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The mouse β-globin locus control region: hypersensitive sites 3 and 4

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ABSTRACT

The human β-globin LCR plays a key role in the transcriptional regulation of the β-globin locus and comprises four erythroid specific DNase I hypersensitive sites, designated 5'HS1 – 4. We have now isolated genomic clones containing 5'HS3 and 5'HS4 of the mouse β-globin LCR. 5'HS3 and 5'HS4 are located 15 kb and 22 kb upstream of the mouse ϵ^y-globin gene, respectively. Sequence analysis of murine 5'HS3 and 5'HS4 reveals a significant degree of sequence conservation with their human homologues, including the presence of recognition sites for functionally relevant transcription factors. 5'HS3 and 5'HS4 regions were found to form hypersensitive sites in nuclei from murine erythroid cells, but not in nuclei from a variety of nonerythroid haematopoietic cell lines. Analysis of different mouse strains revealed the existence of a polymorphism that alters the spacing between 5'HS3 and 5'HS4. Taken together, our results emphasize the extent of evolutionary conservation and complexity of mammalian β-globin LCRs. Finally, the cloning of mouse 5'HS3 and 5'HS4 will facilitate the molecular analysis of LCR function in the mouse model.

INTRODUCTION

In transgenic mice, the level of expression of the transferred genes varies from mouse to mouse, and sometimes the transgene is not expressed at all. This is attributed to the fact that the expression of the exogenous gene is strongly dependent on the chromatin environment at the particular site(s) of integration (1,2). Locus control regions (LCRs) are a novel class of *cis*-acting DNA regulatory elements which are operationally defined by their ability to confer high level expression on *cis*-linked genes independently of their position of integration within the genome (3). Thus, when constructs containing a gene linked to LCR sequences are used to produce transgenic mice, the level of expression of the gene is directly proportional to the number of gene copies integrated. This suggests that every copy of the transgene is being expressed and, therefore, that LCR sequences are able to overcome the influence of the host chromatin at the site(s) of integration. It is believed that LCR sequences exert their effects through the stabilization of an open chromatin organization in their vicinity (4,5).

The archetypal LCR is located upstream of the human β-globin locus (6). The human β-globin LCR is a complex regulatory element spanning some 15 kb of DNA. Naturally occurring deletions that remove β-globin LCR sequences render the remaining β-globin genes transcriptionally quiescent, late replicating, and in an inactive chromatin configuration (4,7–10). The LCR is also involved in controlling the differential pattern of expression that the β-globin genes exhibit during different stages of ontogeny (11–14). It therefore appears that the β-globin LCR is not an enhancer in the classical sense, but represents a novel type of regulatory element controlling the genetic activity of the entire β-globin domain.

The active sequences within the β-globin LCR are contained within four erythroid specific DNase I hypersensitive sites (15–19). These sites are located 6.1, 10.9, 14.7 and 18 kb upstream of the human ε-globin gene and designated 5'HS1, 2, 3 and 4, respectively (20–22). When constructs containing different combinations of these sites linked to globin as well as heterologous genes are used to produce transgenic mice, high level, copy number dependent, erythroid specific expression of those genes is obtained (3,16,17,23–26). These experiments have shown that 5'HS2, 3 and 4 can individually act as transcriptional potentiators; 5'HS1 is essentially inactive in this particular type of assay. 5'HS2 and 3 have also been shown to confer high level expression to a linked β-globin gene in stably transfected mouse erythroleukaemia (MEL) cells (15,27). Finally, 5'HS2 exhibits classical enhancer activity in transiently transfected cells (28). Taken together these results illustrate the complexity of the β-globin LCR, with multiple functions probably residing in the different sites.

The characterization of the sequence elements and mechanisms by which the β-globin LCR exerts its function has been made difficult by its extraordinary complexity. Since functionally important DNA sequences are presumably under constant evolutionary selection, one approach to the analysis of the β-globin LCR is to compare this region in evolutionary diverged species. Sequences homologous to the human β-globin 5'HSs

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have been found in other mammals; 5'HS1 in rabbits, mice and goats (29–32), 5'HS2 in goats and mice (31–33, T.E. *et al.*, unpublished), and 5'HS3 in goats (31–32). To date, information concerning the evolutionary conservation of 5'HS4 was not available. Here we report the cloning and characterization of sequences corresponding to 5'HS3 and 4 of the mouse β-globin LCR.

MATERIALS AND METHODS

Cloning and sequencing

An EMBL-3 SP6/T7 Balb/c mouse genomic library (Clontech) was screened with a 5'HS3 probe by routine procedures (34). Restriction fragments containing either 5'HS3 or 5'HS4 regions were subcloned into pBluescript (Stratagene), and plasmid or phagemid DNA was prepared for sequencing. DNA sequences were determined in both strands using the dideoxy chain termination method of Sanger. The sequences were analyzed using MICROGENIE (Beckman) and GeneWorks (IntelliGenetics) software packages.

Cells and tissues

MEL 585 cells (35) (kind gift of T.Papayannopoulou) were cultured in RPMI 1640 medium containing 10% FCS. The pre-B cell line 18.8 (36) (kindly provided by F.Alt) was maintained in RPMI 1640 medium supplemented with 10% FCS and 50 μM β-mercaptoethanol. The pre-T cell line EL4 (ATCC TIB 39) was maintained in Dulbecco's modified Eagle's medium containing 10% horse serum. The monocyte cell line WEHI-274.1 (ATCC CRL 1679) was maintained in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose, 10% FCS and 50 μM β-mercaptoethanol. The monocyte-macrophage cell line J774A.1 (ATCC TIB 67) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS. Spleen and thymus were isolated from adult and 3-week old mice respectively. Foetal livers were isolated from day-14 mouse embryos produced by a B6D2F_1 (C57BL/6J×DBA/2 F_1 hybrid)×C57BL/6J mating and pooled for analysis. Spleen, thymus and foetal liver tissues were dispersed mechanically and filtered through a nylon mesh to obtain single cell suspensions.

DNase I analysis

Nuclei were isolated and treated with DNase I essentially as described elsewhere (37), except that volumes were scaled down to accommodate ~5×10^7 cells. DNase I concentrations in the digested samples ranged from 0 to 6 μg/ml. Purified DNA from each sample was digested with appropriate restriction enzymes, separated on 0.8% agarose gels, blotted onto nitrocellulose membranes, and hybridized with [α-^{32}P]dCTP-oligolabelled probes as described (34). After hybridization, the membranes were washed in 0.2×SSC, 0.5% SDS at 65°C for ~1 h and autoradiographed.

RESULTS AND DISCUSSION

By using a genomic probe derived from the 5'HS2 region of the human β-globin LCR, we and others have previously isolated genomic λ clones containing 5'HS2 of the mouse LCR (33, T.E. *et al.*, unpublished). To see whether sequences homologous to human 5'HS3 were also present in such clones, phage DNA was prepared, digested with different restriction enzymes and hybridized under low stringency conditions to a 0.7 kb PstI fragment from human 5'HS3. This analysis identified a 1.8 kb cross-hybridising SacI fragment located ~5 kb upstream of the mouse 5'HS2 region (not shown), which was selected for further characterisation. Sequence analysis of this fragment revealed extensive homology to human 5'HS3 sequences (not shown). To see whether these 5'HS3-homologous sequences actually form a DNase I hypersensitive site in the nuclear chromatin of erythroid cells, nuclei from murine erythroleukaemia (MEL) cells were isolated and subjected to DNase I analysis. As shown in figure 1, two DNase I sub-bands of 1.8 kb and 8.5 kb were detected in this experiment. The 1.8 kb sub-band corresponds to a hypersensitive site that maps within the 5'HS3-homologous region. The 8.5 kb sub-band indicates the existence of an additional site located some 7 kb upstream of 5'HS3, which presumably corresponds to the mouse homologue of human 5'HS4. Further analysis of this region in both the λ clone and Balb/c genomic DNA indicated that i) this upstream site was not present in the l clone, and ii) that the λ clone was rearranged in the region immediately upstream of the highly conserved 5'HS3 region (not shown). To obtain clones that were unrearranged upstream of 5'HS3 and also contained the upstream hypersensitive site, we screened a genomic library using a conserved fragment of murine 5'HS3 as a probe. Of the several clones isolated, one, λ-mLCR9, was chosen for the analyses presented below.

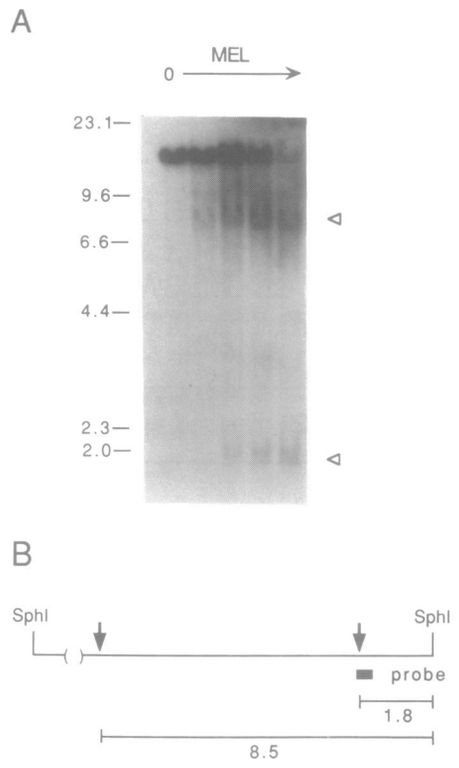

Figure 1. Mapping of DNase I hypersensitive sites upstream of 5'HS2 in the mouse β-globin LCR. **A)** DNase I hypersensitive site analysis in MEL cells. DNase I concentrations increase from left to right; the first lane marked 0 contains no added DNase I. DNase I sub-bands are indicated by open arrowheads and dashes show the relative migration of DNA molecular weight markers in kb. **B)** Diagram showing the DNase I strategy employed and the locations of the hypersensitive sites identified; numbers are in kb.

Analysis of 5'HS3

A 5.0 kb BamHI-EcoRI restriction fragment from λ-mLCR9 was subcloned and used to determine the sequence of a 1051 bp interval containing mouse 5'HS3 (EMBL accession number

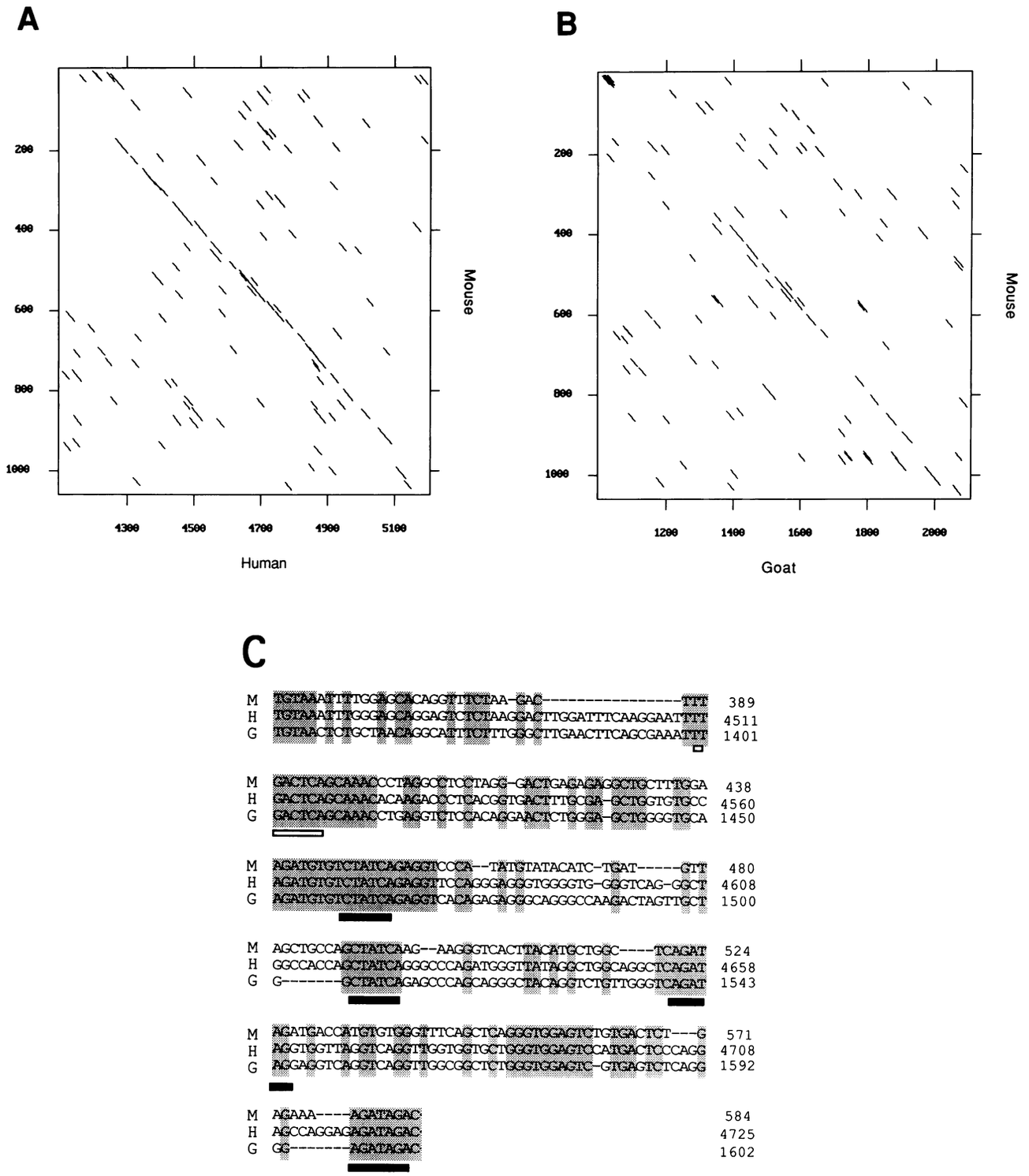

Figure 2. DNA sequence analysis of 5′HS3. **A** and **B)** Dot matrix comparisons of human versus mouse 5′HS3 and goat versus mouse 5′HS3, respectively. Dots indicate a greater than 65% homology over a range of 14 nucleotides. **C)** Alignment of mouse (M), human (H) and goat (G) sequences in the evolutionarily conserved core region of 5HS3. Bases conserved in all three species are shaded. Filled rectangles indicate consensus GATA binding sites; the open rectangle indicates a consensus NFE-2 binding site.

X66475); the 3′ boundary of this interval is defined by an EcoRV site located approximately 15.5 kb upstream of the ϵ^{y}-globin gene cap site. Dot matrix comparisons of this sequence with 5′HS3 sequences from human (38) and goat (31,32) are shown in figures 2A and 2B respectively. Mice, humans and goats have been diverging from each other for roughly the same amount of time, i.e., 80−90 million years (39). The analysis revealed that mouse 5′HS3 shares a more extended region of homology with human 5′HS3 than with goat 5′HS3. The most extensive conservation is between human and goat 5′HS3 (not shown),

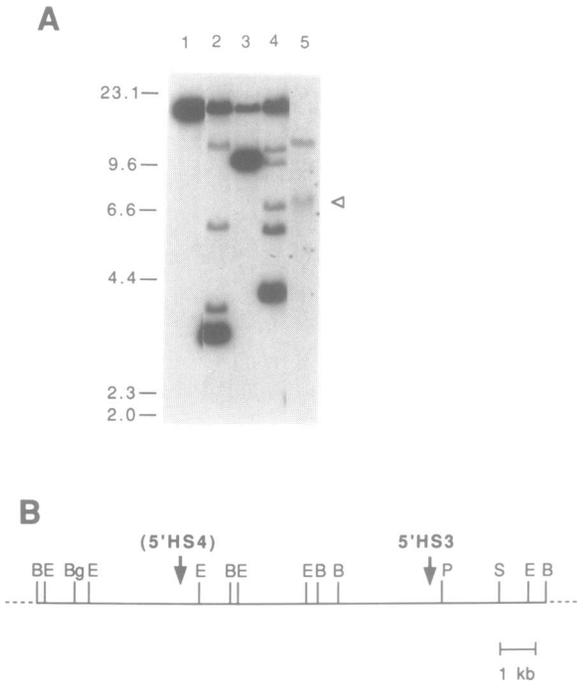

Figure 3. Mapping of λ-mLCR9. **A)** Indirect end labelling analysis. All samples were limit restricted with PstI prior to Southern blot analysis using a 0.4 kb StyI-PstI fragment from mouse 5′HS3 as a probe. 1) λ-mLCR9 DNA; 2−4) λ-mLCR DNA partially restricted with BamHI, BglII and EcoRI, respectively; 5) A sample from a DNase I series from MEL cell nuclei; the DNase I sub-band is indicated by an open arrowhead. Dashes indicate the relative size of molecular weight markers in kb. **B)** Partial restriction map of λ-mLCR9 showing the locations of 5′HS3 and the hypersensitive site located approximately 7 kb further upstream (presumed to be the mouse homologue of human 5′HS4). B, BamHI; E, EcoRI; Bg, BglII; P, PstI; S, SphI. The BamHI site at the 5′ end of the clone probably results from the cloning procedure and does not exist in the Balb/c genome.

consistent with a faster rate of nucleotide substitution in rodents than in primates and artiodactyls (40). Alignment of 5′HS3 sequences revealed a core element of some 250 bp that is evolutionarily conserved in all three species (figure 2C). In this core region, mouse 5′HS3 share 61% homology with human 5′HS3 and 62% homology with goat 5′HS3. Motifs in human 5′HS3 shown to bind GATA-1 or NFE-2 *in vitro* (16) are located within this human 5′HS3 core defined by evolutionary comparison; these factor binding motifs are conserved in both mouse and goat. Note also that the conserved core of human 5′HS3 (coordinates 4462−4725) overlaps with the core of this site defined functionally through gene transfer experiments in transgenic mice (16) and MEL cells (27) (coordinates 4552−4776). Taken together, our results suggest that 5′HS3 has been highly conserved during mammalian evolution and that the GATA and NFE-2 binding sites in the core region are critical to 5′HS3 function.

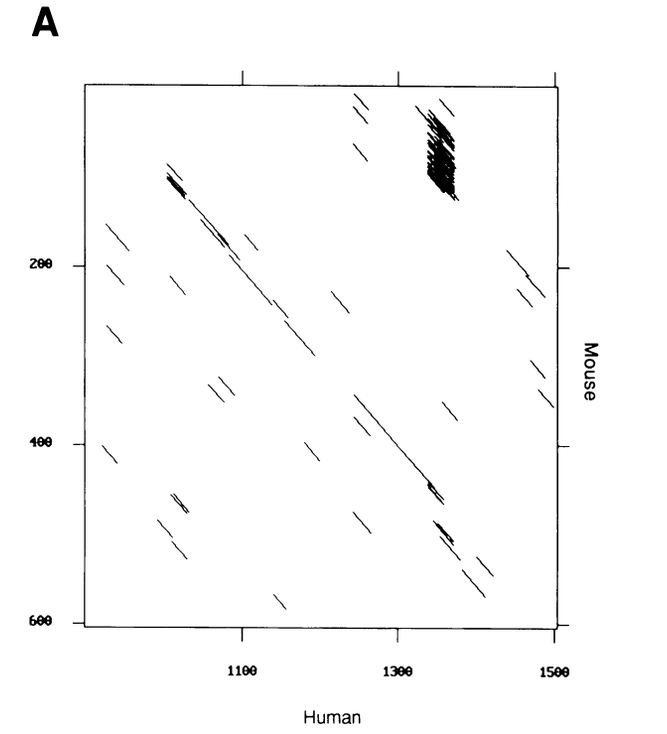

Figure 4. DNA sequence analysis of 5′HS4. **A)** Dot matrix comparison of mouse versus human 5′HS4 sequences. Dots indicate greater than 65% homology over a range of 14 nucleotides. **B)** Alignment of mouse (M) and human (H) 5′HS4 in the evolutionarily conserved core region of 5′HS4. Conserved bases are shaded. Filled rectangles indicate consensus GATA binding sites; the open rectangle indicates a consensus NFE-2 binding site.

Analysis of 5′HS4

Our DNase I analysis on MEL cells revealed an additional hypersensitive site located 6.7 kb upstream of 5′HS3. Believing that this site was the murine homologue of human 5′HS4, we hybridised restriction fragments from λ-mLCR9 to a 1.4 kb BamHI-SphI probe from the human 5′HS4 region. However, under the hybridisation conditions used, no cross-hybridising fragments were detected (not shown). We therefore physically mapped the region corresponding to this hypersensitive site by the indirect end labelling analysis presented in figure 3. λ-mLCR9 was limit digested with PstI and then partially cleaved with either BamHI, EcoRI or BglII. These samples, together with a PstI restricted sample from a MEL cell DNaseI series, were Southern blotted and probed with a 0.4 kb StyI-PstI fragment from mouse 5′HS3. This analysis localised the hypersensitive site to one end

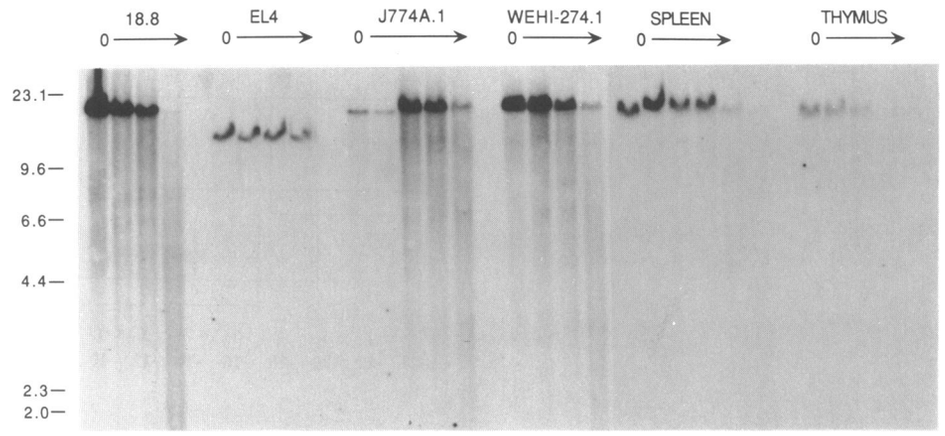

Figure 5. DNase I analysis of nonerythroid haematopoietic cells. The cell lines used lines represent pre-B (18.8), pre-T (EL-4) and myelo-monocytic (J774A.1 and WEHI-274.1) cells. Immunophenotypic analysis of tissue samples showed that the ratio of T- to B-cells was approximately 1:2 in the spleen, and that the thymus was composed almost exclusively (>95%) of T-cells. Note the absence of DNase I sub-bands in all series as well as the smaller size of the main band in the EL-4 DNase I series. Strategy and methods are as in legend to figure 1. Dashes indicate molecular weight markers in kb.

of a 2.8 kb EcoRI fragment which was subsequently subcloned and used for DNA sequencing. Dot matrix comparison of the 600 bp sequence obtained (EMBL accession number X66476) with the human 5'HS4 sequence (38) revealed the existence of a significant degree of similarity (figure 4A), suggesting that this hypersensitive site did indeed correspond to 5'HS4. Alignment of mouse and human 5'HS4 sequences revealed a core region of approximately 250 bp which is 62% conserved between the two species (figure 4B). This core of human 5'HS4 defined by evolutionary conservation overlaps the core defined by functional studies in transgenic mice (19), and the 101 bp region containing the minimum elements necessary for hypersensitive site formation (41). The alignment of mouse and human sequences in the 5'HS4 region shows the conservation of two adjacent and opposing GATA binding motifs. The significance of these so-called 'double sites' is not fully understood but it is thought that they may play an important role in position independent expression (19,42). The evolutionary conservation of this arrangement in human and mouse 5'HS4 supports the notion that these 'double sites' are functionally significant. In addition to the double GATA site, the human 5'HS4 core also contains a consensus motif for NFE-2 and AP-1 transcription factors. This motif binds both proteins *in vitro*, and has been suggested to act as an enhancing element (19,41). As shown in figure 4B, this recognition site is not well conserved in the mouse sequence, suggesting that this element may not be essential for the function of 5'HS4 in the mouse. However, a more detailed characterisation of the DNA/protein interactions mediating 5'HS4 activity will be necessary to assess the functional significance of this species difference.

Erythroid specificity of 5'HS3 and 5'HS4 formation

Human β-globin LCR hypersensitive sites were initially described as being restricted to erythroid cells (20–22). However, later studies have documented the presence of some of these sites in transformed cell lines of nonerythroid origin (43). In order to test the erythroid specificity of mouse 5'HS3 and 4, we analysed the chromatin structure of these sites in nonerythroid haematopoietic cell lines. The results of these experiments are presented in figure 5, and show that 5'HS3 and 4 are not formed in either nonerythroid-myeloid cell lines (J774.2, WEHI 274) or in lymphoid cell lines representing pre-T (EL4) and pre-B (18.8) compartments. Analysis of the chromatin structure of 5'HS3 and 5'HS4 in normal murine lymphocytes obtained from spleen (a source of mature B- and T-cells) and thymus (a source of immature T-cells) yielded similar results to those obtained with the non-erythroid haematopoietic cell lines (figure 5). Taken together these data suggest that in the mouse these sites are formed specifically in erythroid cells.

Spatial alterations in the LCR

We noted that the size of the main band in the DNase I series of the EL-4 cell line was smaller than expected (see figure 5). To test whether this represented an alteration peculiar to EL-4 or a genuine structural polymorphism in the mouse strain from which this cell line was derived (C57BL/6J), we analysed the region containing 5'HS3 and 5'HS4 in different mouse strains. This analysis revealed the presence of several strain-specific RFLPs in the 5'HS3–5'HS4 region. So far we have identified two alleles at the mouse β-globin LCR; allele *a* is shared by a number of different strains including BALB/c and DBA/2, whereas allele *b* has as yet only been found in C57BL/6J (see figure 6A).

Careful examination of the RFLPs suggested that an insertion/deletion event altering the relative spacing of 5'HS3 and 4 had occurred after the derivation of the different strains. By DNase I hypersensitive site analysis of erythroid cells from mice with different LCR alleles, we directly compared the spacing between 5'HS3 and 5'HS4 in the *a* and *b* alleles. Foetal liver cells from gestational day-14 embryos derived from an $(a/b)\times(b/b)$ mating (see materials and methods) were isolated and pooled for the DNase I analysis shown in figure 6B. This cross results in the preferential representation of the *b* allele in the pooled samples analysed, with the major DNase I sub-band observed corresponding to 5'HS4 in the *b* allele. This sub-band migrates 2 kb faster than the minor sub-band, which corresponds to 5'HS4 in the *a* allele; MEL cells derived from a/a homozygous mice (DBA/2) provide an additional control for the location of 5'HS4 in the *a* allele. These results show that the spacing of 5'HS4 relative to 5'HS3 is altered in different alleles of the mouse β-globin LCR (figure 6C); the spacing between these sites in the

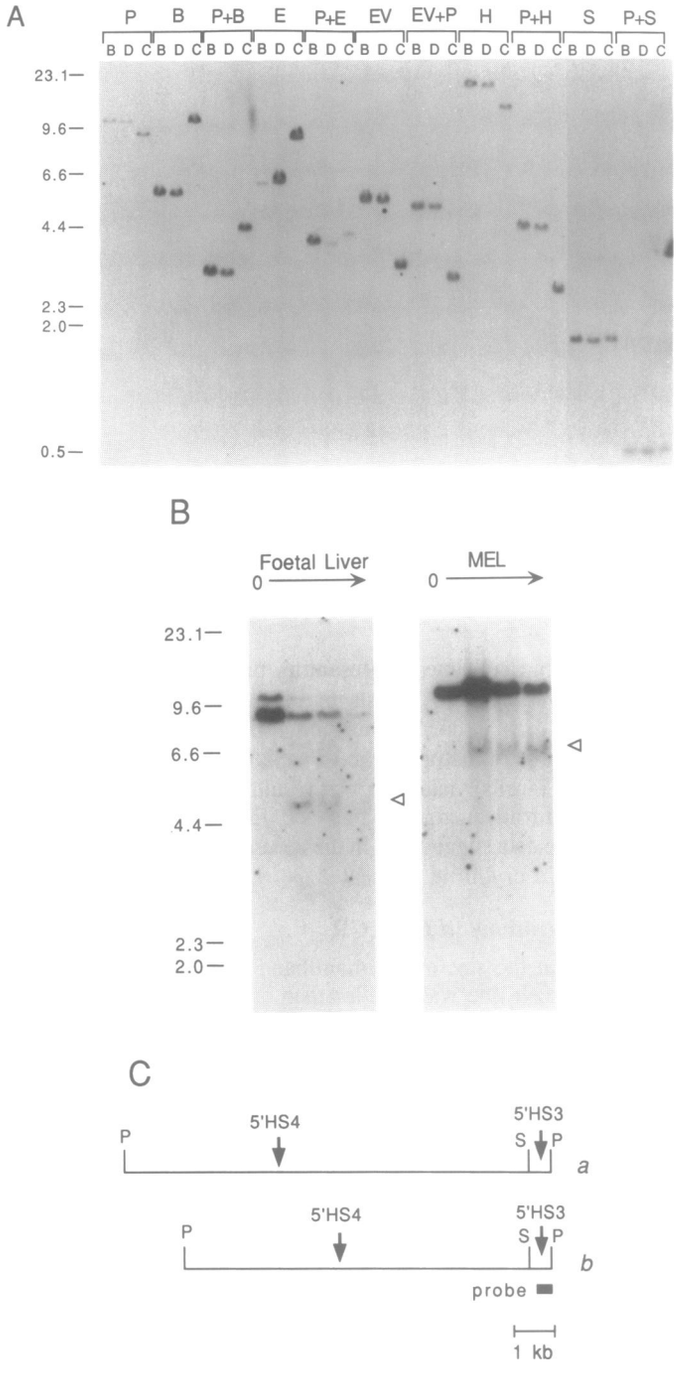

Figure 6. Naturally occurring spacing mutations in the mouse β-globin LCR. **A)** Identification of strain-specific RFLPs. DNA samples from BALB/c (B), DBA/2 (D) and C57BL/6J (C) were digested as indicated (P, PstI; B, BamHI; E, EcoRI; EV, EcoRV; H, HpaI; S, SacI) and analysed by Southern blot using a 0.4 kb StyI-PstI fragment from 5'HS3 as a probe. Note that the restriction pattern in the immediate vicinity of 5'HS3 (see DNA samples digested with SacI or SacI + PstI) appears to be the same in all three strains. Outside this region, the C57BL/6J strain differs from BALB/c and DBA/2 strains, which appear to be identical. Dashes indicate molecular weight markers in kb. **B)** DNase I analyses showing allelic alterations in the spacing between 5'HS3 and 5'HS4. The left hand panel shows the analysis of gestational day-14 foetal liver cells. Foetal erythroid cells from *a/b* and *b/b* embryos were pooled for DNase I analysis (see text for further explanation). The major sub-band is marked by a open arrowhead and corresponds to 5'HS4 in the *b* allele. The right hand panel shows the analysis of MEL cells derived from an *a/a* mouse. The sub-band corresponding to 5'HS4 (*a* allele) is indicated by an open arrowhead. Both DNase I series were digested with PstI and probed using a 0.4 kb StyI-PstI fragment from 5'HS3. Dashes indicate molecular weight markers in kb. **C)** Schematic representation of the analyses shown in panel B. Arrows show the position of the hypersensitive sites indicated. P, PstI; S, SacI.

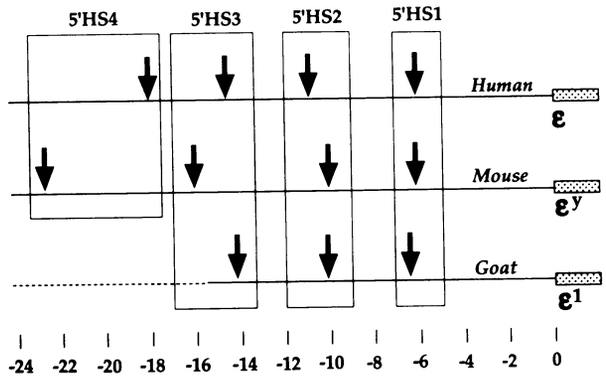

Figure 7. Comparison of LCRs from Human, Mouse and Goat β-globin gene clusters. The LCR 5'HSs is indicated by the vertical arrows. 5'HS that are evolutionary homologues are boxed. The scale is in kb, 0 denoting the position of the cap site of the 5' most embryonic gene in each cluster. Dotted lines indicate regions that have not yet been analysed. The mouse LCR diagrammed was cloned from the Balb/c mouse. The spatial organisation of the Balb/c LCR appears to be shared by several other mouse strains with the notable exception of C57BL/6J (see text) where the spacing between 5'HSs is somewhat different.

b allele (5 kb) more closely resembling the organization of the human β-globin LCR, where 5'HS4 and 5'HS3 are separated by 3.3 kb. The existence of apparently nondeleterious, naturally-occurring, spacing mutations within the mouse β-globin LCR, suggests that changes in the spacing between the different elements of the LCR can be functionally tolerated *in vivo*.

Perspective

With their ability to organise the genetic activity of entire transcriptional domains, LCRs represent a novel and important class of eukaryotic *cis*-acting transcriptional regulators. Whilst valuable, analyses of the β-globin LCR through functional assays have been hampered by its structural complexity, the multiplicity of its functions and the large physical range over which it acts. Evolutionary approaches to the analysis of the LCR obviate such obstacles and identify the conserved DNA sequences deemed relevant by natural selection itself. In this context, the comparison of the organisation and structure of β-globin LCRs from different organisms may provide significant insights into their mechanism of action; the organisation of the mammalian β-globin LCRs thus far cloned is shown in figure 7. The cloning and characterisation of 5'HS3 and 5'HS4 of the mouse β-globin LCR will facilitate evolutionary studies of this sort, as well as analyses of LCR function using the mouse model. It will now be possible to *in vivo* footprint 5'HS3 and 5'HS4 in murine haematopoietic cells and cell lines, and test the function of these sites in a phylogenetically homologous transgenic system. But, most importantly, using the mouse embryonic stem cell model, targeted mutations can be made in 5'HS3 and 5'HS4 of the mouse β-globin LCR by homologous recombination (see refs. 6 and 44). Such mutations can be propagated through the germ line and their effect may be scored in the context of an otherwise native β-globin locus, in which the spatial relationships and functional integrity of the remaining LCR hypersensitive sites and globin genes remain unaltered.

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