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Adjacent DNA elements dominantly restrict the ubiquitous activity of a novel chromatin-opening region to specific tissues

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Locus control regions (LCRs) are thought to provide a dominant tissue-specific open chromatin domain that allows for proper gene regulation by enhancers/silencers and their associated transcription factors. Expression of the T-cell receptor alpha (TCRα) gene is limited to T cells and its locus exists in different chromatin configurations in expressing and non-expressing cell types. Here we show that eight DNase I-hypersensitive sites in the TCRα locus comprise an LCR that confers T-cell compartment-specific expression upon a linked heterologous transgene. Removal of the three 5′-most hypersensitive sites of this LCR, containing TCRα enhancers/silencers, abolishes tissue-differential chromatin structure and results in transgene expression in all tissues examined. The remaining five DNase I-hypersensitive sites therefore constitute a novel control element possessing a widely active chromatin-opening function that allows for ubiquitous expression of a linked transgene in all transgenic founder mice. Furthermore, these data show that *cis*-acting elements without inherent LCR activity can dominantly modulate chromatin structure to determine tissue-specific gene expression *in vivo*.

Keywords: chromatin/locus control region/T cell receptor α

Introduction

A locus control region (LCR) is a DNA regulatory element that imparts position-independent, high-level, copy number-dependent, tissue-specific expression of a linked transgene in chromatin (Grosveld *et al*., 1987; reviewed in Orkin, 1990; Felsenfeld, 1992). Without an LCR, transgene expression is subject to 'position effect variegation' and can be absent if the transgene is integrated into inactive chromatin (reviewed in Palmiter and Brinster, 1986). It is widely believed that LCRs function by providing an open chromatin environment, which is necessary for proper expression of a linked transgene independently of the state of surrounding chromatin at the locus of integration (Festenstein *et al*., 1996; Milot *et al*., 1996). This chromatin-opening ability is thought to be inherently tissue-specific in nature. It has been observed that the activity of an LCR is restricted to the tissues in which its locus of origin is normally expressed (Grosveld *et al*., 1987; Lang *et al*., 1988; Aronow *et al*., 1992; Bonifer *et al*., 1994; Madisen and Groudine, 1994; Jones *et al*., 1995) even when it is linked to heterologous transgenes (Greaves *et al*., 1989). LCRs are thought to be important in the determination of tissue-specific gene expression in the context of chromatin. Classical transcriptional regulatory elements (enhancers/silencers), with their associated nuclear factors, have also been implicated in tissue-specific gene regulation, as some of them are cell type-specific in their activity (Faisst and Meyer, 1992). The role of these elements is thought to be the modulation of RNA polymerase activity (reviewed in Singer and Berg, 1991). Their action is thought to be subsequent to LCR-mediated chromatin opening, which allows nuclear factor access to their DNA-binding sites.

Pre-rearranged T-cell receptor alpha (TCRα) transgenes under endogenous controls are expressed only in T cell-bearing tissues, such as thymus and spleen, but not in other organs, such as the liver and heart (Diaz *et al*., 1994). Moreover, the endogenous TCRα locus exists in differential chromatin configurations in expressing and non-expressing tissues (Hong *et al*., 1997). We aim to find the *in vivo* sequence requirements for the determination of this T cell-specific chromatin structure and gene expression of the TCRα locus.

In T-cell lines, eight DNase I-hypersensitive sites (HS) have been identified in the 3′ region of the TCRα locus. HS1 maps to the well characterized TCRα enhancer (Winoto and Baltimore, 1989a; reviewed in Leiden, 1993). HS2–6 lie 3′ of HS1 in the locus while HS7 and 8 are 5′ to HS1 (Diaz *et al*., 1994). HS1 and HS6 are the predominant HS observed in normal thymocytes (Hong *et al*., 1997). Rearranged TCRα transgenes containing a TCRα variable region promoter and 3′ sequences extending only through the α-enhancer (containing HS7, 8 and 1) are variably and inefficiently expressed (von Boehmer, 1990; Diaz *et al*., 1994). This indicates that these three HS alone do not comprise an LCR. Inclusion of HS2–6 in such a TCR transgene results in high-level, position-independent, copy number-dependent and T cell-specific expression (Diaz *et al*., 1994). Therefore, HS1–8 appears to constitute a T cell-specific LCR.

Here we report that a 6 kb subfragment of this LCR contains a chromatin-opening activity that is not restricted to T cells. A reporter transgene linked to this fragment is expressed in all transgenic founder mice, though not in a strictly copy number-dependent manner. Unexpectedly, expression is seen in all tissues examined. Therefore, this fragment constitutes a novel non-tissue-restricted chromatin-opening element with a subset of LCR-like properties. The remaining 3 kb of immediate upstream sequence in the LCR contain previously characterized enhancer/silencer elements (Winoto and Baltimore,

1989a,b) and several HS without inherent LCR activity. We further show that these *cis*-acting elements, when included in the above transgene, alter the chromatin configuration of neighboring sequences and restrict transgene activity to T cell-bearing tissues. Thus, these sequences provide tissue specificity to the downstream chromatin-opening activity. These data support a novel role for non-LCR transcriptional regulatory elements as modulators of chromatin structure in the determination of tissue-specific gene expression *in vivo.*

Results

A transgenic reporter system for TCRα LCR analysis

To define and characterize the TCRα LCR further, two transgenic constructs were made (Figure 1). Both include a 4.9 kb *Bgl*II fragment of the human β-globin locus as a 'reporter gene'. This fragment contains the β-globin exons, introns, promoter and enhancer (Greaves *et al*., 1989). Extensive previous work has established that this β-globin fragment is only expressed sporadically and at varying levels in erythroid cells (Chada *et al*., 1985; Townes *et al*., 1985; Grosveld *et al*., 1987; Greaves *et al*., 1989). Addition of the β-globin LCR leads to its high-level, erythroid-specific expression (Grosveld *et al*., 1987), while addition of the human CD2 LCR to this fragment results in high-level reporter β-globin expression only in T cells (Greaves *et al*., 1989). These results indicate that this is a suitable reporter system for our LCR studies. We linked the β-globin fragment to either the 9 kb region of the TCRα locus containing all eight HS identified in the locus (β:1–8) or to the 6 kb subregion containing HS2, 3, 4, 5 and 6 (β:2–6). Transgenic mice were prepared using these constructs, and multiple independent founder lines were analyzed for each. Transgene transcription was measured by RNase protection. A 220 base RNA probe was used to detect human β-globin mRNA. As a loading control, a 135 base RNA probe to γ-actin was added to the hybridization reactions (Enoch *et al*., 1986). RNase protection of thymus and spleen RNA from β:1–8 transgenic mice showed that all eight independent lines of varying copy number efficiently expressed the human β-globin mRNA (five lines shown in Figure 2A and B). PhosphorImager analysis showed that expression, in general, trends upwards with increasing copy number (Figure 2C). Small deviations from copy-related expression seen in this construct (e.g. line 48 spleen) may be partially explained by the heterologous nature of the reporter gene. Such minor deviations from copy-dependent expression were reported in other heterologous LCR systems (Greaves *et al*., 1989). In addition, spleen is not a static organ in terms of T-cell populations and this could contribute to these deviations.

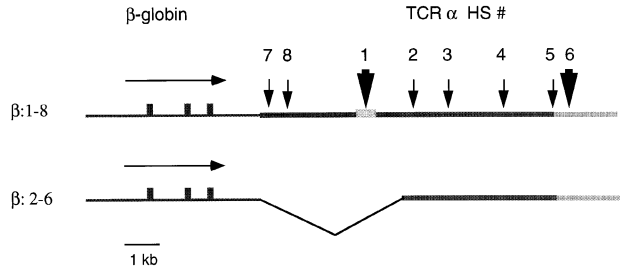

Fig. 1. Diagram of transgenes used in this study, described in the text, with DNase I-hypersensitive sites of the TCRα locus (observed in T-cell lines) labeled with vertical arrows. Large arrows indicate predominant HS found in thymocytes. Horizontal arrows indicate the transcriptional orientation of the β-globin reporter gene.

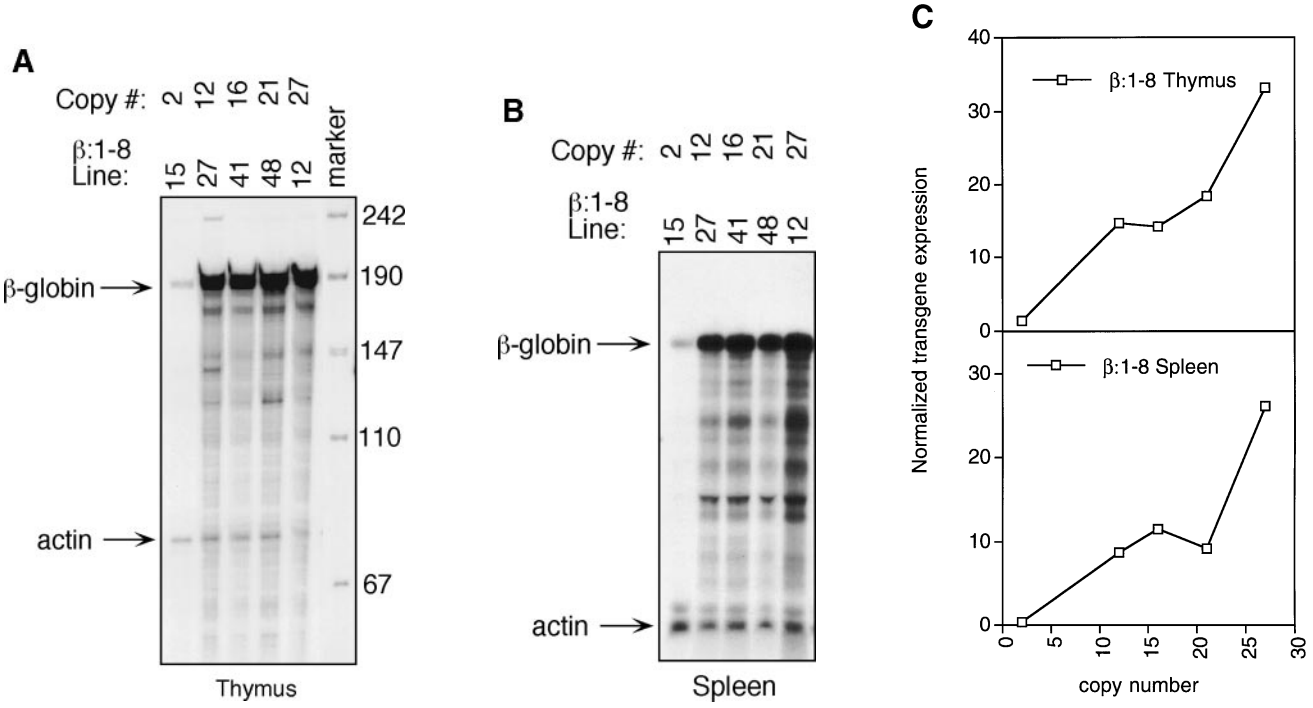

Fig. 2. Expression of the β:1–8 transgene is present in all transgenic founders. (**A**) RNase protection assay on thymus RNA from five independent β:1–8 transgenic lines and (**B**) on spleen RNA from the same lines. Line numbers and their estimated copy number are indicated. Arrows indicate signals from human β-globin transgene and actin mRNA. (**C**) PhosphorImager analysis of β:1–8 expression in thymus (upper) and spleen (lower). The β-globin signal was normalized to the actin signal and given an arbitrary number.

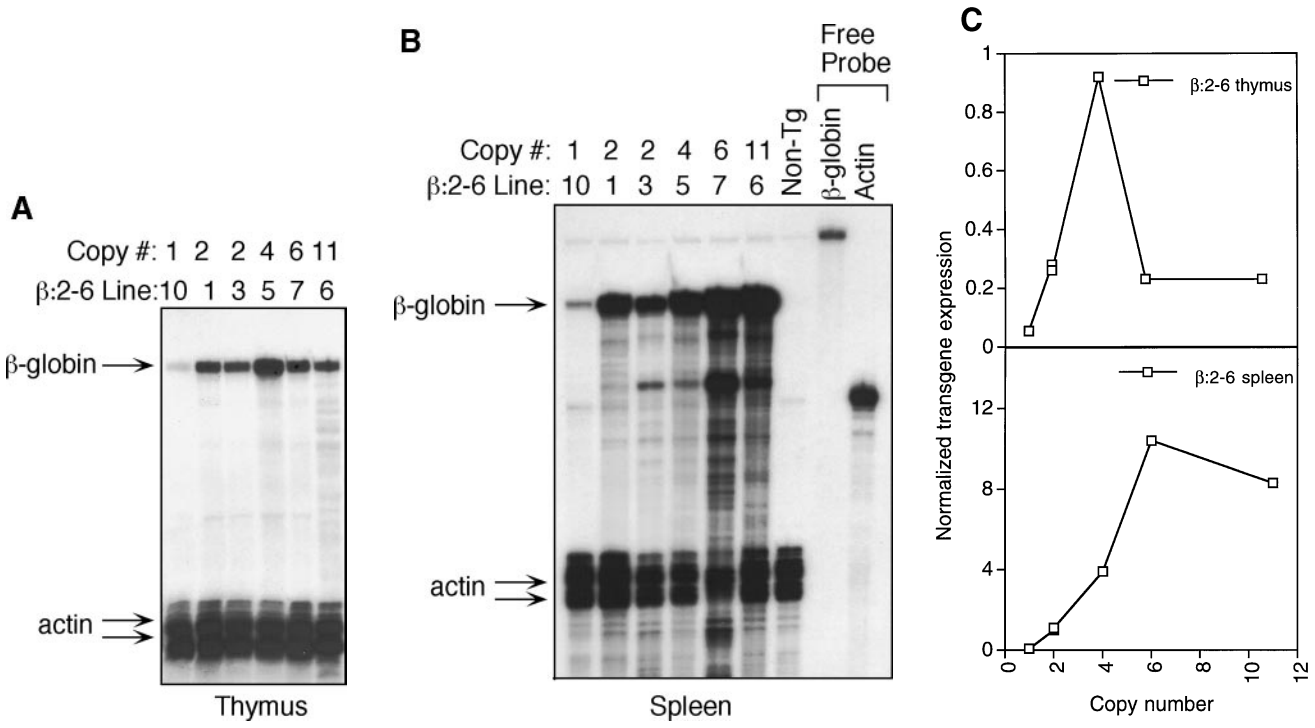

Fig. 3. Expression of the β:2–6 transgene is present in all transgenic founders. (**A**) RNase protection assay on thymus RNA from six independent β:2–6 transgenic lines and (**B**) on spleen RNA from the same lines. Line numbers and their estimated copy number are indicated. Arrows indicate signals from the human β-globin transgene and actin mRNA. (**C**) PhosphorImager analysis of β:2–6 expression in thymus (upper) and spleen (lower). The β-globin signal was normalized to the actin signal and given an arbitrary number. Arrows indicate mRNA signals from the β-globin transgene and control actin.

The β-globin expression under the control of HS1–8 is high. In the lowest copy line (#15), β-globin expression, per copy, is >200% of the endogenous TCRα expression (data not shown). These data demonstrate that the HS1–8 sequences, linked to a heterologous transgene and present in multicopy transgene concatemers, retain most of their LCR properties. They will therefore be referred to hereinafter as the TCRα LCR.

Similarly to the β:1–8 transgene, the β:2–6 transgene was expressed in the thymus and spleen of all six independent transgenic lines (Figure 3A and B). However, several important differences were observed between the two transgenes. The β:2–6 transgene does not maintain the upward trend in expression with increasing copy number seen with β:1–8 in thymus. Splenic expression of this transgene does show a better relationship with copy number than thymic expression in five of six lines (line 7 gives higher than predicted splenic expression) (Figure 3C). It also appears that removal of the HS 7, 8 and 1 region from the transgene causes a severe reduction in thymic expression of the reporter transgene (note the *y*-axes of thymus panels in Figures 2C and 3C). This reduction is probably due to the absence of the TCRα transcriptional enhancer in β:2–6. This shows that the deleted region is necessary for high-level, copy-related expression in thymus. In contrast to the thymus, the degree of splenic expression per copy is more similar for both β:1–8 and β:2–6 transgenes. Non-T cell expression of β:2–6 in the spleen might mask the reduction in T-cell transgene expression observed in the thymus and produce the observed result. As the spleen contains mostly non-T cells, these results suggest that deletion of HS7, 8 and 1 changes the cell type distribution of transgene expression.

HS1–8 is T cell restricted while HS2–6 is ubiquitously active

To examine the distribution of human β-globin mRNA driven by the two transgenes, total RNA was prepared from various transgenic mouse tissues. High-level mRNA production by the β:1–8 construct was restricted to thymus and spleen, two organs with significant numbers of T cells (Figure 4A and B). This transgene's expression was very low to absent in non-lymphoid tissues. Surprisingly, the β:2–6 transgene was expressed efficiently in all tissues analyzed (Figure 4C). Expression was seen in the thymus, heart, spleen, kidney, liver and lung. The same ubiquitous distribution was seen in organs (specifically liver, heart, lung, kidney and thymus) that had been perfused to remove circulating blood (data not shown). Thus, HS2–6 appears to be ubiquitously functional. The expression of the β:2–6 transgene in non-lymphoid organs, in general, tends upward with increasing copy number (Figure 5A and B). However, as in thymus and spleen, it is not absolutely copy-number dependent. This is particularly obvious in the low-copy lines. These data show that addition of the 3 kb HS7, 8, 1 region appears to inhibit reporter transcription in non-lymphoid organs, while greatly increasing thymic transgene expression. Thus, these *cis*-acting elements restrict the activity of the TCRα LCR, limiting high-level transgene expression to T-cell compartments.

HS2–6 opens chromatin equivalently in lymphoid and non-lymphoid organs

Since LCRs are thought to act at the level of chromatin structure, we examined the chromatin configuration of the two β-globin transgenic constructs. The DNase I hypersensitivity assay (DHA) was used to probe the

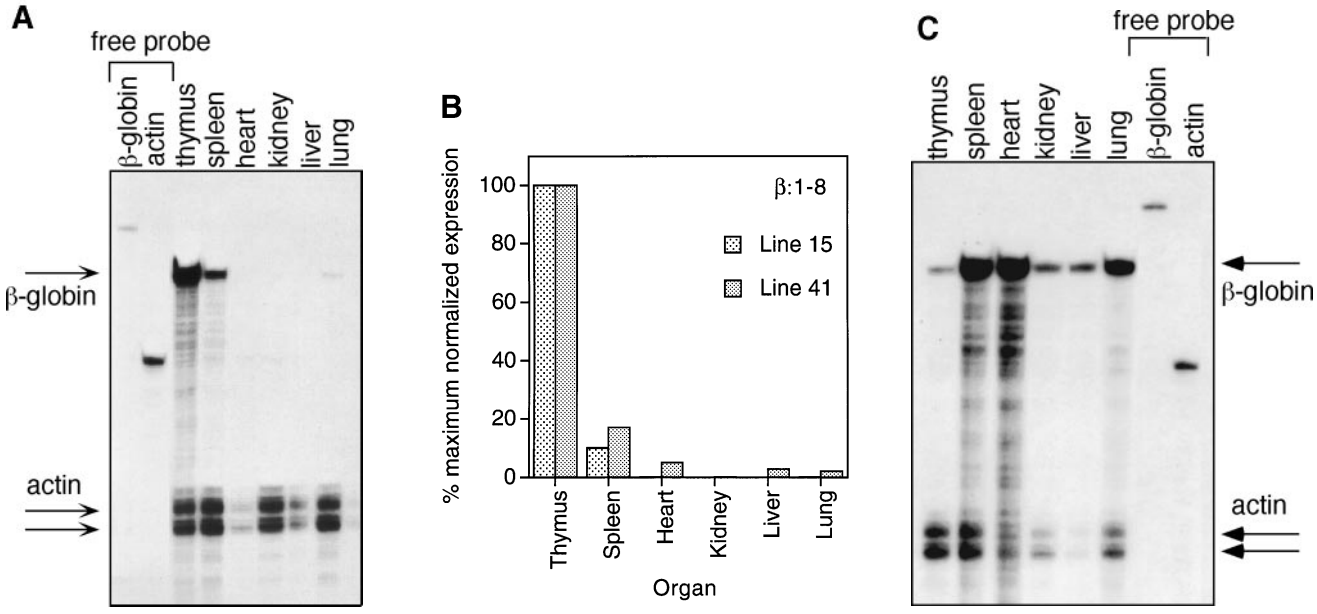

Fig. 4. Tissue distribution of β:2–6 and β:1–8 transgene expression. (**A**) RNase protection assay on RNA from various tissues of β:1–8, line 15 transgenic mice. Arrows indicate mRNA signals from the β-globin transgene and control actin. (**B**) PhosphorImager analysis of two separate RNase protection experiments from β:1–8 lines 15 and 41. Data are expressed as the percentage of maximum expression observed within the experiment, after normalizing to the actin signal. (**C**) RNase protection assay on RNA from organs of β:2–6, line 6 transgenic mice. Arrows indicate mRNA signals from the β-globin transgene and control actin.

chromatin conformation of the transgene loci. We analyzed different mouse tissues to look for correlation between the chromatin structure and mRNA expression patterns. Figure 6A shows a DHA of the β:2–6 transgene locus in thymus (lymphoid) and liver (non-lymphoid). The DNase I hypersensitivity pattern appears equivalent in both tissues, and indicates a chromatin conformation that leaves the region broadly accessible to nucleases. Distinct bands in the region of HS5 and HS6 are observed. There are additional prominent smears where HS2 and HS3 would be expected. A weak smear is seen in the HS4 region. In one line, the HS4 smear is as strong as the others in both tissues (data not shown). There are additional HS smears in the β-globin gene itself. The open, and equivalent, chromatin configurations apparent in both tissues are different from the HS patterns observed in the endogenous locus (Hong *et al.*, 1997), but correlate with the efficient expression of the transgene in those organs.

HS7, 8 and 1 determine tissue-specific chromatin structure

Figure 6B shows a DHA performed on a β:1–8 transgenic mouse. The addition to β:2–6 of 3 kb of 5′-flanking DNA altered the chromatin configuration formed at the HS2–6 region, making it more similar to the endogenous HS pattern (Hong *et al.*, 1997). In the TCRα locus, HS6 is much stronger in thymus than in liver. HS2–5, 7 and 8 are generally much weaker in both organs than is observed in T-cell lines (Diaz *et al.*, 1994). The α-enhancer-associated HS1 is very strong in thymus. In liver, and other non-lymphoid organs, HS1 is replaced by a strong hypersensitive site further 3′ which we call HS1′. HS1 itself is not detected in these organs. Similarly, in the β:1–8 transgene, DNase sensitivity of the HS2–5 region was suppressed in both thymus and liver. Thymic nuclei displayed a stronger HS6 than liver nuclei. The added HS7, 8, 1 region also assumed different chromatin configurations in thymus and liver (Figure 6B). HS8 is much stronger in thymus than in liver. This correlates with the higher transgene expression observed in thymus. As in the endogenous locus, thymic nuclei displayed a strong HS1 on the β:1–8 transgene that mapped to the TCRα enhancer region. Liver nuclei from multiple independent β:1–8 lines showed an HS pair located 3′ of HS1 that corresponds to HS1′. To confirm that HS1 and HS1′ were indeed different, they were mapped relative to a *Bgl*II site in their vicinity (Figure 7). Genomic DNA samples isolated from DNase-treated liver and thymic nuclei were divided into equal fractions. All samples were digested with *Mfe*I restriction enzyme to generate the parent restriction fragment. One fraction from each tissue was then digested further with *Bgl*II. By comparing the relative mobility of DNase-generated subfragments with the double restriction enzyme-digested fragment, we show that the HS1′ pair is 3′ of the *Bgl*II. In contrast, the HS1 cluster in thymus appears to include the *Bgl*II site and extend further 5′ and slightly 3′. These two HS clusters localize similarly in the endogenous TCRα locus (data not shown). A map of the locations of HS1 and HS1′ is shown (Figure 7). These data show that a 3 kb region of the TCRα locus containing multiple *cis*-acting elements can modulate the chromatin-opening activity of the neighboring HS2–6 region, causing the region to assume different configurations in thymus and liver.

Discussion

It is important to understand how tissue-specific gene expression is achieved in the context of chromatin. We have developed a transgenic mouse model system for understanding how classical transcriptional control elements and LCRs cooperate in determination of the cell

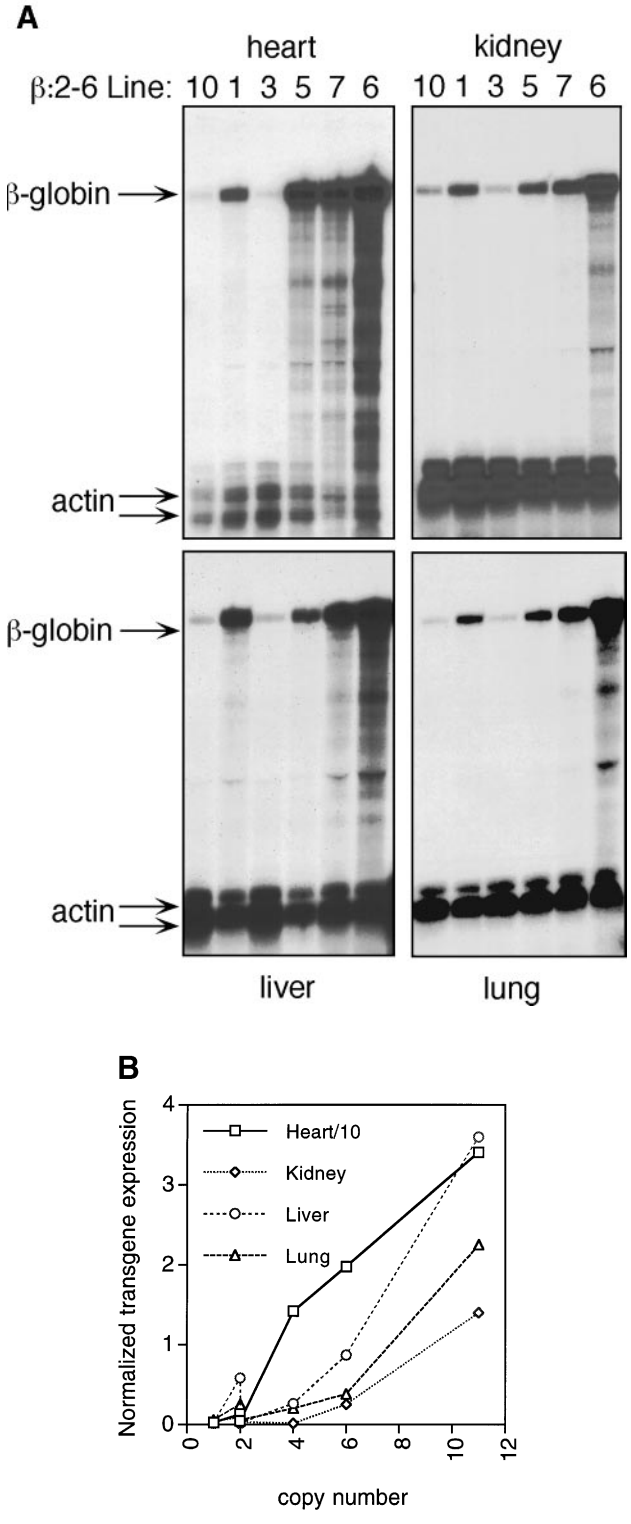

Fig. 5. β:2–6 expression in non-lymphoid organs. (**A**) RNase protection with RNA from the indicated organs from six independent β:2–6 lines. (**B**) PhosphorImager analysis of the RNase protection experiments shown in (A). Transgene expression is normalized to the actin signal and given an arbitrary number. Heart expression is divided by 10 to facilitate presentation of all the data on one graph.

type-specific expression and chromatin structure of the TCRα locus. Knowledge of the roles of various transcriptional control elements implicated in this process, and the nature of their cooperation, may reveal the molecular mechanisms governing cell type differentiation *in vivo*. This information is also crucial to the design of gene therapy strategies.

Differential chromatin structure and gene expression

In contrast to the open state of chromatin at active loci, closed chromatin structure (i.e. not DNase I hypersensitive) is usually observed at silenced gene (and transgene) loci (Grosveld *et al*., 1987; Felsenfeld, 1992; Elliot *et al*., 1995; Festenstein *et al*., 1996; Milot *et al*., 1996). In our TCRα LCR model system, interaction of HS7, 8 and 1 sequences with the HS2–6 region does not close the chromatin in non-lymphoid organs but, rather, alters the open state of the chromatin. This change correlates with a dramatic change in the distribution of transgene expression. These data indicate that discrete changes in chromatin structure, rather than complete chromatin closure, can also accomplish gene silencing to achieve tissue-specific transcription. Our recent discovery of the ubiquitously expressed *Dad1* gene (Apte *et al*., 1992; Nakashima *et al*., 1993) 3 kb 3′ of the TCRα LCR (Hong *et al*., 1997) provides a rationale for why HS2–6 interaction with 5′ *cis*-acting elements would not completely shut down the LCR. The alternative 'open' conformation of the LCR in non-T cells may be indicative of a state in which it is able to communicate with the downstream *Dad1* gene, but not the upstream TCRα gene. In this model, the *Dad1* gene may be dependent on elements of the TCRα LCR for its expression. We currently are testing this possibility.

A model for tissue-specific expression of the TCRα locus in chromatin

The differential patterns of DNase I hypersensitivity in thymus and liver suggest a possible model for how T cell restriction is imposed in the β:1–8 transgene. HS1′, formed in liver, could constitute a negative element (for example, a silencer or boundary) that prevents cooperation between the LCR and upstream enhancers/promoters. Boundary elements have been described in both human and chicken globin genes (Chung *et al*., 1993; Li and Stamatoyannopoulos, 1994). Silencers have been described in the CD4 gene (Sawada *et al*., 1994, Siu *et al*., 1994). This negative element would prevent induction of strong hypersensitivity at HS1, 8 and 6 inhibiting transcription in liver (and presumably in other non-T cell-bearing organs). In T cells, the negative element would be overcome by the presence of the nearby TCRα enhancer. The binding of lymphoid-specific proteins to their recognition sites in this region would render the site hypersensitive in lymphoid organs, altering the chromatin structure so as to limit the activity of the negative element. This could be accomplished by the lymphoid-specific protein LEF-1, an essential component of the TCRα enhancer (Giese *et al*., 1995). LEF-1 has the ability to bend DNA, facilitating interactions between other proteins that bind nearby. This property, which induces formation of a nucleoprotein–DNA complex, could in principle alter chromatin structure (Grosschedl *et al*., 1994). Several other studies have documented the ability of transcription factors to effect changes in local chromatin structure (Stamatoyannopoulos *et al*., 1995; Boyes and Felsenfeld, 1996). Furthermore, it has been proposed that enhancer elements counteract

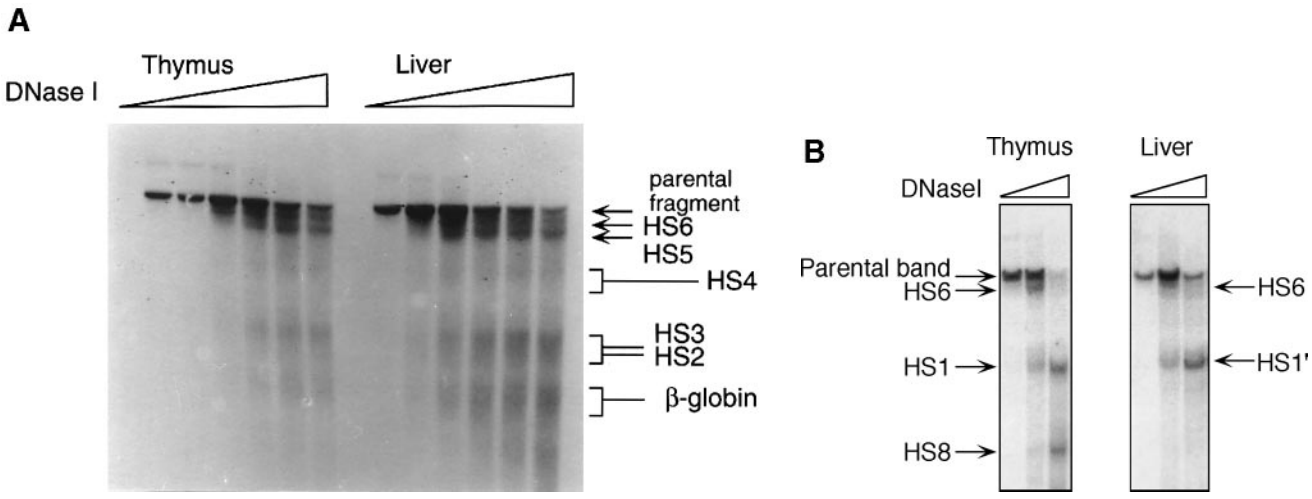

Fig. 6. Chromatin configuration at the β:2–6 and β:1–8 transgene loci. DNase I hypersensitivity assays at the (**A**) β:2–6 transgene, line 6 and (**B**) β:1–8 transgene, line 12. The parental restriction fragment and distinct DNase I HS-generated subfragments are indicated by arrows. Brackets indicate indistinct smeary DNase I-generated subfragments and the HS that would be expected in those regions. Slopes indicate increasing DNase I concentration (general range: 0.0–4.0 mg/ml).

repressive chromatin structures as a component of their action (Jenuwein *et al*., 1993; Barton and Emerson, 1994; Walters *et al*., 1996). Establishment of cooperation between the downstream and upstream elements within the TCRα LCR may render HS6 more sensitive to DNase I and relatively suppress the nuclease sensitivity of the HS2–5 region. In the β:2–6 transgene, the proposed negative element and TCRα enhancer elements are absent, leaving the HS2–6 region free to interact with upstream elements in the human β-globin reporter gene and drive transcription. This is evidenced by the extensive nuclease sensitivity of this construct in both thymus and liver.

Differential chromatin structures which exist in thymus and liver at the endogenous TCRα locus are strikingly similar to those formed on the β:1–8 transgene. Therefore, the proposed model applies to endogenous T cell-specific control of TCRα gene expression. This supports an important role for the regulation of differential chromatin structure and LCR activity by other *cis*-acting elements in the determination of cell type-specific gene expression *in vivo*. The model gives some non-LCR transcriptional control elements a dual role in determining tissue-specific gene expression as modifiers of both RNA polymerase activity and LCR function. A similar 'dual role' hypothesis has been invoked to explain characteristics of enhancer activity *in vitro* and in stably transfected cell lines (Jenuwein *et al*., 1993; Barton and Emerson, 1994; Walters *et al*., 1996).

A novel, non-tissue-restricted chromatin-opening element: a ubiquitous subunit of LCRs?

The discovery of a ubiquitous chromatin-opening activity is novel. The TCRα LCR HS2–6 region should be a useful tool for driving expression of genes systemically in transgenic mouse models. This element may also be helpful in the design of vectors for gene therapy applications. It may be possible to use HS2–6 together with particular combinations of other *cis*-acting elements to direct transgene expression to specific tissues. It appears, however, that not all tissue-specific *cis*-acting elements can dominantly restrict this region's activity. Although human β-globin is erythrocyte specific in its expression,

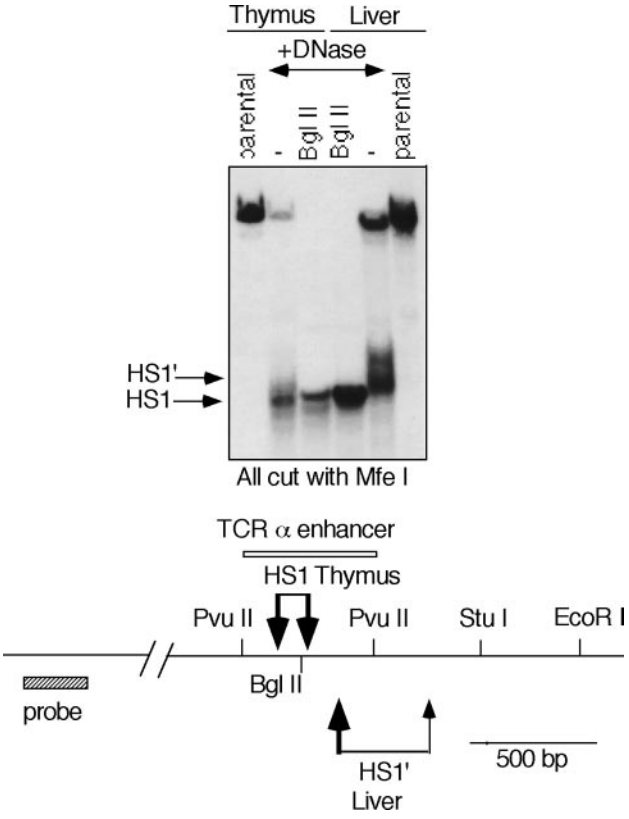

Fig. 7. Fine mapping of HS1 and HS1′. DNase I hypersensitivity assay on liver and thymus nuclei from β:1–8, line 12. 'Parental' lane indicates *Mfe*I-digested, non-DNase-treated sample. The DNase-treated samples were divided and treated as described in the text. Restriction enzymes used in double digestion are indicated. This experiment was run on a 0.6% agarose gel. HS1 and HS1′ are indicated with arrows. The localization of HS1 and HS1′ clusters (indicated by vertical arrows) relative to restriction sites is shown below.

and its enhancers are considered to be erythrocyte specific (Behringer *et al*., 1987; Antoniou *et al*., 1988), we (this study) and others (Greaves *et al*., 1989) have shown that these elements do not drive erythroid-specific expression under the influence of heterologous LCRs. It is possible

that our chromatin-opening element enables the human β-globin control sequences to use the transcriptional regulatory machinery of the various organs sufficiently to drive RNA polymerase, whereas alone, these elements appear to drive erythroid-specific expression (Chada *et al*., 1985; Townes *et al*., 1985).

The HS2–6 element described here has many characteristics of an LCR. It permits efficient transgene expression in all founders and drives quite high levels of expression in some organs that trend upward with increasing copy number. It provides open chromatin for a linked transgene and, to some degree, helps it to overcome position effects that would silence it in a proportion of transgenic founders. It differs from LCRs in two important ways. First, it does not provide absolute copy number dependence in all the organs in which it is active. This is particularly evident in the thymus of high-copy β:2–6 lines and the non-lymphoid organs of the low-copy lines. Secondly, complete LCRs should function in single copy (Ellis *et al*., 1996). Our only single-copy β:2–6 line gives less expression per copy than the multicopy lines, indicating that the HS2–6 region may be functioning as a 'partial LCR' (Ellis *et al*., 1996; Milot *et al*., 1996). Elements within the β-globin reporter gene appear to cooperate somewhat with HS2–6 to partially restore some degree of copy-related expression in non-lymphoid organs. However, copy-related expression in thymus clearly requires the T cell-specific elements contained in the HS7, 8 and 1 region. We propose that the HS2–6 region represents a novel element that provides open chromatin and linked transgene expression in all founder mice. This element is a ubiquitously active subunit of the TCRα LCR. This activity, in combination with the upstream tissue-restrictive elements, comprise, at least in multiple copies, a T cell-specific LCR.

Perhaps what most distinguishes the HS2–6 element from LCRs is its ubiquitous activity. LCRs have been described in many tissue-specific gene loci, including human β-globin (Grosveld *et al*., 1987), chicken macrophage-specific lysozyme (Bonifer *et al*., 1994), human CD2 (Greaves *et al*., 1989), human growth hormone (Jones *et al*., 1995), human adenosine deaminase (Aronow *et al*., 1992) and the immunoglobulin heavy chain locus (Madisen and Groudine, 1994). These LCRs have all been shown to drive tissue-specific expression of linked transgenes in mice and/or cell lines. Furthermore, these LCRs contain within them elements with enhancer activity (Tuan *et al*., 1989; Lake *et al*., 1990; Aronow *et al*., 1992; Bonifer *et al*., 1994; Madisen and Groudine, 1994; Jones *et al*., 1995). In contrast, HS2–6 appears to be devoid of classical transcriptional enhancer activity (Diaz *et al*., 1994).

The most extensively characterized LCR is that for β-globin (reviewed in Dillon and Grosveld, 1993; Engel, 1993; Orkin, 1995; Martin *et al*., 1996). It consists of four HS located 6–22 kb upstream of the fetal ε-globin gene. LCR activity has been mapped to three 200–300 bp core fragments of HS2, HS3 and HS4. In this LCR, separate elements conferring tissue specificity and chromatin opening have not been observed. HS2 contains the classical enhancer activity while HS3 contains the dominant 'chromatin-opening' activity responsible for position independence (Ellis *et al*., 1996). The HS3 core element contains several binding sites for the erythroid-specific GATA-1 factor as well as G-rich sequences (GT boxes) known to bind the ubiquitous Sp-1 family proteins. A combination of both of these *cis*-acting elements is essential for the activity of HS3, suggesting that the chromatin-opening activity of the β-globin LCR requires both ubiquitous and tissue-specific factors (Philipsen *et al*., 1993).

5'
*CAGCTG*TAAACTGAAACAAACCCTTTCCTCCCATGGGCTTCCACCCCCCACCCCACCCCATGGCGTTTTATCACAGCAATGAAAACCCTAAGATAAATCTTAAGTCATGAGGCACCCAAACACTAGCCTAGACTTCCAGTCCACAACCCACCAGAACTACAACTGAGCTAGTAGCTTCCAGGTGCTGGAAACTAGCTGATGCTGGCTGGCTTGCTGACTTTCCCAAAGAGGGAACAATCTCTGTCACTTCCCAAGTATGTTCCAACAATGTAACAGACCGCTTTGTGGGCCAAGATCACAGACGGATCAAGAGCAGAGGTGCAGGCTGCCGGGAGAGGAAGCAGGAGGAACATTTGCATGCCGTGGTGACAGGAAGTGTGTAGGCAGAGGGTGCAAGGGCTGTTTCCCACCACTGCCGATGCCGAGCAAGGAACAATGTACAGTAGTTGTGGTAAATGTTGCAGCCCCACAGATTGAACACAGGAAATAAAAATAACCCCCCCCCCCACACACACACACAGAGGAGGTGTGAGCTGAAGCTGCAGAAACAGCAGACATCCATCCCTCAGCCCATACAGTACCTGAGTCAGCAAAACCAACCCCTCCCGGG
3'

Fig. 8. Sequence of the *Pvu*II–*Sma*I fragment of the TCRα LCR containing HS6. G-rich sequences (when read on the opposite strand) are underlined. *Pvu*II and *Sma*I restriction sites are indicated by italic type. The sequence of the HS2–6 region has been deposited in GenBank, accession number AF 000941.

In the TCRα locus, we show separable elements controlling tissue specificity and chromatin opening. In addition, we show that non-LCR, TCRα *cis*-acting elements are dominant in controlling the chromatin structure, turning the ubiquitously active HS2–6 chromatin-opening region into a T cell-specific LCR. HS6 is the most prominent HS in this region. The sequence of the 610 bp *Pvu*II–*Sma*I fragment containing HS6 is shown in Figure 8. This region contains many G-rich sequences (GT boxes) similar to those found in the minimal β-globin LCR HS regions. This novel element, in general, or these G-rich motifs specifically, may be an important common component of the chromatin-opening activity for the TCRα, β-globin and perhaps other LCRs. The ubiquitous activity of the TCRα HS2–6 region may be due to the presence of these elements, whose activity would be modulated by the 5′ *cis*-acting elements (HS7, 8, 1 and 1′) to enforce T cell specificity. Perhaps LCR-integral, tissue-specific elements play analogous roles within other LCRs. Alternatively, the wide activity of this chromatin-opening region may reflect the presence of other uncharacterized elements that are not present in tissue-specific LCRs. Further mutational analysis and identification of proteins mediating the HS2–6 region activity and comparison with other systems should lead to a better general understanding of the molecular basis for LCR function.

Materials and methods

Transgenic mice

DNA fragments for microinjection were purified by double gel purification on low-melting point agarose (Seaplaque-FMC) followed by digestion with β-agarase (New England Biolabs). DNA was microinjected into the pronucleus of (C57Bl×CBA)F1 fertilized mouse eggs, and transferred into pseudo-pregnant CD1 foster mothers. Transgenic mice were identified by Southern blot analysis on tail DNA and/or PCR on ear-punch DNA. Copy number was determined for each line by analysis of at least two Southern blots by PhosphorImager analysis (Molecular Dynamics)

DNA constructs

The HS1–8 fragment was constructed as follows: a 564 bp *Alu*I fragment containing HS7 was blunt ended and cloned into the *Pvu*II site of pSP72

(Promega). This HS7 fragment was liberated as a *Bgl*II–*Sal*I fragment and was cloned back into the *Sal*I–*Bam*HI sites of pSP72. A 9.0 kb *Stu*I (partial digest)–*Sac*I fragment representing HS8, 1–6 was then cloned into this plasmid digested with *Sma*I and *Sac*I. The 4.9 kb *Bgl*II β-globin fragment was blunt ended and cloned into the *Pvu*II site of pSP72. It was then liberated using *Sal*I and *Xho*I sites and cloned into the *Sal*I site of the HS1–8 plasmid. The entire insert was isolated using *Sal*I and *Cla*I for microinjection. The β:2–6 was made similarly with a 6.0 kb *Eco*RI–*Sac*I fragment representing TCRα locus HS2–6 instead of the HS1–8 fragment.

RNA analysis

RNA was prepared according to the 'one-step' protocol (Chomczynski and Sacchi, 1987) from transgenic mouse tissues that were dissected of fat, minced (except for thymus and spleen) and rinsed extensively with phosphate-buffered saline to minimize contamination by blood. Five to 10 μg of RNA were used for analysis. RNase protection was performed according to the protocol of Gilman (Ausubel *et al*., 1991) RNA probes were labeled with [^{32}P]GTP and SP6 RNA polymerase as follows. For β-globin, a 2.0 kb *Bam*HI fragment spanning exons 1 and 2 was cloned into pSP72 (Promega) in the opposite orientation with respect to the SP6 promoter. The plasmid was linearized with *Ava*II to generate an RNA probe to exon 2. For γ-actin, plasmid was made and linearized with *Hin*fI (Enoch *et al*., 1986). Resulting RNA probes were acrylamide gel-purified prior to hybridization.

DNase hypersensitivity assays

Nuclei from liver (Wu, 1989) and thymus (Enver *et al*., 1985) were prepared then resuspended in DNase digestion buffer (Siebenlist *et al*., 1984) at 10^8 nuclei/ml. Nuclei were digested for 10 min on ice then stopped with 1/10 volume 5% SDS/100 mM EDTA. Genomic DNA was digested with *Sna*BI and *Sac*I to generate a fragment beginning 5′ of the β-globin coding region to 3′ of HS6. The digested, DNase I-treated DNA samples were subjected to electrophoresis through 0.8% agarose (unless otherwise specified). Southern blots were prepared on Hybond-N membrane (Amersham). Hybridization was performed using 'Quickhyb' solution (Stratagene). The probe used was a *Sna*BI and *Eco*RI restriction fragment of the β-globin gene. For the HS1 mapping experiment, the primary digest was as described in the text and generates an 11.2 kb parent fragment extending from the β-globin gene to 5′ of HS6. The probe was an *Mfe*I–*Eco*RI fragment of the β-globin gene. This experiment was run on a 0.6% agarose gel.

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