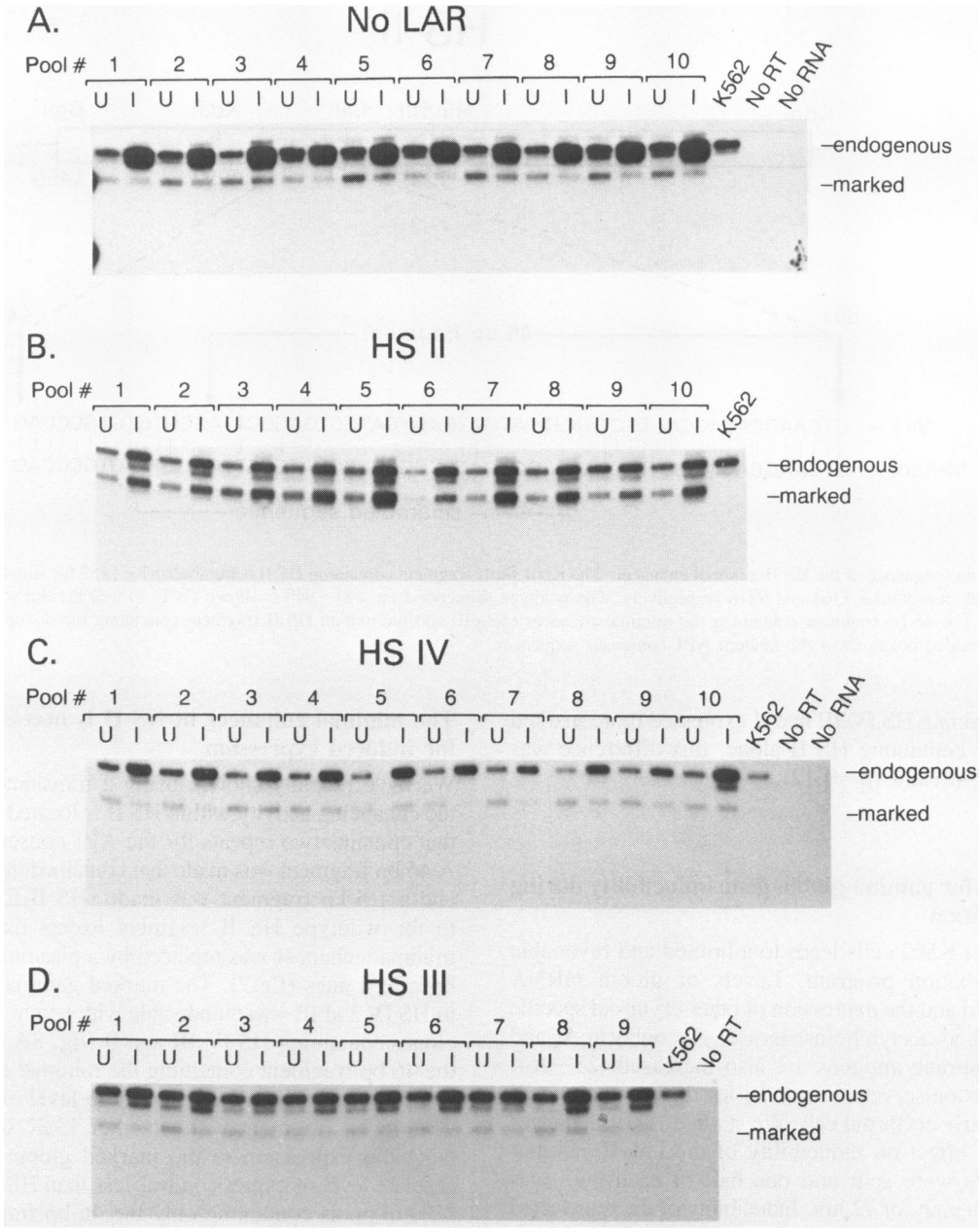


Figure 6. RNA analysis of uninduced and hemin induced K562 cell pools of clones. Each pool of clones was split and half of the cells were stimulated with hemin for 3 days as described. Panels A, B, C, and D show pools containing the indicated LAR fragments linked to the marked globin gene. For each pool, 'U' indicates the uninduced aliquot and 'I' indicates the hemin induced aliqout. Endogenous and marked signals are indicated on the right and control lanes are shown as in figure 3.

within the ^{A}gamma-globin gene in each construct so that digestion of genomic DNA with XhoI yields an internal fragment with a size dependent on the cloned upstream LAR elements. The signal given by the construct was compared, by scanning densitometry, to the signal given by the three endogenous ^{A}gamma-globin genes occurring at 4.9 kb. Copy numbers ranged from 4.0 copies per cell for pools containing HS II to 13.2 for pools containing no LAR fragments. No detectable rearrangements were noted within any given pool.

Karyotype analysis showed that our K562 cell line is triploid with three number 11 chromosomes and Southern blot analysis confirmed the presence of six endogenous gamma-globin genes (data not shown). Transfected gene expression was normalized for comparison with a single endogenous gene by multiplying the RNA ratios by 6 and dividing by the transfected gene copy number.

HS II alone or in combination with the other upstream sites was analyzed in uninduced K562 cell pools (fig 5). Constructs with no LAR fragments expressed the marked gamma-globin gene at 11% that of an endogenous gene. Addition of HS II increased this ratio to 61% demonstrating a significant increase over the no LAR baseline ($p < .001$). HS IV and III in combination were relatively inactive (23%) but addition of site II increased activity to 84% ($p < .001$). Differences in expression of the marked gene with these LAR constructs were statistically significant only when pools of clones were analyzed. Although

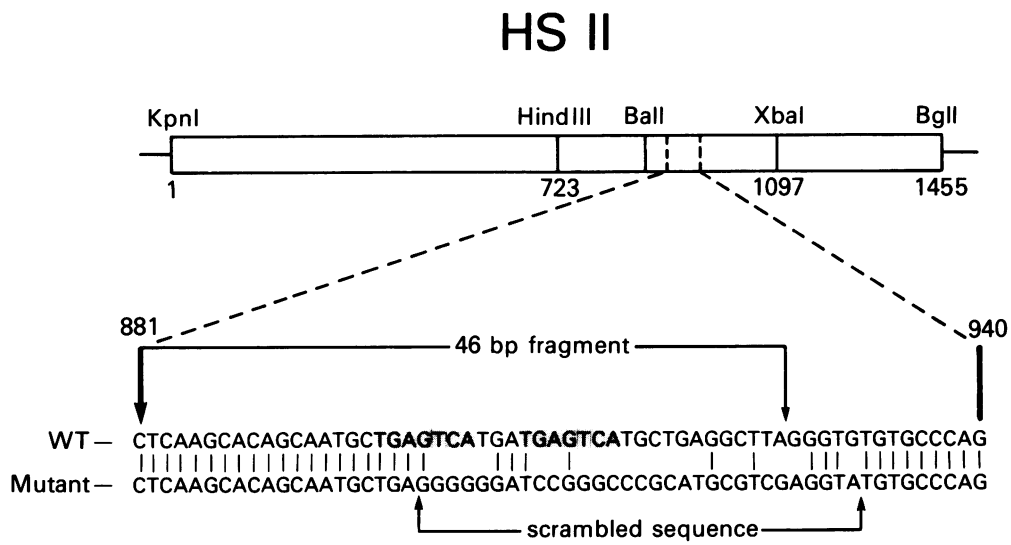


Figure 7. The location and sequence of the HS II minimal enhancer. The KpnI-BglII fragment containing HS II is numbered 1 – 1455 for simplicity but these numbers correspond to gene bank coordinates 7764 and 9218 respectively. The wildtype sequence from 881 – 940 is shown (WT) to indicate the position and sequence of the minimal enhancer. The 46 bp fragment containing the minimal enhancer (46bpE) and the mutant HS II fragment containing the disrupted enhancer (II-E) are also shown. The two shaded boxes show the tandem AP1 consensus sequences.

pooled clones containing HS IV,III and II expressed more marked mRNA than those containing HS II alone, this difference was not statistically significant ($p=0.12$).

HS II is required for gamma-globin gene inducibility during erythroid maturation

Hemin induction of K562 cells leads to a limited and reversible erythroid differentiation program. Levels of globin mRNA increase several fold and the expression of other erythroid specific gene products such as acetylcholinesterase, glycophorin A, and fetal red cell membrane antigens are also increased (23 – 26). Thus, hemin induction serves as a model for differentiation and maturation of an early erythroid cell. We studied LAR sequences to determine their effect on inducibility of the linked gamma-globin gene. Pools were split and one half of each pool was cultured in 20 μM hemin for 72 hrs. Inducibility of the transfected marked gene was compared to endogenous globin gene inducibility by comparing the marked to endogenous RNA ratios from uninduced versus induced pools. This analysis clearly shows that the transfected marked gene is inducible only when HS II is present in the construct (fig. 6). Pooled clones transfected with constructs containing either no LAR, HS IV, or HS III show no increase in marked gene expression with hemin induction in contrast to the large increase in endogenous gamma-globin expression (figs 6A,C,and D). These pools show a significant decrease in the marked to endogenous RNA ratios with hemin induction when calculated by densitometry. In contrast, pools transfected with the 1.5 kb fragment containing HS II show induction of both the transfected marked gene and the endogenous gamma-globin genes (fig. 6B). Densitometry shows the average RNA ratio to be slightly increased with hemin induction demonstrating that the marked transfected gene induces at least to the same degree as that of the endogenous genes. Constructs containing HS II in combination with sites III or IV also exhibit inducibility of the marked gene (data not shown).

The minimal enhancer in HS II is necessary and sufficient for induced expression

We have recently shown, using a transient assay system, that the enhancing activity within HS II is located in a 20 bp sequence that contains two repeats for the AP1 consensus sequence (12). A 46 bp fragment was made that contained this sequence (46bpE) and a 1.5 kb fragment was made (HS II-E) that was identical to the wildtype HS II fragment except that a portion of the minimal enhancer was replaced by a plasmid polylinker ablating both AP1 sites (fig.7). The marked gamma-globin gene linked to HS IV and III was uninducible with hemin in contrast to pooled clones containing HS IV,III and II (fig. 8A and B). Addition of the 46 bp fragment containing the minimal enhancer to sites IV and III reconstituted inducible, high level expression (fig. 8D). The 46 bp fragment without other LAR sequences conferred inducible expression to the marked globin gene although the absolute level of expression was less than HS IV, III, 46bpE (fig. 8E). In pools containing only the 46 bp fragment linked to the globin gene, marked to endogenous RNA ratios showed no significant change with hemin induction and averaged about 17%. In contrast, the globin gene linked to HS IV, III and II-E failed to induce and was expressed at very low levels in induced cells (fig. 8C). Pools of clones containing this construct showed a significant decrease in averaged RNA ratios from 21% ± 8.4% in uninduced cells to 7.6% ± 4.0% in hemin induced populations ($p=0.001$). This data clearly shows that the 46 bp minimal enhancer is both necessary and sufficient for gamma-globin gene induction with hemin induced erythroid maturation in K562 cells.

DISCUSSION

We have shown that the minimal enhancer in HS II is a critical element in the human beta-globin LAR. HS II increases expression of correctly initiated gamma-globin mRNA in K562 cells six fold over baseline and at 61% that of endogenous levels. Exogenous mRNA is detected at relatively low levels without

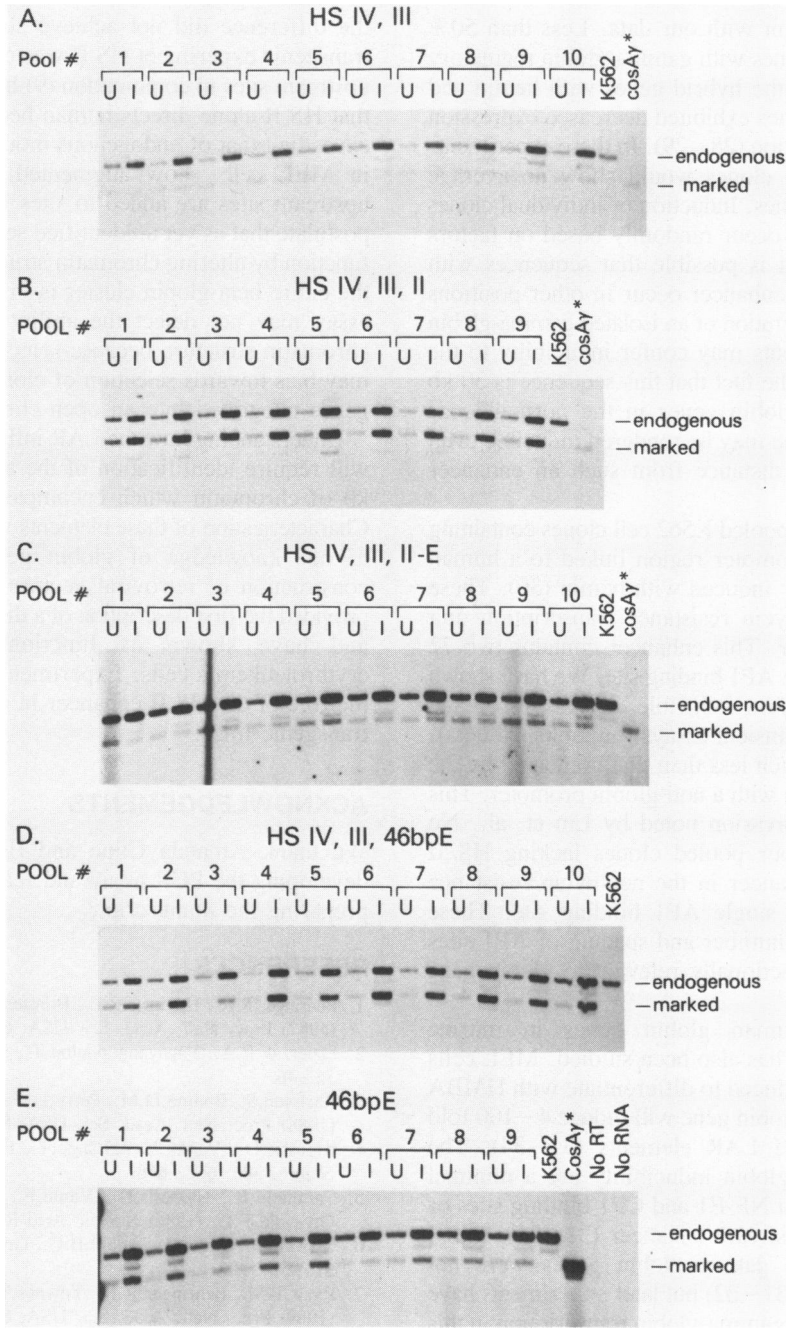


Figure 8. The effect of the HS II minimal enhancer on gamma-globin gene inducibility. Panels A–E show RNA assays on uninduced and induced pools transfected with the indicated LAR fragments. Lanes are labelled as in figure 6.

HS II, even when the HS IV and HS III are cloned upstream of the exogenous gene. The absolute requirement of the 46 bp enhancer for inducibility of the exogenous gene further establishes the importance of this element. These data suggest that this small and discrete enhancer sequence located more than 30 kb from the gamma-globin genes acts as a powerful, maturationally specific transcriptional activator.

We and others have noted clonal variability in globin expression when testing constructs containing LAR elements. Although Grosveld et. al. initially reported a direct correlation between copy number and human beta-globin expression that was independent of integration site within the transgenic mouse genome (6), others have reported clonal variability in both transgenic mice and tissue culture systems. Ryan et. al. have recently shown that copy number corrected human beta-globin expression ranged from 16% to 200% that of mouse beta-globin in 13 transgenic mice using a 30 kb construct containing the four upstream sites (9). Expression ranged from 6% to 84% when a 5.8 kb fragment containing HS II was tested. Similar clonal variability has been noted in MEL cell clones (8,27). These findings substantiate the importance of investigating large numbers of clones in pooled populations when using tissue culture cells and of studying a sufficient number of animals in transgenic experiments. Studies reporting activity in a few isolated clones without rigorous statistical analysis are unlikely to be informative.

Previous studies of gamma-globin gene inducibility in isolated

K562 cell clones are consistent with our data. Less than 50% of clones containing hybrid genes with gamma-globin regulatory sequences show induction of the hybrid genes with hemin and a significant proportion of clones exhibited decreased expression of the hybrid genes with induction (28-29). In these experiments it seems likely that pools of clones would show no average induction of the exogenous genes. Induction of individual clones without LAR sequences may occur randomly based on factors such as site of integration. It is possible that sequences with similar function to the HS II enhancer occur in other positions in the human genome and integration of an isolated gamma-globin gene near one of these elements may confer inducibilty to the integrated exogenous gene. The fact that this sequence is 30 kb upstream from the gamma-globin genes in the normal locus suggests that a transfected gene may be rendered inducible even if integrated at considerable distance from such an enhancer element.

Lin et. al. have shown that pooled K562 cell clones containing a gamma-globin upstream promoter region linked to a human beta-globin gene are modestly induced with hemin (30). These constructs contained a neomycin resistance transcription unit containing the SV40 enhancer. This enhancer contains two 72 bp repeats, each containing one AP1 binding site. We have shown that the SV40 enhancer confers inducible expression on the gamma-globin promoter in transient assays (unpublished data). The level of inducibility is much less than that conferred by the HS II enhancer and is not seen with a non-globin promoter. This may explain the induced expression noted by Lin et. al.. No induction was observed in our pooled clones lacking HS II although the polyoma A enhancer in the neomycin resistance transcription unit contains a single AP1 binding site. These observations suggest that the number and spacing of AP1 sites in each enhancer may be functionally relevant to globin gene inducibility.

Inducibility of cloned human globin genes in murine erythroleukemia (MEL) cells has also been studied. MEL cells express adult globins when induced to differentiate with HMBA or DMSO. The human beta-globin gene will induce 4-100 fold in MEL cells without linked LAR elements (31-34). The sequences required for beta-globin inducibility are a minimal promoter with either upstream NF-E1 and CP1 binding sites or NF-E1 elements in the downstream enhancer (32-34). Initial studies reported that human gamma-globin genes were not inducible in MEL cell clones (31-32) but later experiments have shown variable inducibility of gamma-globin transcription in this system (35-36). Requirements for inducibility may differ between beta and gamma-globin genes and between K562 and MEL cells.

Activation of globin gene transcription by the HS II enhancer is likely mediated by transcription factors that bind to this sequence. We have recently shown that a protein complex forms on the enhancer in induced K562 cells and that complex formation correlates with high level inducible activity and occupancy of both AP1 sites (12). It is of interest that a recently described erythroid specific protein designated NF-E2 binds to an AP1 consensus sequence within the erythroid promoter of the human prophobilinogen deaminase gene and that NF-E2 binding is necessary for inducibility of this erythroid specific gene (37-38).

We have shown that the HS II enhancer is an important element in the LAR but the functional significance of the other sites is less well defined. Our assay shows an increase in expression from 61% to 84% when HS IV and HS III are added to HS II but the difference did not achieve statistical significance. In one transgenic experiment HS II was only 40% as active as all four upstream sites in combination (9) however a recent study reports that HS II alone directs human beta-globin expression at levels exceeding that of endogenous mouse alpha-globin (39). Studies in MEL cells show augmented expression when the distal upstream sites are added to sites II and I (27). We and others postulate that as yet unidentified sequences within the LAR may function by altering chromatin structure so that in erythroid cells the entire beta-globin cluster is in an active configuration. Our assay may not detect the ability of LAR sequences to alter chromatin structure because selection with a dominant marker may bias towards selection of clones containing the transfected genes integrated into an open chromatin region.

Understanding how the LAR influences globin gene expression will require identification of the active elements within the 30 kb of chromatin which encompasses the hypersensitive sites. Characterization of these elements will add important information to our knowledge of globin gene regulation and assist in construction of retroviral vectors for gene transfer. We have provided the first description of a discrete element within the LAR and have shown its functional significance in human erythroleukemia cells. Experiments are in progress to confirm the role of the HS II enhancer in normal erythroid cells within transgenic mice.

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