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, εγ4γ8β, 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 βh1 and βmai 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, 1990; Blom et al. 1990; Enver et al. 1989, 1990; Behringer et al. 1990; Lindenbaum and Grosveld 1990), this led to the 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 heterocellular βO- and βO- 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 (μLRC) 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.
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 (mβα 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 μββ construct, whereas in the converse μαβ 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 mβα. 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 βεβ gene [not shown]. The relative expression of the β-globin gene compared with human α- and mouse βεβ-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

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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 mβα line has two copies of the Hβ gene but only one copy of the Hα gene [data not shown].
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 µαβ 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 [Müller et al. 1989; Bickel and Pirrotta 1990].
As well as opening up the chromatin, the LCR dramatically
increases the expression of linked globin genes over dis-
tances of up to 50 kb [Klousis et al. 1983; Forrester et al.
1990], and it seems reasonable to assume that this en-
hancing effect is the result of direct physical contact be-
tween 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 be-
tween 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 dis-
tance 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 fre-
cency 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
distal genes must interact with the nearest LCR (Fig.
1). The one exception is μ < α < β where the genes are
at a very similar distance from the upstream and down-
stream LCRs. This difference in effective volumes, and
therefore in frequency of interaction, provides a poten-
tial explanation for the observation that a proximal lo-
cation 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 em-
byronic stage is caused by a combination of stronger in-
teraction with the LCR and a greater frequency of con-
tact. When the γ-globin gene is placed in the distal po-

tion, 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 interac-
tion have a major effect on competition for activation by
the LCR, it predicts that in the μ < α < β construct,
both will be expressed in embryonic yolk sac, and this is
what is actually observed. In these experiments, quanti-
tation 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 be-
cause of random integration sites in the host genome,
and in multicity 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 e-, 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 HPPH support the
existence of polar competition in vivo [Dillon et al.
1991]. Second, there is evidence to suggest that the hu-
mam β-globin cluster is located on one large chromatin
loop. In erythroid cells the entire β-globin locus is part of
a region of DNase sensitivity that is dependent on the
presence of the LCR [Klousis 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 attach-
ment regions (MAR) sites have been mapped within the
locus [Jarman and Higgs 1988], and these could poten-
tially 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 lo-
cus border elements [LBE] [Stieff et al. 1989; Kellum and
Schedl 1991]. Additional evidence for directional com-
petition 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 βmax and βmin globin genes [Skow et al. 1983]. The
difference in relative distances from the LCR (Moon and
Ley 1990) predicts that the proximal gene (βmax) should
have an advantage over the distal gene (βmin). The data of
Skow et al. (1983) not only shows that this is the case but
also that deletion of the βmax globin gene in thalassemic
mice results in an increase in the levels of βmin 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.

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 HindIII–EcoRI fragment of the human γ-globin gene was cloned into the μ-locus vector 1417 (Talbot et al. 1989). A 5.0-kb BglII fragment of the human β-globin gene was cloned 3’ to γ for μγβ and a 4.1-kb SphI–BglII 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 β-globin genes as for μβγ and μγβ.

μβα and μαβ A 7.0-kb BglII–Asp718 fragment of the human α1- and β-globin genes was cloned into the μ-locus vector 1417. A 4.1-kb SphI–BglII fragment of the human β-globin gene was cloned 5’ to the α- and β-globin genes as for μβγ. A 3.0-kb BglII–XbaI fragment of human α-globin was cloned into the μ-locus vector 1417. A 5.0-kb BglII 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 Sall fragments and microlocus SstII fragments were purified by electrophoresis from agarose gels. DNA was injected at a concentration of 1 μg/ml into the pronuclei of
Globin gene order and expression

[S1 nuclease analysis

Globin gene RNA was assayed by S1 nuclease protection (Berk and Sharp 1979). Probes were end-labeled with T4 polynucleotide kinase $\beta_{\text{major}}$, $\beta_{\text{h1}}$, and human $\beta$- and $\gamma$-globin genes or reverse transcription [human $\alpha$-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% (recrystallized) 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 532-bp AccI fragment [human $\beta$-globin gene], 700-bp HindIII–NcoI fragment $[\beta_{\text{major}}, \beta_{\text{globin}}$, globin gene], 225-bp Hind fragment $[\beta_{\text{h1}}]$, 900-bp BamHI fragment [human $\gamma$-globin gene], and 750-bp BstEII fragment [human $\alpha$-globin gene].

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