Regulation of Human Globin Gene Switching

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The β-globin multigene cluster contains five developmentally regulated genes in the order 5′-ϵ,γ_G,γ_A,δ,β-3′. In the early stages of human development, the embryonic yolk sac is the hematopoietic tissue and expresses the ϵ-globin gene. This is followed by a switch to the γ-globin gene in the fetal liver and the δ- and β-globin genes in adult bone marrow (for review, see Collins and Weissman 1984).

The entire β-like gene locus has been sequenced, and a large number of structural defects, collectively known as the β-thalassemias, have been documented in and around the β-globin gene (for review, see Collins and Weissman 1984; Poncz et al. 1988). In a related condition, hereditary persistence of fetal hemoglobin (HPFH), γ-globin gene expression and hence HbF (fetal hemoglobin) production persist into adult life. These diseases are not only clinically important, but they also provide natural models for the study of transcriptional regulation during development (see below). The entire locus is regulated by the locus control region (LCR), which first became apparent from the study of a Dutch thalassemia (Kioussis et al. 1983) and later a Hispanic thalassemia (Driscoll et al. 1989). The LCR is characterized by a set of developmentally stable, hypersensitive sites, 5′ HS1, 2, 3, and 4, and is distributed over approximately 15 kb located upstream of the ϵ-globin gene (Fig. 1) (Tuan et al. 1985; Forrester et al. 1987; Grosveld et al. 1987). The deletion of the LCR in these thalassemias showed not only that it is required for the expression of the β-like globin genes, but also that its presence affects chromatin structure over a distance of at least 150 kb (Forrester et al. 1990). The functional significance of the LCR became clear when it was used to drive the expression of the human globin genes in transgenic mice.

When the regulation of the individual globin genes in the absence of the LCR was studied in transgenic mice, it showed that the γ- and β-globin genes were expressed in a developmentally specific manner, albeit at low levels and dependent on the position of integration in the host genome (Magram et al. 1985; Townes et al. 1985; Chada et al. 1986; Kollias et al. 1986). When the β-globin gene was studied in the context of the complete LCR region in transgenic mice, a completely different pattern was observed: The gene was expressed at levels comparable to that of the endogenous mouse globin genes, and it became independent of the position of integration in the mouse genome. In addition, the level of expression was dependent on the number of copies of the introduced transgene (Grosveld et al. 1987).

Structure and LCR Function

Position independence and copy number dependence can theoretically be explained by at least two independent mechanisms: Either positive activation by the LCR is always achieved and can result in very high levels of expression which obscures small position effects that may still be present in the background, or (and) the region contains elements that insulate it from neighboring regions, providing a locus border element (LBE), as has been described in *Drosophila* (Kellum and Schedl 1991). However, preliminary experiments indicate that this is not the case and that such a border may be located further upstream (S. Pruzina et al., unpubl.). In addition, small fragments containing any one of the hypersensitive sites can also give rise to copy-number-dependent expression independent of the site of integration, albeit at lower levels than the full LCR (Forrester et al. 1989; Ryan et al. 1989; Talbot et al. 1989; Fraser et al. 1990). This makes it highly unlikely that insulating sequences form the basis of the effect. It therefore appears that the position-independence we observe is (at least in part) due to the fact that the LCR achieves activation of transcription in some dominant fashion, perhaps by creating very stable interactions between the LCR and the genes (see below). Consequently, positive position effects would only be present as part of the background and would only become apparent in situations where the linked gene is suppressed (Dillon and Grosveld 1991; see below for discussion).

The main activity of the LCR is associated with HS2, 3, and 4 (Forrester et al. 1989; Ryan et al. 1989; Tuan et al. 1989; Collis et al. 1990; Fraser et al. 1990, 1993; Lowrey et al. 1992), in agreement with the deletion observed in a Hispanic $\gamma\beta$ thalassemia (Fig. 1) (Driscoll et al. 1989). Each of the sites contains elements capable of activating a linked transgene, independent of the site of integration. A number of erythroid-

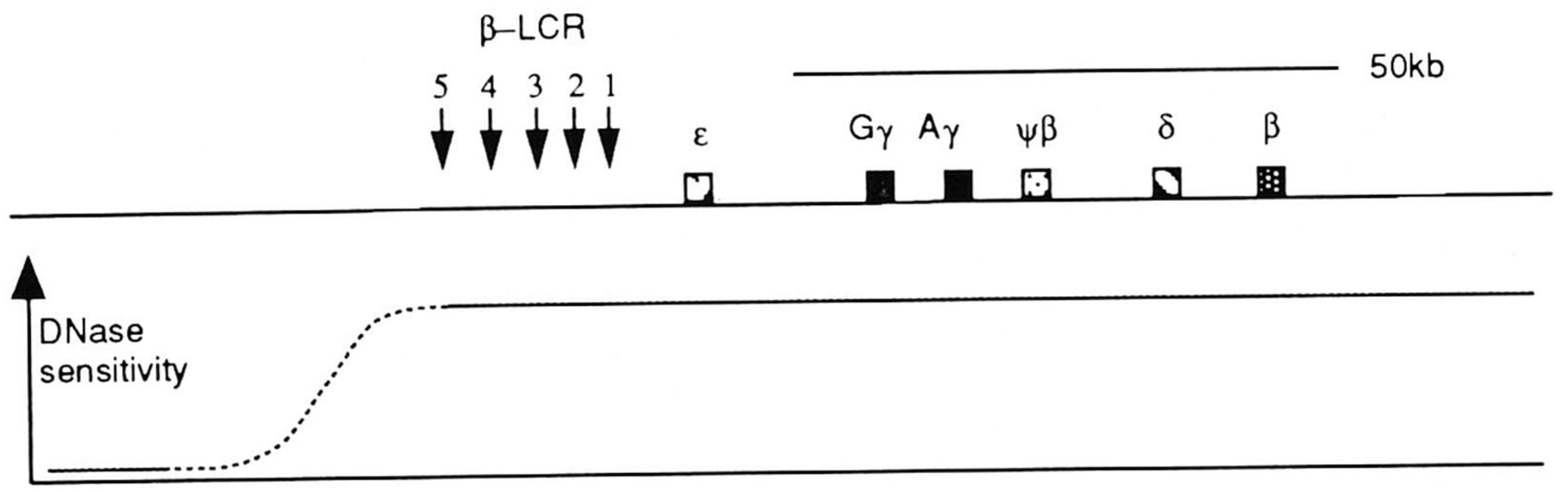

Figure 1. The human β locus and its expression during human development. The human β-globin locus is shown on top. The LCR is represented by five arrows showing the hypersensitive sites. The genes are represented by boxes. The middle panel shows the general DNase sensitivity in erythroid cells (dotted line indicates the as-yet-unknown transition). The bottom panel shows the developmental expression pattern of the genes.

specific and ubiquitously expressed protein-binding sites have been mapped to these fragments. Several of these specific binding sites are present in all three of the active HS regions (Fig. 2) (Philipsen et al. 1990; Talbot et al. 1990; Pruzina et al. 1991). Very striking are closely spaced binding sites for the erythroid- and megakaryocytic-specific factor GATA1 (Martin and Orkin 1990; Romeo et al. 1990), which has been shown to be essential for erythroid development (Pevny et al. 1991). Deletion of GATA1-binding sites in the promoter prevents erythroid-specific induction of the β-globin gene (de Boer et al. 1988), and the protein has been shown to have transcriptional activation properties (Martin and Orkin 1990). Our recent experiments with HS3 in transgenic mice indicate that the minimal combination required for position-independent expression is in actual fact not the presence of two closely spaced GATA1 sites, but two GATA1 sites immediately flanking a G-rich motif that binds Sp1 in vitro (Fig. 2) (Philipsen et al. 1993). This is in perfect agreement with the binding sites that appear to be occupied in vivo (Strauss and Orkin 1992). Sp1 may therefore play an important role in erythroid-specific transcriptional activation. It would be an attractive candidate because it has been shown to be able to loop DNA (Li et al. 1991; Mastrangelo et al. 1991; Su et al. 1991), a process thought to be central to gene activation in general (for review, see Ptashne 1988) and to the interactions between the LCR and the globin genes in particular (Hanscombe et al. 1991).

A different result is obtained from the detailed functional analysis of HS2. Transient transfection experiments show that classic enhancer activity is associated with this site only (Tuan et al. 1989; Ney et al. 1990), and not with the others. Dissection of the HS2 showed that a number of proteins are bound to the core fragment (Fig. 2) (Talbot et al. 1990). Attention has been focused on a double consensus sequence for the Jun/Fos family of DNA-binding proteins which appeared crucial for HS2 activity (Ney et al. 1990; Sorrentino et al. 1990; Talbot et al. 1990). Two NF-E2 molecules (Mignotte et al. 1989a,b) and at least one

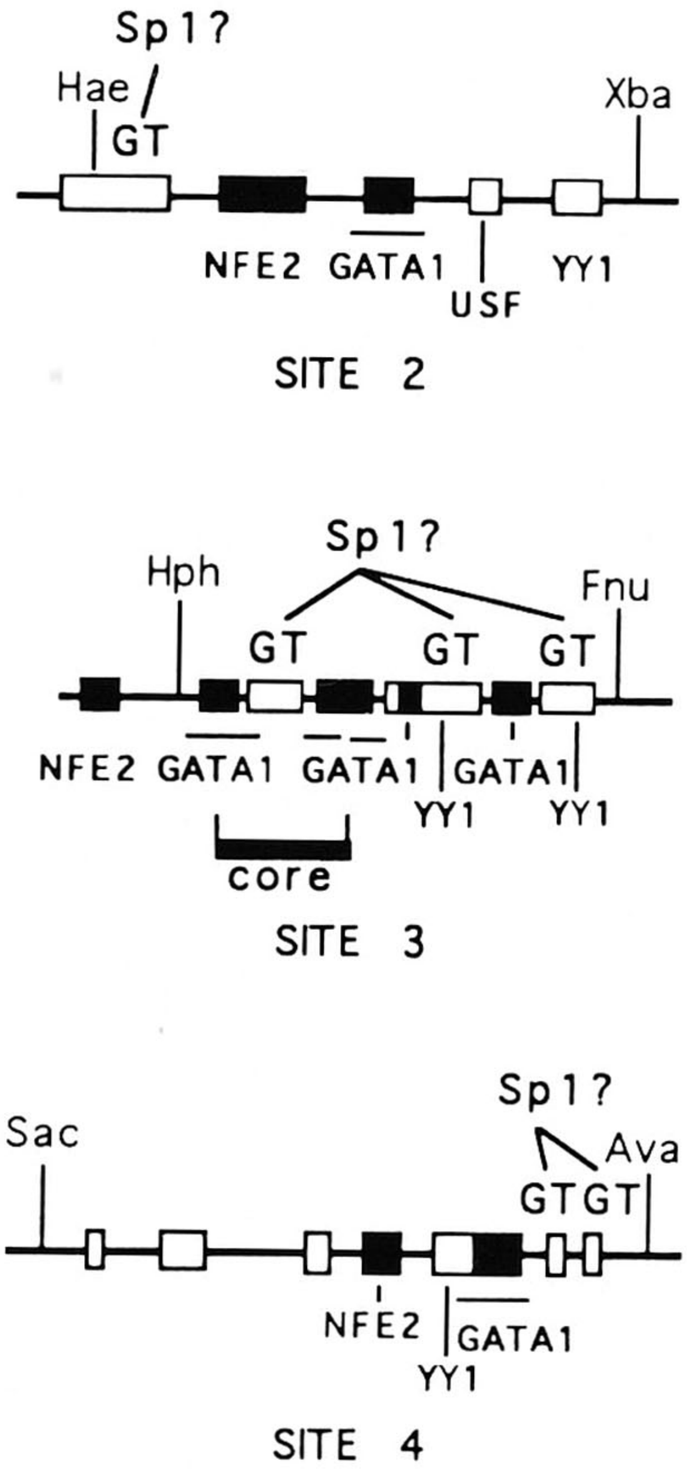

Figure 2. Summary of the protein-binding sites HS2, 3, and 4 in the human β-globin LCR. Black boxes indicate erythroid-specific factor binding, open boxes the binding of ubiquitously expressed factors. The minimal functional core of HS3 is indicated by a thick black line (Philipsen et al. 1993).

other protein binding at two nonequivalent sites are involved in HS2 function (Talbot and Grosveld 1991). However, the presence of this double NF-E2 sequence alone is insufficient to provide high levels of expression (Talbot et al. 1990), and when the Jun/Fos-binding site is removed from the 300-bp core fragment, HS2 retains the ability to activate a linked β-globin gene in a copy-number-dependent fashion, albeit at low levels (Talbot and Grosveld 1991). We therefore conclude that although the 5′ HS2 NF-E2 region has strong enhancer activity (Ney et al. 1990) and is important in obtaining high levels of globin-gene expression, it does not appear to be necessary for position-independent globin-gene activation.

Combinations of deletions of the GATA1 and the ubiquitous factor binding sites of HS2 (USF and YY1; Talbot and Grosveld 1991; Ellis et al. 1993) showed that it was impossible to find any particular binding site combination that was crucial for LCR activity (Ellis et al. 1993). However, when using these minimal HS2 fragments, it became apparent that LCR activity was only obtained when multiple copies (Ellis et al. 1993), but not single copies, of the transgene constructs had integrated (Fig. 3). This suggests that LCR activity was only restored when these minimal sites could interact with each other, presumably by loop formation. This in turn could explain how the complete LCR might work in vivo; namely, by the formation of a loop complex between the LCR elements to form a single larger complex that would be capable of interacting with any of the genes. Such a mechanism would also explain why the genes compete with each other for interaction with the LCR, an observation which is difficult to reconcile

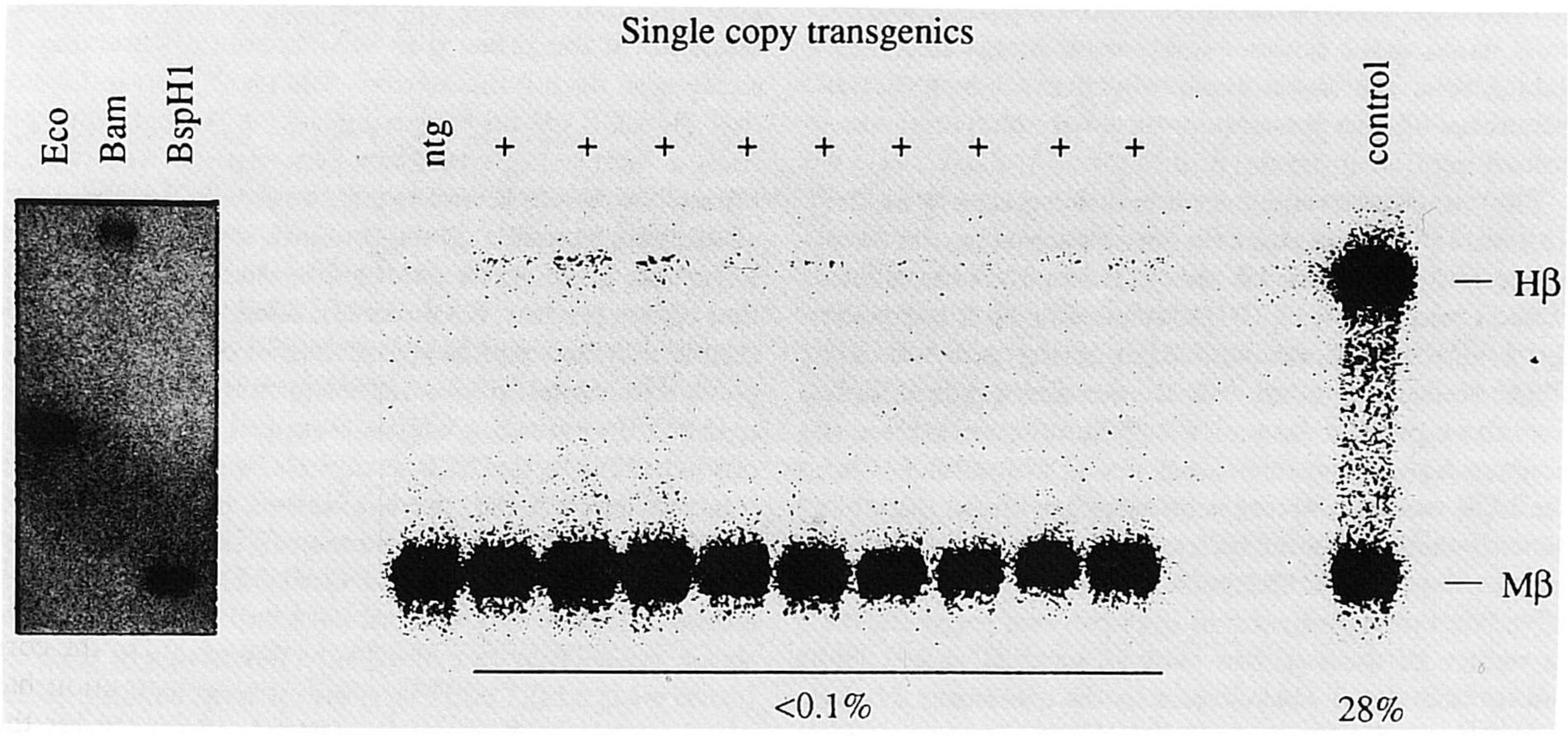

Figure 3. S1 nuclease protection and Southern blot analysis of a single-copy HS3 β-globin transgene. The Southern blot on the left shows single end fragments for the transgene insert indicating that only a single copy has been inserted into the genome. The S1 analysis on the right shows the result of a transgenic litter from the same line as in the Southern blot. The human β-globin gene is expressed at levels $<0.1\%$ when compared to the mouse βmaj gene. The lane on the right shows a control mouse expressing at 28%.

with a mechanism involving different genes interacting with different elements of the LCR at the same time (see below).

Developmental Regulation of the β-like Globin Genes in Transgenic Mice

When the developmental regulation of the individual globin genes in the absence of the LCR was studied in transgenic mice, the ε gene was inactive (Shih et al. 1990), but the γ- and β-globin genes were expressed in a developmentally specific manner, albeit at low levels and dependent on the position of integration in the host genome (Magram et al. 1985; Townes et al. 1985; Chada et al. 1986; Kollias et al. 1986). When the genes were studied in the context of the complete LCR region in transgenic mice, it was found that the ε gene was expressed at the embryonic stage only (P. Fraser, unpubl.), in agreement with the data published for the ε gene linked to part of the LCR (Raich et al. 1990; Shih et al. 1990). The γ-globin gene, like its murine structural homolog the βh1 gene, is expressed in the embryonic yolk sac. However, in contrast to βh1, the γ gene is expressed in the early fetal liver and is only silenced after day 16 of development. It is not expressed in the adult (Dillon and Grosveld 1991). Although it has not been directly tested in transgenic mice, an individual β gene linked to the LCR is expressed prematurely in differentiating embryonic stem cells, although not at maximal levels. Like the murine β gene, the human β gene is expressed at high levels in the fetal liver and adult (Grosveld et al. 1987; Behringer et al. 1990; Enver et al. 1990). This suggests that a large part (but not all) of the developmental regulation of the globin genes is specified by the regions immediately flanking the genes. However, it is also clear that the LCR is not developmentally neutral and that it influences the expression pattern of the genes (see below).

The simplest model based on these data was the proposal that stage-specific suppressors are the very important regulators of the individual globin genes (Dillon and Grosveld 1991). According to this model, the LCR can interact with each of the genes at any stage of development, but it interacts preferentially with the ε gene in the yolk sac. When the interaction is negated by stage-specific suppressors in the fetal liver, the LCR would interact preferentially with the γ-globin genes, which in turn are suppressed in the adult. At least two candidate binding sites for suppressor factors have been proposed, one in the upstream region of the promoter of the ε-globin gene (Cao et al. 1989) and one at the distal CAAT box in the promoter of the γ-globin genes (Mantovani et al. 1989). However, this model does not explain how the later-expressing γ and β genes are kept silent at the early stages of human erythroid development. Evidence from natural mutations and transgenic mouse experiments with combinations of genes suggest that competition of the genes for the LCR plays an important role in this process, in particular for the γ and β genes (see below).

Developmental Specificity of the LCR

Even though the LCR contains several hypersensitive elements with LCR activity that are distributed over 15 kb of DNA, the different globin genes appear to compete with each other for the interaction with the LCR (see below). This could be explained if the individual HS elements would interact with each other (see above for HS2) to form a larger complex, which in turn would interact with the genes. Individual HS sites in the larger complex could have a different developmental or gene specificity and the genes could be competing for a specific element in the larger complex at a given developmental stage. We have therefore tested whether the individual HS regions, which make up the complete LCR, interact differently with the genes and whether each HS has a different developmental specificity. Each HS region was linked to a set of human γ- and β-globin genes and introduced into transgenic mice, and the expression pattern of the different human globin genes during development was determined (Fig. 4) (Fraser et al. 1993). The results showed that the individual sites behave differently with the γ- or β-globin gene, which has a number of implications for the mechanism of LCR function and showed that the LCR is not developmentally neutral, but that it influences the expression pattern of the genes. Most striking was the result that HS3 is the only part of the LCR that is capable of directing the expression of the γ-globin gene in the fetal liver. This result suggests that this site is the part of the LCR for which the γ and β genes compete during the fetal stage of development. Since all of the other sites are capable of directing β expression during this period, this result also indicates that the LCR elements may interact with each other to form a larger single complex (see also above), which interacts with the genes. Interestingly, HS4 is the most active site in adult β expression, suggesting that it forms the most stable interaction with the β gene at that stage (Fraser et al. 1992). This combination of results indicates that a switch from HS3 to HS4 takes place at the fetal/adult switch and that the γ promoter is capable of forming stable complexes with HS3 but not with HS4 in the LCR complex. From these results, it is not clear whether the HS regions are gene-specific, but the HS3 and HS4 difference offers an opportunity to test this possibility. The Greek HPFH γ gene is expressed effectively in adult mice and able to compete the β gene (Berry et al. 1992). When this γ gene is tested with a HS3 or HS4 alone, it may become clear whether the single point mutation leads to a positive change in the ability of γ to interact with HS4 in the adult or whether the γ gene would still interact with HS3. The latter would suggest gene specificity rather than developmental specificity to be associated with the individual HS of the LCR.

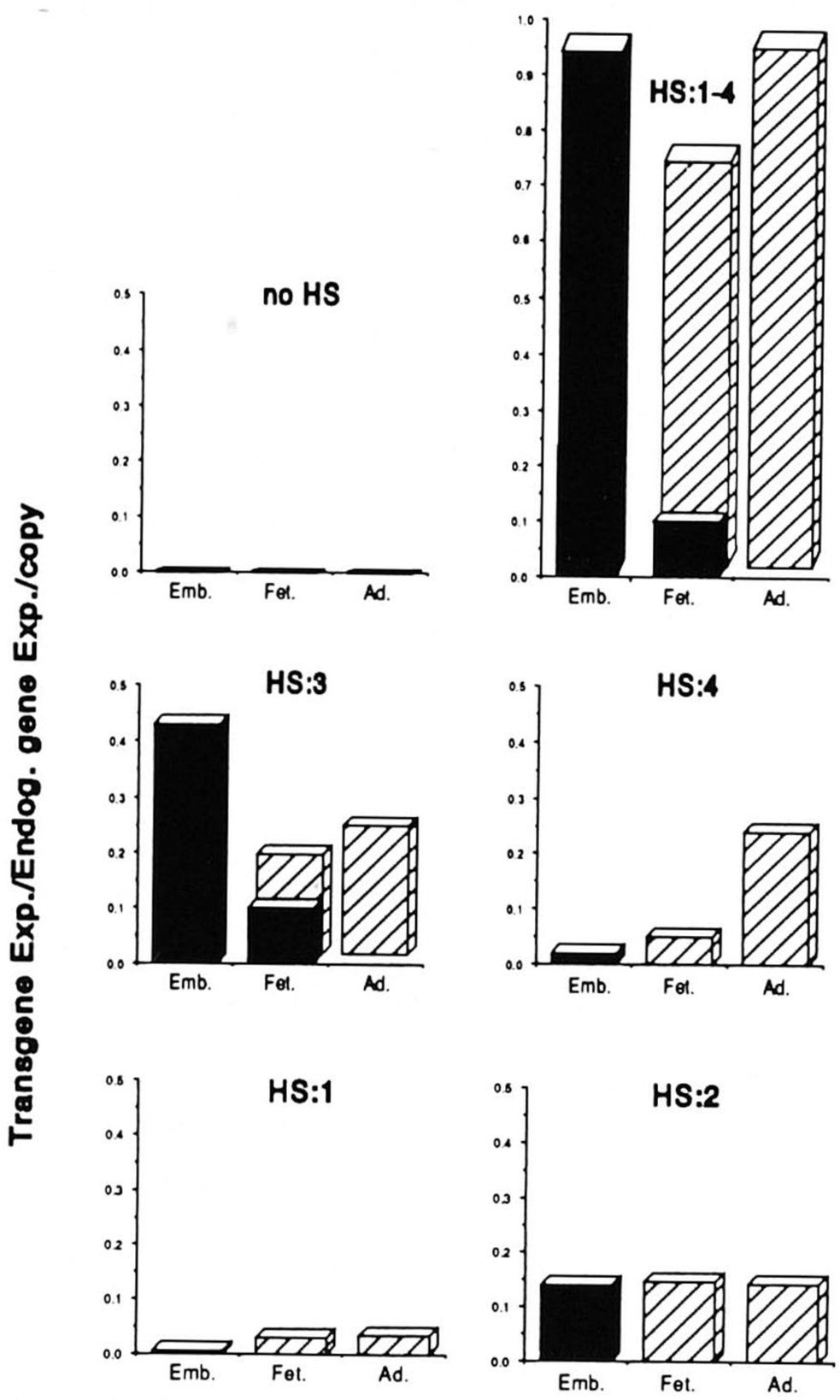

Figure 4. Histogram of the expression level of the human γ and β transgenes at different times of development when coupled to all or individual HS. The diagram at the top shows the cosmid construct inserted into the various transgenic mice. The expression level of the γ gene is shown in the black boxes, that of the β gene in hatched boxes.

Competition of the Globin Genes in Transgenic Mice

Competition between the genes became apparent when combinations of genes were used in transgenic mouse experiments. The premature expression of the β gene when it was linked to the LCR and expressed in transgenic mice could be abrogated by competition for the LCR with another globin gene, e.g., γ or α (Behringer et al. 1990; Enver et al. 1990; Hanscombe et al. 1991). This competition appears to operate in a polar fashion, providing an advantage to a gene that is proximal to the LCR and a disadvantage to a gene that is distal to the LCR (Hanscombe et al. 1991). These results were subsequently confirmed by the expression of an HPFH γ and β gene (Berry et al. 1992).

Why would the expression of the upstream genes prevent expression of those located downstream but not vice versa? Existing competition models are based on the idea that the high-level expression of the genes in the β-globin locus is potentiated by direct interaction of each gene with the LCR. There is strong evidence that enhancers work through such interactions (Muller et al. 1989; Bickel and Pirotta 1990 and references therein). The polar competition that appears to operate in the locus would be explained if the LCR had an intrinsic preference for interaction with a more proximally located gene. A mechanism by which the LCR searches along the chromosome and interacts with the first gene it encounters would require energy and very complex machinery. A second possibility would be the formation of chromatin structures that would bring gene and LCR together, but these would require specific and developmentally regulated functions for the spacer regions in the locus. A comparison of the phenotypes of different deletions in the locus (for review, see Poncz et al. 1988), together with the transgenic mouse data, argues against such a role. An alternative and much simpler possibility is that the determining parameter is the relative frequency of contact between genes and LCR (Hanscombe et al. 1991). Where two genes each retain a significant capacity to form stable interactions with the LCR, a difference in their frequency of contact with the LCR would dramatically affect competition between them. During the fetal stage, the interaction of the γ genes with the LCR would be much more frequent than that of the β gene and, owing to the action of stage-specific factors, would also be stronger. The combination of these two parameters acting together would allow the γ genes to compete out β expression. In the adult stage, although the β gene would now have the stronger interaction with the LCR, its much lower frequency of contact would make it difficult for it to compete out a γ gene that retained a significant capability to form such interactions. It is possible that this effect could be achieved by a very strong β interaction, but it seems unlikely that this would have evolved specifically to silence γ expression. The more likely alternative mechanism would be one of promoter-mediated silencing of the early genes by stage-specific factors; the transgenic mouse data indicate that this is the one which has in fact evolved. Such a competition mechanism may have evolved to ensure that the switchover from γ- to β-globin expression is smooth and that the total output of the β gene locus is kept constant and in balance with the output from the α locus.

To test the looping/stochastic mechanism in the complete β-globin locus, we decided to use a marked β gene in different positions and measure its transcriptional output relative to the other genes during development. Our preliminary data (N. Dillon et al., unpubl.) indicate that a looping/stochastic mechanism may indeed be active when the genes are transcribed. It is not clear yet how the β-globin locus is first activated and whether this would also involve looping mechanisms. It could also be a scanning type mechanism such as a transcription event throughout the locus, which could open the locus by altering the structure of the chromatin to an active form. However, these types of mechanisms may also be distinguished by using marker genes in novel positions inside and outside the locus in transgenic mice.

The model described above would explain the fact that the order of the genes in the β-globin locus is largely conserved among mammals. However, it does not predict that the order of expression during development will follow that of the genes in the locus in all species. For example, a gene located proximal to a LCR could be subject to factor-mediated repression early in development and then be activated at a later stage. This activation would bring competition into play and would result in a switch from a distal to a proximal gene.

Our experiments with a complete human β-globin locus in transgenic mice indicate that such a mechanism may be operating in the early expression period in the mouse. The more distal γ-globin genes are expressed and at a higher level than the more proximal ε gene in the yolk sac, which cannot be explained by a simple order/competition model only (Strouboulis et al. 1992). Preliminary experiments indicate that the ε and γ genes compete with each other (P. Fraser et al., unpubl.). This shows that the ε and γ genes behave differently in humans and transgenic mice and suggests that an important change in at least one transcription factor (an ε activator and/or γ suppressor) must have taken place in the process of fetal recruitment of the γ genes in humans (Strouboulis et al. 1992). Another possible way to change the patterns of expression of the genes would be to change the position of the LCR. This would also alter the parameters affecting competition. In the chicken β-globin locus there is evidence that part of the LCR lies between the β and ε genes (Reitman et al. 1990).

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