The EMBO Journal vol.15 no.3 pp.562–568, 1996

A dominant chromatin-opening activity in 5′ hypersensitive site 3 of the human β-globin locus control region

James Ellis[1], Kian C.Tan-Un[2], Alex Harper[3], David Michalovich[4], Nikos Yannoutsos[4], Sjaak Philipsen[4] and Frank Grosveld[4,5]

Laboratory of Gene Structure and Expression, National Institute for Medical Research, London, NW7 1AA, UK

[1]Present address: Department of Genetics, Research Institute, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada
[2]Present address: School of Professional and Continuing Education, Knowles Building, University of Hong Kong, Hong Kong
[3]Present address: Transgenic Research Unit, The Rayne Institute, St Thomas' Hospital, London, SE1 7EH, UK
[4]Present address: Department of Cell Biology and Genetics, Erasmus University Rotterdam, PO Box 1738, 3000 DR Rotterdam, The Netherlands

[5]Corresponding author

Single-copy human β-globin transgenes are very susceptible to suppression by position effects of surrounding closed chromatin. However, these position effects are overcome by a 20 kbp DNA fragment containing the locus control region (LCR). Here we show that the 6.5 kbp microlocus LCR cassette reproducibly directs full expression from independent single-copy β-globin transgenes. By testing individual DNase I-hypersensitive sites (HS) present in the microlocus cassette, we demonstrate that the 1.5 kbp 5′HS2 enhancer fragment does not direct β-globin expression from single-copy transgenes. In contrast, the 1.9 kbp 5′HS3 fragment directs β-globin expression in five independent single-copy transgenic mouse lines. Moreover, the 5′HS3 core element and β-globin proximal promoter sequences are DNase I hypersensitive in fetal liver nuclei of these expressing transgenic lines. Taken together, these results demonstrate that LCR activity is the culmination of at least two separable functions including: (i) a novel activity located in 5′HS3 that dominantly opens and remodels chromatin structure; and (ii) a recessive enhancer activity residing in 5′HS2. We postulate that the different elements of the LCR form a 'holocomplex' that interacts with the individual globin genes.

Keywords: chromatin/β-globin/locus control region

Introduction

The human β-globin locus is located in active or open chromatin in cells of the erythroid lineage (reviewed in Felsenfeld, 1992; Dillon and Grosveld, 1993). The entire locus is in a DNase I-sensitive chromatin conformation in erythroid cells (Groudine *et al.*, 1983), and the β-globin gene is expressed to very high levels. In addition, nucleosomes located within the β-globin locus in chicken erythroid cells are hyperacetylated (Hebbes *et al.*, 1994), suggesting that they are accessible to *trans*-acting factors (Lee *et al.*, 1993). In contrast, the chromatin structure of the locus is closed in non-erythroid tissue and the β-globin gene is completely inactive. A similarly inactive locus is present in erythroid cells of a subset of β-thalassaemias in which components of the locus control region (LCR) located 5′ of the cluster are deleted (Kioussis *et al.*, 1983; Driscoll *et al.*, 1989; Forrester *et al.*, 1990). The LCR is composed of four erythroid-specific DNase I-hypersensitive sites (HS) on a 20 kbp fragment (Tuan *et al.*, 1985; Forrester *et al.*, 1986) and directs copy number-dependent, position-independent expression of a linked human β-globin gene in transgenic mice (Grosveld *et al.*, 1987; Ryan *et al.*, 1989). These findings suggest that the LCR is the primary regulator of chromatin structure in the locus.

The copy number-dependent expression activity of the LCR in transgenic mice has been mapped to the 200–300 bp cores of 5′HS2 (Talbot *et al.*, 1990; Caterina *et al.*, 1991; Talbot and Grosveld, 1991), 5′HS3 (Philipsen *et al.*, 1990) and 5′HS4 (Pruzina *et al.*, 1991). This activity is a property of redundant combinations of *trans*-acting binding sites and not an effect of a single DNA binding factor (Ellis *et al.*, 1993; Philipsen *et al.*, 1993). In contrast, the only enhancer activity identified within the LCR by transient transfection assays is located in 5′HS2 and is dependent on specific NF-E2 factor binding sites (Tuan *et al.*, 1989; Moi and Kan, 1990; Ney *et al.*, 1990).

Previously, we noted that a 215 bp 5′HS2 core element directed copy number-dependent expression of multicopy transgenes present in a concatamer but failed to activate single-copy β-globin transgenes (Ellis and Grosveld, 1993; Ellis *et al.*, 1993). These data suggested that single-copy β-globin transgenes are more sensitive to suppressive position effects than multicopy transgene concatamers. In order for an LCR reproducibly to direct significant levels of expression from single-copy transgenes at every integration site, it must possess a chromatin-opening activity to override the suppressive position effects of surrounding closed chromatin. Therefore, reproducible expression from independent single-copy transgenes serves as a sensitive assay for chromatin-opening activity.

Reports of single-copy β-globin transgenic mouse lines are in agreement that fragments containing the 20 kbp LCR reproducibly direct 100% human β-globin expression compared with endogenous mouse globin levels (Dillon and Grosveld, 1991; Berry *et al.*, 1992; Strouboulis *et al.*, 1992; Gaensler *et al.*, 1993; Peterson *et al.*, 1993). In this paper, we investigate whether reproducible single-copy transgene expression requires the presence of all four HS. We show that the 6.5 kbp microlocus LCR cassette (Talbot *et al.*, 1989) directs full expression of independent single-copy transgenes, and we map the chromatin-opening activity to the 1.9 kbp 5′HS3 fragment. Moreover, we

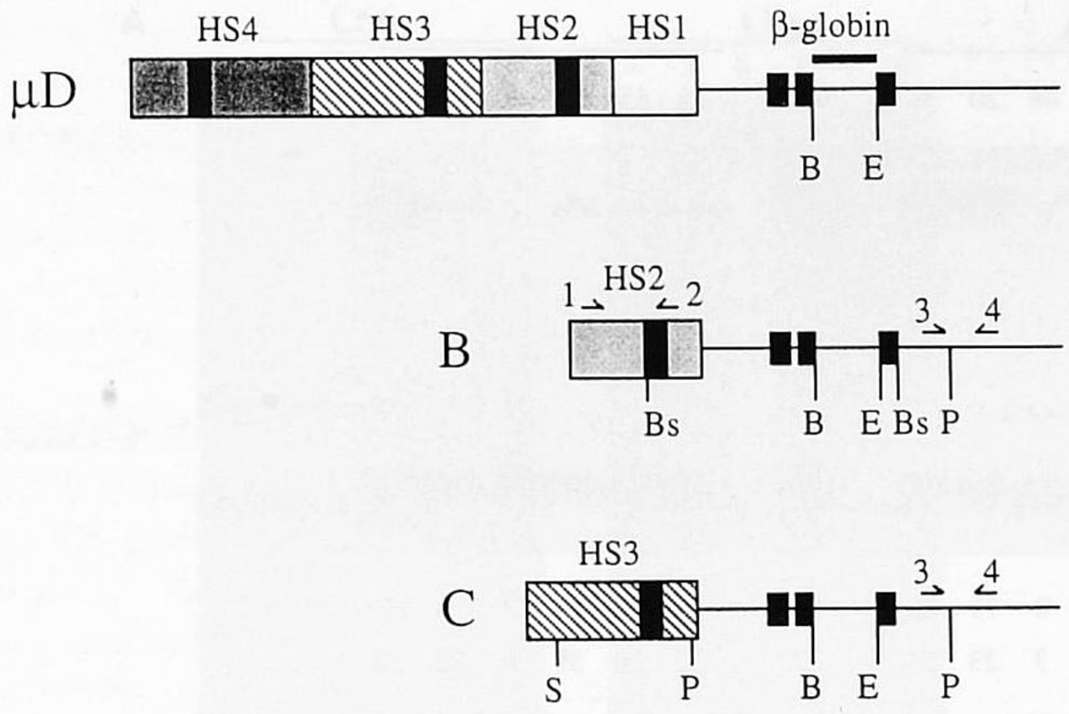

Fig. 1. Maps of the human β-globin constructs used for transgenesis. The microlocus LCR (construct μD) contains all four HS in a 6.5 kbp cassette (Talbot *et al.*, 1989) containing the following sequences: 1.0 kbp partial *SSt*I–*Hin*dIII 5′HS1 (open box); 1.5 kbp *Asp*718–*Bgl*II 5′HS2 (light grey box); 1.9 kbp *Hin*dIII 5′HS3 (striped box); and 2.1 kbp *Bam*HI–*Xba*I 5′HS4 (dark grey box). The cores of 5′HS2 (Talbot and Grosveld, 1991), 5′HS3 (Philipsen *et al.*, 1993) and 5′HS4 (Pruzina *et al.*, 1991) are shown as black boxes. Constructs B and C contain only the 5′HS2 fragment or the 5′HS3 fragment respectively, each cloned 5′ of the 800 bp human β-globin promoter and gene sequences (exons shown in small black boxes). The narrow black box is the βivs2 probe. Transgene intactness was demonstrated by restriction site mapping on Southern blots and by PCR with primers 1–4 (arrows). B, *Bam*HI; Bs, *Bsp*HI; E, *Eco*RI; P, *Pst*I; S, *Stu*I.

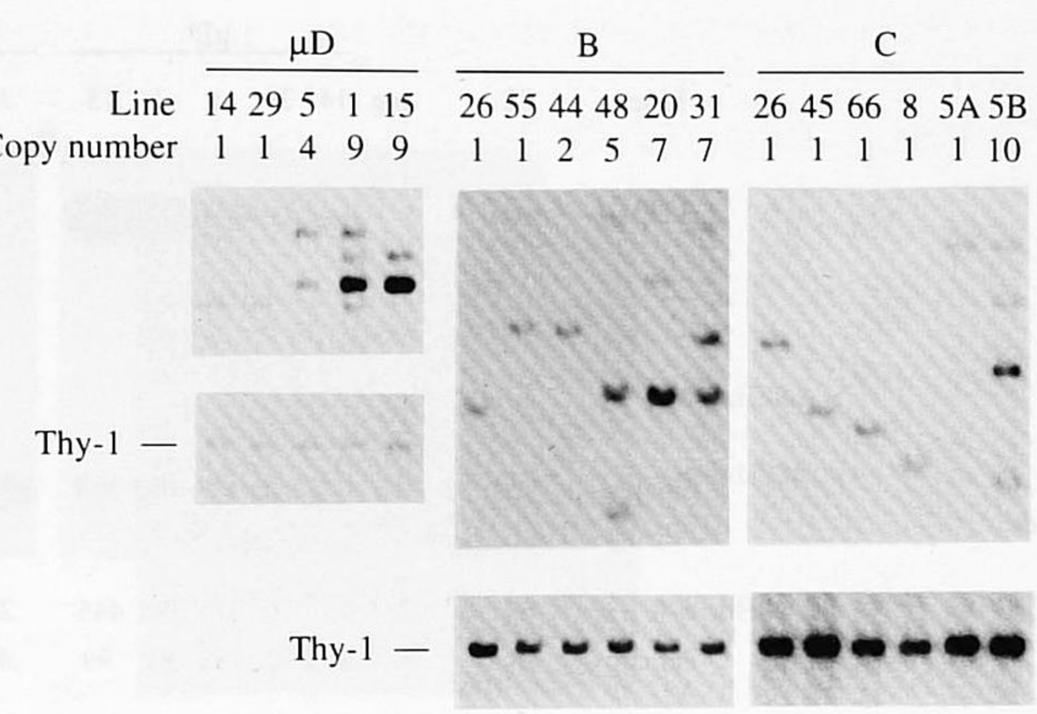

Fig. 2. Copy numbers of transgenic mouse lines determined by Southern blot analysis of F_1 fetal head DNA singly digested with *Eco*RI (μD and B lines) or *Bam*HI (C lines) to reveal 5′ and 3′ end fragments respectively when hybridized to the βivs2 probe (Figure 1). Copy numbers were confirmed by digesting μD and B lines with *Bam*HI, and C lines with *Eco*RI (data not shown). Although the two-copy B44 line contained only one 5′ end fragment, two 3′ end fragments were observed indicating two independent single-copy integration sites (data not shown). Loading controls of the same blots hybridized to an *Apa*I-digested mouse Thy-1 probe are shown below.

show that the 1.5 kbp 5′HS2 enhancer fragment is not active in single-copy transgenic mice. Since the only construct that directed full levels of single-copy transgene expression is the microlocus LCR cassette, we propose that LCR activity requires the separable functions of both the chromatin-opening activity in 5′HS3 and the enhancer activity in 5′HS2.

Results

Generation of single-copy β-globin transgenes in mice

In order to characterize the chromatin-opening activity located within the 20 kbp LCR fragment, we tested three LCR constructs in single-copy transgenic mice. Unfortunately there is no efficient method available for the generation of single copy transgenes. We therefore had to inject large numbers of fertilized mouse eggs (several thousand) with DNA fragments at a low concentration (0.25 μg/ml) to obtain sufficient animals with intact single-copy integrations of the different constructs. The microlocus LCR construct contains all four HS on a 6.5 kbp cassette linked to the human β-globin gene (Figure 1). The B and C constructs are derivatives of the microlocus LCR and contain the 1.5 kbp 5′HS2 fragment and the 1.9 kbp 5′HS3 fragment respectively. Founder (F_0) transgenic mice were generated with these LCR constructs and screened from non-transgenic littermates by Southern blots on tail DNA. However, the F_0 generation copy number can be difficult to establish reliably because founder mice are occasionally a mosaic of transgenic and non-transgenic cells, which can lead to an incorrect interpretation of the results, or of cells containing different transgene integration events and copy numbers. Therefore we bred the founders to non-transgenic animals to obtain non-mosaic F_1 fetuses.

The copy number of these lines was determined by Southern blot analysis of F_1 fetal head DNA digested at a single internal restriction site and hybridized with a probe specific for human β-globin. In this manner, cleavage of single-copy transgenes with *Eco*RI occurs downstream of the βivs2 probe and reveals a 5′ end fragment that extends into flanking genomic sequences. Likewise, single-copy transgenes digested with *Bam*HI are visualized as 3′ end fragments. Higher transgene copy numbers usually contain one end fragment and a multicopy band indicating a head-to-tail transgene concatamer. Single-copy lines must contain only one unique 5′ end fragment and one unique 3′ end fragment of single-copy intensity.

By following this procedure of determining copy number and demonstrating equal DNA loading by reprobing for mouse Thy-1, we established that the μD14 and μD29 lines contained a single copy of the microlocus construct, the B26 and B55 lines contained a single copy of the 5′HS2 construct, and five C lines (C5A, C8, C26, C45, C66) contained a single copy of the 5′HS3 construct (Figure 2). Although the two-copy B44 line contained only one 5′ end fragment and no concatamer-sized band (Figure 2), two 3′ end fragments were observed indicating two independent single-copy integration sites (data not shown). Transgene intactness was shown by Southern blot and PCR analyses of the transgene termini and the β-globin 3′ enhancer element (Figure 1).

5′HS3 directs reproducible expression from single-copy transgenes

To examine the expression status of the transgenes relative to the endogenous mouse β-major genes, we extracted total RNA from 13.5-day F_1 fetal livers and performed S1 nuclease analysis (Figure 3). As a positive control for expression we used fetal liver RNA from Line 72 which contains a single-copy of the 70 kbp human β-globin

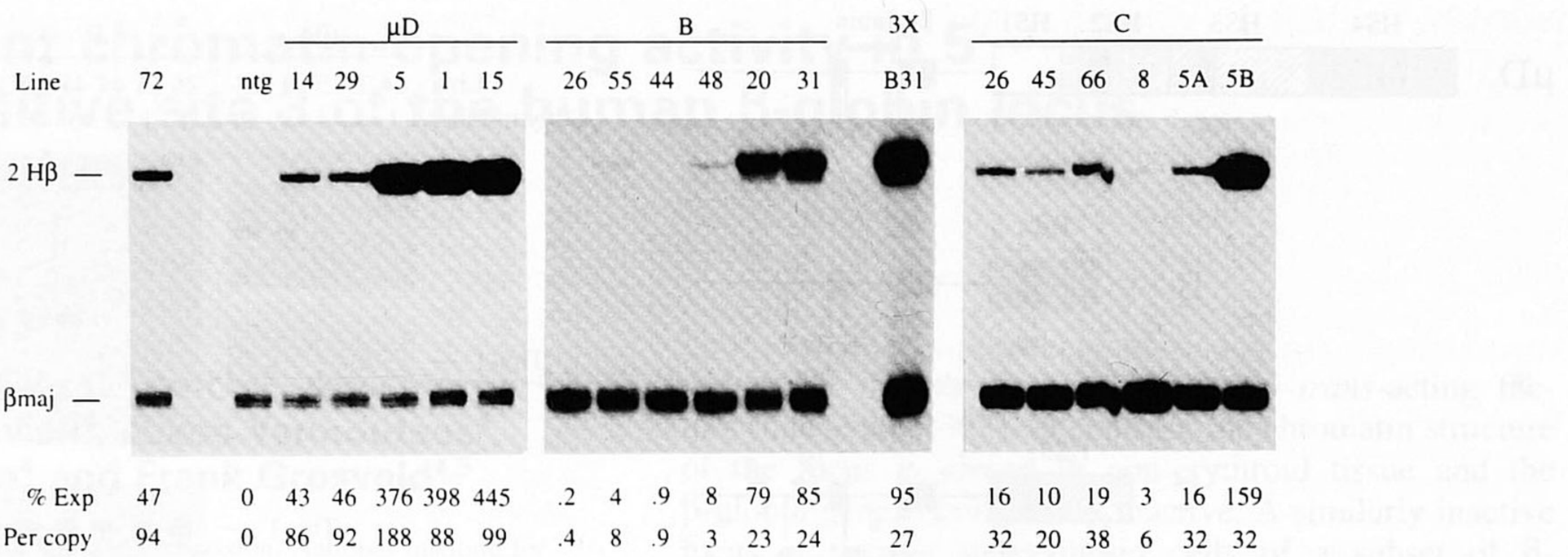

Fig. 3. Quantitation of β-globin expression in transgenic lines by S1 nuclease analysis of 13.5-day fetal liver RNA. The μD, B and C transgenic samples are loaded in the same order as Figure 2. Specific activity of the human β (Hβ) and mouse β-major (βmaj) probes was 2:1. ntg, non-transgenic; 72, Line 72; 3X, probe excess control of the adjacent seven-copy B line.

locus (Strouboulis *et al.*, 1992), and expresses human β-globin mRNA at 47% of the level produced by the two mouse β-major genes, or 94% per copy. The single-copy μD14 and μD29 lines expressed human β-globin at a mean average of 89% per copy. These data demonstrate that the microlocus LCR fully activates single-copy transgene expression in every mouse and therefore contains a putative chromatin-opening activity.

To determine whether this activity requires the presence of all four HS, we evaluated expression from single-copy transgenes containing the 1.5 kbp 5′HS2 fragment. In contrast to the full levels of expression produced by the microlocus LCR construct in the μD lines, β-globin transgenes alone express non-reproducibly and at <1% per copy (Ryan *et al.*, 1989; Philipsen *et al.*, 1993). S1 nuclease analysis of fetal liver RNA from the B lines detected human β-globin RNA at <1% per copy in the single-copy B26 and the two-copy B44 lines, although significant levels of 8% were detected in the other single-copy B55 line (Figure 3). Similar results have been obtained by other groups (Curtin *et al.*, 1989; N.Raich, personal communication). These data demonstrate that expression directed by the 1.5 kbp 5′HS2 fragment, which contains the only classical enhancer element in the LCR (Tuan *et al.*, 1989; Moi and Kan, 1990; Ney *et al.*, 1990), can be suppressed by surrounding closed chromatin. In agreement with our earlier data (Fraser *et al.*, 1990), expression of 23% per copy was seen in both the seven-copy B20 and B31 lines, confirming that 5′HS2 activates multicopy transgenes present in a concatamer (Ellis and Grosveld, 1993; Ellis *et al.*, 1993). The five-copy B48 mouse appears to be an exception and is not in agreement with our earlier data on 5′HS2 (Fraser *et al.*, 1990, 1993). These results therefore show a typical pattern for a construct that intrinsically is unable to express but that is strongly influenced by position effects.

Evaluation of single-copy transgene expression regulated by the 1.9 kbp 5′HS3 fragment was performed by S1 nuclease analysis on fetal liver RNA from the C lines. In contrast to the non-reproducible expression directed by the 5′HS2 enhancer at single copy, the C lines expressed significant human β-globin levels in all five single-copy lines with a mean average of 26% per copy (Figure 3). These data indicate that 5′HS3 contains an activity which overrides the suppressive effects of surrounding closed chromatin and activates significant expression from independent integration sites.

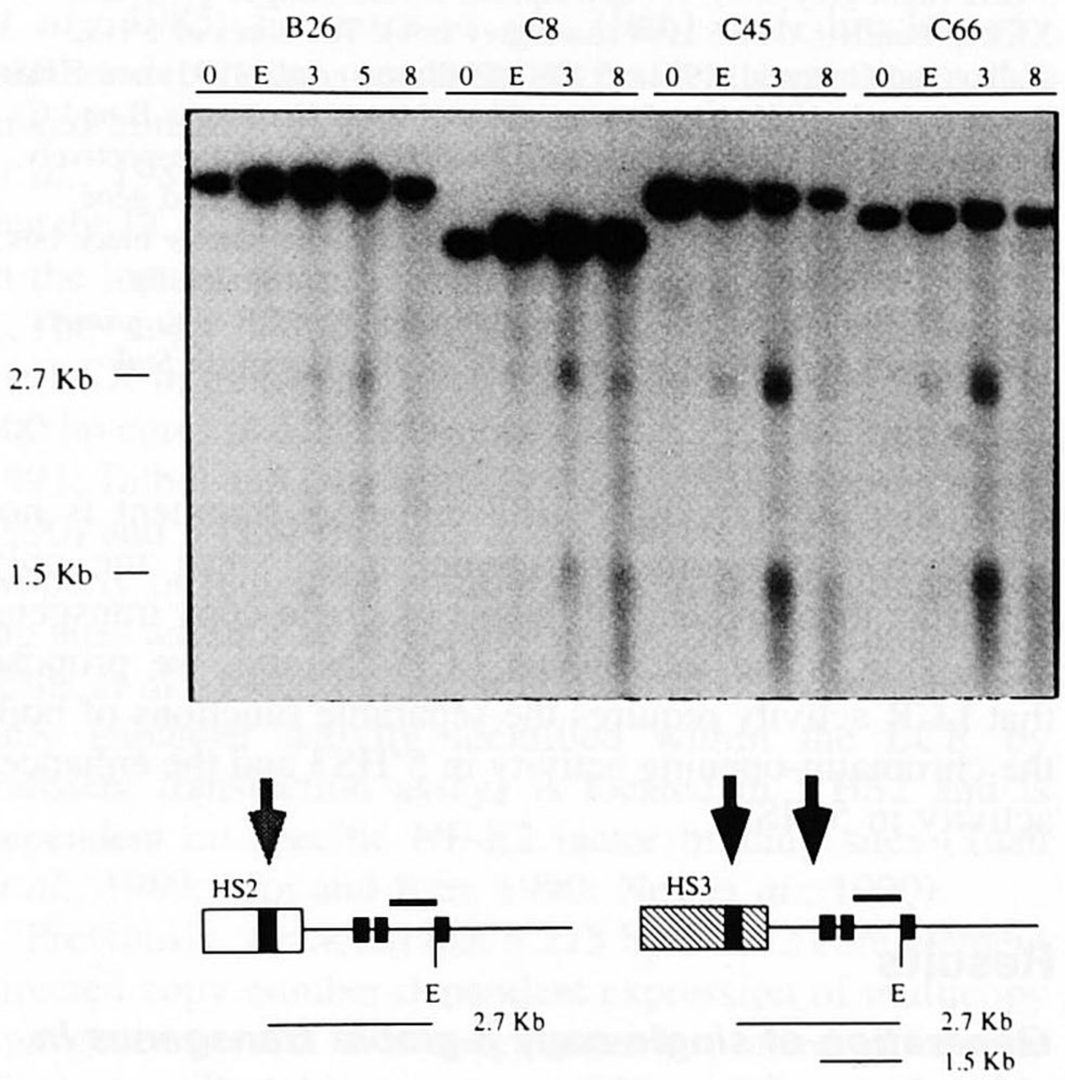

Fig. 4. DNase I-hypersensitive site mapping of single-copy human β-globin transgenes in fetal liver nuclei of B and C construct transgenic lines. Samples were digested with increasing concentrations of DNase I, genomic DNA extracted and subsequently digested with *Eco*RI before hybridization with the βivs2 probe. Maps of the B and C constructs are shown below. Black and grey arrows correspond to strong and weak HS respectively. 0, zero time point; E, endogenous DNase; 3, 5, 8, samples digested with 3, 5 and 8 μl DNase I respectively.

5′HS3 remodels the chromatin structure of single-copy transgenes

To investigate whether the 5′HS3 sequences in the C lines are in an open chromatin conformation, we performed DNase I HS mapping on nuclei prepared from 13.5-day transgenic fetal livers obtained from the single-copy transgenic C26, C45 and the lowest expressing line C8 (Figure 4). As expected for expressing transgenes, we

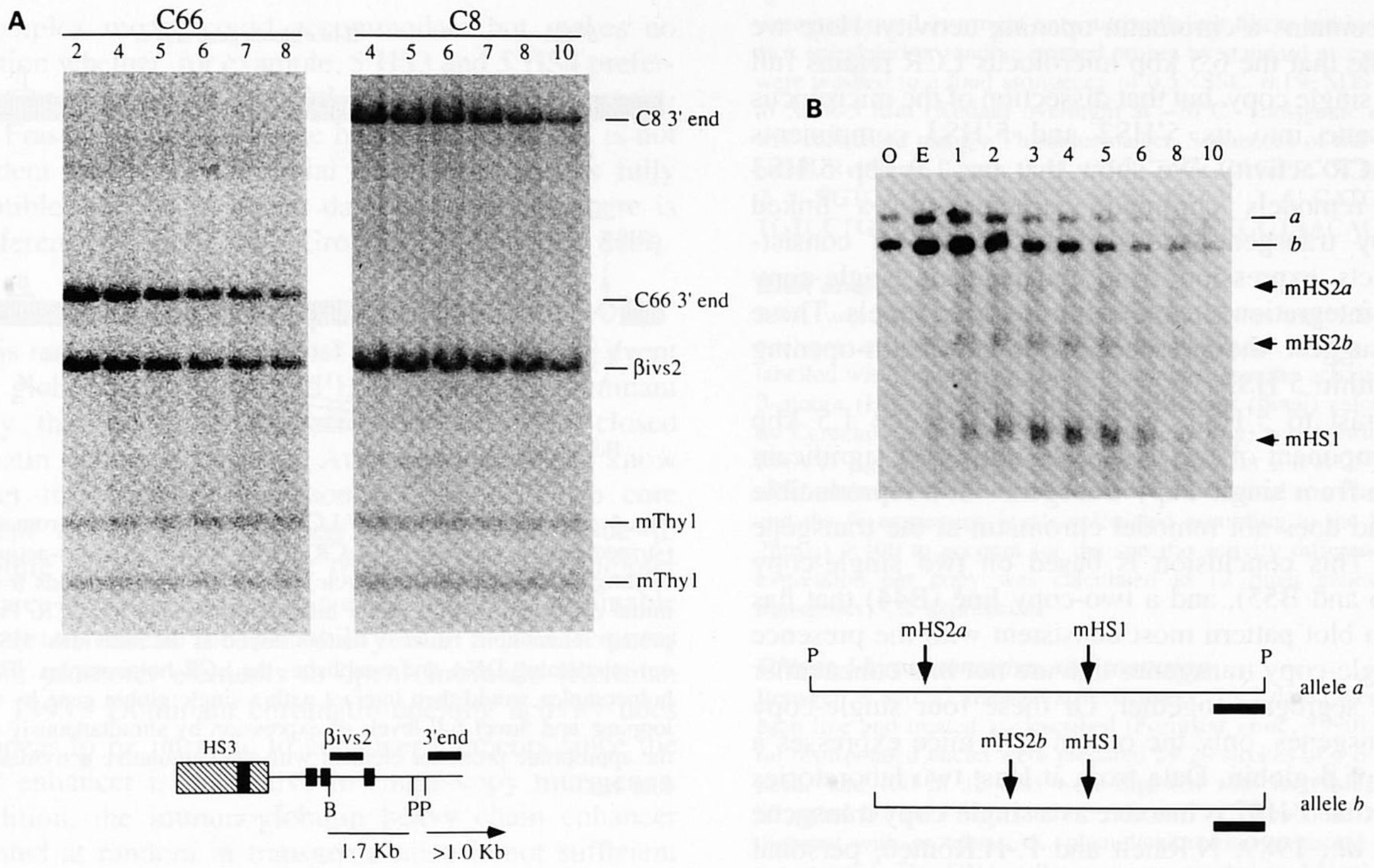

Fig. 5. (**A**) DNase I sensitivity of the intron and flanking region of the human β-globin transgene in lines C8 and C66. The genomic DNA was digested with *Bam*HI and *Pst*I before hybridization. The probes were a *Bam*HI–*Eco*RI fragment covering intron 2 and an *Eco*RV–*Pst*I fragment from the 3′ end of the transgene construct. The numbers indicate the same DNase I digestions as in Figure 4. The control probe is from the endogenous mouse Thy1 gene (Spanopoulou *et al.*, 1988). (**B**) DNase I-hypersensitive site mapping of the endogenous mouse β-globin LCR in 13.5-day fetal liver nuclei of the non-expressing B26 transgenic line. Aliquots of the same DNase I-treated samples shown in Figure 4 were digested with *Pst*I before hybridization with a 1.1 kbp *Eco*RI–*Pst*I probe (Jiminez *et al.*, 1992). 0, zero time point; E, endogenous DNase; 1–10, samples digested with 1 to 10 μl DNase I respectively.

observed HS at 2.7 and 1.5 kbp 5′ of the *Eco*RI site in the β-globin transgene corresponding to the 5′HS3 core (Philipsen *et al.*, 1990) and the proximal β-globin promoter sequences respectively (Antoniou *et al.*, 1988). Interestingly, the C8 transgene band is itself more resistant to DNase I cleavage than the other transgene bands (Figure 4), suggesting that it is located in particularly inaccessible chromatin (Rivier and Pillus, 1994). Nevertheless, chromatin at the C8 transgene promoter is correctly remodelled (Wallrath *et al.*, 1994), and the transgene is expressed, albeit at a reduced level. We have also tested whether general DNase I sensitivity is established inside and beyond the introduced transgene (Figure 5A), by using an intron-specific probe and a probe at the very 3′ end of the transgene. The latter probe measures well into the flanking region and the *Pst*I digest ensures that we only examine sequences past any known HS (Forrester *et al.*, 1986). Both probes show that the DNA is sensitive to digestion. These data are consistent with the presence of a dominant chromatin-opening activity residing in the 1.9 kbp 5′HS3 fragment that not only forms HS on the same sequences as those detected *in vivo* (Tuan *et al.*, 1985; Forrester *et al.*, 1986; Philipsen *et al.*, 1990), but also directs significant single-copy transgene expression.

DNase I-hypersensitive site mapping of the 5′HS2 sequences in the non-expressing B26 single-copy line (Figure 4) identified weak hypersensitivity at the expected position 2.7 kbp 5′ of the *Eco*RI site corresponding to the 5′HS2 core (Talbot *et al.*, 1990; Talbot and Grosveld, 1991). Such a weak hypersensitive site is normally observed at 5′HS2 in non-erythroid cells (Forrester *et al.*, 1987; Talbot *et al.*, 1990). This weak sensitivity does not correlate with gene expression and clearly it is unable to induce the hypersensitive site corresponding to the active β-globin promoter. To demonstrate that strong DNase I hypersensitivity could be detected in these same DNA preparations, we digested samples with *Pst*I and hybridized with a probe specific for the endogenous mouse β-globin LCR (Figure 5B). This analysis revealed hypersensitivity at mouse 5′HS1 and both alleles of mouse 5′HS2 as previously described (Jiminez *et al.*, 1992).

These results suggest that *trans*-acting factors can bind to the 5′HS2 enhancer sequences of single-copy transgenes *in vivo*, but that the effect is not a dominant chromatin-opening activity because the promoter chromatin is not correctly remodelled and the β-globin transgene is not expressed to a significant level. Taken together, our results demonstrate that the chromatin-opening activity in 5′HS3 can be functionally separated from the enhancer activity in 5′HS2, and suggest a multistep model of LCR activation.

Discussion

5′HS3 contains a dominant chromatin-opening activity

We have used expression from independent single-copy transgenes as a sensitive assay for chromatin-opening activity. This approach has confirmed that full levels of human β-globin expression are reproducibly obtained from LCR constructs containing all four HS and indicates that

the LCR contains a chromatin-opening activity. Here we demonstrate that the 6.5 kbp microlocus LCR retains full activity at single copy, but that dissection of the microlocus LCR cassette into its 5′HS2 and 5′HS3 components disturbs LCR activity. We show that the 1.9 kbp 5′HS3 fragment remodels chromatin structure of a linked single-copy transgene and flanking region. It consistently directs expression from independent single-copy transgene integration sites, albeit at reduced levels. These findings suggest the presence of a chromatin-opening activity within 5′HS3.

In contrast to 5′HS3, we conclude that the 1.5 kbp 5′HS2 component of the LCR does not direct significant expression from single-copy transgenes in a reproducible manner and does not remodel chromatin at the transgene promoter. This conclusion is based on two single-copy lines (B26 and B55), and a two-copy line (B44) that has a Southern blot pattern most consistent with the presence of two single-copy transgenes that are not in a concatamer but which segregate together. Of these four single-copy 5′HS2 transgenes, only the one in B55 mice expresses a low level of β-globin. Data from at least two laboratories also show that 5′HS2 is inactive as a single-copy transgene (Curtin *et al.*, 1989; N.Raich and P.-H.Romeo, personal communication). Inclusion of these data strongly supports the notion that 5′HS2 and 5′HS3 behave very differently in single-copy transgenic mice; this difference is statistically significant ($P \leqslant 0.016$). Taken together with our previous report that the 215 bp 5′HS2 core element is not functional in single-copy founder transgenic animals, these findings provide strong evidence that 5′HS2 does not contain a chromatin-opening activity. However, 5′HS2 directs high level expression from multicopy transgenes and is a strong enhancer in transient transfection assays.

Full levels of expression in single-copy transgenic mice were only obtained from the microlocus LCR cassette, suggesting that the chromatin-opening domain in 5′HS3 and the enhancer element in 5′HS2 have distinct functions that cooperate to produce LCR activity. Despite many injections we have generated only two 5′HS4 single-copy mice (one intact construct and one lacking the 3′ β-globin enhancer). The data from these 5′HS4 mice are therefore still preliminary, but they show that 5′HS4 also does not express the transgene. It is known that this fragment has no enhancer activity in transient transfections but does contribute to the overall β-globin expression level in stable assays (Fraser *et al.*, 1990; Pruzina *et al.*, 1991). Interestingly, Lowrey *et al.* (1992) have shown that 5′HS4 reforms a hypersensitive site when introduced (without a gene) into multicopy transgenic mice. It is, however, not clear whether 5′HS4 has the capacity to remodel the chromatin when present in a single copy.

Holocomplex model of LCR activation

On the basis of our data we suggest that the LCR is composed of multiple HS with separable functions. A possible *in vivo* mechanism for LCR activity is that the chromatin-opening domain within 5′HS3 is responsible for the initial formation of DNase I hypersensitivity. Such a central role appears to be confirmed by the fact that a 5′HS3 deletion affects the expression of the entire locus (J.Strouboulis *et al.*, unpublished results). Open chromatin would then spread from 5′HS3, permitting *trans*-acting factor access to the other HS and revealing their transcriptional enhancement activities. We propose that this enhancement is secondary to the essential 5′HS3 element and may be mediated by DNA looping (Ptashne, 1986) to act closely together or even form a LCR 'holocomplex' (Figure 6). The Sp1 *trans*-acting factor is certainly a candidate for this role as it binds to all the HS cores (Philipsen *et al.*, 1990; Talbot *et al.*, 1990; Pruzina *et al.*, 1991) and has been shown to loop DNA (Mastrangelo *et al.*, 1991; Su *et al.*, 1991), but accessory bridging or other factors may participate in this putative loop formation. In order to form loops, it is likely that the HS core elements must be separated by spacer DNA. The LCR holocomplex would accomplish globin gene activation and switching by increasing the accessibility of *trans*-acting factors to their binding sites in the adjacent globin promoters. These promoter-bound factors would interact with the LCR holocomplex by DNA looping to align the cumulative transcriptional enhancement activity of the LCR with the promoters and stimulate mRNA initiation by the general transcription machinery. Expression of the early genes (ε and γ) would continue until the interaction between the LCR holocomplex and the promoter is interrupted by stage-specific silencing factors (Gumucio *et al.*, 1992). At this point the only productive DNA loop that can be formed is between the LCR holocomplex and the nearest unsilenced promoter to direct γ-globin expression in the fetus or δ- and β-globin expression in the adult.

By forming a single LCR holocomplex from the four HS, this model can account for the fact that globin genes are in competition for the LCR in a polar fashion (Hanscombe *et al.*, 1991), that the activities of the individual HS are additive, and that all the HS must be present to obtain full levels of β-globin gene expression (Fraser *et al.*, 1990). The model is also consistent with the observation that all the HS of the LCR are developmentally stable. These properties are difficult to explain by models that do not invoke a holocomplex and that are based on specific HS–promoter interactions (Engel, 1993). The

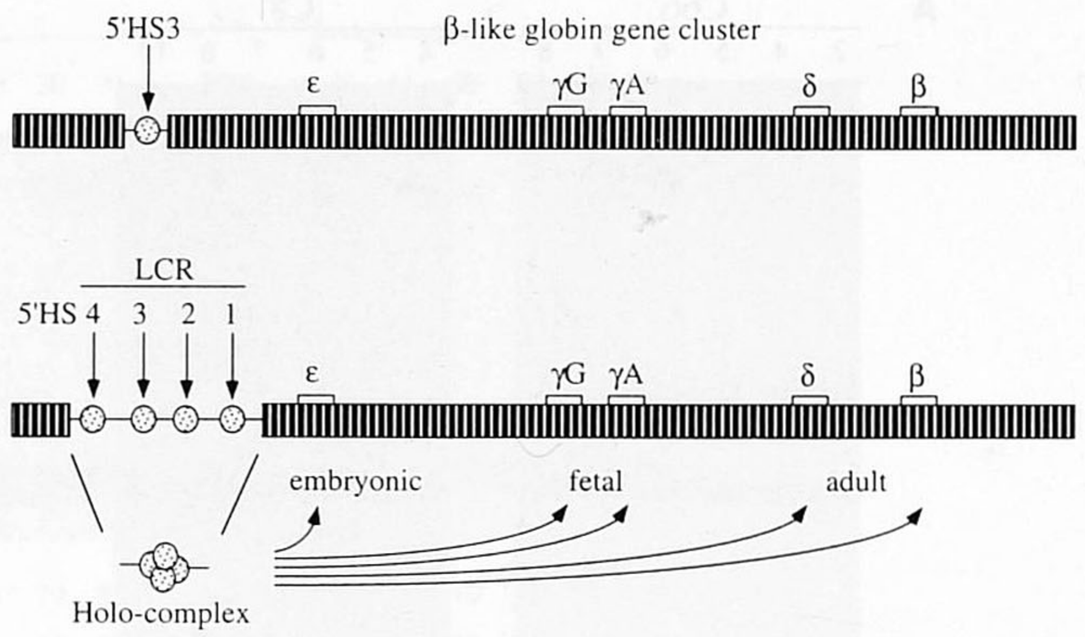

Fig. 6. Holocomplex model of LCR activation. Closed chromatin (striped box) at the β-globin LCR is first opened by *trans*-acting factor binding at 5′HS3 (speckled circle). Open chromatin spreads from this initial event and permits factor binding throughout the LCR. Protein–protein interactions between factors bound at the individual HS loops out intervening DNA and establishes the LCR holocomplex. The holocomplex would then interact with a single globin gene by DNA looping, and direct full levels of expression by simultaneously aligning the appropriate promoter element with the cumulative activities of all four HS.

holocomplex model could accommodate but makes no prediction whether, for example, 5′HS3 and 5′HS4 preferentially interact with the γ- and β-globin promoters respectively (Fraser *et al.*, 1993). The holocomplex model is not dependent on such preferential interactions and is fully compatible with more recent data showing that there is no preference (P.Fraser and F.Grosveld, unpublished data).

Chromatin-opening activity

What is required for the original chromatin-opening event in the globin LCR? Clearly 5′HS3 contains a dominant activity that overrides the effects of adjacent closed chromatin in transgenic mice. At present we do not know whether this domain corresponds to the 225 bp core fragment of 5′HS3 (Philipsen *et al.*, 1990). Due to the nature of the single-copy transgene assay, promoter sequences are required to obtain expression. We are unable therefore to assess the possibility that 5′HS3 requires adjacent promoter elements to open chromatin (Reitman *et al.*, 1993). Dominant chromatin-opening activity does not appear to be intrinsic to enhancer elements since the 5′HS2 enhancer is not active in single-copy transgenes. In addition, the immunoglobulin heavy chain enhancer integrated at random in transgenic mice is not sufficient to form a DNase I-hypersensitive site nor activate transcription of a linked promoter (Jenuwein *et al.*, 1993). Nevertheless, enhancer and chromatin-opening activities may not be mutually exclusive because a liver-specific enhancer element can be bound *in vivo* by *trans*-acting factors that displace nucleosomes and precisely position adjacent nucleosomes, resulting in a remodelling of chromatin structure (McPherson *et al.*, 1993).

Our finding that 5′HS2 is not functional at low copy numbers but activates high levels of expression from multicopy transgene concatamers suggests that multiple 5′HS2 elements may interact to open chromatin, or that the spread of closed chromatin is limited by distance and is unable to suppress transgenes that are centrally located in the concatamer. By testing additional 5′HS2 constructs we have ruled out the former possibility (J.Ellis *et al.*, manuscript in preparation), again suggesting that chromatin-opening activity is not related to the sheer number of HS present but rather to a specific domain in 5′HS3. Future experiments will fine map this dominant chromatin-opening domain and characterize the *trans*-acting factors that bind to the region. Such a domain may be suitable for regulating expression from retrovirus or adeno-associated virus-based gene therapy vectors that integrate at single copy.

Materials and methods

Transgenic mice

DNA fragments were gel-purified by Gene Clean (Bio 101) extraction and Elutip (Schleicher and Schuell) columns. DNA concentration was adjusted to 0.25 ng/μl, microinjected into the pronucleus of (C57Bl×CBA)F_1 fertilized mouse eggs, and transferred into pseudo-pregnant F_1 foster mothers as described (Kollias *et al.*, 1986). Transgenic mice were identified by Southern blot analysis and PCR on DNA extracted from tail biopsies. Founder mice were bred to non-transgenic F_1 mice to obtain 13.5-day transgenic fetuses.

DNA analysis

Tail and fetal head DNA was extracted, digested with restriction enzymes and 5 μg of each sample separated on 0.7% agarose gels. Southern blots were performed overnight onto nitrocellulose filters and hybridized with nick translated or random primed probes by standard procedures. Filters were washed to a final stringency of 0.5×SSC, 0.1% SDS and exposed to XAR-5 film (Kodak) overnight at –70°C. Transgenic copy number was calculated using a PhosphorImager. Sequences of the primers used for PCR (Figure 1) are: 1. 5′-GTCTTAGCCAGTTCCTTACAGCT-3′; 2. 5′-TGTCACATTCTGTCTCAGGCATC-3′; 3. 5′-CATGGTTTGACTGTCCTGTGAGC-3′; 4. 5′-GGTGGTTGATGGTAACACTATGC-3′

RNA analysis

RNA was extracted from 13.5-day fetal livers and subjected to S1 nuclease analysis as described (Antoniou *et al.*, 1988). Probes were labelled with T4 polynucleotide kinase and specific activities of human β-globin (Hβ) relative to the mouse β-major (βmaj) calculated as 2:1 by Cerenkov counting. Labelled probe (20 ng) was hybridized to total RNA (1 μg) overnight at 54°C and digested for 2 h at 25°C with 75 U of S1 nuclease. The protected bands were quantified on a PhosphorImager and the % expression levels calculated according to the formula (Hβ/2βmaj) ×100 to account for the specific activity differences. Per cent expression per copy was calculated as (2 βmaj genes/number Hβ transgenes)×% expression.

DNase I-hypersensitive site mapping

Between 8 and 12 transgenic fetal livers (13.5 days) were pooled for each line and treated as described (Forrester *et al.*, 1990). In brief, 1.2 ml resuspended nuclei were prepared by 20 strokes of a B-type Dounce pestle and 100 μl aliquots were digested with increasing volumes of 80 μg/ml DNase I for exactly 3 min at 37°C. Reactions were stopped, digested with proteinase K, phenol/chloroform extracted and ethanol-precipitated. The DNA pellet was resuspended in 100 μl water, 20 μl was digested with *Eco*RI or *Pst*I before Southern blot analysis and hybridization to the human βivs2 probe or mouse β-globin LCR probe respectively. Autoradiography was for 4 days. General DNase I sensitivity was determined by digesting 10 μl of the resuspended DNA pellet with *Bam*HI and *Pst*I before Southern analysis using the human βivs2, 3′ end and a mouse Thy1 probe as probes.

Acknowledgements

We thank S.Raguz-Bolognesi for advice on DNase I-hypersensitive site mapping and for reagents, T.Enver for the mouse β-globin LCR probe, J.Strouboulis for line 72 RNA, N.Solomon and C.Wilson for animal care, Th.Stijnen for help with the statistical calculations and A.Bygrave and others for tailing mice. J.E. was supported by an MRC (Canada) Fellowship. This work was funded by MRC (UK), the NWO (The Netherlands) and the Howard Hughes Foundation (USA).

References

Antoniou,M., deBoer,E., Habets,G. and Grosveld,F. (1988) The human β-globin gene contains multiple regulatory regions: identification of one promoter and two downstream enhancers. *EMBO J.*, **7**, 377–384.

Berry,M., Grosveld,F. and Dillon,N. (1992) A single point mutation is the cause of the Greek form of hereditary persistence of fetal haemoglobin. *Nature*, **358**, 499–502.

Caterina,J., Ryan,T., Pawlik,K., Palmiter,R., Brinster,R., Behringer,R. and Townes,T. (1991) Human β-globin locus control region: analysis of the 5′ DNase I hypersensitive site HS2 in transgenic mice. *Proc. Natl Acad. Sci. USA*, **88**, 1626–1630.

Curtin,P., Liu,D., Liu,W., Chang,J. and Kan,Y.W. (1989) Human β-globin expression is enhanced by a distant DNase I hypersensitive site. *Proc. Natl Acad. Sci. USA*, **86**,7082–7086.

Dillon,N. and Grosveld,F. (1991) Human γ-globin genes silenced independently of other genes in the β-globin locus. *Nature*, **350**, 252–254.

Dillon,N. and Grosveld,F. (1993) Transcriptional regulation of multigene loci: multi-level control. *Trends Genet.*, **9**, 134–137.

Driscoll,M., Dobkin,C. and Alter,B. (1989) β-Thalassemia due to a de novo mutation deleting the 5′ β-globin gene activation-region hypersensitive sites. *Proc. Natl Acad. Sci. USA*, **86**, 7470–7474.

Ellis,J. and Grosveld,F. (1993) Nuclear factors regulating erythroid progenitor cell differentiation and gene expression. *Semin. Dev. Biol.*, **4**, 359–369.

Ellis,J., Talbot,D., Dillon,N. and Grosveld,F. (1993) Synthetic human β-globin 5′HS2 constructs function as locus control regions only in multicopy transgene concatamers. *EMBO J.*, **12**, 127–134.

Engel,J.D. (1993) Developmental regulation of human β-globin gene transcription: a switch of loyalties? *Trends Genet.*, **9**, 304–309.

Felsenfeld,G. (1992) Chromatin as an essential part of the transcriptional mechanism. *Nature*, **355**, 219–224.

Forrester,W., Thompson,C., Elder,J. and Groudine,M. (1986) A developmentally stable chromatin structure in the human β-globin gene cluster. *Proc. Natl Acad. Sci. USA*, **83**, 1359–1363.

Forrester,W.C., Epner,E., Driscoll,M.C., Enver,T., Brice,M., Papayannopoulou,T. and Groudine,M. (1990) A deletion of the human β-globin locus activation region causes a major alteration in chromatin structure and replication across the entire β-globin locus. *Genes Dev.*, **4**, 1637–1649.

Fraser,P., Hurst,J., Collis,P. and Grosveld,F. (1990) DNase I hypersensitive sites 1, 2 and 3 of the human β-globin dominant control region direct position-independent expression. *Nucleic Acids Res.*, **18**, 3503–3508.

Fraser,P., Pruzina,S., Antoniou,M. and Grosveld,F. (1993) Each hypersensitive site of the human β-globin locus control region confers a different developmental pattern of expression on the globin genes. *Genes Dev.*, **7**, 106–113.

Gaensler,K.M.L., Kitamura,M. and Kan,Y.W. (1993) Germ-line transmission and developmental regulation of a 150-kb yeast artificial chromosome containing the human β-globin locus in transgenic mice. *Proc. Natl Acad. Sci. USA*, **90**, 11381–11385.

Grosveld,F., Blom van Assendelft,G., Greaves,D.R. and Kollias,G. (1987) Position-independent, high-level expression of the human β-globin gene in transgenic mice. *Cell*, **51**, 975–985.

Groudine,M., Kohwi-Shigematsu,T., Gelinas,R., Stamatoyannopoulou,G. and Papayannopoulou,T. (1983) Human fetal to adult hemoglobin switching: changes in chromatin structure of the β-globin gene locus. *Proc. Natl Acad. Sci. USA*, **80**, 7551–7555.

Gumucio,D.L., Heilstadt-Williamson,H., Gray,T.A., Tarle,S.A., Shelton,D.A., Tagle,D.A., Slightom,J.L., Goodman,M. and Collins,F.S. (1992) Phylogenetic footprinting reveals a nuclear protein which binds to silencer sequences in the human γ and ε globin genes. *Mol. Cell. Biol.*, **12**, 4919–4929.

Hanscombe,O., Whyatt,D., Fraser,P., Yannoutsos,N., Greaves,D., Dillon,N. and Grosveld,F. (1991) Importance of globin gene order for correct developmental expression. *Genes Dev.*, **5**, 1387–1394.

Hebbes,T.R., Clayton,A.L., Thorne,A.W. and Crane-Robertson,C. (1994) Core histone hyperacetylation co-maps with generalized DNase I hypersensitivity in the chicken β-globin chromosomal domain. *EMBO J.*, **13**, 1823–1830.

Jenuwein,T., Forrester,W.C., Qiu,R.-G. and Grosschedl,R. (1993) The immunoglobulin μ enhancer core establishes local factor access in nuclear chromatin independent of transcriptional stimulation. *Genes Dev.*, **7**, 2016–2032.

Jiminez,G., Griffiths,S., Ford,A., Greaves,M. and Enver,T. (1992) Activation of the β-globin locus control region precedes commitment to the erythroid lineage. *Proc. Natl Acad. Sci. USA*, **89**, 10618–10622.

Kioussis,D., Vanin,E., deLange,T., Flavell,R.A. and Grosveld,F. (1983) β-globin gene inactivation by DNA translocation. *Nature*, **306**, 662–666.

Kollias,G., Wrighton,N., Hurst,J. and Grosveld,F. (1986) Regulated expression of human $^{A}\gamma$-, β- and hybrid γβ-globin genes in transgenic mice: manipulation of the developmental expression patterns. *Cell*, **46**, 89–94.

Lee,D.Y., Hayes,J.L., Pruss,D. and Wolffe,A. (1993) A positive role for histone acetylation in transcription factor access to nucleosomal DNA. *Cell*, **72**, 73–84.

Lowrey,C., Bodine,D. and Nienhuis,A. (1992) Mechanism of DNase I hypersensitive site formation with the human β globin locus control region. *Proc. Natl Acad. Sci. USA*, **89**, 1143–1149.

Mastrangelo,I., Courey,A., Wall,J., Jackson,S. and Hough,P. (1991) DNA looping and Sp1 multimer links: a mechanism for transcriptional synergism and enhancement. *Proc. Natl Acad. Sci. USA*, **88**, 5670–5674.

McPherson,C.E., Shim,E.-Y., Friedman,D.C. and Zaret,K.S. (1993) An active tissue-specific enhancer and bound transcription factors existing in a precisely positioned nucleosomal array. *Cell*, **75**, 387–398.

Moi,P. and Kan,Y.W. (1990) Synergistic enhancement of globin gene expression by activator protein-1-like proteins. *Proc. Natl Acad. Sci. USA*, **87**, 9000–9004.

Ney,P.A., Sorrentino,B.P., Lowrey,C.H. and Nienhuis,A.W. (1990) Inducibility of the HSII enhancer depends on the binding of an erythroid specific nuclear protein. *Nucleic Acids Res.*, **18**, 6011–6017.

Peterson,K.R., Clegg,C.H., Huxley,C., Josephson,B.M., Haugen,H.S., Furukawa,T. and Stamatoyannopoulos,G. (1993) Transgenic mice containing a 248-kb yeast artificial chromosome carrying the human β-globin locus display proper developmental control of human β-globin genes. *Proc. Natl Acad. Sci. USA*, **90**, 7593–7597.

Philipsen,S., Talbot,D., Fraser,P. and Grosveld,F. (1990) The β-globin dominant control region: hypersensitive site 2. *EMBO J.*, **9**, 2159–2167.

Philipsen,S., Pruzina,S. and Grosveld,F. (1993) The minimal requirements for activity in transgenic mice of hypersensitive site 3 of the β-globin locus control region. *EMBO J.*, **12**, 1077–1085.

Pruzina,S., Hanscombe,O., Whyatt,D., Grosveld,F. and Philipsen,S. (1991) Hypersensitive site 4 of the human β globin locus control region. *Nucleic Acids Res.*, **19**, 1413–1419.

Ptashne,M. (1986) Gene regulation by proteins acting nearby or at a distance. *Nature*, **322**, 697–701.

Reitman,M., Lee,E., Westphal,H. and Felsenfeld,G. (1993) An enhancer/locus control region is not sufficient to open chromatin. *Mol. Cell. Biol.*, **13**, 3990–3998.

Rivier,D.H. and Pillus,L. (1994) Silencing speaks up. *Cell*, **76**, 963–966.

Ryan,T.M., Behringer,R.R., Martin,N.C., Townes,T.M., Palmiter,R.D. and Brinster,R. (1989) A single erythroid specific DNase I super-hypersensitive site activates high levels of human β-globin gene expression in transgenic mice. *Genes Dev.*, **3**, 314–323.

Spanopoulou,E., Giguere,V. and Grosveld,F.G. (1988) The transcriptional unit of the murine Thy-1 gene unit: Differential distribution of transcriptional initiation sites in brain. *Mol. Cell. Biol.*, **8**, 3847–3856.

Strouboulis,J., Dillon,N. and Grosveld,F. (1992) Developmental regulation of a complete 70 kb human β-globin locus in transgenic mice. *Genes Dev.*, **6**, 1857–1864.

Su,W., Jackson,S., Tjian,R. and Echols,H. (1991) DNA looping between sites for transcriptional activation: self-association of DNA bound Sp1. *Genes Dev.*, **5**, 820–826.

Talbot,D. and Grosveld,F. (1991) The 5′HS2 of the β-globin locus control region enhances transcription through the interaction of a multimeric complex binding at two functionally distinct NF-E2 binding sites. *EMBO J.*, **10**, 1391–1398.

Talbot,D., Collis,P., Antoniou,M., Vidal,M., Grosveld,F. and Greaves,D. (1989) A dominant control region from the human β-globin locus conferring integration site-independent gene expression. *Nature*, **338**, 352–355.

Talbot,D., Philipsen,S., Fraser,P. and Grosveld,F. (1990) Detailed analysis of the site 3 region of the human β-globin dominant control region. *EMBO J.*, **9**, 2169–2178.

Tuan,D., Solomon,W., Li,Q. and London,I. (1985). The 'β-like-globin' gene domain in human erythroid cells. *Proc. Natl Acad. Sci. USA*, **82**, 6384–6388.

Tuan,D.Y.H., Solomon,W.B., London,I.M. and Lee,D.P. (1989) An erythroid-specific, developmental-stage-independent enhancer far upstream of the human 'β-like globin' genes. *Proc. Natl Acad. Sci. USA*, **86**, 2554–2558.

Wallrath,L.L., Lu,Q., Granok,H. and Elgin,S.C.R. (1994) Architectural variations and inducible eukaryotic promoters: preset and remodeling chromatin structures. *BioEssays*, **16**, 165–170.

Received on August 10, 1995; revised on October 16, 1995