Tandem AP-1-binding sites within the human β-globin dominant control region function as an inducible enhancer in erythroid cells

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A powerful enhancer has been mapped to an 18-bp DNA segment located 11 kb 5′ to the human ε-globin gene within the dominant control or locus-activating region. This enhancer is inducible in K562 human erythroleukemia cells, increasing linked γ-globin promoter/luciferase gene expression to 170-fold over an enhancerless construct. The enhancer consists of tandem AP-1-binding sites, phased 10 bp apart, which are both required for full activity. DNA–protein binding assays with nuclear extracts from induced cells demonstrate a high molecular weight complex on the enhancer. The formation of this complex also requires both AP-1 sites and correlates with maximal enhancer activity. Induction of the enhancer may have a role in the increase in globin gene transcription that characterizes erythroid maturation. Enhancer activity appears to be mediated by the binding of a complex of proteins from the jun and fos families to tandem AP-1 consensus sequences.

[Key Words: AP-1; erythroid; inducible; enhancer; DCR; hypersensitive site]

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Human hemoglobins are tetramers composed of two pairs of globins, with one pair encoded by a gene within the α-like cluster on chromosome 16 and the other pair encoded by a gene within the β-like cluster on chromosome 11. The β-gene cluster contains five functional genes and one pseudogene (ε, βγ, Λγ, Σβ, δ, and β) that are distributed over 40 kb of DNA. Expression of the β-like globin genes is both tissue and developmentally stage specific. ε, γ-, and β-globins are produced in embryonic, fetal, and adult erythroid cells, respectively. Globin genes at each developmental stage exhibit a marked increase in the level of expression during maturation of erythroid cells in contrast to diminished expression of most other genes (Karlsson and Nienhuis 1985; Stamatoyannopoulos and Nienhuis 1987).

Regulation of genes within the β-cluster occurs, at least in part, through interaction of trans-acting proteins with cis-acting regulatory elements (Dynan 1989; Mitchell and Tjian 1989). The promoters of the individual globin genes are tissue specific and may contribute to preferential expression during development (Lin et al. 1987; Rutherford and Nienhuis 1987). Intragenic elements and enhancers 3′ to the β and Λγ genes may also provide specificity (Behringer et al. 1987; Bodine and Ley 1987; Kollias et al. 1987; Trudel and Costantini 1987; Antoniou et al. 1988). The promoters and enhancers are marked by tissue-specific DNase I hypersensitive sites as assayed in isolated nuclei. Formation of such sites at the promoters is developmentally stage specific (Groudine et al. 1983; Tuan and London 1984). Several proteins have been shown to interact with sequence motifs within globin promoters and enhancers (de Boer et al. 1988; Gumucio et al. 1988; Mantovani et al. 1988a,b, Catala et al. 1989), including one such factor found only in erythroid cells, designated nuclear factor-erythroid 1 [NF-E1] or globin factor-1 [GF-1] (Wall et al. 1988; Tsai et al. 1989).

Study of naturally occurring deletion mutants of the β-globin cluster, the γδβ-thalassemias, suggested the existence of additional regulatory elements. An intact β-globin gene on a chromosome with a large upstream deletion was unexpressed, methylated, and in an inactive chromatin conformation (Kioussis et al. 1983). Major DNase I hypersensitive sites have been discovered 5′ and 3′ to the β-globin gene cluster, identifying regions that may contain these regulatory elements (Tuan and London 1984; Tuan et al. 1985; Forrester et al. 1986). Four erythroid-specific hypersensitive sites (HS I–HS IV), distributed over 20 kb, 5′ to the ε-globin gene, are present in nuclear chromatin from erythroid cells of all developmental stages. A fifth site located even farther 5′ to the ε gene is also present in other hematopoietic cells. The sixth site (HS VI) is located 21.8 kb 3′ to the β-globin gene. Other γδβ-thalassemia mutations have been characterized (Curtin et al. 1985), including one that begins between HS I and HS II, 9.5 kb 5′ to the ε
gene, leaving HS I and the entire cluster intact [Driscoll et al. 1989]. The β gene on this chromosome was not expressed. Thus, the flanking HS sites, particularly those 5' to the cluster, have emerged as potential regulatory elements that may control expression of the entire gene cluster in erythroid cells.

Direct experimental evidence for an important functional role of these regulatory elements was obtained in transgenic mice. In earlier studies the human β-globin gene was expressed at low levels in transgenic animals with substantial variation among strains derived from different founder animals [Magram et al. 1985; Townes et al. 1985; Kollias et al. 1986]. Grosveld et al. [1987] assembled a 38-kb segment of DNA that included the human β-globin gene flanked by fragments containing the 5' and 3' major DNase I hypersensitive sites. Within the context of these regulatory elements, the human β-globin gene was expressed at a high level, equivalent to that of an endogenous mouse gene, and independent of chromosomal integration position. Because of its activity in transgenic mice and ability to confer erythroid specificity on heterologous promoters, Grosveld and his colleagues designated the 5' region containing major DNase I hypersensitive sites as the dominant control region (DCR) [Blom van Assendelft et al. 1989; Talbot et al. 1989]. An alternative designation, that of the locus-activating region (LAR), is based on the apparent ability of regulatory elements within this region to establish an active chromatin structure of the β-globin gene cluster [Forrester et al. 1987].

Progress has been made in localizing the functional components within the DCR/LAR, although the sequence motifs and interacting proteins required for DCR/LAR activity have not yet been defined. A 6.5-kb fragment containing the four erythroid-specific 5' HS sites has full activity in both transgenic mice and murine erythroleukemia (MEL) cells [Talbot et al. 1989]. Forrester et al. [1989] have demonstrated that a 2.5-kb fragment containing these sites also has full DCR/LAR activity in MEL cells. A fragment containing only HS II has been shown to confer tissue-specific, high-level expression on a β-globin gene in transgenic mice at 40–50% of the level achieved with all four sites [Ryan et al. 1989]. Furthermore, HS II contains a powerful enhancer, active in transient assays performed with human erythroleukemia cells [Tuan et al. 1989]. Our results suggest that this enhancer is an important regulatory element within the DCR/LAR. We have now mapped the HS II enhancer to an 18-bp segment. Its activity increases with induction of erythroid maturation in human erythroleukemia cells and depends on interaction with proteins that bind to tandem AP-1 consensus sequences within the 18-bp element.

Results

Localization of the HS II enhancer

The distribution of the DNase I hypersensitive sites 5' to the e-globin gene that mark the DCR are shown in Figure 1. HS II has been localized to 10.9 kb (± 0.1 kb) 5' to the e-globin gene transcriptional start site [Forrester et al. 1987]. In mapping the enhancer, we initiated our studies with a 1455-bp KpnI–BglII fragment containing HS II. We designated the first nucleotide within the KpnI site as nucleotide 1, making the first nucleotide within the BglII site nucleotide 1455. A 732-bp HindIII–BglII fragment (nucleotide 723–1455) has been shown to contain the enhancing activity of HS II [Tuan et al. 1989]. We utilized a larger KpnI–BglII fragment to include another hypersensitive site present in nuclei of erythroleukemia cells [Forrester et al. 1987]. Our reporter plasmid consisted of a -260 γ-globin gene promoter, in a pUC-based plasmid, linked 5' to the coding sequence of the luciferase gene [DeWet et al. 1987]. Mutants of the HS II fragment or oligonucleotides were subcloned 5' of the chimeric γ-globin promoter/luciferase gene and assayed for enhancing activity. Transient assays were performed in K562 cells, a human erythroleukemia cell line [Lozzio and Lozzio 1975]. K562 cells express the β- and γ-globin, but not β-globin genes and therefore have been used as a model for the embryonic/fetal stage of development [Rutherford et al. 1981; Dean et al. 1983]. Addition of chemicals such as hemin to K562 cells induces an erythroid phenotype, as manifested by increased expression of globin and other erythroid-specific genes [Hoffman et al. 1980; Dean et al. 1983; McGinniss and Dean 1985; Bartha et al. 1987].

The 5' and 3' truncation mutants of the HSII fragment were made by exonuclease III digestion. These mutants were cotransfected into K562 cells with a B19 promoter/chloramphenicol acetyltransferase [B19–CAT] reporter construct as an internal control. Relative activity was derived by dividing the luciferase activity of a given mut-
tant by that of the enhancerless reporter plasmid, each having been corrected for transfection efficiency with the internal control. Study of the initial set of truncation mutants demonstrated that the enhancer was within a 174-bp fragment (807–981) [Fig. 2]. A second series of closely spaced truncation mutants established the 3' border of the enhancer at nucleotide 916 [data not shown]. To define the 5' boundary of the enhancer, a series of 5 oligonucleotides were synthesized and subcloned into the expression plasmid (Fig. 3). Each of these had full enhancer activity, including the smallest of only 20 bp [896–916].

DNase I protection assays were done to identify regions of sequence-specific, DNA–protein interactions by utilizing a restriction fragment extending from nucleotide 723 to 977 as probe. With K562 nuclear extracts, a very prominent footprint was present on both strands directly over the functionally defined minimal enhancer (Fig. 4). This footprint was also present with HeLa cell nuclear extract. Nuclear extracts from uninduced and hemin-induced K562 cells gave similar footprinting patterns; however, the footprint over the enhancer appeared at lower concentrations of induced nuclear extract. Uninduced and induced K562 nuclear extracts were standardized by comparing their Sp 1-binding activity in a gel mobility-shift assay, which was equivalent at equivalent protein concentrations (data not shown). At higher concentrations of K562 or HeLa cell nuclear extract, another footprint, flanked by sites of increased DNase I sensitivity, appeared from ~10–30 bp 5' of the enhancer footprint. However, this region and other sequences outside the minimal enhancer were not necessary for enhancer activity and were not studied further.

To verify that no other sequences within the KpnI–BglII HS II fragment had enhancer activity, we scrambled 31 nucleotides (900–931) while leaving the remainder of the fragment unaltered. This mutated fragment was designated HS II enh−. A 16-bp stretch of the 20-bp enhancer [901–916] was mutated by this construction. Our earlier analysis of 3' truncation mutants indicated that sequences between 916 and 931, also rearranged in this construction, had no role in the activity of the enhancer. The HS II enh− fragment had no activity in transient assays in K562 cells (Fig. 5). The footprint present over the active enhancer was not present when the corresponding restriction fragment from HS II enh− was used as a probe [Fig. 4B]. When the same constructs were transfected into HeLa cells, the HS II enhancer had modest activity (relative activity: no HS II, 1.0 ± 0.1; HS II [1455 bp], 2.8 ± 0.8; HS II [46 bp], 3.9 ± 0.1; HS II enh−, 0.7 ± 0.1. In summary, our data establish that a 20-bp segment (896–916) has the full enhancing activity of HS II and that no other sequences within the KpnI–BglII fragment contribute to this activity.

**Figure 2.** Enhancing activity of HS II truncation mutants. The KpnI–BglII fragment containing HS II used in our studies is shown at top. The first nucleotide of the KpnI site is 1 (7764 in GenBank) and the first nucleotide of the BglII site is 1455. Truncation mutants 5' and 3' of HS II are depicted below. The results from three separate experiments are shown at right. Relative activity was determined by comparing the luciferase activity of a given mutant to that of an enhancerless reporter plasmid (no HS II). The relative activity of truncation mutants in each experiment should be compared to wild type from the same experiment. Cotransfected B19–CAT reporter construct served as an internal control in all experiments. Each mutant was tested at least twice, and the relative activities shown are representative.
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The HS II enhancer is induced with erythroid maturation

We designed experiments to determine whether the HS II enhancer has a role in the increase in globin gene expression that occurs with hemin-induced maturation of K562 cells. In accordance with the protocol of Lloyd et al. (1989), K562 cells were grown in the presence of 20 μM hemin for 24 hr and transfected with reporter plasmids with or without an HS II enhancer. After an additional 48 hr in the presence of hemin, extracts of these cells were prepared and assayed for luciferase and CAT activity. Comparison was made to results obtained with standard transfections in uninduced K562 cells.

In induced K562 cells, the HS II enhancer increased expression of the reporter gene > 100-fold (Table 1). This contrasted to the 10-fold increase in activity conferred by the enhancer in uninduced cells. The HS II enhancer fragment was inactive in both induced and uninduced cells. A construct containing only 46 bp of HS II (880–926), including the enhancer, was at least as active as one containing the full HS II fragment. Both constructs expressed 10- to 15-fold higher in induced compared to uninduced cells. By primer extension assay we demonstrated that the increase in luciferase gene expression in induced K562 cells, conferred by the enhancer, was due to an increase in luciferase transcripts correctly initiated from the 5′ border of the enhancer. The results from two experiments are shown at right. Relative activity was determined by comparison to an enhancerless reporter plasmid (no HS II). Cotransfected B19-CAT served as an internal control in all experiments. Every oligonucleotide was tested at least twice, and the relative activities shown are representative.

The enhancer contains two AP-1-binding sites, both of which are required for full activity

Inspection of the 20-bp enhancer revealed tandem, direct 9-bp repeats, each containing a full match to the 7-bp activating protein-1 (AP-1) consensus binding sequence, TGAGTCA (Fig. 8A) (Jones et al. 1988). AP-1 was first identified as the product of the c-jun gene (Bohmann et al. 1987), but now the designation AP-1 refers to the jun and fos families of proteins that bind as dimers to the AP-1 consensus sequence (Curran and Franz 1988). We used DNA fragments containing authentic AP-1-binding sites as competitors in gel mobility-shift assays (Fig. 7B).
The retarded bands observed in extracts from both uninduced and induced K562 cells with the HS II enhancer probe were effectively competed by three known AP-1-binding sites (Angel et al. 1987; Lee et al. 1987). In contrast, no competition was observed with a fragment containing authentic binding sites for NF-E1, the erythroid-specific factor (Tsai et al. 1989).

To study binding by members of the jun and fos families to the enhancer further, gel mobility-shift assays were performed with erythroid and nonerythroid nuclear extracts and with in vitro-synthesized c-Jun and c-Fos (Fig. 7C). Consistent with the ubiquitous distribution of members of the jun and fos families, the HS II enhancer was bound by proteins present in all nuclear extracts tested [lanes 1, 3, 5, 7, and 9]. When present, the less retarded bands [b] comigrated with a single AP-1 site probe [lanes 2, 4, 6, 8, and 10], suggesting that they represented binding to one AP-1 site and that the highly retarded band [a] represented binding to both AP-1 sites in the enhancer. The latter was supported by in situ phenanthroline/copper footprinting of the highly retarded band seen with induced K562 nuclear extract. A footprint was apparent in the region 893–918, encompassing both AP-1 sites [data not shown].

In vitro-synthesized c-Jun/c-Fos heterodimer also bound the enhancer and migrated adjacent to one of the less retarded bands seen with HeLa cell nuclear extract and slightly above those seen with uninduced K562 nuclear extract. This band corresponded to single AP-1 site occupancy [lanes 11–16]. By increasing the concentration of the c-Jun/c-Fos heterodimer, a band can be elicited that comigrates with the highly retarded band seen with nuclear extracts [data not shown]. c-Jun or c-Fos alone shows little or no binding to the enhancer, respectively. These results demonstrate clearly that members of the jun and fos families are able to bind the HS II enhancer. We interpret these data to suggest that the comigrating band formed in extracts of HeLa cells is a c-Jun/c-Fos heterodimer. The absence of this band in extracts of uninduced K562 cells suggests that the complex...
Figure 5. Mutation of enhancer sequence within HS II eliminates all activity. Thirty-one bp (900–931) of the full HS II fragment (1455 bp) was scrambled, including 16 of the 20 bp of the minimal enhancer. This construct was designated HS II enh−. The location of the scrambled sequence within HS II enh− is expanded. The enhancing activity of this construct was compared to wild-type HS II and to an enhancerless reporter plasmid (no HS II), as shown at bottom. Cotransfected B19-CAT served as an internal control. On repetition, this experiment gave virtually identical results.

Table 1. The HS II enhancer is inducible

| Uninduced | Hemin induced |
|-----------|---------------|
|           | luciferase activity (per 1000 light units) | CAT activity (per 1000 cpm) | luciferase/CAT* | relative activity | luciferase activity (per 1000 light units) | CAT activity (per 1000 cpm) | luciferase/CAT* | relative activity |
| No HS II  | 2.9           | 10.5          | 0.4          | 1.0          | 3.3             | 22.8           | 0.2          | 0.4          |
| HS II (1455 bp) | 38.4      | 12.5          | 4.0          | 10.8         | 746x          | 20.5           | 42.7         | 115         |
| HS II (46 bp) | 73.0      | 21.0          | 4.0          | 10.9         | 1,527x        | 27.1           | 63.4         | 171         |
| HS II enh− | 1.1              | 10.7          | 0.1          | 0.4          | 4.0             | 47.4           | 0.1          | 0.2          |
| No extract | 0.1            | 3.0           | —            | —            | 0.1            | 3.0           | —            | —            |

*Luciferase/CAT activity ratio, after subtracting background (no extract) from each.

xSamples giving values >100,000 light units were diluted, reassayed, and corrected for dilution by the appropriate factor. These represent corrected values.

binding the enhancer in uninduced K562 cells is not the same as that in HeLa cells but represents either a modification or a different dimer from the Jun/Fos dimer families.

To determine the contribution of the individual AP-1-binding sites to enhancer function and protein complex formation, the enhancer was scanned with mutants in 3-bp intervals [Fig. 8A]. These triplet scanning mutants were assayed for activity in uninduced and induced K562 cells. Mutation of either AP-1 consensus sequence [mutants 1–6] caused a loss in activity to 20–58% of that observed with the wild-type enhancer in the uninduced state and 6–34% of that seen in the induced state [Fig. 8B]. Mutant 7, which falls outside both AP-1 consensus sequences, was as active as the wild-type enhancer in the uninduced state and slightly less active in the induced state. Those mutants with the lowest activity also tended to have the lowest inducibility, although inducibility per se was preserved with even the least active mutants. However, the activity of the least active mutants of either AP-1 site (mutants 2, 3, and 5) was far below that of the wild-type enhancer in the induced state. These data lend support to our hypothesis that complex formation involving both AP-1 sites is necessary for full enhancer activity.

The functional activity of the enhancer mutants correlated quite well with their ability to form the highly retarded multimeric complex in a gel mobility-shift assay with induced K562 nuclear extract [Fig. 9]. With mutants of either AP-1 site (mutants 1–6), the band corresponding to single-site occupancy [b] predominated. In contrast, the wild-type enhancer and mutant 7, which leave both AP-1 consensus sequences intact, generated the band corresponding to occupancy of both AP-1 sites [a]. Gel mobility-shift assay of the enhancer mutants with HeLa cell nuclear extract gave a very similar pattern, but the bands corresponding to single-site occupancy [b] are closer together than with induced K562 nuclear extract. These results establish that formation of the multimeric complex [and full activity of the enhancer] requires both AP-1 sites.

Discussion

The enhancer within HS II has been localized to two tandemly repeated AP-1 consensus binding sites within an 18-bp segment. These binding sites are phased 10 bp apart, placing them on successive helical turns of the DNA molecule. An unexpected finding was the increase in enhancer activity as human erythroleukemia cells matured in response to chemical induction. These observations led us to propose a physiological role for the enhancer in contributing to the increase in globin gene...
expected in our laboratory when we observed that the regulatory elements, marked by the four 5’ HS sites, that are required for tissue-specific, high-level expression of the individual globin genes in erythroid cells.

The enhancer was mapped by utilizing short-term (transient) assays of unintegrated reporter genes, but it is also active when stably integrated. The role of the enhancer in the induction of globin genes was first suspected in our laboratory when we observed that the 1455-bp HS II fragment was required for induced expression of a stably integrated δ/γ-globin gene in K562 cells, as reflected by the concentration of correctly initiated δ-globin mRNA (Sorrentino et al. 1990). The 46-bp enhancer was shown to confer inducibility, whereas an integrated δ-γ-globin gene linked to the HS II enhancer gave only a threefold increase in reporter gene expression in K562 cells without the HS II enhancer (Lin et al. 1987). The human δ-globin gene linked to a nonglobin promoter, or the promoter linked to a nonglobin gene stably integrated into MEL cells, is variably expressed but most often highly inducible without the HS II enhancer (Chao et al. 1983; Wright et al. 1983, 1984). Inducibility of the δ-globin promoter depends on the presence of a recognition site for a CCAAT-binding protein (CP1) and one of two NF-E1 sites in the δ-globin promoter (de Boer et al. 1988). The basis for this difference in the requirement for the HS II enhancer for globin gene inducibility between mouse and human erythroleukemia cells is uncertain at present. Perhaps there is redundancy in regulatory mechanisms, as has been observed in other systems. Experiments are in progress to define the function of the enhancer and its effect on human globin gene expression in transgenic mice.

The jun and fos families of proteins, collectively referred to as AP-1 (Curran and Franzka 1988), share a highly homologous domain composed of a basic segment and a leucine zipper (Gentz et al. 1989; Kouzarides and Ziff 1989; Ransone et al. 1989; Turner and Tjian 1989). Members of the jun family bind with high affinity to DNA as a heterodimeric complex with members of the fos family (Halazonetis et al. 1988; Cohen et al. 1989). The dimer binds to a single AP-1 site. We therefore infer that the tandem sites that make up the HS II enhancer bind two dimers forming a multimeric complex of at least four polypeptide chains.

The jun and fos families are expressed ubiquitously, although individual genes may be differentially expressed in specific cell types. This has been shown for the jun genes in the tissues of adult mice [Hirai et al. 1989]. Similarly, the enhancer was bound by proteins expressed in all cell types tested, but the composition of the complex binding to the enhancer need not be the same in extracts from different cell types. Consistent with this, on gel mobility-shift assay, we found differences in mobility between uninduced K562 and HeLa cell nuclear extracts relative to in vitro-synthesized c-Jun/c-Fos heterodimer. We also found differences between induced K562 and HeLa cell nuclear extracts when single-site occupancy bands were generated by enhancer mutants with one AP-1 site ablated.

Tuan et al. [1989] have reported that the HS II enhancer is erythroid specific. In our experiments, the enhancer gave only a threefold increase in reporter gene expression in HeLa cells but more than a 100-fold increase in induced K562 cells. The promoter in our experiments may have contributed to this tissue specificity, although Tuan et al. [1989] reported tissue specificity of

expression that is characteristic of erythroid cell maturation. This enhancer seems likely to be one of several regulatory elements, marked by the four 5’ HS sites, that are required for tissue-specific, high-level expression of the individual globin genes in erythroid cells.

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the enhancer with a nonglobin promoter. In transgenic mice, an 882-bp fragment of HS II, which contains the enhancer, has been reported to be sufficient for high-level, tissue-specific expression of the β-globin gene (Curtin et al. 1989).

How can the apparent erythroid specificity of the HS II enhancer be equated with the presence of tandem AP-1-binding sites? As noted above, the polypeptides present in the complex formed on these sites may differ in various cells. Recent data suggest that there is an erythroid-specific DNA-binding protein, designated NF-E2, that binds to a sequence, GCTGAGTCA, which contains the AP-1 consensus sequence (Mignotte et al. 1989a,b). An NF-E2-binding site is required for inducibility of the porphobilinogen gene erythroid promoter in MEL cells. The sequence identified as being required for NF-E2 binding is present in the HS II enhancer. The potential role for NF-E2 in the apparent erythroid specificity of the HS II enhancer can only be a matter of speculation at present.

The c-jun and c-fos genes are among a class, designated as early response genes, that is part of the immediate response of various cells to hormone or serum stimulation. These genes appear to be components of a general signaling system used to regulate specific genes in various cell types (Curran and Franz 1988). The c-fos gene is expressed at high levels in late-stage erythroblasts (Caubet et al. 1989). The c-fos, c-jun, and jun-B genes have been shown to be expressed in K562 cells in response to transforming growth factor β (Pertovaara et al. 1989). We have preliminary data that hemin stimulation results in increases in c-jun and c-fos mRNA levels in K562 cells (R. Redner, pers. comm.). Also of interest will be the expression of the physiological regulator erythropoietin on expression of these genes in normal erythroid cells.

There is precedence for the importance of AP-1-binding sites in enhancer function. c-jun and c-fos, as part of the early response to various stimuli, allow AP-1-binding sites to confer inducibility on promoter function (Angel et al. 1987, Lee et al. 1987). Functionally important AP-1-binding sites have been found in the enhancers of the chicken β-globin (Reitman and Felsenfeld 1988), metallothionein II (Lee et al. 1987), and collagenase genes (Angel et al. 1987), and the enhancer of a strain of polyomavirus (Dyman 1989). Mutation of the chicken β-globin enhancer AP-1-binding site reduces enhancer activity to 10% of wild type. The structure of this enhancer, like the HS II enhancer, appears to be relatively simple. Two footprinted regions, one including the AP-1 site and the other consisting of two Eryf-1 sites (the chicken equivalent of NF-E1), are sufficient for positive regulation by the chicken β-globin enhancer. The role of this AP-1 site in maturational induction of the chicken β-like globin genes is unknown. This AP-1 site is relatively inactive alone, but a concatamer of three has 60-fold enhancing activity.

It has been shown recently that efficient trans-activation of a CAT reporter plasmid by Jun-B requires two or more adjacent AP-1 sites (Chiu et al. 1989). We speculate that the close spacing of AP-1 sites in the human HS II enhancer allows formation of a unique, highly active complex. Hemin induction of K562 cells appears to lead to complex formation on the enhancer and a dramatic increase in activity. Whether this is due to changes in the concentration of proteins available to bind the enhancer or to other changes favoring complex formation is unresolved.

How could the HS II enhancer influence the level of expression of individual globin genes during development and erythroid maturation? It is located nearly 11 kb from the nearest gene α and >50 kb from the most distal δ. We envision a model whereby a loop structure is formed that brings the enhancer and individual promoters into close proximity through operation of selector elements analogous to the model proposed by Choi and Engel (1988). In this model, enhancer action on selected promoters at various stages of development depends on the protein and DNA interactions that result in formation of a particular loop structure bringing the enhancer in proximity to the transcription initiation complex.

### Methods

**DNA construction**

**Reporter plasmid** pUC007, a pUC-based plasmid with a novel poly linker, was used as the vector in these constructions. pUC007 consists of the following poly linker subcloned into the HindIII and EcoRI sites of pUC9: Xhol, BglII, Xhol, HindIII, Smal, BamHI, Apal, SpII, and SalI. The γ-globin promoter was subcloned as a HindIII (−260 to the cap site)–AluI (+36) fragment into the BglII and HindIII sites of pUC007 (via an intermediate subcloning step into the HindIII site of pUC9). The coding region of the firefly luciferase gene (pSV232-LA85'; DeWet et al. 1987) was then linked 3′ to the γ-globin promoter as a HindIII–BamHI fragment in pUC007. This enhancerless plasmid was used to establish the level of reporter gene expression with a γ-globin promoter alone. An Xhol–AatII fragment

**Figure 7.** Gel mobility-shift assays of the HS II enhancer. (A) An SpI probe from SV40 was used to standardize uninduced and induced K562 nuclear extracts [lanes 1 and 2]. The relative SpI-binding activity is shown at top. A 46-bp oligonucleotide consisting of HS II sequence (880–926), including the enhancer (896–916), was used as probe with increasing concentrations of uninduced and induced K562 nuclear extracts [lanes 3–13]. (B) The HS II enhancer-containing probe described above was used as probe with 4.4 μg of uninduced or induced K562 nuclear extracts. Synthetic oligonucleotides were used as unlabeled competitors in 200-fold excess. The NF-E1 competitor is described in Methods. The AP-1 competitors were derived from the collagenase gene and SV40 and metallothionein II gene enhancers. (C) The HS II enhancer-containing probe [HS II] described above and a single AP-1 site-containing probe [AP-1] were compared in alternate lanes. The latter was derived from region II of the chicken β-globin enhancer. These probes were studied against a panel of nuclear extracts ([3–5 μg per reaction) and in vitro-synthesized c-jun [rat] and c-Fos [rat]. The nuclear extracts were made from K562-uninduced [K562U] or K562-induced [K562I] cells, MEL cells, HeLa cells, and COS cells, a monkey kidney cell line. Reticulocyte lysate without added RNA (NA) served as a control for in vitro-synthesized c-jun and c-Fos.
Figure 8. Scanning mutants of the HS II enhancer. The wild-type and mutant enhancers were synthesized as 46-bp oligonucleotides, subcloned into the reporter plasmid, and assayed for enhancing activity. (A) The wild-type 46-bp fragment containing the HS II enhancer is shown at top. Tandem AP-1-binding sites are boxed. Seven mutants scanning the enhancer in 3-bp intervals are shown beneath the 46-bp wild-type sequence. Only mutant 7 falls completely outside both AP-1-binding sites. (B) Activity of the wild-type and mutant enhancers is shown relative to an enhancerless reporter plasmid in uninduced (open bars) and induced (solid bars) K562 cells. The fold increase with induction is shown for the wild-type and mutant enhancers over the solid bars. Each was assayed in duplicate and normalized based on the activity of a cotransfected B19-CAT reporter construct as an internal control.

of this plasmid containing the chimeric β-globin promoter/luciferase gene, was subcloned into the SalI and Smal sites of pUC007, 3' of various mutants of HS II. This strategy allowed for the rapid construction and testing of mutants of HS II.

**Truncation mutants of HS II**  HS II was subcloned as a KpnI (blunted)–BglII fragment into the BglII (blunted) and BamHI sites of pUC007 (~pUC007–HS II). Truncation mutants of HS II were made by exonuclease III digestion from either the 5' or 3' end of HS II. A modification of the Erase-a-Base protocol (Promega) was used. Truncations (3') were made as follows: 40 µg of pUC007–HS II DNA was linearized at the SalI site, phenol/chloroform-extracted, ethanol-precipitated, and resuspended in 450 µl of buffer [66 mM TrisHCl and 0.66 mM MgCl₂ (pH 8.0)]. The linearized DNA was digested with 3000 units of exonuclease III at 32°C for 6 min. At 30-sec intervals, 36-µl aliquots (12 total) were taken and added to 108 µl of S1 buffer with 30 units of S1 nuclease, on ice. The samples were incubated with S1 nuclease at 25°C for 30 min. S1 nuclease was inactivated by the addition of 14 µl of S1 stop buffer and incubation at 70°C for 10 min. To blunt the truncated ends, 14 µl of Klenow buffer, containing 2.5 units of Klenow DNA polymerase and 14 µl of 0.125 mM dATP, dCTP, dGTP, and dTTP, was added. The DNA was purified by using Gene Clean (Bio 101) and digested with XhoI. The 3'-truncated HS II fragments were then gel-purified and subcloned as an XhoI-blunted fragment into pUC007 opened at the Xhol and Smal sites. The extent of 3' truncation was determined precisely by sequencing. Truncation mutants (5') were made in the same way, except pUC007–HS II was initially opened at the Xhol site and truncation mutants were subcloned as a SalI-blunted fragment into pUC007 opened at SalI and Smal. The extent of the 5' truncation was determined by restriction mapping.

**Scrambled enhancer construct**  An enh⁻ version of HS II was made by replacing 16 of the 20 bp of the enhancer with polylinker from a 3' truncation mutant of HS II. An oligonucleotide was synthesized (924–974), consisting of wild-type HS II sequence from 932 to 974, with HindIII and XhoI sites on the 5' end and an EcoRI site on the 3' end. This oligonucleotide was subcloned into the HindIII and EcoRI sites of pUC9, sequenced, and cut out again as an XhoI–AatII fragment. This fragment was subcloned into the SalI and AatII sites of pUC007 downstream of a 3' truncation mutant, consisting of wild-type HS II sequence from nucleotide 1 to 900 and a polylinker sequence from 901 to 923. This plasmid was opened at PpuM1 and AatII, and a wild-type HS II sequence from 975 to 1455 was subcloned in as a PpuM1–AatII fragment. This construct effectively replaced wild-type HS II sequence 901–931 with polylinker.
probes, as described in the legend to Fig. 8, in conjunction with induced K562 nuclear extract (3.4 μg) or HeLa cell nuclear extract (4.7 μg).

Figure 9. Gel mobility-shift assays of enhancer mutants. Assays were done by use of the wild-type and mutant enhancer probes, as described in the legend to Fig. 8, in conjunction with induced K562 nuclear extract (3.4 μg, top) or HeLa cell nuclear extract (4.7 μg, bottom).

**Oligonucleotides**

Oligonucleotides were synthesized on an Applied Biosystems DNA Synthesizer model 380B, by use of phosphoramidite chemistry. The oligonucleotides were purified on OEC cartridges (Applied Biosystems). Prior to subcloning or use as competitors, complementary strands were annealed in a buffer of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 10 mM MgCl₂ by heating to 95°C for 2 min and cooling to room temperature for >1 hr. Oligonucleotides were made with 5' HindIII and 3' BamHI ends. They were subconed into these sites of pUC007 and sequenced by the chain-termination method (Sequenase, U.S. Biochemical) [Smith 1980]. The chimeric γ-globin promoter/luciferase reporter gene was inserted into the Sall and AatII sites of these plasmids as an Xhol–AatII fragment.

**Tissue culture and cell transfections**

Human erythroleukemia K562 cells and adherent HeLa cells were grown in improved minimal essential medium (MEM, Biofluids) with 10% fetal calf serum. Plasmid DNA was prepared by the method of Dignam (Dignam et al. 1983), in the presence of PMSF, aprotinin, pepstatin, and leupeptin. The plasmid was dialyzed against a buffer (buffer D) composed of 20 mM HEPES (pH 7.8), 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 20% glycerol and stored in small aliquots at −70°C. Protein concentration was determined by the Bradford method (Bradford 1976), with a kit from Bio-Rad and bovine serum albumin as a standard. The protein concentration was generally 5–6 mg/ml. Hemin-induced K562 nuclear extracts were prepared by the addition of hemin to the culture medium (20 μM final concentration) 36 hr prior to harvest.

**Analysis of DNA-binding proteins**

**Nuclear extracts** Cells were cultured in suspension in microcarrier flasks [Wheaton] and harvested in late log phase. Extracts were prepared by the method of Dignam (Dignam et al. 1983), with the presence of PMSF, aprotinin, pepstatin, and leupeptin. The extract was dialyzed against a buffer (buffer D) composed of 20 mM HEPES (pH 7.8), 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 20% glycerol and stored in small aliquots at −70°C. Protein concentration was determined by the Bradford method (Bradford 1976), with a kit from Bio-Rad and bovine serum albumin as a standard. The protein concentration was generally 5–6 mg/ml. Hemin-induced K562 nuclear extracts were prepared by the addition of hemin to the culture medium (20 μM final concentration) 36 hr prior to harvest.

**In vitro-synthesized c-Jun (rat) and c-Fos (rat)** c-Jun and c-Fos were provided by Frank Rauscher and Tom Curran. The in vitro association of c-Jun and c-Fos and reaction conditions for gel mobility-shift assay have been described by Rauscher et al. (1988).

**DNase I protection assay** Probes for the DNase I protection assay were made from pUC007–HS II and pUC007–HS II enh−. These plasmids were linearized at PvuI (974) and end-labeled with [32P]polyadenylate kinase (lower strand) or Klenow DNA polymerase (upper strand). The probe was released by digestion with HindIII and purified from agarose by Gene Clean.

Each footprint reaction [Jones et al. 1985] contained 10,000 cpm of end-labeled probe, varying amounts of nuclear extract, and 1 μg of poly[d(I-C)] in a volume of 30 μl. The final iodine composition was 20 mM HEPES (pH 7.8), 60 mM KCl, 0.2 mM EDTA, 1.3 mM MgCl₂, 0.5 mM DTT, 10% glycerol, and 6% polyethylene glycol (avg. M, 8000). The reaction was incubated at 4°C for 15 min, followed by 25°C for 15 min. After adding CaCl₂ and MgCl₂ to a final concentration of 3 mM, DNase I digestion was performed at 25°C for 1 min. The amount of DNase I used in each reaction was the amount needed to give equivalent degrees of probe digestion. The reaction was termi-
nated with a stop buffer containing EDTA, SDS, and proteinase K. Proteinase K digestion proceeded at 37°C for 30 min. The samples were then extracted twice with phenol/chloroform, precipitated twice with ethanol, and analyzed on a 5% sequencing gel.

In situ phenanthroline/copper footprinting After gel mobility-shift assay, as described below, in situ phenanthroline/copper footprinting was performed according to the method of Kuwabara and Sigman [1987].

Gel mobility-shift assay The HS II enhancer and chicken β-globin enhancer probes were prepared from oligonucleotides subcloned into pUC007, released by HindIII and BamHI digestion, labeled with Klenow DNA polymerase, and purified over 12% acrylamide gels. The SV40 (Sp1) probe was provided by Todd Evans and Gary Felsenfeld. Gel mobility-shift assays were performed (Fried and Crothers 1981; Strauss and Varshavsky 1984) with 20,000 cpm of probe added last to a 20-μl reaction containing varying amounts of nuclear extracts and 1 μg of poly[d(I-C)]. The final ionic conditions were identical to the samples were run on a 4% nondenaturing acrylamide gel in the presence of 5% glycerol and 2% SDS. The SV40 (Spl) probe was provided by Todd Angel, P., I. Baumann, B. Stein, H. Delius, H.J. Rahmsdorf, and P. Herrlich. 1987. 12-O-Tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5'-flanking region. Mol. Cell. Biol. 7: 2256–2266.

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