TCR Alpha LCR and non-LCR cis-elements contributing to tissue specific expression of the TCR Alpha gene in thymic and peripheral T cells

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TCR ALPHA LCR AND NON-LCR CIS-ELEMENTS CONTRIBUTING TO TISSUE SPECIFIC EXPRESSION OF THE TCR ALPHA GENE IN THYMIC AND PERIPHERAL T CELLS

by

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Abstract

TCR ALPHA LCR AND NON-LCR CIS-ELEMENTS CONTRIBUTING TO TISSUE SPECIFIC EXPRESSION OF THE TCR ALPHA GENE IN THYMIC AND PERIPHERAL T CELLS

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Martina Kučerová-Levisohn

Adviser: Dr. Benjamin Ortiz

Orchestrated expression of multiple genes residing in the complex TCRα/δ/Dad1 locus requires tight control from multiple cis-acting elements. The TCRα locus control region (LCR), is positioned between TCRα and Dad1 gene, and has been implicated in the differential expression of both genes. In this study, we focus our work on the hypersensitive site (HS)1 prime (HS1’), located 3’ of the classical Eα enhancer, within the TCRα LCR. We investigated its non-redundant role in TCRα expression in thymic and peripheral T cells as assayed by in vivo and in vitro studies. Furthermore, formation of HS1’ in both lymphoid and non-lymphoid tissue raised the possibility of HS1’ playing a dual role in regulating both the upstream (TCRα) as well as the downstream (Dad1) genes. To answer this question, we created wild type and mutant HS1’ dual-reporter BACs utilizing human and rat CD2 reporter genes in the position of TCRα and Dad1, respectively. We find HS1’ important for TCRα expression in thymus and spleen T cells, but dispensable for Dad1 expression. We widened our focus to include sequences outside of the TCRα LCR. Specifically, DNase I hypersensitivity assay revealed a cluster of active chromatin just 5’ of the constant region Cα exons. Analysis of this 3.9-kb region using a BAC transgenic mouse model reveals its importance for TCRα gene expression in thymic and splenic T cells.
Interestingly, this novel DNase hypersensitive regulatory complex will remain present upon the Va-Jα rearrangement of the TCRα gene given its location 3’ of the most downstream functional joining (J) segment, Jα2. Therefore, the novel cis-acting region may contribute to endogenous TCRα gene activity.
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Dedicated to the loving memory of my father.
# TABLE OF CONTENTS

## CHAPTER 1: INTRODUCTION

1.1 Murine TCRα/TCRΔ/DAD1 Locus ................................................................. 1
1.2 TCRα Locus Control Region ........................................................................... 2
1.3 Gene regulation of the TCRα locus ................................................................ 5
1.4 LCRs ............................................................................................................... 7

## CHAPTER 2: MATERIALS AND METHODS ......................................................... 8

2.1 DNA constructs .............................................................................................. 8
2.2 Transgenic mice ............................................................................................. 10
2.3 Embryonic stem cell (ESC) culture, transfection and in vitro differentiation . 11
2.4 Bone marrow derived hematopoietic stem cell and in vitro differentiation on OP9-DL1 cells .................................................................................................. 13
2.5 Flow cytometry .............................................................................................. 13
2.6 RNA isolation and detection .......................................................................... 15
2.7 DNaseI Hypersensitivity Assay ...................................................................... 16

## CHAPTER 3: IN VIVO AND IN VITRO MODEL SYSTEMS ................................. 18

3.1 Transgenic Mouse Model and in vitro ES cell differentiation model .......... 18
3.2 20 days in a dish, the journey from ES cell to T cell ..................................... 19
3.3 Monitoring in vitro co-culture progress by flow cytometry ......................... 22

## CHAPTER 4: MIMICKING THE COMPLEXITY OF THE TCRα/DAD1 LOCUS BY UTILIZING A DUAL-REPORTER BAC CONSTRUCT ........................................ 25

4.1 Construct design of wild type dual-reporter BAC ........................................ 25
4.2 Wild type dual reporter BAC carrying TCRα LCR exhibits integration site independent expression ................................................................. 26
4.3 Tissue/cell type specificity of the wild type BAC .................................................. 28
4.4 TCRα/Dad1 dual-reporter BAC follows correct developmental timing ............... 31
4.5 High expression of hCD2 reporter in BAC is comparable in thymic and peripheral T cells ...................................................................................... 34

CHAPTER 5: NON-REDUNDANT ROLE OF HS1’ IN THYMIC AND PERIPHERAL

EXPRESSION OF THE TCRα GENE ........................................................................ 38

5.1 Introducing the HS1’ site of the TCRα LCR ......................................................... 38
5.2 The mutant delta HS1’ dual-reporter BAC ........................................................... 40
5.3 hCD2 reporter gene expression is impaired in the absence of HS1’ site .............. 41
5.4 Role of HS1’ in tissue specific expression in the context of the TCRα/Dad1 BAC46
5.5 Developmental timing of the dHS1’ BAC ................................................................ 49

CHAPTER 6: NOVEL TCRα GENE CIS-REGULATORY REGION ............................. 53

6.1 Introduction ........................................................................................................... 53
6.2 Multiple DNaseI hypersensitive sites are detected 3’ of Jα2 ................................. 54
6.3 TCRα/Dad1 bacterial artificial chromosome (BAC) with dual-reporter transgenes for analyses of functionality of HS sites cluster ........................................ 55
6.4 Flow cytometry detection of phenotypic differences in reporter gene expression between wild type and ΔJα3-Cα1 mutant BAC transgenic T cells .................. 56
6.5 Deletion of the Jα3-Cα1 region impairs Vα promoter-driven hCD2 reporter gene expression in thymocytes and spleen T cells .................................................. 58
CHAPTER 7: DISCUSSION AND SIGNIFICANCE ................................................................. 62

7.1 ROLE OF HS1’ IN THE TCRα LOCUS ........................................................................ 62

7.2 NOVEL TCRα GENE CIS-REGULATORY REGION ..................................................... 67

REFERENCES ..................................................................................................................... 69
TABLE OF FIGURES

FIGURE 1. Undifferentiated mouse ESC .............................................................. 19
FIGURE 2. Mesoderm formations ........................................................................ 20
FIGURE 3. Morphology of mesoderm .................................................................. 21
FIGURE 4. Diagram of the steps of the mESC-OP9 co-culture procedure .................. 24
FIGURE 5. A dual-reporter BAC .......................................................................... 26
FIGURE 6. A TCRα/Dad 1 gene locus-derived dual reporter BAC construct is expressed independently of genomic integration site .............................................................. 27
FIGURE 7. Expression pattern of tissue distribution of the dual-reporter transgene in the wild type BAC. .............................................................................................. 29
FIGURE 8. Cell type distribution of TCRα/Dad1 BAC reporter gene activity .......... 30
FIGURE 9. Flow cytometry detection of the developmental timing TCRα reporter (hCD2) expression ........................................................................................................ 32
FIGURE 10. Proper TCRα-like timing of the hCD2 reporter gene is observed during T cell development in the wild type BAC ........................................................................... 34
FIGURE 11. Reporter and endogenous TCRα mRNA levels in thymic and peripheral T cells .................................................................................................................. 36
FIGURE 12. Diagram (drawn not to scale) of the TCRα/Dad1 gene locus-derived dual reporter BAC construct with HS1’ deletion ............................................................................. 40
FIGURE 13. Impaired hCD2 reporter expression is evident in four, independent HS1’ mutant BAC transgenic mouse lines .................................................................................. 41
Figure 14. Impaired hCD2 reporter expression in thymocytes of HS1’ mutant BAC transgenic mice...

Figure 15. Peripheral T cells exhibit reduced hCD2 transgene expression in HS1’ mutants

Figure 16. Tissue distribution of hCD2 and rCD2 transgene expression remains unaltered in HS1’ mutant

Figure 17. Diagram of single reporter transgene constructs used in vitro ESC differentiation experiments

Figure 18. Flow cytometry analyses of copy number matched ESC clones during in vitro ESC differentiation

Figure 19. DNase I hypersensitivity sites (HS) within the Jα2 to Cα1 region

Figure 20. Diagram (not to scale) of the ΔJα3-Cα1 mutant TCRα/Dad1 dual-reporter BAC construct

Figure 21. Impaired hCD2 reporter expression in the absence of the deleted region

Figure 22. Absence of the deleted region impairs upstream, but not downstream reporter gene expression in thymocytes

Figure 23. The deleted region is functional in peripheral T cells

Figure 24. HS1’ is not required for Dad1 regulation
Chapter 1: INTRODUCTION

1.1 Murine TCR\(\alpha\)/TCR\(\delta\)/Dad1 Locus

Mouse chromosome 14 contains the complex T-cell receptor (TCR)-\(\alpha/\delta\)/Defender against Death (Dad)-1 locus that displays a unique layout of differentially expressed genes. Tight gene regulation must be orchestrated during development to lead to functional rearrangement and tissue specific expression of TCR\(\alpha/\delta\) genes and ubiquitous expression of Dad1 gene. TCR\(\alpha/\delta\) genes encode the TCR\(\alpha\) and TCR\(\delta\) protein subunits of the T cell receptor of two distinct lineages of T cells, \(\alpha\beta\) and \(\gamma\delta\). V(D)J rearrangement of the TCR\(\beta\) and TCR\(\delta\) gene precedes rearrangement of the TCR\(\alpha\) gene. TCR\(\delta\) rearranges at double negative DN2 stage [1] that is marked by absence of the CD4 and CD8 cell surface markers, whereas TCR\(\alpha\) rearranges at a later time point, between DN4 [2-4] and DP (CD4\(^+\)/CD8\(^+\)) stage [2,4]. TCR\(\alpha\) activation coincides with onset of the TCR\(\alpha\) LCR activity [5]. The \(\alpha\) chain of the TCR is formed by recombination of one variable (V) and one joining (J) gene segment to the constant region C\(\alpha\). The \(\delta\) chain has additional diversity (D) gene segments; therefore the \(\delta\) chain rearranges the V(D)J gene segments to C\(\delta\). The majority of circulating T cells belong to the \(\alpha\beta\) lineage. Successful rearrangement of the \(\alpha\) chain deletes \(\delta\) gene segments since the V\(\delta\), D\(\delta\), J\(\delta\), C\(\delta\) are located between the V\(\alpha\) and J\(\alpha\) segments. The rearrangement of the TCR\(\delta\) and TCR\(\alpha\) gene is catalyzed by RAG1/2 enzymes and is controlled in cis [6].

Defender against death 1 (Dad1) is an anti-apoptotic gene that was discovered in a temperature sensitive mutant cell line [7]. Later on, Dad1 was confirmed to encode a mammalian subunit of oligosaccharyltransferase (OST) enzyme, that is involved in N-linked glycosylation [8]. As
polypeptides are synthesized from the mRNA they are translocated across the endoplasmic reticulum (ER) membrane into the lumen, where they are modified by the OST enzyme. N-linked glycosylation is crucial for the efficient folding and assembly of the newly synthesized polypeptides [9]. Without Dad1, the essential cellular functions fail, resulting in programmed cell death [8,9]. Deletion of Dad1 gene results in early embryonic lethality [10,11]. Dad1 is highly conserved in vertebrates, invertebrates and plants [7,9,10]. Dad1 is ubiquitously expressed with Dad1 mRNA transcripts detected as early as day E6.5 [11]. Furthermore, the expression of Dad1 gene is regulated during the course of the T cell development, with highest expression levels occurring at the SP stage of T cells [11]. However, it is unknown, what exact role Dad1 plays in the T cell development. Overexpression of Dad1 leads to increased proliferation of peripheral T cells upon antigen stimuli [11].

Since TCRα and Dad1 gene have different gene expression profiles and functions, they might be controlled by a cis-acting regulatory element called a Locus Control Region (LCR) that resides between the two genes. The TCRα LCR’s influence on the TCRα is known, however regulation of Dad1 gene has not yet been determined.

1.2 TCRα Locus control region

The TCRα locus control region (LCR), residing between the TCRα and Dad1 gene [12], is a powerful cis-acting DNA element that has been studied in context of a linked heterologous transgene. The LCR manifests its powers by providing high expression levels of the transgene at all integration sites (integration site-independence) of transgenic animal that confer to the spatiotemporal control of the given LCR [13,14]. TCRα LCR allows the TCR genes to be expressed specifically at T cells at time of DN to DP stage transition. Lastly, the expression of
the transgene will positively correlate with the transgene’s copy number that the particular transgenic mouse line carries [13,14].

TCRα LCR consists of nine DNaseI hypersensitive sites (HS) that, as assayed, contain highly accessible form of chromatin that stretches over a ~13.5-kb large region [12,15]. The known function of individual sites (if already determined) will be reported in the order of their position starting from the 5’ end.

**HS7 and HS8**, located on the most 5’ end of the TCRα LCR are thought to contain silencer elements [16], however, they are not required for full LCR activity [15].

**HS1** was discovered by transient transfection assay [16] and is the classical enhancer Eα that is an integral part of the LCR since its deletion leads to a loss of strict copy number-dependency, one of the characteristic traits of LCRs [15]. It also ensures the highest-level thymic expression of an LCR-linked transgene [15].

**HS1 prime** (HS1’) was discovered as a cluster of a few HS sites that forms strongly in both lymphoid and non-lymphoid tissues [15,17] and is highly enriched in histone H3 acetylation [18]. CCCTC-binding factor (CTCF) dependent enhancer blocking activity was observed in HS1’ [18] and its role in LCR activity was already tested *in vivo*. It was found that the CTCF and 5’CTCF-like containing sequences are not required for full LCR activity. Only minor effects on transgene expression levels (per copy) were observed with maintained tissue-specificity [19].

HS1’ site is a focus of one of my projects and is further described in chapter 5.

**HS2-6** of the TCRα LCR provides an open chromatin environment and this region is crucial for protection from the position effect variegation (PEV) that results in gene silencing and absence
of transgene expression if integration occurs at a site of heterochromatin [20]. Therefore, this 3’ portion of the LCR (HS2 through HS6) carries the hallmark of the LCR’s integration site independence. Without the 5’ portion (HS1 and HS1’) of the LCR that specifically increases expression of the transgene in the thymus and inhibits non-lymphoid organ transgenic expression, chromatin conformation assayed by the DNaseI hypersensitivity assay remains in open configuration in both non-lymphoid and lymphoid tissue [20].

**HS4** is located ~3.8-kb away from HS1’, it is differentially methylated in lymphoid and non-lymphoid organs. In lymphoid organ HS4 site is hypomethylated [21] and highly enriched in histone acetylation [18]. Both modifications are associated with open chromatin state. Transgenic mice with an internal deletion of the HS4 region exhibit decreased transgene expression in thymus and abnormal expression in different organs with large variability [19]. Therefore, HS4 region is needed to protect transgene expression from PEV. Interestingly, HS4 differential methylation is lost upon HS1’ site removal, linking these two sites.

**HS6** is a well-characterized site of the TCRα LCR by *in vivo* and *in vitro* assays. HS6 site alone can suppress PEV and is a source for wide chromatin opening activity [22]. Furthermore, *in vivo* occupied factor-binding sites were identified in thymus (thymic footprint (TF) 1-2-3) and were determined to bind AML-1/RUNX and Elf-1 [22], proteins known for their interaction with chromatin remodeling complexes [23,24]. Once again, HS1’ was required for tissue specific factor occupancy in the identified footprint. Removal of TF1-2-3 sites resulted in decreased expression of the reporter gene (per copy) as assayed in a transgenic mouse model and in the formation of inaccessible chromatin configuration where HS4 site was not formed and HS1 and HS1’ were weak [25]. Furthermore, *in vitro* assay in NIH 3T3 cells identified an additional site
3’ of the TF 1-2-3 (site HS6-316) that is functional in support of wide chromatin opening activity. Together, this data indicate a great importance of HS6, its dependence on HS1’ and HS6 influence on HS4 and HS1, HS1’ regions [25].

From the structure/function analyses of the TCRα LCR, a scenario emerges of a great interplay among the individual HS sites that function in harmonic synergy to bring about the developmentally appropriate, tissue specific expression of the TCRα gene and Dad1 gene.

1.3 Gene regulation of the TCRα locus

Regulation of gene expression in thymocytes is a complex process involving multiple cis elements such as promoters, enhancers and locus control regions. Additionally, trans elements including transcription factors, chromatin-remodeling complexes and histone-modification enzymes are essential for proper gene regulation [26]. For example, T cell development requires Notch signaling that initiates (T vs. B cells) [27] and sustains differentiation in addition to transcription factors such as GATA-3, E2A/HEB, c-Myb, Runx1, TCF-1 and Ikaros that are expressed at different times during differentiation [28].

Enhancers, originally defined by transient transfection gene assays using differentiated cell lines, function in an orientation independent manner. Enhancers increase transcriptional initiation from promoters and may be located near or far away from promoters, docking site of RNA Polymerase II. Enhancers might directly interact with promoters leading to transcriptional activation, for example in our locus Eα has been shown to directly interact with TEA promoter [29] in order to initiate germline expression of J regions. This model is termed “looping” and
was described in late 1990’s by the Groudine lab [30] and received abundant support from 3C (chromosome conformation capture) [29] and 3D-FISH studies [31-33].

Transcription and recombination of the locus is greatly influenced by DNA packaging into chromatin in the nucleus. The nucleosome is the fundamental unit of chromatin. 147 bp large segments of DNA are wrapped tightly around the histone octamer (H2A, H2B, H3 and H4). Multiple, reversible covalent modifications (e.g. acetylation and methylation, phosphorylation and ubiquitination) can be introduced post-transcriptionally to histone tails that alter chromatin structure and/or recruit proteins [34]. Chromatin then can exist in either condensed chromatin (heterochromatin) associated with silent genes, or loose, decondensed (euchromatin) configuration occurring in expressed genes. It has been determined that inaccessibility of gene locus to nuclease (e.g. DNaseI) and DNA methylation, catalyzed by methyltransferase enzyme, are associated with silenced genes [35]. On the other hand, hypomethylation, DNase I accessibility and histone acetylation are associated with actively transcribing genes. Moreover, “active” markers (e.g. H3K4me3) or “repressed” (e.g. H3K27me3) marks contribute to chromatin configuration. However, histone marking cannot always be interpreted in such a straightforward manner. Furthermore, it has been shown that a “poised” state exists for some enhancers where presence of H3K4me1 or H3K4me2 in absence of H3K27ac is noted on the histone tail of progenitor cells [36]. Bivalent chromatin structure with both activating H3K4me3 and repressing H3K27me3 modifications at the same site has been shown in embryonic stem cells and is thought to provide flexible platform for later developmental state [37].
TCRα gene is a developmentally regulated gene that will become active at the ~ DP stage of the T cell development and undergo V(D)J rearrangement. Differentiating T cells can be easily monitored and separated based on their expression of cell surface markers (see chapter 3).

1.4 LCRs

The first discovered LCR came from the human β-globin gene locus. Puzzling over the lack of transcription of intact β-globin genes in transgenic mice [38-40], and certain thalassemia patients [41,42], an important regulatory element (eventually termed an LCR) was discovered 5’ of the human β-globin genes [43]. Since then, a few additional LCRs and LCR-like elements have been found (reviewed in [13,14]). Immunologically relevant genes have been a fruitful source of LCRs: human CD2 [44], perforin (PRF1) [45], human MHC class I HLA-B7 [46], macrophage lysozyme [47], mouse TCRγ [48], human T cell-specific adenosine deaminase [49] are a few examples. Our work presented below comes from the TCRα LCR [12] and its surrounding locus.
Chapter 2: MATERIALS AND METHODS

2.1 DNA constructs

TCRα/Dad1 bacterial artificial chromosome (BAC) dual-reporter constructs

Wild type BAC

The BAC utilized in this study is based on a TCRα/Dad1 genomic region-containing sub-fragment [50] of clone RP23-94114 (BACPAC Resources, Oakland, CA). Our BAC fragment spanned from the extreme 3’- Jα region to approximately 38-kb downstream of the Dad1 exons. BAC modifications were done using Red/ET recombination technology (Gene Bridges) following the manufacturers instructions. The (approximately 5.4-kb) genomic human CD2 (ghCD2) reporter gene [51] driven by a 429-bp Vα17 promoter sequence (minimal promoter established by Dr. Armin Lahiji, Vα17 promoter was a kind gift from Dr. Derek Sant’Angelo), was inserted approximately 3.7-kb upstream of exon 1 of the TCRα constant region in the transcriptional orientation of the TCRα gene. The second reporter gene was a 703-bp cDNA of the rat CD2 (rCD2) gene [52] linked to an SV40 poly-adenylation signal (from pEYFP-C1). This reporter was recombined in frame to ATG in exon 1 of the Dad1 gene. Thus, the Dad1 promoter drives transcription of the rCD2 reporter gene. Prior to transfection, the dual-reporter BAC construct fragment was released from the pBACE3.6 vector backbone using NotI and FseI restriction enzymes and separated by Field-Inversion Gel Electrophoreses (FIGE). The 76.2-kb band was isolated from the gel by electro-elution into TE buffer followed by phenol/chloroform extraction and ethanol precipitation.
**Mutant BACs (ΔHS1’ BAC and ΔJα3_Cα1 BAC)**

Two mutant versions of the wild type BAC were created using Red/ET recombination technology (Gene Bridges). Both mutant versions utilize the same dual-reporter genes (ghCD2 and rCD2) as described above. Deleted regions are as follows: one, HS1’ site (BglII–BamHI) of the TCRα LCR was removed, creating a deletion of 826-bp, that lead to the ΔHS1’ BAC construct. Second, the Jα3-Cα1 mutant BAC has a deletion of 3.9-kb region spanning from 38-bp 5’ of a SacI site [53] (located between Jα4 and Jα3) to 9-bp 3’ of an EcoRV site within the Cα constant region exon 1. This deletion removed the identified cluster of DNaseI hypersensitive sites described in chapter 6. The ~75.4-kb (ΔHS1’ BAC construct) and ~72.5-kb (ΔJα3_Cα1 BAC construct) bands were isolated from the gel by electro-elution into TE buffer followed by phenol/chloroform extraction and ethanol precipitation.

**hCD2:1-8 and hCD2:1-8ΔHS1’ constructs**

hCD2:1-8 construct was previously described [5]. Briefly, ~10.5-kb Sall–BamHI fragment of the hCD2 reporter gene (lacking the cytoplasmic tail responsible for signaling of the CD2 molecule) was positioned 5’ of the 10.2-kb Sall–SacI fragment of TCRα LCR (all 9 HS of the TCRα LCR are included). hCD2:1-8ΔHS1’ construct has 826-bp of BglII–BamHI sequence removed, corresponding to the HS1’ site of the TCRα LCR. DNA fragments containing transgene cassettes for transfections were liberated from the pBluescript (Stratagene) vector by digestion with Sall and NotI restriction enzymes. In order to obtain purified fragments for transfection, samples were run on 1% low-melting-point agarose (Sea Plaque), excised and followed by β-agarose digestion (NEB) and ethanol precipitation.
**Neomycin-G418 resistance cassette**

A 1.6-kb Neomycin-G418 resistance cassette driven by the SV40 promoter was excised from the pEYFP-C1 vector (Clontech) using SspI and EcoO109I restriction enzymes, run on 0.8% agarose gel and the DNA fragment was column purified (Qiagen). The gel purified Neomycin-G418 resistance cassette was used for co-transfection purposes in equimolar amounts to the construct during ESC transfections.

### 2.2 Transgenic mice

The purified DNA constructs were microinjected into male pronuclei of (C57BL X CBA) F1 fertilized eggs and transferred to pseudo-pregnant females at the MSKCC facility. Transgenic founders were identified by Southern blot and PCR screening. Founders were then outcrossed to C57BL/6 mice (Taconic) to establish individual transgenic mouse lines. The relative transgene copy numbers among the individual mouse lines were determined by Southern blot and qPCR. ~10 µg of tail genomic DNA was digested with AfII restriction enzyme overnight. After electrophoresis and transfer to nitrocellulose membrane, Southern blot was performed. A 508-bp large probe corresponding to 3’ end AfII fragment was prepared by PCR and double labeled with [α-32P] dATP and [α-32P] dCTP using RadPrime DNA Labeling System (Invitrogen by Life Technologies) before hybridization. Relative copy number for each BAC transgenic mouse line was determined from three Southern blots, simultaneously detecting transgene and endogenous locus (band of different sizes) and quantified by PhosphorImager.

Samples of mouse tail genomic DNA (5ng) were prepared with mix of DyNAmo HS SYBR Green qPCR Kit (New England BioLabs) and qPCR was done in an Applied Biosystems 7500
RT-PCR device. Relative copy numbers were determined using the comparative \( \Delta \Delta C(t) \) method with mouse GAPDH as a normalizer. The primers used were as follows: mGAPDH forward: 5’-cctctggecccttgagctagga-3’ mGAPDH reverse: 5’-cacaagaagatgcggcggctc-3’. To detect BAC, primers were designed at the most 5’ end of the construct (V\( \alpha \)17 promoter forward, reverse) and the 3’ end region (Dad1 forward, reverse). Since primers amplify endogenous as well as transgene sequence, any DNA sequence detected above the non-transgenic sample correspond to additional sequences due to presence of the transgene. The non-transgenic control value obtained represents two (endogenous) copies and was set to 1. The primers used were as follows: V\( \alpha \)17 fw1: 5’-ctgettaagatctcttaccag-3’, rev: 5’-caactgcacttctgagctgc-3’, Dad1 fw: 5’-gagcagcatttccaccege-3’ Dad1 rev: 5’-ttccacatccccacctctac-3’. Sample values from three experiments for each primer combination in duplicates were averaged to obtain relative copy numbers for individual transgenic mouse lines. Results from the three Southern blots and from the qPCR analyses were similar.

2.3 Embryonic stem cell (ESC) culture, transfection and in vitro differentiation

Undifferentiated mouse embryonic stem cells (ESR1) were cultured on top of Mitomycin C arrested mouse embryonic fibroblasts (Millipore) monolayer in Dulbecco’s Modification of Eagle’s Medium (DMEM) with high glucose and sodium pyruvate (Corning) with additional supplement of 20% ES cell qualified FBS (Fetal bovine serum) (Gemini), 1% Glutagro (Corning), 1% Penicillin/Streptomycin (Corning), 1% HEPES (Millipore), 1% nonessential amino acids (Millipore), 0.1% gentamicin (Life Technologies), 0.1% (55 \( \mu \)M) \( \beta \)-mercaptoethanol (Life Technologies), and 10 ng/ml LIF (Millipore).
About 1.0 X 10^7 ES cells were transfected using a Bio-Rad Gene Pulser at the setting of 0.24 kV and 500 µF in 0.5 ml electroporation buffer (Millipore) with 12 µg of BAC or 10 µg of the hCD2:1-8ΔHS1’ fragment and each of the reporter gene constructs were co-transfected with an equimolar amount of the neomycin-G418 resistance cassette. The G418 selection was done 24-hours post transfection at the concentration of 0.35 mg/ml for first 2 days followed by decrease to 0.175 mg/ml of G418 for the rest of the selection; with change of selection media daily. Individual ESC colonies were picked after 7-10 days and were clonally propagated. Presence of the hCD2 transgene in drug-selected ES cell clones was detected by PCR using the following primers: fw: 5’-gaggaaaccaacccctaagatgag-3’ and rev: 5’-cgtaatcttttggagactgcacc-3’ detecting the 5’-end portion of the hCD2 gene. PCR positive clones were further screened on Southern blot for intactness of the transgene. The copy number of individual ES clones was determined from at least three Southern blots after digestion of genomic DNA samples with BglII and probed from 3’-end with 827-bp probe [5] located in HS6 region of the TCRα LCR to detect intact head to tail configuration of the transgene and quantified using PhosphorImager. For BAC ES cells, screening was done with Vα17-forward primer: 5’-atctgtcactctagctagcc-3’; hCD2ΔrT-reverse: 5’-cgtaatctctttggagactgcacc-3’ and rCD2 specific primers (see below). Southern blots were done to confirm presence of transgene and estimate relative copy number.

In vitro differentiation of ESC to T cells was previously described [54] and our modification to the existing protocol (see chapter 3) was described in detail in literature [55,56] as well as in a video-documented journal [57]. Selected ESC clones carrying hCD2:1-8ΔHS1’construct were differentiated into T cells alongside a non-transfected ESR1 as a negative control and previously published [55] hCD2:1-8 ESC (clones A1, B5, D6) served as positive controls and expression
baseline for comparing mutant clones.

2.4 Bone marrow derived hematopoietic stem cell and in vitro differentiation on OP9-DL1 cells

_in vitro_ differentiation of HSC purified from bone marrow was performed as previously described [54]. WT BAC line 36 and non-transgenic littermate control were used for the experiment. Bone marrow (from femur and tibia of 8 weeks-old mice) was flushed out using a syringe and recovered cells were MACS purified using Lineage\(^{\text{neg}}\) cell kit (Milteneyi). Lineage\(^{\text{neg}}\) (CD4, CD8, CD11b, CD19, CD45R, CD161, Gr.1, Ter119) cells were seeded on OP9-DL1 monolayers and supplemented with 5 ng/mL Flt-3L and 1 ng/mL IL-7 and passaged every 4-5 days to fresh OP9-DL1 monolayer. Day 11 and day 12 of the co-culture, the IL-7 cytokine concentration was lowered to 0.5 ng/mL and 0.25 ng/mL, respectively and cells with both cytokine concentrations were kept in parallel. The progress of the co-culture was monitored by flow cytometry.

2.5 Flow cytometry

_Mice_

\(~1\times10^6\) of single cell suspension thymocytes or splenocytes from BAC transgenic mouse lines were pretreated in with 100 µL of FACS staining medium (RPMI 1640 supplemented with 3% FBS and 10mM HEPES buffer) for 10 min at 4°C along with 1µg of rat anti-CD16/32 (Clone 2.4G2, Life Technologies) to block Fc receptors. Afterwards, 0.2-0.5 µg of the Abs (from BD Biosciences) were added and incubated for 20 min at 4°C, followed by three washes with FACS
staining medium. For detection of our reporter genes on the cell surface, FITC conjugated mouse anti-human CD2 (clone S5.2) or mouse anti-rat CD2 (clone OX-34) were used in combination with rat anti- mouse CD4 (clones GK1.5 or RM4-5) conjugated to APC and rat anti-mouse CD8α (clone 53-6.7) conjugated to PE; or hamster anti-mouse TCRβ chain (clone H57-597) conjugated to APC and rat anti-mouse CD19 (clone1D3) conjugated to PE. Samples were acquired using FACSCalibur (BD Biosciences) device and collected data was analyzed with FlowJo (Tree Star) software.

Co-culture

The progress of the ESC differentiation toward the T cells was monitored by flow cytometry on crucial days of the co-culture: day 12, 15/16 and 20/21 using 5 channels of the FACScan device. All antibodies used were obtained from BD Biosciences or Life Technologies. First, the Fc receptors were blocked by pretreating the cells with anti-CD16/32 (Clone 2.4G2) for 10-20’; followed by addition of antibodies conjugated to a particular fluorochrome. The following antibodies were used: anti-human CD2 (clone S5.2) or anti-rat CD2 (clone OX-34) conjugated to FITC for reporter gene detection on cell surface and anti-mouse CD45 (Clone 30-F11) conjugated to PE or APC, CD44 (Clone IM7) conjugated AF700, CD25 (Clones 3C7 or PC61) conjugated to PE or APC, CD8 (Clone 53-6.7) conjugated to PE, CD4 (Clones GK1.5 or RM4-5) conjugated to AF700, CD11b (clone M1/70.15) conjugated to PE, Ter119 (clone TER119) conjugated to AF700 to distinguish particular stages of hematopoiesis during ESC differentiation. Dead cell discriminator (DCD) from Invitrogen was used to label the non-viable cells and remove them from the subsequent analyses with FlowJo (Tree Star) software. Samples were acquired on the FACScan device.
2.6 RNA isolation and detection

**BAC transgenic mice: RNA Analysis by Northern blots**

RNA was prepared from PBS-rinsed mouse organs (thymus, kidney, lung, liver and heart) using the single step RNA isolation protocol [58]. 5-10 µg of total thymic RNA was run on 0.8% agarose gel and transferred to neutral nylon membrane (Amersham) for Northern blot analysis. Hybridization probes were labeled with [$\alpha$-32P] dATP and [$\alpha$-32P] dCTP using RadPrime DNA Labeling System (Invitrogen by Life Technologies). To detect hCD2 transgene RNA, a labeled 500-bp EcoRV-PstI fragment of hCD2 exon 2 was added during hybridization. A 699-bp NcoI-SalI fragment was used for rCD2 transgene RNA detection. Both Northern blots were normalized to 18S rRNA to control for loading and efficiency of transfer. Ten pmol of the highly specific 18S rRNA 20-mer 5’-cggaactacaaggtatctg-3’ probe [59] was end-labeled using $\gamma$-32P ATP and T4 polynucleotide kinase (New England Biolabs). PhosphorImager analyses were used to obtain normalized transgene expression levels (per copy) for individual mouse lines.

**BAC transgenic mice: RNA Analysis by qRT-PCR**

RNA samples from thymus and purified spleen T cells were isolated using RNeasy Mini Kit (Qiagen). Spleen T cells of >91% purity were obtained using magnetically activated cell separation (MACS, Miltenyi Biotec) system. cDNAs was synthesized from RNA using the QuantiTect Reverse Transcription Kit (Qiagen) with genomic DNA Wipeout Buffer or the ProtoScript kit (NEB). Expression of the hCD2 transgene was detected with hCD2-specific primers described previously [55]. Levels of rCD2 expression was detected with the following rCD2-specific primers: fw: 5’-ccagtgccttgctaggatacg-3’, rev: 5’- ggagttttcttttgctttcagcc-3’.
Endogenous Dad1 expression levels were used as a normalizer in these experiments using a forward primer specific for exon 1 that are not present in the BAC reporter: (5’-tgcatctgccctgtcctc- 3’) and a reverse primer complimentary to Dad1 exon3 (5’-ggaaagtaaggctacagtggagg-3’). Samples of cDNA were prepared with mix of DyNAmo HS SYBR Green qPCR Kit (New England BioLabs) and qRT-PCR experiments were carried out in ViiA7 system (Applied Biosystem) or Applied Biosystems 7500 device.

**OP9 co-cultulture derived T cells: RNA Analysis by qRT-PCR**

For each clone, on the final day 20/21 of the co-culture, RNA was isolated by utilizing the RNeasy Micro Kit (Qiagen). 0.5-1.0 µg total RNA was reverse transcribed to c-DNA using ProtoScript kit (NEB). Expression of human CD2 transgene was detected with hCD2 primers [55], TCRα primers [50] were used to normalize for loading variation, content and stage of differentiation of T cells in co-culture. Relative hCD2 transgene levels were determined by the comparative ΔΔC(t) method as previously described [55,56]. Samples were mixed with DyNAmo HS SYBR Green qPCR Kit (New England BioLabs), run in duplicates and three repetitions were performed in ViiA7 qPCR system (Applied Biosystem).

**2.7 DNasel Hypersensitivity Assay**

Nuclei of MACS purified spleen T cells from C57BL/6 mice were subjected to DNasel titration (Worthington). The reaction was performed on ice and stopped after 10 min by adding 1/10 volume of stop buffer (5% SDS, 100mM EDTA). Proteinase K (200 µg/ml) was added to samples and incubated overnight at 55°C. This was followed by phenol/chloroform extraction and ethanol precipitation of DNasel-treated genomic DNA. These samples were then digested
with the NdeI restriction enzyme to generate a 6.7-kb parental fragment of the endogenous Jα-Cα region. Samples were loaded on 0.8% agarose gel, run and transferred to positively charged nylon membrane (Nytran SPC) for Southern blot analysis. A 669-bp probe was generated by PCR using the parent BAC clone as a template and the following primers: fw: 5’-atggctgagggaaaggtctacg-3’ and rev: 5’-agaaaagtctctggaactggtc-3’. The probe was labeled with [α-32P] dCTP and/or [α-32P] dATP using the RadPrime DNA Labeling System (Life Technologies).
Chapter 3: *In vivo and in vitro* model systems

### 3.1 Transgenic Mouse Model and *in vitro* ES cell differentiation model

Two methods of analyzing the activity of the TCRα LCR and non-LCR elements were employed in these studies. The gold standard in the field of LCRs for many decades has been the transgenic mouse model. This method provides a very powerful approach, in which individual transgenic lines are established, each representing an independent genomic integration point of the tested construct. However, this model can be rather slow, expensive and “tricky” if the transgenic founders don’t transmit or stop breeding. Unfortunately, this statement foreshadows some of our results.

A second method, an *in vitro* embryonic stem cell (ESC) differentiation, was validated just last year in our lab [55]. All hallmarks of the LCR (integration independent expression in copy number dependent manner of the linked reporter gene with tissue specific and correct developmental timing) were manifested. Therefore, this model can stand on its own, however, for this study, it is used as supplemental method for our *in vivo* study. The OP9-DL1 system for *in vitro* differentiating ESC to T cells [54] is an elegant, faster approach and supplements our transgenic studies, where each generated ES clone is “equivalent” to individual mouse, carrying the construct at a different integration site.
3.2 20 days in a dish, the journey from ES cell to T cell

Embryonic stem cells (ES cells) are harvested from the inner cell mass (ICM) of the blastocyst. ES cells are self-renewing, totipotent cells that can contribute to all tissue types (germ cells included) of the animal if injected back to the blastocyst [60]. We utilized the murine R1/E ES cells that were derived from 3.5 day blastocyst of the 129 mouse strains cross and were kind gift from the J. C. Zúñiga-Pflücker lab (University of Toronto). ES cells are selected and propagated on a confluent monolayer of irradiated or Mitomycin C arrested mouse embryonic fibroblast (MEFs) with addition of the leukemia inhibitory factor (LIF) [61] to keep cells in an undifferentiated state (Figure 1).

**Figure 1. Undifferentiated mouse ESC.** Phase contrast microscopy image of undifferentiated mESCs (sharp edged colonies) growing on top of a MEF monolayer (100x magnification). (Originally published in JoVE [57].)

We start the process of the in vitro differentiation with drug-selected, stably transfected ES cells that carry intact reporter gene cassettes. ESC clones are seeded on a bone marrow stromal cell line, OP9 that does not expresses the macrophage colony-stimulating factor (M-CSF) [62] due to M-CSF gene mutation [63], and thereby allows commitment to the hematopoietic lineage [64,65]. These cells are marked by cell surface expression of Flk-1 [66].
If ES cells are left “without direction”, they proliferate and spontaneously differentiate into embryoid bodies (EB) - cystic structures that are characterized by semi-organized germ layers tissues [67], including the mesoderm that will give rise to hematopoietic precursors [68-70]. Therefore, ES cells are left (free of external, additional cytokines) to form mesoderm for length of 5 to 7 days [56,57]. This is one of the crucial steps in this co-culture method, since the majority (80-90%) of ES cells colonies need to be visually inspected to confirm mesoderm formation before proceeding with the co-culture (Figure 2).

**Figure 2. Mesoderm formations.** Phase contrast microscopy images of mesoderm-like colony formation at “day 5” of co-culture. (A) 40x and (B) 100x views of co-culture plates with >90% mesodermal colony differentiation. These plates are ready for day 5 passage. (C-F) Examples of Day 5 co-culture plates that require 1-2 day postponing before transfer (C&E, 40x magnification, D&F, 100x magnification). (Originally published JoVE [57]).
It’s important to visually monitor the progress of the co-culture and develop an eye for recognizing mesoderm formation (either “wagon wheel” or “florets” can form) [57,71] (Figure 3).

**Figure 3. Morphology of mesoderm.** Phase contrast microscopy images of the variety of mesoderm-like colony formations at day 5 of co-culture. (A) The colony morphology referred to as “craters” or “wagon wheel.” (B) The colony morphology referred to as “starburst” or “florets” (200x magnification). (Originally published JoVE [57].)

Culturing ES cells on an OP9 monolayer would not achieve full-scale differentiation to T cells [72], rather it would drive differentiation towards monocytic, erythroid and B cell lineages. T cells do not develop in the bone marrow; T cell precursors migrate to thymus to complete their development. It is therefore important to “mimic” the thymic environment with its essential factors in the in vitro studies as well. This was accomplished by retroviral transduction of the OP9 cell line with the Notch ligand Delta-like 1 (OP9-DL1) that provides necessary signals for full T cells differentiation [73]. In addition, an optimal lot of fetal bovine serum (FBS) and cytokines are necessary for successful and robust differentiation.
In vitro differentiation cultures are provided with fms-like tyrosine kinase 3 ligand (Flt3-L) starting on “day 5 passage” since Flt-3L is important for differentiation to lymphohematopoietic progenitors [65]. By day 8, semi-adherent hematopoietic progenitor cells (HPC) are at the crossroad for myeloid and B cell or T lymphoid lineage. HPCs are collected and cells that will be induced to T cell lineage are transferred to OP9-DL1 monolayers [73], whereas for monocytic, erythroid or B cell lineage, cells are kept on OP9 monolayers [65]. In both cases, in vitro co-cultures are supplemented with Flt-3L and, starting from day 8, interleukin 7 (IL-7).

3.3 Monitoring in vitro co-culture progress by flow cytometry

Flow cytometry analyses can be utilized to monitor the progress of the co-culture. All cells are initially gated with forward and side-scatter on lymphocyte population, followed by live-gate (DCD\textsuperscript{neg} or DAPI\textsuperscript{neg}) before analyses.

Cells undergoing differentiation on the OP9 monolayer will yield erythroid (CD45\textsuperscript{neg}, TER119\textsuperscript{+}) and monocytic (CD45\textsuperscript{+}, CD11b\textsuperscript{hi}) lineages on day 12 and B cells (CD45\textsuperscript{+}, CD19\textsuperscript{+}) by day 16.

Cells differentiating on OP9-DL1 monolayers will first go through double negative (DN) stages of T cell development that are characterized by absence of the CD4 and CD8 cell markers and can be further divided to four stages DN1-4. Day 12 yields DN1 (CD44\textsuperscript{+}, CD25\textsuperscript{neg}, CD4\textsuperscript{neg}, CD8\textsuperscript{neg}) and DN2 (CD44\textsuperscript{+}, CD25\textsuperscript{+}, CD4\textsuperscript{neg}, CD8\textsuperscript{neg}) stage T cells, day 16 yields DN3 (CD44\textsuperscript{neg}, CD25\textsuperscript{+}, CD4\textsuperscript{neg}, CD8\textsuperscript{neg}) and DN4 (CD44\textsuperscript{neg}, CD25\textsuperscript{neg}, CD4\textsuperscript{neg}, CD8\textsuperscript{neg}) stages. Some DP (CD4\textsuperscript{+}, CD8\textsuperscript{+}) T cells can also begin emerging by day 16 but large amounts of DP T cells and single positive (SP) CD8\textsuperscript{+} T cells are not present until day 20 of the co-culture. SP CD4\textsuperscript{+} T cells do not form in this system. This limitation is thought to be partially due to the lack of major
histocompatibility complex (MHC) Class II molecule on cell surface of the OP9 cells [74] that are necessary for positive selection of helper T cells (CD4⁺) [75].

**Figure 4** summarizes the key steps of the *in vitro* differentiation procedure, suggested analysis time points and lineage expectations during the time of co-culture.
**Figure 4. Diagram of the steps of the mESC-OP9 co-culture procedure.** (A) The key cell transfer steps during the first eight days of co-culture. The approximate number of cells seeded on OP9 cells at days zero and five are indicated. (B) The day 8 transfer step and the expected cellular differentiation products detected by flow cytometry at key time points of co-culture. Please see text for detailed descriptions of the immunophenotypes. (Originally published JoVE [57].)
Chapter 4: Mimicking the complexity of the TCRα/Dad1 locus by utilizing a dual-reporter BAC construct.

4.1 Construct design of wild type dual-reporter BAC

Uniquely positioned and differentially expressed genes in the TCRα/Dad1 locus call for complex and tight gene regulation that must be orchestrated during development to lead to functional rearrangement and tissue specific expression of TCRα and TCRδ genes, and ubiquitous expression of Dad1 gene.

Previously, single reporter genes have been utilized in TCRα LCR transgenic mouse studies. Originally, the 4.9-kb BglII fragment of the human β-globin (including the promoter, exons, introns and 3’ enhancer) linked to the TCRα LCR [15,19,20,25] was utilized. This reporter was replaced by cell surface-detectable ~10.5-kb SalI-BamHI fragment of genomic hCD2 reporter [5,55,56]. In addition, 6.5-kb EcoRI genomic fragment of the human leukocyte antigen-B7 (HLA-B7) reporter gene [46] was also utilized but unfortunately did not surface for antibody detection [50]. All reporters gene listed had a desired pre-requisite; poor expression without additional elements [38-40,44,46,76,77] that makes them greatly suitable for LCR studies.

Prototype of the dual-reporter BAC system [50] developed by Dr. Stefan Knirr gave expected, T cell specific expression pattern to the upstream reporter and ubiquitous expression to the downstream reporter. In addition, low (rather than high) ectopic B cell expression was seen (improvement from previous single reporter genes). Unfortunately, aberrant splicing occurred rendering the Va11.1 driven hCD2 reporter gene (c-DNA) undetectable on the cell surface [50].
Our latest model (work done with Dr. Stefan Knirr), a BAC dual-reporter gene version 2 (see material and method section) is used in this study. We are utilizing the genomic human CD2 (ghCD2) reporter gene [51] driven by a 429-bp Va17 minimal promoter sequence positioned to ~3.7-kb upstream of exon 1 of the TCRα constant region in the transcriptional orientation of the TCRα gene. The second reporter gene is a 703-bp cDNA of the rat CD2 (rCD2) gene [52] that utilizes the Dad1 promoter for its transcription (Figure 5).

**FIGURE 5. A dual-reporter BAC.** Diagram (not to scale) of the dual-reporter BAC construct for detecting the products of a Va17 promoter driven human CD2 reporter gene (Va17 ghCD2), and a Dad1 promoter-driven rat CD2 (rCD2) reporter cDNA (Dad1 rCD2) [52]. The numbered, dark boxes indicate the exons of the hCD2 reporter gene, TCRα constant region (Cα) and Dad1 gene. The ‘dot’ in Exon 5 of the hCD2 gene indicates a premature stop codon that results in production of a non-signaling hCD2 protein [51]. The light box indicates the TCRα LCR sequences. The numbered, vertical arrows indicate DNase I hypersensitive sites in the region of the LCR. Eα refers to the classical transcriptional enhancer element of the TCRα gene. The position of the FseI and NolI restriction sites used to excised the BAC reporter fragment for microinjection (or before transfection) are shown. (Originally published in Journal of Immunological Methods [56].)

### 4.2 Wild type dual reporter BAC carrying TCRα LCR exhibits integration site independent expression

Since our dual-reporter wild type BAC construct carries the full length TCRα LCR with additional surrounding sequences (5’-end reaching Jα3 proximal SacI site [53], the 3’-end ~38-kb downstream of Dad1 gene) totaling in ~77-kb, we fully expect uniform, integration site
independent expression of the upstream (TCRα reporting) hCD2 gene as per defining LCR property of position-effect suppression that was demonstrated numerous times [5,20,50,55]. The downstream (Dad1 reporting) rCD2 gene is expected to be ubiquitously active [50].

Four independent BAC transgenic mice lines (lines 36, 42, 62 and 71) carrying the TCRα/Dad1 dual-reporter BAC constructs in 4, 7, 3 and 2 copies; as well as three independent ESC clones (clones 15, 17 and 68) carrying the exact same construct but with 2, 4, 3 copies, are analyzed here by flow cytometry. All seven independent integration sites are permissive for hCD2 expression. rCD2 expression is expected rendering active Dad 1 promoter. rCD2 expression is detected in all mouse lines and clones, albeit at low levels; this may be due to cDNA rCD2 reporter gene rather than genomic version (Figure 6).

**Figure 6.** A TCRα/Dad 1 gene locus-derived dual reporter BAC construct is expressed independently of genomic integration site. Flow cytometry detection of TCRα reporter (hCD2) and Dad1 reporter (rCD2) activity. (A) CD4/8 DP T cells (dark curves) derived in vitro from three independent, BAC-transfected ESC clones. The light curves represent the signals from control, non-transfected ESR-1 cell-derived DP T cells assayed in parallel. (B) Spleen T
cells (TCRβ+) from the indicated, independent wild type (Wt) dual-reporter BAC transgenic mouse lines. Reporter gene expression in transgenic (solid line) and non-transgenic control (dashed line) cells is shown. (Panel A originally published in Journal of Immunological Methods [56].)

4.3 Tissue/cell type specificity of the Wild type BAC

Furthermore, thymic and in vitro derived T cell specific expression is expected of the hCD2 reporter gene under the influence of the TCRα LCR. rCD2 reporter gene is expected to be expressed in all tissues/organisms examined, as well as to be detectable in all phenotypic cells emerging from differentiating co-culture.

qRT-PCR analyses of the hCD2 reporter mRNA levels from organs/tissues of four WT BAC transgenic mice revealed expected tissue distribution with the maximal thymic expression (set to 100%). Very low expression (<4% of the thymic levels) was detected in the non-lymphoid organs (ranging from 1.9% in kidney to 3.8% in heart). Transgene mRNA levels were normalized to β-actin (Figure 7A). This thymus specific tissue distribution of the hCD2 transgene under the TCRα LCR influence concurs with previously published data [5,20,50].

Northern blot analyses were carried out to examine the tissue distribution of the rCD2 reporter gene. rCD2 mRNA levels were normalized to 18S ribosomal RNA signal. Figure 7B shows two representative transgenic lines (line 36 and 42) with the ubiquitous distribution of the rCD2 expression. PhosphorImager analyses of the rCD2 mRNA levels and its expression pattern in organs of four wild type BAC transgenic mice are shown in Figure 7C.
FIGURE 7. Expression pattern of tissue distribution of the dual-reporter transgene in the wild type BAC. Analyses of reporter mRNA levels in lymphoid (thymus) and non-lymphoid (kidney, lung, liver, heart) organs of Wt transgenic mice. (A) qRT-PCR analyses of human CD2 (hCD2) reporter gene (black bars) normalized to β-actin in the indicated tissues. Observed mRNA levels are expressed as a percent of that observed in thymus. The average relative mRNA levels of the four wild type BAC transgenic mouse lines are plotted. (B) Northern blot analyses of the rat CD2 (rCD2) reporter gene in indicated organs from representative Wt BAC transgenic mouse (line 36 and 42). 18S rRNA is used as a loading control. (C) PhosphorImager analyses of Northern blots assessing the rCD2 expression (white bars) in different organs of the wild type BACs (n=4). Transgene levels are normalized to 18S rRNA and expressed as a percentage of the organ with the highest level of reporter expression for each line.
Similarly, the three ESC clones differentiated in vitro to T cells show no expression of hCD2 outside of T lymphocytes (expressing DP T cell population is shown). No hCD2 expression was detected in monocytic or erythroid progenitor cells (Figure 8). On the other hand, as expected, the rCD2 expression was detected (at low but uniform levels) in all examined cells (Figure 8).

**Figure 8.** Cell type distribution of TCRα/Dad1 BAC reporter gene activity. Flow cytometry detection of TCRα reporter (hCD2) and Dad1 reporter (rCD2) in the indicated in vitro differentiation progeny of a representative dual-reporter BAC transfected ESC clone Wt68. Representative gating (shown at left) of day 12 co-cultures (for erythroid, monocytic and DN1 T cells) and day 20 co-cultures (DP T cells). Signals from the progeny of transfected cells are shown by the dark curves. The light curves represent the signals from control, non-transfected ESR-1 cell-derived progeny assayed in parallel with transfected clones. (Originally published in Journal of Immunological Methods [56]).
From the above data, we conclude that the tissue specific expression of the upstream hCD2 and ubiquitous expression of the downstream rCD2 reporter is maintained in the WT BAC, mimicking the endogenous TCRα/Dad1 expression pattern.

4.4 TCRα/Dad1 dual-reporter BAC follows correct developmental timing

See chapter 3 for detailed explanation of cell surface marker distribution. Briefly, as the early common lymphoid progenitors (CLP) seed the thymus, they undergo developmental progression through the immature stages that are marked by absence of the CD4 and CD8 cell markers and are commonly referred to as double negative (DN) cells. This developmental stage can be further divided based on expression of CD25 and CD44 molecules to DN1-DN4. TCRα genes are the last to rearrange (in doing so, the TCRδ genes embedded between the Vα and Jα segments are removed) to replace the pre-Tα surrogate leading to αβ TCRs. The rearrangement of the TCRα occurs in transition between the DN to DP stage of the T cell development [4]. We therefore expect to see up regulation of the hCD2 reporter gene at this transitional stage. Similar kinetics are shown with a single reporter gene linked to the TCRα LCR in vitro [55] and in vivo [5]. In sharp contrast to hCD2 reporter, we expect rCD2 to exhibit early onset and widespread pattern of expression (shown in figure 8), since Dad1 gene is ubiquitously expressed, with mRNA transcripts detected as early as day 6.5 of mouse embryonic development [11].

To determine the developmental timing of the onset of the reporter genes, one can isolate the small population of double negative (DN) CD4neg/CD8neg cells from the mouse thymocytes utilizing magnetically activated cell sorting (MACS), and stain for CD44 and CD25 cell surface markers that will distinguish early stages of T cell development. Alternatively, the onset of
expression can be observed in the differentiating co-culture by flow cytometry. The co-culture can be initiated from WT BAC-transfected ESC clones as described in chapter 3 or initiated from HSC-derived from the bone-marrow of the transgenic animal [54]. (See material and methods section for brief description of this procedure.)

Developmental progress of the three independent WT BAC ESC clones was monitored in the *in vitro* co-culture assay and cells collected to detect various developmental stages (FACS day 12, 16 and 20). We show the expected upregulation of the hCD2 reporter at the DN3 stage of the T cell development (Figure 9).

**Figure 9. Flow cytometry detection of the developmental timing TCRα reporter (hCD2) expression.** Analyses of differentiating T cell progeny of a representative dual-reporter BAC transfected ESC clones. Representative gating of day 12 (DN1 and DN2), day 16 (DN3) and day 20 (DP) ESC-OP9DL1 co-cultures is shown at left. Reporter hCD2 gene signals from the
indicated T cell progeny of transfected cells are shown by the dark curves. The light curves represent the signals from control, non-transfected ESR-1 cell-derived progeny assayed in parallel. (Clone WT15 was part of a figure published Journal of Immunological Methods [56].)

Similarly, in vitro differentiation of HSC obtained from bone marrow of WT BAC transgenic line 36 (and non-transgenic littermate control) on the OP9-DL1 stromal monolayer shows upregulation of the hCD2 reporter gene at the same stage (DN3) (Figure 10). This experiment was performed together with Ph.D. student, Joe Giovinazzo, rotating in our lab and sharing his expertise in bone marrow removal. Optimization of the IL-7 concentration is necessary for the bone marrow-derived HSC differentiation [54]. Standard amount of 1ng/ml of IL-7 added starting day 8 is kept constant during in vitro ESC to T cell differentiation. However, for the bone marrow derived HSC in vitro differentiation, the co-culture progress benefits greatly from a decrease of IL-7 starting day 12 [54]. Decreasing of the IL-7 concentration is necessary for more rapid progress of earlier progenitors to enter DP (CD4⁺/CD8⁺) stage of differentiation [54]. In our hands, the 0.25 ng/mL IL-7 concentration was optimal and lead to increased number of DP T cells compared to the co-culture carried with higher IL-7 concentration (Figure 10 far right).
**Figure 10. Proper TCRα-like timing of the hCD2 reporter gene is observed during T cell development in the wild type BAC.** (Top) Flow cytometric analysis of hCD2 transgene expression during thymocyte development initiated from a bone marrow of WT BAC (line 36) and non-transgenic littermate control using the in vitro OP9-DL1 differentiation system. Representative gates are shown for each collected time-point. Day 7 represents T cells in DN1 and DN2 stages of the T cell development. Day 11 revealed DN2 and DN3 and by day 21 T cells progress to various numbers of DP (CD4⁺/CD8⁺) cells depending on the concentration of IL-7 (see text). Please see text for detailed descriptions of the immunophenotypes. (Bottom) Histograms displaying hCD2 reporter gene expression during DN1 to DP stage transition of developing T cell from the wild type BAC (dark curve) and its non-transgenic littermate control (light curve).

4.5 High expression of hCD2 reporter in BAC is comparable in thymic and peripheral T cells

Size is the one great advantage and disadvantage of the BAC constructs. Since BACs can accommodate large portions of DNA, we were able to include a large portion of the TCRα/Dad1 locus. However, this makes BACs hard to manipulate. Previously, in peripheral T cells, lower than expected levels of simple TCRα-LCR driven reporter gene expression were detected.
(ranging from 15-46% of thymic expression levels), whereas, the endogenous TCRα mRNA levels were upregulated in peripheral T cells [5]. Since two different reporters were utilized (β-globin and hCD2), an argument was made against reporter gene-specific effects. It was thought that other elements, in the wider TCRα locus, are responsible for supporting the highest-level peripheral TCRα expression [5].

Utilizing our WT BAC that spans from the extreme 3’- Jα region to approximately 38-kb downstream of the Dad1 exons, we took the opportunity to return to the question of the peripheral T cell expression. We compared hCD2 expression levels in thymocytes and purified spleen T cells of the four WT BAC transgenic mice. qRT-PCR analyses show that the β-actin normalized hCD2 expression level of peripheral T cells were comparable to thymic levels. On average, ~ 71% of thymic levels were detected in spleen T cells (Figure 11). This level of peripheral T cell expression of hCD2 from the BAC transgene (relative to the thymic levels) seems to be improved over that seen from TCRα-LCR driven transgenes [5]. However, it is necessary to point out that direct comparisons of hCD2 reporter gene expression from the BAC construct and the TCRα-LCR driven transgenes have not yet been made.
**Figure 11.** Reporter and endogenous TCRα mRNA levels in thymic and peripheral T cells. (Left) qRT-PCR analyses of mRNA levels of (A) hCD2 reporter gene and (B) endogenous TCRα gene in thymus (black bars) and purified spleen T cells (white bars) of indicated transgenic BAC mice. Signals normalized to β-actin. Expression is graphed within each line as a percentage of the thymic expression (set to 1.0). (Right) Graph on the right represents averaged values from four BAC transgenic lines. Statistical analyses of the data performed by using the two-tailed student’s t-test. No significant difference in (A) hCD2 or (B) endogenous TCRα mRNA levels between thymus and spleen T cells is detected (p=0.115) and (p=0.2106), respectively.

The endogenous TCRα expression in peripheral T cells, although not significantly higher than thymic TCRα mRNA levels, reaches, on average, above the thymic levels, whereas peripheral T cell expression of the transgene does not. Although we can’t rule out that the BAC is missing some additional, yet unidentified, peripheral T cell “specific” cis-element(s) from the ~1.6-Mb wide TCRα/δ locus [78], we currently do not favor this hypothesis. First, our WT BAC spans to Jα2. Upon rearrangement of the most V-distal Jα region (Jα2) [79,80], the sequence upstream of Jα2 is removed via recombination, yet the TCRα is efficiently transcribed; therefore any indispensable element should be present downstream of Jα2 [53]. Second, a Rec-HY transgenic
model closely mimics endogenous TCRα expression pattern including the periphery [81]. This construct utilized modified pTαCass [53] vector, therefore includes equal sequence downstream of Jα2, containing the entire TCR Cα region and full TCRα LCR (same as our WT BAC). Contrary to our original hypothesis, the added intronic Jα2-Cα region, although clearly functional in peripheral T cell expression (see chapter 6), does not seem differentially contribute to peripheral vs. thymic expression. Our preferred hypothesis involves differential post-transcription regulation of the TCRα gene in thymus and periphery. Post-transcription regulation occurs by short, cis-acting RNA elements, likely located in the 5’UTRs or 3’UTRs. Such elements add additional complexity in regulation of gene expression by controlling the stability of the mRNA [82]. Small, noncoding RNAs termed microRNAs (miRNAs) are able to target their complementary sequences and alter expression of the gene. Therefore, if the endogenous TCRα gene and hCD2 transgene are subject to different post-transcription regulation, we might be detecting these small differences. However, this hypothesis needs further investigation.

In summary, our dual-reporter WT BAC shows appropriate spatiotemporal, TCRα-like expression kinetics and ubiquitous, Dad1-like expression for its upstream and downstream reporter, respectively, making it an ideal model for investigating the role of HS1’ in TCRα and Dad1 expression.
Chapter 5: Non-redundant role of HS1’ in thymic and peripheral expression of the TCRα gene

5.1 Introducing the HS1’ site of the TCRα LCR

HS1’ is located 3’ of the classical enhancer Eα and the fine mapping positioned the HS1’ site 3’ of the BglII restriction site [20]. It does not display any enhancer activity as documented by transient transfection experiments [16] and forms in both lymphoid and non-lymphoid organs [17,20]. Transgenic mice analyses implicate the HS1’ region in maintaining the tissue-differential chromatin structure (preferential, strong HS6 formation in thymus) and in counteracting the wide chromatin-opening capacity of the 3’-end of the TCRα LCR located in HS2-6 region, mainly in HS6 [17,20,22]. HS1’ is thought to possess tissue specificity that allows for lymphoid-specific expression of the TCRα gene [15,20].

In 1997, the HS1’ region was included in the TCRα enhancer knock out (EαKO) mice that were created to address function of the Eα in the regulation of the TCRα gene rearrangement and expression [83]. The targeted region consisted of Eα enhancer and the neighboring cluster of DNaseI HS, named HS1’ region [15,20]. This targeted deletion of the HS1 (Eα) and HS1’ sequences led to developmental block in thymocytes at the DP stage. This block is not complete since small numbers (~20-fold reduction compare to wild type) of αβ T cells appear in periphery. The repertoire of these cells is limited, utilizing mainly members of Va2 family [83]. Furthermore, the deleted region is important for germline transcription from the T early alpha
(TEA) promoter, Vα-Jα rearrangement and its transcription and proper expression of rearranged TCRδ transcripts in γδ T cells [83].

The attempt to rescue the severe phenotype of the EaKO mice was made by returning sequence of the murine Ea core back to the original EaKO allele in Ea core knock in mice (EaCRKI) [84]. Ea core sequence was chosen based on the homology to the human 116-bp (BstXI to DraI) Ea sequence [85] rather than the somewhat larger 226-bp (PvuII to BglII) originally defined murine Ea [16]. Results showed slightly enhanced Vα-Jα rearrangement, slightly elevated usage of the 5’ Vα gene segments but overall, EaCRKI mice do not show any significant improvement over the original EaKO (HS1 and HS1’) phenotype [84]. This strongly argues for a great significance of the HS1’ region.

In order to examine the non-redundant role of the HS1’ region we made a clean, internal deletion of the HS1’ site in our dual-reporter BAC (Figure 12) allowing us to determine the role HS1’ plays in TCRα expression as well as in Dad1 regulation. The TCRα LCR’s influence on the regulation of Dad1 gene has not yet been determined, but HS1’ site of the TCRα LCR is a suitable candidate for its role in Dad1 regulation for the following reasons: its lies on the cusp of the 5’-end (tissue specificity) [20] and 3’-end (suppression position effect/chromatin opening capacity) [22]; it forms in both lymphoid and non-lymphoid organs [17,20]; and the unusual arrangement of the non-homologous genes (TCRα vs. Dad1) is evolutionarily conserved (human, mouse, chicken) [78]. Furthermore, both TCRα and Dad1 have regulated gene expression during the course of the T cell development, with highest expression levels occurring at the SP stage of T cells [86].
5.2 The mutant delta HS1’ dual-reporter BAC

In this study, we are utilizing a mutant version of our wild type BAC that has a internal deletion of 826-bp between the BgII and BamHI restriction sites, removing the HS1’ site of the TCRα LCR (Figure 12).

Figure 12. Diagram (drawn not to scale) of the TCRα/Dad1 gene locus-derived dual reporter BAC construct with HS1’ deletion. Two reporter genes in opposite transcriptional orientations (indicated by horizontal arrows), genomic human CD2 (ghCD2) and rat CD2 (rCD2) are driven by the cognate Vα17 and Dad1 promoters, respectively. Individual exons of the ghCD2 reporter gene, TCRα constant region (Cα) and Dad1 gene are numbered and depicted as dark boxes in the diagram. The large, light box represents the TCRα LCR sequences consisting of nine DNaseI hypersensitive sites (HS). The numbered, vertical arrows mark positions of individual HS of the TCRα LCR, namely HS 1-8. HS1 is the classical transcriptional enhancer element of the TCRα gene, Eα. 3’ of the Eα is HS1 prime (HS1’). Wild type and mutant delta HS1’ version were created. BgII and BamHI restriction sites mark the borders of removed sequence in the HS1’ BAC mutant. The positions of the FseI and NotI restriction sites used to excise the BAC reporter fragments for microinjection are also shown in the diagram.

Initially, four wild type BACs and four mutant dHS1’ BAC transgenic lines were available. However, two dHS1’ BAC founders failed to breed and left us with only two established mutant lines. We are confident that our results are a representative sample since the initial flow cytometry analyses of all four mutant dHS1’ lines are in agreement.
5.3 hCD2 reporter gene expression is impaired in the absence of HS1’ site

Flow cytometry analysis was used to detect cell surface expression of the hCD2 reporter gene in thymic cells from wild type and mutant dHS1’ BAC transgenic mice. The mean fluorescence intensity (MFI) was used as a measure of the reporter gene expression. Thymic cells were gated to the DP (CD4+/CD8+) population and MFI values for hCD2 reporter were divided by copy number and normalized to MFI of the CD4+ cells. All four dHS1’ mutant BAC transgenic lines 5F, 20, 24F and 38 (copy number 7, 2, 15 and 3) showed copy number dependent hCD2 expression. The raw hCD2 MFI values per copy were within narrow 1.7 fold difference (data not shown). Removal of HS1’ region leads to reduced levels of hCD2 expression (Figure 13). On average, only 30% of the wild type levels (per copy) are observed in dHS1’ mutant.

**Figure 13.** Impaired hCD2 reporter expression is evident in four, independent HS1’ mutant BAC transgenic mouse lines. Mean fluorescence intensity (MFI) of hCD2 reporter (per transgene copy) in gated DP thymocytes of wild type (black bars) and dHS1’ mutant (white bars) BAC transgenics. Per copy reporter MFI was normalized to MFI of the CD4 and graphed as percent of the maximal value. Two of the four mutant lines (M5F and M24F) are analyzed founders that failed to establish transgenic line.
Since LCRs are active at the transcriptional level, Northern blot and/or qRT-PCR assays are more suitable methods, and are therefore employed for further analyses. We used both methods to examine the effect of the HS1’ deletion on the expression levels of our reporter genes by comparing mutant levels to wild type in thymus (Figure 14) and peripheral T cells isolated from spleen (Figure 15).

In thymus, using Northern blot and PhoshorImager analyses, detected hCD2 and rCD2 mRNA levels are normalized to the 18S rRNA levels, divided by the estimated relative copy number and graphed as percentage of the highest expressing line. We detected a significant decrease of the hCD2 reporter mRNA levels (per transgene copy) in mutant dHS1’ BAC that are on average under 40% of the wild type BAC levels (2.6-fold lower). The second reporter gene, rCD2 is not influenced by the deletion (Figure 14).
**Figure 14.** Impaired hCD2 reporter expression in thymocytes of HS1’ mutant BAC transgenic mice. (A) Northern blot analyses detecting the hCD2 and rCD2 reporter transgene expression in thymus of multiple transgenic lines bearing either the wild type (n=4) or delta HS1’ (n=2) BAC constructs. The non-transgenic littermate (ntg) control is included. Reporter gene signal is normalized to 18S rRNA. Relative copy numbers of the individual transgenic
mouse lines are indicated. (B&C) PhosphorImager analysis of Northern blot experiments in A. The normalized, per copy mRNA levels of (B) hCD2 and (C) rCD2 reporter gene from wild type (black bars) and HS1’ mutant (white bars) BAC are graphed as percentage of maximal expression. (D) Statistical analyses of the above data using the two-tailed student’s t-test. The asterisk indicates the statistical significance (p=0.0265) of the difference in hCD2 reporter expression between the wild type and mutant. Whereas, no significant difference (p=0.44) in rCD2 reporter expression was detected.

qRT-PCR analyses were utilized to determine the role HS1’ region plays in peripheral T cells. MACS purified spleen T cells (purity >92%) were used for these analyses. We employed endogenous mRNA expression levels of TCRα and Dad1 genes as our normalization controls for the hCD2 and rCD2 reporter, respectively. Results are graphed in Figure 15 and show that average transgene mRNA levels (per copy) of the upstream reporter (hCD2) are reduced over 3.2-fold from the wild type BAC levels when the HS1’ region is removed. Once again, removing HS1’ doesn’t alter the levels of the rCD2 reporter mRNA as assayed per transgene copy.
Figure 15. Peripheral T cells exhibit reduced hCD2 transgene expression in HS1’ mutants. qRT-PCR analyses of (A) human and (B) rat CD2 reporter transgene expression in purified spleen T (SpT) cells isolated from wild type (black bars) and HS1’ mutant (white bars) BAC transgenic mice. Per copy and normalized transgene mRNA levels are graphed relative to the highest expressing line (as % maximum). The endogenous TCRα and Dad1 mRNA levels were used as normalizers for the hCD2 and rCD2 reporter mRNA levels, respectively. Three experiments performed in duplicates are presented. (C) Statistical analyses of the above data using the two-tailed student’s t-test. Statistical significance of the difference in hCD2 mRNA levels between wild type and HS1’ mutant BAC is marked by asterisk (p=0.027). In contrast, no significant difference in rCD2 mRNA levels was detected (p=0.79).
In summary, from our *in vivo* analyses of the dual-reporter BAC transgenic mice carrying the HS1’ region deletion, we report that deletion has effects upstream but not downstream, impairing the upstream hCD2 transgene activity in thymic and peripheral T cells but does not have a direct role in Dad1 regulation.

5.4 Role of HS1’ in tissue specific expression in the context of the TCRα/Dad1 BAC

Endogenous expression of the TCRα gene is highly tissue specific, as is the hCD2 reporter gene mRNA production under the control of the TCRα LCR [5,50]. In order to determine the role of the HS1’ in tissue specificity in the context of the TCRα/Dad1 BAC, we compared mRNA samples from lymphoid (thymus) and non-lymphoid tissue/organs of wild type and mutant BAC transgenic mouse lines on Northern blots (expression of the rCD2 reporter c-DNA) or assayed by qRT-PCR (ghCD2 expression).

Both wild type and dHS1’ BACs displayed the wild type tissue distribution of hCD2 mRNA production, with highest expression in thymus and very low expression in non-lymphoid organs, under 5% of the thymic expression (**Figure 16A**). The highest expression among the non-lymphoid representatives was observed in liver of the HS1’ BAC mutant, however its value was merely 4.1% of thymic expression levels, it is well within the expected values. To our surprise, we have not observed increased expression of the reporter gene in heart (or as a matter of fact, any other examined organ) as was previously reported for single reporter transgenic analyses [15]. It is possible that previously observed phenotypes resulted from “reverse” synergy of multiple removed elements (specifically HS7, 8, Eα and HS1’) of the TCRα LCR. Present
studies examine clean, uni-site deletion, leaving rest of the locus intact. However, this discrepancy is most likely due to difference in reporter gene usage (β-globin vs. hCD2) and/or choice of transgenic cassette (single reporter vs. BAC). Furthermore, transgenic analyses of the β-globin transgene under influence of the HS2-6 elements from the TCRα LCR indicated more favorable environment for the transgene expression in heart [20].

Positioning the rat CD2 reporter gene into the 1st exon of the Dad1 gene [50], allowed us to determine any influence of the deleted region on Dad1 gene expression. Rat CD2 mRNA was detected on Northern blot in all examined organs (Figure 16B), displaying a similar pattern of ubiquitous expression in both wild type and mutant transgenic lines. Therefore, the transgene expression pattern in mutant does not deviate from the consistent transgene expression pattern of the wild type.
**Figure 16.** Tissue distribution of hCD2 and rCD2 transgene expression remains unaltered in HS1’ mutant. (A) qRT-PCR analysis of hCD2 transgene expression in the indicated organs/tissue (thymus, kidney, lung, liver and heart). Averaged, β-actin normalized hCD2 transgene mRNA levels are graphed as percentage of thymic expression (set to 1.00) in wild type (black bars) n=4 and HS1’ mutant (white bars) n=2 BAC transgenic mouse lines. (B) PhosphorImager analyses of the rCD2 reporter mRNA signals from the indicated tissue/organ detected on the Northern blot. Rat CD2 transgene levels are normalized to 18S rRNA and expressed as a percentage of the organ with the highest level of reporter expression (% maximum) within each transgenic line.
5.5 Developmental timing of the dHS1’ BAC

We have not yet directly examined the onset of the hCD2 reporter gene in mutant dHS1’ BAC transgenic mice. We did, however address this question using dHS1’ BAC ESC (clone D10). This mutant ESC clone did not deviate in the onset of the hCD2 reporter gene expression from its wild type counterpart. The onset of hCD2 expression was observed at the DN3 stage (data not shown). Unfortunately, more positive dHS1’ BAC ESC clones with intact transgenes were not generated, as the size of the BAC and the difficulties in its manipulation re-surfaces.

To overcome this obstacle, we utilized simpler and shorter transgene cassettes. The hCD2:1-8 construct was previously described in both in vivo [5] and in vitro [55] studies. As a matter of fact, this particular construct was the one that was validated in the in vitro ESC differentiation system for LCRs studies [55]. Therefore, control ESC clones carrying the hCD2:1-8 transgene, generated by Dr. Armin Lahiji were ready for immediate use. We created constructs that had deletion of the HS1’ region (Figure 17) and established independent ESC clones (see chapter 2).
**Figure 17. Diagram of single reporter transgene constructs used in vitro ESC differentiation experiments.** Scaled diagram of the hCD2:1-8 and hCD2:1-8dHS1’ transgene constructs. The hCD2 reporter gene fragment is positioned 5’ of the TCRα LCR. White box represents the Eα enhancer. Dark boxes represent individual exons of the hCD2 gene. Exon V of the hCD2 gene has a premature stop codon (marked by x) that results in non-signaling CD2 molecule [5,51]. Horizontal arrow indicates the transcriptional orientation of the hCD2 reporter gene. The vertical arrows mark individual hypersensitive sites (HS) consisting the TCRα LCR. Full length TCRα LCR (all 9 HSs) or mutant delta HS1’ constructs were used. Deletion of 826-bp sequence (HS1’ region) between BglII and BamHI is marked by breakpoint in continuity of line within the TCRα LCR.

Seven hCD2:1-8dHS1’ ESC clones were *in vitro* differentiated alongside a non-transfected ESR1 (negative control) and wild type hCD2:1-8 control (clones FA1, FB5 and FD6 [55]. All seven clones showed no hCD2 expression in monocytic or erythroid cells (Figure 18 and data not shown) as expected. Six out of seven clones display TCRα-like expression of the reporter gene with expression onset at the DN3 stage of the T cell development (Figure 18 and data not shown). We have obtained one ESC clone (N3) that exhibits aberrant, early onset of the hCD2 reporter gene expression at DN1 stage. Since this phenotype occurred only in a single clone, we believe that this particular clone is experiencing a positive position effect at the site of integration. The endogenous hCD2 gene (under control of its own regulatory elements) starts expression at DN1 stage [87].
**Figure 18.** Flow cytometry analyses of copy number matched ESC clones during *in vitro* ESC differentiation. hCD2 reporter transgene activity of hCD2:1-8 construct (clone FB5, FA1) [55] and mutant hCD2:1-8ΔHS1' construct (clones N1, N3 and L2) are shown. FB5 and N1, N3 carry 2 copies (left); FA1 and L2 are a single copy integrants (right). Representative gating is shown far left. Cells collected on day 12, 16 and 20 of co-culture. hCD2 reporter gene expression in transfected (dark line) and non-transfected (light line) *in vitro* derived cells is shown in each histogram.
In summary, neither the *in vivo* nor *in vitro* analyses of constructs with HS1’ deletion revealed aberrant tissue distribution of the hCD2 reporter gene expression. The transfected ESC clones we have in hand at present only enable *in vitro* comparisons of spatiotemporal expression patterns between the wild type and HS1’ mutant reporter transgene constructs. Further quantitative analyses comparing the expression level of the reporter gene (per transgene copy) in the wild type and dHS1’ ESC clones will require creation of additional wild type ESC clones with higher copy numbers that better match the range obtained for the mutant ESC clones. This is needed to rule out the possibility that transgene expression becomes saturated at high copy in our *in vitro* system.
6.1 Introduction

The great diversity of TCRs is achieved through the V(D)J recombination process that utilizes multiple variable (V), diversity (D) and joining (J) gene segments of the TCRβ and TCRα loci, residing on mouse chromosomes 6 and 14, respectively. Alpha and beta chains combine to form complete mature αβ T cell receptors (TCRs) that the vast majority of T cells carry. The TCRα genes share their locus with TCRδ genes that are embedded between the Vα and Jα segments. Upon rearrangement, TCRδ segments are deleted. The complex TCRα/δ gene locus is tightly cis-regulated by the elements within the TCRα Locus control region (LCR) located 3’ of the constant region (Cα). LCRs are thought to act on the chromatin level to achieve the appropriate expression mode employing epigenetic markings [21,29,88].

HS1 and HS1’ regions are key players to achieve enhanced T cell specific transcription of the TCRα gene [15,83] and to regulate the V(D)J recombination, transcription of the germline sequences and of the rearranged genes [83]. Removal of the HS1 and HS1’ sites has a hard impact on these processes as discussed in chapter 5. However, limited TCRα gene recombination is still observed even without the Ea/HS1’ elements. Peripheral populations of αβ T cells, though of limited antigenic variety, are observed. Expression of the mature TCRα transcripts is lowered but present [83]. Therefore as suggested, other elements of the TCRα/δ locus are responsible for allowing accessibility and transcriptional activation. Enhancer Eδ must be excluded from the potential pool of elements since it’s excised upon Vα-Jα rearrangement as mention above.
Since the TCRα LCR consists of 9 HS sites (see introduction), the remaining elements might have been possible candidates. However, wider search for appropriate candidate is necessary since it has been documented that although powerful in bring TCRα-like expression to the heterologous transgenes in thymus [5,55] it’s lacking in periphery [5]. Therefore, a wider scope in the search for additional elements is necessary.

6.2 Multiple DNaseI hypersensitive sites are detected 3’ of Jα2

In rearranging thymocytes, the most Vα-distal functional joining (J) region that undergoes rearrangement is Jα2 [79,80]. Upon V-J rearrangement of the TCRα gene, sequences 5’ of the Jα2 are subject to deletion. Therefore, regulatory regions required for proper TCRα transcription should reside downstream of Jα2 [53]. To identify potential regulatory DNA within this region, we performed DNaseI hypersensitivity assays (DHA) on isolated nuclei from purified spleen T cells. A cluster of hypersensitive sites (HS) was revealed in a DNA region stretching from Jα2 to Cα1 (Figure 19). The most prominent of these is located near the Jα1 pseudogene (HS-J1). Flanking HS-J1 are four weaker HS. Two of these approximately map to the Jα2-Jα1 region. The third HS lies within the Jα1-Cα1 intron and the fourth localizes to the Cα1 exon (Figure 19 bottom). Since the Jα1 region is a non-functional pseudogene and is not transcribed due to defective DNA recombination signals and RNA splicing signals [79], the detected array of active chromatin would remain in all functionally rearranged TCRα genes.
**Figure 19. DNase I hypersensitivity sites (HS) within the Jα2 to Cα1 region.** (A) Spleen T cell nuclei from C57BL/6N mouse were subjected to DNasel titration. Arrows indicate the positions of the 6.7-kb NdeI parent fragment and HS. Results from two independent, representative experiments are shown. (B) Scaled diagram of the approximate locations of detected HS (arrows). The thick arrow near Jα1 indicates the most prominent HS.

6.3 TCRα/Dad1 bacterial artificial chromosome (BAC) with dual-reporter transgenes for analyses of functionality of HS sites cluster

To assess the contribution of the deleted sequence to the expression of the TCRα and Dad1 genes, we created a mutant version of our wild type dual-reporter TCRα/Dad1 BAC construct (WT described in chapter 3). The mutant BAC (named Va17ghCD2_rCD2_ΔJα3-Cα1) is missing ~3.9-kb sequence (~SacI to EcoRV, covering region from upstream of Jα3 to Cα1) that includes the five HS identified in the cluster (Figure 20). Independent transgenic mouse lines of ΔJα3-Cα1 mutant BAC were obtained.
**Figure 20. Diagram (not to scale) of the ΔJα3-Cα1 mutant TCRα/Dad1 dual-reporter BAC construct.** Horizontal arrows indicate the orientation of the two reporter genes. A Vα17 promoter drives expression of a genomic human CD2 reporter gene (ghCD2). The rat CD2 reporter is driven by the Dad1 promoter. Vertical arrows indicate the location of the HS of the TCRα LCR (including the TCRα enhancer, Eα). The 3.9-kb region deletion in the mutant BAC is marked and runs from 38-bp 5’ of the SaeI site through 30-bp 5’ of the end of Cα exon 1.

### 6.4 Flow cytometry detection of phenotypic differences in reporter gene expression between wild type and ΔJα3-Cα1 mutant BAC transgenic T cells

Flow cytometry analyses were used to detect cell surface expression of both human and rat CD2 reporter genes in T cells from wild type and mutant BAC transgenic mouse lines. Four wild type and four mutant lines were assayed on per cell bases. Expression of both reporters was observed in all transgenic mouse lines (Figure 21). However, hCD2 reporter expression seemed impaired in T cells from the mutant BAC transgenic lines (Figure 21 3rd column from left). The observed hCD2 expression levels on a per cell basis variegated in the mutant BACs, whereas the wild type BACs showed tighter, uniform per cell hCD2 expression. The expression of the rCD2 reporter gene was detected at low but uniform levels, indicating no involvement of the deleted region in the Dad1 expression. To further investigate the severity of the observed variegated expression phenotype of the hCD2 reporter gene at the mRNA level, additional quantitative analyses were performed and transgene expression assayed on per copy basis.
**Figure 21. Impaired hCD2 reporter expression in the absence of the deleted region.** Flow cytometry analyses of human CD2 (hCD2) and rat CD2 (rCD2) reporter gene expression in spleen T cells (TCRβ⁺) from the indicated, independent wild type (Wt) and mutant (Mt) dual-reporter BAC transgenic mouse lines. Reporter gene expression in transgenic (solid line) and non-transgenic control (dashed line) cells is shown.
6.5 Deletion of the Jα3-Cα1 region impairs Vα promoter-driven hCD2 reporter gene expression in thymocytes and spleen T cells

We compared reporter gene mRNA expression levels observed in T cells (both thymic and peripheral) isolated from four wild type and four Jα3-Cα1 mutant BAC transgenic lines using Northern blot (Figure 22) and qRT-PCR analyses (Figure 23).

Human and rat CD2 mRNA levels were normalized to 18S ribosomal RNA and quantified by the PhosphorImager. We detected decreased mRNA expression levels (per transgene copy) of the hCD2 reporter in the mutant BAC transgenic lines that was on average 3.3 times lower than the counterpart wild type (Figure 22). In contrast, the rCD2 reporter mRNA expression levels (per transgene copy) appear unaffected.
**FIGURE 22. Absence of the deleted region impairs upstream, but not downstream reporter gene expression in thymocytes.** (A) Northern blot analyses of human and rat CD2 reporter gene mRNA levels in thymocytes from the indicated lines of wild type and mutant reporter BAC transgenic mice. 18S rRNA signals are used as a loading control. Relative transgene copy number for each mouse line is indicated. Panels B and C depict PhosphorImager analyses of the human CD2 (B) and rat CD2 (C) reporter mRNA signals detected by northern blots. The normalized mRNA levels (per transgene copy) from each wild type (black bars) and mutant (white bars) transgenic mouse line are graphed relative to each other (as % maximum). (D) Statistical analyses of the above data using the two-tailed student’s t-test. The asterisk indicates the statistical significance of the difference in hCD2 mRNA levels between wild type and mutant BAC (p=0.012). In contrast, no significant difference in rCD2 mRNA levels was detected (p=0.942).
Similar results were obtained from mRNA analyses performed on the purified spleen T cells (Figure 23). Levels of hCD2 mRNA (per copy of the transgene) in the mutant ΔJα3-Cα1 BAC were on average 4.9 times lower than the wild type. The mRNA expression levels (per transgene copy) of the second reporter gene rCD2, were not affected by the deleted region. These data indicate that the deleted Jα3-Cα1 sequence plays a role in TCRα gene regulation in thymus as well as in periphery.
**Figure 23. The deleted region is functional in peripheral T cells.** qRT-PCR analyses of human (A) and rat (B) CD2 reporter gene mRNA levels in isolated spleen T cells (SpT) from the indicated lines of wild type (black bars) and mutant (white bars) transgenic mice. Observed reporter mRNA levels per copy from each transgenic line are graphed relative to each other (as % maximum). hCD2 reporter mRNA levels were normalized to endogenous TCRα mRNA levels, and rCD2 expression were normalized to endogenous Dad1 mRNA levels using primers that detect sequences not present in the reporter BAC. Three experiments were performed in duplicates. (C) Statistical analyses of the above data using the two-tailed student’s t-test. The asterisk indicates the statistical significance of the difference in hCD2 mRNA levels between wild type and mutant BAC (p=0.0159). In contrast, no significant difference in rCD2 mRNA levels was detected (p=0.8188).
Chapter 7: Discussion and Significance

7.1 Role of HS1' in the TCRα locus

HS1’ differs from HS1 (Eα). The latter exhibits classical enhancer activity [16], whereas the former does not and is thought to function in chromosomal context, in that it restricts the open chromatin conformation of the TCRα locus to the lymphoid organs [15]. Yet, much of the information accumulated comes from data where both elements were included (as discussed in detail in chapter 5). Moreover, deletion analysis of TCRα LCR (progressing from the 5’ to 3’ and removing HS7, HS8; HS1; and finally HS1’) in transgenic mice characterizes HS1’ function to be of great importance for high-level, lymphoid-specific gene expression [15]. Since both HS1 and HS1’ were removed, these analyses resulted in loss of copy number dependence (Eα removal) and very low levels of reporter expression in thymus, reaching only <1% of endogenous TCRα signal for two-single copy transgenic mouse lines; 14-60% for the multicopy lines [15]. Therefore, our dual-BAC model provided the opportunity to characterize the HS1’ site in a non-redundant fashion. Furthermore, including reporter genes on either side of the TCRα LCR, thereby mimicking the native locus, gave us the opportunity to determine the role of HS1’ in Dad1 expression.

Our dHS1’ BAC transgenic mice allowed us to examined the non-redundant role of HS1’ region in transcriptional regulation of the TCRα locus. We have shown that deletion of the HS1’ region leads to lower expression of the TCRα reporter (reduction 2.6-fold in thymus and 3.2-fold in peripheral T cells). While markedly decreased expression of the TCRα reporter was observed,
tissue specificity and integration site independence remained unaltered. While these data clearly
demonstrate the non-redundant, and significant, function of the HS1’ region, it is surprising,
given prior data, that the transcription deficits in the absence of HS1’ are not more severe.

Previous studies demonstrated the significant importance of the 1.15-kb (*Pvu*II to *Bam*HI) region
(that includes both HS1 and HS1’ sites) for T cell development progression, rearrangement and
TCRα expression (germline as well as mature transcripts) [83]. Specifically, Northern blot
analyses of total RNA from the HS1/HS1’ KO thymocytes failed to detect germline transcripts
initiated from the TEA as well as Cα-hybridizing transcripts. Mature transcripts (from spleen)
were detected but were about 6-fold lower than the wild type [83]. Since these EaKO studies
lacked both elements, it was impossible to discern which HS site is responsible for the defects.
Returning the 116-bp murine Eα core region to the EaKO allele, approximated HS1’ deletion in
the Eα core knock in mice (EαCRKI) [84]. This allele also bears an additional deletion of ~110-
bp of the non-core Eα region. Since EαCRKI mice show only slight improvement (in an
increased utilization repertoire of variable gene segments, mainly from the Vα2 family [84]),
over the defects seen in EaKO, the apparent importance of the HS1’ region and/or the remaining
~110-bp of the non-core Eα region emerges. Since we examined the effects of the removal of the
HS1’ region alone in BAC transgenic mice, and did not observe as dramatic an impairment as
prior data predicted, our data points into a new direction and calls for further dissection of the
potential role of 110-bp large sequence outside of the murine Eα core in TCRα gene regulation.

Furthermore, although our BAC study did not address rearrangement per se, HS1’ region and the
CTCF site residing in emerges as crucial for rearrangement of the TCRα locus, and the
mechanism is described in the below paragraphs.
HS1’ has CTCF-dependent enhancer blocking insulator activity [18]. CTCF is a ubiquitously expressed zinc-finger protein, involved in looping and long-range chromosomal interactions; a common theme involved in insulation [89]. Co-localization of cohesin and CTCF has been shown in many genomic sites [90]. Indeed, cohesin colocalizes with CTCF present in the HS1’ site as well [91,92]. Cohesin, originally known for its role in keeping sister chromatids together during cell division [93], was also shown to play a role in rearrangement of the TCRα locus [91,92,94]. Cohesin bindings sites were found in TEA, multiple V gene promoters and at Dad1 gene. In addition to binding to CTCF site in HS1’, cohesin has also been found to bind in a region between TCRα and Dad1 genes [92], where CTCF-independent enhancer-blocking activity was found (HS2-6 region) [18]. Binding of cohesin in these two insulator regions signifies its importance and calls for further exploration of its role in this locus. In order to understand the role of CTCF and cohesin, one must appreciate the fact that although CTCF binding can be controlled by DNA methylation status [95], CTCF is ubiquitously expressed and therefore not stage-specific or cell/tissue specific.

Cohesin, which displays a more stage-specific binding pattern [96] is most likely responsible for the developmental stage-specific contraction of the TCRα locus as shown by 3D-FISH analyses [31,33].

The role of cohesin in TCRα rearrangement was addressed in an elegant experiment, where Rad 21 (major subunit of cohesin) was conditionally deleted at the DP stage of T cell development. T cells pause their cell cycle at the DP stage in order to undergo TCRα chain rearrangement, therefore deletion of cohesin at this stage allowed for separation of its role in cell division from that in rearrangement [92]. The results of these experiments added to the existing evidence for
role of CTCF and cohesin in TCRα rearrangement.

Cohesin-deficient T cells showed reduction of germline Jα transcription that in turn led to reduction of histone H3 lysine 4 trimethylation (H3K4me3) mark [92]. Methylation marks can be deposited to the J arrays of TCRα locus during germline transcription initiated at TEA since histone methyltransferases can travel with elongating RNA Pol II complex [97]. Germline transcription and trimethylation of H3K4 has been shown to induce accessibility of the TCRα/δ locus to the recombination machinery [98]. RAG2 binds to H3K4me3 [99], hence decrease in H3K4me3 leads to reduced recruitment of RAG2 and impairment of rearrangement. Therefore, CTCF and cohesin control contraction of the TCRα locus, that in linear DNA sequence would be too far apart, allows for orderly rearrangement.

Location of HS1’ between the 5’ tissue specific and 3’ chromatin opening sub-elements of this locus, presence of CTCF and cohesin sites, and enhancer blocking activity points to the role of HS1’ in prevention of inappropriate cross talk between multiple elements of the complex TCRα/δ/Dad 1 locus. However, it is not clear yet how and what individual elements of the TCRα LCR contribute in insulating the two differentially expressed genes residing on either side. We hypothesized the role of HS1’ in regulation of Dad1 gene (see chapter 5). However, our results did not show a difference in rCD2 expression (at the position of Dad1) upon removal of HS1’. One possible reason for our results is that there may be redundancy in this locus since both genes play a vital role.

It has been shown that deletion of cohesin results in increased readout of Dad1 and Cδ transcript at the expense of Cα transcript [92]. Therefore, the functional separation between TCRα and Dad1 genes was lost in cohesin depleted T cells [91,92]. Since our BAC investigated the HS1’
role in Dad1 regulation and no increased expression of rCD2 reporter (Dad1) was found, this observed increase of Dad1 transcripts must be due to lost boundary other than HS1’; therefore, other site(s) in the HS2-6 region are responsible for this insulator function. Two additional sites, HS4 and HS6 showed dependence on HS1’, in that HS1’ was necessary for lymphoid specific hypomethylation of HS4 [21] and for the occupancy of tissue specific factors in HS6 [22] are strong candidates for additional function. Cohesin is required for formation of this boundary that limits Eα enhancer activity to TCRα locus in DP T cells [91,92].

**Figure 24.** HS1’ is not required for Dad1 regulation. In the absence of HS1’ site (dHS1’ BAC construct), no altered rCD2 (Dad 1) is observed. Therefore, other element(s) within the HS2-6 are capable of insulator (enhancer blocker) function.

Dad1 gene expression is upregulated at the SP stage of the T cell development [86], as is the endogenous expression of TCRα. It has been also indicated that N-linked glycosylation is necessary for optimal cell surface expression of TCRα [100]. However, due to the relevance of apoptosis during T cell development, the upregulation of Dad1 (anti-apoptotic gene) at the SP T cell stage (after positive selection that removes majority of unsuitable thymocytes) might be under the control of the TCRα LCR. It would be interesting to investigate the element(s) of the TCRα LCR that (and in what combination) are important players in insulating the expression of these two genes in a developmentally orchestrated manner.
7.2 Novel TCRα gene cis-regulatory region

Utilizing the DNaseI hypersensitivity assay, we have identified region of active chromatin 5’ of the Cα constant region. The identified cluster comprises of a few HS sites with the most dominant site adjacent to the non-functional pseudogene Ja1 [79,80]. Previous studies indicated presence of additional element(s) to the Eα and HS1’ regions in the TCRα/δ locus that would be able to facilitate accessibility and transcriptional activation of the locus [83] (see chapter 6). The discovery of a novel cluster of HS sites in this region may provide an explanation for the surprising residual TCRα gene activity observed in the EαKO mice. The location of this cluster is interesting from a few perspectives. First, it is located 3’ of the most Vα-distal functional J segment (Ja2) [79,80]. Therefore it will remain present in the locus upon any functional V-J rearrangement. Second, its position 5’ of the Cα completes the “flanking” of Cα from both sides: our novel cis-acting element occupies the 5’ end of the Cα while the classical Eα enhancer is located on the 3’ end. This particular arrangement of cis-acting enhancer-like elements 5’ and 3’ of the constant region exons is also observed in many large antigen loci, e.g. the IgH [101], Igκ [102], TCRγ [103] and TCRδ [6].

The location of the μ and κ enhancers within the intronic Ja-Cα region inspired the search for the human TCRα enhancer ~25 years ago [104]. Utilizing a CAT assay, enhancer activity was reported for the Ja_Cα intronic region in the human TCRα locus [104]. Subsequently, the enhancers for the mouse and human TCRα locus were found on the 3’ end of the Cα [16,85]. Individual segments 5’ of the Cα1 were included in testing for classical enhancer activity but failed to reveal any, categorizing our element as a cis-acting DNA element without classical
enhancer activity. We have demonstrated its functionality *in vivo* in the context of transgenic animals.

A single locus can harbor multiple *cis* elements playing unique or redundant roles as seen in a particularly intriguing example coming from the IgH locus. The intronic Eμ enhancer plays a key role in the V(D)J rearrangement [101] whereas the 3’ enhancer is implemented in class switching. However, it was also found that Eμ plays a nonessential role in both class-switch recombination and somatic hypermutation [105], exhibiting an overlapping function with the 3’ regulatory element [106]. Furthermore, relieving Eμ’s responsibilities in V(D)J recombination (by inserting pre-rearrange cassette) revealed Eμ’s role in allelic exclusion [107]. Analyses of the VDJ knock-in/Eμ KO mice [107] pointed to faulty clonal selection and generation of autoimmune B cell clones [108], detrimental to an organism’s immunity. Therefore the above example not only points to a complexity of the regulation of the antigen receptor loci but also to great importance of identifying and functionally dissecting multiple cis-acting elements, residing in a locus (regardless of its overlapping or redundant appearance). The *in vivo* assayed novel cis-acting regulatory complex 5’ of Cα adds yet other region of the TCRα locus that has a potential to participate in complex gene regulation of assembly, timing and expression level leading to functional, antigen recognizing immune cells, key players of adaptive immunity.
REFERENCES


