that the activating regions detected in our screens are exclusively of the acidic type. □

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Human γ-globin genes silenced independently of other genes in the β-globin locus

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ERYTHROPOIESIS during human development is characterized by switches in expression of β-like globin genes during the transition from the embryonic through fetal to adult stages. Activation and high-level expression of the genes is directed by the locus control region (LCR), located 5′ to the ε gene[1–3]. The location of the LCR and its role in directing high-level expression of the globin genes has led to the suggestion that competition from the β gene for interaction with the LCR has a major role in silencing the fetal γ genes during adult life[4,5]. We have now constructed lines of transgenic mice containing the human $A\gamma$ globin gene linked to the LCR. We observe high-level expression of the transgene in the embryonic stages but silencing of the gene in adult animals. We conclude that the γ gene is not deregulated by the presence of the LCR and that competition from the β gene is not required for silencing of the γ genes in adult life. The silencing is therefore likely to be mediated by stage-specific factors binding to sequences immediately flanking the genes.

Two constructs (Fig. 1) were injected into mouse oocytes. Mini-LCR γ contained the $A\gamma$ gene and its enhancer region cloned between the complete 5′ LCR sequence and the 3′ hypersensitive site-1 region[1], whereas $\Delta 3'\gamma$ lacked the 3′ site-1 region. Fifty-five founder animals were generated. To avoid artefacts from high copy numbers, end-blotting was used to exclude animals carrying more than two copies of the transgene. Six such lines were analysed as well as one line carrying three copies. Southern blots showed all integrated fragments were intact and arranged in a tandem repeat where more than one copy was present. Yolk sac RNA from 10.5-day embryos and blood RNA from adult animals was analysed for the presence of γ transcripts. Expression in embryonic yolk sac was compared with that of the mouse embryonic $\beta h1$ gene, whereas expression in adults was compared with the mouse β_{maj} gene (Figs 1 and 2*a*, and Table 1). In embryonic yolk sac the γ gene expressed on a per-copy basis at between one and two times the level of the $\beta h1$ gene. The relative level of embryonic γ transcription cannot be precisely quantitated as it was for the human adult β gene[1] because erythropoiesis at 10.5 days is in a dynamic state compared with the steady state of adult erythropoiesis. Two mouse β-like genes $\beta h1$ and εy are expressed in the yolk sac at 10.5 days. Between 10 and 12 days $\beta h1$ expression drops sharply, whereas εy expression increases before declining and disappearing from the circulation by 16.5 days[6]. The human γ-globin gene is normally expressed as a fetal gene so it is not possible to tell which of the mouse genes it should most closely follow in patterns of expression. It does not seem to follow either $\beta h1$ or εy completely as expression is still detectable in 16.5-day fetal liver at levels of up to 20% of β_{maj} per copy (Fig. 2*b*).

Analysis of blood from adult animals bred from each of the γ transgenic lines showed that three lines expressed the transgene at less than 0.5% of the expression observed for the mouse β_{maj} gene (Fig. 2*a* and Table 1). Three other lines expressed at around 1% of total β_{maj} or 2% when corrected for the copy number of the β_{maj} gene. The line that contained three copies of the construct ($\Delta 3'\gamma$ line 4) expressed the transgene at a level of 6% per copy. Although the six lines carrying one or two copies express the γ gene at very low levels, it is clear that there is considerable variation in this basal level (see Table 1). These differences are reproducible for each line (two animals per line) and are not related to copy number. For example, the single copy mini-LCR line 2 shows higher levels of expression than the two-copy mini-LCR line 1. We conclude that these differences are due to integration position effects. Analysis of these two lines during the fetal stages (Fig. 2*b*) shows that γ transcripts are present in fetal liver, but at a lower level than in embryonic yolk sac (15–50% of mouse β_{maj}), and expression declines during development to the very low adult level. The difference in γ expression between the two lines in adults is also present in the fetal stages. Our results show that a γ-globin gene linked to the LCR is silenced in transgenic mice without a linked β-globin gene. But it is also clear that the γ-gene is susceptible to positive position effects which affect the basal level and, in the three-copy

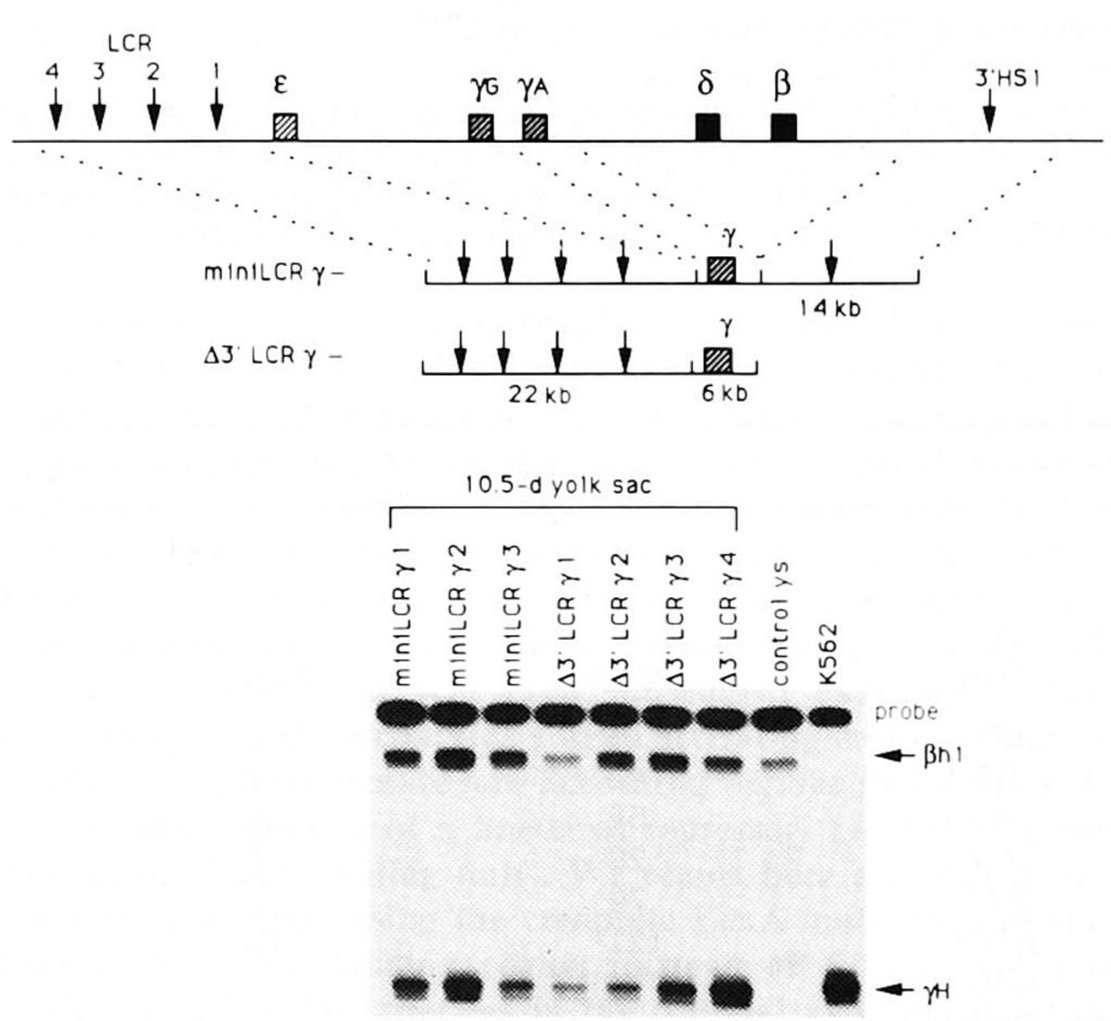

FIG. 1 S1-nuclease analysis of γ-globin transcripts in 10.5-d yolk sac from transgenic mouse lines containing the constructs shown. The probe mix contained two 5′ end-labelled fragments, a *Bst*NI fragment (190 bp) mapping to the $A\gamma$ cap site, and a mouse $\beta h1$ *Hin*fI fragment mapping to the 5′ end of exon 3. Probes were of equivalent specific activity.
METHODS. The mini-LCR γ construct was made by cloning a 5.6-kb fragment extending from −1,340 relative to the $A\gamma$ cap site to +4,308 into a polylinker between *Cla*I and *Kpn*I sites and then exchanging the fragment for the β globin *Cla*I–*Kpn*I fragment of the minilocus[1]. A *Sal*I fragment containing the entire insert and a *Sal*I–*Kpn*I fragment lacking the 3′ hypersensitive site 1 region were injected into mouse oocytes. Copy number of the resulting transgenic mouse lines was determined by Southern blot analysis of end fragments. The copy numbers of the lines are as follows: mini-LCR γ lines 2 and 3 and $\Delta 3'$LCR γ lines 1, 2 and 3, 1 copy; mini-LCR line 1, 2 copies; $\Delta 3'$ line 4, 3 copies. The new nomenclature and numbering used is that agreed at the Seventh Conference on Hemoglobin Switching, Airlie House, 1990.

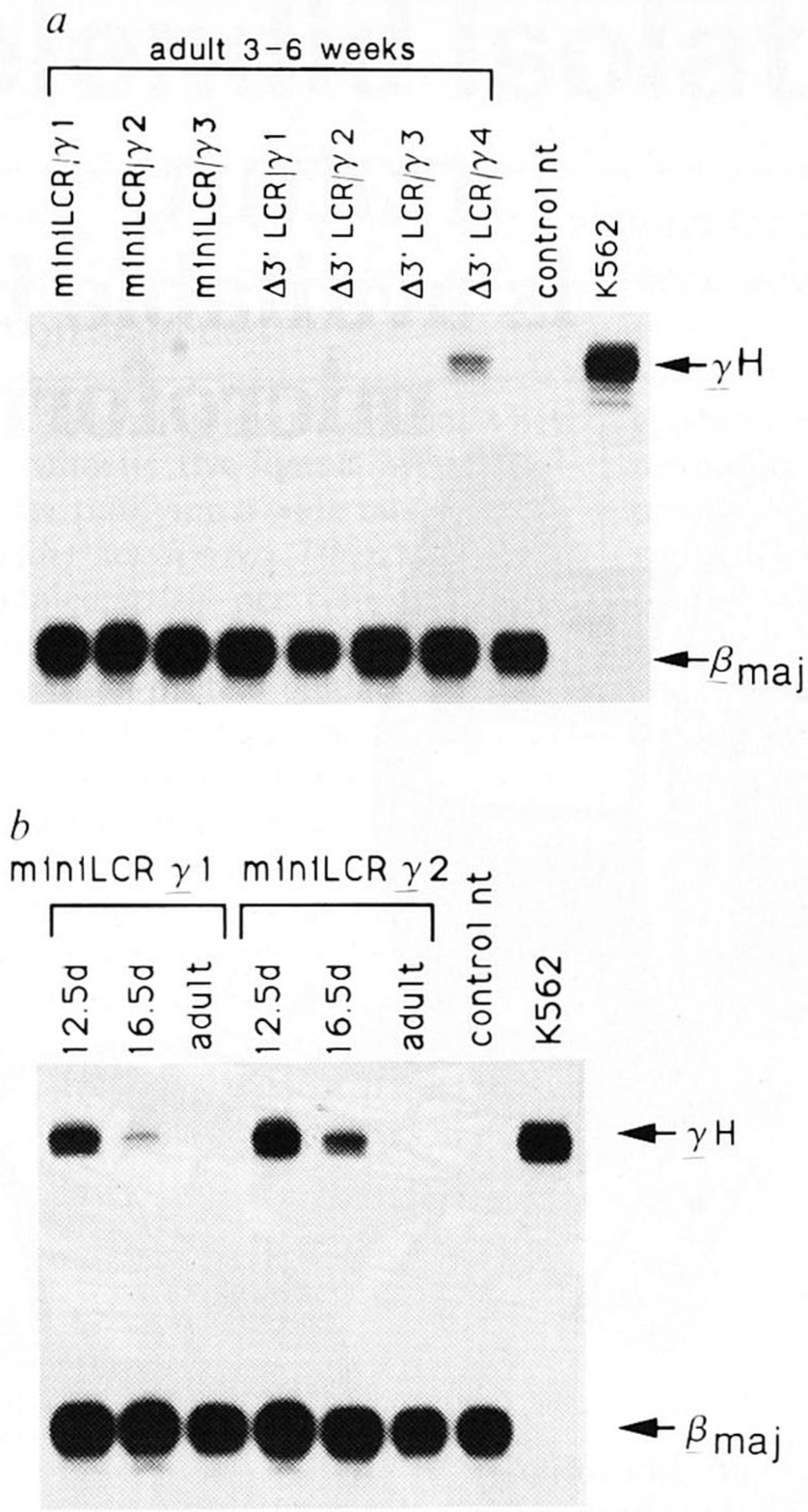

FIG. 2 S1-nuclease analysis of adult and fetal expression of γ-globin RNA in the transgenic lines described in Fig. 1. The same γ probe was used together with a 5′ end-labelled *Nco*I–*Hin*dIII fragment from the mouse β_{maj} gene (700 bp) which maps the 5′ boundary of exon 2. *a*, Analysis of blood RNA from adults aged 3–6 weeks bred from the same lines as in Fig. 1. *b*, Analysis of RNA from fetal liver (12.5 and 16.5 d gestation) and adult blood from two mini-LCR γ lines.

line, give rise to a significant level of adult expression. It should be noted that these position effects affect the silencing of the gene which is likely to be a promoter-mediated function. It has been suggested that the enhancer-like sequences located at the breakpoints in several of the deletional hereditary persistence of fetal haemoglobin (HPFH) syndromes interfere with γ silencing causing the high-level expression observed in these conditions. The position effects observed by us may be analagous to these[8,9].

TABLE 1 Relative levels of γ transcripts compared with $\beta h1$ levels at 10.5 d and β_{maj} levels in adults

	10.5 days $\gamma/\beta h1$/copy (%)	Adult γ/β_{maj}/copy (%)
mini LCR γ1	105	<0.5
mini LCR γ2	200	2
mini LCR γ3	128	<0.5
Δ3′ LCR γ1	200	2
Δ3′ LCR γ2	104	2
Δ3′ LCR γ3	200	<0.5
Δ3′ LCR γ4	170	6

See also Figs 1 and 2*a*. Quotation was by densitometric scanning and RNA dilution.

Our findings contradict earlier reports[4,5] that the γ genes are deregulated in adult transgenic mice by the LCR and that competition from the β gene is required for silencing of γ transcription in adult stages. Several features of these studies might explain the observed adult expression. Enver *et al.*[4] used a γ gene linked to a 2.6-kilobase (kb) micro-LCR construct, which is missing all of site 4 (S. Pruzina *et al.*, manuscript submitted) and part of site 3 (ref. 19). The construct does contain the complete site 2, which is the only site known to contain a strong enhancer activity[10–12]. The small size of the construct resulted in the site-2 enhancer being ~2 kb upstream from the γ cap site compared with 11 kb in our construct and 25 kb in the normal human locus. Behringer *et al.*[5], analysed adult expression in two lines co-injected with human α, β and γ genes. We have found that the structures adopted by co-injected genes are too complex to be resolved by conventional Southern blotting (P. Fraser, D. Whyatt and N.D., unpublished results). Co-injection of fetal and adult genes as used by Behringer *et al.* may bring the 3′ end of the γ gene close to activating sequences associated with the adult α and β genes (for example, the β-globin 3′ enhancer). In addition, in both of those studies, increases in adult γ expression may have been amplified in multicopy animals. The three-copy line in this study (Δ3′ line 4) demonstrates how a mild position effect can be exaggerated by even a relatively low copy number to give an apparently significant level of adult expression. Finally, the constructs used in those studies lack the region located at the 3′ end of the Aγ gene which shows enhancer activity in transient expression assays[13]. It is present in our constructs suggesting that it might have a role in silencing the γ genes.

We have found that there is no deregulation of a γ gene in transgenic mice as a result of linkage to the LCR and that the presence of a β-globin gene in *cis* is not required for silencing of γ expression. The phenotypes of some human conditions involving deletions of the β gene have been proposed as support for a competition model[14]. Small deletions of the β promoter

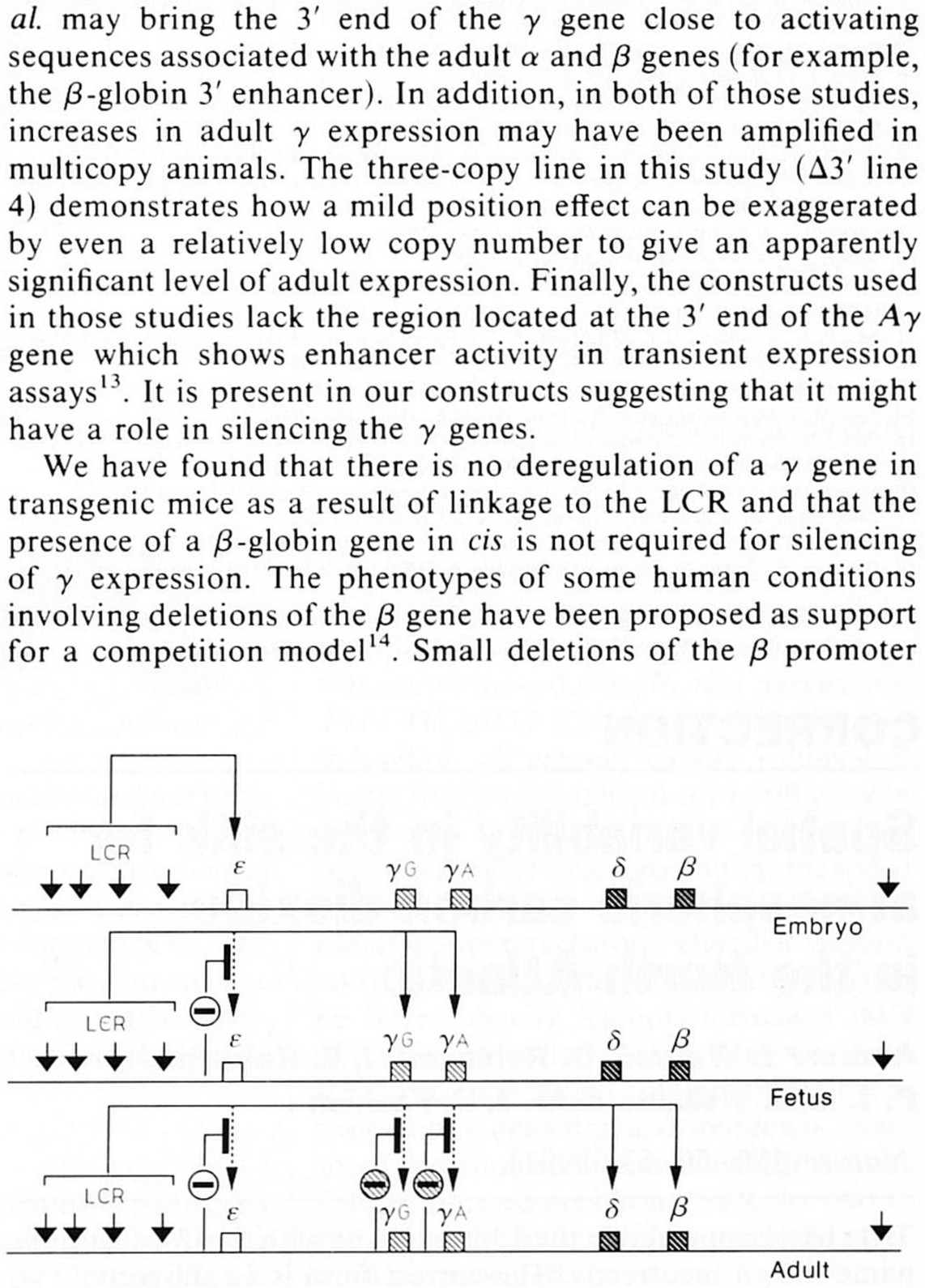

FIG. 3 Model for stage-specific regulation of the genes of the β-globin locus. Solid lines indicate activation of genes by the LCR. ⊖, Stage-specific negative factors silencing the gene. The location of these is not accurate and there may be more than one factor for each gene.

that abolish β transcription produce levels of γ expression averaging less than 5% (reviewed in ref. 15). Higher levels (averaged 10%) are only seen with much larger deletions (>10 kb) associated with the $\delta\beta$ thalassaemias[15] and the distribution is heterogeneous with γ chains not detectable in many cells using existing staining methods[16]. This heterocellular distribution is problematic for a competition model based on the proposition that deletion of the β gene allows the LCR to exert a dominant positive effect on the γ genes in an adult environment. Changes in chromatin structure and/or position effects caused by these large deletions may directly interfere with promoter-mediated silencing. This seems more likely than a competition model. The high-level pancellular expression observed in the deletion HPFH conditions suggests that extra mechanisms operate in these syndromes. One possible mechanism is that the expression is the result of enhancer-like sequences which have been found close to several of the HPFH deletion breakpoints[7,8].

From our data, and other results for the ε gene (refs 17 and 18; and P. Watt and P. Fraser, unpublished results), it seems likely that both of these genes are silenced by sequences located in their promoters. But the relative ease with which silencing of the γ genes can be perturbed suggests that the precise mechanisms of operation of these sequences may differ. When the β gene is placed in a position relative to the LCR which is similar to that normally occupied by ε, it is expressed in the embryonic stage in transgenic mice (ref. 5, and O. Hanscombe *et al.*, manuscript submitted). Therefore early silencing of the β gene may depend on its precise position in the locus and on the presence of active genes between it and the LCR (O. Hanscombe *et al.*, manuscript submitted). A model based on these findings is illustrated in Fig. 3. □

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CORRECTION

Spatial variability in the sink for atmospheric carbon dioxide in the North Atlantic

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Nature **350,** 50–53 (1991).

THIS letter appeared in the 7 March issue with the third author's name shown incorrectly. The correct form is as above.