Cell, Vol. 87, 105–114, October 4, 1996, Copyright ©1996 by Cell Press

Heterochromatin Effects on the Frequency and Duration of LCR-Mediated Gene Transcription

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Summary

Locus control regions (LCRs) are responsible for initiating and maintaining a stable tissue-specific open chromatin structure of a locus. In transgenic mice, LCRs confer high level expression on linked genes independent of position in the mouse genome. Here we show that an incomplete LCR loses this property when integrated into heterochromatic regions. Two disruption mechanisms were observed. One is classical position-effect variegation, resulting in continuous transcription in a clonal subpopulation of cells. The other is a novel mechanism resulting in intermittent gene transcription in all cells. We conclude that only a complete LCR fully overcomes heterochromatin silencing and that it controls the level of transcription by ensuring activity in all cells at all times rather than directly controlling the rate of transcription.

Introduction

Nuclear processes such as transcription, replication, and recombination can be affected by chromatin structure (e.g., Pillus and Grunstein, 1995). The genome can be roughly divided in two major chromatin states based on cytological observation (Heintz, 1928; Eissenberg et al., 1995). Euchromatin appears decondensed in interphase, whereas heterochromatin is highly condensed throughout the cell cycle. Euchromatic regions that contain most of the genes and unique sequences replicate early in S phase. Heterochromatin replicates late in S phase (Holmquist, 1987) and contains middle and highly repetitive sequences (John and Miklos, 1979). The largest regions of heterochromatin appear near the centromeres and in the inactivated X chromosome. Studies in Drosophila and yeast have shown that heterochromatin can have effects on juxtaposed euchromatic regions, suggesting that chromatin condensation can spread from heterochromatin into regions of euchromatin (Wilson et al., 1990). Stochastic heterochromatinization of juxtaposed euchromatic regions, which results in stably inherited gene silencing in a clonal subpopulation of cells, is known as position-effect variegation (PEV; for review see Karpen, 1994). Evidence that PEV indeed results from heterochromatinization has come from genetic studies of specific genes that encode proteins involved in heterochromatin formation and have been shown to enhance or suppress these position effects (Pirrotta, 1995; Orlando and Paro, 1995). Although the dynamics of formation of the open and closed states of a locus are still obscure, it is clear that some genomic elements and nuclear factors can influence the chromatin configuration. In mammals, position effects that result in the stable silencing of genes in a clonal subpopulation of cells have also been observed (Elliot et al., 1995; Festenstein et al., 1996).

Another type of position effect frequently observed in transgenic experiments is thought to be due to the action of regulatory elements at the site of integration. This can result in both positive and negative effects on the transgene. Alternatively, the regulatory elements on the transgenic construct may interact with a gene located at the site of integration and result in a lower level of transgene expression (see Milot et al., 1996). These non-PEV effects result in a change in the level of expression in all of the expressing cells.

An increasing number of gene loci and gene clusters have been shown to contain *cis* elements known as locus control regions (LCRs), which are thought to be responsible for initiating or maintaining (or both) a cell type–specific open chromatin structure within a specified domain. The most thoroughly characterized LCR is that of the human β-globin locus (Grosveld et al., 1993). It consists of a series of five DNase I hypersensitive site (HS) regions located upstream of the globin cluster (Figure 1) each containing a number of binding sites for ubiquitous and erythroid-specific DNA-binding factors. Expression of β-globin transgenes without an LCR were found to be low, variable, and dependent on the position of integration in the host genome. Inclusion of LCR sequences in β-globin transgene constructs led to reliable, high level, copy number–dependent expression irrespective of position in the host genome (Grosveld et al., 1987). This is in agreement with the study of patients with deletions of the LCR. The globin genes in the otherwise normal locus were found to be insensitive to DNase I, transcriptionally inactive (Kioussis et al., 1983; Forrester et al., 1990), and replicated late in S phase (Aladjem et al., 1995).

The human β-globin LCR is thought to overcome position effects through a dominant positive activity rather than through an insulator function (Schedl and Grosveld, 1995). Recent studies demonstrate that the individual globin genes compete for LCR function and that the LCR activates only one gene at a time (Wijgerde et al., 1995), probably through direct interaction between the LCR and the gene via DNA looping (Dillon et al., submitted). These results suggested that the relative levels of

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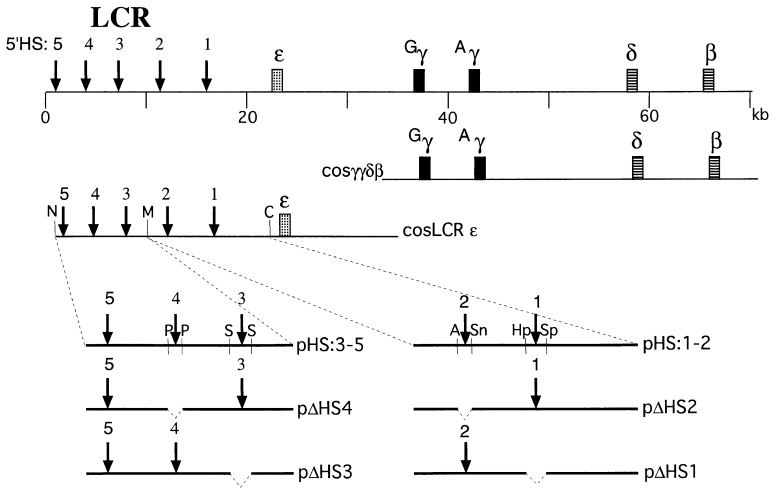

Figure 1. Construction of 70 kb Globin Locus LCR Deletion Mutants

The top line shows the 70 kb human globin locus construct. The individual HS regions of the LCR (5′HS:1–5) are designated with arrows and the globin genes with boxes. The two cosmids (cos LCR ϵ and cos γγδβ; Strouboulis et al., 1992a, 1992b) used to create the 70 kb construct are shown, as are the positions of restriction sites used to make the LCR plasmid subclones shown at the bottom. Individual HS deletions were created in the plasmids pHS:3–5 and pHS:1–2 using the restriction sites shown. Individual HS deletion plasmids were incorporated into cos LCR ϵ, which was then linked to cos γγδβ to create the injection constructs as described by Strouboulis et al. (1992b). Restriction sites are as follows: N, NotI; M, MluI; C, ClaI; P, Pm1I; S, SauI; A, ApaLI; Sn, SnaBI; Hp, HpaI; Sp, SpeI.

gene expression in an active locus is (at least in part) determined by the duration of the interaction of the LCR with each of the genes. In this paper, we report the results obtained with transgenic mice containing the entire globin locus with LCR HS deletions. The results show that deletion of an individual HS from the LCR leads to severely reduced expression of the globin genes in some mouse lines. In situ hybridization analysis reveals that this loss of position independence is caused by integration at or near the centromere, resulting in pancellular and heterocellular position effects. We conclude that the LCR must be complete to overcome heterochromatin silencing and that it does so by ensuring that the locus is active all of the time in all of the (red) cells.

Results

Generation of LCR Deletion Transgenic Mice

The preparation of 70 kb LCR deletion DNA fragments for microinjection was carried out according to the cosmid linking method of Strouboulis et al. (1992a, 1992b). Four different locus constructs were produced with small deletions of HS:1, HS:2, HS:3, or HS:4 (Figure 1). The constructs were microinjected into the pronuclei of fertilized mouse eggs to produce transgenic founder mice. Tail DNA from founder mice was analyzed on Southern blots by probing with the entire 70 kb locus construct (data not shown). Those founder mice, which contained all of the EcoRI fragments characteristic of the entire locus, were selected for further breeding. F1 and F2 mice were similarly analyzed, and, in addition, end fragment, junction fragment, and internal probes were used to verify the structure and estimate the copy number. Transgenic line 72 was used as the control. This line contains a single copy of the human globin locus and expresses the human β-globin transgene at a level similar to that of the endogenous mouse globin genes (Strouboulis et al., 1992a). The mouse Thy-1 gene was used as an internal loading control. Copy numbers ranged from 1 to approximately 12 copies.

We prepared RNA from F2 transgenic 10.5 day whole embryos, 12.5, 14.5, and 16.5 day fetal livers, and adult blood and determined the level of mRNA by S1 nuclease protection assays. Each sample was assayed with the same mixture of six probes for mouse, ϵy, βh1, β-major, and human, ϵ, γ, and β (see Experimental Procedures). S1 analyses are shown in Figure 2 for one transgenic line from each construct as well as transgenic line 72, which contains the full locus as a single integrated copy. The intensity of each S1 protected band was corrected for probe-specific activity and copy number and is presented in Figure 3 as a percentage of endogenous globin gene expression.

HS Deletions Affect Globin Gene Expression

The two HS:1 deletion lines (Δ1A, containing 1 copy of the human locus, and Δ1B, containing 2 copies) show widely varying levels of expression of the globin genes per copy, indicating a loss of copy number dependence. Δ1A globin gene expression is very low, ranging from 2%–10% per copy, whereas Δ1B expresses near normal levels when compared with line 72 (Figures 2 and 3). These results indicate that deletion of HS:1 may result in expression that is sensitive to the position of integration in the mouse genome. Δ1B shows the level of expression that would be expected on the basis of the previous data obtained for HS:1. HS:1 itself is known to have very little if any transcriptional activity (Collis et al., 1990; Fraser et al., 1990), and a patient with a deletion of HS:1 from the LCR has normal levels of β-globin expression (Kulozik et al., 1991). More interesting is the result obtained for the Δ1A line, which suggests that 5′HS:1 is nevertheless an essential component of the LCR to provide position-independent expression of the globin locus when taken out of the context of its normal chromosomal position (see below).

Based on previous data with the individual HS:2 (Talbot et al., 1989, 1990; Curtin et al., 1989; Ryan et al., 1989; Fraser et al., 1990, 1993; Morley et al., 1992) and the deletion of the mouse β-major globin HS:2 by homologous recombination (Fiering et al., 1995), we expected that the deletion of HS:2 would result in a mild (20%–30%) reduction in transcription of the genes in the locus. However, the four lines containing the HS:2 deletion construct (Δ2A, 8 copies; Δ2B, 3 copies; Δ2C, 1 copy;

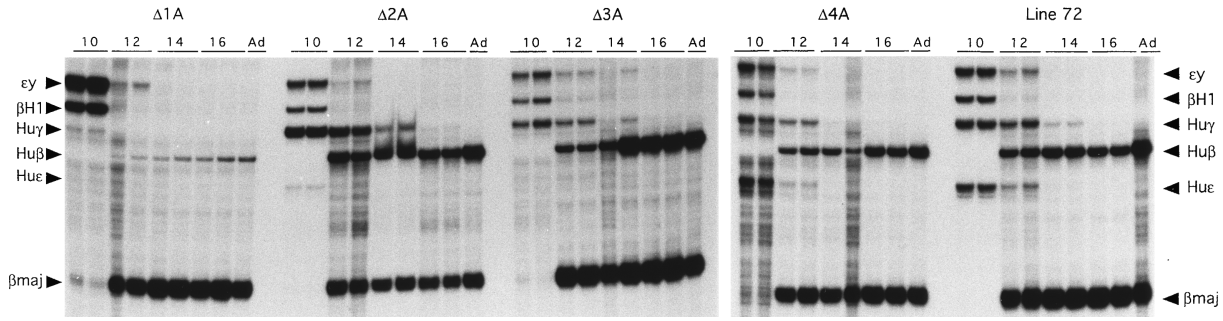

Figure 2. S1 Nuclease Protection Assays

S1 nuclease protection assays are shown for one transgenic line for each of the deletion constructs (Δ1A, 1 copy; Δ2A, 8 copies; Δ3A, 4 copies; Δ4A, 1 copy) used and line 72 (1 copy). RNA was prepared from transgenic 10 day whole embryos, 12, 14, and 16 day fetal livers, and adult blood as described in Experimental Procedures. Two samples for each timepoint except adult blood are shown. All samples were assayed with the same mixture of six radiolabeled probes for mouse εy, βH1, and β-major and human ε, γ, and β; the ratio of probe-specific activities was 0.8:0.8:0.8:3.3:1:1, respectively. The position of the protected fragments for each probe are indicated.

and Δ2D, 1 copy) show varying levels of expression per copy, indicating a loss of copy number dependence and hence position dependence (Figures 2 and 3). The most striking examples of position sensitivity of the Δ2 lines are Δ2C and Δ2D because they show a different effect for different genes in the locus. Δ2D shows appreciable levels of γ-globin expression in the embryonic period, but very low levels of human β-globin in the fetal liver and adult stages. In contrast, transgene expression in the Δ2C line is detectable only in the adult, where human β-globin expression is approximately 6% of the mouse β-major globin. The other two Δ2 lines (Δ2A and Δ2B) show a reduction in expression of all the human globin genes at all stages. Hence, HS:2 also appears to be crucial for the function of the LCR when integrated at another position in the genome (see below).

The transgenic mice containing the HS:3 deletion construct (Δ3A, 4 copies; Δ3B, 4 copies; Δ3C, 3 copies; and

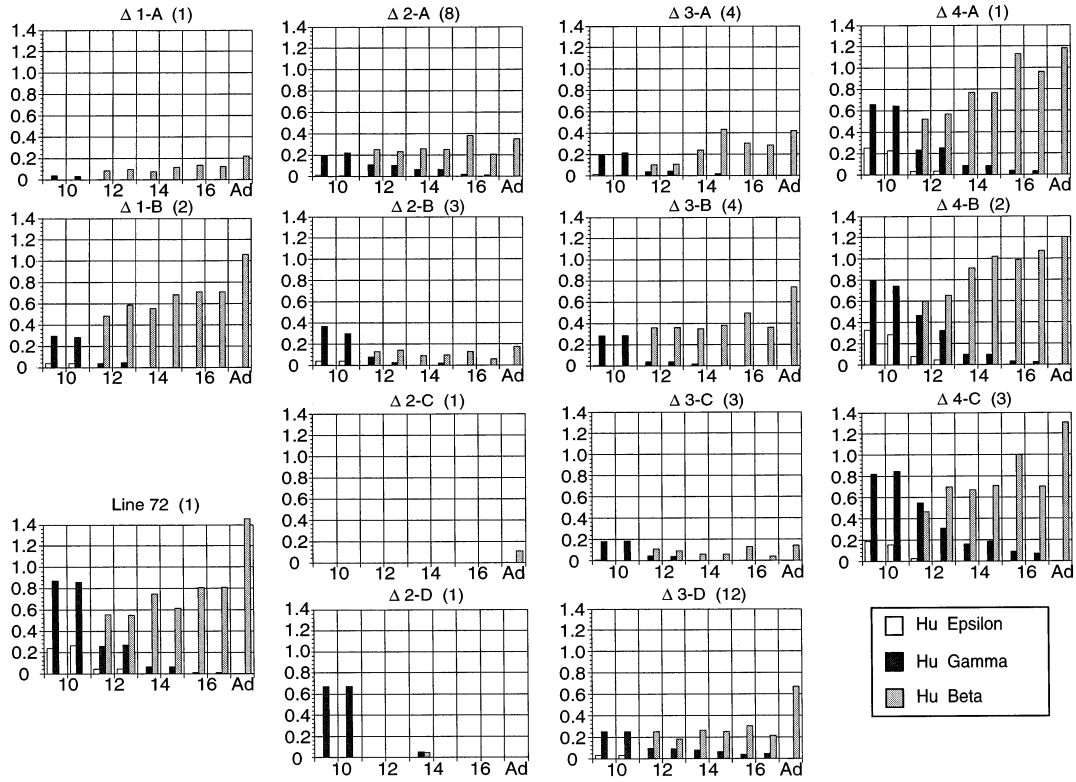

Figure 3. Quantitation of S1 Assays for All Transgenic Lines

S1 assays were quantitated by phosphorimage analysis and corrected for probe-specific activity and copy number of the transgenic line. Copy numbers are indicated in parentheses. The values shown are the per copy levels of expression of the human genes as a ratio of the endogenous mouse globin genes (human gene/copy number/[εy + βH1 + β-major]).

Δ3D, 12 copies) were expected to show at least the same or greater reduction in expression, in particular of the embryonic and fetal genes (Fraser et al., 1990, 1993; Philipsen et al., 1990, 1993; Hug et al., 1996). Indeed, the results show a general reduction in expression of all the human genes, but also a lack of copy number dependence (Figures 2 and 3). Δ3C in particular is expressing at less than half the level per copy of the other Δ3 lines. This suggests that deletion of HS:3 from the LCR also causes susceptibility to position effects (see below).

Three lines contain the HS:4 deletion construct (Δ4A, Δ4B, and Δ4C) with copy numbers of 1, 2, and 3, respectively. These lines appear to express the globin genes in a position-independent and copy number–dependent manner, as the level of expression per copy is similar to line 72.

Chromosomal Position of Transgenes

The results of the S1 analysis showed that the LCR deletion transgenic lines express the human globin genes at lower levels than expected. In addition, the levels of expression do not correlate with the number of copies integrated in the mouse genome. In particular, transgenic lines Δ1A, Δ2B, Δ2C, Δ2D, and Δ3C show extremely low levels of expression per copy when compared with line 72 or the other deletion lines. Studies in Drosophila and yeast have shown that chromosomal position can influence gene expression. To determine whether reduced gene expression in these mice can be correlated with chromosomal position, we performed fluorescence in situ hybridization (FISH) analysis on metaphase chromosomes from ten LCR deletion transgenic lines (lines Δ1B and Δ2D were lost before FISH analysis; Δ3D was not done). All four of the very low expressing lines (Δ1A, Δ2B, Δ2C, and Δ3C; Figure 3) were integrated at or near the centromere (Figure 4A). This suggests that pericentromeric integration of an incomplete LCR construct results in severely reduced gene expression. Such a position effect may be caused by heterochromatin spreading as is thought to occur in PEV. Since FISH on metaphase chromosomes is a crude method for determining the precise chromosomal location of a transgene, we refined these results by using a second probe for the mouse α-satellite repeat (Figure 4B), which is located primarily in centromeres. All four lines Δ1A, Δ2B, Δ2C, and Δ3C are integrated very close to or contacting the α-satellite DNA, whereas the next closest integrant (Δ2A) is clearly separate (Figure 4B).

The accumulated data from a number of laboratories present a large number of complete LCR transgenics that express their linked transgenes in a copy number–dependent, position-independent manner, suggesting that the full LCR is able to overcome centromeric position effects. This would not be expected on a statistical basis if centromeric silencing of the complete LCR were to occur. However, the chromosomal position of full LCR transgenic lines had not previously been reported, and hence we analyzed the chromosomal position of the eight full LCR transgenic lines available in our laboratory (Strouboulis et al., 1992a; Dillon et al., submitted; J. Gribnau et al., unpublished data). All were integrated in noncentromeric locations with the exception of line 2 (Strouboulis et al., 1992a), which is integrated close to a centromere (Figures 4A and 4B). This line, which contains a single copy of the 70 kb locus, expresses the human globin genes normally.

In Situ mRNA Analysis

In view of the well-characterized PEV observed in Drosophila and yeast, we next addressed the question of whether the position effect we observed in the LCR deletion lines was due to a reduction of the number of cells that express the transgene (as expected for PEV) or whether the expression level in each cell had been affected. To this end, in situ hybridization and indirect immunofluorescence were performed on disrupted fetal liver cells from the four position-effect lines as well as on other LCR-deletion and complete LCR lines with specific probes for human and mouse β-globin mRNA. The results show that in all lines tested, with the exception of Δ2B and Δ2C (Figure 5), the human transgene is expressed in all erythroid cells, since every cell that contains mouse β-major globin mRNA also contains human β-globin mRNA (Figure 5). In the two position-effect LCR deletion lines, Δ2B and Δ2C, the transgene shows variegated expression patterns, as human β-globin mRNA is present in only 25% and 4% of the cells, respectively, of every erythroid fetal liver examined. In situ hybridization of individual colonies from methyl cellulose cultures of line Δ2B fetal liver cells indicates that expression status is clonally inherited (data not shown). We conclude that the reduced level of transgene expression seen in these two lines results from clonally inherited PEV caused by centromeric heterochromatinization.

DNase I and Restriction Enzyme Sensitivity

Since the FISH and in situ mRNA analyses revealed that Δ2B and Δ2C were in heterochromatic regions and subject to PEV, whereas all other lines expressed the genes in all erythroid cells, it could be expected that a difference in DNase I sensitivity would be observed between the different lines. Expression of the locus in erythroid cells normally correlates with the presence of DNase I HS in the LCR and an HS in the promoter of the β-globin gene (Tuan et al., 1985; Forrester et al., 1987; Grosveld et al., 1987). We therefore tested for the presence of the erythroid-specific HS:5 (Zafarana et al., 1995) in the LCR (present in all constructs) and the HS found in the promoter of the β-globin gene using the mouse β-major gene promoter as a control (Table 1). Only lines Δ2B and Δ2C (the PEV lines) showed severely reduced or an absence of sensitivity, whereas all other lines, including Δ1A and Δ3C (severe PE), showed the LCR and promoter sites. We also assayed restriction enzyme sensitivity using ApaLI digestion in isolated nuclei from lines Δ1A and Δ2B and line 72. ApaLI cuts at +50 in exon 1 of the human β-globin gene. As expected from the DNase I results, ApaLI sensitivity in line 72 and Δ1A appeared similar, with approximately 60% digested, whereas the PEV line Δ2B showed a reduction in ApaLI sensitivity, with less than 20% digested (data not shown). These results suggest that the position effect observed in Δ2B and Δ2C is indeed caused by a

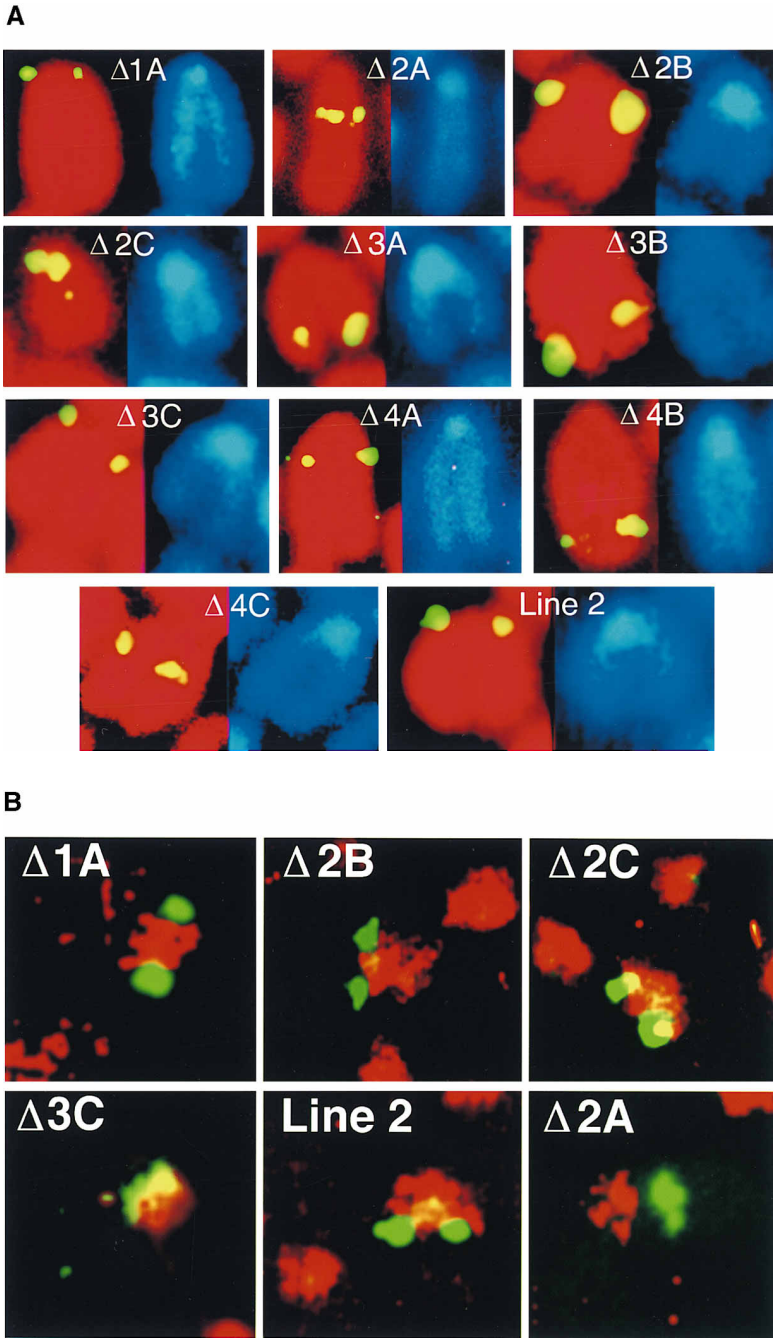

Figure 4. FISH Analysis of Transgenic Lines

(A) Left panels (red) show the human β-globin transgene locus detected by in situ hybridization of metaphase chromosomes of adult bone marrow cells with the complete insert as a probe (see Experimental Procedures). Right panels show DAPI staining (blue); centromeres correspond to the light blue areas. Transgenic line numbers are indicated on top of each sample.

(B) Double-label FISH of bone marrow metaphase chromosomes from the pericentromeric lines using probes for the human globin locus (green) and mouse α-satellite DNA (red).

closed chromatin conformation such as seen in heterochromatin, but they do not give an indication about the nature of the position effect observed in Δ1A and Δ3C.

In Situ Primary Transcript Analysis

Similar to the full LCR line 72, the two low expressing pericentromeric lines Δ1A and Δ3C express the human transgene in all erythroid cells. We decided to investigate the reduced expression levels of these two lines further by comparing the transcriptional status from several lines. We performed primary transcript in situ hybridizations with gene-specific intron probes for human β-globin and mouse β-major globin in 13.5 day fetal liver cells (Wijgerde et al., 1995). Lines Δ1A and Δ3C have significantly lower percentages (25% and 75%, respectively) of erythroid cells with human β-globin primary transcript signals (Figure 6; Table 2). The fact that all erythroid cells in these lines have human β-globin mRNA in their cytoplasm (Figure 5) demonstrates that the transgenes are active in each cell. The low percentage of transcriptionally active cells indicates that each transgene is transcribed for only a fraction of the normal time.

As expected, the PEV line Δ2B had human primary transcript signals in only 25% of erythroid fetal liver cells (Table 2), consistent with the percentage of erythroid

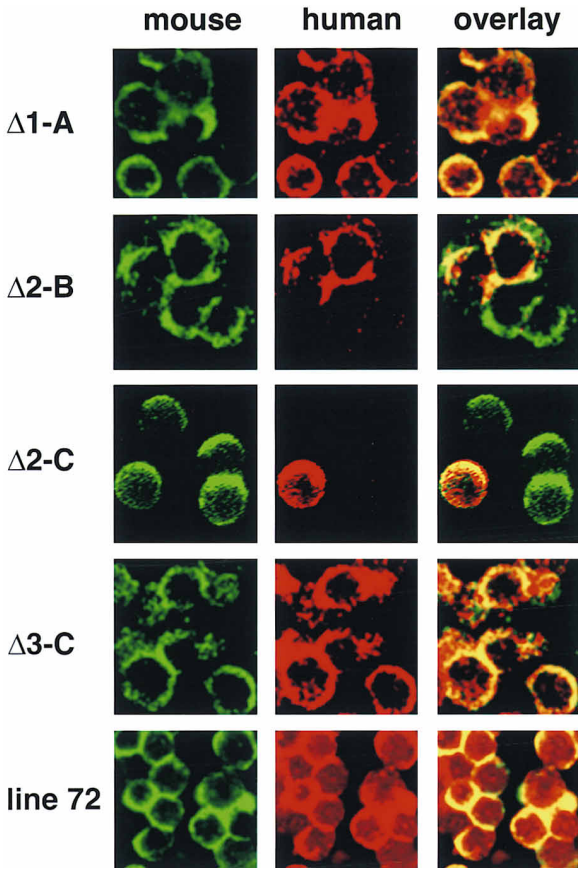

Figure 5. mRNA In Situ Hybridization

Left panels (green) show the in situ hybridization of 13.5 day fetal liver cells using a mouse β-major mRNA digoxigenin-labeled oligonucleotide from the third exon as the probe (Wijgerde et al., 1995). Middle panels show detection in the same cells of a deoxyribonucleoprotein (DNP)-labeled human β-globin third exon oligonucleotide probe (Wijgerde et al., 1995). Right panels show the overlay of the two images. Transgenic mouse line numbers are shown on the left.

cells that contained human β-globin mRNA (Figure 5). The full LCR control line 72 had β gene transcription signals in 87% of the erythroid cells as expected (10%–15% of loci transcribe the γ- or δ-globin genes at this stage [M. W. et al., unpublished data]). The highly expressing LCR deletion lines (Δ4A, Δ4B, and Δ4C) had positive transgene transcription signals in a high percentage of erythroid cells (Table 2). In the single-copy line Δ4A, the percentage of cells positive for β-globin transcription is similar to the single-copy line 72. The percentages approach 100% in the multicopy Δ4 lines (Δ4B and Δ4C) as expected, suggesting that the β-globin loci in these lines are continually active. Of potential significance is the fact that the multicopy lines Δ2A and Δ3B do not approach 100%, as would be expected if they were transcribing normally as in the Δ4 lines. This result suggests that they too may be transcribed for only part of the time.

Stochastic versus Timing Phenomenon

In an attempt to characterize further the novel position effect observed in Δ1A and Δ3C, we bred the PEV line Δ2B and the timing effect line Δ1A to homozygosity. These two lines were chosen because the percentage of erythroid cells that had human globin transcription foci were the same in both lines (25%). If transgene activation is random, as is expected for PEV (Elliot et al., 1995), and the individual loci in a homozygous cell are stochastically activated or silenced, then the number of transcriptionally positive erythroid cells should increase from 25% to 44%. Most of these cells should have only one active locus and approximately 6% should have both transgene loci active. Transcription was analyzed via primary transcript in situ hybridizations for human and mouse β-globin. As expected for the PEV line (Δ2B), the percentage of transgene-expressing cells increased significantly in the homozygous fetal liver, from 25% to 43%. Most of these cells (74%) had only one chromosome actively transcribing the human genes. A quarter of these cells (26%) showed active transcription originating from both transgene chromosomes. These results are what would be expected from a stochastic activation of the globin transgene. In contrast, the percentage of transcription-positive cells in the Δ1A line did not increase significantly in the homozygotes (25%–31%), and surprisingly 80% of these cells show active transcription from both transgene loci. This result is the complete opposite of the PEV result. Therefore, locus activation in Δ1A appears to be a cellular timing phenomenon, since both chromosomes are activated simultaneously in a single cell and not stochastically at the level of the individual locus as in PEV. These results suggest that differentiation stage or cell cycle phase may be important in controlling heterochromatin-mediated silencing of the Δ1A transgene.

Table 1. DNase I Sensitivity

Transgenic Line	Human LCR HS:5	Human β-Globin Promoter	Mouse β-Major Globin Promoter
Line 72[a]	+	+	+
Δ1A[b]	+	+	+
Δ2A	+	+	+
Δ2B[b, c]	–	–	+
Δ2C[b, c]	–	–	+
Δ3B	+	+	+
Δ3C[b]	+	+	+
Δ4B	+	+	+
Δ4C	+	+	+

The DNase I hypersensitivity analysis was carried out on isolated nuclei from 13.5 day fetal liver cells. The presence or absence of LCR HS:5 or the HS in the human or mouse β-globin promoters is indicated by a plus or minus sign. The left column shows the transgenic mouse line number. The probes used are described in Experimental Procedures.

[a] Complete LCR.

[b] Pericentromeric location.

[c] PEV lines.

Discussion

We have analyzed the developmental expression pattern of the 70 kb human β-globin locus constructs in transgenic mice after deletion of individual HS from the LCR. Of 13 transgenic lines presented, at least 5 exhibit

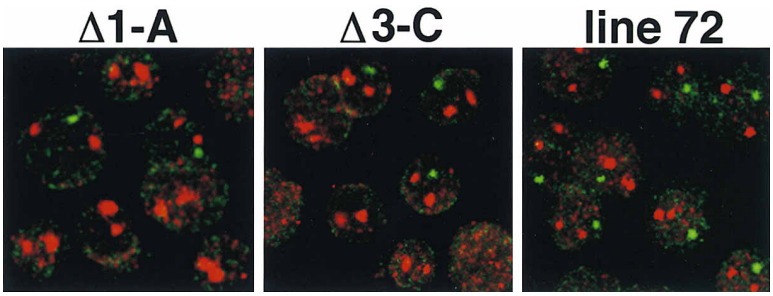

Figure 6. Primary Transcript In Situ Hybridization Analysis

In situ hybridization with gene-specific intron probes as described by Wijgerde et al. (1995). Red foci represent mouse β-major globin primary transcript (DNP labeled); green foci represent human β-globin primary transcript (digoxigenin). Transgenic mouse line numbers are indicated at the top.

extremely low levels of expression per copy. This demonstrates that deletion of a single HS can result in loss of position independence and copy number dependence, which are the major distinguishing features in the functional definition of an LCR (Grosveld et al., 1987), and suggests that expression of these transgenes is being influenced by the chromatin environment of the integration locus. The exceptions to this loss of function are the HS:4 deletion lines, which appear to show copy number–dependent and position-independent expression. However, with only three lines, it is possible that the transgenes in these mice have fortuitously landed in favorable chromosomal environments, and as a result the expression patterns observed appear position insensitive (Figure 4). However, others have shown that deletion of HS:4 from the locus can also result in loss of function (Bungert et al., 1995).

FISH analysis revealed that the transgenes in the lowest expressing lines were integrated at or near the centromere in each case. This type of pericentromeric localization of genes has been shown to lead to heterochromatin-induced silencing in Drosophila and yeast and, more recently, in mammals in the case of the CD2 LCR (Festenstein et al., 1996). We show through in situ analyses that reduced expression of pericentromeric transgenes can be accomplished by at least two functionally distinct mechanisms. The first is PEV, which results in a clonal subpopulation of erythroid cells that express the human globin genes. As a consequence, the levels of mRNA, DNase sensitivity, and restriction enzyme sensitivity have been severely decreased in the total population of cells. The second type of position effect exhibits low levels of transgene expression in all erythroid cells as evidenced by the S1 and mRNA in situ analysis. However, this low level of expression appears not to be caused by a general decrease in the rate of transcription of the individual genes. Rather, precursor RNA analysis reveals that only a fraction of the erythroid cells are transcribing the transgene at any moment, suggesting that low expression in these lines is caused by a decrease in the amount of time that the transgene is transcriptionally active in a particular cell. DNase I hypersensitivity and restriction enzyme sensitivity are maintained in these lines, suggesting that this position effect is caused by a chromatin conformation that differs from PEV. This result also suggests that DNase I hypersensitivity is not proportionally linked to transcription. Analysis of homozygous animals confirms that transcriptional activation is a cell-timing phenomenon and is not stochastic as in PEV. This presents two intriguing possibilities. First, we may be observing a window of reorganization of the genome during the cell cycle. Thus, the transgenes may be switching on briefly after disruption of higher order structure and then off again as heterochromatin spreads into the locus, or vice versa. Second, the transgene locus may be silenced prior to terminal differentiation of the red cells, which could account for the difference in transcriptional frequency observed between the transgene and endogenous loci.

Table 2. Percentage of Erythroid Cells Positive for Human Globin Transcription Foci

Transgenic Line	Copy Number	$\frac{\text{Human } \beta \text{ Foci}}{\text{Mouse } \beta \text{ Foci}} \times 100$
Line 72[a]	1	87
Δ1A[b]	1	25
Δ2A	8	89
Δ2B[b, c]	3	25
Δ3B	4	83
Δ3C[b]	3	75
Δ4A	1	86
Δ4B	2	97
Δ4C	3	95

Transcription foci were detected for mouse and human β-globin genes in heterozygous 13.5 day fetal liver cells as in Figure 6. The percentage of erythroid cells (i.e., cells positive for β-major transcription) that show transcription foci for the human β-globin gene are shown for various transgenic lines.

[a] Complete LCR.

[b] Pericentromeric location.

[c] PEV line.

The cell-timing effect may also be seen to a lesser extent in the noncentromeric LCR deletion transgenes (Δ2A [8 copies] and Δ3B [4 copies]). For example, transcription in situ analysis of line Δ3B shows that even though this line contains 4 copies of the transgenic locus, the percentage of erythroid cells that are transcriptionally positive for human β-globin is slightly lower than the single-copy full LCR line 72. This suggests that the LCR of each locus in these multicopy deletion lines spends a considerable amount of time uncoupled from the genes. The length of time that the LCR remains uncoupled appears to be exaggerated in the pericentromeric lines, which suggests that heterochromatin formation is somehow interfering with LCR activation, leading to the severe reduction in expression. Clearly this process is very different from PEV.

The results to date have shown that the individual HS of the LCR appear additive, as most or all were necessary for full expression (see Grosveld et al., 1993). The data presented here suggest that this additive effect is not accomplished through an increase in the density of

polymerases on an individual gene, but (at least in part) through an increase in the stability of the LCR–gene complex leading to increased frequency and duration of transcription periods. These results may seem to be in contradiction with the recently published analysis of deletions of the mouse β-major globin HS:2 and HS:3 in their native positions in the mouse genome (Fiering et al., 1995; Hug et al., 1996), both of which result in a 30% reduction in the expression of the mouse adult β genes only. Those experiments cannot measure position independence, since the mouse β-globin locus is maintained at its original position in the genome. Our observations suggest that the reduced expression seen in those knockout lines may be caused by a decrease in the stability of the LCR–gene complex leading to a reduction in frequency or duration of transcription. In this respect, it is worth noting that the individual deletions have essentially the same effect and that the effect is greatest on the most distal genes.

The constructs described here are subject to an inherent selection since the constructs are capable of very high levels of expression. As a result, the integration of multiple copies in areas of the genome that permit expression is lethal (Hanscombe et al., 1989). Thus, there is a bias toward survival of transgenic lines in which integration occurs in areas of the genome that reduce the level of expression. This is supported by the inherent difficulty in obtaining mouse lines that contain intact multiple copies of the complete locus (see also Strouboulis et al., 1992a; Dillon et al., submitted) and may explain why a higher than expected number of our lines have insertions in or near the centromere.

The results have important implications for the proposed mechanisms of LCR activation of gene expression. We have previously proposed that the different HS regions of the LCR may act together as a single functional unit that interacts with a single gene in the locus at any given time. This may be through formation of a holocomplex in which the individual HS must first interact among themselves to form a larger complex, which becomes the limiting element in gene competition. Clearly, deletion of one of the HS from the LCR results in position sensitivity and loss of copy number dependence, which suggests that the deletions may disrupt the normal interactions between the HS and the genes or formation of a holocomplex. This disruption appears to prevent the normal dominant positive effect of the LCR from overcoming negative effects that are thought to occur in heterochromatic regions of the genome.

Hence, we suggest that initiation of transcription from a competent gene in the "open" chromatin domain of the globin locus originates only through direct complex formation with the LCR and maintenance of that transcription requires continued association (see also Wijgerde et al., 1995). Deletions that decrease the stability of LCR–gene interactions decrease the frequency or shorten the duration (or both) of such associations, thereby reducing the transcriptional output from a given gene by decreasing the period of activity. This effect is most obvious in heterochromatic regions of the genome where the LCR spends a significant amount of time uncoupled (i.e., not activating any globin gene). Therefore, the complete LCR determines the level of gene expression by determining the frequency and the duration of transcription periods, rather than only controlling the rate of transcription.

Experimental Procedures

Construction of Human β-Globin Locus LCR Deletion Constructs

The human β-globin LCR was subcloned in two parts in pBR322. HS:4 was removed from the plasmid containing the 5′ half of the LCR by deletion of an 875 bp Pm1I fragment that included the entire HS:4 core fragment as defined by Pruzina et al. (1991) to give plasmid ΔHS:4. HS:3 was deleted by removal of a 1.38 kb SauI fragment that includes the HS:3 core as defined by Philipsen et al. (1990) to give plasmid ΔHS:3. The HS:2 core (Talbot et al., 1990) was removed by deletion of a 742 bp ApaLI–SnaBI fragment to give plasmid ΔHS:2. The ΔHS:1 plasmid was created by deletion of a 1.7 kb HpaI–SpeI fragment that includes 919 bp from the 1.02 kb HS:1 fragment used by Talbot et al. (1989). The four 70 kb LCR deletion constructs were prepared by incorporating the deletion plasmids into cosmid (cos) LCR ϵ, which was linked to the 3′ cosmid (cos γγδβ) and purified for microinjection as described by Strouboulis et al. (1992).

Transgenic Mice

Purified fragments were microinjected into the pronuclei of FVB fertilized mouse eggs and transferred into the oviducts of pseudopregnant (CAB × C57B1) F1 female mice. Transgenic founders were identified via Southern blotting of tail DNA. Transgenic DNA was probed with the 70 kb injection fragment on Southern blots. Those that contained all the EcoRI fragments characteristic of the entire locus were selected for further breeding and analysis. Copy numbers were determined by quantitation of Southern blots via phosphorimage analysis. End fragment, junction fragment, and internal probes were used to determine the copy numbers and verify the structure of the transgenes.

Preparation of RNA and S1 Nuclease Protection Assays

RNA was prepared from whole, frozen, transgenic 10.5 day embryos and fetal livers from 12.5, 14.5, and 16.5 day and adult blood and subjected to S1 nuclease protection assays as previously described (Fraser et al., 1990). The probes used were those described by Lindenbaum and Grosveld (1990) with the exception of the human γ-globin probe. The human γ-globin probe was a 315 bp AvaII fragment taken from a plasmid containing the human A γ-globin cDNA ligated to the human A γ-globin 5′ flanking region. Human γ-globin RNA protects a 165 bp fragment of this probe from S1 nuclease cleavage. Protected bands on the S1 gels were quantitated via phosphorimage analysis (Molecular Dynamics).

DNA FISH Analysis

Extracted bone marrows were cultivated for 24 hr in RPMI 1640 medium (GIBCO BRL). Chromosome preparations were made according to standard procedures. FISH was carried out as described by Mulder et al. (1995). The probes used were the linked human β-globin locus (70 kb fragment) or a mouse minor satellite dimer (gift of A. Mitchell). The probes were labeled with biotin and digoxygenin and immunochemically detected with fluorescein or Texas red. The DNA was counterstained with DAPI.

HS Assay

Ten transgenic fetal livers (13.5 days) for each line were treated as described by Forrester et al. (1990). Suspension of nuclei was performed by 20 strokes of a Dounce pestle (type B) and 100 ml aliquots were digested for 3 min at 37°C with an increasing amount of DNase I. Reactions were stopped and treated with proteinase K and extracted with phenol–chlorophorm. After ethanol precipitation, the pellet was resuspended in 100 μl of water. We then digested 30 μl with EcoRI, followed by Southern blot analysis. Hybridizations were performed with the EcoRI fragment covering 5′HS:5, BamHI–EcoRI human βIVS II, or with an XbaI–Sau3A fragment covering the mouse β-major promoter.

mRNA and Primary Transcript In Situ Hybridization

Primary transcript and mRNA in situ hybridizations to detect transcriptional activity and expression of the human and mouse β-globin genes in fetal liver cells were performed as described by Wijgerde et al. (1995). Quantitation of transcription signals was done by counting at least 1000 cells from each line with an epifluorescence microscope. The figures presented were created with a laser scanning confocal microscope.

Acknowledgments

We are grateful to Lien Braam, Joost Gribnau, David Whyatt, David Michalovich, and Sara Pruzina. We thank A. Mitchel for the mouse satellite probe. E. M. was supported by a Medical Research Council (MRC) (Canada) postdoctoral fellowship and J. S. by the MRC (United Kingdom). This work was supported by the NWO (Netherlands), the Howard Hughes Foundation, and the European Union (Biotech).

Received January 22, 1996; revised August 8, 1996.

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