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Complete TCRα gene locus control region activity in T cells derived *in vitro* from embryonic stem cells

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Abstract

Locus Control Regions (LCR) are cis-acting gene regulatory elements with the unique, integration site-independent ability to transfer the characteristics of their locus-of-origin's gene expression pattern to a linked transgene in mice. LCR activities have been discovered in numerous T cell lineage expressed gene loci. These elements can be adapted to the design of stem cell gene therapy vectors that direct robust therapeutic gene expression to the T cell progeny of engineered stem cells. Currently, transgenic mice provide the only experimental approach that wholly supports all the critical aspects of LCR activity. Herein we report manifestation of all key features of mouse T cell receptor (TCR)-α gene LCR function in T cells derived *in vitro* from mouse embryonic stem cells (ESC). High level, copy number-related TCRα LCR-linked reporter gene expression levels are cell type-restricted in this system, and upregulated during the expected stage transition of T cell development. We further report that *de novo* introduction of TCRα LCR linked transgenes into existing T cell lines yields incomplete LCR activity. Together, these data indicate that establishing full TCRα LCR activity requires critical molecular events occurring prior to final T-lineage determination. This study additionally validates a novel, tractable and more rapid approach for the study of LCR activity in T cells, and its translation to therapeutic genetic engineering.

Introduction

Locus control regions (LCR) have been discovered in numerous gene loci that are selectively active in T cells. An LCR is a cis-acting DNA element capable of transferring most aspects of the expression pattern of its gene locus of origin to a linked transgene in mice (1). These aspects include a predictable mRNA production level that also displays locus-of-origin appropriate developmental timing and tissue restriction. Furthermore, unlike most known cis-acting elements, an LCR can accomplish this at virtually any ectopic site of integration in the genome. Transgenic analyses of LCRs have clearly demonstrated their ability to overcome integration site-dependent position effects that can silence a transgene at a subset of ectopic genomic locations (2, 3). Thus, LCR driven transgene expression is present in the appropriate tissues of all transgene positive mice at levels that are roughly transgene copy number-dependent (4). The integration site-independent ability of the LCR to robustly and predictably regulate a linked heterologous transgene in time and space makes it a prime target in the search for DNA elements with the power to increase the specificity and robustness of therapeutic gene expression from lentiviral vectors. The number and

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variety of LCR activities that are active in T cells is unusually large. They are derived from functionally important gene loci that feature a diverse array of developmental expression patterns during T cell generation and function. These gene loci include human CD2 (5), human adenosine deaminase (6), mouse T cell receptor (TCR)-α (7), mouse interleukin-2 (8), mouse CD4 (9), human perforin (10) and the mouse TH2 cytokine cluster (11). Thus, the continued study of LCR activity is of particularly high significance to the understanding of T cell biology. In addition, these LCRs provide a potentially rich source of cis-acting DNA tools for creating vectors that can drive high level therapeutic cargo gene expression with developmentally directed characteristics in T cells.

T cells are a highly significant cell type to target for gene therapy. The αβ T cell receptor (TCR) complex is used by most circulating T cells to recognize antigen and initiate immune responses. T cells can be genetically modified to contain a specific, cloned TCR (12) or engineered chimeric antigen receptor (CAR) cDNAs (13) that encode receptors enabling them to initiate a desired immunotherapeutic response. Current efforts in this vein have treated hematologic malignancies by introducing CAR-encoding vectors directly into fully developed T cells (14). However, it is also possible, and desirable, to introduce therapeutic antigen receptor gene constructions into embryonic stem cells (ESC), induced pluripotential stem cells (iPSC) and hematopoietic stem cells (HSC) using lentiviral vectors. Such stem cell genetic engineering represents a promising approach for providing an individual with a longer-term source of T cells producing an introduced therapeutic antigen receptor gene product.

Naturally, ESC, iPSC and HSC populations all give rise to multiple cell lineages in addition to T cells, each of which executes a unique program of gene expression. The safest outcome of the above-described stem cell gene therapy approach would require restricting high-level production of the introduced TCR/CAR protein to the T cell progeny of the genetically engineered stem cells. Achieving this important goal will require major advances in the understanding of the cis-acting DNA sequence requirements for predictable spatiotemporal gene regulation in native chromatin during, and after, T cell differentiation from stem cells. It will further require increased knowledge of gene regulatory DNA with the capacity to insulate therapeutic genes from the silencing effects of the genome wide heterochromatin changes likely to accompany T cell development from stem cell precursors. The multiple functions of the LCRs active in T cells seem to hold the key to addressing both of these critical issues.

To date, the transgenic mouse is the only experimental model shown to support all aspects of an LCR's complex activity at ectopic integration sites. While a powerful approach, experiments in transgenic mice are resource intensive and involve protracted timetables. Basic structure/function analyses of LCRs, and translation of this information to gene therapy vector design, can be greatly accelerated by the development of a cell culture model capable of supporting the many facets of LCR activity. However, early attempts to develop such a system for the β-globin LCR indicated that *de novo* introduction of LCR driven transgenes into differentiated cell lines does not support full LCR activity (15). Subsequent studies involving cell fusion have further suggested that the development of full LCR activity may require the LCR DNA to be present in the genome prior to cell lineage commitment (16).

We study the LCR present in the mouse TCRα gene locus (Figure 1A). The TCRα LCR was originally identified as a cluster of nine DNAse I hypersensitive sites (HS) (7) located in between the Cα constant region exons and the downstream Dad1 gene (17). It has been amply demonstrated, using multiple reporter transgenes, that the TCRα LCR drives copy number-related levels of linked transgene expression (18–20). Using randomly integrated

transgenes in mice, we have identified five distinct functional sub-regions of this LCR. Two of these sub-regions are required for the LCRs spatiotemporal specificity (21). The others seem to provide a form of insulation capacity that prevents integration site-dependent position effects on TCRα LCR function (22, 23). Informed by our prior TCRα LCR studies *in vivo*, we sought to develop an experimental system to assay for complete TCRα LCR activity that was not dependent on transgenic mice.

Here we report the finding of a cell culture model that supports all aspects of TCRα LCR activity observed in whole animals. The model involves transfection of mouse embryonic stem cells with a TCRα LCR linked reporter gene construct. Transfected embryonic stem cell clones are then induced to differentiate into T cells (and other hematopoietic progeny) *in vitro*. This approach permits the examination of reporter gene expression levels per transgene copy, as well as the developmental timing and cell type-restriction of reporter gene expression. As is observed in transgenic mice, TCRα LCR-linked reporter mRNA expression in this system correlates with integrated transgene copy number. Furthermore, high-level transgene expression is cell-type restricted, and is activated at the expected T cell developmental stage transition. These efforts validate *in vitro* ESC differentiation as an effective experimental model for the further study and translation of TCRα LCR activity. By comparing TCRα LCR activity in this system to that observed in directly transfected T cell lines, the present study also provides new evidence that the establishment of full LCR activity in lineage-committed cells requires molecular components acting prior to cell lineage differentiation.

Materials and Methods

Reporter gene constructs

The hCD2ΔT transgene (24) was excised from the pBluescript SK vector using *Sal* I and *Bam* HI. The hCD2:1-8 transgene (19) was excised using *Kpn* I and *Not* I. The SV40 promoter-driven Neomycin-G418 resistance cassette was excised from the pEYFP-C1 vector (Clontech) using *Ssp* I and *Eco*O109 I enzymes.

T cell line culture and transfection

T-cell lines VL3-3M2 (25) and C6VLB (26) were cultured in RPMI 1640 with 5% FBS and 10% FBS respectively, supplemented with 1% Penicillin-Streptomycin (Cellgro), 1% Glutagro (Cellgro) and 54μM β-mercaptoethanol (Sigma). Cells were transfected using a BioRad Gene Pulser (0.3 kV and 960 μF). Approximately 1×10^7 cells were re-suspended in 0.5 ml of Electroporation Buffer (Millipore) with 10 μg of hCD2:1-8 transgene fragment, or 5 μg of hCD2ΔT fragment. An equimolar amount of a Neomycin G418 resistance cassette was co-transfected with the reporter transgene. 24 hours post-transfection, Neomycin-G418 was added at a concentration of 0.4 mg/ml for VL3-3M2 and 0.35 mg/ml for C6VLB. Individual clones were obtained by serial dilution.

Embryonic stem cell (ESC) culture and transfection

The mouse ESR1 cell line was co-cultured with Mitomycin C arrested Mouse Embryonic Fibroblasts (MEFs) (Millipore) in Dulbeco's Modified Eagle Media (DMEM) (Cellgro) supplemented with 20% FBS (Gemini), 1% Glutagro (Cellgro), 1% Penicillin/Streptomycin (Cellgro), 1% HEPES (Millipore), 1% Non Essential Amino Acids (Millipore), 0.1% Gentamycin (Life Technologies), 0.1% β-mercaptoethanol (Life Technologies), and 10 ng/ml of Leukemia Inhibitory Factor (LIF) (Millipore). Cells were transfected by BioRad Gene Pulser (0.24kV and 500μF). Approximately 1×10^7 ESCs were re-suspended in 0.5 ml of Electroporation Buffer (Millipore) with 15 μg of hCD2:1-8 transgene fragment, or 7.5 μg of hCD2ΔT fragment. An equimolar amount of a Neomycin-G418 resistance cassette was co-

transfected with the reporter transgene. 48 hours post-transfection, G418 was added at a concentration of 0.18 mg/ml. Selection media was changed daily. Individual colonies were picked after 10 days and clonally propagated.

Both ESC and T cell transfectant clones were initially screened for transgene integration by PCR using primers complimentary to the hCD2 promoter region (*Forward*: 5′-GAGGAAAC CAACCCCTAAGATGAG-3′ *Reverse*: 5′-CGTAATCTCTTTGGAGACTGCACC-3′). Intact transgene copy number was subsequently determined via Southern blot using an 800 bp *Bgl* II probe from the HS6 region of the TCRα LCR, as described previously (19). Copy number estimates were determined by PhosphorImager analyses of at least three Southern blots for each set of clones. All clones directly compared in assays were analyzed for relative copy number on the same Southern blots. Enzymes and probes chosen enabled simultaneous detection of distinct sized fragments from both the endogenous TCRα locus and the transgene. Transgene signals were normalized to the endogenous signal within each sample.

In vitro ESC differentiation

The protocol for *in vitro* derivation of T cells, and other hematopoietic cell types, from mouse ESC was carried out as previously described (27). Emerging hematopoietic stem cells from day 8 co-cultures were harvested and transferred onto OP9-DL1 cell monolayers (to derive T-cells) (28) or OP9 cell monolayers (to derive monocytic, erythroid, or B-cells) (29). In a typical experiment, multiple, independent, transfected ESC clones were differentiated in parallel with a non-transfected ESR1 control co-culture. The ESR1 derived progeny were used as negative controls for the corresponding differentiation products of the multiple transfected ESC clones assessed in the same experiment. Cells were analyzed by flow cytometry on Day 12 of co-culture (to detect monocytic, erythroid, or early-stage developing T cells), Day 16 (to detect B-cells), and Day 18 (to detect later stage developing T cells). Co-cultures were harvested on Day 20 for RNA extraction, cDNA synthesis and Quantitative real time PCR (qRT-PCR) analysis.

Flow cytometry

FACSCalibur and FACSVantage devices were used. Antibodies used include Fluorescein isothiocyanate (FITC) conjugated, anti-hCD2 (clone S5.2), R-Phycoerythrin/cyanine dye 7 (PE-Cy7) conjugated anti-CD45 (Clone 30F-11), Allophycocyanin (APC) conjugated anti-CD44 (Clone IM7), PE-conjugated anti-CD25 (Clone 3C7), PE conjugated anti-CD8 (Clone 53–6.7), Anti CD16/32 (Clone 2.4G2), (all from BD Biosciences) and CD4 APC (Clone RM4-5), CD11b (RM2805), Ter119 (mTer04) and CD19 (RM7705) (Life Technologies). Dead Cell Discriminator (DCD) or 4′, 6-diamidino-2-phenylindole (DAPI) (Life Technologies) was used to label non-viable cells. Before staining, cells were pre-treated with anti CD16/32 antibody (to block Fc receptors). Cells were stained with fluorochrome-conjugated antibodies for 20 minutes and washed three times. For analyses, live cells were gated based on forward and side scatter and lack of DAPI or DCD signal. CD45 was additionally used to gate on white blood cell types derived in co-culture. FlowJo (Tree Star, Inc.) software was used for data analyses.

Quantitative real time PCR

On day 20 of ESC-OP9-DL1 co-culture, total RNA was extracted from one well of a 6 well plate per clone (Qiagen RNeasy micro kit). RNA for VL3-3M2 and C6VLB clones was extracted from 1×10^7 cells (Qiagen RNeasy mini kit). cDNA was synthesized from 1μg of each of these RNA samples (NEB Protoscript cDNA synthesis kit). qRT-PCR was performed using an Applied Biosystems 7500 device. Samples were prepared using the Dynamo SYBR green qPCR kit, (New England Biolabs). TCRα primers (20) were used to

normalize for both loading and T cell content of the co-cultures. hCD2 primers were used to detect reporter gene expression (Forward: 5′-CCTTTCTGCTGGTGAACTTGTG-3′ Reverse: 5′-TCAACACAACCCTGACCTGTG-3′). Relative levels of hCD2 gene products were calculated as follows: Non-transfected ESR1 C_t (cycle threshold) value was used as the "calibrator". C_t values observed using TCRα primers were used as our normalizing control, as TCRα is the "reference gene" in this assay. C_t values observed with hCD2 primers were all subtracted by the C_t values obtained for TCRα to obtain the normalized hCD2 ΔC_t for each co-culture. Next, all ΔC_t values were then subtracted by ΔC_t of the calibrator to obtain $\Delta\Delta C_t$. Normalized relative hCD2 expression in T cells derived from each clone, was calculated by using the $2^{-\Delta\Delta CT}$ method. The resulting values were then divided by the transgene copy number determined for each clone. In an experiment, relative mRNA levels per transgene copy were determined from triplicate samples. The highest level observed in a given experiment was designated as 1.0. The relative mRNA levels observed in the three separate experiments were then averaged and graphed.

Results

Applying in vitro embryonic stem cell differentiation to the study of the TCRα LCR

Recent advances have enabled quantitative differentiation of various hematopoietic cell types from mouse embryonic stem cells (ESCs) in co-culture with a bone marrow derived feeder cell line (OP9) (29). ESCs-OP9 co-culture yields hematopoietic stem cells (HSCs) when the growth media is supplemented with fms-like tyrosine kinase receptor-3 ligand (Flt-3L) (30). Subsequent addition of interleukin (IL)-7 supports HSC differentiation into cells of monocytic ($CD11b^{hi}$), erythroid ($TER119^{+}$) and B lymphocyte ($CD19^{+}$) lineages. HSCs can be driven towards development of T-lymphocytes by using OP9 cells transfected with a delta like ligand (DLL-1 or DLL-4), of the notch receptor (31). This model has proven valuable to the study of various aspects of T cell development that were formerly only approachable in whole animal models (32–34). We investigated if this *in vitro* ESC differentiation system would support the properties of the TCRα LCR that have been well demonstrated in transgenic mice. As in our previous work (19), here we use a reporter transgene fragment encoding a non-signaling variant of the human CD2 protein (hCD2ΔT) (24). Transgenic mice carrying the unlinked hCD2 reporter gene alone did not display detectable hCD2 reporter protein expression (35). Thus, this fragment was linked to HS 1-8 the TCRα LCR (Figure 1A) to create the hCD2:1-8 transgene construct that has been a suitable reporter for TCRα LCR activity (19, 20).

We generated ESC clones transfected with hCD2ΔT or hCD2:1-8 (Figure 1B). Each independently generated ESC clone represents an independent assay of a construct's transcriptional activity. hCD2:1-8 and hCD2ΔT transfected ESC clones were differentiated into CD4, CD8 double positive (DP) and CD8 single positive (SP) T-lineage cells. All of the hCD2ΔT transfected clones yielded hCD2 negative DP and CD8 SP T-cells (Figure 2A). In contrast, all of the hCD2:1-8 clones produced hCD2 positive DP and CD8 SP T-cells (Figure 2B). Thus, hCD2 gene expression in this system is TCRα LCR-dependent and integration site-independent.

TCRα LCR does not drive consistent, high level hCD2:1-8 transgene expression in non-T lineage cell types derived in vitro

In transgenic mice, a reporter gene linked to the TCRα LCR is expressed primarily in lymphoid organs and not other tissues (18, 19). In the OP9 co-culture system, ESCs can be differentiated into various hematopoietic cell types. To test cell type distribution of TCRα LCR activity in the system, we differentiated the hCD2:1-8 transfected ESC clones into either $TER119^{+}$ cells (indicative of erythroid lineage) or $CD11b^{hi}$ cells (monocytic lineage).

These populations of cells were analyzed via flow cytometry for hCD2 expression. The hCD2 reporter gene signal was low to absent in these differentiation products after 12 days of co-culture on OP9 stroma (Figure 3). Longer co-culture time (16 days) did not result in hCD2 reporter gene upregulation in non-lymphoid cells (data not shown). We were able to generate B cells from five of the hCD2:1-8 transfected ESC clones after 16 days of OP9 co-culture. Congruent with the ectopic B-cell expression observed in hCD2:1-8 transgenic mice (19), reporter hCD2 expression was detected in three of the five clones (data not shown).

TCRα LCR linked reporter gene is expressed with endogenous TCRα gene-like kinetics during T-cell development in vitro

The endogenous TCRα gene is activated at or during the double negative (DN)3 ($CD4^-$,$CD8^-$, $CD25^+$,$CD44^-$) to DP stage transition of T-cell development (36). In transgenic mice, the hCD2:1-8 transgene is upregulated with similar kinetics (19). In contrast, the hCD2 reporter gene linked to its cognate LCR is activated at the DN1 stage of T cell development (37).

Representative hCD2:1-8 transfectant ESC clones were analyzed for hCD2 expression during the DN stages. Congruent with the expected timing of endogenous TCRα gene activation *in vivo,* we generally observed hCD2 reporter gene upregulation at DN3 or during the DN3 to DP transition *in vitro* (Figure 4). One clone (1-8:A1) produced DN2 stage cells displaying a low hCD2 signal reminiscent of the low activity occasionally observed for this transgene at the DN2 stage *in vivo* (19). Nevertheless, in the main, these data indicate that the TCRα LCR can confer the developmental timing of its locus of origin to a linked heterologous reporter gene during *in vitro* T cell differentiation.

The hCD2:1-8 transgene linked to TCRα LCR is expressed in a copy number dependent manner by T cells derived in vitro

A hallmark manifestation of TCRα LCR activity *in vivo* is its ability to drive transgene copy number related mRNA levels from a linked reporter gene. To determine if the *in vitro* ESC differentiation system supported this aspect of TCRα LCR activity, we extracted total RNA from 20-day ESC-OP9-DL1 co-cultures that are rich in DP and CD8 single positive (SP) T-cells (27). The extracted RNA was analyzed for hCD2 expression levels using quantitative, real-time PCR (qRT-PCR). TCRα primers were used to normalize for loading variation and T cell content variability among the co-cultures. Figure 5 shows qRT-PCR data from analyses of two separate groups of TCRα-LCR linked reporter gene transfected ESC clones. Each group of clones was derived from independent transfections. RNA from T cells derived *in vitro* from each clone was analyzed in triplicate, and experiments on each of these two groups were repeated three times. The data demonstrate that reporter mRNA expression levels per copy vary within a very narrow 1.6-fold range in both sets of experiments (Figure 5A, 5C) and, thus, correlate strongly and significantly with the integrated transgene copy number (Figure 5B, 5D). These data are consistent with the full TCRα LCR activity observed *in vivo*. These data indicate that the TCRα LCR provides a degree of integration site-independence in this system comparable to that observed in transgenic mice. Taken together, the data described above indicate that the *in vitro* ESC differentiation system supports the full range of TCRα LCR activity seen *in vivo*.

Incomplete TCRα LCR activity after de novo introduction into lineage-committed T cell lines

As mentioned previously, prior data had suggested that the development of full LCR activity may require the LCR DNA to be present in the genome of a cell prior to cell lineage commitment (16). We decided to directly test this hypothesis by assessing TCRα LCR activity after its *de novo* introduction into T cell lines. To do this, we used the identical

reporter gene constructs and transfection/selection approach that yielded full TCRα LCR activity above in T cells derived *in vitro* from ESCs. Figure 6 shows qRT-PCR analyses of hCD2:1-8 reporter gene expression in transfected clones of two different T cell lines at distinct developmental stages. The VL3-3M2 (25) cell line represents a DP thymocyte stage, while the C6VLB (26) cell line represents a circulating CD4 SP stage. The graph depicts relative hCD2 reporter mRNA levels (analyzed in triplicate) per intact integrated transgene copy, as observed in three separate experiments for each cell line. In both cell lines, the expression levels per copy (5.5-fold for VL3 and 83.5-fold for C6VLB) fall outside the range of copy number-dependence that would be expected of full LCR activity (Figure 6A, 6C) (38). In sharp contrast to the strong single copy reporter gene expression seen in the T cells derived from transfected ESC clones (see Figure 5 clones A1 and D9), the single copy C6VLB clone (CL6) is highly sensitive to silencing. Even excluding the CL6 clone, the range of mRNA expression levels per transgene copy would be nearly 5-fold. While this might be indicative of "partial LCR" activity (38), this result would still fall outside the range characteristic of full LCR activity *in vivo*. Thus, in these cell lines, reporter expression does not correlate significantly with the integrated transgene copy number (Figure 6B, 6D). There are no material differences in the methods of transfection, selection and analyses of both the ESC and cell line clones used to generate TCRα LCR reporter gene positive T cells. Therefore, the data presented here support the above-stated hypothesis.

Discussion

The ability of an LCR to consistently establish an independently (and predictably) regulated gene locus at an *ectopic* site in the genome, has been linked to the prevention of heterochromatin-induced position effects (2, 22) and epigenetic modifications (39, 40) in multiple systems. At least three complete LCRs have been isolated and shown to dominantly and predictably regulate a linked, heterologous transgene when randomly integrated into the genome (18, 41, 42). As such, continued study of the unique properties of LCRs manifested at ectopic genomic locations is of high significance. LCR-driven transcription units provide valuable models for investigating the nature and activity of cis-acting gene regulatory DNA during development in a manner that is free of native locus-derived functional redundancy. They further provide critical opportunities to explore how these cis-acting DNA elements might be applied to the improvement of therapeutic transgenesis (43, 44).

A combination of somatic cell fusion (45, 46) and whole animal transgenic (4, 42) approaches identified the first described LCR in the human β-globin locus. Transgenic mice have been particularly important in the characterization of numerous LCRs and all the multiple dimensions of their regulatory activities [reviewed in (1)]. Nevertheless, the high cost and long timelines inherent in this approach inspired various efforts to develop additional models for the study of β-globin LCR activity. These ranged from "cell free" *in vitro* transcription (47) to somatic cell genetics (48) to direct, *de novo* introduction of LCR-driven reporter transgenes into established cell lines at pre-determined (49) or random (15) sites in the genome. The latter approach was proven unable to support the complete β-globin LCR activity seen in transgenic mice. In particular, integration-site independence was incomplete, as manifested by the absence of transgene copy number-related reporter gene expression levels (15). Introduction of β-globin LCR-driven reporter genes first into embryonic fibroblasts, followed by fibroblast cell fusion with erythroid cells seemed to rescue this deficiency (16). While this approach does not recapitulate normal erythroid cell development, these experiments suggested that β-globin LCR DNA must pre-exist in an undifferentiated environment before it can establish its full activity in differentiated cells. Pre-differentiation molecular "priming" events at the β-globin locus have been described that would support this notion (50–52). Epigenetic pre-priming of T cell expressed gene loci has also been discovered to occur as early as the ESC stage (53). These events may be

critical to establishing proper transcriptional activation upon cellular differentiation (54) and may be part of the reason why differentiated cell lines seem unable to support full LCR activity *de novo*.

The development of protocols for *in vitro* differentiation of T cells from mouse ESC (27, 31) seemed to provide a system that, in principle, could incorporate the input of any early epigenetic priming events on gene regulation. We thus sought in this study to determine if complete TCRα LCR activity could be established in T cells derived via this technology. This approach was used once before to test the cell type specificity of the human Perforin gene and LCR contained in a bacterial artificial chromosome (10). We have now demonstrated here that the *in vitro* ESC to T cell differentiation system supports all known aspects of full TCRα LCR activity. The developmental timing, cell type-specificity and copy number-relatedness of TCRα LCR-driven reporter transgenes in this system are all comparable to that observed in transgenic mice. In contrast, the identical LCR-driven reporter constructs, transfection, selection and mRNA analysis approaches revealed that two distinct T cell lines were unable to support the full integration site-independence of TCRα LCR activity *de novo*. Together these data indicate that during the development of T cells from embryonic stem cell precursors, critical events prior to final T cell lineage differentiation are required for the establishment of complete TCRα LCR activity.

Studies of the human CD2 gene locus provided the first example of an LCR active in T cells (5). Both transgenic (2, 41, 55) and knock-in (56, 57) mouse studies of this LCR have demonstrated its impact on the ongoing establishment of chromatin and gene expression states during T cell development. A recent application of microcell mediated chromosome transfer to the study of selected aspects of Perforin LCR activity notwithstanding (10), the overwhelming majority of studies of the numerous other T cell-active LCRs have been similarly dependent on whole animal models. The present study now validates *in vitro* ESC to T cell differentiation as a novel experimental model for the study of LCR activity at ectopic sites. This system bears a close resemblance to normal cellular differentiation. Furthermore, it enables examination of all key aspects of LCR activity in T cells (integration site-independence, developmental timing, cell type-specificity) without the use of transgenic mice.

Combining TCRα LCR activity with *in vitro* T cell derivation from ESC can facilitate studies involving genetic manipulation of T cell development and function. It has been previously demonstrated that TCRα gene constructs containing the full TCRα LCR lead to robust cell surface transgenic TCR expression in mice with proper developmental kinetics (58). These mice were free of the T cell abnormalities that result from premature expression of the TCR during thymocyte development (59). The work described here indicates that it will be possible to generate similarly normal TCR transgenic T cells via transfection of ESCs followed by *in vitro* differentiation in OP9-DL1 co-culture. It has also recently been shown that CD8 T cells generated *in vitro* and adoptively transferred into syngeneic mice are able to generate antigen specific responses without graft-versus-host pathology (60). Thus, the present study also now augurs the feasibility of using TCRα LCR linked transgenes to create genetically modified CD8 T cells *in vitro*, whose activity can then be examined *in vivo* after transfer. Finally, *in vitro* hematopoiesis from ESC precursors should, in principle, support the further study of the numerous other LCRs previously identified in gene loci expressed in cells of the immune system. This approach will provide a rapid screening system for adapting the activity of the many T cell-active LCRs identified to gene therapy. These efforts should improve the efficacy and temporospatial specificity of therapeutic gene expression from vectors proposed for use in stem cell based approaches to T cell genetic engineering.

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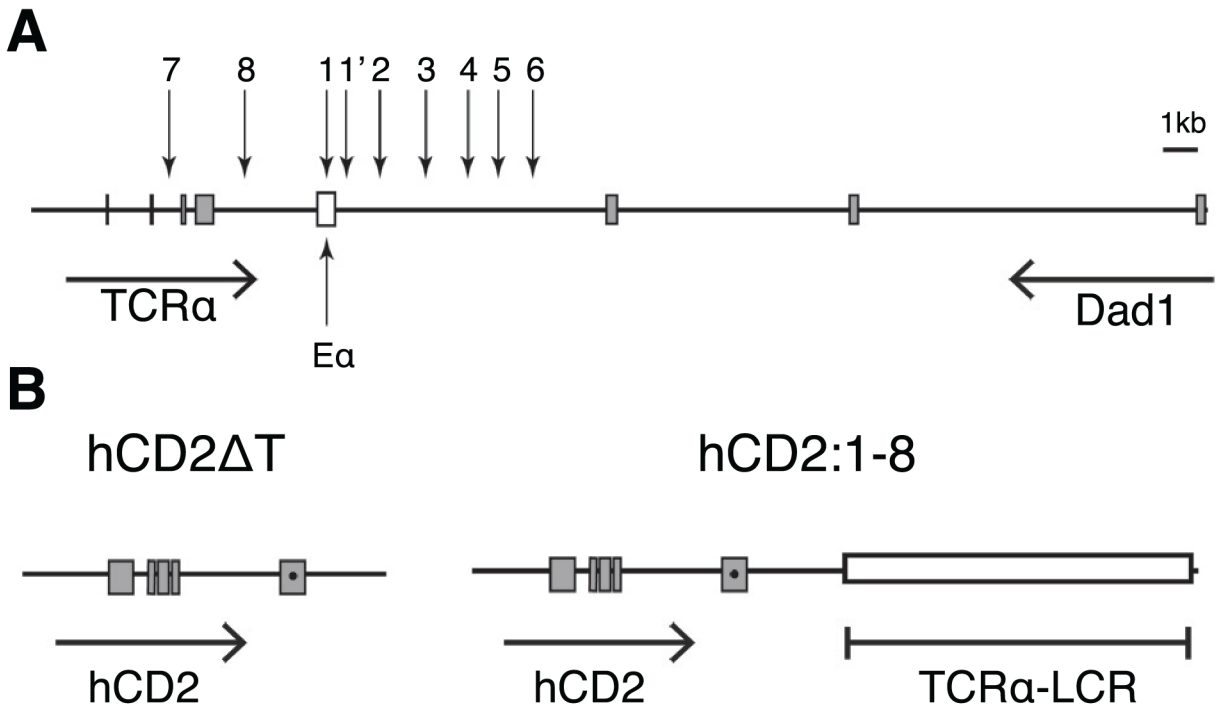

Figure 1. The TCRα LCR genomic region and reporter transgenes

(A) Diagram of the TCRα/Dad1 locus. Vertical arrows depict DNase hypersensitive sites (HS)1-8 of the TCRα LCR. The open box marks the Eα classical transcriptional enhancer. All other boxes indicate exons of their respective genes. Horizontal arrows indicate the transcription orientation of the genes. Diagram is drawn to scale. (B) Depiction of the hCD2ΔT and hCD2:1-8 transgenes. A premature stop codon (●) was introduced in exon V prior to the codons of the cytoplasmic tail (24) In hCD2:1-8, the TCRα LCR cassette (18) (open box) containing an exon-free HS1-8 fragment is linked to hCD2ΔT gene fragment.

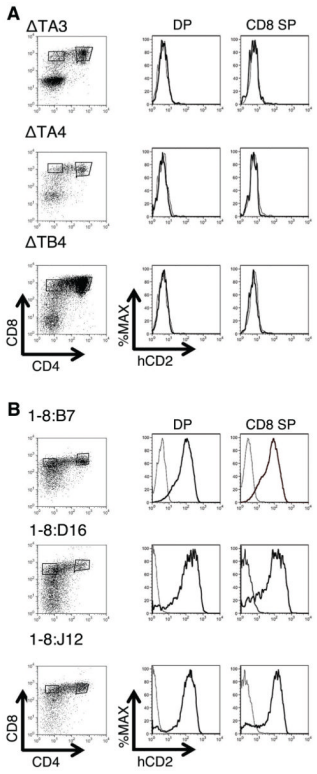

Figure 2. TCRα LCR-dependent hCD2 reporter protein expression in T-cells differentiated from ES cell clones

(A) Flow cytometry analyses of hCD2 expression in three representative independent clones of ESC transfected with hCD2ΔT (ΔT) and subsequently differentiated into CD4,CD8 double positive (DP) and CD8 single positive (SP) T-cells. None of the clones produce T cells that are hCD2 positive (n=6). (B) DP and CD8 SP T cells derived from three representative independent ESC clones transfected with hCD2:1-8 are positive for hCD2 reporter protein. All hCD2:1-8 ESC clones (1-8:) produce T cells that express the hCD2 protein on their cell surface (n>12). Cell population gates are shown at left. hCD2 expression in transfected (dark curve) and non-transfected (light curve) gated cells is shown at right.

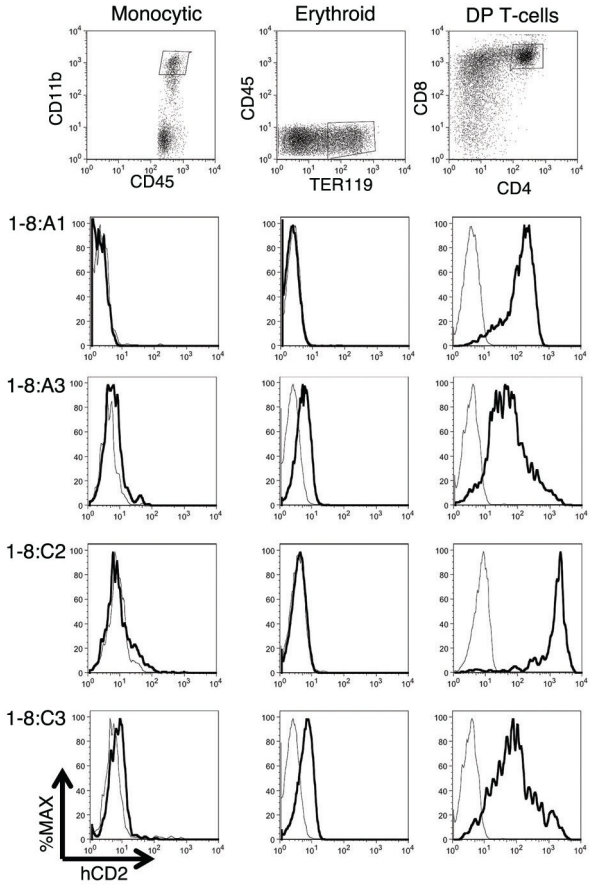

Figure 3. Cell type restriction on high-level hCD2:1-8 reporter transgene expression during *in vitro* hematopoiesis from ESCs

Flow cytometric analysis of *in vitro* differentiated hematopoeietic progeny of four representative hCD2:1-8ESC clones (1-8:). Monocytic ($CD11b^{hi}CD45^{+}$) and Erythroid ($TER119^{+}CD45^{neg}$) cells were harvested on day 12 of OP9+ESC co-culture and were low to negative for hCD2. DP T-cells ($CD4^{+}CD8^{+}$) were harvested on Day 18 of OP9DL1+ESC co-culture and were strongly positive for hCD2. Representative target cell population gates are shown at top. hCD2 expression in gated transfected (dark curve) and non-transfected (light curve) cells is shown below in each column.

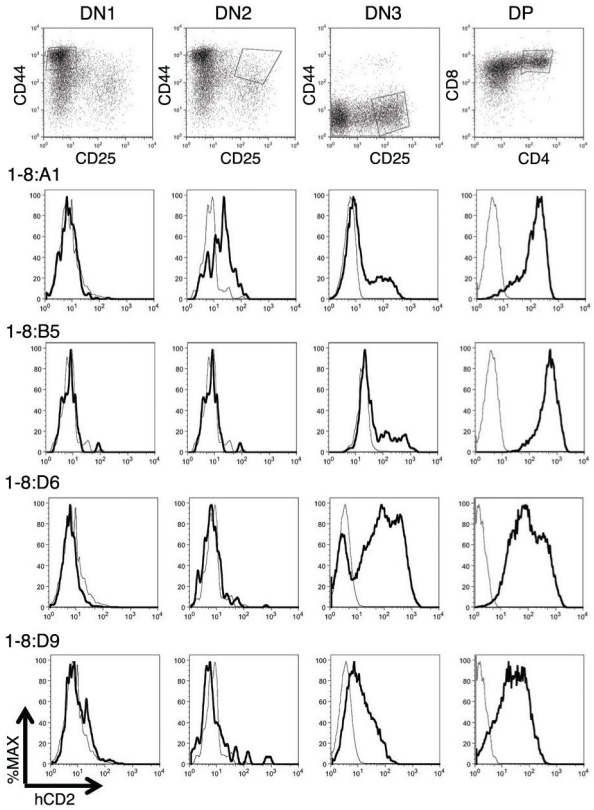

Figure 4. Appropriate upregulation of the hCD2:1-8 reporter gene at DN3 or during the DN3 to DP stage transition of *in vitro* T cell development

Flow cytometry analysis of T cell development from four representative hCD2:1-8ESC clones (1-8:) differentiated in the OP9DL1 co-culture system. Cells were harvested on day 12 of co-culture to detect CD4, CD8 double negative (DN)1 ($CD44^{+}CD25^{neg}$) and DN2 ($CD44^{+}CD25^{+}$) stage T cells, or day 18 to examine DN3 ($CD44^{neg}CD25^{+}$) and DP ($CD4^{+}CD8^{+}$) cells. Note that clone 1-8:A1 also appears in Figure 3. Representative target cell population gates are shown at top. hCD2 expression in gated transfected (dark curve) and non-transfected (light curve) cells is shown below in each column.

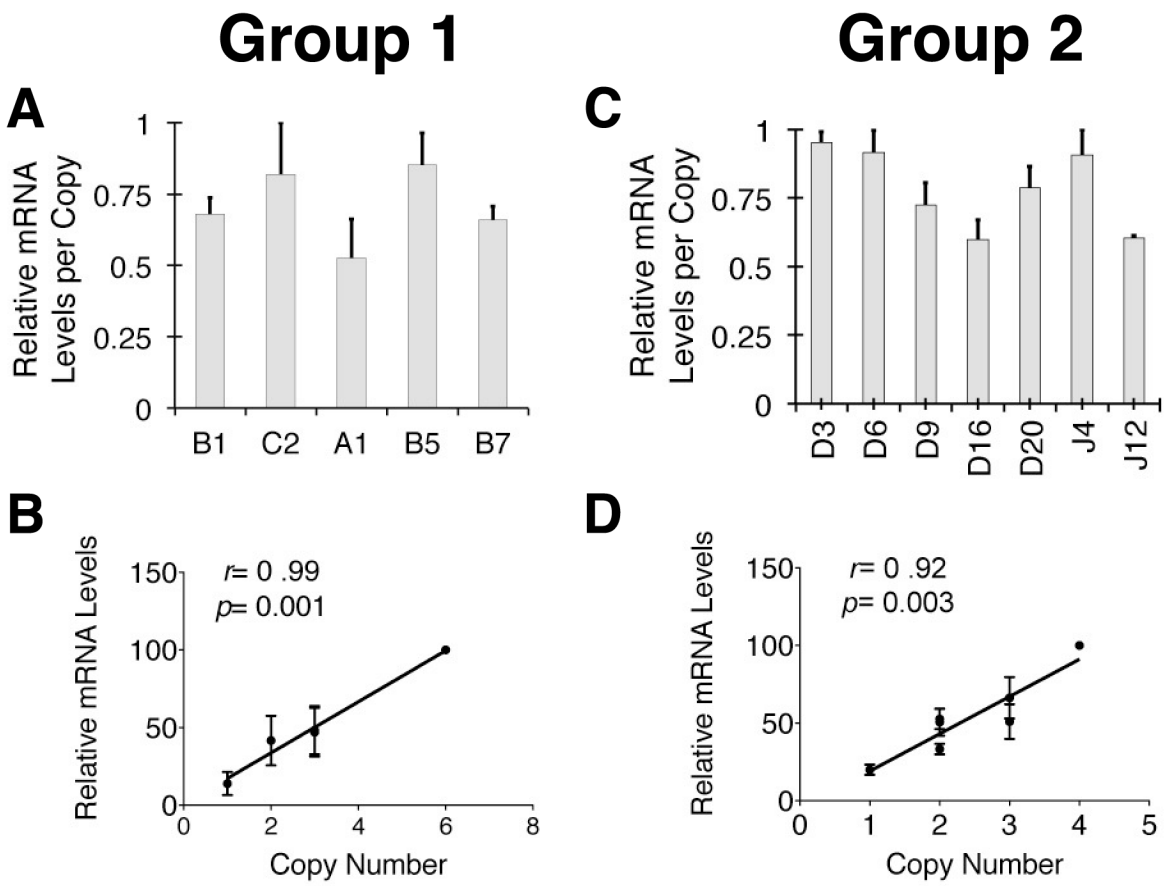

Figure 5. TCRα LCR drives copy number-related hCD2 reporter mRNA levels in T cells derived *in vitro* from ESCs

Quantitative (q)RT-PCR analysis of *in vitro* differentiated T cell progeny of representative hCD2:1-8ESC clones. Cells were harvested on day 20 of OP9DL1-ESC co-culture. (A) qRT-PCR results from T cells derived from five individual, independent hCD2:1-8ESC clones generated from an ESC transfection (Group 1). Copy number estimates are (L to R) 3, 6, 1, 2 and 3. Individual samples were run in triplicate in the qRT-PCR experiments. The y-axis signifies the relative mRNA levels detected in a given experiment with the highest level observed designated as “1.0”. Bars represent averages of three independent experiments (see Materials and Methods). The range of mRNA levels per transgene copy is 1.6-fold. (B) Graph of the correlation between relative mRNA level and transgene copy number. Prism (Graphpad) software was used to calculate x-y value correlation (r) and significance (p) noted on the graphs. The p-value was derived from an “F test” on the linear relationship between x and y values. (C) qRT-PCR results [analyzed and depicted as in (A)] from T cells derived from seven additional individual, independent hCD2:1-8ESC clones generated from an additional, independent ESC transfection (Group 2). Copy number estimates are (L to R) 2, 4, 1, 3, 3, 2 and 2. The range of mRNA levels per transgene copy is 1.6-fold. (D) Graph of correlation between relative mRNA level and transgene copy number [analyzed and depicted as in (B)].

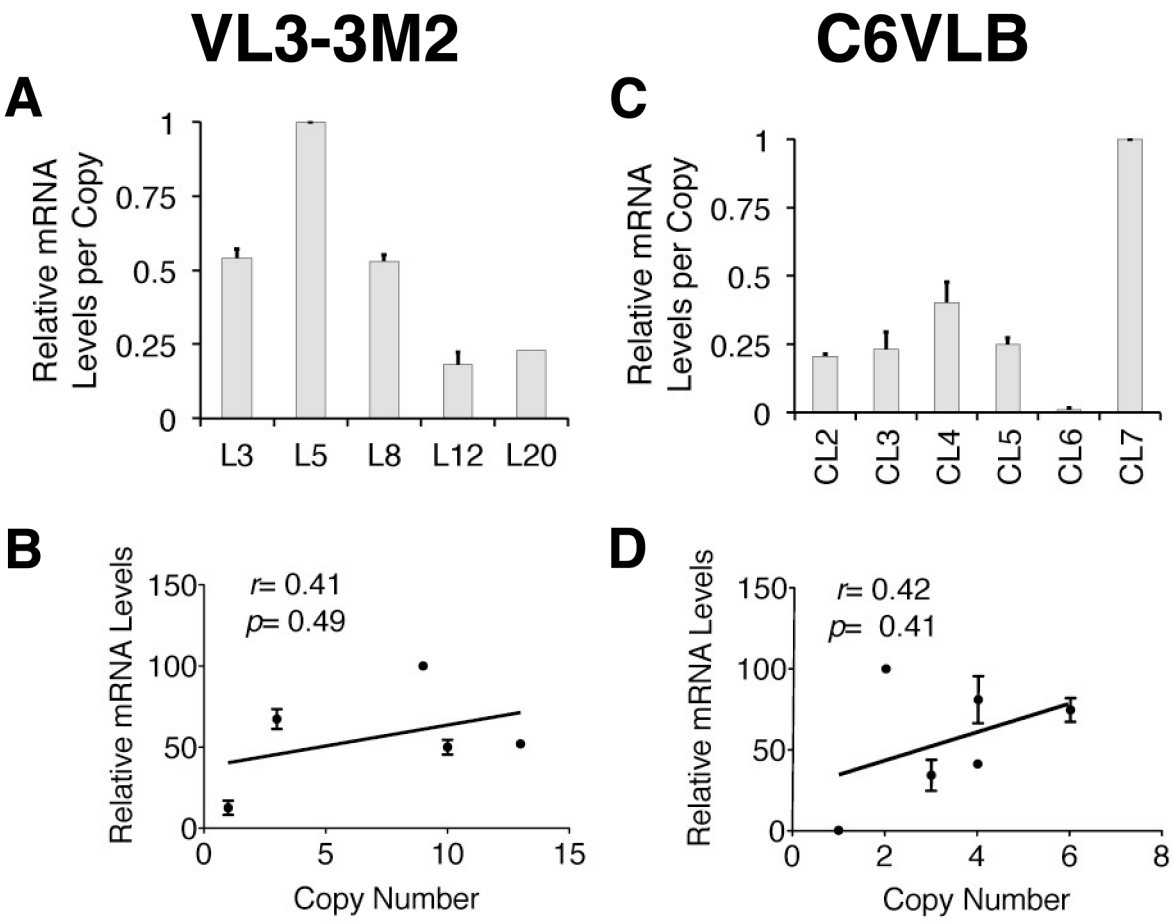

Figure 6. TCRα LCR driven reporter mRNA levels are not copy number-related after *de novo* transfection into established T-cell lines

(A) qRT-PCR analysis of clones of a CD4, CD8 DP T cell line, VL3-3M2, transfected with the hCD2:1-8 reporter gene. Copy number estimates are (L to R) 8, 3, 1, 13 and 10. Individual samples were run in triplicate in the qRTPCR experiments. The y-axis shows relative mRNA levels detected in a given experiment with the highest level observed designated as "1.0". Bars represent averages of three independent experiments (see Materials and Methods). The range of mRNA levels per copy is 5.5-fold. (B) Graph of the correlation between relative mRNA level and transgene copy number [analyzed and depicted as in Figure 5B, 5D]. (C) qRT-PCR results [analyzed and depicted as in (A)] of clones of C6VLB (a CD4+ T cell line) transfected with the hCD2:1-8 reporter gene. Copy number estimates are (L to R) 4, 3, 4, 6, 1, and 2. The range of mRNA levels per transgene copy is over 80-fold. (D) Graph of the correlation between relative mRNA level and transgene copy number [analyzed and depicted as in Figure 5B, 5D].