Importance of globin gene order for correct developmental expression

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We have used transgenic mice to study the influence of position of the human globin genes relative to the locus control region (LCR) on their expression pattern during development. The LCR, which is located 5′ of the globin gene cluster, is normally required for the activation of all the genes. When the human β-globin gene is linked as a single gene to the LCR it is activated prematurely in the embryonic yolk sac. We show that the correct timing of β gene activation is restored when it is placed farther from the LCR than a competing human γ- or α-globin gene. Correct timing is not restored when β is the globin gene closest to the LCR. Similarly, the human γ-globin gene is silenced earlier when present farthest from the LCR. On the basis of this result, we propose a model of developmental gene control based on stage-specific elements immediately flanking the genes and on polarity in the locus. We suggest that the difference in relative distance to the LCR, which is a consequence of the ordered arrangement of the genes, results in nonreciprocal competition between the genes for activation by the LCR.

[*Key Words*: Human globin genes; locus control region; transgenic mice; developmental expression]

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The human β-like globin genes lie as a cluster, $\epsilon\gamma^G\gamma^A\delta\beta$, on chromosome 11 in a 5′ → 3′ order that reflects their developmental expression; ε is expressed in the embryonic yolk sac, γ in the fetal liver, and δ and β mainly in the adult bone marrow. Although there are some exceptions because of duplications and gene conversions (Collins and Weissman 1984; Margot et al. 1989), the order of the genes is conserved in mammals. A similar conservation of order has been observed in other multigene loci, for example, the order of segment-identity homeo-box genes has been conserved from fly to human (Gaunt and Singh 1990). The entire set of β-like globin genes is controlled by the locus control region (LCR), situated 5′ of the ε-globin gene and >50 kb away from the β-globin gene (Forrester et al. 1987; Grosveld et al. 1987). In the absence of the LCR, human γ- or β-globin transgenes are expressed at the same developmental stage as the murine β_{h1}- and β_{maj}-globin genes, that is, at the embryonic and fetal/adult stages, respectively (Magram et al. 1985; Townes et al. 1985; Chada et al. 1986; Kollias et al. 1986). The level of transgene expression is low, however, and it varies between mice because of integration position effects. Nevertheless, these experiments suggested that each gene and its immediate flanking region (~3 kb) contain sufficient information for developmentally correct expression. The addition of the LCR confers high-level expression and position independence on human globin transgenes in mice and cultured erythroid cells (Grosveld et al. 1987; Blom et al. 1989). Initial reports suggested that linkage of γ-globin gene alone to the LCR resulted in γ expression at all developmental stages (Enver et al. 1989, 1990; Behringer et al. 1990). Together with the observation of premature expression of an LCR-linked β-globin gene (Blom et al. 1989; Enver et al. 1989, 1990; Behringer et al. 1990; Lindenbaum and Grosveld 1990), this led to a proposal that γ- to β-globin switching is regulated by a reciprocal competition (Townes and Behringer 1990).

However, genetic data, particularly that for individuals with heterocellullar $\delta\beta^0$- and β^0- thalassemia, argue against the requirement of the β-globin gene for γ-globin gene silencing (Dillon et al. 1991). In addition, when the single γ-globin gene experiment was carried out on a number of transgenic lines carrying only one or two copies of the LCR–γ-globin gene construct, a different result was obtained. Although γ-globin gene expression persisted in the early fetal liver, it was silenced at adult stages, independent of the presence of the β-globin gene (Dillon and Grosveld 1991). Similar results have been obtained with the ε-globin gene (Lindenbaum and Grosveld 1990; Raich et al. 1990; Shih et al. 1990; Watt et al. 1991), supporting the notion that even in the presence of the LCR the ε- and γ-globin genes are suppressed at later stages of development in the absence of competition from other genes. This effect is mediated by the stage-specific sequences immediately flanking the genes and is independent of the presence of the β-globin gene, removing the basis of the argument for a reciprocal competition model.

In this paper we use a combination of the α-, γ-, and

β-globin genes to test competition in the globin locus. The results show that the developmental expression pattern of the γ- and β-globin genes is affected by their positions relative to the LCR. As a result, we propose a novel model of developmental regulation for the globin genes involving nonreciprocal competition. This competition is influenced by the sequences immediately flanking the genes and the positions of the genes relative to the LCR. The implication of gene order as an important parameter in developmental regulation could be important for our understanding of the developmental regulation of a number of other multigene loci.

Results

We decided to test the competition hypothesis by altering the order of the genes relative to the LCR and following expression during the early stages of development in transgenic mice. The human γ- and β-globin genes were linked to the small version of the globin LCR (Talbot et al. 1989) in two different orders, resulting in constructs μγβ and μβγ (Fig. 1). Each of these was injected into fertilized mouse eggs, and the level of human γ- and β-globin was measured by S1 nuclease protection analysis in transgenic embryos at 9.5 days of gestation or in fetal livers at 13.5 days (Fig. 2A). At these time points there is a clear difference between the expression levels of the mouse embryonic and fetal/adult genes (Fig. 2B), which were checked for each of the mice (not shown). DNA from each of the embryos and fetuses was also Southern blotted and shown to contain multiple copies of the transgene construct, as determined by the relative density of an internal restriction fragment compared with end fragments and the endogenous mouse Thy-1-gene. γ- and β-globin signals were quantitated by densitometry and expressed as the ratio Hβ/Hγ (Table 1). The result shows that the order of the genes alters their relative expression levels completely. When the β-globin gene is in the 5′ position (μβγ), it is expressed during the embryonic and fetal periods, whereas when it is present in the 3′ position (μγβ), it is barely detectable during the embryonic period. Expression of the γ-globin gene is also affected; expression is lower at 13.5 days when it is present in the 3′ position, rather than the 5′ position (μβγ vs. μγβ). Because of severe globin chain imbalance, it is difficult to obtain adult lines that pass on the transgenes. Thus, embryos were taken directly after injection for this experiment. For this reason, we cannot exclude the possibility that some of the animals are mosaic for the transgenes. As a consequence, only relative, not absolute, expression levels of the transgenes can be obtained from this experiment. Nevertheless, the results clearly show that the relative distance of the genes to the LCR influences their developmental expression pattern and that competition, if it occurs, would be dependent on gene order.

In an attempt to obtain germ-line transgenic mice and to see whether a different gene not normally linked to

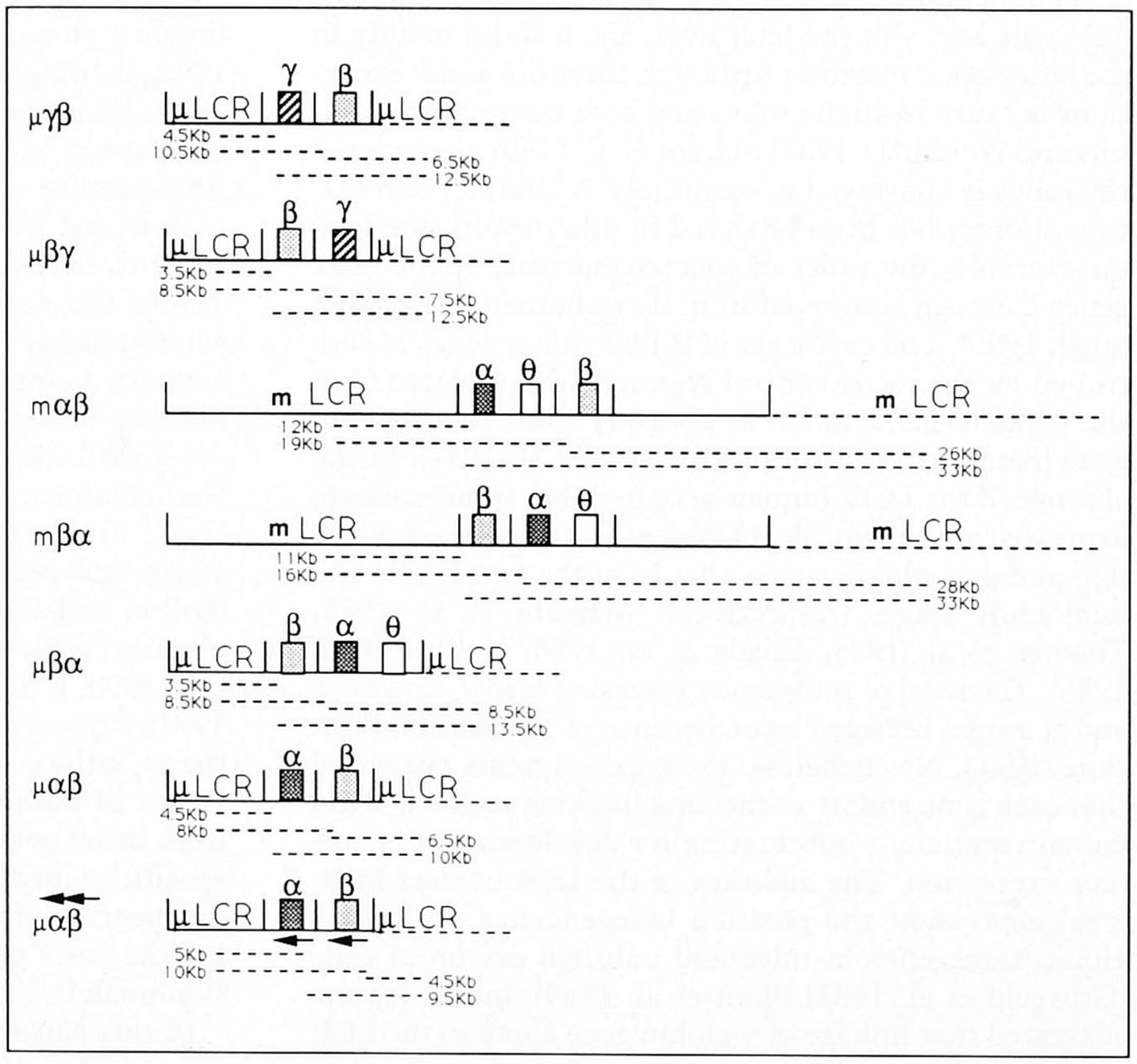

Figure 1. LCR minilocus (mLCR) and microlocus (μLCR) constructs. Plasmid vector sequences are not shown. Genes are represented as shaded boxes. All genes are in the same transcriptional orientation, 5′ → 3′, with respect to each other and the LCR, with the exception of the last construct, where the orientation of the genes has been reversed relative to the LCR (arrows). The broken LCR lines indicate the situation in multicopy animals, where copies of the construct are integrated in a tandem array. The distance from a promoter to a 5′ and 3′ LCR is indicated by broken lines below the constructs.

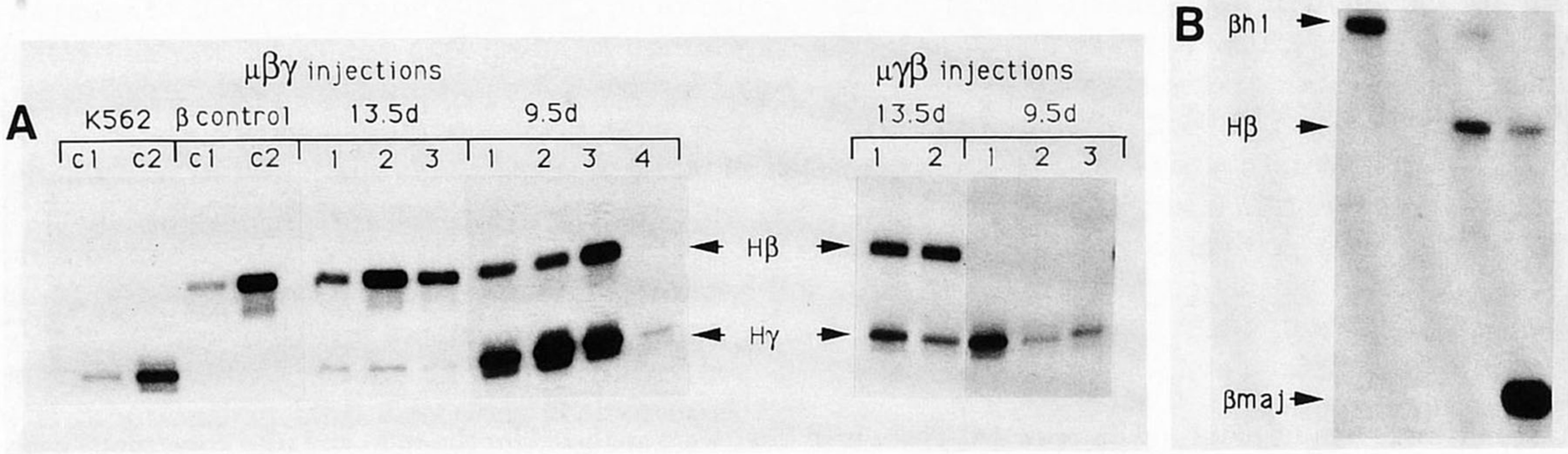

Figure 2. S1 nuclease analysis of μγβ and μβγ mice. Whole embryo RNA or fetal liver RNA was isolated 9.5 or 13.5 days after injection. Transgenic embryos were identified, and copy number and intactness of the construct were determined by Southern blot analysis as described previously (Hanscombe et al. 1989) (not shown). (*A*) Analysis of the μβγ and μγβ injections. Each lane represents an independent injection analyzed with human γ- and β-globin gene S1 probes. The 9.5-day sample of μβγ4 has very low signals due to a low transgene contribution to this mosaic embryo. The low signal in 9.5-day μγβ3, which runs close to the position expected for the β-protected fragment, is a partial S1 probe degradation product of slightly slower mobility. In separate control S1 protection experiments, mouse embryonic β_{h1} or fetal/adult β_{maj} RNA was analyzed. An example of this is shown (*B*) for 9.5-day μγβ1, 9.5-day μβγ5, and 13.5-day μγβ1. The controls were a no-RNA sample (control), a γ-globin gene RNA control from human K562 cells, and a β-globin gene RNA control from Hull cells.

the β-globin gene has the same effect, we tested the β-globin gene in combination with the human α-globin gene (Fig. 1). Two types of constructs were tested: one set in the (so-called) minilocus (mαβ and mβα), and the second set in the microlocus as for the γ- and β-globin genes described above (μαβ and μβα). The minilocus contains the complete 5′ LCR and the region 3′ of the β-globin gene containing the 3′-hypersensitive site 1 (Grosveld et al. 1987). In multicopy animals the β- and α-globin genes are flanked by LCR sequences. However, measuring from a point in the middle of the LCR between the two most active 5′-hypersensitive sites (HS2 and HS3; for review, see Dillon et al. 1991), the promoters of both the α- and β-globin genes are closer to the 5′ LCR than to the LCR brought in at the 3′ side by the next copy of the same construct (see Fig. 1). The human θ gene was included in the construct to prevent possible transcriptional interference by transcriptional readthrough from the α-globin gene (Proudfoot 1990). The θ-globin gene was not included in the μαβ construct, whereas in the converse μβα construct its presence is irrelevant, as only a single-copy breeding mouse line was obtained (Table 1). In all of the microlocus constructs the distances between the promoters of the genes and the middle of the LCR are reduced considerably (Fig. 1).

We obtained two breeding mouse lines for the mβα and one line for the μβα. S1 analyses show that the human β-globin gene is expressed during the embryonic stage at levels comparable to that of the human α-globin gene (Fig. 3A; Table 1). However, this level is only 10–30% of the mouse β_{h1} gene (not shown). The relative expression of the β-globin gene compared with human α- and mouse β_{maj}-globin genes increases after the switch to the fetal liver stage, demonstrating that part of the stage specificity of the genes is maintained. The human

Table 1. *Summary of expression data*

Construct	Ratio	Embryo		Fetus		Adult
μβγ	Hβ/Hγ	0.22		3.3		
		0.10		7.2		
		0.21		8.9		
		0.26				
μγβ	Hβ/Hγ	0.00		1.02		
		0.00		1.58		
		0.01				
mβα	Hβ/Hα (2)	0.43	→	6.65	→	6.65
	(5)	0.15	→	1.78	→	2.27
μβα	Hβ/Hα (1)	1.05	→	2.57	→	2.57
mαβ	Hβ/Hα	0.06		0.47		
		0.00		0.28		
		0.02		0.38		
		0.01				
μαβ	Hβ/Hα (20)	0.01	→	1.26		
	(4)	0.03				
◆◆ μαβ	Hβ/Hα	0.26		0.42		
		0.53		0.26		
		0.09				

Data are shown in Figs. 2 and 3. The different columns show the ratio of Hβ/Hγ or Hβ/Hα for each construct at different stages of development. The values were obtained from scanning autoradiographs or cutting out and counting S1 nuclease-protected bands, followed by correction for specific activities of the probes. Numbers in parentheses indicate copy numbers of the constructs; arrows indicate bred lines. The mβα1 line has two copies of the Hβ gene but only one copy of the Hα gene (data not shown).

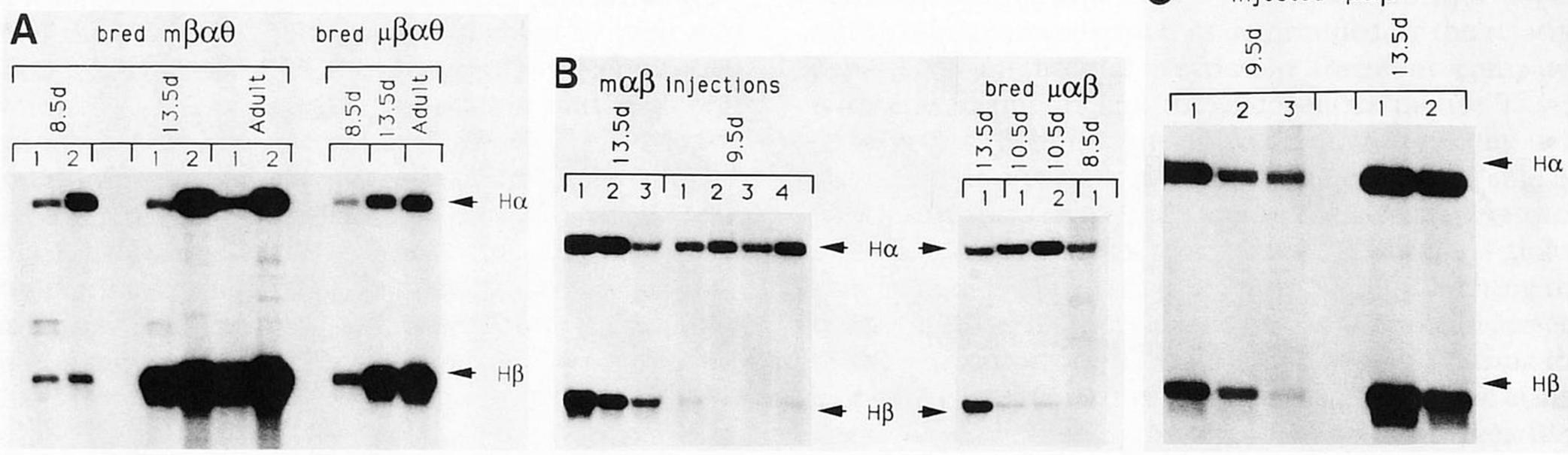

Figure 3. S1 nuclease analysis of αβ and βα mice. (*A*) Three bred lines were analyzed for the mβα and μβα constructs; each line was analyzed at 8.5-day (equivalent to 9.5-day stage in an analysis immediately after injection), 13.5-day, and adult stages. (*B*) Only two highly mosaic lines were obtained that passed on the transgene at low frequency for the μαβ construct. For μαβ1 we obtained 8.5-10.5-, and 13.5-day samples; for μαβ2, only a 10.5-day sample was obtained. No lines were obtained for the mαβ construct; the expression of this construct was therefore analyzed immediately after injection, with each lane representing a different injection. (*C*) Three 9.5-day and two 13.5-day transgenic embryos containing the injected μ locus with the inverted α- and β-globin genes analyzed.

α-globin gene, which would be expected to express at all stages of mouse development, is already expressed at the embryonic stage, even when it is present 3′ to the β-globin gene (Fig. 3A; Table 1). The fact that both α- and β-globin genes are expressed at reasonably similar levels at the embryonic and fetal stages makes it possible to obtain breeding lines for this type of construct (Hanscombe et al. 1989), although this is greatly facilitated by the inclusion of another α-globin gene (Greaves et al. 1990). When the gene order is reversed, the β-globin gene is expressed at very low levels at the embryonic stage and is activated to high levels only after the switch to the fetal stage (Fig. 3B; Table 1). This again leads to a chain imbalance in early development, particularly in multicopy animals, which makes it difficult to obtain fully transgenic breeding lines. The two lines (μαβ1 and μαβ2) that we obtained were both mosaic mice, which passed the transgene only at very low frequency. The mαβ construct did not result in any lines and was therefore only analyzed in embryonic yolk sacs and fetal liver samples taken directly after injection. Comparison of the results for the αβ and βα constructs agrees with those for the γβ and βγ constructs and shows a very similar pattern of changes in developmental regulation.

We can therefore draw a number of conclusions. The difference in transcriptional efficiency is unlikely to be caused by transcriptional interference (Proudfoot 1990), as this should not be influenced by developmental stage in the case of the α-globin gene, and inclusion of the θ-globin gene (which is itself only expressed at very low levels) (Hanscombe et al. 1989) does not change the result. Distance per se also does not appear to be very important, because changing the distance between the promoters and the LCR at its 5′ end or the LCR of the next copy does not substantially alter the basic result. Instead, it appears that the relative order of the genes to the LCR is an important parameter and that the LCR has a preference for the nearest promoter, particularly at the embryonic stage of development. This is reminiscent of the effects observed when multiple genes were linked to a single enhancer sequence in transient transfection experiments (deVillers et al. 1983; Wasylyk et al. 1983). To test this possibility and change the relative distance without adding or removing sequences, we inverted the orientation of the genes relative to that of the LCR (μ < α < β; Fig. 1). Because we have shown that the LCR is active in both orientations (Talbot et al. 1989) the net effect of this alteration is a change of distances in the multicopy constructs. In contrast to the μαβ construct, the β-globin gene promoter is now slightly closer to the two most active elements of the LCR (5′ HS2 and HS3), namely those of the next copy (Fig. 1). The result of the S1 analysis (Fig. 3C) of the expression of this construct in the embryonic yolk sac shows that the β-globin gene is now expressed at a higher level, relative to the α-globin gene, when compared with the result for μαβ, further indicating that the LCR has a preference for acting on the closest promoter at the embryonic stage.

Discussion

The possibility that competition between the genes of the β-globin locus for the activating function of the LCR might play a role in stage-specific regulation has been discussed extensively in the literature (Blom et al. 1989; Enver et al. 1990; Townes and Behringer 1990). Competition from a γ-globin gene appears to be required for early silencing of the β-globin gene, but recent data showing that the ε- and γ-globin genes are silenced autonomously in the adult suggests that the parameters affecting competition in the locus are more complex than was originally thought. The experiments described in this paper were designed to examine the effect of one

such parameter, namely, the differences in the relative distances of the genes from the LCR, which arise from the ordered organization of the genes extending in one direction away from the LCR. We have found that the relative distance of a gene from the LCR has a profound effect on its ability to affect the expression of another gene. A γ-globin (or α-globin) gene located proximal to the LCR can completely block embryonic expression of a distal β-globin gene but loses the ability to do so when it is located distally from the nearest LCR.

These results show that linkage of another gene is not sufficient to allow competition and argues strongly against a reciprocal competition model. Instead, it shows that competition is polar. Previous data have shown that the LCR is functional in both directions (Talbot et al. 1989). This creates an apparent contradiction, namely, how a nonpolar element (the LCR) has a polar effect on multiple genes. Current ideas on the mechanisms by which regulatory elements such as enhancers exert their effect across long stretches of intervening DNA involve direct physical interaction through the formation of loops (Muller et al. 1989; Bickel and Pirotta 1990). As well as opening up the chromatin, the LCR dramatically increases the expression of linked globin genes over distances of up to 50 kb (Kioussis et al. 1983; Forrester et al. 1990), and it seems reasonable to assume that this enhancing effect is the result of direct physical contact between LCR and gene promoter. We suggest that the key to understanding the polar effect that we have observed lies in the parameters that govern the interaction between the genes and the LCR. One of the parameters affecting interaction will be the frequency of contact, and this will be critical in a situation where two genes are competing for one regulatory region. A gene is likely to achieve competitive dominance when it interacts more frequently and more strongly with the LCR than other competing genes.

The frequency with which contact will occur between two points on a stretch of DNA will be determined by the effective volume to which they are restricted relative to one another. If the DNA is part of a large open loop that allows freedom of movement in three dimensions, this leads to a theoretical prediction that the effective volume will be that of a sphere whose radius is the distance between the two elements and will be proportional to the cube of that distance (Dillon et al. 1991). In an idealized model, doubling the distance between a gene and the LCR would increase the effective volume by eightfold, resulting in an eightfold reduction in the frequency of interaction and competitive ability. In all but one of the constructs used in this study, there is a large difference in the volumes within which the proximal and distal genes must interact with the nearest LCR (Fig. 1). The one exception is $\mu < \alpha < \beta$ where the genes are at a very similar distance from the upstream and downstream LCRs. This difference in effective volumes, and therefore in frequency of interaction, provides a potential explanation for the observation that a proximal location favors the ability of a gene to compete while a distal location prevents it from competing effectively. The proximal dominance of the γ-globin gene in the embryonic stage is caused by a combination of stronger interaction with the LCR and a greater frequency of contact. When the γ-globin gene is placed in the distal position, it has a much lower frequency of interaction compared with the proximal β-globin gene; this cancels out the effect of its stronger interaction and results in the expression of both genes. In the fetal liver, the β-globin gene has a stronger interaction with the LCR, and when it is in the proximal position it interacts more frequently and largely competes out the γ-globin gene. When the γ-globin gene is in the proximal position, both genes are expected to express in the fetal liver. Because this model proposes that effective volume and frequency of interaction have a major effect on competition for activation by the LCR, it predicts that in the $\mu < \alpha < \beta$ construct, both will be expressed in embryonic yolk sac, and this is what is actually observed. In these experiments, quantitation of relative expression levels and relating these back to calculated effective volumes is less than ideal for a number of reasons; for example, loop sizes vary because of random integration sites in the host genome, and in multicopy integrations the most distal gene in the most distal copy of an array contributes much less to the expression levels than a distal gene in the middle of an array.

Would this model also apply to the intact β-globin locus in vivo, where distances from the LCR to the ε-, γ-, and β-globin genes differ by a factor of 2 or more? Several lines of evidence in addition to our results indicate that this may be the case. First, the genetic data obtained for human β-thalassemia and nondeletion HPFH support the existence of polar competition in vivo (Dillon et al. 1991). Second, there is evidence to suggest that the human β-globin cluster is located on one large chromatin loop. In erythroid cells the entire β-gobin locus is part of a region of DNase sensitivity that is dependent on the presence of the LCR (Kioussis et al. 1983; Forrester et al. 1990) and extends at least 150 kb beyond the β-globin gene (Forrester et al. 1990). A number of matrix attachment regions (MAR) sites have been mapped within the locus (Jarman and Higgs 1988), and these could potentially prevent the formation of a large loop. However, our preliminary data indicate that none of these sites have the in vivo functional properties described for other locus border elements (LBE) (Stief et al. 1989; Kellum and Schedl 1991). Additional evidence for directional competition comes from the mouse β-globin locus. This gene cluster occurs in two forms, one containing a single adult β-globin gene and one containing two adult genes, the β_{maj}- and β_{min}-globin genes (Skow et al. 1983). The difference in relative distances from the LCR (Moon and Ley 1990) predicts that the proximal gene (β_{maj}) should have an advantage over the distal gene β_{min}. The data of Skow et al. (1983) not only shows that this is the case but also that deletion of the β_{maj}-globin gene in thalassemic mice results in an increase in the levels of β_{min}-globin gene expression in accordance with our model.

Thus, the results reported in this paper and the genetic evidence allow us to propose a model for the develop-

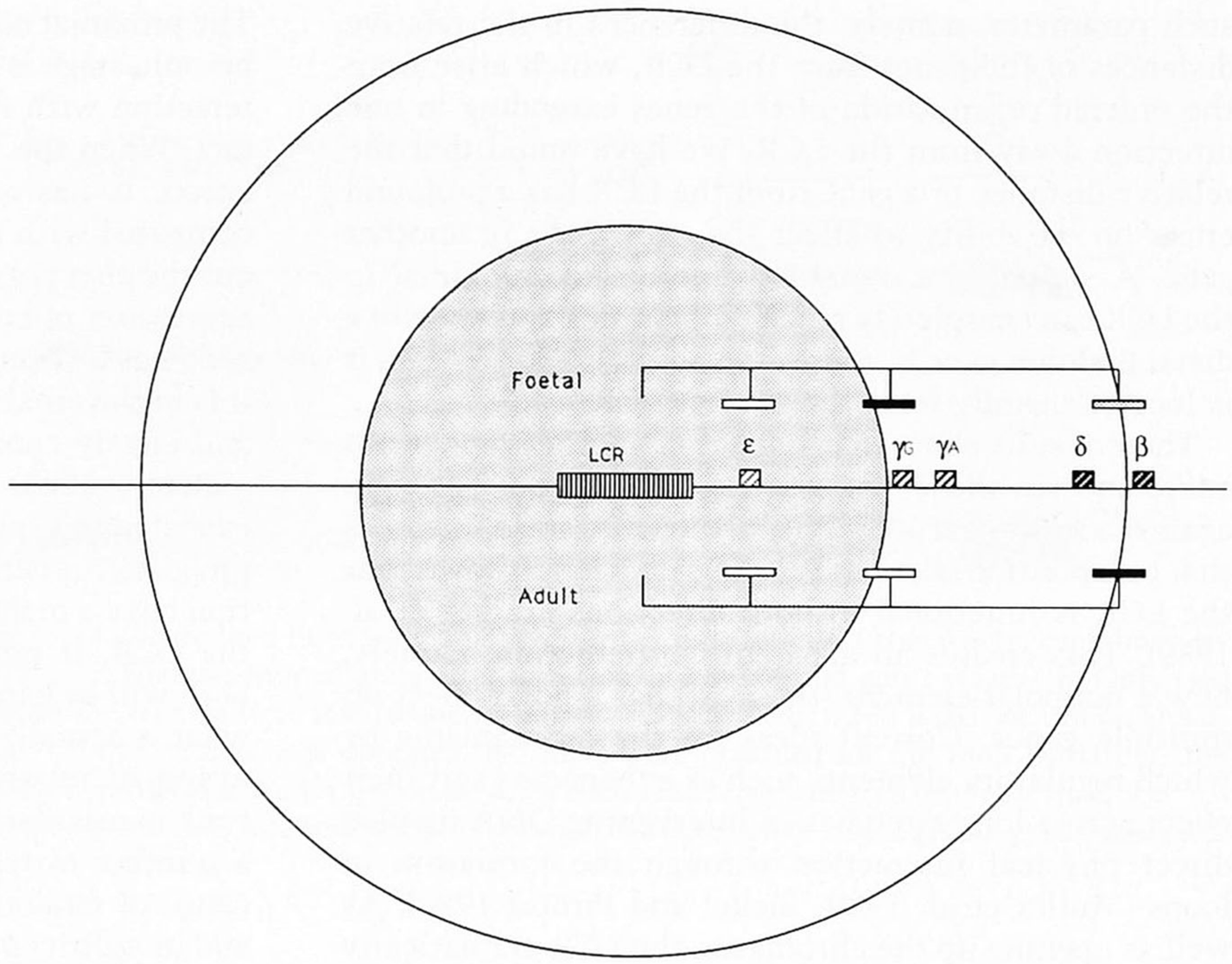

Figure 4. Model for regulation of γ- and β-globin gene expression during fetal and adult stages of development. The LCR, indicated by a lined box, lies 5′ of the entire β-like globin gene locus. β-Like globin genes are indicated by hatched boxes. Interaction between the LCR and individual genes is represented by bars: An open bar indicates no interaction; a shaded bar indicates a weak interaction; a solid bar indicates a strong interaction. The volumes in which the LCR–β- or LCR–γ-globin genes operate are represented by two-dimensional circles. As the distance between the LCR and the gene increases linearly, this volume increases by the cube of the distance.

mental regulation of the locus (Fig. 4), which is a combination of local control sequences and polar competition. The much greater frequency of interaction of the proximal genes with the LCR would allow them to compete effectively with the distal genes and prevent premature expression. However, the same effect would prevent the distal genes from competing effectively in the later stages. Instead, it would be necessary to evolve promoter-mediated silencing for the proximal genes, and this is observed for the ϵ- and γ-globin genes. The specification of the developmental profile by a combination of stage-specific repressors and gene order would explain why the order is largely conserved among mammals. However, it is important to note that our model does not predict that conservation of gene order will be absolute, because a change in the location of regulatory elements or in factor profile could have considerable effects. For example, factor-mediated repression might block expression of a proximal gene early in development. Release of that repression at a later stage would bring competition into play and result in a distal-to-proximal switch. A change in the position of the LCR would also be expected to have a considerable effect, and such a situation may well occur in the chicken globin locus, where competition was first proposed on the basis of transient transfection experiments (Choi and Engel 1988). Recent experiments in transgenic mice (Reitman et al. 1991) suggest that the chicken ϵ-globin gene is part of an ancient translocation that not only moved the ϵ-globin gene but also part of the LCR distal to the β-globin gene. The observation that relative distance from a shared regulatory element plays a role in developmental regulation could be important for a number of multigene loci, many of which have conserved their arrangement during evolution.

The model that we have put forward is based on certain assumptions. Detailed testing of the effect of large differences in relative distance between genes in the intact locus should permit assessment of the validity of these assumptions. In particular, we can test whether the genes and LCR are located on a single, large, open DNA loop that is free to move in solution or whether constraints such as physical attachment sites close to the genes are operating in vivo.

Materials and methods

Constructs

μγβ and βγ A 5.6-kb *Hin*dIII–*Eco*RI fragment of the human ^{A}γ-globin gene was cloned into the μ-locus vector 1417 (Talbot et al. 1989). A 5.0-kb *Bgl*II fragment of the human β-globin gene was cloned 3′ to γ for μγβ and a 4.1-kb *Sph*I–*Bgl*II fragment was cloned 5′ of γ for μβγ.

mαβ and mβα Human β-globin gene fragments were cloned into construct 1254 (Hanscombe et al. 1989) 5′ and 3′ to the α- and θ-globin genes as for μβγ and μγβ.

μβα and μαβ A 7.0-kb *Bgl*II–*Asp*718 fragment of the human α1- and θ-globin genes was cloned into the μ-locus vector 1417. A 4.1-kb *Sph*I–*Bgl*II fragment of the human β-globin gene was cloned 5′ to the α- and θ-globin genes as for μβγ. A 3.0-kb *Bgl*II–*Xba*I fragment of human α-globin was cloned into the μ-locus vector 1417. A 5.0-kb *Bgl*II fragment of the human β-globin gene was cloned 3′ of the α-globin gene as for μγβ.

Microinjection and identification of transgenic mice

Minilocus *Sal*I fragments and microlocus *Sst*II fragments were purified by electroelution from agarose gels. DNA was injected at a concentration of 1 μg/ml into the pronuclei of

(C57Bl × CBA)F_1 fertilized eggs (Hogan et al. 1986). Injected eggs were transferred on the same day to day-0.5 pseudopregnant F_1 foster mothers. Embryos were analyzed at 9.5 days and 13.5 days directly after injection. Live mice were obtained for mβα and μβα and were bred to nontransgenic F_1 mice to obtain 8.5-, 10.5-, and 13.5-day embryos. Embryos and pups were analyzed for the presence of the injected fragment by slot blot analysis of placental and tail DNA, respectively, with a 0.9-kb *Bam*HI–*Eco*RI fragment of the human β-globin gene as a probe.

Constructs were shown to be intact, and multicopy mice were identified by Southern blotting (Southern 1975); probes used were a 0.75-kb *Bst*EII fragment of the human α-globin gene, a 3.3-kb *Eco*RI fragment, and a 0.46-kb *Eco*RI–*Bgl*II fragment from the minilocus construct.

S1 nuclease analysis

Globin gene RNA was assayed by S1 nuclease protection (Berk and Sharp 1979). Probes were end-labeled with T4 polynucleotide kinase (β_{maj}-, β_{h1}-, and human β- and γ-globin genes) or reverse transcription (human α-globin gene), and specific activities were estimated by Cerenkov counting. Labeled probe (10 ng) was hybridized to total RNA in 20 μl of 40 mM PIPES (pH 6.4), 400 mM NaCl, 1 mM EDTA, and 80% (recrystalized) formamide overnight at 52°C. Samples were digested for 2 hr at 25°C with 150 units of S1 nuclease in 250 μl of 200 mM NaCl, 30 mM NaO acetate (pH 4.5), and 2 mM $ZnSO_4$. Protected fragments were separated on 6% urea–polyacrylamide gels.

Probes used were 525-bp *Acc*I fragment (human β-globin gene), 700-bp *Hin*dIII–*Nco*I fragment (β_{maj}-globin gene), 225-bp *Hin*F fragment (β_{h1}), 900-bp *Bam*HI fragment (human γ-globin gene), and 750-bp *Bst*EII fragment (human α-globin gene).

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