Synergistic regulation of human β-globin gene switching by locus control region elements HS3 and HS4

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Proper tissue- and developmental stage-specific transcriptional control over the five genes of the human β-globin locus is elicited in part by the locus control region (LCR), but the molecular mechanisms that dictate this determined pattern of gene expression during human development are still controversial. By use of homologous recombination in yeast to generate mutations in the LCR within a yeast artificial chromosome (YAC) bearing the entire human β-globin gene locus, followed by injection of each of the mutated YACs into murine ova, we addressed the function of LCR hypersensitive site (HS) elements 3 and 4 in human β-globin gene switching. The experiments revealed a number of unexpected properties that are directly attributable to LCR function. First, deletion of either HS3 or HS4 core elements from an otherwise intact YAC results in catastrophic disruption of globin gene expression at all erythroid developmental stages, despite the presence of all other HS elements in the YAC transgenes. If HS3 is used to replace HS4, gene expression is normal at all developmental stages. Conversely, insertion of the HS4 element in place of HS3 results in significant expression changes at every developmental stage, indicating that individual LCR HS elements play distinct roles in stage-specific β-type globin gene activation. Although the HS4 duplication leads to alteration in the levels of ε- and γ-globin mRNAs during embryonic erythropoiesis, total β-type globin mRNA synthesis is balanced, thereby leading to the conclusion that all of the human β-locus genes are competitively regulated. In summary, the human β-globin HS elements appear to form a single, synergistic functional entity called the LCR, and HS3 and HS4 appear to be individually indispensable to the integrity of this macromolecular complex.

[Key Words: LCR; competition; HS3, HS4]

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Each of the genes in the human β-globin locus is sequentially activated during embryonic development: the 5' most (embryonic) ε-globin gene is expressed during the first trimester of gestation, the two γ-globin genes during the second and third trimesters, and, shortly after birth, the 3' adult δ- (minor) and β-globin proteins replace γ-globin chains in tetrameric hemoglobin (Stamatoyannopoulos and Neinhuis 1994). The β-type globin genes are all regulated by the locus control region (LCR), positioned far 5' of the structural genes themselves (Forrester et al. 1987; Grosveld et al. 1987). The LCR is composed of one constitutive and four tissue-specific DNaseI hypersensitive sites (designated HS1, closest to ε-globin, to the constitutive element HS5, lying furthest away; see Orkin 1990, for nomenclature).

The LCR (or smaller units containing the HSs, termed mini- or micro-LCRs) was originally studied as a single contiguous structure, where its effects on single genes or a subset of the genes within the β-globin locus were assayed (e.g., Talbot et al. 1989). Individual LCR elements HS2, HS3, and HS4 were then shown to be independently capable of conferring high level, tissue-specific transcription to linked human β-locus genes in transgenic mice (Curtin et al. 1989; Ryan et al. 1989; Ney et al. 1990; Talbot et al. 1990; Pruzina et al. 1991; Talbot and Grosveld 1991; Lloyd et al. 1992; Morley et al. 1992). Subsequently, it was shown that the HS elements individually elicited markedly different developmental stage-specific activities in constructs harboring several β-locus genes (Fraser et al. 1993). Thus LCR elements were shown to be more than passive amplifiers of globin gene transcription, and rather, were intrinsically capable of conferring a discriminating developmental response in transcriptional control over individual β-locus genes. While the LCR is now generally acknowledged to be an active participant in this program of temporal gene activation, gene-proximal regulatory sequences have also...
The nomenclature used to describe the individual behavior of different globin genes during development (hemoglobin switching) was originally applied to the chicken embryonic ε- or adult β-globin genes. Autonomous regulatory genes are both activated and suppressed in proper developmental time with only an enhancer in cis, whereas competitive regulation requires other cis elements to be present in a particular construct for proper temporal gene activation and suppression (Choi and Engel 1988). Enver et al. (1990) first showed that competition was also applicable to describing gene regulation in the human β-globin locus. More recent evidence supporting the notion that β-globin locus genes are regulated by a competitive mechanism showed that LCR function is grossly disrupted if a strong transcription unit is placed between human HS1 and HS2 (Kim et al. 1992); however, deletion of HS2 alone from the endogenous mouse β-globin locus results in only a mild phenotype (Fiering et al. 1995). Transgenic experiments examining fragments of the LCR linked to human β-globin genes led to the conclusions that ε-globin was autonomously regulated (Raich et al. 1990; Shih et al. 1990), while adult β-globin transcription was competitive (Enver et al. 1990). Evidence for control of the γ-globin genes was, however, contradictory: Two early reports suggested that γ-globin transcription was regulated competitively, whereas subsequent observations provided compelling evidence for autonomy (Behringer et al. 1990; Enver et al. 1990, Dillon and Grosveld 1991).

The disparity in the results analyzing human γ-globin gene regulation could be attributed to differences in the arrangement and number of cis-regulatory elements surrounding the gene. Such contradictions arising from the analysis of slightly different DNA constructions could reflect a requirement for mutant transgenes to be examined within a context where all known (as well as perhaps currently unidentified) regulatory elements are present. Thus transformation of mice with YACs containing large segments of contiguous genomic DNA came as a timely technical advance. In independent reports, YAC transformation of the murine germ line resulted in the recovery of tissue-specific control of the tyrosinase or α1(II) collagen genes, where earlier studies had failed to demonstrate complete complementation with smaller (genomic λ or cosmid DNA) clones (Schedl et al. 1993b; Strauss et al. 1993). In contemporary experiments, it was shown that YACs bearing the entire human β-globin locus also resulted in appropriate developmental regulation of the human genes after introduction into the mouse germ line (Gaensler et al. 1993; Peterson et al. 1993). We therefore devised a strategy to create LCR mutations in a YAC bearing the whole human β-globin locus by use of homologous recombination in yeast. YAC DNAs were then isolated from pulse-field gels and injected into fertilized ova to generate transgenic mice. Pups containing intact, unarranged, single-copy YACs were monitored for the expression of each human β-globin gene at different stages of erythroid development by use of a reverse-transcription–PCR (RT–PCR) assay.

In this report, we describe several explicit tests of a regulatory sequence competition hypothesis formulated for the human β-globin gene locus (Engel 1993). In essence, the model predicted that the stage-specific activation of the genes within the human locus would be achieved by preferential binary association of individual HS sites with (known or theoretical) gene-proximal regulatory elements at various times during erythroid development. DNA looping and direct juxtaposition of distal and proximal elements were proposed to mediate these interactions (Choi and Engel 1988, Gallarda et al. 1989). Each of the mutations revealed new insight into the complex roles that the HS3 and HS4 elements play in the generation of LCR function and in developmental stage-specific competition between human β-globin locus genes and LCR hypersensitive sites.

The present experiments show that deletion of either HS3 or HS4 fundamentally impairs expression of all the β-type globin genes at all erythroid developmental stages, despite the fact that other powerful LCR elements (for example in the case of the HS4 core element–deletion mutant, HS2 and HS3) remain in the YAC transgene. The replacement of HS4 by HS3 appears to fully complement HS4 function at every developmental stage. The converse, however, is not true; when HS4 is substituted for HS3, the expression of every gene at all stages of erythropoiesis is altered, and thus HS4 cannot fully compensate for HS3 function. Moreover, when the wild-type and the HS4 substitution mutant transgenes are compared, alteration of the transcript level of human ε- and γ-globin mRNAs in the embryonic yolk sac is reciprocal. This suggests that HS3 and HS4 competitively and collaboratively control the expression of these two genes at the embryonic stage of erythroid development. Taken together, these data reveal that the LCR behaves as a single cooperative unit and that, not only is this synergistic macromolecular complex comprised of the individual HS elements, but also the elements are not always uniquely specified for discrete functions in the complex.

Results
Generation of human β-globin YAC mutants
A201F4 is a 155-kb YAC that contains the entire human β-globin locus (Gaensler et al. 1991, 1993). The YAC initially segregated unstably in the parent yeast strain, and therefore, a clone which stably mitosed was isolated by mating and sporulation. The YAC DNA was then cloned into a bacteriophage λ vector (Maniatis et al. 1982), and individual recombinant phage containing overlapping segments of the β-globin locus were isolated (Fritsch et al. 1980). Recombinants specifying the
β-globin LCR HS elements and the individual genes were further subcloned into plasmid vectors to facilitate mutagenesis. The minimal HS4 core element has been localized to a 280-bp SacI–AvaI segment of a 3.2-kb parental EcoRI–HindIII fragment (Pruzina et al. 1991), while the minimal HS3 core element was defined as a 225-bp HindIII fragment within a larger 1.9-kb HindIII fragment (Philipson et al. 1990, Fig. 1). For the creation of the HS3 replacement mutant, the HS4 core element was amplified by PCR from the parental HS4 subclone with primers that incorporated unique XbaI and XhoI sites at the ends. The subcloned products of all PCR reactions were verified by sequencing. This 280-bp HS4 minimal element was then used to replace the corresponding HS3 core sequence within the 1.9-kb HS3 HindIII subclone, which generated the mutant hypersensitive site designated HS343 (i.e., a 280-bp HS4 core element embedded in HS3-flanking DNA; Fig. 1). A plasmid incorporating the HS3 core element surrounded by HS4-flanking sequences (HS434) was generated by use of a conceptually identical strategy. Two other plasmids containing deletions of each of the two HS core elements within the bodies of the otherwise unmodified parental fragments were constructed at the same time (see Materials and methods).

To facilitate the generation of mutant β-locus YACs, two manipulations were performed. First, the URA3 selectable marker gene in the right arm of A201F4 was retrofitted with the LYS2 gene, thereby inactivating URA3 in the YAC by homologous recombination (Srivastava and Schlessinger 1991). At the same time, each of the four mutated HS plasmids [described above] was subcloned into the yeast integrative plasmid vector pRS306, containing the URA3 gene (Sikorski and Hieter 1989). Each pRS306 subclone was then used to transform the [LYS2-modified] wild-type human β-globin YAC. For example, HS343 (Fig. 1) subcloned in pRS306 was digested with SauI and then used to transform yeast bearing the LYS2-retrofitted YAC clone (Fig. 2; see Materials and methods). The majority of the yeast colonies growing on selective [trp- lys- ura-] medium contained the targeting plasmid integrated at the homologous human β-globin HS site within the YAC, thereby creating an intermediate with the LCR elements arranged (e.g., in replacing HS3 with HS4) in the order: 5′-HS4–[HS343]–URA3–HS3–HS2–3′ (Fig. 2). Selective excision of the targeting plasmid was mediated by growth on medium containing uracil, followed by counterselection on 5-fluoroorotic acid (FOA)-containing plates [lethal to cells retaining the URA3 marker; see Materials and methods]. Excision of pRS306 resulted in reversion to either the parental YAC structure or replacement of the parental HS site by the desired mutant element (Fig. 2, Winston et al. 1983). Thus the number of YACs to be tested for transgene expression characteristics in mice were five (Fig. 3): wild-type [the LYS2-retrofitted YAC, designated HS4321], an HS3 duplication (HS3321; incorporating HS434), an HS3 core element deletion (HS4021; incorporating HS343), an HS4 duplication (HS4421; incorporating HS3321), and the HS4 core element deletion (HS0321; incorporating HS434). The structures of the four mutants and the parental wild-type YAC were verified by Southern blotting on both conventional and pulse-field gels (Fig. 4).

Transgenic mice

Two methods have been used to introduce YACs into the mouse germ line. One involved microinjection into

**Figure 1.** Diagram of the human β-globin LCR and generation of the HS3 and HS4 replacement mutants. The top line indicates the positions of the hypersensitive sites in the human β-globin LCR (Talbot et al. 1989). The second line depicts the two subclones (HS3 and HS4) that were used to initiate the yeast targeting mutagenesis strategy (see Materials and methods). These two HS element subclones were used as substrates for PCR amplification of the individual core elements and flanking sequences. The PCR amplification was carried out with primers that incorporated new, unique restriction enzyme sites at a number of positions to aid in subsequent cloning steps (Table 1). Both the HS3 and HS4 core elements [line 3] were amplified from the respective parental plasmids using these primers to incorporate new sites into the cores [line 4], and finally the two core elements were exchanged between the two appropriately PCR-adapted parent clones to yield the desired mutant elements HS343 and HS434 [line 5].
Figure 2. Generation of LCR HS replacement and deletion mutants by use of homologous recombination. In this example, the HS343 fragment (generated as outlined in Fig. 1) was subcloned into yeast shuttle vector pRS306 (Sikorski and Hieter 1989). After digestion with Sall (top line), it was then used to transform [LYS2-modified; see Materials and methods] yeast clone A201F4, harboring the 155-kb human β-globin YAC (line 2; Gaensler et al. 1991). Colonies containing the targeting vector (i.e., growth on Lys−, Trp−, Ura− medium) were tested for homologous integration by Southern blot analysis (line 3). Individual clones that had undergone homologous recombination were replated on Ura+ medium and then replica plated onto FOA plates (see Materials and methods). Clones growing on FOA were tested individually for the presence of the original YAC (HS4321) vs. the replacement mutant structure (HS4421; line 4) by Southern blots (see Fig. 4).

fertilized murine eggs (Schedl et al. 1993a, b), whereas another involved lipofection-mediated transfer of purified YAC DNA into embryonic stem (ES) cells, followed by blastocyst injection to generate chimeric mice (Strauss et al. 1993). We chose to use the former method because microinjection is reported to result in more frequent integration of intact YAC transgene DNA (Gnirke et al. 1993), and because we planned to use these same mutant YACs for transgenic studies after further mutagenesis.

Wild-type and mutated YACs were isolated after pulse-field gel electrophoresis (PFGE), purified, and microinjected into fertilized murine ova (see Materials and methods; Schedl et al. 1993a,b; Peterson et al. 1993).

After initial PCR identification and then Southern blotting of founder tail DNA to confirm the presence of the transgene, all positive animals were bred to ensure that the transgenes could be stably transmitted to progeny. At least two transgenic lines were analyzed for each of the YACs, arbitrarily referred to below as lines a and b (an additional line, c, was analyzed, which contained the HS4421 YAC mutant transgene). Southern blotting of each of the F1 or F2 lines, with probes both flanking and internal to the locus (RI 1.8, RI 3.3, RK29, HS4, HS3, HS2, ε-, γ-, and β-globin; Gaensler et al. 1991; Fig. 3) showed that each contained restriction fragments of the expected size. Fragmented YACs discovered through this analysis were excluded from further consideration. The a

Figure 3. Diagrammatic representation of the LCR mutant YACs. Diagrammatic representations of the five individual human β-globin YACs generated for this analysis are shown. The bottom five lines depict the structures of the parental and four mutant YACs studied here, whereas the top line depicts the approximate position of all these elements within the YAC borne by A201F4. In addition, other sequences flanking the gene and LCR elements were used as probes in Southern blot analysis to characterize the physical integrity of the locus before and after transgene integration into the germ line of mice (see Results; Fig. 4; data not shown; Gaensler et al. 1991).
Human β-globin switching in YAC transgenic mice

Figure 4. Characterization of the A201F4 YAC HS mutations. All four YAC mutants (HS3321, lanes 2, 7; HS0321, lanes 3, 8; HS4421, lanes 4, 9; and HS4021, lanes 5, 10) generated by use of the strategy outlined in Fig. 2 and depicted diagrammatically in Fig. 3, were analyzed by Southern blotting. (A) Confirmation of targeted mutagenesis in each of the YACs was first investigated by PFGE. Examination of ethidium bromide-stained pulse-field gels of the parental and mutated human β-globin YACs showed that the mutant YAC chromosomes are unaltered in size. (B) The pulse-field gel shown in A was transferred to a nylon membrane and then hybridized with radiolabeled HS4 (lanes 1–5) or HS3 (lanes 6–10) core element probes. (C) DNA prepared from the wild-type and each of the mutant YACs was digested with EcoRI, transferred to nylon filters, and then hybridized to radiolabeled HS4, adult β-globin and embryonic ε-globin probes (lanes 1–5) or HS3, fetal γ-globin and 3′ flanking marker RK29 probes (lanes 6–10; Fig. 3, Gaensler et al. 1991). Abbreviations representing each of the YACs are (+) HS4321 (lanes 1, 6; the wild-type YAC; Fig. 3); (A4–*3) HS3321; (A4) HS0321; (3−* 4) HS4421; and (A3) HS4021.

line animals used for breeding and subsequent mRNA analysis were found to contain a single copy of the YAC left and right vector arms, except a single transgenic line in which HS3 had been deleted (HS4021a, Figs. 3 and 7, below), which contained neither (not shown). The transgenic lines also contained all markers within the locus on contiguous restriction enzyme fragments of the expected size, and virtually identical band intensities. In summary, these data indicated that all of the animals subjected to detailed analysis here contained intact, single-copy YAC transgenes that were transmissible through the germ line.

Human β-globin multiplex PCR assay

To determine the pattern, timing, and abundance of expression of each of the human β-globin locus genes in transgenic mice, RNA isolated from the yolk sacs, fetal livers, or adult spleens of transgenic embryos or animals (see Materials and methods) was analyzed by semiquantitative RT–PCR (Foley and Engel 1992; Foley et al. 1993; Leonard et al. 1993). The level of ε-, γ-, and β-globin mRNAs were compared to an internal control, mouse α-globin mRNA, which remains relatively constant during murine gestation (Whitelaw et al. 1990). This RNA analysis method relied on the fact that unique primers for each of the human β-type globin genes could be defined (Fig. 5A; Table 1), that they would give rise to specific amplicons that differed from each other in size, and that these primers would not cross-react, either with one another or with cDNAs produced after reverse transcription of mouse β-type globin mRNAs. We demonstrated that these expectations could be fulfilled in control experiments (Fig. 5B), thus simplifying the analysis by allowing simultaneous (multiplex) assay for accumulation of all three human β-globin transcripts (huε, huγ, and huβ) and the endogenous mouse α-globin control (muα) in each RNA sample at every stage of murine erythroid development. The assay is only semi-quantitative, however, because primer sets for each gene may differ subtly in annealing efficiency and could therefore differ in the amount of isotope-labeled deoxynucleotide incorporated (the products are of different length and G + C content), thus the absolute abundance of one transcript compared to another at a different stage of development is not quantitative, but the relative abundance of any individual PCR product at specific developmental stages can be directly compared after normalization to the intensity of the muα globin internal control, the length and G + C content of the PCR products, and the transgene copy number in comparison to the endogenous mouse α-globin genes (see Materials and methods; Table 2).

RNAs were prepared by use of standard procedures (Chomczynski and Sacchi 1987), from transgenic F2 or F3 embryos at 9.5 days post-coitus (dpc) [yolk sac] and 14.5 dpc (liver) or from the spleens of 2- to 6-month-old anemic animals, representing embryonic, fetal or adult de-
Figure 5. Developing a multiplex RT-PCR assay for human β-type globin gene expression in transgenic mice. [A] The human β-globin gene locus DNA sequences [EMBL data base] were compared to one another and to the mouse β-globin locus sequences to define primers [Table 1] that would both be specific for each human globin gene and additionally would not cross-react with mouse β-type globins; the approximate positions and anticipated product lengths as cDNA are shown. When possible, we chose primer positions spanning introns to ensure that products arise originally from mRNA and not contaminating genomic DNA. [B] Control PCR reactions were performed to determine the temperature optima and substrate specificity for each set of human β-globin and mouse α-globin gene primers. In every reaction shown here, all four globin gene-specific primer sets [Table 1] were included; in addition, each lane represents the inclusion of different individual cloned substrate globin cDNAs (30 pg each, shown in the table below the diagram) in the PCR reactions (see Materials and methods).
**Table 1. Oligonucleotides used for amplification of core enhancers and flanking sequences**

| Name                  | Sequence                                      |
|-----------------------|-----------------------------------------------|
| HS4-core US:          | 5'-CGCATCTAGAGGACCCCAATGACAAGAGG-3'           |
| HS4-core DS:          | 5'-AGGGCTCAGTCCGGGAAAGGAGGAGGAGG-3'           |
| HS3-core US:          | 5'-GCAGCTCTAGATGGCAGTGGTCTCTAAAGGTGAT-3'      |
| HS3-core DS:          | 5'-GGGCTCAGTGGTCTCTGCTGCTCTCCC-3'             |
| HS4-5' flank:         | 5'-CCGATCTAGAGGAGGACAGCAGTGGAT-3'             |
| HS4-3' flank:         | 5'-CAGGCTCAGTGCTGCTTGTGCTTTC-3'               |
| HS3-5' flank:         | 5'-GAAGTCTAGAGGAGGACAGCAGTGGAT-3'             |
| HS3-3' flank:         | 5'-TGTAAGTGGAGGACAGCAGTGGAT-3'                |
| R-arm US:             | 5'-TCCGTAATCTTGAGATCGGGCGT-3'                 |
| R-arm DS:             | 5'-GGTGATGTCGGCGATATAGGCGCCAGCAAC-3'          |
| L-arm US:             | 5'-GTGATAAATTAAAGTCTTGCGCCCTAAACCC-3'         |
| L-arm DS:             | 5'-GCTACTTGGGAGCAGACATCGACTACGCGAT-3'         |
| hoglobin. US:         | 5'-CTTTGGAGATGCTATATAAACACATG-3'              |
| hoglobin. DS:         | 5'-CCAGAGATATACCCATACCATCCTAC-3'              |
| hyglob. US:           | 5'-GACCCTTTGGAATACCATCCATTC-3'                |
| hyglob. DS:           | 5'-GATTTGCTGACAGCAGACATCAGAGT-3'              |
| hβglob. US:           | 5'-ACACAATCTGTTCTAGCAGCACTCA-3'               |
| hβglob. DS:           | 5'-GGTTGCCCATACACAGACATCAGAGT-3'              |
| maglob. US:           | 5'-GAATCTGACAGCAGCAGACATCAGAGA-3'             |
| maglob. DS:           | 5'-CCTTTGACAGACATCAGCTCAGATAT-3'              |

Oligonucleotides were designed according to published sequences (GenBank/EMBL). Bold nucleotides represent restriction sites that were generated to facilitate subcloning. Oligonucleotides specific for the right and left vector arms of the YAC were adopted from Peterson et al. (1993). Oligonucleotides used for RT-PCR (human ε-, γ-, β-, and mouse α-globins) were designed according to sequences from the GenBank/EMBL data bank.

Uniquely specified, constituent elements. An appropriately constituted LCR also appears to be capable of discriminating between the individual genes by presently obscure competitive regulatory interactions at every stage of erythroid development.

**Human β-globin YAC transformation of mice**

All of the transgenic animals studied here are, to the best of our knowledge, single copy and unarranged for the microinjected YAC, and thus any regulatory pattern alterations reported here that differ from the parental YAC transgene expression pattern cannot be due to effects from neighboring, multiply integrated YAC transgene loci. This was initially a cause for concern because it has been shown that multicopy transgenes may behave quite differently than single-copy constructs harboring uniquely specified, constituent elements. An appropriately constituted LCR also appears to be capable of discriminating between the individual genes by presently obscure competitive regulatory interactions at every stage of erythroid development.

**Table 2. Quantitative analysis of HS3 and HS4 human β-globin LCR mutations**

| Genotype | Embryonic yolk sac (9.5 dpc) | Fetal liver (14.5 dpc) | Ad. spleen |
|----------|------------------------------|------------------------|------------|
|          | ε | γ | total ys | γ | β | total fl | β |
| HS4321   | 91/97 | 96/124 | 188/221 | 6.7/8.4 | 64/78 | 71/86 | 46/(N.D.) |
| HS3321   | 117/91 | 111/94 | 228/185 | 6.5/7.5 | 70/71 | 76/78 | 39/47 |
| HS3032   | 2.1/1.2 | 2.3/5.8 | 4.4/7.0 | 1.7/2.1 | 1.3/2.1 | 3.0/4.2 | 5.7/9.8 |
| HS4421   | 8.4/6.9/7.5 | 168/185/195 | 176/192/202 | 1.5/2.7/2.6 | 0/1.9/3.2 | 1.5/4.6/5.8 | 13/14/(N.D.) |
| HS4021   | 8.7/6.4 | 0/43 | 8.7/49 | 0/3.2 | 0/3.8 | 0/7.0 | 7.3/15 |

Expression of human β-type globin transgenes from the wild-type (HS4321) and mutated (HS3321, HS3032, HS4421, HS4021) YACs in transgenic mice, normalized to expression of the mouse α-globin gene (four copies, arbitrarily set as 100%). The data were obtained from quantitation of multiple PCR analyses (as represented by the data shown in Fig. 6) on a PhosphorImager as described in Materials and methods. The first number shown in each box is from transgenic line a, the second from line b, and (for HS4421) the third from line c, for each YAC. (N.D.) not determined.
Figure 6. Erythroid expression of the human \(\alpha\)-, \(\gamma\)-, and \(\beta\)-globin genes in HS3 and HS4 YAC mutant transgenic mice. RNA was prepared from transgenic embryos or adult mouse erythroid tissues, reverse transcribed, amplified into the linear range by PCR and then electrophoresed as described (see Materials and methods). Erythroid tissues representing embryonic, fetal and adult stages (Strouboulis et al. 1992; Gaensler et al. 1993) were analyzed. (Lanes 1–5) 9.5-day embryonic yolk sac RNA, (lanes 6–10) 14.5-day embryonic liver RNA and (lanes 11–16) adult spleen RNA. The expression of the following YACs (Fig. 3) was examined in each erythroid tissue: HS4321 (lanes 1,6,12), HS3321 (lanes 2,7,13), HS0321 (lanes 3,8,14), HS3321 (lanes 4,9,15), and HS4021 (lanes 5,10,16). Lane 11 is an RNA sample from a nontransgenic mouse spleen.

\(\beta\)-globin locus HS regulatory elements (Ellis et al. 1993). We found that all transgenic lines were single copy, even though there appears to be no \textit{a priori} restriction to the integration of multiple YAC transgenes (Schedl 1993b). Perhaps this should not have been surprising considering the physiological circumstances, because one of the defining characteristics of the LCR is the ability to confer transgene copy number-dependent, tissue-specific, high-level expression to genes under its regulatory control (see below). A consequence of tandem YAC integration might therefore have been predicted to result in the synthesis of equivalent quantities of transgenic human and endogenous (diploid) mouse \(\beta\)-globin mRNAs. The physiological consequences of such a multicopy integration event might have been expected to result in a considerable \(\beta\)-globin polypeptide chain imbalance, and hence severe anemia; thus, in this role, the LCR may have provided a natural selection against multicopy transgenic animals in these studies. This argument, however, cannot explain the single-copy integration of the two deletion mutants (HS0321 and HS4021), because both display significantly impaired human \(\beta\)-type globin synthesis at every developmental stage.

HS3 and HS4 mutations fundamentally alter LCR activity

Deletion of HS3 (mutant HS4021, Fig. 3) or elimination of HS4 (HS0321) resulted in fundamentally impaired globin-gene expression at all stages of erythropoiesis (Table 2). Although the two HS4 deletion mutant lines both behaved similarly, the two lines representing the HS3 deletion mutant differed significantly in expression characteristics from one another at all erythroid developmental stages. The divergent expression characteristics of the two independent HS4021 transgenic lines may indicate that HS3 ablation leads to loss of the integration site position-independence normally conferred by the LCR. Alternatively, the explanation for the difference in these two lines may be that hitherto undetected human \(\beta\)-globin transcriptional regulatory elements lie between the boundaries described by the two transgenes [remember that although both lines appear to be normal in organization and contiguity, HS4021 lane a is missing both YAC vector arms, presumably including some segments of human DNA lying between these and the extreme 5' (RI 3.3) and 3' (RK29) markers used to characterize the integrity of the locus; Gaensler et al. 1991]. These two alternatives should be easily resolved through the analysis of further transgenic lines bearing this YAC mutant.

When the same two LCR HS elements were substituted for one another, HS3 quantitatively complemented HS4 function at all stages of erythroid development (mutant HS3321), whereas HS4 only partially compensated for HS3 loss at the same stages (mutant HS4421),...
Fig. 7). Although HS3 is required for γ-globin transcription in the yolk sac, its loss can be complemented by HS4 at this stage (Fig. 7), but not during fetal liver or adult erythropoiesis. The HS4 duplication mutant additionally exhibited gene-specific transcriptional alterations and only partially rescued HS3 activity, whereas the HS3 duplication was entirely competent when compared to wild type at every stage of erythroid development. In the fetal liver, γ-globin mRNA synthesis was restored to the same extent in either the HS3 or HS4 deletion or HS4 duplication mutants, while at the same time β-globin synthesis was significantly diminished by the HS3 duplication was entirely competent when compared to wild type at every stage of erythroid development. In the fetal liver, γ-globin mRNA synthesis was reduced to the same extent in either the HS3 or HS4 deletion or HS4 duplication mutants, while at the same time β-globin synthesis was significantly diminished by the HS3 duplication was entirely competent when compared to wild type at every stage of erythroid development.

The LCR appears to act as a synergistic functional unit

The LCR was originally identified as a group of DNaseI hypersensitive sites in erythroid chromatin 5' to the β-globin gene locus (Tuan and London 1984; Tuan et al. 1985; Forrester et al. 1986) and is known to be activated in early erythroid precursors and to organize the entire locus into an open chromatin configuration (Forrester et al. 1990). Promoter-, silencer-, and enhancer-binding transcription factors present in the erythroid milieu of the yolk sac, fetal liver, or bone marrow presumably positively or negatively influence the interactions that subsequently determine gene-specific developmental activation. The expression level of each gene achieved by individual HS elements is lower than that observed with the intact LCR (Grosven et al. 1987; Strouboulis et al. 1992; Fraser et al. 1993), initially suggesting that HS elements must interact either with one another or with other, more gene-proximal, elements within the locus to realize wild-type levels of globin-gene expression.

One attractive mechanism for such a cooperative effect is that the whole LCR is organized as a single, integral macromolecular complex that can discriminate between interactions of specific HS elements with a particular gene (or set of genes) at specific developmental stages. We proposed a model for the regulation of human β-globin gene switching that was a conceptual extension of observations describing the mechanisms controlling chicken β/e-globin gene switching (Choi and Engel 1988; Foley and Engel 1992). This hypothesis included both the molecular analysis of human hemoglobinopathies and the growing body of evidence provided by analysis of transgenic animals (Engel 1993). This nonexclusive model suggested that the appropriate registry of cis-regulatory sequences occurs in a simple combinatorial fashion and is sufficient to elicit full activity from each of the HS elements.

Surprisingly, deletion of HS3 or HS4 core elements disrupted human β-globin gene expression at all developmental stages, despite the presence of other powerful regulatory elements (HS2/HS4 or HS2/HS3, respectively, in mutants HS4021 or HS0321) in the YACs. These data suggest several alternative interpretations. The first is that the LCR acts as an integral functional unit, and hence any attempt to dissect the multiple activities attributed to the unit cannot be achieved without fundamentally impairing the structure (and hence the function) of the LCR. The observation that deletion of either HS4 or HS3 leads to a catastrophic disruption of LCR function at each stage of erythropoiesis lends support to this hypothesis.

A second tenable hypothesis is one that would view the effect of deletion of any single HS core element as a contribution to the collapse of the entire accessible chromatin structure of the locus, much like the loss of HS2 through HS5 (and beyond) exhibited in a documented Hispanic γβ-thalassemia (Driscoll et al. 1989; Forrester et al. 1990). One might predict from this second hypothesis that the effects on chromatin throughout (and extending beyond) the locus would be altered, whereas if the deletion of a single HS element disrupts a hypothetical (and possibly much more localized) LCR structural unit, the chromatin effects may consequently be much more locally restricted and could lay entirely within the LCR. Furthermore, because transcription of all the genes is impaired in HS4 deletion mutant (HS0321) mice, if DNaseI hypersensitivity is retained at, for example, the HS2 and HS3 sites, this would certainly demonstrate that hypersensitivity does not equate to functional activity, a subject of considerable recent interest (e.g., Reitman et al. 1993; Stamatoyannopoulos et al. 1995).

Finally, when one considers the overall impression from these observations (that control of the entire locus appears to be completely compromised after deletion of only the very small HS3 or HS4 core elements), a third explanation for the dramatic effects of either mutant is that residual transcription factor-binding sites (flanking the core elements) could elicit a dominant-negative phenotype on the rest of the locus. The concept that such residual activity resides outside of the cores can be easily envisioned, because the core elements were originally defined primarily on the basis of their individual transcriptional activation functions, rather than the multiple other activities (e.g., chromatin domain opening, transgene integration-site independence and copy-number dependence for expression) that have been ascribed to the LCR. Although the nature of the mechanism by which such a hypothetical dominant-negative effect might compromise the whole LCR is presently obscure, one prediction of this hypothesis is that even though the individual core element-deletion mutants lead to a loss of the transcriptional activation functions, the DNaseI hypersensitivity at the position of the deleted core elements should be retained. We are currently testing these various predictions.

Stage-specific β-type globin gene regulation by HS3 and HS4

In previous studies, Shih et al. (1990) showed that when
a human e-globin transgene was linked in cis to HS1 plus HS2, the transgene was appropriately regulated (i.e., activated and extinguished) at the proper time and site of hematoipoiesis during murine development. Similarly, when the γ-globin gene was joined to a miniature version of the LCR, γ-globin synthesis was also appropriately activated and suppressed (Dillon and Grosveld 1991). On the basis of these experiments, both groups concluded that the embryonic and fetal human β-locus genes were autonomously regulated.

The present experiments show that completely autonomous e-globin gene control may not be elicited solely by the HS1/HS2 plus e-globin gene-proximal regulatory sequences alone, because all of these elements are present in all the YAC transgenes examined here, and in three of the four YAC LCR mutants, e-globin synthesis is grossly misregulated (see below). Similarly, the conclusion that γ-globin is autonomously regulated may also have been premature. The transgenes examined in the previous study contained multiple HS elements in cis to the gene, and therefore competitive regulation (for example, between multiple hypersensitive elements and gene-proximal transcriptional control elements) could have masked possible competitive interactions. Even though the identity of the competing elements have not been defined through the present analysis, the fact that all three HS4421 transgenic lines exhibit competitive complementation in the embryonic yolk sac stage means that all of the human β-locus genes (the e-, γ-, and β-globins; this work and Enver et al. 1990) are competitively regulated.

Competitive gene regulation

A significant aspect of the human globin competition model was the inherent assumption that stage-specific transcriptional silencing would play a prominent role in regulating the human β-globin locus genes (Engel 1993), but only one LCR mutation resulted in the kind of elevated gene expression that one might have predicted from a loss of gene silencer activity: γ-Globin transcription in the yolk sac is significantly induced in the HS4421 mutant relative to the parental locus (Fig. 7). This mutant, however, also displays a simultaneous decrease in e-globin gene transcription at the same developmental stage. Therefore the total human β-type globin mRNA abundance in the embryonic yolk sac of HS4421 mutant transgenic animals is the same as that in the wild-type locus YAC transgenics (Table 2; Fig. 7).

This observation suggests that the elevated yolk sac γ-globin gene transcription observed in the HS4 duplication mutants does not stem from a relief from silencing, but rather that any combination of two HS3 or HS4 core elements generates a discrete quantum of LCR transcriptional activity in the embryonic yolk sac. Achieving comparable levels of accumulated mRNA in compensating regulatory sequence mutants was originally a hallmark for competitive interactions in the chicken β-globin gene locus (Foley and Engel 1992). The conceptually analogous result described here suggests strongly that the HS4421 LCR mutants, as well as the wild-type parental LCR, are competing for some limiting cis elements shared between the human e- and γ-globin genes at the yolk sac stage of mouse erythropoiesis. Given the numerous transcriptional regulatory sequences that have been implicated in controlling the human β-globin gene locus, it would be misleading at the present time to suggest a mechanism for how this competition arises. Nonetheless, these observations strongly support the contention that both the human e- and γ-globin genes are competitively regulated during the yolk sac stage. We suspect that other regulatory sequence element mutations, when examined in the context of an otherwise intact locus (as exemplified in the YAC mutant studies described here) may shed additional light on the identity of the specific competing regulatory elements.

Competition among cis-regulatory transcriptional control elements was first invoked as a simple epigenetic regulatory mechanism regulating differential chicken β- and e-globin gene transcription (Choi and Engel 1988) and was subsequently confirmed through detailed mutational analysis (Foley and Engel 1992). The first indication that genes within the human β-globin locus might be regulated by use of a similar strategy emerged shortly thereafter (Enver et al. 1990; Kim et al. 1992). Subsequently, indications that regulatory sequence competition might be a more general biological regulatory phenomenon began to emerge. The Drosophila HOM-C complex genes (as well as possibly their vertebrate counterparts) were proposed to be regulated in a competitive manner (Lewis 1992), and a conceptually similar scheme to chicken β/e-globin gene competitive interactions has been invoked to explain the developmental behavior of the alternatively imprinted Igf2 and H19 genes (Sasaki et al. 1992; Bartolomei et al. 1993). Clearly any control network where complex regulatory patterns must be elicited from multiple cis elements must now be considered as possible focal points for the involvement of competitive transcriptional interactions.

Human β-globin gene transcription has long been the subject of intense scrutiny, not only because the human β-globin gene was among the first eucaryotic genes to be molecularly cloned, but also because defects in this locus offer one of the most promising targets for potential intervention by somatic gene therapy. The frequency of deficiencies in hemoglobin production in the human population, coupled to the ability to isolate hematopoietic stem cells, suggest that clinical intervention could be among the first successful somatic therapies to affect the longevity and quality of life of afflicted individuals. Clearly, understanding the precise mechanisms controlling gene expression in this locus is a precedent to achieving success in defining effective clinical genetic strategies, and thus we anticipate that full elucidation of the mechanisms underlying proper globin-gene switching will contribute in a fundamental way to this ultimate goal.
Materials and methods

Recloning the human β-globin gene locus

The yeast clone A201F4, containing the entire human β-globin gene locus including extensive flanking sequences, was generously provided by Dr. R. Myers [Gaensler et al. 1991]. High molecular weight genomic DNA from this clone containing the 155-kb β-globin YAC was prepared and partially digested with the restriction enzyme Sau3A1. Fragments 15–20 kb in length were isolated from sucrose gradients and ligated to λEMBL4 arms [Stratagene]. After in vitro packaging, the phage library (~10^6 complexity) was amplified on Escherichia coli LE392. Phage DNA from the amplified library was transferred to filters that were then hybridized to specific probes to identify human β-globin locus clones [Maniatis et al. 1982]. After rescreening of positive plaques to purity, DNA from the phage containing globin locus sequences was isolated. Recombinants contained overlapping globin gene and LCR sequences representing the entire locus [Fritsch et al. 1980].

The core sequences of individual hypersensitive sites of the LCR have been previously well defined in functional as well as biochemical assays [Philipson et al. 1990; Talbot et al. 1990; Pruitt et al. 1991]. Individual activities localized to those 200- to 400-bp fragments are referred to as core elements. These core elements, together with substantial flanking DNA, were initially isolated as either wild-type 1.9-kb HindIII [HS3] or 3.2-kb EcoRI–HindIII [HS4] fragments from individual λEMBL4 recombinants subcloned into pGEM7 [Promega].

The HS3 and HS4 core element mutations were individually reconstructed from these two plasmids by PCR. Unique restriction enzyme sites were incorporated into the ends of all of the primers to allow the eventual ordered ligation of the fragments in a yeast integrative plasmid shuttle vector [Table 1]. The following order of fragments was used for ligation to generate the two final substitution mutant subclones: HS3 5′, BstXI/XbaI; HS4, core, XbaI/XhoI; HS3 3′, XhoI/ClaI; and HS4 5′, EcoRI–XbaI; HS3 core, XbaI/XhoI; HS4 3′, XhoI/HindIII [Fig. 1].

To generate the targeted YAC substitution mutants, the HS3 and HS4 duplication mutants (HS4°4 and HS3°3, respectively; Fig. 1) were subcloned into yeast integrative plasmid vector pRS306 [Sikorski and Hieter 1989]. To prepare HS4°4, PCR fragments corresponding to the 5′ EcoRI–SacI fragment of HS4 [converting the SacI site to an XbaI site by PCR; Fig. 1], the core element of HS3 [exchanging the natural HphI and Fnu4HI sites for XbaI and XhoI sites, respectively] and the 3′ Aval (converted to XhoI by PCR) to HindIII fragment of HS4 were digested with the appropriate restriction enzymes and ligated in a single re-action to pRS306 that had already been digested with EcoRI and HindIII. HS3°3 was prepared by use of a conceptually identical strategy. For the generation of the two core element-deletion mutants, the HS3 and HS4 5′- and 3′-flanking sequence PCR fragments were first digested with XbaI and XhoI, filled in with Klenow polymerase, and digested with the enzymes recognizing the PCR-generated 5′ and 3′ ends before ligation to pRS306 that had been digested with the same enzymes. The DNA fragments used to prepare each of the four YAC substitution mutants were sequenced to verify that no mutations were introduced during PCR or subcloning.

Homologous targeting of the HS element mutants in yeast cells

Most of the yeast methods used here were derived from a single reference source [Guthrie and Fink 1991]. pRS306 contains a URA3 selectable marker used to monitor integration into the yeast genome [Sikorski and Hieter 1989]. To use pRS306 as the targeting vector, we first disrupted URA3 by inserting the LYS2 gene into the resident URA3 gene in the right arm of the A201F4 YAC by homologous recombination using pRV1 [at the same time incorporating the neomycin resistance gene [neo], also carried on pRV1, Srivastava and Schlessinger 1991]. Correct integration of this URA3-disruption plasmid was monitored by Southern blot hybridization [not shown] and the ability of appropriately targeted cells to grow on Lys−, but no longer on Ura− medium.

Generation of HS mutant β-globin YACs was carried out in a two-step procedure involving site-specific integration of pRS306, harboring one of the four mutated HS sequences, into the A201F4 YAC, followed by excision of the wild-type copy (together with the URA3 marker gene) mediated by yeast homologous recombination [Winston et al. 1983]. After transformation of the [LYS2-modified] yeast clone A201F4 with the linearized targeting vectors [HS4°4 and HS4°4 with BamHI], HS3°3 and HS3°3 with SacI, yeast colonies were replated onto selective [Lys−, Ura−, Trp−] medium [Guthrie and Fink 1991]. After colony purification, homologous integration was verified by restriction enzyme digestion and Southern blot hybridization. Individual colonies in which the targeting vector was integrated correctly were then transferred to Lys−, Trp−, Ura− medium to allow reversion [which leaves either the wild-type or the mutant HS copy]. After 2 days on Lys−, Trp− medium, colonies were replated onto medium containing FOA (Jersey Lab Supply) to select against the growth of clones that had retained the URA3 marker. Excision of the wild type sequence in surviving colonies was verified by in situ yeast colony hybridization [Guthrie and Fink 1991], screening for the presence or absence of the wild-type core elements [HS3 or HS4].

The integrity of the mutated β-globin YACs after homologous recombination was assayed by restriction digestion of yeast DNA and subsequent Southern blotting with probes spanning the human β-globin gene locus [Figs. 3 and 4; Gaensler et al. 1991]. In addition, the size of the intact, mutated YACs was verified by pulse-field gel electrophoresis and Southern blot hybridization with the HS3 and HS4 core elements as probes [Fig. 4].

Isolation of YAC DNA for microinjection

Yeast was grown to high density on selective YNB medium [465 mls of double deonized H2O and 25 ml of 40% glucose was added to 0.8 gram of yeast nitrogen base, 1 gram Lys−, Trp−, amino acid mix, and 2.5 grams of [NH4]2SO4]. Cells were pelleted by centrifugation and washed once with 50 mm EDTA [pH 8.0]. The cell pellet was then resuspended in a few drops of lyticase solution [85 μl of 2 mM DTT and 20 mg of lyticase (70,000 U/gram; ICN), and added to 5 ml of SCE (1 M sorbitol; 0.1 M Na-citrate, 10 mM EDTA at pH 7.0)]. After a 20-min incubation at 37°C, an equal volume of 1% LMP agarose (Bio-Rad) in 0.125 mM EDTA, pH 8.0 [preheated to 47°C] was added. The solution was mixed and immediately poured into a plug-forming mold, then allowed to solidify at 4°C for 1 hr. The yeast plugs were then transferred to 5 ml of buffer containing 0.5 mM EDTA [pH 8.0], 100 mM Tris-HCl [pH 8.0], and 50 mM DTT for 4 hr at 37°C and then incubated overnight at 50°C in 0.5 mM EDTA [pH 8.0], 100 mM Tris-HCl [pH 8.0], 1% sarcosyl, and 0.6 mg/ml of proteinase K. After several washes in 75 mM NaCl, 25 mM EDTA [pH 8.0], the plugs were stored at 4°C in 0.25 mM EDTA [pH 8.0] until use.

YAC DNA was isolated from pulse-field gels according to standard protocols [Peterson et al. 1993; Schedl et al. 1993a] with the following modifications. Agarose blocks containing
YAC DNA were excised from the pulse-field gel, equilibrated at 4°C for 2 hr with injection buffer [10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA (pH 7.5), 100 mM NaCl, 3 μM spermine, 7 μM spermidine], including one buffer change. After incubation with β-agarase (NEB), the DNA solution was concentrated to 40 μl by centrifugation through a Millipore MC-filter (Ultrafree MC Polysulphone Type PTHK) at 3500 rpm. After allowing the DNA to resuspend on the filters for 2 hr at 4°C, the solutions were combined and an aliquot (30–40 μl) of the DNA solution to be injected was loaded onto a pulse-field gel alongside a control YAC yeast plug. The gels were then blotted and hybridized to β-globin locus probes to verify the integrity of the recovered DNA. YAC DNA was generally recovered at a concentration of 2–5 ng/μl, and was diluted to a final concentration of 1 ng/μl immediately before injection.

Transgenic mice

YAC DNA was injected into fertilized mouse eggs (CD1, Charles River) and then transferred to pseudopregnant foster mothers (B6D2F1/J) as described [Hogan et al. 1986]. Tail DNAs from offspring were first analyzed for the presence of left and right YAC vector arm sequences by PCR and then for the integrity of the transgenic β-globin locus by Southern blot hybridization of restriction enzyme-digested F1 or F2 tail DNA with probes spanning the locus [Gaensler et al. 1991; Figs. 3 and 4].

Semiquantitative multiplex RT–PCR analysis

The ε-globin cDNA clone was isolated from a human K562 human erythroleukemia cell cDNA library (generously provided by S. Orkin). The ends of the clones were sequenced to verify its authenticity in comparison to the published genomic sequence [Baralle et al. 1980]. Recombinant cDNAs representing the human γ- and β-globin mRNAs [Wilson et al. 1979] were also used to determine the temperature optimum for the multiplex PCR analysis [Fig. 5B, detailed below].

RNA was isolated from embryonic yolk sacs (day 9.5), from fetal livers (day 14.5) or from anemic adult spleens by acid guanidinium–isoctoanuric acid cesium chloride–extraction method (Chomczynski and Sacchi 1987). To generate anemic adult animals, mice were injected with N-acetyl-phenylhydrazine (40 mg/gram body weight at 0, 8, and 24 hr). RNA was isolated from the spleens of these anemic animals 6 days after the first injection.

cDNA synthesis was performed essentially as described [Leonard et al. 1993]. Total RNA [1 μg] from the various erythroid cell stages was resuspended in 7 μl of DEPC-treated H2O, heated for 15 min at 65°C and quenched on ice for 5 min. Reverse transcription mix [10 μl, containing 10 mM KCl, 20 mM Tris-HCl (pH 8.4), 10 mM DTT, 5 mM MgCl2, 1 mM each dNTP, and 1 unit of RNAsin, Promega], as well as 1 μl of d[N1] primer [at 10 A260 U/ml] and 2 μl of M-MuLV reverse transcriptase [200 U/ml, BRL] were added to the RNA solution, which was then incubated at room temperature for 15 min. After further incubation for 90 min at 42°C, the samples were either immediately subjected to PCR or stored at –20°C.

PCR reactions were each 50-μl final volumes containing: 1 μl [5%] of the cDNA reaction, 10 mM Tris-HCl (pH 8.4), 10 mM KCl, 2.5 mM MgCl2, 50 μM each dNTP, 2 units of Vent polymerase (Exonuclease NEB), 2 μCi [α-32P]dCTP, and 0.4 pmole each of the four sets of globin primer pairs (Table 1). Each PCR reaction was initiated by a denaturation step for 2 min at 94°C, 1 min at 58°C (for primer annealing), extension for 1 min at 72°C, finally followed by a denaturation step for 1 min at 94°C after each cycle. Titration of cycle number was performed on each cDNA sample to verify that the amplification of all globin templates was within the linear range. An aliquot (2–4 μl) of each PCR reaction was electrophoresed on 8% neutral polyacylamide gels at 160 V, dried, and then subjected to autoradiography. Individual band intensities were quantified (Table 2) on a Molecular Dynamics PhosphorImager (Foley and Engel 1992). Multiple samples representing each stage of erythropoiesis in each transgenic line were analyzed in this manner with different initial RNA preparations as well as different cDNA synthesis reactions.

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