# DEVELOPMENTAL-STAGE AND CELL-TYPE SPECIFIC ACTIVITIES OF THE MOUSE T CELL RECEPTOR-ALPHA LOCUS CONTROL REGION

**by**

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## ABSTRACT

DEVELOPMENTAL-STAGE AND CELL-TYPE SPECIFIC ACTIVITIES OF THE MOUSE T CELL RECEPTOR-ALPHA LOCUS CONTROL REGION

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The genomes of multicellular eukaryotes encode tens of thousands of genes, with but a small proportion of these being expressed in any given cell-type. An understanding of the mechanisms governing the spatial patterns and the developmental timing of gene expression is of particular interest to biologists and is a prerequisite for the development of effective forms of treatment of genetic disorders. The work presented here focuses on the regulation of the T cell-specifically expressed mouse T cell receptor alpha (TCRα) gene. The TCRα locus contains a powerful cis-acting transcriptional regulatory element known as a locus control region (LCR). An LCR is defined by its ability to confer upon linked reporter transgenes high-level, tissue-specific and copy number-dependent expression, independent of the site of chromosomal integration. The TCRα LCR is flanked in the genome by three differentially expressed genes.

It is of particular interest to determine the role of the LCR in the expression of the genes in its locus as well as understand the molecular basis for its powerful activity at ectopic sites. Transgenic mouse models have been employed here to investigate the functions of

key LCR elements as well as activities of the LCR as a whole. These studies have lead to the identification of discrete sequences involved in regulating chromatin accessibility. This work has also produced evidence of the importance of LCR function during thymic development and, surprisingly suggests the involvement additional, as yet uncharacterized elements in the specification of peripheral patterns of gene expression in the locus. It will be important to identify the elements involved in the differential mechanisms of thymic and peripheral T cell gene regulation described here, and to determine the overall significance of these mechanisms to T cell biology.

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# CHAPTER 1

# INTRODUCTION

## 1.1 T cells and T cell Receptors

Adaptive immune responses involve the specific recognition and subsequent elimination of foreign antigens by the lymphocytes, T cells and B cells. The specificity of antigen recognition is dependent on unique antigen receptors expressed by these cells. The T cell receptors (TCRs) are expressed as heterodimeric chains on the surface of T cells. TCRalpha (TCRα) and TCR beta (TCRβ) are expressed exclusively in αβ T cells, while TCRgamma (TCRγ) and TCRdelta (TCRδ) are expressed in γδ T cells. The development of functional T cells is strictly dependent on the proper regulation of these TCR genes.

## 1.2 T cell development

The self-renewing hematopoietic stem cell (HSC) resides in the adult bone marrow and is capable of differentiating into all the various blood-cell types of both lymphoid and myeloid lineages. The lineage fate of a HSC involves highly coordinated interactions between transcription factors and cis-acting gene regulatory elements. Essentially all circulating T cells develop from a common lymphoid progenitor (also capable of B cell and natural killer cell differentiation) that has differentiated from the HSC (Fig. 1), which then migrates to the thymus. Once in the thymus, T cell development is marked by the expression of a series of stage-specific markers. These include the expression of the

various T cell receptor chains and co-receptors. Likewise, the differential expression of protein factors at these developmental stages, gives rise to the separate T cell lineages.

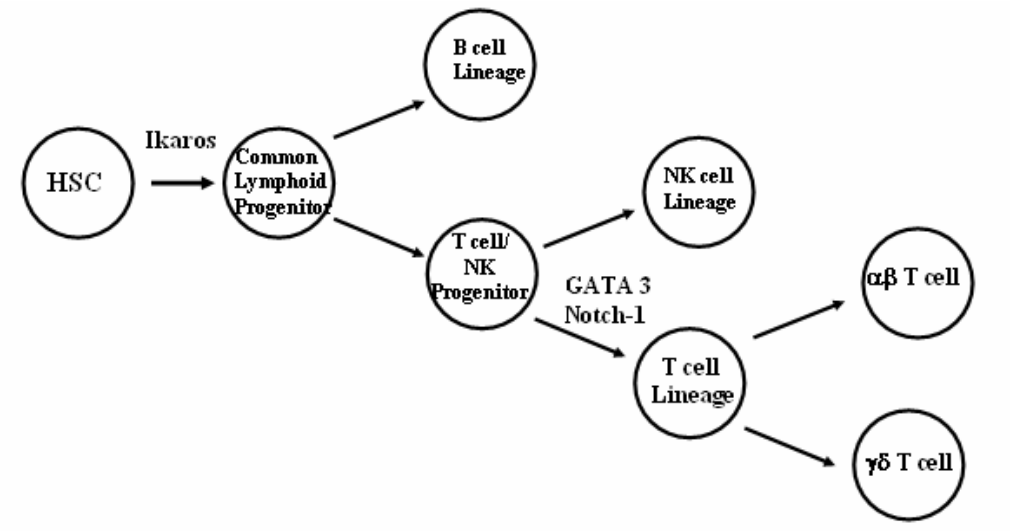



**Figure 1. Lymphocyte development.** Outline of the development of B and T lymphocytes and natural killer (NK) cells from the hematopoietic stem cell (HSC). Key transcription factors involved in lineage decisions are indicated.

## 1.3 Regulation of T cell development

### 1.3.1 Transcription Factors

Through gene targeted mutational analysis, several key regulators of T cell development have been identified (Rothenberg and Taghon, 2005). Some of the most important factors include members of the Ikaros, Runx and Ets transcription factor families, GATA-3, the signaling receptor Notch1 and the TCF/LEF factors. While these factors have all been implicated in early T cell development, many have also been shown to be involved in

lineage decisions at later stages. Runx1/AML1 is required for the development of all hematopoietic lineages from the fetal liver and adult bone marrow. The Ikaros family of transcription factors is necessary for lymphocyte (T cell and B cell) development. The Notch1 signaling pathway and T cell specific GATA-3 are both important for T cell-lineage specification, while the TCF/LEF factors are primarily involved in cell proliferation at specific T cell developmental stages. Many of these transcription factors have been implicated in the regulation of TCRα gene expression, through interactions with the TCRα enhancer (Eα) (Balmelle et al., 2004; Spicuglia et al., 2000).

### 1.3.2 Cis-acting elements

Prior to the 1990's studies of cis-acting transcriptional regulatory elements were largely focused on the classical DNA sequence elements such as enhancers, silencers and promoters. These elements are all united in their ability to regulate transcription by directly influencing the activity of RNA polymerase II. The activities and transcription factor interactions of these elements have been extensively studied since the 1980's. For the most part, these studies utilized cell-based transient transfections of reporter gene constructs. Although transient transfection assays provided a wealth of information regarding the factor interactions and sequence requirements of these classical gene regulators, it became apparent that additional elements were required to achieve reliable, high-level expression of randomly integrated transgenes in mice. Researchers would later recognize the importance of a different class of cis-acting regulatory elements, whose activity is dependent on a chromosomal context.

Although the mechanistic details of chromatin based modes of transcriptional regulation are still largely unknown, it is clear that chromatin remodeling factors and covalent modifications of DNA and its associated histones are important determinants of chromatin structure and gene expression (Jenuwein and Allis, 2001; Narlikar et al., 2002). In addition, a few examples of cis-acting elements seeming to act at the chromatin-level have been described. These include matrix attachment regions or MARs (also known as scaffold attachment regions), insulators and locus control regions (LCRs). MARs have been described as AT-rich elements that attach to the nuclear matrix to form DNA loops that separate independent transcriptional domains. Insulator elements provide a protective function, preventing the inappropriate silencing of genes, by inhibiting the propagation of heterochromatin from neighboring loci. Insulators also block promoter activation by an enhancer when positioned between the two elements. Lastly, LCRs, originally viewed as "super enhancers", are large DNA elements composed of several DNase I hypersensitive sites that are capable of transferring all of the characteristics of its locus of origin to a linked reporter gene in a chromosomal context. The work described here focuses on the regulation of gene expression by an LCR element in the mouse TCRα locus.

## 1.4 Locus Control Regions

A Locus Control Region (LCR) is defined by its ability to impart upon a linked transgene physiological levels of expression in a copy number-dependent and tissue-specific manner (specified by the LCR locus of origin), independent of the site of chromosomal integration (Li et al., 2002). LCRs are characterized by clusters of DNase I hypersensitive

sites that work cooperatively for complete LCR activity. The hypersensitive sites within LCRs usually represent individual cis-acting elements with activities that are characteristic of either classical (e.g. enhancers and silencers) or chromatin-based regulatory elements. Falling into the latter category are elements capable of modifying chromatin structure and suppressing the silencing of genes integrated into transcriptionally inactive loci.

The LCR of the human β-globin gene was the first to be identified (Grosveld et al., 1987). In early transgenic studies of human β-globin, genomic fragments that included regulatory sequences immediately flanking the β-globin gene produced sub-physiological levels of expression in only a small fraction of transgenic mice (Kollias et al., 1986; Magram et al., 1985; Townes et al., 1985). These findings implicated additional regulatory elements in the proper regulation of β-globin gene expression. An indication as to the identity of these elements came from the observation that in some forms of β-thalassemia (a human disease characterized by severe anemia due to significantly lowered or absent β-globin expression) in which the β-globin gene was completely intact, there were large naturally occurring deletions upstream of the gene (Kioussis et al., 1983; Taramelli et al., 1986). The importance of these sequences in the proper regulation of β-globin gene expression was confirmed by Grosveld and colleagues in a transgenic mouse study that reported high-level, position-independent expression of a human β-globin transgene upon inclusion of sequences normally found 50-kb upstream of the β-globin gene.

While the human β-globin LCR remains the most extensively studied LCR to date, LCRs have now been implicated in the regulation of many other genes in humans and mice. Several of these LCR elements are found in immunologically relevant gene loci. In humans, these include the loci of the T cell-specific human CD2 (Greaves et al., 1989) and adenosine deaminase (Aronow et al., 1992) genes and the MHC class I HLA-B7 gene (Kushida et al., 1997). The LCR in the human growth hormone locus (Jones et al., 1995) has also been extensively studied. Examples of genes regulated by LCRs in mice include the β-globin (Moon and Ley, 1990) and TCRγ (Baker et al., 1999) genes. Partial LCR activity has also been implicated in the regulation of the mouse interleukin-2 (Yui et al., 2001) and immunoglobulin μ heavy chain (Jenuwein et al., 1993). For a comprehensive list and review of LCRs refer to Li et al. (2002). The LCR of the mouse T cell receptor alpha gene was first described in a publication by Diaz et al.(1994).

## 1.5 The TCRα/δ/Dad1 Locus

### 1.5.1 Organization of the TCRα/δ/Dad1 Locus

In a manner similar to immunoglobulin (B cell receptor) gene rearrangement, TCR genes are assembled from variable (V), diversity (D) and joining (J) gene segments, by the RAG-1 and RAG-2 recombinase enzymes (Bassing et al., 2002). The TCRα gene segments (Vα and Jα) are located on mouse chromosome 14. Gene regulation at this locus is complicated by the presence of two other genes with very different expression profiles (Fig. 2). Interestingly, in the germline configuration, the TCRδ genes (Vδ, Dδ Jδ and Cδ) are located between the TCRα Vα and Jα genes. This ensures that upon

rearrangement of TCRα, the TCRδ genes are excised from the locus. The TCRα and TCRδ are expressed mutually exclusively in αβ and γδ T cells. Moreover, the initiation of rearrangement and expression of these genes occur at different stages of thymocyte development. At the 3' end of the locus is a third gene, Defender against Death 1 (Dad1). Dad1 was originally described as a suppressor of apoptosis (Nakashima et al., 1993) and was subsequently identified as a subunit of the oligosaccharyl transferase enzyme complex (Makishima et al., 1997). In contrast to the cell-type specific expression of the TCR genes, Dad1 is expressed in all tissues (Hong et al., 1997), including non-lymphoid organs. It is of particular interest to determine how the differential gene expression patterns of these three genes in a common genomic locus, are achieved. Spanning a 12-kb region between the TCRα gene on the 5'end and Dad1 on the 3' end are the 9 DNase I hypersensitive sites (HS) that make up the TCRα LCR. There is evidence to suggest that the LCR may be involved in the differential regulation of all three genes in this locus.

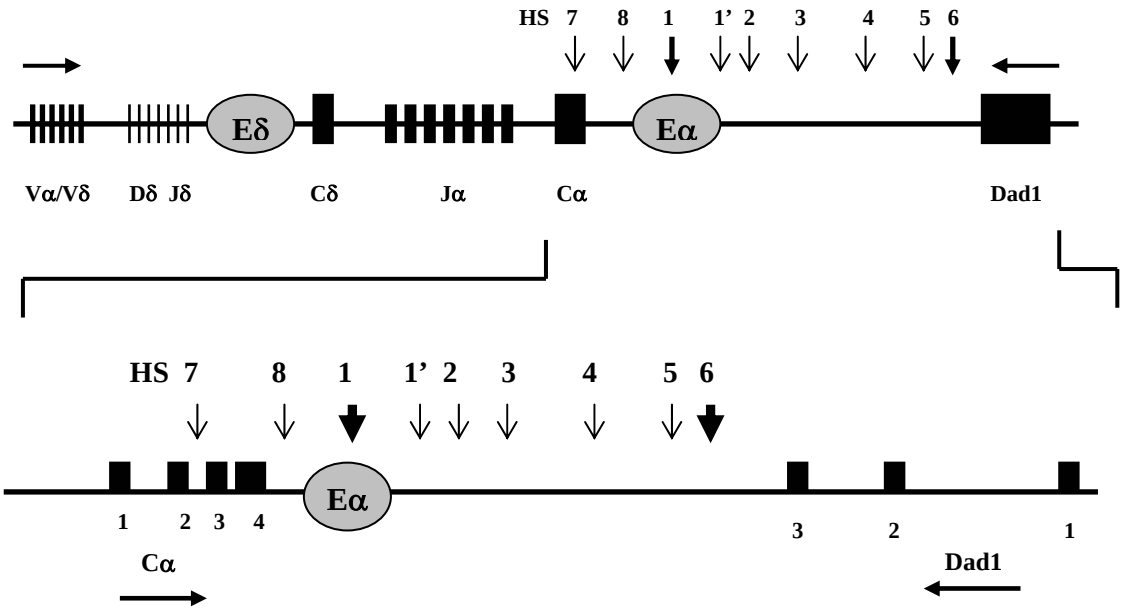



**Figure 2**. **The mouse TCRα/δ/Dad1 locus.** The LCR is represented as 9 DNase I hypersensitive sites (HS) and their relative positions are indicated by vertical arrows. Horizontal arrows indicate the transcriptional orientations of TCRα and Dad1 genes. The TCRα (Eα) enhancer and TCRδ (Eδ) enhancer are represented as grey ovals. This diagram is not drawn to scale and does not represent the correct numbers of variable (V), diversity (D), and joining (J) gene segments. The diagram of the LCR at bottom is shown for detail. Numbered black rectangles represent exons of the genes indicated.

## 1.5.2 The TCRα locus control region

The mouse TCRα LCR was initially characterized as an element made up of 8 HSs (HS 1-8) that directed lymphoid organ-specific, position-independent and copy number-dependent expression of a TCRα reporter gene in transgenic mice (Diaz et al., 1994). Subsequently, an additional hypersensitive site, HS1', was identified (Ortiz et al., 1997). These nine HSs extend from the TCRα constant region to a position 3-kb downstream of the Dad1 gene. HS1 maps to the extensively-studied TCRα enhancer (Eα) (Winoto and Baltimore, 1989). HS7 and HS8 are located 5' of HS1 and colocalize with sequences that were previously characterized as transcriptional silencers in transient transfection

experiments (Winoto and Baltimore, 1989). HS1', lies immediately 3' of Eα. The region containing HSs 2-6 is located 3' of HS1'.

The DNase I hypersensitivity assay is used as an indirect assessment of chromatin structure in a locus. The hypersensitive sites of the TCRα LCR were originally identified in the EL4 αβ T cell line (Diaz et al., 1994). Chromatin structure was also examined at the endogenous TCRα locus. Differential patterns of HS formation at the TCRα locus were observed between TCRα expressing and non-expressing tissues (Hong et al., 1997). In the thymus, HS1 and HS6 are the most prominent HSs detected, while HS1' is also forms, albeit more weakly. In non-lymphoid organs HS1' is the only prominent HS.

## 1.6 Functions of the TCRα LCR and its sub-elements

### 1.6.1 A transgenic approach to the study of TCRα LCR function

Much of our understanding of TCRα LCR function has come from transgenic mouse studies. These studies involve the introduction of transgenic DNA constructs into single cell embryos. As mature chromatin has not yet formed at this stage, transgenes tend to integrate randomly into the genome. Therefore, each resulting transgenic line represents integration into a unique transcriptional environment.

One of the early transgenes used to study TCRα LCR function consisted of a human β-globin reporter gene linked 3' of an LCR fragment containing all nine HSs. This transgene was called β:1-8. In addition to reproducing the expression characteristics of

the endogenous TCRα gene, this transgene also assumed a chromatin configuration (assayed by DNase I hypersensitivity) identical to that of the endogenous locus (Ortiz et al., 1997). Its ability to reproduce the characteristics of TCRα gene expression and the chromatin structure of the endogenous locus made this transgenic model suitable for the investigation of the activities of the LCR and its sub-elements.

### 1.6.2 Silencers HS7 and HS8

Sequences that correspond to HS7 and HS8 were found to silence enhancer activity in multiple non-T cell and γδ T cell lines, but not in αβ T cell lines (Winoto and Baltimore, 1989). These results suggested that the function of these sequences is to specifically silence the activity of the TCRα Eα in non-αβ T cells, thereby preventing the ectopic expression of the TCRα gene in other tissues. A 5' deletion of HS7 and HS8 in the context of the β-globin transgene (producing the β:1-6 construct) had no effect on LCR activity (Ortiz et al., 1999). In the absence of these sequences lymphoid organ-specific expression was still maintained. These results would indicate that HS-7 and -8 are not required for restricting the activity of Eα to T cells and that their contribution to LCR activity is negligible.

### 1.6.3 TCRα Enhancer (Eα) and HS1'

Well before the mouse TCRα LCR was characterized, the T cell specific Eα element was localized to a 230-bp PvuII-BglII fragment located 3' of the Cα region. This region which co-localizes to HS1 was able to activate reporter gene expression in T cell lines

(Winoto and Baltimore, 1989). The highly homologous sequences of the mouse Eα and the human Eα, both have been the focus of extensive investigation with regards to factor interactions and the regulation of TCR rearrangement and expression. The human TCRα Eα has been shown to require the corporative binding of several transcription factors into a multi-protein complex (Balmelle et al., 2004; Hernandez-Munain et al., 1998). Some of these factors include TCF-1, LEF-1, Runx1/Aml1, Ikaros and GATA-3, all of which have been shown to be regulators of T cell differentiation (discussed above). The consensus sequences for all of these protein factors have also been identified at the mouse Eα (Spicuglia et al., 2000).

Targeted gene knockout studies in mice have been conducted to address the role of Eα in the regulation of TCRα gene expression. A targeted deletion of sequences known to contain both Eα and HS1' resulted in an overall block in thymocyte development at the $CD4^+CD8^+$ double positive (DP) stage with only few mature peripheral αβ T cells that expressed a limited repertoire Vα genes (Sleckman et al., 1997). Surprisingly, this deletion also produced lower than normal levels of TCRδ gene transcription in γδ T cells, suggesting that the Eα/HS1 elements also contribute to TCRδ gene regulation. Since both Eα and HS1' were deleted in these experiments it is difficult to attribute the observed phenotypes to the absence of either element.

In the β-globin transgenic model, a 5'deletion of Eα demonstrated its importance in directing high-level, copy number-dependent expression in the thymus (Ortiz et al., 1999). Surprisingly, lymphoid-specific expression and the tissue-differential chromatin

structure were not perturbed in the absence of the enhancer. However, a further 5' deletion that eliminated HS1', resulted in the loss of tissue-specific expression and an equivalently highly accessible chromatin structure in both lymphoid and non-lymphoid tissues (Ortiz et al., 1997).

Another interesting property of HS1' is its involvement in tissue differential DNA methylation patterns at the TCRα locus. At the endogenous locus and in the wild type LCR transgene, sequences at HS4 are specifically demethylated in the lymphoid organs (Santoso et al., 2000). This demethylation event is abolished in the absence of HS1'.

It is important to note that HS1' is not a classical enhancer, as the corresponding region failed to display any enhancer activity in transient transfection experiments (Winoto and Baltimore, 1989). It is clear that the activities of HS1' are distinct from those of the adjacent Eα. In general, HS1' seems to play a central role in cell-type specific expression, possibly through its regulation of differential chromatin structure in TCR-expressing and non-expressing tissues. These activities of HS1' are likely to involve very complex molecular mechanisms.

### 1.6.4 The HS2-HS6 region

HS2-6 activity has been studied using transgenic animals. In the absence of the upstream HS1/HS1'clusters, HS2-6 established a highly accessible chromatin configuration that was equivalent in the lymphoid and non-lymphoid tissues of these animals, but differed from the chromatin structures observed at the endogenous locus (Ortiz et al., 1997). In

accordance with this finding, tissue-unrestricted expression of the transgene was observed. Although strict copy number-dependence was lost under the regulation of HS2-6, expression was observed in all transgenic lines, indicating that sequences within the HS2-6 region are able to suppress site-of-integration silencing of integrated transgenes.

A targeted deletion of sequences containing HS2-6 did not affect the expression of TCRα gene expression *per se*, but reduced the number of mature thymocytes expressing the gene (Hong et al., 1997). It should be noted, however that this deletion also removed the third exon of the Dad1 gene, and therefore, the observed effects cannot be attributed solely to the absence of the LCR sequences. The interpretation of these results was further complicated by the presence of a neomycin resistance gene retained from the gene targeting event. This gene was driven by a phosphoglycerate kinase promoter, which may have had an effect on TCRα gene expression.

## 1.7 **Defender against death1 (Dad1)**

The Defender against death 1 (Dad1) gene is located 3-kb upstream of HS6. Dad1 is ubiquitously expressed and a null mutation of this gene results in embryonic lethality (Hong et al., 1997). Dad1 was originally described as an anti-apoptosis gene in the BHK21 hamster cell line (Nakashima et al., 1993), and was subsequently found to bear homology to a subunit of the oligosaccharyl transferase enzyme (Makishima et al., 1997), which is involved in N-linked glycosylation. Interestingly, Dad1 expression is regulated during thymocyte development (Hong et al., 1999), raising the possibility of a role for this gene in T cell differentiation. The double positive ($CD4^+$ $CD8^+$) thymic stage is

accompanied by a high incidence of apoptosis of cells that fail the positive selection process (ensures TCRα recognition of foreign antigen in the context of self antigen presentation molecules). It is interesting that relatively low-level expression of the anti-apoptotic Dad1 gene coincides with this double positive stage of thymic development. Dad1 expression is significantly upregulated in the most mature thymocytes, a stage at which high levels of TCRα are also expressed. As the optimal surface expression of TCRα has been reported to rely on N-linked glycosylation (Strong et al., 1994), this suggests that Dad1 may indeed play a role in the N-linked glycosylation of TCRα.

## 1.8 Significance

For many years studies of transcriptional control were limited to the investigation of the functions of classical control elements such as promoters and enhancers. It is now generally accepted that the activities of these elements do not fully support the temporal and spatial patterns of expression of genes in chromatin. The emergence of chromatin-based regulators of transcription has brought to light the complexity of gene regulation, but has also presented the opportunity to elucidate the mechanisms involved. The immune system provides numerous examples of genes that are strictly regulated in terms of the cell-type and developmental-stage specificity of expression. These genes therefore provide ideal systems to gain a better understanding of these important biological mechanisms. Examination of the TCRα locus addresses questions regarding T cell specific expression and the developmental timing of expression throughout T cell development. Certain elements within the TCRα LCR have been implicated in both of these activities.

A clear understanding of the mechanisms of transcriptional control in T cells is also important for therapeutic purposes. Viral vector gene therapy currently holds promise for the treatment of T cell-based diseases such as the severe combined immunodeficencies (SCIDs) and AIDS. Some of the problems that currently plague viral gene therapy trials include low-level expression, gene silencing and the specificity of expression. It is currently well accepted that an ideal vector for gene therapy would need to incorporate chromosomal regulatory elements that direct therapeutic levels of gene expression in the appropriate cell type at the desired time. As this TCRα LCR and others all incorporate elements that counter these negative transcription effects, the determination of the minimal sequence requirements for the activities of these elements will be useful in the design of gene therapy vectors.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Reporter transgene constructs

A 4.9-kb BglII genomic fragment of the human β-globin gene, which includes the β-globin promoter, 3'enhancer, exons and introns (Greaves et al., 1989), was used as a reporter gene for the construction of the β:1-8, β:1-6, β:6, β:1-6ΔTF123 and β:1-6ΔCTCF transgenes described in this work. The β:1-8 transgene has been previously described (Ortiz et al., 1997). Briefly, the human β-globin reporter gene was linked 5' of a fragment containing HS1-8 of the TCRα LCR. The β:1-6 transgene has also been described (Ortiz et al., 1999). Briefly, the human β-globin reporter gene was linked 5' of a 7.4-kb XhoI-SacI LCR fragment containing HS1-6. The β:6 transgene consists of the β-globin reporter gene linked 5' of a 1.6-kb MfeI-SacI HS6-containing fragment (Ortiz et al., 2001). The β:1-6ΔTF123 transgene was made in the context of the β:1-6 transgene and contains an effective 283-bp BclI-PstI deletion within HS6 that contains the previously described TF1, TF2 and TF3 sites (Harrow et al., 2004). The β:1-6ΔCTCF transgene was also made in the context of the β:1-6 transgene and contains a 94-bp AvrII-SacI deletion containing the HS1' 5' and 3' CTCF sites.

The "tailless" hCD2 reporter gene (hCD2ΔT) has been previously described (Melton et al., 1996). Briefly, a stop codon was introduced into exon V of the gene resulting in a molecule that lacks 116 amino acids of the cytoplasmic tail normally required for signaling. The hCD2:1-8 transgene was constructed in pBluescript (Stratagene, La Jolla

CA) with a 10.5-kb SalI-BamHI fragment of this "tailless" hCD2 gene that includes the hCD2 promoter and five exons ($2^{nd}$ and $3^{rd}$ introns have been removed) linked 5' of a 10.2-kb SalI-SacI fragment containing TCRα LCR HS1-8.

The HLA-B7:1-8 transgene was constructed by linking a 6.5-kb EcoRI genomic fragment of the human HLA-B7 gene (containing the native HLA-B7 gene and promoter) (Chamberlain et al., 1988) 3' of a 10.2-kb SalI-SacI fragment containing TCRα LCR HS1-8 (Ortiz et al., 1997). The transcriptional orientation of the HLA-B7 gene in this construct is such that the 3' end of the gene is linked to the 3' end of the LCR, thus mimicking the transcriptional orientation of the endogenous Dad1 gene.

## 2.2 Transgenic mice

DNA fragments for microinjection were twice gel purified from low melting agarose (Seaplaque) digested with β-agarase (New England Biolabs, Beverly, MA). Purified DNA was injected into the male pronucleus of F1 (C57BL/6 x CBA) fertilized mouse eggs that were then injected into pseudo pregnant female mice. Potential transgenic founders were screened by Polymerase Chain Reaction and/or southern blot analyses of DNA derived from ear-punch or tail-tip biopsy. Transgenic founders were outcrossed to C57BL/6 (Taconic Germantown, NY) to establish individual lines. Heterozygous transgenic offspring were used for experimental analyses. Relative transgene copy-number was determined by PhosphorImager (Molecular Dynamics) analysis of Southern blots. A minimum of three DNA samples purified from tails of individual mice within each line were treated with identical restriction enzymes and hybridized to the same

probe on a single Southern blot. Enzymes chosen for restriction analysis and probe preparation allowed for the simultaneous detection of non-overlapping fragments from the endogenous TCRα locus and the transgene. To control for differences in loading, transgene signals were normalized to the endogenous TCRα signal within each sample.

## 2.3 RNA analyses

RNA was purified according to the single-step RNA isolation protocol (Chomczynski and Sacchi, 1987) from mouse organs that were rinsed in PBS (to remove excess blood) and homogenized. RNA was also purified from T cells and B cells isolated from spleen through magnetic bead separation (described below). Five to ten μg of total RNA per sample were run on a 0.8% agarose gel and transferred onto a nylon membrane for hybridization. To detect the human β-globin transgene mRNA, a 428-bp BamHI-NcoI genomic fragment of the human β-globin gene coding region was used as a probe. A 0.5-kb EcoRV-PstI fragment from exon II of the human CD2 gene was used as a probe for to detect hCD2 transgene mRNA. HLA-B7 mRNA was detected using a 2-kb BglII genomic fragment. A 0.5-kb Sau3AI fragment from the TCRα constant region cDNA was used as a probe to detect endogenous TCRα mRNA. To control for differences in loading and transfer efficiency, a probe to 18S rRNA (Ambion, Austin, TX) was used. The TCRα constant region probe was also used as a loading control for the comparison of transgene expression within a single organ-type between transgenic lines. All probes were labeled with [α-32P]dATP and/or [α-32P]dCTP using random primers (Invitrogen). Transgene and endogenous TCRα mRNA were quantified and normalized to 18S rRNA by PhosphorImager (Molecular Dynamics) analysis.

## 2.4 DNase I hypersensitivity assays

Nuclei from liver (Wu, 1989) and thymus (Enver et al., 1985) were prepared as previously described. Prepared nuclei were resuspended in DNase digestion buffer (Siebenlist et al., 1984) at $5x10^7$ nuclei/ml. Nuclei were digested for 10 minutes on ice with increasing amounts (0-1.25 μg/$2x10^7$nuclei) of DNase I (Worthington) and reactions were stopped with 1/10 volume of 5% SDS/100mM EDTA. Nuclei were treated overnight at 55°C with 200 μg/ml proteinase K. Genomic DNA was then purified by phenol/chloroform extraction.

For analysis of hypersensitivity at the β:1-6ΔTF123 transgene locus, DNase I-treated samples were digested with SwaI and Sac I to generate a 9.2-kb parent fragment of the transgene. Separate aliquots of the same DNase I treated samples were digested with NheI to generate an 11-kb parent TCRα locus fragment for analysis of hypersensitivity at the endogenous TCRα gene. Samples were run on 0.8% agarose and transferred to nylon membrane for Southern blot analysis. The transgene locus was probed from the 5'-end with a 547-bp SwaI-PstI human β-globin fragment. The endogenous TCRα locus was also probed from the 5'-end with a 1.2-kb NheI to XhoI TCRα locus fragment that is not present in the transgene.

## 2.5 *In vivo* DNase I footprint analysis of HS1'

Nuclei from liver and thymus from a C57BL/6 mouse were prepared and DNase I-treated as described above. The amount of DNase I (Worthington Biochemical, Lakewood, NJ) used for nuclei digestion ranged from 0.0 to 2.0 μg/$2x10^7$nuclei. Plain genomic DNA was

purified from a C57BL/6 mouse thymus. Aliquots of genomic DNA were treated with amounts of DNase I ranging from 0 to1.0 ng per 35 μg of DNA. Genomic DNA was purified by phenol/chloroform extraction. The DNA was then subjected to ligation-mediated (LM)-PCR (Mueller et al., 1987). Gene-specific oligonucleotides (Genset, San Diego, CA) were selected, synthesized, and acrylamide gel purified. The products of these reactions were separated on 5% denaturing acrylamide urea sequencing-type gels.

## 2.6 Nuclear extracts preparation and electrophoretic mobility shift assay (EMSA)

Thymic and NIH 3T3 nuclei were prepared as they were for DNase I hypersensitivity experiments (described above). Nuclear pellets were resuspended in extraction buffer C (Staal et al., 1990) and rocked for 30 min followed by microcentrifugation at top speed. The supernatants were frozen in aliquots at -80°C. Nuclear extract (0.5-3 mg) was used in EMSA with 40,000 cpm (dry) of 32P-labeled oligonucleotide probe. The final binding reaction conditions were as follows: 15 mM HEPES pH 7.9, 80 mM NaCl, 15 mM KCl, 0.02 mM EDTA, 1 mM DTT, 1 mM PMSF, and 3% glycerol. Incubations were on ice for 45 min. For binding site competition assays, a 50- to 100-fold molar excess of unlabeled oligonucleotide was added to the binding reaction before the addition of labeled probe.

The sequences of the oligonucleotide probes used in the HS1' experiments are as follows: 5'CTCF-CCACTCTGTTGTAGTG, 3'CTCF-CCACACGGGGGCAGCA, β-globin FII-CCCAGGGATGTAATTACGTCCCTCCCCCGCTAGGGGGCAGCA (Bell et al., 1999).

The sequences of the oligonucleotide probes used in the HS6 experiments have been published (Ortiz et al., 2001) and are as follows:
thymic footprint (TF)1-TGCCGTGGCGACAGGAAGTG, TF2-TGTACAGTAGTTGT GGTAAATG, TF3-AGCTTCCACAGATTGAACACAGGAAATA, core binding factor (CBF)-TCGACTCCCGCAGAAGCCACATG, Ets-1-TCGACCTCTGGAAAGAGGAG, GATA3-TCGAGTAGAGATAAGATC, E-BOX-TCGAGGGCCACGTGCCCAG, GT-BOX-GCAGAGGTGGGTGGAGTTTCG.

For antibody supershift/blocking assays, 2-10 μg of antibody were added to the binding reaction, 1 hour into the incubation. The following antibodies for supershifitng HS6 complexes were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): anti-AML-1 N-20 (sc-8563), anti-AML-1 C-19 (sc-8564), anti-Elf-1 (sc-631), anti-Ets-1/Ets-2 (sc-112). For CTCF supershift experiments the following antibodies were obtained from Santa Cruz: anti-CTCF C-20 (sc-15914), anti-CTCF N-17 (sc-15916) and anti-CTCF E-14 (sc-15913). Anti-CTCF (06-917) was obtained from Upstate, Lake Placid, NY.

## 2.7 **Flow cytometry analysis**

Single cell suspensions of thymocytes and spleen cells were stained in FACS staining medium (RPMI 1640 medium supplemented with 3% fetal bovine serum and 10 mM HEPES buffer solution). For each sample $10^6$ cells per 100 μl volume were stained with 0.2 to 1.0 μg of the respective antibodies for 20 minutes at $4^oC$. For staining with antibodies derived from mouse hybridomas, samples were first blocked for 20 minutes at $4^oC$ with a 10-fold excess of normal mouse IgG and/or rat anti-mouse CD16/32 (Caltag

laboratories Burlingame, CA) per $10^6$ cells to reduce background staining. To detect surface expression of hCD2 the following antibodies were used: mouse anti-hCD2 from clone S5.2 conjugated to FITC or PE-Cy7 (BD Pharmingen, San Jose, CA) and mouse anti-hCD2 from clone S5.5 conjugated to PE-Cy5 or PE-Cy5.5 (Caltag). For analysis of thymic subsets, rat anti-mouse CD4 (GK1.5) conjugated to FITC, and rat anti-mouse CD8α (53-6.7) conjugated to PE (both from BD Pharmingen) were used. Rat anti-mouse CD44 conjugated to PE and rat anti-mouse CD25 conjugated to allophycocyanin (both from Caltag) were used to analyze thymic DN subsets. To analyze spleen subsets, the following PE-conjugated antibodies were used: rat anti-mouse CD19 (BD Pharmingen), mouse anti-mouse CD90.2 (Caltag), rat anti-mouse F4/80 (Caltag) and rat anti mouse Gr-1 (Caltag). PE-conjugated hamster-anti mouse γδ TCR (GL3) (BD Pharmingen) was used for analysis of γδ T cells. Flow cytometry acquisition was performed on FACScalibur (Becton Dickinson) for 4-color analyses and FACScan (Becton Dickinson) for two- and three-color analyses. Flow cytometry data were analyzed using Cell Quest Pro.

## 2.8 Magnetic bead cell purification

The Magnetically Activated Cell Sorter (MACS) and all kits used for the purification of cell subsets from thymus and spleen were obtained from Miltenyi Biotec (Auburn, CA). Enriched cells of at least 95% purity were used in experiments. The Pan T cell Isolation and the B cell Isolation kits were used to isolate mouse T cells and B cells, respectively. Rat anti-mouse CD19 and mouse anti-mouse CD90.2 were used to confirm purity of spleen B cells and T cells, respectively. To purify DN thymocytes for DN subset analysis, a single cell suspension of thymocytes in FACS staining medium was first stained with

the FITC-conjugated versions of rat anti-mouse CD8α (53-6.7), rat anti-mouse CD4 (GK1.5) and hamster anti-mouse γδ TCR (GL3) (all obtained from BD Pharmingen), followed by removal of labeled cells with an anti-FITC MicroBeads kit. For analysis of thymic γδ T cells, DN T cells were isolated as described except that FITC-conjugated anti-mouse γδ TCR (GL3) was not used in the initial step. Flow cytometry was used to confirm the purity of DN thymocytes by using FITC-conjugated versions of rat anti-mouse CD8β (53-5.8) and rat anti-mouse CD4 (RM4-4), both of which are derived from clones producing antibodies recognizing different epitopes than those used for purification. FITC positive cells were gated out for flow cytometry analysis of purified DN thymocytes.

# CHAPTER 3

# HS1'

## 3.1 In vivo factor occupancy and function of HS1' sequences

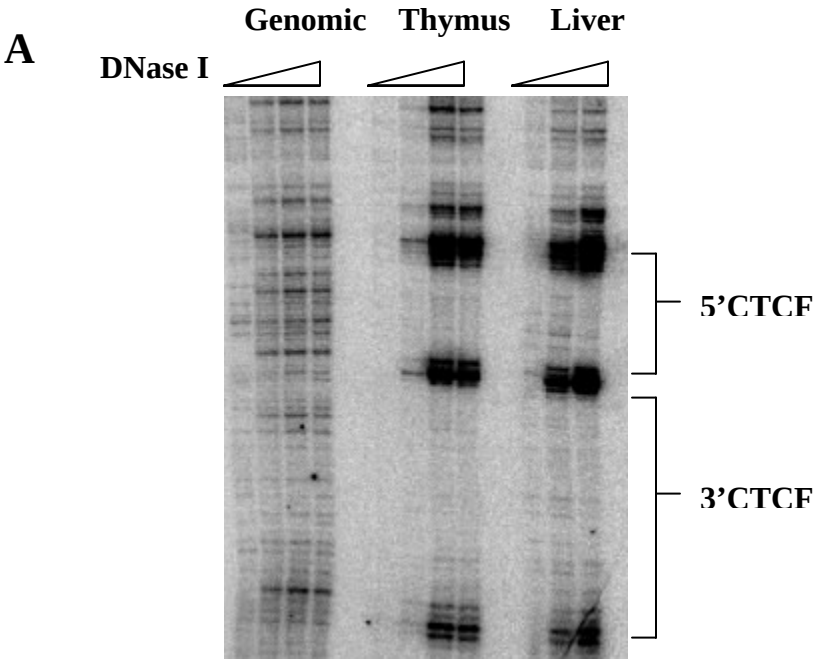
### 3.1.1 Background

Through mutational analysis of LCR activity in transgenic mice, HS1' has been shown to contribute to high-level lymphoid-specific gene expression (Ortiz et al., 1999). HS1' has also been implicated in the regulation of the differential chromatin structures found between lymphoid and non-lymphoid tissues, as a deletion of this element resulted in an equivalently accessible chromatin conformation in the thymus and non-lymphoid liver of transgenic mice (Ortiz et al., 1997). Another interesting property of HS1', is its involvement in the lymphoid-specific demethylation of sequences at HS4, almost 4-kb away (Santoso et al., 2000).

### 3.1.2 HS1' contains CTCF binding sequences

As HS1' is the only prominent hypersensitive site in non-lymphoid organs (Hong et al., 1997), this suggested that this element supports at least some activities that are not specific to T cells. To gain a better understanding of HS1' function, I have used *in vivo* footprint techniques to study the differences in factor occupancy between lymphoid (thymic) and non-lymphoid (liver) tissues. Several small regions of differential factor occupancy were identified between the two tissues (data not shown). The most obvious feature, however, was a large protected region that appears equivalently occupied in both

organs (Fig. 3A). Examination of this large footprint revealed two adjacent sequences that bear homology to the consensus CTCF (CCCTC binding factor) binding sequence (Fig. 3B). CTCF, is a ubiquitously expressed protein that has been shown to be required for the enhancer-blocking activities of vertebrate insulators (Bell et al., 1999). These two CTCF sequences were named 5'CTCF and 3'CTCF, and became the primary focus of the investigation of HS1'.




**B**

| | |
|---|---|
| **FII β-globin consensus** | **CCGCTAGGGGGCAG** |
| **HS1' 3'CTCF** | **CCACACGGGGGCAG** |
| **HS1' 5'CTCF** | **CCACTCTGTTGTAG** |

**Figure 3**. ***In vivo* footprint of HS1'of the TCRα LCR. *A*,.** Genomic DNA and nuclei of the tissues indicated were treated with increasing amounts of DNase I and the region of interest was amplified by ligation-mediated PCR. Regions corresponding to 3'CTCF and 5' CTCF sequences are indicated. ***B***, Alignment of 3'CTCF and 5'CTCF to the consensus chicken β-globin FII CTCF site. Shading indicates consensus with the FII sequence.

To examine complex formation at these CTCF sequences, electrophoretic mobility shift assays (EMSAs) were performed using two 16-bp oligonucleotides that bore the putative HS1' CTCF binding sites, 5'CTCF and 3'CTCF. As a positive control for CTCF binding, a 42-bp oligonucleotide that contains the CTCF binding site from the chicken β-globin 5'HS4 insulator (FII) was used (Bell et al., 1999).

The 5'CTCF and 3'CTCF oligonucleotides were incubated with either thymic or NIH 3T3 fibroblast nuclear protein extracts. Both extracts formed a single protein complex at 5'CTCF and 3'CTCF (Fig. 4). The similarities in the mobility rates of the complexes formed from both thymic and fibroblast extracts, suggested that these complexes were equivalent. These observations would be consistent with the presence of the ubiquitous CTCF protein in both tissue types. Due to the apparent similarities between thymic and NIH 3T3 complexes all further experiments were conducted using thymic nuclear extracts only. The specificity of complex formation at both 3'CTCF and 5'CTCF was confirmed by competitions with a molar excess of the respective unlabelled homologous competitors (Fig. 5). Interestingly, 3'CTCF and 5'CTCF cross compete for complex formation. Moreover, the CTCF sequence from the chicken β-globin 5' HS4 insulator, FII, efficiently displaced both of the 3'CTCF and 5'CTCF protein complexes. The HS1' CTCF sequences, however, did not displace the FII CTCF complex (Fig. 5).

As an alternative approach to demonstrate the presence of the CTCF binding to HS1', EMSA supershift assays were conducted using various antibodies directed against various CTCF epitopes. After several attempts under variable reaction conditions these

antibodies failed to supershift either of the HS1' "CTCF" complexes (data not shown). As these antibodies also failed to supershift the control chicken 5'HS4 CTCF complex, it was not possible to draw any conclusions regarding CTCF binding to the HS1' sequences from these experiments.

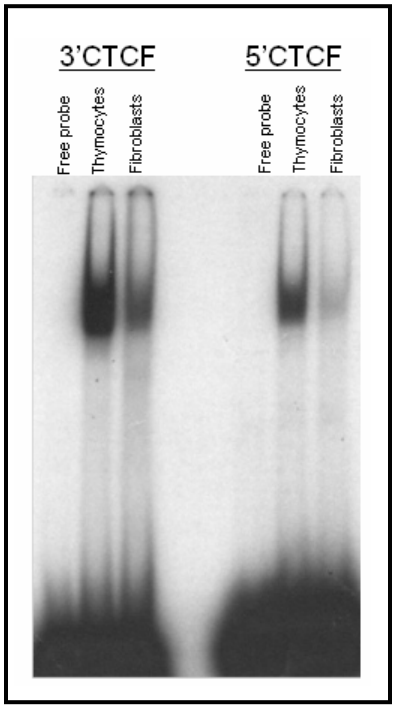



**Figure 4. Complex formation at HS1' 3'CTCF and 5'CTCF sites.** EMSA experiments showing the formation of thymic and fibroblast complexes at 3'CTCF and 5'CTCF.

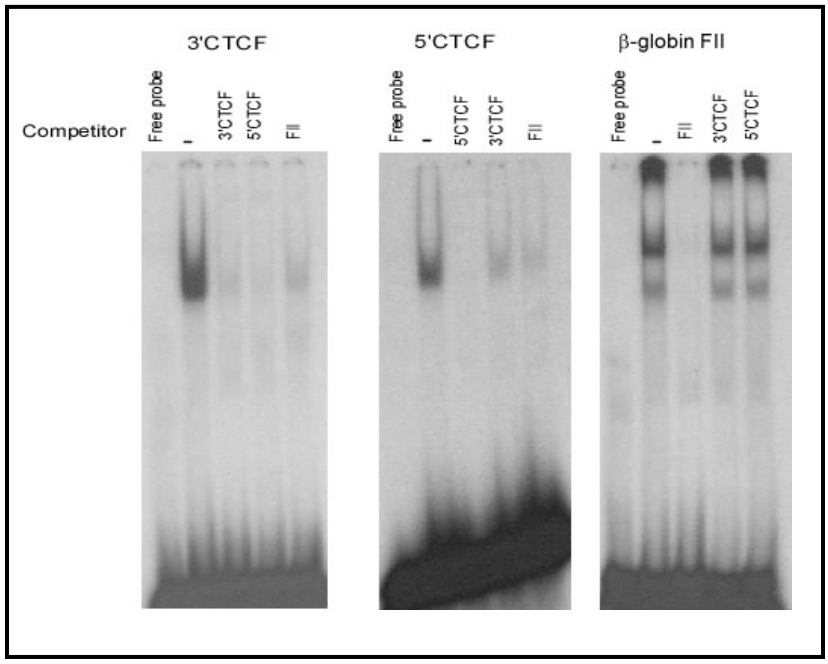



**Figure 5. Sequence–specific complex formation at HS1' CTCF sites.** Mouse thymic nuclear extract is used in EMSA competitions between HS1' 3'CTCF and 5'CTCF and chicken β-globin FII sequences.

The presence of a single CTCF site within HS1' was later reported in a publication by Magdinier et al., (2004). This site was named TCR Alpha Dad1 (TAD1). TAD1 is a 14-bp sequence that maps to the 3'CTCF site described here. The sequences corresponding to 5'CTCF were not examined. In these studies, CTCF binding to TAD1 was demonstrated *in vivo*, through chromatin immunoprecipitation (ChIP) using nuclei from the cultured cell lines AKR1 (a T cell line) and NIH 3T3 fibroblasts. EMSA supershift and competition experiments in which nuclear protein extracts from these same cell lines were used also produced evidence of CTCF binding to TAD1.

The findings of these gel shift experiments are in contrast to the observations described in this work. Differences between the protein compositions of the nuclear extracts may account for the inconsistencies between the results. Magdinier and colleagues conducted EMSA competitions and supershifts using nuclear extracts form immortal cell lines (AKR1 and NIH 3T3), while thymic nuclear extracts are used here in similar experiments. Interactions with factors present in the thymus (but absent from the cell lines) may stabilize the binding of CTCF at FII such that the HS1' sequences are unable to displace CTCF. The absence of these same factor interactions at the HS1' CTCF oligonucleotides (due to differences in length and nucleotide sequence) may allow FII to efficiently compete for the less stable 3'CTCF and 5'CTCF complexes. Interactions between CTCF and other thymic factors may have also altered or made inaccessible CTCF epitopes normally recognized by antibodies used in supershift experiments.

### 3.1.3 HS1' displays CTCF-dependent enhancer-blocking activity

The functionality of TAD1 was also tested by Magdinier et al. (2004) in an *in vitro* enhancer blocking assay in the erythroleukemia cell line K562. TAD1 blocked the activation of a human Aγ-globin promoter-driven neomycin reporter by the 5' HS2 β-globin enhancer. These studies produced the first evidence to support an insulator function of HS1'and left open the opportunity to test whether this region functions *in vivo*, and how it may contribute to LCR activity.

### 3.1.4 A transgene model for the study of HS1' CTCF activity

To further investigate the role of the 3'CTCF/TAD1 and adjacent 5'CTCF sequences in a context that more closely resembles the endogenous TCRα locus, I have established four independent transgenic mouse lines in which a 94-bp AvrII-SacI fragment containing both of these CTCF sites, has been deleted. This deletion was made in the context of the previously analyzed β:1-6 transgene that includes the human β-globin reporter linked 5' of an LCR fragment that contains HSs 1 through 6 (Fig. 6). This new construct was named β-1-6ΔCTCF. In general, preliminary examination of transgene expression in these lines shows preferential expression of the β-globin gene in the thymus and spleen, with little to no expression the other organs examined. It appears that this CTCF deletion does not severely disrupt the tissue-specificity of LCR activity. Although the lymphoid-specific expression in these mutant transgenic lines does not appear to be perturbed, the effect of the CTCF deletion on transgene expression levels (per copy) has yet to be assessed. Using this transgene system, studies will also be undertaken to examine of the effects of the CTCF deletion on chromatin structure. It will also be of interest to determine whether the CTCF elements play a role in the lymphoid-specific demethylation event at HS4.

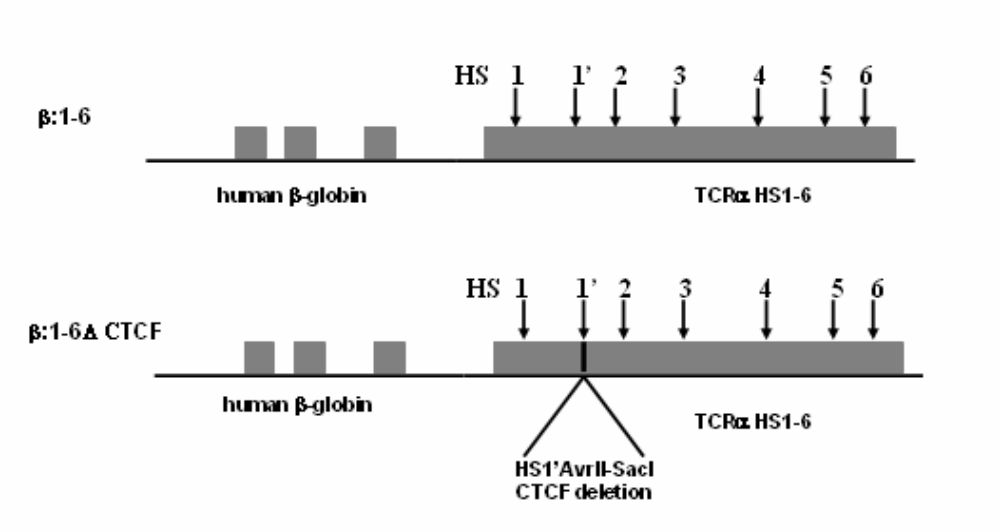



**Figure 6**. **Transgene constructs used for the study of CTCF function *in vivo*.** The LCR is represented as 7 DNase I hypersensitive sites (HS) and their relative positions are indicated by vertical arrows. β:1-6 contains the human β-globin linked to the wild type LCR HS1-6 (top). β:1-6ΔCTCF (bottom) is identical to the β:1-6 transgene except that it contains a 94-bp AvrII-SacI deletion that removed the 3'CTCF and 5' CTCF sequences.

## 3.2 HS1' Discussion

### 3.2.1 HS1' is a non-classical transcriptional regulator with complex activities

HS1' belongs to a more recently recognized group of chromatin-based cis-acting elements whose transcriptional activities are distinct from those of classical control elements such as enhancers and promoters. Some of these elements have been classified based on similarities in their transcriptional behavior. For example, the 3'CTCF /TAD1 sequence within HS1' has recently been classified as an insulator, based on its interaction with the CTCF factor and is ability to block enhancer/promoter interactions (Magdinier et al., 2004). Many of these non-classical elements, however, display other unique characteristics that may be specific to gene regulation at their respective loci. Apart from its apparent enhancer-blocking property, HS1' has also been implicated in the regulation of tissue-differential chromatin structure and lymphoid-specific gene expression at the TCRα locus (Ortiz et al., 1997; Ortiz et al., 1999).

The newly described CTCF-dependent enhancer blocking property of 3'-CTCF/TAD1 suggests that this element may function as an enhancer-blocker at the endogenous TCRα/δ/Dad1 locus to separate the tissue-specific activities of the 5' Eα from the ubiquitously active downstream region. HS1' may therefore prevent the inappropriate activation of the T cell receptor genes by Dad1 regulatory elements in non-T cells. In support of this hypothesis, both HS1' 5'CTCF and 3'CTCF sites are occupied *in vivo* in thymic as well as in non-lymphoid tissues (Magdinier et al., 2004). Furthermore, experiments performed in the lab have produced evidence of additional, as yet

uncharacterized HSs in the Dad1 region (unpublished data) that may potentially (and inappropriately) regulate TCRα gene expression.

Interestingly, Dad1 expression is regulated during thymic development (Hong et al., 1999), suggesting that a relationship exists between this gene and T cell development. Upon transit from the DP to the SP stage Dad1 expression is significantly upregulated. The Dad1 promoter region is located within 30-kb 3' of the Eα/HS1' elements. Since the Eα is known to mediate transcription over large distances of over 70-kb at the endogenous TCRα locus (Sleckman et al., 1997), it could conceivably regulate Dad1 expression in T cells. HS1' may therefore function to prevent the inappropriate activation of transcription from the Dad1 promoter by Eα.

### 3.2.2 Proposed models for the selective control of HS1' CTCF-dependent insulator activity at the TCRα/δ/Dad1 locus

Although a requirement for enhancer blocking activity between the T cell-specific Eα and the ubiquitously-active downstream regulatory elements seems reasonable, this model does not conform to the complex patterns of gene regulation at the endogenous locus. For example, evidence suggests that HS6 plays a role in the regulation of TCRα gene expression (Harrow et al., 2004) and may therefore be required to interact with the upstream LCR elements (5' of HS1'). The activity of an insulator at HS1' in this locus would need to be spatially modified in T cells to allow access of these upstream elements to HS6.

A model involving temporally selective modification of HS1' insulator activity would also be plausible in the context of Dad1 gene regulation. Relatively low-level Dad1 transcription at the earliest thymic stages presumably relies on Dad1-specific gene regulatory elements. At the DP stage where the majority of thymocytes undergo apoptosis, it may be necessary to maintain low levels of Dad1 due to its anti-apoptotic functions. However the upregulation of Dad1 expression in SP thymocytes suggests that it may, at this stage, fall under the regulation of Eα, since this element is active in SP thymocytes. The DP to SP thymocyte transition would therefore need to be accompanied by a relief of HS1' enhancer-blocking activity.

Modification of HS1' insulator function may involve CTCF dissociation or interactions with other stage- or cell type-specific factors. Examples of insulator activity modulation have been described in other systems. The 5'HS5 element of the human β-globin LCR has been classified as a CTCF-dependent insulator. The enhancer-blocking activities of this element appear to be specified by the stage of erythroid development (Wai et al., 2003). Moreover, the various genes of the β-globin cluster seem to be differentially affected by 5'HS5 activity. Transgenic studies comparing the expression of the various human β-globin genes at different erythroid developmental stages showed that a gene that is normally less efficiently expressed a particular stage is more susceptible to the enhancer-blocking effects of 5'HS5 than one that is normally expressed at higher levels (Tanimoto et al., 2003). Another example of modifiable insulator activity is seen at the imprinted Igf2/H19 locus. The parental-specific methylation status of the CTCF sites within the Igf2/H19 internal control region (ICR) insulator element, determines whether

the CTCF protein binds, and this in turn determines which of the two genes is expressed (Bartolomei et al., 1993; Hark et al., 2000). On the maternally-inherited allele CTCF binding blocks a downstream enhancer from activating the upstream Igf2 gene, thereby preventing its expression. Alternatively, the H19 gene is expressed. On the paternally-inherited allele, CTCF binding is blocked by the methylation of its binding sites within the ICR, thus permitting the expression of Igf2.

Since DP thymocytes account for over 80% of the thymus population, the *in vivo* footprint performed in this study more than likely represents factor occupancy in at this stage of development. It would be interesting to determine if any differences in HS1' factor occupancy (specifically at 3'CTCF/TAD1 and surrounding sequences) occur upon DN to DP transition when the TCRα gene is activated, or similarly, upon the DP to SP transition when Dad1 expression is upregulated. Stage-specific differences in the binding of CTCF or other factors that modify HS1' activity may be revealed in these experiments.

### 3.2.3 HS1' CTCF element may regulate long-range DNA methylation status

The occupancy of HS1' by CTCF is interesting for yet another reason. HS1' has been shown to be involved in the regulation of a lymphoid-specific DNA demethylation event at HS4, almost 4-kb away (Santoso et al., 2000). It is possible that this demethylation event depends on the CTCF factor. Such a long-range effect of an insulator on DNA methylation status has been described. It has been reported that at the Igf2/H19 locus, the ICR insulator (also referred to as the differentially methylated region or DMR) on the

maternally-inherited allele is important for the prevention of *de novo* methylation at specific sites several kilobases away (Lopes et al., 2003; Rand et al., 2004), and it has been suggested that this function may depend on the CTCF binding at the ICR.

### 3.2.4 *In vivo* CTCF function

It had previously been shown that HS1' contributes to the regulation of lymphoid-specific gene expression. In the newly established β:1-6ΔCTCF transgenic lines it appeared that the absence of CTCF binding had no effect on the preferential expression in thymus and spleen. It appears that the HS1' sequences involved in regulating lymphoid-specific expression reside outside the CTCF region. This transgene system may yet prove useful for the examination of the effects of the CTCF deletion on other aspects of HS1' activity, including gene expression levels in the lymphoid organs and the regulation of differential chromatin structure and methylation status of sequences near HS4.

# CHAPTER 4

# HS6

## 4.1 In vivo factor occupancy and non-classical gene regulatory functions of HS6

### 4.1.1 Functions of the HS2-6 region

The observed tissue-unrestricted properties of the HS2-6 region suggested that certain elements within this incomplete LCR element may regulate, at least in part, the expression of the nearby ubiquitous Dad1 gene located at the 3' end of the locus. In the interest of defining sequences that are important for these tissue-unrestricted activities, experiments were focused on HS6. These initial studies of HS6 described here have been published (Ortiz et al., 2001).

### 4.1.2 The widespread and PEV-suppressing activities of HS2-6 are contained within HS6

A new transgene that consisted of the human β-globin gene linked to a fragment containing HS6 (named β:6), was established to study the activities of HS6 in the absence of all other LCR elements. The tissue distribution of transgene expression in these β:6 lines, closely resembled the tissue-unrestricted pattern of expression that had previously been described for the β:2-6 transgene (Ortiz et al., 2001). Moreover, the ability of HS6 to suppress the variability of transgene expression was also evident in this study. Taken together, these results demonstrated that the tissue-unrestricted and position effect

variegation (PEV)-suppressing activities of the HS2-6 region were largely attributable to HS6.

### 4.1.3 Differences in HS6 factor occupancy between lymphoid and non-lymphoid tissues

In contrast to the widespread activity of HS6, there was also evidence of its contribution to the tissue-specific activities of the LCR. In the absence of these widely-active downstream sequences, the Eα is not sufficient to overcome gene silencing when integrated into transcriptionally silent chromatin (von Boehmer, 1990). Moreover, in the context of the wild type LCR, the HS6 region is hypersensitive in lymphoid organs only (Hong et al., 1997). To investigate the tissue-specific activities of HS6, *in vivo* footprint (IVFP) experiments were used to examine factor occupancy within this element. These experiments revealed three interesting regions of differential factor occupancy between thymus and non-lymphoid liver. These sites were named thymic footprint (TF)1, TF2 and TF3 (Ortiz et al., 2001).

### 4.1.4 HS6 binds lymphoid-specific factors AML-1 and Elf-1

My contribution to this initial study of HS6 involved the examination of *in vitro* factor binding to the TF1, TF2 and TF3 sites using electrophoretic mobility shift assay (EMSA) experiments. In these experiments nuclear protein extracts from mouse thymus or NIH 3T3 fibroblasts (representative of non-lymphoid tissue) were incubated with radiolabeled oligonucleotides representing the TF1, TF2 and TF3 sequences. The results of there experiments are shown in figure 7.

Incubation of TF1 with either of the thymic or fibroblast nuclear extracts resulted in the formation of a single protein complex. These TF1 complexes appeared to be equivalent based on their similar rates of mobility. TF2 bound two thymic and two fibroblast complexes. While the lower TF2 complexes appeared equivalent in both tissue types, the upper complexes were different, as the thymic complex migrated more quickly than the fibroblast complex. Finally, the TF3 oligonucleotide bound only one well defined thymic complex that was absent from fibroblasts.

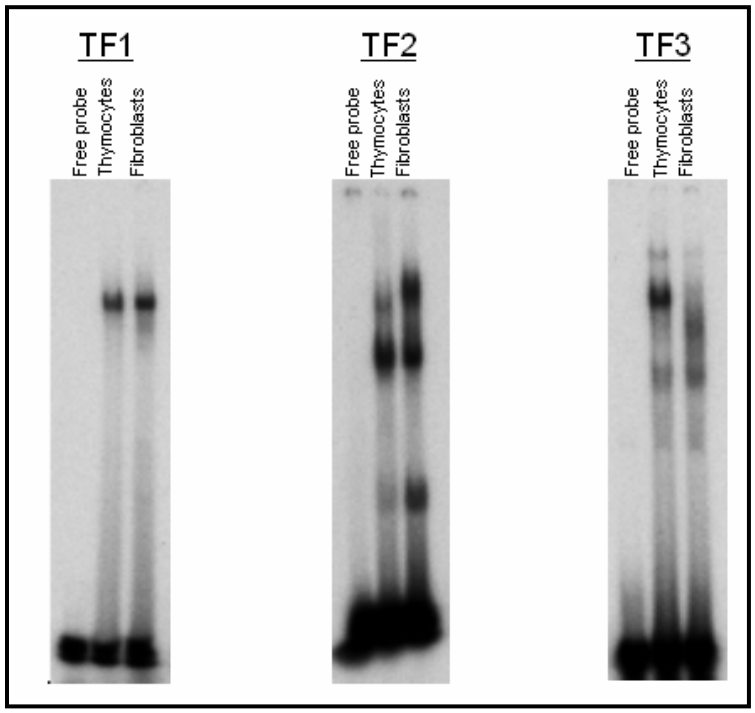



**Figure 7. Complex formation at HS6 TF1, TF2, and TF3 sequences.** EMSA experiments showing the formation complexes at TF1, TF2 and TF3 using nuclear extracts derived from mouse thymocytes and NIH 3T3 fibroblasts. This figure has been reprinted from Ortiz et al. (2001).

Complex formation at TF1, TF2 and TF3 was efficiently competed for by their respective homologous sequences, confirming the specificity of binding (Fig. 8 and not shown). Several oligonucleotides that contain consensus binding sites of factors known to be important for T cell development were used in competition assays. These competitors included the consensus binding sites of CBF, EBox, Ets-1, GATA-3 and GT box. Competitions were observed for the formation of the upper TF2 complexes only (Fig. 8A). CBF is a heterodimer of the lymphoid specific factors AML-1 (CBFα2) and CBFβ. Interestingly, the CBF sequence displaced the upper TF2 complexes formed with both thymic and fibroblast extracts, although fibroblasts do not normally express these lymphoid-specific CBF factors.

To confirm the interaction of the CBF complex with TF2, EMSA supershift experiments were performed using antibodies targeted to the N-terminal and C-terminal of AML-1 (Fig. 8B). The C-terminal antibody supershifted neither the thymic nor the upper fibroblast complex. However, a supershift of the thymic (but not the fibroblast) complex was achieved with the addition of the N-terminal antibody, confirming the presence of AML-1 in the upper TF2 complex.

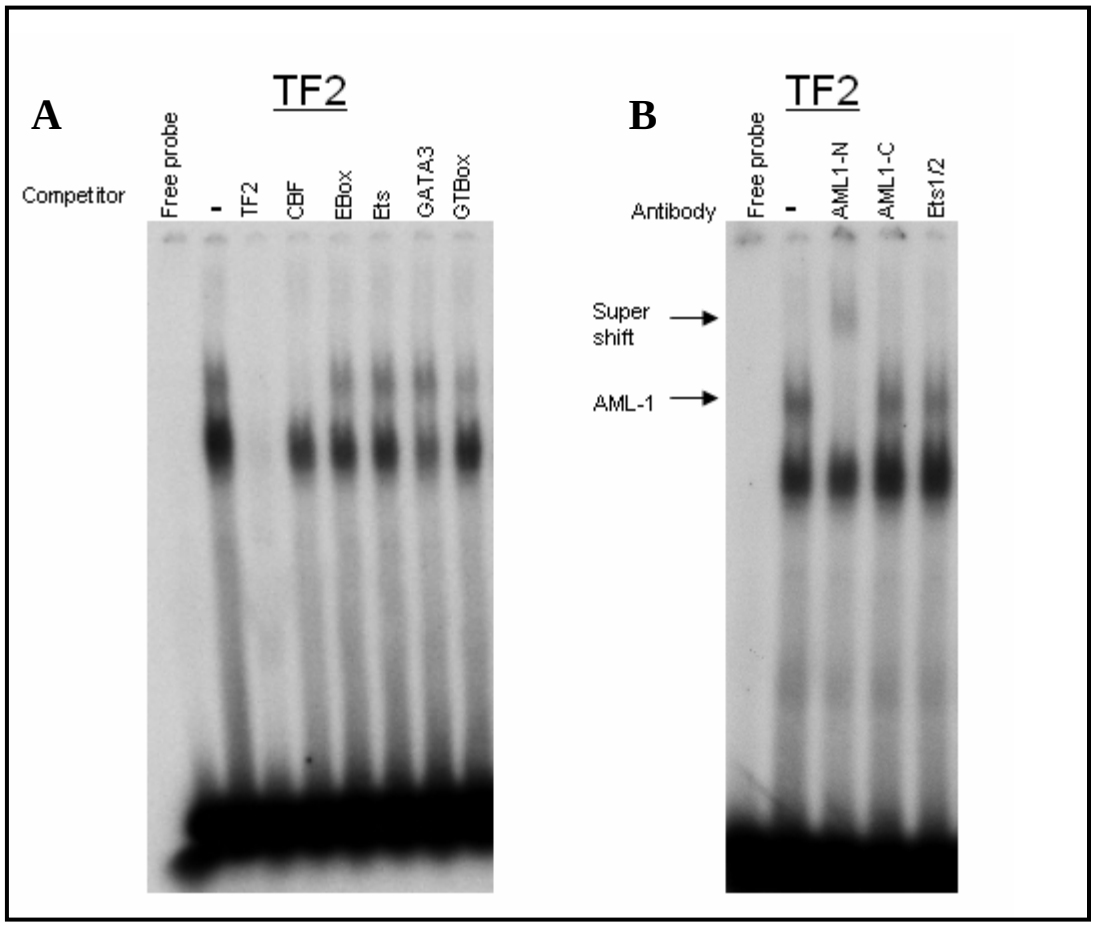



**Figure 8. TF2 binds AML-1.** ***A***, EMSA competition using indicated competing oligonucleotides and thymic nuclear extract. CBF consensus-sequence competes for TF2 thymic upper complex. ***B***, EMSA supershift of TF2 thymic upper complex by antibody to AML-1. This figure has been reprinted from Ortiz et al. (2001).

Upon visual examination of the TF3 site, sequence homology to the consensus Elf-1 binding site was observed. Elf-1 is a member of the Ets family of transcription factors. In competition experiments, an oligonucleotide containing the Ets binding site did not compete for the formation of the TF3 thymic complex. Moreover, an antibody directed against Ets 1 and 2 had no effect on this complex (Fig. 9). However, incubation with an antibody targeted against the related factor Elf-1, blocked the formation of the TF3 thymic complex, demonstrating the binding of this specific Ets family member to TF3.

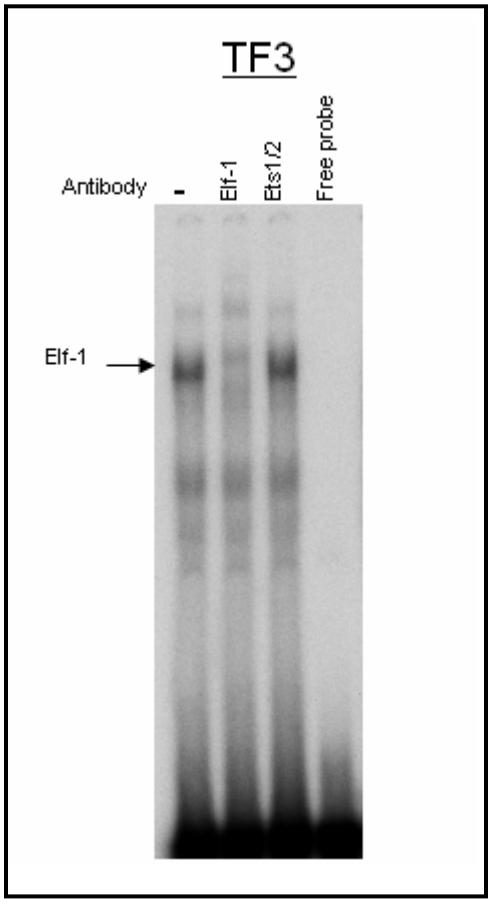



**Figure 9. TF3 binds Elf-1.** EMSA supershift/blocking assay showing the blocking of TF3 thymic complex formation by an antibody directed against Elf-1. This figure has been reprinted from Ortiz et al. (2001).

### 4.1.5 A transgenic model for the study of TF1-2-3 activity

Demonstration of the binding of the lymphoid factors AML-1 to TF2 and Elf-1 to TF3 provided further evidence for the tissue specific activities of HS6. To determine whether the TF1, TF2 and TF3 sequences (hereafter, collectively referred to as the TF1-2-3 region) were functional *in vivo,* further transgenic studies of HS6 were undertaken. The results of these studies have been reported (Harrow et al., 2004). A 238-bp BclI-PstI sequence deletion that contained the entire TF1-2-3 region was made in the context of the previously analyzed β:1-6 transgene (Fig. 10). This new construct named β:1-6ΔTF123 was carried by five independent mouse lines whose reporter mRNA expression patterns were analyzed by Jeanne Amuta. My first contribution to these analyses was the determination of the transgene copy numbers, which ranged from 1 (line 3) to 44 (line 5) copies. As a control for wild type LCR function two of the previously analyzed β:1-6 transgenic lines were used in this study.

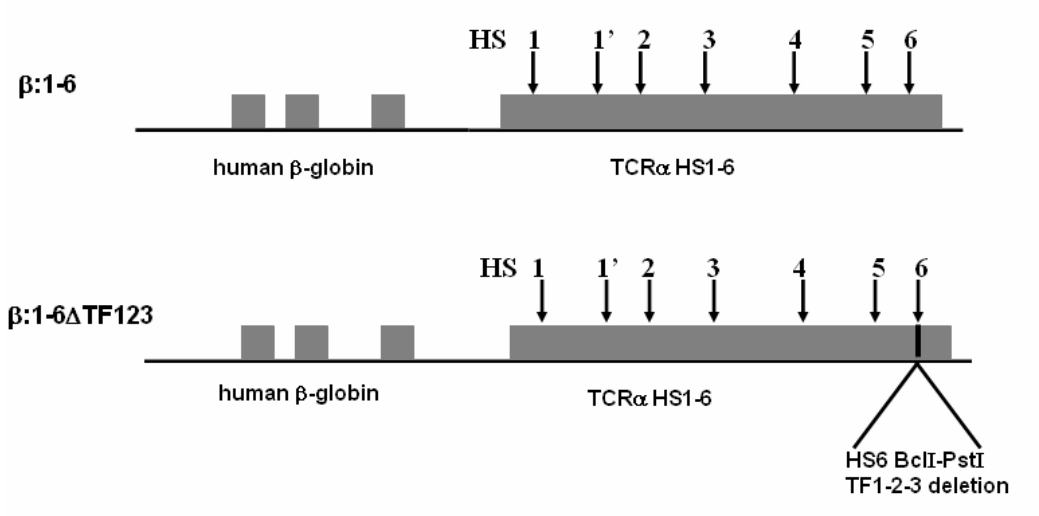



**Figure 10**. **Transgene constructs used for the study of TF1-2-3 function.** The LCR is represented as 7 DNase I hypersensitive sites (HS) and their relative positions are indicated by vertical arrows. β:1-6 contains the human β-globin gene linked to the wild type LCR HS1-6 fragment (top). The β:1-6ΔTF123 transgene (bottom) differs from β:1-6 in that it contains a 238-bp BclI-PstI deletion that includes the TF1-2-3 sequences.

### 4.1.6 The TF1-2-3 region increases lymphoid expression *in vivo*

Northern blot experiments were performed by Jeanne Amuta to examine the effect of the TF1-2-3 deletion on lymphoid expression levels and on the tissue distribution of transgene expression. The amount of β-globin transgene mRNA per copy was measured in the thymus and spleen of all lines carrying mutant and wild type LCR transgenes (Fig.11A). Phosphorimager analysis was used to measure the β-globin signal intensity, and this was normalized to the endogenous TCRα expression (Fig. 11B). In the thymus, the normalized β-globin transgene expression in the mutant β:1-6ΔTF123 lines was significantly reduced by 2.0- to 5.1- fold in comparison to the expression in the β:1-6 animals. Moreover, transgene expression in the spleen was reduced by 3.4- to 17- fold in four out of five lines carrying the TF1-2-3 deletion.

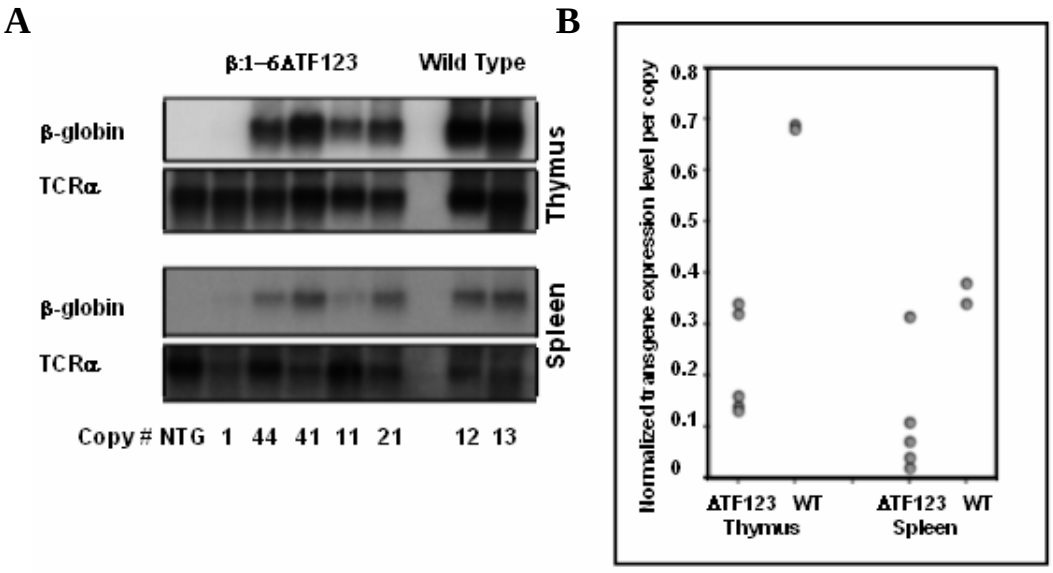



**Figure 11. TF1-2-3 deletion results in reduced levels of lymphoid expression.** ***A***, Northern blot analysis comparing the amounts transgene expression per copy in thymus and spleen of β:1-6ΔTF123 mutant transgenic lines with wild type transgenic β:1-6 lines. The β-globin signal is normalized to the endogenous TCRα signal and divided by the copy number for each line. Copy numbers are indicated for β:1-6ΔTF123 lines 3, 5, 6, 30 and 33 from left to right. ***B***, Phosphorimager analysis of normalized transgene levels is shown in a graph where each dot represents an independent transgenic line. This figure has been reprinted from Harrow et al. (2004).

In transgenic lines that carry the wild type LCR, thymic transgene expression is typically the highest (Diaz et al., 1994; Ortiz et al., 1997). The expression in spleen ranges between 10-30% of thymus, with low to undetectable expression in non-lymphoid organs. With the exception of the β:1-6ΔTF123 line 3, the tissue distribution of transgene expression in all lines carrying the mutant LCR was not severely perturbed (Fig. 12). In contrast, β:1-6ΔTF123 line 3 produced the highest levels of transgene expression in the spleen with unusually low levels of thymic expression (13% of spleen), comparable those seen in

non-lymphoid organs. These results would suggest that the transgene integration site in line produces a positive position effect that favors splenic expression.

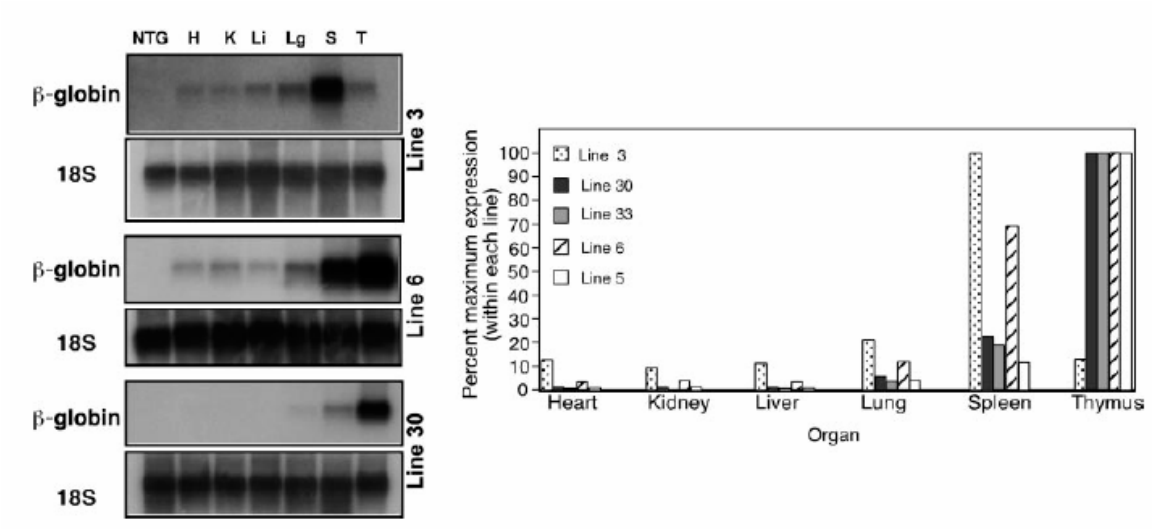



**Figure 12. Deletion of the TF1-2-3 region results in variable tissue distribution of transgene expression**. Northern blot analysis of transgene mRNA levels in various organs of the three indicated β:1-6ΔTF123 lines (left). β-globin mRNA is normalized to 18S rRNA. Phosphorimager analysis of β-globin mRNA levels in all 5 β:1-6ΔTF123 lines in shown graphically (right). This figure has been reprinted from Harrow et al. (2004).

### 4.1.7 Deletion of TF1-2-3 reduces chromatin accessibility throughout the transgene locus

The DNase I hypersensitivity assay may be used as an indirect assessment of the chromatin structure of a locus. To determine the effect of the TF1-2-3 deletion on chromatin structure I performed DNase I hypersensitivity assays on thymocytes derived from two independent mutant β:1-6ΔTF123 lines (lines 5 and 6). The DNase I hypersensitivity of the mutant transgene was compared to that of the endogenous LCR. Figure 13 (right panel) shows the hypersensitivity at the endogenous TCRα locus in β:1-6ΔTF123 line 5 (similar observations were made in β:1-6ΔTF123 line 6). The disappearance of the parent fragment at the higher DNase I titration points at the endogenous LCR, is an indication that in this assay the DNase I reaction went to completion. All the hypersensitive sites (HSs 6, 4, 1' and 1) that normally form at this locus were clearly visible.

Separate aliquots of the same DNase I-treated samples from the transgenic mouse thymocytes were cut with a different set of restriction enzymes and hybridized with a different probe that allowed for examination of the hypersensitivity of the mutant transgene locus (Fig. 13, left panel). It appeared that in the absence of the TF1-2-3 region, the hypersensitivity across the transgene locus was compromised. The absence of HS6 formation was not surprising as the deleted sequences are normally found within this hypersensitive site. Interestingly, the hypersensitivity of the HS1 and HS1'clusters was impaired and HS4 was not detected. It should be noted that the hypersensitivity pattern of wild type LCR transgenes resembles that of the endogenous LCR (Ortiz et al., 1997). The

diminished hypersensitivity across the mutant transgene locus indicates that the majority of transgene integrants exist in an abnormally inaccessible chromatin configuration. This inaccessible structure very likely accounts for the observed reduction in lymphoid transcription.

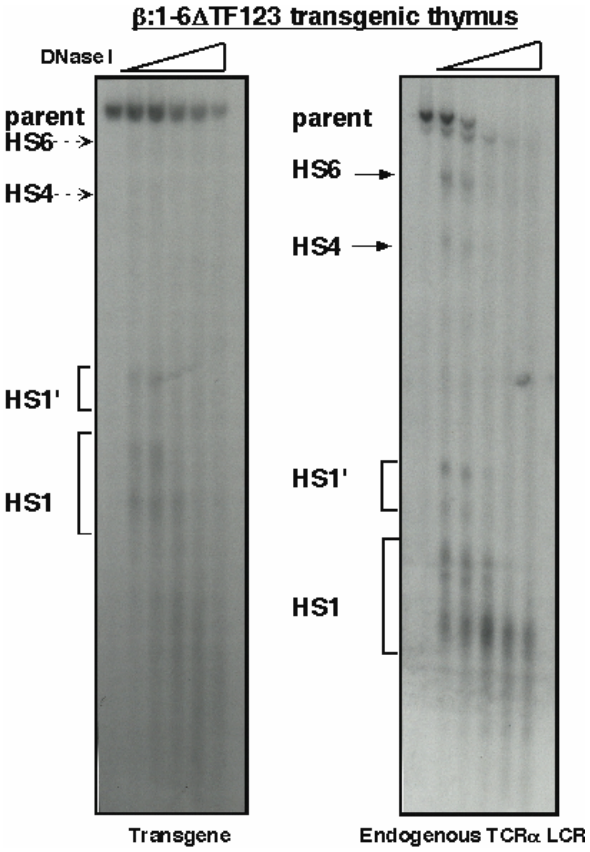



**Figure 13. The TF1-2-3 deletion reduces chromatin accessibility throughout the transgene locus.** Southern blot of DNase hypersensitivity at the endogenous TCRα LCR and transgene locus of a representative mouse from β:1-6ΔTF123 line 5. Separate aliquots of the same DNase I treated thymic nuclei were digested with separate enzymes and hybridized to separate probes to allow for detection of the transgene and endogenous loci (as described in materials and methods). Similar results were observed in β:1-6ΔTF123 line 6. This figure has been reprinted from Harrow et al. (2004).

## 4.2 HS6 Discussion

### 4.2.1 HS6 prevents position-effect gene silencing

Expression of the human β-globin reporter under the control of HS6 was observed in all four β:6 transgenic lines, which is an indication of the ability of this element to prevent position-effect gene silencing at ectopic integration sites. Furthermore, it was also demonstrated that HS6 reduces variability in expression levels in all organs examined. It is interesting to note that HS6, in isolation, is capable of driving substantial levels of transgene expression in the absence of the TCRα Eα (Ortiz et al., 2001). However, HS6 is not an enhancer as this region does not display any such activity in transient transfections (Winoto and Baltimore, 1989). HS6 function is entirely dependent on a chromosomal context. Similar activities have been described for the 5'HS3 and HS4 elements of the human β-globin LCR (Navas et al., 2001; Navas et al., 1998). These elements do not function transiently, but activate expression when stably integrated into chromatin. Moreover, 5'HS3, in the absence of all other human β-globin LCR elements, has been shown to suppress PEV in transgenic animals (Ellis et al., 1996). The ability to overcome heterochromatin-induced gene silencing has also been attributed to other LCR elements. These include the human CD2 HS3 (Zhuma et al., 1999) and the facilitator elements of the human adenosine deaminase LCR (Aronow et al., 1995). Although these elements are united in their position effect suppressing abilities, they bear no sequence homology, and their mechanisms of action are unknown. The PEV- suppressing activities of the human CD2 HS3 has been associated with the ubiquitously expressed HMG box transcription factor, HBP1 (Zhuma et al., 1999). The HS6 sequences of the TCRα LCR do not possess binding sites for this protein; however, it would be reasonable to assume

that such ubiquitously-expressed factors would be similarly involved in the PEV-suppressive functions of HS6, since this element is active in all tissues.

### 4.2.2 HS6 regulates chromatin accessibility in the thymus

Although HS6 directs transgene expression in a variety of tissues, in the context of the wild type LCR this element is hypersensitive only in T cell-bearing organs (Hong et al., 1997). This would suggest that HS6 contributes to the tissue-specific functions of the LCR. Differences between *in vivo* factor occupancy of HS6 in the thymus and liver lead to the identification of binding sites for the lymphoid-specific factors, AML-1 and Elf-1. An internal LCR deletion of the TF1-2-3 sequences containing the binding sites for these factors resulted in an overall reduction in transgene expression in thymus and spleen. Interestingly, this deletion also resulted in a locus-wide reduction in DNase I hypersensitivity of the transgene in the thymus. This then raises questions regarding the significance of AML-1 and Efl-1 in the regulation of transcription and/or chromatin structure.

Elf-1 belongs to the Ets family of winged helix transcription factors. Certain members of this family play essential roles at the earliest stages of T cell differentiation, while other family members are expressed throughout thymocyte development and in mature T cells (Anderson et al., 1999). Elf-1 has been implicated in the regulation of certain T cell-specific genes including CD4 (Sarafova and Siu, 1999) and the IL-2 receptor (John et al., 1995) genes. AML-1 (Runx1) is a member of the Runx family of transcription factors (for a review see (Rothenberg and Taghon, 2005) and references therein). Runx factors

are functional throughout T cell development. In addition, AML-1 is indispensable for the generation of all hematopoietic lineages from the fetal liver and adult bone marrow.

Neither Elf-1 nor AML-1 has been directly implicated in the regulation of chromatin structure. However, Elf-1 does contain the winged helix motif that is shared by other factors, namely HNF3 and linker histone H5, which are known to participate in nucleosome positioning (Chipev and Wolffe, 1992; McPherson et al., 1993). The structural similarities between these factors and Elf-1 are interesting, as this presents a potential mechanism by which Elf-1 may regulate chromatin structure.

The presence of adjacent binding sites for AML-1 and Elf-1 in HS6 is an interesting finding. AML-1 in known cooperate with various Ets family members to mediate transcription of several immunologically important genes. Interactions between AML-1 and Ets-1 have been shown to be important for the activation of transcription from the TCRα, TCRβ and IgH μ enhancers (Erman et al., 1998; Giese et al., 1995; Wotton et al., 1994). Functional interactions between AML-1 and members of the Elf subclass of Ets factors, have been identified at the B lymphoid Kinase (blk) promoter (Cho et al., 2004). Although HS6 function fulfills the definition of neither a classical enhancer nor a promoter, an AML-1/Elf-1 interaction at the HS6 element may potentially contribute to high-level lymphoid expression through non-classical regulatory mechanisms.

LCRs are generally thought to overcome the silencing effects of heterochromatin by creating an open chromatin structure at ectopic integration sites (Li et al., 2002).

However, the importance of these elements in regulating the accessibility of chromatin at endogenous loci has been brought into question. Deletions of endogenous LCR sequences at the β-globin gene loci in mice and human erythroid cell lines, reportedly resulted in no change in the hypersensitivity of the remaining elements in these loci (Epner et al., 1998; Reik et al., 1998). While it is difficult to reconcile these observations with the many examples of chromatin opening activities of LCR containing transgenes, it does not undermine the importance of elements such as HS6 in overcoming heterochromatin-induced gene silencing at ectopic integration sites.

### 4.2.3 HS6 activity is context-dependent

These studies have been valuable in characterizing HS6 with respect to its functions in the absence of all other LCR elements. It's combined PEV-suppressing and tissue-unrestricted transcriptional activities may potentially be applied to heterologous genes when widespread, position-independent expression is desired. The absence of HS6 formation in non-lymphoid organs does bring into question its hypothesized contribution to the expression of ubiquitously-expressed Dad1. However, it is possible that HS6 may regulate, at least in part, the expression of Dad1 in T cells, while other Dad1-associated elements function in non-lymphoid tissues.

Evidence suggests that HS6 function is modified in the presence of the upstream LCR elements to participate in lymphoid-specific activities. Its ability to establish an accessible chromatin environment suggests that HS6 supports Eα (which is susceptible to PEV in a transgenic context) in the regulation of TCRα gene expression in T cells. These

T cell specific activities of HS6 may very likely involve interactions with the lymphoid-specific factors AML-1 and Elf-1.

# CHAPTER 5.

## Developmental-stage and fine-cell type specificity of LCR activity

### 5.1 A transgene model for single-cell analysis of TCRα LCR activity

Previously, transgenic studies of the TCRα LCR made use of a 4.9-kb BglII human β-globin fragment as a reporter (Ortiz et al., 1997; Ortiz et al., 1999). Included in this fragment are the β-globin exons, introns, the promoter and 3' enhancer. This fragment which has also been used by others in transgenic studies is poorly expressed in the absence of additional control elements (Kollias et al., 1986; Magram et al., 1985; Townes et al., 1985). Under the control of the TCRα LCR, this human β-globin gene was expressed lymphoid-specifically (in thymus and spleen), in a copy number-dependent and site-of-integration independent manner (Ortiz et al., 1997). Until recently, much of the studies of TCRα LCR activity utilized this transgene model. Unfortunately, this system was limited in its ability to generate information regarding LCR activity at the single cell level. β-globin expression was only detectable at the mRNA level in whole organs.

To overcome the limitations of the β-globin system I generated a new transgene model that used the human CD2 (hCD2) gene as a reporter. The hCD2 fragment which is used here includes the 5 exons of the gene (introns 2 and 3 have been removed) flanked on the 5' end by the 5-kb promoter region (Melton et al., 1996). In the absence of additional regulatory elements, the hCD2 gene has been shown to be poorly expressed as it is susceptible to site-of-integration silencing in transgenic animals (Festenstein et al., 1996; Lang et al., 1991). In its natural locus hCD2 is regulated by its own LCR (Greaves et al.,

1989). In this study, we therefore attribute the expression of this gene to the activities of the TCRα LCR. Due to the surface expression of the hCD2 molecule I was able to use flow cytometry to analyze LCR activity in individual cells of various hematopoietic lineage, at different stages of T cell development.

In humans hCD2 is primarily expressed in thymocytes and peripheral T cells, and has also been detected in a subset of natural killer cells (Bierer et al., 1989) (Howard et al., 1981). The normal functions of hCD2 include mediating T cell adhesion and the transduction of signals through its cytoplasmic domain (Chang et al., 1989; Moingeon et al., 1989). The version of hCD2 used in this study contains a stop codon that has been introduced by PCR into the last exon of the gene (Melton et al., 1996). This modified gene produces a truncated form of the hCD2 molecule that lacks a significant portion of the cytoplasmic tail, which is normally required for signaling. In the absence of cytoplasmic signaling, transgenic mice expressing this form of hCD2 displayed normal T cell development.

### 5.1.1 The hCD2:1-8 transgene model

The hCD2:1-8 transgene used in these studies consists of the modified hCD2 gene (described above) linked 5' of a fragment containing all nine HSs of the TCRα LCR (Fig. 14). The identical LCR fragment has previously been linked to the human β-globin reporter forming the β:1-8 construct (Ortiz et al., 1997). Four independent transgenic lines (numbered 4, 15, 29 and 44) carrying the hCD2:1-8 construct, were established. Consistent with previous findings (Ortiz et al., 1997) and with the position-effect

suppressive activities of the LCR, all four lines expressed hCD2 under the control of this element. However, using flow cytometry to examine surface expression in these lines, it was observed that in line 15, expression was extensively variegated in both the thymus and spleen. This result differed from the expression patterns seen in the other three lines, which all produced more uniform levels of expression in whole thymic and splenic populations. Using southern blots to detect the transgene locus in these lines, it was revealed that in line 15 the majority of transgene integrants were truncated. This lead to the conclusion that these partial transgene fragments, presumably lacking complete LCR activity, resulted in the observed variability in hCD2 expression. For this reason line 15 was excluded from further transgenic analyses.

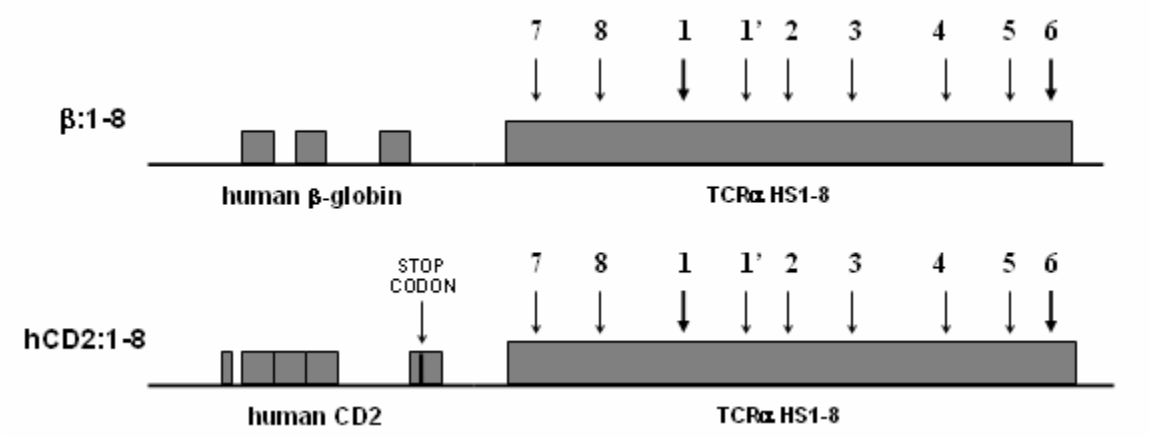



**Figure 14**. **Diagrams of the β:1-8 and hCD2:1-8 transgenes**. The β:1-8 transgene (top) consists of the human β-globin reporter gene linked 5' of the wild type TCRα LCR HS1-8 fragment. The hCD2:1-8 transgene (bottom) consists of the human CD2 reporter gene linked 5' of the identical wild type LCR HS1-8 fragment. The premature stop codon in exon V of the hCD2 gene is represented. The positions of hypersensitive sites are indicated by vertical arrows.

### 5.1.2 Lymphoid-organ specific expression of the hCD2:1-8 transgene

The TCRα LCR had previously been shown to direct lymphoid organ-specific of linked reporter genes (Diaz et al., 1994; Ortiz et al., 1997). Transgene mRNA levels are normally highest in the thymus, while spleen expression ranges from 15 to 30 % of that of thymus. Typically, in all other organs expression is either very low or not detected. I used northern blots to analyze the tissue distribution of transgene expression in the organs of the three hCD2:1-8 lines (lines 4, 29 and 44). Transgene mRNA levels were normalized to the 18S ribosomal RNA signal. Figure 15A shows the typical distribution of transgene mRNA expression in a representative line carrying this hCD2:1-8 transgene. Phosphorimager analysis of the mRNA expression patterns in a representative animal from each line is shown in figure 15B. In agreement with previous findings, the mRNA levels in spleen ranged from 13% to 41% of thymic levels, with little (< 3%) to no expression detected in all the other organs examined.

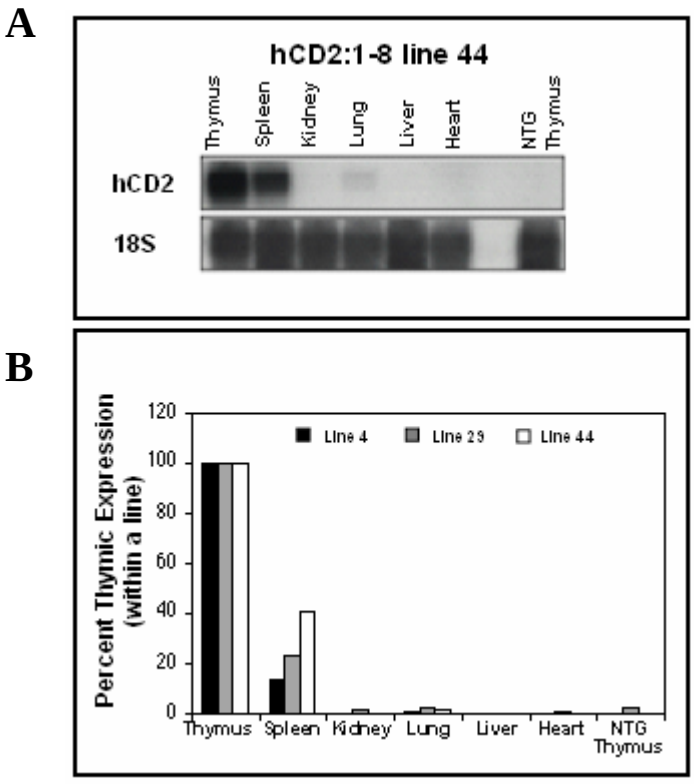



**Figure 15. The TCRα LCR drives lymphoid organ-specific expression of the hCD2 gene.** ***A***, Northern blot experiments showing the tissue distribution of transgene expression in a representative hCD2:1-8 line (line 44). hCD2 signals were normalized to 18S rRNA. ***B***, Phosphorimager analysis of a representative animal from each hCD2:1-8 line is shown graphically. Transgene mRNA levels are expressed as a percentage of the organ with the highest levels of expression. This figure has been reprinted from Harrow, F. and Ortiz, B.D. (In press).

The relative numbers of intact transgene copies in the hCD2:1-8 lines were determined by southern blot (not shown). These numbers were 22, 19 and 21 in lines 4, 29 and 44, respectively. Northern blots were used to measure the amounts of hCD2 expression per transgene copy in the thymus and spleen of all lines (not shown). In the thymus of these three lines, the range in the amounts of expression per transgene copy was within 1.4-fold. In the spleen this range was within 1.3-fold. In both organs the variability in

expression between the lines is well within the range for copy number-dependent transgenes driven by this (Ortiz et al., 1997) and other LCRs (Li et al., 1999).

At the level of transcription, the activities of the LCR in this new hCD2 transgene model were similar to those observed in the β-globin system (β:1-8). The hCD2:1-8 and β:1-8 transgenes contain the identical wild type LCR fragment differing only in the linked reporter gene. In this study of the LCR, a representative β:1-8 line (line 12) is used as a control to rule out any possible hCD2 promoter-specific effects on transgene activity.

### 5.1.3 Ectopic expression of the LCR-driven hCD2 reporter gene

The use of fluorochrome-conjugated antibodies directed against hCD2, has enabled the examination the fine cell-type distribution of LCR activity in the hCD2:1-8 lines. Whole cell populations of thymus and spleen from adult transgenic mice were stained with anti-hCD2 and analyzed by fluorescence activated cell sorting (FACS). In all experiments non-transgenic littermates were used as negative controls for hCD2 staining. The mean fluorescence intensity was used as a measure of the amounts of gene expression. Surface expression of hCD2 in a representative transgenic line (line 44) is shown in figure 16. A comparison of the relative amounts of hCD2 surface expression between thymus and spleen revealed a similar pattern as seen at the mRNA level. Between the three lines, splenic expression ranged from 20% to 36% of thymus.

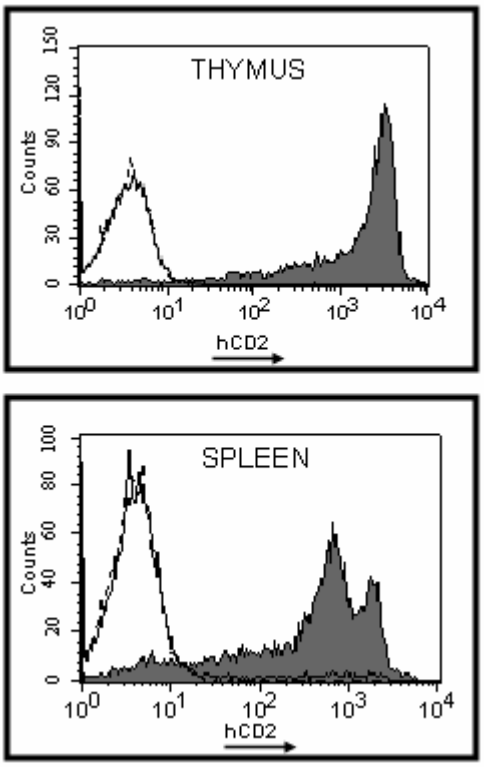



**Figure 16. Relative surface expression of hCD2 transgene in whole populations of thymus and spleen**. Flow cytometric analysis of hCD2 transgene expression in the thymus (top) and spleen (bottom) of an individual of a representative transgenic line (hCD2:1-8 line 44). Cells from whole thymus and whole spleen were stained with anti-hCD2 antibody. hCD2 expression is represented as a grey -filled line in the transgenic individual and as a solid open line in the non-transgenic littermate control. This figure has been reprinted from Harrow, F. and Ortiz, B.D. (In press).

Examination of the relative amounts of transgene mRNA in the hCD2:1-8 lines, and in the previously analyzed β:1-8 lines, suggested that these transgenes were being expressed specifically in T cells, as these cells account for roughly 25% of the spleen population. However, FACS analysis of hCD2 expression in whole spleen indicated that T cell-specific expression was not likely since there was no distinct non-expressing population, which would be expected if most of the cells were negative for hCD2 (Fig.16). To confirm these suspicions, surface markers for T cells and B cells were used to examine

hCD2 expression in the spleen sub-populations. These results are shown in figure 17. As expected, hCD2 was expressed in virtually all thy1$^{+}$ spleen T cells. Moreover, hCD2 expression was also detected in essentially all of the CD19$^{+}$ B cells. Using antibodies directed against Gr1 and F/480 surface markers, I also examined hCD2 expression in spleen granulocytes and monocytes, respectively. Interestingly, variegated expression of hCD2 was evident in both of these populations (not shown).

γδ T cells were also examined for the expression of the hCD2 transgene. These cells are included as a very small population within the double negative or DN (CD4$^{-}$CD8$^{-}$) thymocyte compartment. DN thymocytes were first purified using magnetically activated cell sorting (MACS), and stained with anti-γδ TCR and anti-hCD2 antibodies. Similarly, the spleen T cells were purified using MACS and stained with these same antibodies. These data clearly showed that in both thymic and peripheral γδ T cells, the hCD2 transgene is expressed (Fig. 17 C-D). This result is in contrast to an earlier study which reported αβ T cell-specific transcription (assayed by RNase protection) of a TCRα transgene under the control of the LCR (Diaz et al., 1994). This inconsistency may be a result of the different methods used for cell purification and the detection of transgene expression. Diaz and colleagues enriched for thymic γδ T cells by a series of steps (including complement lysis of CD4+ and CD8+ thymocytes) that, in general, greatly reduced cell survival and may therefore have compromised the samples which were later used for isolating γδ T cells and/or measuring transcription. Here we used more direct methods of purification by MACS and simultaneously detected γδTCR and hCD2 by flow cytometry.

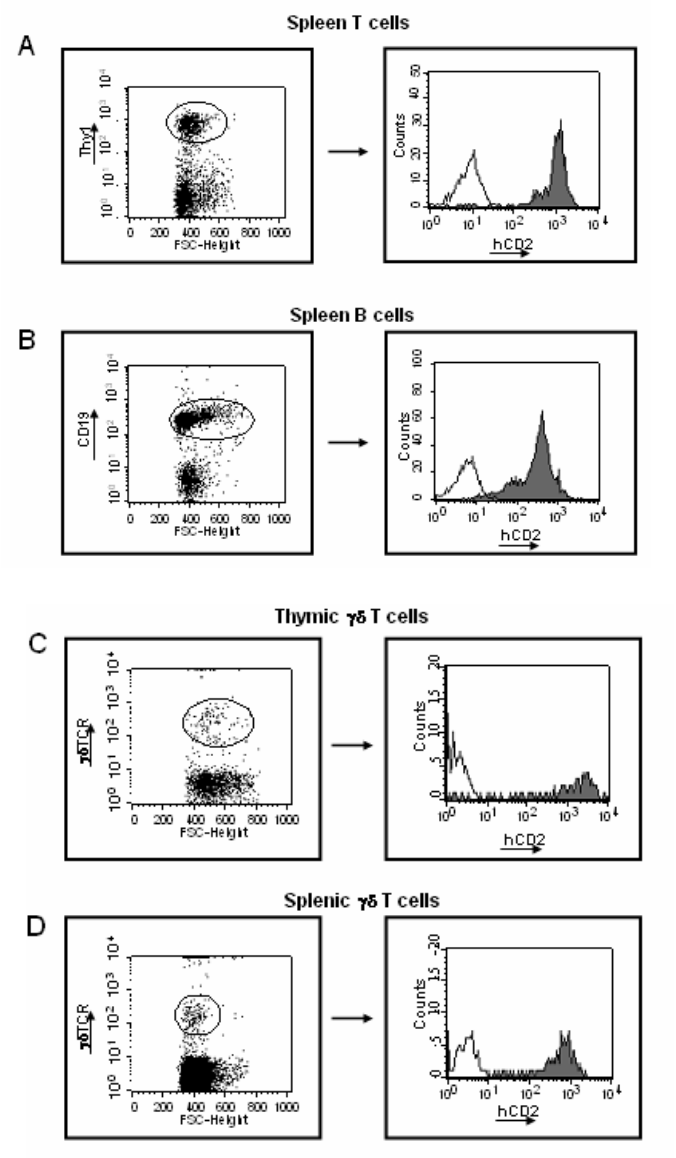



**Figure 17. Ectopic expression of LCR-driven hCD2 transgene.** Flow cytometric analysis of hCD2 expression in the indicated cell types from a representative transgenic line (hCD2:1-8 line 4). Spleen T cell and B cell samples were derived from a single individual. Thymic and spleen γδ T cells were derived from the combined thymi and spleen of two transgenic littermates. ***A***, hCD2 expression in CD90+ spleen T cells. ***B***, hCD2 expression in CD19+ spleen B cells. ***C***, Purified thymic DN cells stained with anti-hCD2 and anti-γδ TCR (to identify γδ T cells). ***D***, Purified spleen T cells stained with anti-hCD2 and anti-γδ TCR. hCD2 expression is represented as a grey filled line in the transgenic individual and as an open line in the non-transgenic littermate control. This figure has been reprinted from Harrow, F. and Ortiz, B.D. (In press).

The observed ectopic expression of the hCD2:1-8 transgene may either be a function of the LCR or the hCD2 promoter element. To distinguish between these two possibilities, spleen B cells were purified from an hCD2:1-8 line and a previously analyzed β:1-8 line, and were examined for the presence of transgene mRNA. The levels of transgene mRNA in the B cells were compared to those of thymocytes form the same animals. Northern blot experiments demonstrated the presence of the respective reporter genes in the B cells of both transgenic lines (Fig. 18A). Phosphorimager analysis of the relative amounts of expression is shown in figure 18B. These results would suggest that the non-T cell expression seen here is not solely a consequence of the hCD2 promoter activity since transgene expression is also detected from the β-globin promoter. It is apparent that the LCR does not sufficiently restrict gene expression to T cells. These observations would also suggest that additional elements, apart from those present in the LCR, are involved in the fine cell-type specification of TCRα expression.

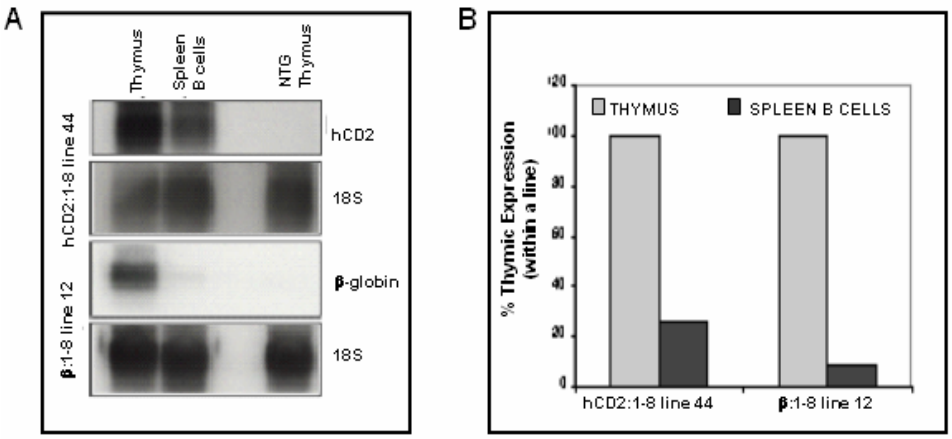



**Figure 18. Transgene expression in spleen B cells.** ***A***, Northern blot analysis comparing transgene expression in thymocytes and purified spleen B cells of a representative hCD2:1-8 line (hCD2:1-8 line 44) and a previously described β:1-8 transgenic line. The hCD2 transgene signal is normalized to 18S rRNA. ***B***, Phosphorimager analysis of northern blot experiments in panel A Transgene expression is represented within each line as a percentage of thymic expression. This figure has been reprinted from Harrow, F. and Ortiz, B.D. (In press).

### 5.1.4 The TCRα LCR supports TCRα-like developmental timing of gene expression in the thymus

The progression of thymocyte development is accompanied by the temporal expression of molecular markers. The most immature or double negative (DN) thymocytes are so named due to the lack of expression of the surface molecules CD4 and CD8. This DN subset may be further subdivided into four developmental stages based on CD25 and CD44 expression. Rearrangement and expression of the TCRβ, TCRγ and TCRδ genes occur early during thymocyte development at the DN stage. TCRα is the last of these receptors to be rearranged. While this gene extensively rearranged in double positive (DP: $CD4^+$ $CD8^+$) thymocytes (Capone et al., 1998; Wilson et al., 1994), the initial

products of rearrangement and expression have been detected at the final double negative stage, DN4 ($CD25^-CD44^-$) (Pearse et al., 1989; Petrie et al., 1995) . I wanted to determine whether the LCR supports this aspect of TCRα expression.

Whole thymus populations were stained with antibodies to hCD2 and the molecular markers CD4 and CD8. In all three hCD2:1-8 lines a significant increase in hCD2 surface expression was detected at the DP stage, compared to the more immature DN ($CD4^-CD8^-$) thymocytes (Fig.19). Furthermore, these increased levels of hCD2 expression were sustained in the more mature single positive (SP: $CD4^+CD8^-$ and $CD4^-CD8^+$) thymocytes. This upregulation of hCD2 expression in DP thymocytes coincides with the extensive rearrangement and expression of TCRα at this stage.

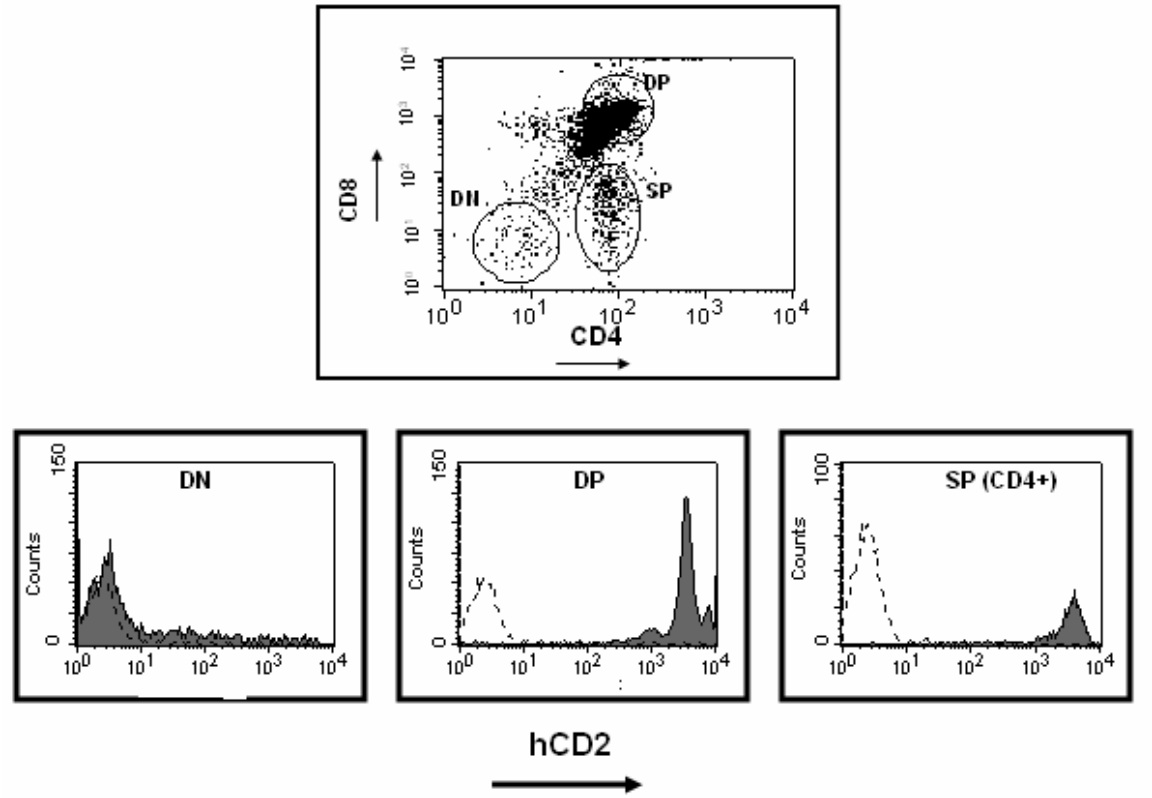



**Figure 19. hCD2 transgene activity is upregulated at the DP thymic stage.** Flow cytometric analysis of hCD2 expression in a representative hCD2:1-8 transgenic line (line 44) during thymocyte development. Thymocytes were stained with anti-CD4 and anti-CD8 to differentiate between the thymic developmental stages: DN (CD4-CD8-), DP (CD4+CD8+) and SP (CD4+CD8-). hCD2 expression is represented as a grey-filled line in the transgenic individual and as an open dashed line in the non-transgenic littermate control. This figure has been reprinted from Harrow, F. and Ortiz, B.D. (In press).

Although the majority of DN cells were found to be negative for hCD2 expression, a small proportion of these cells expressed variegated levels of the transgene. To more closely examine transgene expression during these earliest thymic stages, I used MACS to purify DN thymocytes from the thymi of transgenic mice. These experiments were conducted in two independent hCD2:1-8 lines (lines 4 and 44). The DN thymocytes were

then stained with antibodies to hCD2 and the molecular markers CD25 and CD44. Interestingly, in both transgenic lines examined, hCD2 expression is activated at DN4, precisely when TCRα has been reported to initiate rearrangement and expression (Fig. 20). This activation of hCD2 expression under the control of the TCRα LCR is distinct from the initiation of expression under the control of the hCD2 regulatory elements, which has been observed to occur earlier at DN1 (de Boer et al., 2003).

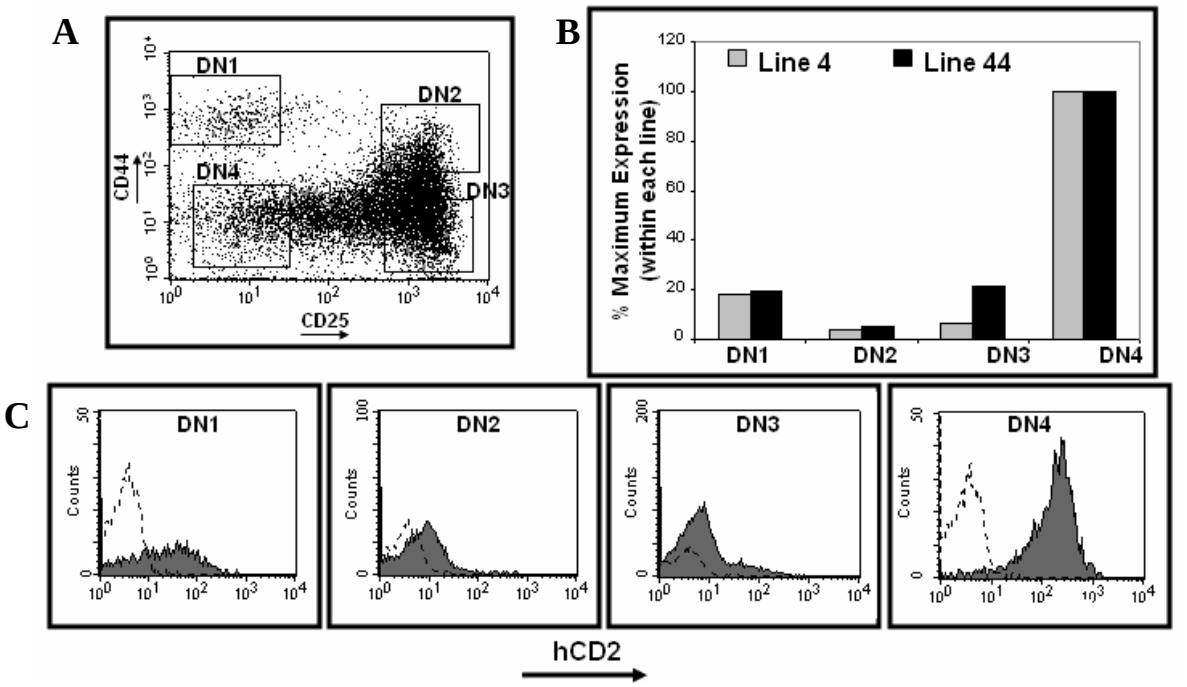



**Figure 20. hCD2 transgene expression is activated at DN4.** Flow cytometric analysis of hCD2 expression during early thymocyte (DN) development. ***A***, Purified DN thymocytes were stained with anti-CD25 and anti-CD44 to differentiate between the DN stages. ***B***, Histograms showing hCD2 expression of a representative hCD2:1-8 transgenic line (line 44) throughout DN development. hCD2 expression is represented as a grey-filled line in the transgenic individual and as an open dashed line in the non-transgenic littermate control. ***C***, Relative mean fluorescence of hCD2 transgene expression during thymocyte DN development in two transgenic individuals (hCD2:1-8 lines 4 and 44), as a percentage of maximum expression. The small percentage of CD4 and/or CD8 positive cells remaining in the purified DN sample was gated out of these analyses by staining with FITC-conjugated anti-CD4 and anti-CD8 antibodies recognizing different epitopes than those used in MACS purification. This figure has been reprinted from Harrow, F. and Ortiz, B.D. (In press).

### 5.1.5 Downregulation of LCR activity in peripheral T cells

Since elements within the LCR appeared to support the developmental regulation of TCRα gene expression in the thymus, I decided to examine LCR activity in the more mature peripheral T cell population. I isolated peripheral T cells from the spleen of transgenic animals using MACS, and compared hCD2 surface expression in these cells to thymocyte expression in the same animals. Surprisingly, in all three hCD2:1-8 lines, a reduction in the amount of transgene expression in spleen T cells was observed (Fig. 21). The levels of spleen hCD2 ranged from 22% to 60% of thymus. These results are in contrast to the normal upregulation of endogenous TCRα gene expression (described below).

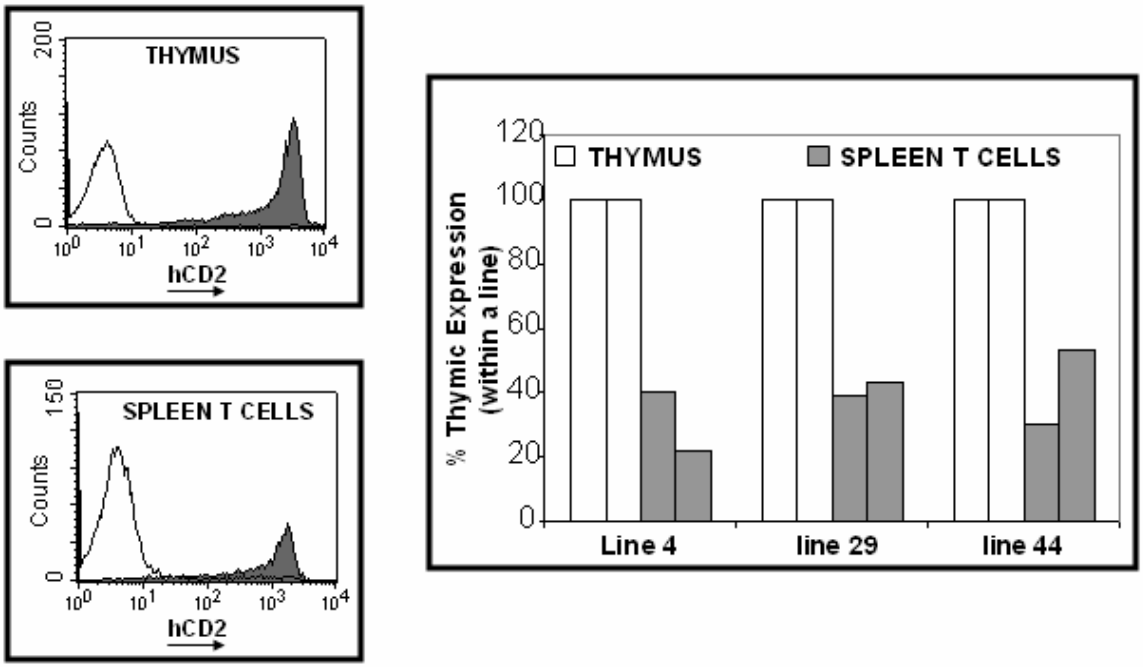



**Figure 21. hCD2 transgene expression is downregulated in peripheral T cells.** Flow cytometric analysis comparing hCD2 transgene expression in thymocytes and spleen T cells of a representative transgenic line (hCD2:1-8 line 44). Thymocytes and purified spleen T cells were stained with anti-hCD2 antibody. hCD2 expression is represented as a grey-filled solid line in the transgenic individual and as an open line in the non-transgenic littermate control. The relative mean fluorescence of hCD2 transgene expression in two representative transgenic individuals from each line (as a percentage of thymic expression within the same individual) is shown graphically. This figure has been reprinted from Harrow, F. and Ortiz, B.D. (In press).

To determine whether this lower level of hCD2 surface expression is a product of lower transgene mRNA levels in the periphery, I compared hCD2 expression in thymocytes and in spleen T cells of transgenic mice. MACS were used to purify T cells from the spleens of mice in two hCD2:1-8 lines (lines 4 and 44). Phosphorimager analysis of the northern blot data is shown in figure 22. In both these lines, lower levels of hCD2 mRNA were observed in spleen T cells compared to the thymus. In line 44 hCD2 transcription was reduced to as little as 46% of thymic levels. These experiments were also conducted in

the β:1-8 line, producing similar results. β-globin transcription was reduced in spleen T cells to 15% of thymic expression levels.

These observations were compared to the transcriptional activity of the TCRα gene at the endogenous locus. A probe to the TCRα constant region was used to examine TCRα mRNA levels on these same northern blots (Fig.22). The endogenous TCRα mRNA levels are significantly higher in peripheral T cells as compared to thymocytes. In hCD2:1-8 line 4, TCRα mRNA was increased by as much as 56%, and by over 100% in the β:1-8 line. Similar results were also observed in a non-transgenic animal (not shown).

The reduced levels of transgene mRNA in the peripheral T cells of two independent lines bearing different reporter genes ruled out the possibility that this downregulation occurred as a result of a reporter gene-specific effect. These results suggested that other elements in the wider TCRα locus, which have yet to be identified, are required to support high-level peripheral TCRα expression.

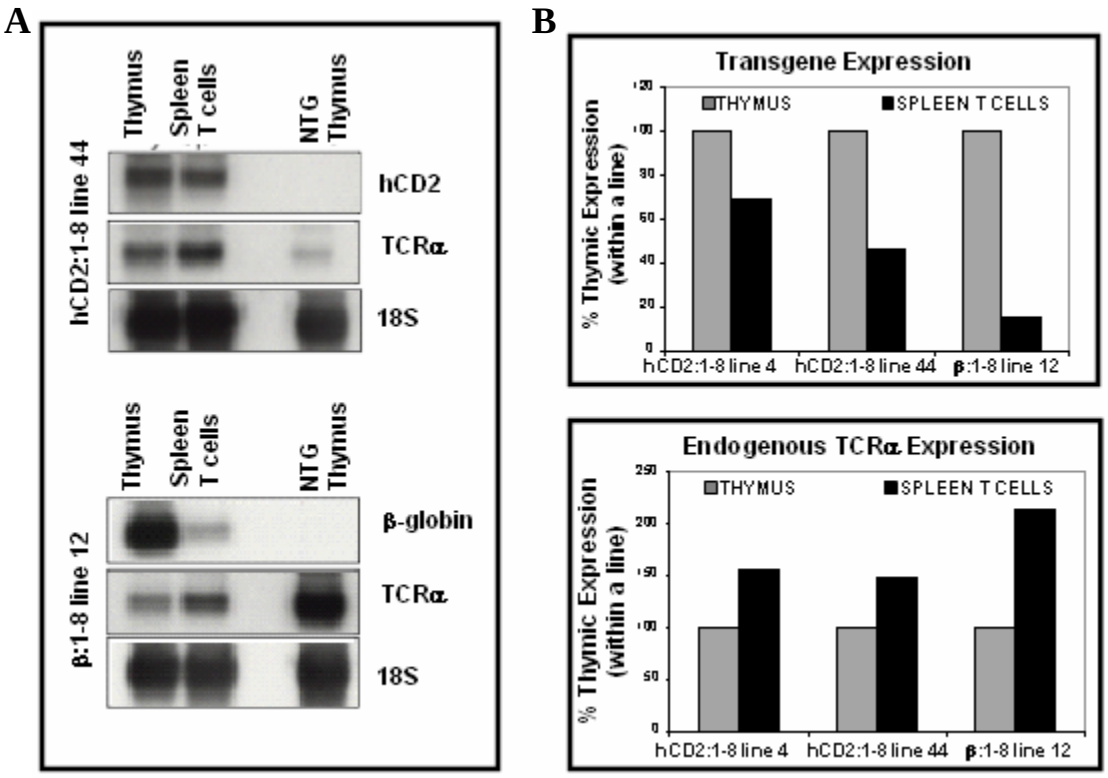



**Figure 22. Peripheral downregulation of LCR transgene activity is in contrast increased levels of endogenous TCRα mRNA.** Northern blot analysis comparing thymocyte and spleen T cell expression of transgene and endogenous TCRα mRNA. ***A***, Transgene mRNA was analyzed in the thymus and purified spleen T cells of two hCD2:1-8 lines (lines 4 and 44) and of a previously described β:1-8 transgenic line. Blots were striped and analyzed for endogenous TCRα. Transgene and TCRα signals were normalized to 18S rRNA. ***B***, Phosphorimager analysis of northern blot experiments in panel *A*. Expression is represented within each line as a percentage of thymic expression. This figure has been reprinted from Harrow, F. and Ortiz, B.D. (In press).

## 5.2 Discussion

Hematopoiesis is an ongoing process throughout adult life involving numerous genes that require complex mechanisms to ensure their cell-type and developmental-stage specific expression. The LCR of the TCRα gene is used here as a model to gain a better understanding of some of these gene regulatory mechanisms. Here I have established a new transgenic model that allows for the analysis of LCR activity in various hematopoietic lineages at different points in development, by the use of the surface-expressed hCD2 as a reporter gene. Consistent with the characteristics of LCR activity at the endogenous locus and in previously analyzed LCR-containing transgenes, the expression of the hCD2 reporter under the control of the wild type TCRα LCR is lymphoid organ-specific. (Ortiz et al., 1997).

### 5.2.1 The LCR supports the temporal expression patterns of the TCRα gene in the thymus

Here I report the activation of TCRα LCR-driven hCD2 expression at the thymic DN4 stage. Interestingly, it is precisely at this stage, DN4, that the initial products of TCRα gene rearrangement and expression have been reported to occur (Pearse et al., 1989; Petrie et al., 1995). To the best of my knowledge this study provides the first description of site-of–integration independent expression that is activated precisely at DN4, under the control of the TCRα LCR. These observations suggest that all the elements necessary for creating a transcriptionally-permissive chromatin environment and regulating thymic stage-specific expression are contained within this LCR fragment. A study by (Capone et

al., 1993) provided evidence of the activation of expression of a TCRβ transgene rearrangement substrate under the control of Eα in the CD25 negative compartment of DN thymocytes (approximates DN4 compartment). These results therefore indicate that Eα is very likely a major contributor to the stage-specific activation of LCR activity. Since Eα-driven transgenes are in general, poorly expressed, the stage-specific activation of gene expression seen here must rely on other LCR elements to counter gene silencing at ectopic integration sites. Accordingly, at the TCRα endogenous locus such elements would be necessary to create a transcriptionally permissive DNA environment. It will be interesting to determine what the minimal LCR sequences are for suppressing gene silencing effects. I propose that sequences within HS6 may largely contribute to these activities (see HS6 discussion).

To facilitate our understanding of T cell development, researchers have made use of transgenic mice carrying pre-rearranged TCRαβ genes. A common problem encountered in these transgene systems is the inappropriate (often premature) timing of TCRα transgene expression, which often leads to abnormal T cell development (Hogquist, 2001; Lacorazza et al., 2001). Here we describe a transgenic system that may be applied to these types of studies to achieve activation of TCRα transgenes at the appropriate stage of T cell development, DN4.

The developmental regulation of TCRα LCR activity may also prove useful for the generation of conditional deletions in mouse studies. Researchers have taken advantage of other stage-specific gene regulatory elements to direct Cre recombinase-mediated

recombination of loxP flanked DNA fragments, at specified stages of T cell development. These include hCD2 LCR elements that activate expression at DN1 (de Boer et al., 2003), the lck gene proximal promoter that activates transcription in the $CD44^+$ compartment prior to DN3 (Lee et al., 2001), regulatory elements from the CD4 locus (promoter, proximal enhancer $E4_{pro}$ and silencers) that activate expression in DP thymocytes and the CD8 enhancer ($E8_{SP}$) which is active in the CD8 SP lineage (Zou et al., 2001). Similarly, the TCRα LCR may be used to activate the expression of Cre recombinase at DN4.

### 5.2.2 The LCR does not sufficiently support the fine cell-type specificity and the upregulation of peripheral TCRα gene expression

In this study, transgene activity was observed in non-αβ T cells, including cells of the γδ T cell lineage. These results are in contrast to an earlier transgenic study that reported αβ T cell-specific expression of a pre-rearranged TCRα gene under the control of the TCRα LCR (Diaz et al., 1994). In this study TCRα mRNA was not detected (by RNase protection assay) in adult γδ T cells. One notable difference between this earlier study and the experiments conducted here is that the TCRα reporter is driven by a Vα-region (Vα11.1) promoter, which would imply that a homologous promoter may be required to confer αβ T cell specific expression. However, this is unlikely as certain Vα-region promoters (including the Vα11.1 promoter) have been shown to be functionally rearranged into both TCRα and TCRδ genes (expressed in αβ and γδ T cells, respectively) (Elliott et al., 1988). Furthermore, a targeted deletion of the Eα-HS1' region

in mice (Sleckman et al., 1997), and examination of hypersensitive site formation at the endogenous LCR in γδ T cells (Ortiz et al., 1999), suggested that elements within the LCR may also play a role in TCRδ gene expression. The LCR-driven hCD2 expression seen here in γδ T cells is not surprising in this regard. I therefore conclude that a Vα-region promoter is not likely a determinant of αβ lineage specificity. The differences in transgene activity reported between the present study and Diaz et al., may be attributed to the greater degree of sensitivity afforded by the flow cytometry techniques employed here.

Until now it was not clear whether the expression of LCR driven transgenes was limited to T cells. Previous studies had implicated TCRα LCR elements in directing ectopic expression of reporter genes. One transgenic study reported B cell expression of a Fas-FADD fusion protein indirectly linked to the LCR through co-integration (Kabra et al., 1999). B cell expression of a T cell receptor transgene linked to an incomplete LCR fragment has also been described (Lobito et al., 2004). The system used here is different, in that it examines the expression of a reporter gene linked directly to the complete wild type LCR fragment and B cell expression was also noted here. Since B cell expression of hCD2 transgenes has been described (de Boer et al., 2003; Law et al., 1994), it was important to determine whether the expression seen here is a property of the hCD2 reporter. The presence of LCR-driven β-globin transcription in spleen B cells (β-globin in not normally be expressed in B cells) of β:1-8 mice confirmed that this was not a reporter gene-specific effect, but that the LCR has some activity in B cells. Although the LCR has been demonstrated to direct lymphoid-specific expression (Ortiz et al., 1997), it

appears that additional elements in the wider TCRα locus are required achieve fine cell-type specificity of expression.

Surprisingly, a downregulation of hCD2:1-8 transgene activity was observed in peripheral T cells. This is in contrast to the apparent upregulation of TCRα gene expression observed at the endogenous locus. Interestingly, these findings may reflect the existence of distinct regulatory mechanisms of TCRα gene expression in the thymus and mature T cells. Similar mechanisms of differential gene regulation in other developmentally regulated gene loci have been described. For example, several developmental-stage specific enhancer clusters found in the mouse CD8 locus, differentially regulate gene expression in DP thymocytes, $CD4^{-}CD8^{+}$ (SP) T cells and extrathymically derived intraepithelial T cells (Hostert et al., 1998). Also the mouse CD4 silencer element specifically represses CD4 expression in DN and $CD4^{-}CD8^{+}$ thymocytes, while heritable chromatin-mediated silencing prevents expression in mature $CD8^{+}$ T cells (Ellmeier et al., 1998). Similar control mechanisms have been described in B cells. At the IgH heavy chain locus the importance of the intronic enhancer, Eμ, during early B cell development has been demonstrated, while the 3' enhancers are not functional until later stages (Arulampalam et al., 1997).

This work described here provides evidence for the importance of LCR activity during thymic differentiation. More specifically, all the elements necessary for activating expression at DN4 are contained within the LCR. However, it appears that other elements

present in the wider TCRα/δ/Dad1 locus are required in to support αβ T lineage specificity and certain aspects of TCRα expression in the periphery.

# CHAPTER 6

# THE SIGNIFICANCE OF GENE POSITION TO GENE EXPRESSION PATTERNS AT THE TCRα LOCUS

## 6.1 The 1-8:B7 transgene model

The presence of three differentially expressed genes in a common locus raises interesting questions regarding the LCRs ability to regulate multiple genes, and the significance of relative gene position in the locus. To address the significance of gene position relative to the LCR and the effect on gene expression patterns, I have established a transgene model utilizing the human leukocyte antigen-B7 (HLA-B7) gene. The HLA-B7 gene is a ubiquitously expressed human class I major histocompatibility (MHC) α chain which engages in antigen presentation. In these studies a 6.5-kb EcoRI genomic fragment of the HLA-B7 gene that includes its exons, introns and promoter is being used as a reporter gene (Chamberlain et al., 1988). In its natural locus the HLA-B7gene relies on additional gene regulatory elements for proper expression (Kushida et al., 1997). We may therefore attribute the expression of this gene in transgenic mice to the presence of the TCRα LCR. The HLA-B7 gene has been placed in a position and transcriptional orientation relative to the wild type LCR fragment similar to that of Dad1. This transgene was named 1-8:B7. Four independent lines bearing this transgene were established.

### 6.1.2 Lymphoid-specific expression of the 1-8:B7 transgene

Northern blots were used to examine transgene expression in the tissues of the 1-8:B7 transgenic lines. Interestingly, in all four lines this gene was expressed lymphoid-organ specifically, with little to no expression in non-lymphoid tissues. The highest levels of

transcription were detected in the thymus, and expression in the spleen ranged from 30% to 60% of thymic expression. The tissue distribution of transgene expression in the 1-8:B7 lines was reminiscent of transcription in the β:1-8 and hCD2:1-8 transgenic lines, both of which have their reporter gene linked 5' of the LCR. The transgene copy numbers of the 1-8:B7 lines have yet to be determined, however, preliminary examination suggests that all these lines bear multiple transgene integrants. The determination of transgene copy-number and expression levels per copy will provide further information on the effects of the LCR on the expression of a gene linked to its 3' end.

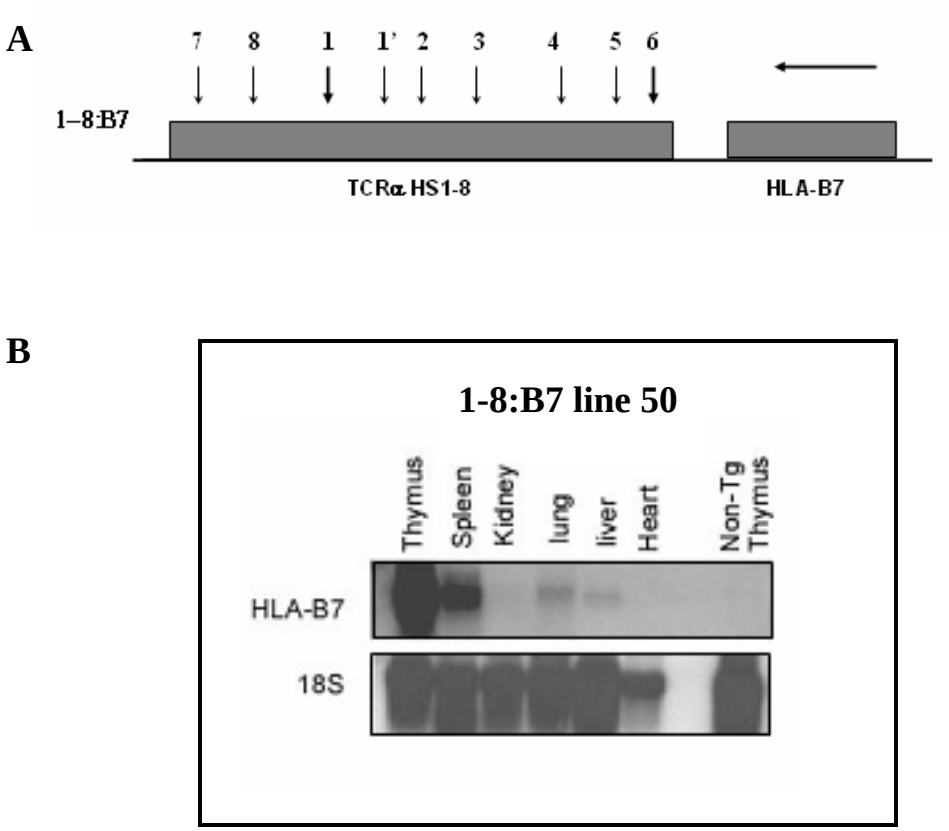



**Figure 23. B7:1-8 transgene expression is lymphoid organ-specific.** *A*, Diagram of the 1-8:B7 transgene. The HLA-B7 gene is linked 3' of the TCRα LCR HS1-8 fragment. The horizontal arrow represents the transcriptional orientation of the gene. Vertical arrows represent the hypersensitive sites. *B,* Northern blot showing tissue distribution of 1-8:B7 transgene expression in a representative line (line50). The HLA-B7 signal is normalized to 18S rRNA.

One of the main reasons for selecting this gene for use in our transgenic model was due to the cell-surface expression of the HLA-B7 molecule. Using an anti-HLA-B7 antibody purified from a mouse B lymphocyte hybridoma (ME 1), HLA-B7 could not be detected on the surface of thymocytes or spleen cells of 1-8:B7 transgenic mice. However, using this same antibody, HLA-B7 was detected on the surface of the JY human B lymphoblastoid cell line, which expresses this molecule.

An unstable HLA-B7 complex may account for its absence on the cell surface of transgenic animals. Normally, the $\beta_2$-microglobulin molecule forms a stable complex through its association with the class I MHC $\alpha$ chain and the antigen. $\beta_2$-microglobulin is actually a requirement for the surface expression of the class I MHC. It appears that the mouse $\beta_2$-microglobulin molecule is incapable of stabilizing the cell-surface HLA-B7 complex. Human $\beta_2$-microglobulin was added to the transgenic mouse thymocytes and spleen cells prior to antibody staining in an attempt to stabilize HLA-B7 complex formation on the surface, however, the complex was still undetected.

## 6.2 Discussion

At the endogenous TCRα locus, the positions of the T cell-specific TCR genes and the ubiquitously expressed Dad1 gene, relative to the LCR, raises the possibility that a relationship exists between gene position and the spatial expression patterns of these genes. In a tandem array of transgene integrants, the LCR is flanked on both ends by the reporter gene, which therefore places one copy of this gene in each of the positions representative of the endogenous 5' TCRα and 3' Dad1 genes. If the relative gene position is a determinant of the spatial expression pattern, then in this configuration the LCR could potentially drive tissue-unrestricted expression of the reporter gene located in the adjacent transcription unit near its 3' end. However, this was not seen as lymphoid organ-specific expression was observed in all transgenic lines (β:1-8 and hCD2:1-8 lines) bearing a 5' linked reporter gene (Ortiz et al., 1997). Directly linking the HLA-B7 reporter gene to the 3' end of the LCR (position of Dad1 at the endogenous locus) also resulted in lymphoid-organ specific expression in all lines carrying this construct.

The observed spatial expression patterns of genes linked both 5' and 3'of the LCR would suggest that there is no directionality to LCR activity and that it drives lymphoid organ-specific expression of a gene regardless of the gene's position. Another possibility is that the LCR may actually be capable of driving tissue-unrestricted expression of a directly linked 3' gene, but that lymphoid-specific expression of the 5' gene from the adjacent integrant dominates. To more definitively address these questions, single copy transgene lines would need to be examined.

# REFERENCES

Anderson, M.K., Hernandez-Hoyos, G., Diamond, R.A. and Rothenberg, E.V. (1999) Precise developmental regulation of Ets family transcription factors during specification and commitment to the T cell lineage. *Development*, **126**, 3131-3148.

Aronow, B.J., Ebert, C.A., Valerius, M.T., Potter, S.S., Wiginton, D.A., Witte, D.P. and Hutton, J.J. (1995) Dissecting a locus control region: facilitation of enhancer function by extended enhancer-flanking sequences. *Mol. Cell. Biol.*, **15**, 1123-1135.

Aronow, B.J., Silbiger, R.N., Dusing, M.R., Stock, J.L., Yager, K.L., Potter, S.S., Hutton, J.J. and Wiginton, D.A. (1992) Functional analysis of the human adenosine deaminase gene thymic regulatory region and its ability to generate position-independent transgene expression. *Mol. Cell. Biol.*, **12**, 4170-4185.

Arulampalam, V., Eckhardt, L. and Pettersson, S. (1997) The enhancer shift: a model to explain the developmental control of IgH gene expression in B-lineage cells. *Immunol. Today*, **18**, 549-554.

Baker, J.E., Kang, J., Xiong, N., Chen, T., Cado, D. and Raulet, D.H. (1999) A novel element upstream of the Vgamma2 gene in the murine T cell receptor gamma locus cooperates with the 3' enhancer to act as a locus control region. *J. Exp. Med.*, **190**, 669-679.

Balmelle, N., Zamarreno, N., Krangel, M.S. and Hernandez-Munain, C. (2004) Developmental activation of the TCR alpha enhancer requires functional collaboration among proteins bound inside and outside the core enhancer. *J. Immunol.*, **173**, 5054-5063.

Bartolomei, M.S., Webber, A.L., Brunkow, M.E. and Tilghman, S.M. (1993) Epigenetic mechanisms underlying the imprinting of the mouse H19 gene. *Genes Dev.*, **7**, 1663-1673.

Bassing, C.H., Swat, W. and Alt, F.W. (2002) The mechanism and regulation of chromosomal V(D)J recombination. *Cell*, **109 Suppl**, S45-55.

Bell, A.C., West, A.G. and Felsenfeld, G. (1999) The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. *Cell*, **98**, 387-396.

Bierer, B.E., Sleckman, B.P., Ratnofsky, S.E. and Burakoff, S.J. (1989) The biologic roles of CD2, CD4, and CD8 in T-cell activation. *Annu. Rev. Immunol.*, **7**, 579-599.

Capone, M., Hockett, R.D., Jr. and Zlotnik, A. (1998) Kinetics of T cell receptor beta, gamma, and delta rearrangements during adult thymic development: T cell receptor rearrangements are present in CD44(+)CD25(+) Pro-T thymocytes. *Proc. Natl. Acad. Sci. U S A*, **95**, 12522-12527.

Capone, M., Watrin, F., Fernex, C., Horvat, B., Krippl, B., Wu, L., Scollay, R. and Ferrier, P. (1993) TCR beta and TCR alpha gene enhancers confer tissue- and stage-specificity on V(D)J recombination events. *EMBO J.*, **12**, 4335-4346.

Chamberlain, J.W., Nolan, J.A., Conrad, P.J., Vasavada, H.A., Vasavada, H.H., Ploegh, H.L., Ganguly, S., Janeway, C.A., Jr. and Weissman, S.M. (1988) Tissue-specific and cell surface expression of human major histocompatibility complex class I heavy (HLA-B7) and light (beta 2-microglobulin) chain genes in transgenic mice. *Proc. Natl. Acad. Sci. U S A*, **85**, 7690-7694.

Chang, H.C., Moingeon, P., Lopez, P., Krasnow, H., Stebbins, C. and Reinherz, E.L. (1989) Dissection of the human CD2 intracellular domain. Identification of a segment required for signal transduction and interleukin 2 production. *J. Exp. Med.*, **169**, 2073-2083.

Chipev, C.C. and Wolffe, A.P. (1992) Chromosomal organization of Xenopus laevis oocyte and somatic 5S rRNA genes in vivo. *Mol. Cell. Biol.*, **12**, 45-55.

Cho, J.Y., Akbarali, Y., Zerbini, L.F., Gu, X., Boltax, J., Wang, Y., Oettgen, P., Zhang, D.E. and Libermann, T.A. (2004) Isoforms of the Ets transcription factor NERF/ELF-2 physically interact with AML1 and mediate opposing effects on AML1-mediated transcription of the B cell-specific blk gene. *J. Biol. Chem.*, **279**, 19512-19522.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156-159.

de Boer, J., Williams, A., Skavdis, G., Harker, N., Coles, M., Tolaini, M., Norton, T., Williams, K., Roderick, K., Potocnik, A.J. and Kioussis, D. (2003) Transgenic mice with hematopoietic and lymphoid specific expression of Cre. *Eur. J. Immunol.*, **33**, 314-325.

Diaz, P., Cado, D. and Winoto, A. (1994) A locus control region in the T cell receptor alpha/delta locus. *Immunity*, **1**, 207-217.

Elliott, J.F., Rock, E.P., Patten, P.A., Davis, M.M. and Chien, Y.H. (1988) The adult T-cell receptor delta-chain is diverse and distinct from that of fetal thymocytes. *Nature*, **331**, 627-631.

Ellis, J., Tan-Un, K.C., Harper, A., Michalovich, D., Yannoutsos, N., Philipsen, S. and Grosveld, F. (1996) A dominant chromatin-opening activity in 5' hypersensitive site 3 of the human beta-globin locus control region. *EMBO J.*, **15**, 562-568.

Ellmeier, W., Sunshine, M.J., Losos, K. and Littman, D.R. (1998) Multiple developmental stage-specific enhancers regulate CD8 expression in developing thymocytes and in thymus-independent T cells. *Immunity*, **9**, 485-496.

Enver, T., Brewer, A.C. and Patient, R.K. (1985) Simian virus 40-mediated cis induction of the Xenopus beta-globin DNase I hypersensitive site. *Nature*, **318**, 680-683.

Epner, E., Reik, A., Cimbora, D., Telling, A., Bender, M.A., Fiering, S., Enver, T., Martin, D.I., Kennedy, M., Keller, G. and Groudine, M. (1998) The beta-globin LCR is not necessary for an open chromatin structure or developmentally regulated transcription of the native mouse beta-globin locus. *Mol. Cell.*, **2**, 447-455.

Erman, B., Cortes, M., Nikolajczyk, B.S., Speck, N.A. and Sen, R. (1998) ETS-core binding factor: a common composite motif in antigen receptor gene enhancers. *Mol. Cell. Biol.*, **18**, 1322-1330.

Festenstein, R., Tolaini, M., Corbella, P., Mamalaki, C., Parrington, J., Fox, M., Miliou, A., Jones, M. and Kioussis, D. (1996) Locus control region function and heterochromatin-induced position effect variegation. *Science*, **271**, 1123-1125.

Giese, K., Kingsley, C., Kirshner, J.R. and Grosschedl, R. (1995) Assembly and function of a TCR alpha enhancer complex is dependent on LEF-1-induced DNA bending and multiple protein-protein interactions. *Genes Dev.*, **9**, 995-1008.

Greaves, D.R., Wilson, F.D., Lang, G. and Kioussis, D. (1989) Human CD2 3'-flanking sequences confer high-level, T cell-specific, position-independent gene expression in transgenic mice. *Cell*, **56**, 979-986.

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

Hark, A.T., Schoenherr, C.J., Katz, D.J., Ingram, R.S., Levorse, J.M. and Tilghman, S.M. (2000) CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. *Nature*, **405**, 486-489.

Harrow, F., Amuta, J.U., Hutchinson, S.R., Akwaa, F. and Ortiz, B.D. (2004) Factors binding a non-classical Cis-element prevent heterochromatin effects on locus control region activity. *J. Biol. Chem.*, **279**, 17842-17849.

Hernandez-Munain, C., Roberts, J.L. and Krangel, M.S. (1998) Cooperation among multiple transcription factors is required for access to minimal T-cell receptor alpha-enhancer chromatin in vivo. *Mol. Cell. Biol.*, **18**, 3223-3233.

Hogquist, K. (2001) Getting TCR transgenics to work on time. *Trends Immunol.*, **22**, 240-241.

Hong, N.A., Cado, D., Mitchell, J., Ortiz, B.D., Hsieh, S.N. and Winoto, A. (1997) A targeted mutation at the T-cell receptor alpha/delta locus impairs T-cell development and reveals the presence of the nearby antiapoptosis gene Dad1. *Mol. Cell. Biol.*, **17**, 2151-2157.

Hong, N.A., Kabra, N.H., Hsieh, S.N., Cado, D. and Winoto, A. (1999) In vivo overexpression of Dad1, the defender against apoptotic death-1, enhances T cell proliferation but does not protect against apoptosis. *J. Immunol.*, **163**, 1888-1893.

Hostert, A., Garefalaki, A., Mavria, G., Tolaini, M., Roderick, K., Norton, T., Mee, P.J., Tybulewicz, V.L., Coles, M. and Kioussis, D. (1998) Hierarchical interactions of control elements determine CD8alpha gene expression in subsets of thymocytes and peripheral T cells. *Immunity*, **9**, 497-508.

Howard, F.D., Ledbetter, J.A., Wong, J., Bieber, C.P., Stinson, E.B. and Herzenberg, L.A. (1981) A human T lymphocyte differentiation marker defined by monoclonal antibodies that block E-rosette formation. *J. Immunol.*, **126**, 2117-2122.

Jenuwein, T. and Allis, C.D. (2001) Translating the histone code. *Science*, **293**, 1074-1080.

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

John, S., Reeves, R.B., Lin, J.X., Child, R., Leiden, J.M., Thompson, C.B. and Leonard, W.J. (1995) Regulation of cell-type-specific interleukin-2 receptor alpha-chain gene expression: potential role of physical interactions between Elf-1, HMG-I(Y), and NF-kappa B family proteins. *Mol. Cell. Biol.*, **15**, 1786-1796.

Jones, B.K., Monks, B.R., Liebhaber, S.A. and Cooke, N.E. (1995) The human growth hormone gene is regulated by a multicomponent locus control region. *Mol. Cell. Biol.*, **15**, 7010-7021.

Kabra, N.H., Cado, D. and Winoto, A. (1999) A tailless fas-FADD death-effector domain chimera is sufficient to execute Fas function in T cells but not B cells of MRL-lpr/lpr mice. *J. Immunol.*, **162**, 2766-2774.

Kioussis, D., Vanin, E., deLange, T., Flavell, R.A. and Grosveld, F.G. (1983) Beta-globin gene inactivation by DNA translocation in gamma beta-thalassaemia. *Nature*, **306**, 662-666.

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

Kushida, M.M., Dey, A., Zhang, X.L., Campbell, J., Heeney, M., Carlyle, J., Ganguly, S., Ozato, K., Vasavada, H. and Chamberlain, J.W. (1997) A 150-base pair 5' region of the MHC class I HLA-B7 gene is sufficient to direct tissue-specific expression and locus control region activity: the alpha site determines efficient expression and in vivo occupancy at multiple cis-active sites throughout this region. *J. Immunol.*, **159**, 4913-4929.

Lacorazza, H.D., Tucek-Szabo, C., Vasovic, L.V., Remus, K. and Nikolich-Zugich, J. (2001) Premature TCR alpha beta expression and signaling in early thymocytes impair thymocyte expansion and partially block their development. *J. Immunol.*, **166**, 3184-3193.

Lang, G., Mamalaki, C., Greenberg, D., Yannoutsos, N. and Kioussis, D. (1991) Deletion analysis of the human CD2 gene locus control region in transgenic mice. *Nucleic Acids Res.*, **19**, 5851-5856.

Law, Y.M., Yeung, R.S., Mamalaki, C., Kioussis, D., Mak, T.W. and Flavell, R.A. (1994) Human CD4 restores normal T cell development and function in mice deficient in murine CD4. *J. Exp. Med.*, **179**, 1233-1242.

Lee, P.P., Fitzpatrick, D.R., Beard, C., Jessup, H.K., Lehar, S., Makar, K.W., Perez-Melgosa, M., Sweetser, M.T., Schlissel, M.S., Nguyen, S., Cherry, S.R., Tsai, J.H., Tucker, S.M., Weaver, W.M., Kelso, A., Jaenisch, R. and Wilson, C.B. (2001) A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity*, **15**, 763-774.

Li, Q., Harju, S. and Peterson, K.R. (1999) Locus control regions: coming of age at a decade plus. *Trends. Genet.*, **15**, 403-408.

Li, Q., Peterson, K.R., Fang, X. and Stamatoyannopoulos, G. (2002) Locus control regions. *Blood*, **100**, 3077-3086.

Lobito, A.A., Lopes, M.F. and Lenardo, M.J. (2004) Ectopic T cell receptor expression causes B cell immunodeficiency in transgenic mice. *Eur. J. Immunol.*, **34**, 890-898.

Lopes, S., Lewis, A., Hajkova, P., Dean, W., Oswald, J., Forne, T., Murrell, A., Constancia, M., Bartolomei, M., Walter, J. and Reik, W. (2003) Epigenetic

modifications in an imprinting cluster are controlled by a hierarchy of DMRs suggesting long-range chromatin interactions. *Hum. Mo.l Genet.*, **12**, 295-305.

Magdinier, F., Yusufzai, T.M. and Felsenfeld, G. (2004) Both CTCF-dependent and -independent insulators are found between the mouse T cell receptor alpha and Dad1 genes. *J. Biol. Chem.*, **279**, 25381-25389.

Magram, J., Chada, K. and Costantini, F. (1985) Developmental regulation of a cloned adult beta-globin gene in transgenic mice. *Nature*, **315**, 338-340.

Makishima, T., Nakashima, T., Nagata-Kuno, K., Fukushima, K., Iida, H., Sakaguchi, M., Ikehara, Y., Komiyama, S. and Nishimoto, T. (1997) The highly conserved DAD1 protein involved in apoptosis is required for N-linked glycosylation. *Genes Cells*, **2**, 129-141.

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

Melton, E., Sarner, N., Torkar, M., van der Merwe, P.A., Russell, J.Q., Budd, R.C., Mamalaki, C., Tolaini, M., Kioussis, D. and Zamoyska, R. (1996) Transgene-encoded human CD2 acts in a dominant negative fashion to modify thymocyte selection signals in mice. *Eur. J. Immunol.*, **26**, 2952-2963.

Moingeon, P., Chang, H.C., Wallner, B.P., Stebbins, C., Frey, A.Z. and Reinherz, E.L. (1989) CD2-mediated adhesion facilitates T lymphocyte antigen recognition function. *Nature*, **339**, 312-314.

Moon, A.M. and Ley, T.J. (1990) Conservation of the primary structure, organization, and function of the human and mouse beta-globin locus-activating regions. *Proc. Natl. Acad. Sci. U S A*, **87**, 7693-7697.

Nakashima, T., Sekiguchi, T., Kuraoka, A., Fukushima, K., Shibata, Y., Komiyama, S. and Nishimoto, T. (1993) Molecular cloning of a human cDNA encoding a novel protein, DAD1, whose defect causes apoptotic cell death in hamster BHK21 cells. *Mol. Cell. Biol.*, **13**, 6367-6374.

Narlikar, G.J., Fan, H.Y. and Kingston, R.E. (2002) Cooperation between complexes that regulate chromatin structure and transcription. *Cell*, **108**, 475-487.

Navas, P.A., Peterson, K.R., Li, Q., McArthur, M. and Stamatoyannopoulos, G. (2001) The 5'HS4 core element of the human beta-globin locus control region is required for high-level globin gene expression in definitive but not in primitive erythropoiesis. *J. Mol. Biol.*, **312**, 17-26.

Navas, P.A., Peterson, K.R., Li, Q., Skarpidi, E., Rohde, A., Shaw, S.E., Clegg, C.H., Asano, H. and Stamatoyannopoulos, G. (1998) Developmental specificity of the interaction between the locus control region and embryonic or fetal globin genes in transgenic mice with an HS3 core deletion. *Mol. Cell. Biol.*, **18**, 4188-4196.

Ortiz, B.D., Cado, D., Chen, V., Diaz, P.W. and Winoto, A. (1997) Adjacent DNA elements dominantly restrict the ubiquitous activity of a novel chromatin-opening region to specific tissues. *EMBO J.*, **16**, 5037-5045.

Ortiz, B.D., Cado, D. and Winoto, A. (1999) A new element within the T-cell receptor alpha locus required for tissue-specific locus control region activity. *Mol. Cell. Biol.*, **19**, 1901-1909.

Ortiz, B.D., Harrow, F., Cado, D., Santoso, B. and Winoto, A. (2001) Function and factor interactions of a locus control region element in the mouse T cell receptor-alpha/Dad1 gene locus. *J. Immunol.*, **167**, 3836-3845.

Pearse, M., Wu, L., Egerton, M., Wilson, A., Shortman, K. and Scollay, R. (1989) A murine early thymocyte developmental sequence is marked by transient expression of the interleukin 2 receptor. *Proc. Natl. Acad. Sci. U S A*, **86**, 1614-1618.

Petrie, H.T., Livak, F., Burtrum, D. and Mazel, S. (1995) T cell receptor gene recombination patterns and mechanisms: cell death, rescue, and T cell production. *J. Exp. Med.*, **182**, 121-127.

Rand, E., Ben-Porath, I., Keshet, I. and Cedar, H. (2004) CTCF elements direct allele-specific undermethylation at the imprinted H19 locus. *Curr. Biol.*, **14**, 1007-1012.

Reik, A., Telling, A., Zitnik, G., Cimbora, D., Epner, E. and Groudine, M. (1998) The locus control region is necessary for gene expression in the human beta-globin locus but not the maintenance of an open chromatin structure in erythroid cells. *Mol. Cell. Biol.*, **18**, 5992-6000.

Rothenberg, E.V. and Taghon, T. (2005) Molecular genetics of T cell development. *Annu. Rev. Immunol.*, **23**, 601-649.

Santoso, B., Ortiz, B.D. and Winoto, A. (2000) Control of organ-specific demethylation by an element of the T-cell receptor-alpha locus control region. *J. Biol. Chem.*, **275**, 1952-1958.

Sarafova, S. and Siu, G. (1999) A potential role for Elf-1 in CD4 promoter function. *J. Biol. Chem.*, **274**, 16126-16134.

Siebenlist, U., Hennighausen, L., Battey, J. and Leder, P. (1984) Chromatin structure and protein binding in the putative regulatory region of the c-myc gene in Burkitt lymphoma. *Cell*, **37**, 381-391.

Sleckman, B.P., Bardon, C.G., Ferrini, R., Davidson, L. and Alt, F.W. (1997) Function of the TCR alpha enhancer in alphabeta and gammadelta T cells. *Immunity*, **7**, 505-515.

Spicuglia, S., Payet, D., Tripathi, R.K., Rameil, P., Verthuy, C., Imbert, J., Ferrier, P. and Hempel, W.M. (2000) TCRalpha enhancer activation occurs via a conformational change of a pre-assembled nucleo-protein complex. *EMBO J.*, **19**, 2034-2045.

Staal, F.J., Roederer, M., Herzenberg, L.A. and Herzenberg, L.A. (1990) Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. *Proc. Natl. Acad. Sci. U S A*, **87**, 9943-9947.

Strong, R.K., Penny, D.M., Feldman, R.M., Weiner, L.P., Boniface, J.J., Davis, M.M. and Bjorkman, P.J. (1994) Engineering and expression of a secreted murine TCR with reduced N-linked glycosylation. *J. Immunol.*, **153**, 4111-4121.

Tanimoto, K., Sugiura, A., Omori, A., Felsenfeld, G., Engel, J.D. and Fukamizu, A. (2003) Human beta-globin locus control region HS5 contains CTCF- and developmental stage-dependent enhancer-blocking activity in erythroid cells. *Mol. Cell. Biol.*, **23**, 8946-8952.

Taramelli, R., Kioussis, D., Vanin, E., Bartram, K., Groffen, J., Hurst, J. and Grosveld, F.G. (1986) Gamma delta beta-thalassaemias 1 and 2 are the result of a 100 kbp deletion in the human beta-globin cluster. *Nucleic Acids Res.*, **14**, 7017-7029.

Townes, T.M., Lingrel, J.B., Chen, H.Y., Brinster, R.L. and Palmiter, R.D. (1985) Erythroid-specific expression of human beta-globin genes in transgenic mice. *EMBO J.*, **4**, 1715-1723.

von Boehmer, H. (1990) Developmental biology of T cells in T cell-receptor transgenic mice. *Annu. Rev. Immunol.*, **8**, 531-556.

Wai, A.W., Gillemans, N., Raguz-Bolognesi, S., Pruzina, S., Zafarana, G., Meijer, D., Philipsen, S. and Grosveld, F. (2003) HS5 of the human beta-globin locus control region: a developmental stage-specific border in erythroid cells. *EMBO J.*, **22**, 4489-4500.

Wilson, A., Held, W. and MacDonald, H.R. (1994) Two waves of recombinase gene expression in developing thymocytes. *J. Exp. Med.*, **179**, 1355-1360.

Winoto, A. and Baltimore, D. (1989) Alpha beta lineage-specific expression of the alpha T cell receptor gene by nearby silencers. *Cell*, **59**, 649-655.

Winoto, A. and Baltimore, D. (1989) A novel, inducible and T cell-specific enhancer located at the 3' end of the T cell receptor alpha locus. *EMBO J.*, **8**, 729-733.

Wotton, D., Ghysdael, J., Wang, S., Speck, N.A. and Owen, M.J. (1994) Cooperative binding of Ets-1 and core binding factor to DNA. *Mol. Cell. Biol.*, **14**, 840-850.

Wu, C. (1989) Analysis of hypersensitive sites in chromatin. *Methods Enzymol.*, **170**, 269-289.

Yui, M.A., Hernandez-Hoyos, G. and Rothenberg, E.V. (2001) A new regulatory region of the IL-2 locus that confers position-independent transgene expression. *J. Immunol.*, **166**, 1730-1739.

Zhuma, T., Tyrrell, R., Sekkali, B., Skavdis, G., Saveliev, A., Tolaini, M., Roderick, K., Norton, T., Smerdon, S., Sedgwick, S., Festenstein, R. and Kioussis, D. (1999) Human HMG box transcription factor HBP1: a role in hCD2 LCR function. *EMBO J.*, **18**, 6396-6406.

Zou, Y.R., Sunshine, M.J., Taniuchi, I., Hatam, F., Killeen, N. and Littman, D.R. (2001) Epigenetic silencing of CD4 in T cells committed to the cytotoxic lineage. *Nat. Genet.*, **29**, 332-336.