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Complete TCR-α Gene Locus Control Region Activity in T Cells Derived In Vitro from Embryonic Stem Cells

Armin Lahiji,*† Martina Kučerová-Levisohn,*† Jordana Lovett,*† Roxanne Holmes,‡ and Benjamin D. Ortiz*†

Locus control regions (LCRs) 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. In this study, we report the manifestation of all key features of mouse TCR-α gene LCR function in T cells derived in vitro from mouse embryonic stem cells. High-level, copy number-related TCR-α 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 also report that de novo introduction of TCR-α LCR-linked transgenes into existing T cell lines yields incomplete LCR activity. These data indicate that establishing full TCR-α LCR activity requires critical molecular events occurring prior to final T lineage determination. This study also validates a novel, tractable, and more rapid approach for the study of LCR activity in T cells, and its translation to therapeutic genetic engineering. The Journal of Immunology, 2013, 191: 472–479.

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 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 TCR-α (7), mouse IL-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 TCR complex is used by most circulating T cells to recognize Ag and initiate immune responses. T cells can be genetically modified to contain a specific, cloned TCR (12) or engineered chimeric Ag 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 Ag-receptor gene constructions into embryonic stem cells (ESC), induced pluripotential stem cells, 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 Ag receptor gene product.

Naturally, ESC, induced pluripotential stem cell, 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. Although this approach is powerful, experiments in transgenic mice are resource intensive and involve protracted timetables. Basic structure and 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 suggested that the development of full LCR activity requires 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 (Fig. 1A). The TCR-α LCR was originally identified as a cluster of nine DNase I hypersensitive sites (HS) (7) located in between the Cα C region exons and the downstream Dad1 gene (17). It has been amply demonstrated, using multiple reporter transgenes, that the TCR-α LCR drives copy number–related levels of linked transgene expression (18–20). Using randomly integrated transgenes in mice, we have identified five distinct functional subregions of this LCR. Two of these subregions 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 TCR-α LCR function (22, 23). Informed by our prior TCR-α LCR studies in vivo, we sought to develop an experimental system to assay for complete TCR-α LCR activity that was not dependent on transgenic mice.

In this study, we report the finding of a cell culture model that supports all aspects of TCR-α LCR activity observed in whole animals. The model involves transfection of mouse embryonic stem cells with a TCR-α 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 observed in transgenic mice, TCR-α 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 study and translation of TCR-α LCR activity. By comparing TCR-α LCR activity in this system to that observed in directly transfected T cell lines, the current 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 hCD2αT transgene (24) was excised from the pBluescript SK vector using SalI and Bam HI. The hCD2αT fragment (19) was excised using Kpn I and Not I. The SV40 promoter-driven Neomycin-G418 resistance cassette was excised from the pEFYFP-C1 vector (Clontech) using Spe I and EcoO109 I enzymes.

#### T cell line culture and transfection

T cell lines VL3-3M2 (25) and G6VLB (26) were cultured in RPMI 1640 with 5% FBS and 10% FBS, respectively, supplemented with 1% penicillin-streptomycin (Cellgro), 1% Glutagio (Cellgro) and 54 μM β-mercaptoethanol (Sigma). Cells were transfected using a BioRad Gene Pulse (0.3 kV and 960 μF). Approximately 1 × 10^7 cells were resuspended in 0.5 ml electroporation buffer (Millipore) with 10 μg hCD2α-1.8 transgene fragment, or 5 μg hCD2αT fragment. An equimolar amount of a Neomycin G418 resistance cassette was cotransfected with the reporter transgene. Twenty-four hours after transfection, Neomycin-G418 was selected at a concentration of 0.4 mg/ml for VL3-3M2 and 0.35 mg/ml for G6VLB. Individual clones were obtained by serial dilution.

#### ESC culture and transfection

The mouse ESR1 cell line was cocultured with mitomycin C–arrested mouse embryonic fibroblasts (Millipore) in DMEM (Cellgro) supplemented with 20% FBS (Gemini), 1% Glutagio (Cellgro), 1% penicillin/streptomycin (Cellgro), 1% HEPES (Millipore), 1% nonessential amino acids (Millipore), 0.1% gentamicin (Life Technologies), 0.1% β-mercaptoethanol (Life Technologies), and 10 ng/ml LIF (Millipore). Cells were transfected with a Bio-Rad Gene Pulse (0.24 kV and 500 μF). Approximately 1 × 10^7 ESCs were resuspended in 0.5 ml electroporation buffer (Millipore) with 15 μg hCD2α-1.8 transgene fragment or 7.5 μg hCD2αT fragment. An equimolar amount of a neomycin-G418 resistance cassette was cotransfected with the reporter transgene. Forty-eight hours after transfection, G418 was added at a concentration of 0.18 mg/ml. Selection media was changed daily. Individual colonies were picked after 10 d and clonally propagated.

Both ESC and T cell transfectant clones were initially screened for transgene integration by PCR using primers complimentary to the hCD2α fragment. An equimolar amount of a neomycin-G418 resistance cassette was cotransfected with the reporter transgene. Forty-eight hours after transfection, G418 was added at a concentration of 0.18 mg/ml. Selection media was changed daily. Individual colonies were picked after 10 d and clonally propagated.

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 described previously (27). Emerging hematopoietic stem cells from day 8 cocultures 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 nontransfected ESR1 control coculture. 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 coculture (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). Cytocides 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. Abs used include FITC conjugated, anti-hCD2 (clone S5.2), R-PE/cyanine dye 7 (PE-Cy7) conjugated anti-CD4 (Clone 30F-11), alkaliphycocyanin-conjugated anti-CD8 (Clone IM7), PE-conjugated anti-CD25 (Clone 3C7), PE conjugated anti-CD5 (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

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or DAPI (Life Technologies) was used to label nonviable cells. Before staining, cells were pretreated with anti CD16/32 Ab (to block Fc receptors). Cells were stained with fluorochrome-conjugated Abs for 20 min and washed three times. For analyses, live cells were gated based on forward and side scatter and lack of DAPI or dead cell discriminator signal. CD45 was also used to gate on WBC types derived in coculture. FlowJo (Tree Star) software was used for data analyses.

**QRT-PCR**

On day 20 of ESC-OP9-DL1 coculture, total RNA was extracted from one well of a six-well plate per clone (Qiagen RNeasy micro kit). RNA for VL3-3ML2 and C6VLB clones was extracted from 1 × 10⁷ cells (Qiagen RNeasy mini kit). cDNA was synthesized from 1 μg of each of these RNA samples (NEB Protocscript cDNA synthesis kit). QRT-PCR was performed using an Applied Biosystems 7500 device. Samples were prepared using the Dynamic SYBR Green qPCR Kit (New England Biolabs), TCR-α primers (20) were used to normalize for both loading and T cell content of the cocultures. hCD2 primers were used to detect reporter gene expression (forward: 5′-CCTTTCGTGCTGTGTAACGTGTG-3′; reverse: 5′-TCACAACACCTGACCTGTG-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 TCR-α primers were used as our normalizing control, as TCR-α is the reference gene in this assay. C t values observed with hCD2 primers were all subtracted by the C t values obtained for TCR-α to obtain the normalized hCD2 △C t for each coculture. Next, all △C t values were then subtracted by △C t of the calibrator to obtain ΔΔC t. Normalized relative hCD2 expression in T cells derived from each clone, was calculated using the 2 -ΔΔC t 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 ESCs in coculture with a bone marrow–derived feeder cell line (OP9) (29). ESC-OP9 coculture yields HSCs when the growth media is supplemented with fms-like tyrosine kinase receptor-3 ligand (Flt-3L) (30). Subsequent addition of IL-7 supports HSC differentiation into cells expressed primarily in lymphoid organs but not in other tissues (18, 19). In the OP9 coculture system, ESCs can be differentiated into various hematopoietic cell types. To test cell type distribution of TCR-α LCR activity in the system, we differentiated the hCD2:1-8 transfected ESC clones into either TER119 + cells (indicated of erythroid lineage) or CD11b + 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 d of coculture on OP9 stroma (Fig. 3). Longer coculture time (16 d) did not result in hCD2 reporter gene upregulation in nonlymphoid cells (data not shown). We were able to generate B cells from five of the hCD2:1-8 transfected ESC clones after 16 d of OP9 coculture. 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).

**TCR-α 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 TCR-α LCR is expressed primarily in lymphoid organs but not in other tissues (18, 19). In the OP9 coculture system, ESCs can be differentiated into various hematopoietic cell types. To test cell type distribution of TCR-α LCR activity in the system, we differentiated the hCD2:1-8 transfected ESC clones into either TER119 + cells (indicated of erythroid lineage) or CD11b + 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 d of coculture on OP9 stroma (Fig. 3). Longer coculture time (16 d) did not result in hCD2 reporter gene upregulation in nonlymphoid cells (data not shown). We were able to generate B cells from five of the hCD2:1-8 transfected ESC clones after 16 d of OP9 coculture. 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).

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

The endogenous TCR-α 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 TCR-α gene activation in vivo, we generally observed hCD2 reporter gene upregulation at DN3 or during the DN3 to DP transition in vitro (Fig. 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, the main, these data indicate that the TCR-α LCR can confer the develop-
mental 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 TCR-α LCR is expressed in a copy number–dependent manner by T cells derived in vitro

A hallmark manifestation of TCR-α LCR activity in vivo is its ability to drive transgene copy number–related mRNA levels from a linked reporter gene. To determine whether the in vitro ESC differentiation system supported this aspect of TCR-α LCR activity, we extracted total RNA from 20-d ESC-OP9-DL1 cocultures that are rich in DP and CD8 SP T cells (27). The extracted RNA was analyzed for hCD2 expression levels using QRT-PCR. TCR-α primers were used to normalize for loading variation and T cell content variability among the cocultures. Fig. 5 shows QRT-PCR data from analyses of two separate groups of TCR-α 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 narrow, 1.6-fold range in both sets of experiments (Fig. 5A, 5C), thus correlating strongly and significantly with the integrated transgene copy number (Fig. 5B, 5D). These data are consistent with the full TCR-α LCR activity observed in vivo. These data indicate that the TCR-α 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 TCR-α LCR activity seen in vivo.

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

As mentioned previously, prior data suggested that the development of full LCR activity could require the LCR DNA to be present in the genome of a cell prior to cell lineage commitment (16). We decided to test this hypothesis directly by assessing TCR-α LCR activity after its de novo introduction into T cell lines. We used the

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**FIGURE 2.** TCR-α LCR-dependent hCD2 reporter protein expression in T cells differentiated from ESC 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 DP and CD8 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 nontransfected (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 hematopoietic progeny of four representative hCD2:1-8ESC clones (1-8). Monocytic (CD11b^CD45^) and Erythroid (TER119^CD45^-^) cells were harvested on day 12 of OP9 plus ESC coculture and were low to negative for hCD2. DP T cells (CD4^CD8^) were harvested on day 18 of OP9DL1 plus ESC coculture and were strongly positive for hCD2. Representative target cell population gates are shown at top. hCD2 expression in gated transfected (dark curve) and nontransfected (light curve) cells is shown below in each column.
identical reporter gene constructs and transfection–selection approach that yielded full TCR-α LCR activity above in T cells derived in vitro from ESCs. Fig. 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, whereas the C6VLB (26) cell line represents a circulating CD4 SP stage. The graph depicts relative hCD2 reporter mRNA levels (analyzed and depicted as in Fig. 3). Representative target cell population gates are shown at top. hCD2 expression in gated transfected (dark curve) and nontransfected (light curve) cells is shown below in each column.

In contrast to the strong single-copy reporter gene expression seen in the T cells derived from transfected ESC clones (Fig. 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. Although 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 (Fig. 6B, 6D).

**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–positive T cells; therefore, the data presented in this study support the aforementioned hypothesis.

![Graph of the correlation between relative mRNA level and transgene copy number.](http://www.jimmunol.org/Downloadedfrom)
multiple dimensions of their regulatory activities (reviewed in Ref. 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 models 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 predetermined (49) or random (15) sites in the genome. The latter approach was proved unable to support the complete β-globin LCR activity seen in transgenic mice. In particular, integration-site independence was incomplete, as shown 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). Although this approach does not recapitulate normal erythroid cell development, these experiments suggested that β-globin LCR DNA must preexist in an undifferentiated environment before it can establish its full activity in differentiated cells. Predifferentiation molecular priming events at the β-globin locus have been described that would support this notion (50–52). Epigenetic prepriming of T cell–expressed gene loci has also been discovered to occur as early as the ESC stage (53). These events could be critical to establishing proper transcriptional activation upon cellular differentiation (54) and might 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 ESCs (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 whether complete TCR-α 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 demonstrated in this study that the in vitro ESC-to–T cell differentiation system supports all known aspects of full TCR-α LCR activity. The developmental timing, cell type specificity, and copy number–relatedness of TCR-α 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 TCR-α 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 TCRs 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 TCR-α LCR activity with in vitro T cell derivation from ESCs can facilitate studies involving genetic manipulation of T cell development and function. It has been demonstrated previously that TCR-α gene constructs containing the full TCR-α 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 in this study 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 coculture. It has also been shown recently that CD8 T cells generated in vitro and adoptively transferred into syngeneic mice are able to generate Ag-specific responses without graft-versus-host pathology (60). Thus, this study also now suggests the feasibility of using TCR-α 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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