

Activation by locus control regions?

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On the basis of homologous recombination experiments to delete the murine β -globin locus control region (LCR) in embryonic stem cells, it was recently suggested that the LCR is not required for the activation of the murine β -globin locus. This conclusion is in direct contradiction to the findings and conclusions that have been obtained with the human β -globin LCR; thus the murine and human LCR may functionally be different or there may be a different interpretation of the results.

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Abbreviations

HS	hypersensitive site
LCR	locus control region
EKLF	erythroid Krüppel-like-factor
ES	embryonic stem
PEV	position effect variegation

Introduction

Locus control regions (LCRs) were functionally defined in transgenic mouse experiments as elements that give rise to full levels of expression of a coupled transgene independent of the site of integration in the host genome [1]. This implied that this element was capable of inducing the changes in chromatin structure that are required for expression of the genes. The first indications that such elements exist was obtained from the analysis of naturally occurring deletions in a number of thalassaemic patients (Figure 1). These deletions removed the upstream part of the β -globin gene locus, leaving the genes intact but silent [2–4]. At the same time, it became clear that individual ϵ , γ or β globin genes when introduced in transgenic mice failed to express at significant levels (for review, see [5]). Taken together, all the functional data indicated the presence of an important regulatory region upstream of the genes. Its location was further indicated by the presence of a set of developmentally stable erythroid specific hypersensitive sites (HSs) in the region 5–25 kb upstream of the ϵ -globin gene in the normal locus [6,7]. Positive evidence for a regulatory role of this region was obtained by coupling it to a β -globin gene and introducing it into transgenic mice, resulting in copy-number dependent, full expression of the gene, independent of the position of integration of the transgene in the host genome [1]. Very similar results were obtained for the T-cell-specific CD2 gene, in this case correlated to a set of HSs on the 3' side of the locus [8]. The indication is that this type of regulatory sequence is found

at many loci and indeed a number of LCRs have now been identified (for review, see [9]).

The important implication of the results in transgenic mice was that an LCR would have the capability to modulate the chromatin structure at the site of integration even when this is normally in a repressed state [10,11]. Most of the studies on LCRs, however, have addressed the process of re-initiation of transcription and only indirectly their ability to modulate chromatin structure to allow transcription to occur. These studies strongly favor a model in which the LCR interacts directly with the genes to initiate transcription (for review, see [5]). However the mode of action of LCRs in chromatin modification is poorly understood and debate has focussed on the issue of whether an LCR is indeed required to open the chromatin structure of the locus and, if it is, how it facilitates access to transcription factors.

Experimental approaches

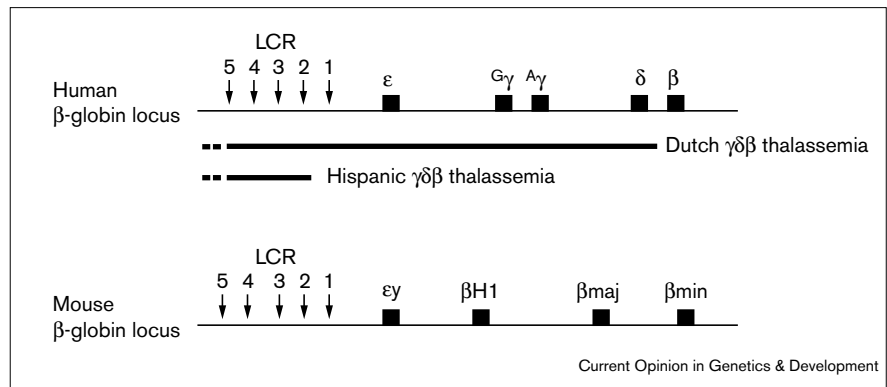
A number of different approaches have been taken to address the issue of LCR function and these can be divided into four categories: first, the analysis of naturally occurring LCR defects in patients (Figure 1); the introduction of manipulated LCR constructs or whole loci into mice (Figure 2); the analysis of an endogenous locus at its natural position in the genome (Figures 2 and 3); the transfer of chromosomes containing altered loci in their natural position and their analysis in appropriate cell types (Figures 2 and 3).

Disease

It is clear that the β globin locus is in an inactive state when the LCR is deleted in disease ([2,12]; Figure 1). The Dutch ($\gamma\delta\beta^0$) thalassaemia has a deletion of 100 kb of DNA including the complete LCR, the ϵ -, γ - and δ -genes, but leaving the β -globin gene together with 2.5 kb of 5' flanking region and the entire 3' side of the locus intact. The gene functions normally *in vitro* [13] but is silent and present in an inactive chromatin structure in the patient *in vivo* [2]. A much smaller deletion was found in the Hispanic $\delta\beta$ -thalassaemia [3,4]; this deletes part of the LCR and its upstream region and ends 8 kb 5' of the ϵ -globin gene, retaining the 3'-most part of the LCR. This locus nevertheless fails to express the globin genes [12]. Recent work on a third LCR deletion patient indicates that the deleted locus with the deletion does not transcribe *in vivo* (M Layton, M Wijgerde, personal communication). The inactive state of the Hispanic thalassaemia is maintained when the locus with its LCR deletion is transferred from a patient's lymphocytes to erythroid cells, whereas a normal locus is activated from its lymphocyte inactive state [12]. Thus these data suggested that the LCR in the human β globin locus is required for the 'opening' of a closed chromatin structure

Figure 1

Schematic representation of the human and mouse β -globin locus. The arrows indicate hypersensitive sites in the locus control regions (LCRs); solid squares, the globin genes; and the solid thick lines, the deletions found in two thalassaemias. The 5' borders of the deletions (broken line) are not drawn and are to the left of the picture.



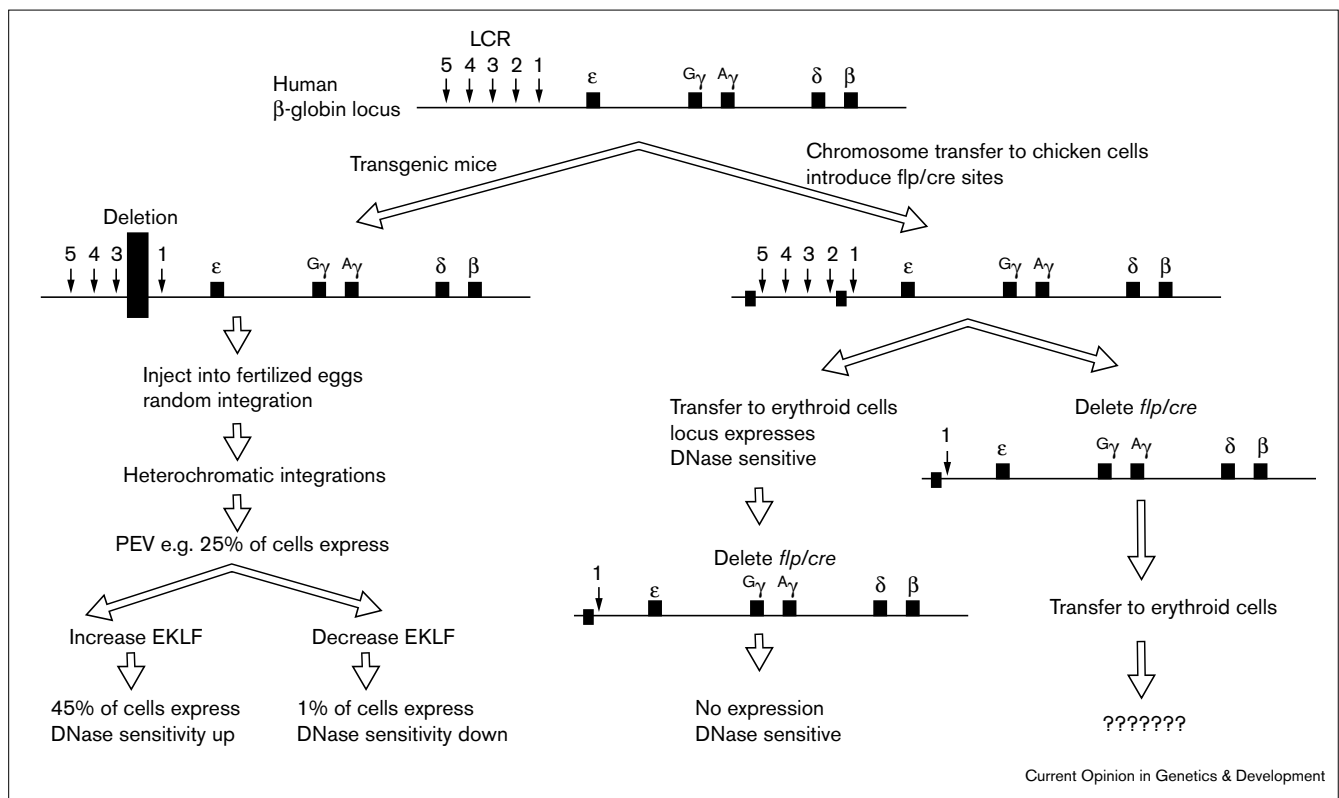
during development and that a failure to do so results in the absence of transcription *in vivo*.

Transgenesis

The property that functionally distinguishes an LCR from a classic enhancer is its capacity to confer integration-posi-

tion independent expression on a gene [1]. Globin genes without an LCR are expressed in only a proportion of transgenic mice and at low levels that do not correlate to copy numbers (up to 3% [14–18]). This type of expression reflects the influence of the chromatin structure and/or the presence of regulatory elements at the site of integration in

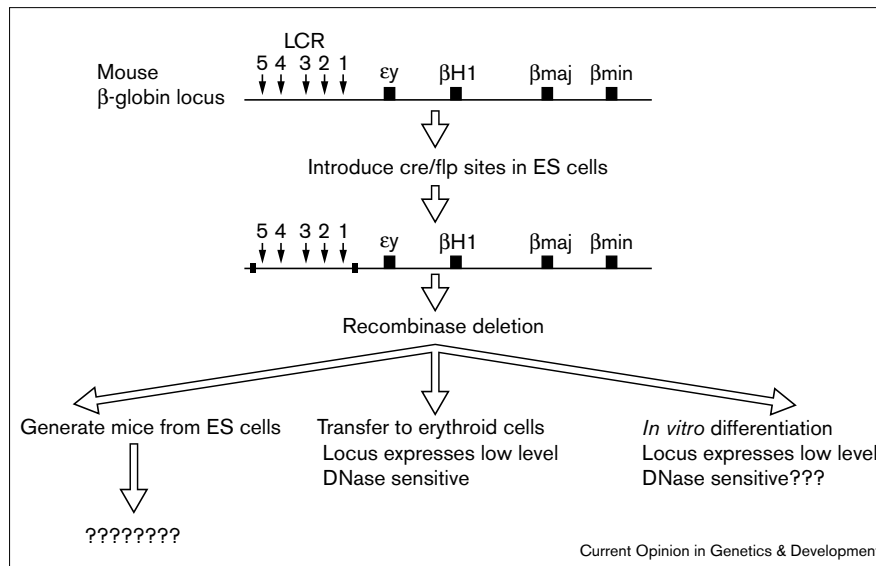
Figure 2



Schematic representation of the experiments carried out with deletions in the human β -globin locus. The left-hand column represents experiments where a human locus containing a deletion in the LCR (represented by a large vertical bar) is introduced into transgenic mice by direct microinjection [11]. The right-hand columns represent the chromosome-transfer experiments where the LCR is deleted before

(right column) or after (left column) the transfer to erythroid cells. Symbols are as in Figure 1; the small solid squares represent the deletion of the sequences located between these sites. Question marks indicate as yet unknown results.

Figure 3



Schematic representation of the recombinease experiment in embryonic stem (ES) cells [35]. Symbols are as in Figure 1; the small solid squares represent recombinease target sites in the locus. Addition of recombinease will result in a deletion of the sequences located between these sites. Question marks indicate as yet unknown results.

the host genome and is generally known as 'position effects'. When integrated in a chromatin structure that is accessible in erythroid cells, the transgene would be expressed, albeit at low levels, whereas it would be silenced when present in a non-accessible or heterochromatic environment. Such position effects (see [19]), appear to be overcome by the LCR in a dominant manner by always establishing an active chromatin structure and activating the nearby genes ([1]; reviewed in [5]). A similar result was obtained for the T-cell-specific CD2 gene and LCR [10]. The action of LCRs differ from that of boundary elements such as the ones described for the *Drosophila* heat-shock genes and the chicken lysozyme or chicken β globin locus [20–22]. Such elements appear to function by insulating the gene from other regulatory sequences present in the host genome. This does not mean that such sequences are absent from the human β -globin locus but suggests that, if present, their main function would not be the activation of the locus. Thus the transgenic experiments show that the primary property of the LCR is an activating function allowing the expression of a linked gene in the appropriate tissues.

Position effects in transgenesis

One disadvantage of the transgenic experiments is that the locus integrates in an unknown chromosomal location and this random site may have an (unknown) effect on the chromatin structure and expression of the integrated locus [19]. At the same time, this phenomenon has yielded some interesting facts about the function of the LCR. When an LCR containing internal deletions is introduced in transgenic mice, position-independent expression is lost [10,11]. In addition to the expected reduction in the efficiency of expression, the transgenic loci become sensitive to position effects when they are integrated in heterochromatic regions of the mouse genome. These effects are not observed with

intact loci [10,11]. Two types of position effects that affect the total output of the locus were observed: a classic position effect variegation (PEV; Figure 2) and a cell-cycle dependent effect [11]. Both of these reductions of expression are different from those that appear to be caused by local regulatory sequences at the site of integration. Such sequences can have an effect on the level of gene expression especially when the affected gene is in a competitive situation and is relatively far from the LCR ([23], see also [19]) but generally have little effect on the total output of the locus. In addition, when the globin LCR is integrated in the X chromosome, it is inactivated and transcription is lost (Whyatt *et al.*, unpublished data). Unfortunately, a large number of experiments have been carried out with incomplete LCRs and/or without a full analysis of the integrated LCR and its integration site. This has resulted in confusing and conflicting data and a number of conclusions on the basis of such incomplete analyses should be treated with caution [24]. Nevertheless it is clear from the transgenic experiments that LCRs appear to be able to activate a transgene independently of the position of integration with the notable exception of X-inactivation.

We have recently carried out a series of experiments to determine whether the LCR is directly engaged in the (re)activation of the locus by crossing mice that contain a β -globin locus that shows PEV with mice that express varying amounts of the erythroid transcription factor EKLF (erythroid Krüppel-like factor). This factor is a zinc finger protein that binds a GT motif in the promoter of the β -globin gene (CAC box [25–27]) and in the LCR [28**] and is required for β -globin gene expression in the definitive stage. The factor interacts with components of the SWI/SNF family of proteins [29**] and is directly involved in modifying the chromatin structure of the LCR *in vivo*, as measured by DNase sensitivity [28**]. When

β -globin PEV mice are crossed to mice that either have a reduced amount of EKLf or that overexpress EKLf, the number of cells activating the human β globin locus decreases or increases respectively (Figure 2, left column). Thus the level of EKLf is directly related to the number of cells that switch on the locus (i.e. activation events) providing direct evidence that the LCR (and promoters?) activates the locus (T McMorrow *et al.*, unpublished data).

Manipulation of the endogenous locus

The function of each of the HSs of the murine LCR (Figure 1) has been studied by removing sites individually or in combination or even by removing the complete LCR by homologous recombination in ES cells [30–32,33•,34•]. This has the advantage that the effects on the locus are studied in its native chromosomal context and not influenced by varying position effects. It should be remembered, however, that this methodology involves selection for the expression of a drug marker that is integrated in the locus and thus inherently selects for loci that are already accessible. When individual sites are removed from the LCR, reductions in expression were observed in the resulting mice. The reduction appears to be caused by a loss in the efficiency of expression and not by PEV such as described for the HS deletions above when the locus is integrated in a HSs environment (T Trimborn, personal communication). A very similar loss of efficiency is also observed in transgenic experiments with the human β -globin locus when individual hypersensitive sites are removed from the LCR and the locus is not integrated in a heterochromatic environment [11,35,36]. Thus, it was concluded that activation of the locus did not depend on individual HSs. When the complete LCR was deleted [34•], much more severe effects were observed than when individual HSs were deleted but these were only studied in ES cells that were differentiated *in vitro* or after chromosome transfer into K562 cells (Figure 3; see below).

Transfer of manipulated chromosomes

The human and mouse β -globin LCR have also been studied by transferring manipulated chromosomes to the appropriate cell type (Figures 2 and 3). The first chromosome-transfer experiments were performed using a normal chromosome and one carrying the Hispanic deletion of the locus. When each one of these loci was transferred from lymphocytes (where both are inactive), to erythroid cells, the normal locus was activated; in contrast, the deleted locus remained silent [12]. In agreement with the transgenic data at the time, this result led to the conclusion that the LCR was required for the activation (and DNase) sensitivity of the locus. The Hispanic deletion, however, is not a clean removal of the LCR; it removes the region upstream of the LCR and leaves HS1 intact.

The HSs 2–5 of the LCR were subsequently deleted by a *cre/loP* recombinase strategy in MEL or GM979 cells after a human chromosome 11 was transferred from the chicken

pre-B-cell line, DT11, that contained a selectable human chromosome 11 for homologous recombination purposes. After transfer from the chicken cells to the erythroid cells, all the HSs appear, the locus becomes DNase sensitive and the globin genes are expressed (Figure 2). When the LCR is subsequently deleted, the human globin genes are no longer expressed but DNase sensitivity is maintained (Figure 2, middle column). On the basis of these results, the authors concluded that the human LCR is required for the maintenance of expression but not for the maintenance of DNase sensitivity, although it should be remembered that HS1 remains present and hypersensitive after the deletion [33•]. This result also strongly supports the notion that the genes are transcribed through a direct interaction with the LCR [37–39] rather than through a binary model where the genes have random access to transcription factors after the locus has been activated [40].

Despite earlier intentions [41], the authors did not report the logical complementary experiment of deleting the LCR in the chicken cells prior to the transfer to erythroid cells to determine whether the LCR is required for the initiation of DNase sensitivity (Figure 2, right column). If a deletion prior to transfer would have failed to induce DNase sensitivity after transfer of the deleted locus in erythroid cells, it would have shown that the LCR is required for the initiation (but not the maintenance) of DNase sensitivity. Conversely, if the deleted locus still would have been DNase sensitive, it would have shown that the LCR is not required for the initiation of sensitivity in an erythroid environment.

The results of the chicken to erythroid cell transfer experiments become particularly relevant in light of the separate set of transfer experiments which were carried out with the mouse β -globin LCR [34•]. Here, the complete LCR was deleted in embryonic stem (ES) cells and transferred to human K562 cells (Figure 3). The result shows that the globin genes are still expressed in a substantial portion of the cells, albeit at a much lower level, and DNase sensitivity was maintained. Moreover, when the ES cells were differentiated *in vitro*, the same result was obtained. This led to the conclusion that the murine LCR is not required for the initiation of DNase sensitivity or expression of the genes and, hence, that an as yet unidentified element may be responsible for activation.

Conclusions?

The conclusions from the ES chromosome transfer experiment are obviously in direct contrast to those obtained from the thalassaemia loci and those obtained from the transgenic mouse experiments. Hence the question remains, is the LCR required for the initial activation of the locus and the initiation of a DNase-sensitive domain? Furthermore, why did these experiments not provide a clear answer? Clearly the results of two experiments should still be awaited. First, it would be helpful to know what happens when the human LCR is deleted in the

chicken cells prior to the transfer into erythroid cells (question marks in Figure 2) and second, what happens when the deleted mouse locus is analysed in mice after transfer of the ES cells through the germline (question marks in Figure 3).

Nevertheless, one explanation of the present contradiction could be that there is an essential difference between the human and the mouse LCR and/or the exact deletions that were made but, with the possible exception of the very recent experiments in ES cells [34**], it still seems reasonable to assume that there is essentially no difference between the LCR of the mouse and human loci. This poses questions: why the different results? Is there a difference in experimental systems addressing essentially different questions or are (some of) the experiments open to different interpretations? Particularly relevant in this respect would be a comparison between the transgenic mouse experiments and the ES cell deletion/transfer experiments. The argument used against the transgenic mouse experiments is that the locus is studied outside of its natural context and that questions about the initiation of DNase sensitivity by definition cannot be addressed in a locus that has been integrated in a position in the genome that is different from the normal position. There is no reason to assume that the process of chromatin activation in general would essentially be different at different locations in the genome, however, because the factors involved (such as acetylases and kinases) are ubiquitous in nature and are used at many different loci. The argument in favour is that the locus is integrated in the genome at a time (fertilized eggs and first cleavages) when the genome is reset (e.g. removal of methylation, with the exception of imprinted genes) and is subsequently taken through the entire process of development and differentiation including somatic modifications and the possibility that the activation of a locus may be fixed by Polycomb-response-like elements and proteins of the Polycomb/trithorax group involved in the 'cellular memory' of epigenetic states [42]. This somatic modification process has not taken place in ES cell manipulated chromosomes in this study, which led to the conclusion that the LCR is not required for chromatin activation [34**]. ES cells have a genome structure that precedes somatic modification (see [43] and references therein) and the mere fact of integrating and expressing a selectable marker shows that the locus is accessible in these cells. It is therefore perhaps not surprising that transferring a locus without the LCR in this 'ES configuration' to an erythroid cell containing all the necessary factors for transcription will result in some transcription in part of the cells. Similarly, the differentiation of the ES cells in culture leads to low expression in part of the cells and is reminiscent of PEV [33**].

LCR activation

The ES cell experiments may bypass the actual function of the LCR, which is to ensure that the locus is activated once the process of somatic development and differentia-

tion has taken place and tissue-specific loci are suppressed throughout the genome. This activation could take place during differentiation as a process of 'mass action' [44], where any of the binding sites for tissue-specific and/or ubiquitous factors in the LCR — or other positions in the locus — are occasionally able to bind the cognate transcription factor in a stochastic manner in inactive chromatin. This binding could have one of two outcomes, either the factor dissociates and the chromatin remains as before or binding results in a chromatin modification increasing the chance of a second factor binding at a neighbouring site. This would increase the chance of the next factor binding and so on until the locus has been activated and has become DNase sensitive, perhaps facilitated by intergenic transcription [45]. In this model, an LCR would be an element that contains a sufficient number of binding sites for factors capable of chromatin modification to ensure activation through a type of chain reaction that maximizes small windows of opportunity for factor binding (T McMorrow *et al.*, unpublished data). Once the locus is activated, the LCR would no longer be required for DNase sensitivity but would be required for the initiation of transcription through a direct interaction with the respective genes.

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