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The mouse TCRα/TCRβ/Dad1 gene locus bears a locus control region (LCR) that drives high-level, position-independent, thymic transgene expression in chromatin. It achieves this through DNA sequences that enhance transcription and protect transgene expression from integration site-dependent position effects. The former activity maps to a classical enhancer region (Eα). In contrast, the elements supporting the latter capacity that suppresses position effects are incompletely understood. Such elements likely play important roles in their native locus and may resemble insulator/boundary sequences. Insulators support enhancer blocking and/or chromatin barrier activity. Most vertebrate enhancer-blocking insulators are dependent on the CTCF transcription factor and its cognate DNA binding site. However, studies have also revealed CTCF-independent enhancer-blocking activity in cell culture. Internal deletion of either of these elements significantly impairs LCR activity. These results reveal that the position-effect suppression region of the TCRα LCR harbors an array of CTCF-independent, positive-acting gene regulatory elements, some of which share characteristics with barrier-type insulators. These elements may help manage the separate regulatory programs of the TCRα and Dad1 genes. The Journal of Immunology, 2007, 179: 1088–1095.

The gene encoding the TCRα chain resides on mouse chromosome 14 in a gene locus containing three important genes, TCRα, TCRβ, and Dad1, whose very close linkage is evolutionarily conserved (1). TCRα and TCRβ genes encode subunits of the αβ and γδ T cell Ag receptors, respectively. Dad1 encodes a protein with antiapoptotic activity (2). Mice bearing Dad1 null mutations die in utero displaying evidence of increased hyperproliferation in response to TCR activation (6). Therefore, in addition to its important functions during embryogenesis, Dad1 may act to modulate the results of T cell stimulation by Ag.

Enforcing proper regulation of TCRα, TCRβ, and Dad1 genes in the same genomic locus is likely a complex task (7). TCRα and TCRβ genes are somatically rearranging and ultimately are solely expressed on either αβ or γδ T cells, respectively. In contrast, the Dad1 gene is ubiquitously expressed (8). The cis-acting control mechanisms ensuring the coordination and separation of these three simultaneous but very different gene regulatory programs are incompletely understood. Proper TCR gene rearrangement/expression and apoptosis are both critical processes in T cell development. Therefore, the identification, characterization, and function of cis-acting transcriptional control elements acting on this important gene locus are of significant interest.

An advance in the understanding of gene regulation in this locus was made with the discovery of a locus control region (LCR)5 in the region of DNA between the TCRα constant region exons and the Dad1 gene (9). In transgenic reporter systems, LCRs are defined by their ability to drive expression of linked genes to physiological levels in a predictable tissue-specific and copy number-related manner irrespective of the site of transgene integration in the mouse genome (10). The TCRα LCR has been consistently shown to direct expression of various reporter transgenes to lymphoid tissues at levels related to copy number (9, 11, 12). This rare characteristic is indicative of the LCR’s ability to completely protect a transgene from position effects on its expression. Position effects occur when sequences at a given integration site either

5 Abbreviations used in this paper: LCR, locus control region; HS, hypersensitive site.
silence or otherwise alter the pattern or level of gene expression expected from a particular transgene construct (13).

Consistent with other LCRs, the TCRα LCR is composed of a group of DNase I hypersensitive sites (HS) named HS1, HS1′, and HS2 through 6. Of these HS, only HS1 (also known as Eα) supports classical transcriptional enhancer activity as defined by transient reporter gene transfection assays (14). Nevertheless, the ~6-kb region downstream of Eα (containing HS1′ and HS2 through 6) is critical for the TCRα LCR’s ability to protect transgenes from position effects (9, 15). This region also appears to play a role in the regulation of the endogenous locus (8, 16). It is especially important to determine the in vivo functional elements of the TCRα LCR responsible for suppressing position effects at ectopic sites of integration in the genome. Such information may give insight into this powerful, but still mostly unexplained, aspect of LCR activity, in general. In addition, these TCRα LCR subelements are very likely to play a role in managing the separate regulatory programs of the TCRα and Dad1 genes that flank it, thus preventing their inappropriate interaction during T cell development. In this way, some of the position effect suppressing subelements of the TCRα LCR may act as insulator/boundary-like elements in vivo.

Insulator sequences are thought to play a role in separating regulatory influence in the genome and support enhancer blocking and/or chromatin barrier activity with the ability to halt the propagation of repressive chromatin states (17). Studies of insulator elements have so far revealed only one functional sequence element that is common to multiple vertebrate enhancer-blocking-type insulators, the binding site for the CTCF transcription factor (18). CTCF is an 11 zinc finger protein that can play multiple roles at the gene loci it regulates, including gene activation, repression, and enhancer-blocking functions (19). In addition, CTCF is activated upon BCR stimulation and thus may play a role in regulating immune responses (20). There is also clear evidence of CTCF-dependent insulator activity in the vertebrate genome and considerable speculation as to their roles in LCR activity (17). As such, there is much interest in the role of CTCF-dependent (21–23) and CTCF-independent (24, 25) insulator-like elements at immunologically important gene loci, including several shown to contain an LCR (reviewed in Ref. 10).

There is considerable evidence that the TCRα LCR harbors subelements with insulator-like properties that may contribute to its ability to suppress position effects in vivo. Assays in cultured cell lines have identified a broad region of TCRα LCR DNA downstream of Eα that supports enhancer-blocking activity (24, 25). In this area, HS1′, HS4, and HS6 are the most prominent HS in both transgenic and native TCRα LCR loci of thymocytes (15, 26). A CTCF site with enhancer-blocking activity has been identified in HS1′ (25). In the present study, we focus our search for DNA elements participating in position effect suppression to these three regions of the TCRα LCR. We previously reported one such element, a 238-bp sequence within HS6 whose deletion significantly impairs, but does not completely abolish, the LCR’s position effect suppression capacity (26). This information, along with the prior enhancer-blocking data (24, 25), clearly suggests that additional elements downstream of Eα contribute to LCR activity. Primary candidates for such elements include the identified CTCF binding site in HS1′ with enhancer-blocking activity (25), the tissue specifically methylated area of HS4 DNA, the function of which is still unknown (27), and a 316-bp segment of the HS6 region at the very 3′ end of the LCR (named HS6-316) whose activity was revealed in cell culture (26). In this study, we describe the functional contributions of these three candidate position-effect suppression elements to TCRα LCR activity in vivo using an integration site-independent transgenic reporter system in mice.

We find that the CTCF region is a very minor contributor to LCR function. In contrast, internal deletion of the HS4 or HS6-316 regions, neither of which interacts with CTCF (25), results in a more significant impairment of LCR activity. Altogether, the data show that the TCRα LCR subregion supporting its position-effect suppression capacity harbors an array of cooperating CTCF-independent, positive-acting functional elements, some of which share properties with barrier insulators. The existence of these multiple elements in between the TCRα and Dad1 genes would, in principle, allow for dynamic regulation of the strength of this intergenic region’s activity. This is an intriguing possibility given the in vivo functions and expression patterns of the TCRα and Dad1 genes that flank it in the genome.

Materials and Methods

In vivo DNase I footprint analyses

In vivo DNase I footprinting was conducted essentially as described in Ref. 28 using nuclei from thymus of C57BL/6 mice and mouse fibroblasts (NHH3T3). Briefly, nuclei were digested with varying amounts of DNase I (Worthington Biochemical) ranging from 0.0 to 4.0 μg/ml. Plain genomic DNA was digested with 0.25–1 μg/ml DNase I. The cleaved genomic DNA was purified by phenol/chloroform/soybean extraction followed by ethanol precipitation. The DNA was then subjected to ligation mediated-polymerase chain reaction (LMP-PCR) using acrylamide gel-purified, gene-specific oligonucleotide primers (Proligo). The amplification products were further analyzed as described previously (28). The primers were complimentary to the following TCRα locus sequences: primer 1, 5′-CTGATCCTCTAGGGAAAAACTTTTCATCAGGC-3′; primer 2, 5′-CTCCCCCCTGGGTGTATTTTGGGAA-3′; and primer 3, 5′-CCAAAAGGCTTTCTCCCTCGGCGTGTT-3′.

Nuclear extract preparation and EMSA

Thymic and NHH3T3 nuclei were prepare previously described (28). Nuclear extract (3 μg) was used in EMSA with 40,000 cpm (dry) of 32P-labeled oligonucleotide probe. The final binding reaction conditions (25) were as follows: 20 mM HEPES (pH 7.9), 5 mM MgCl2, 150 mM KCl, 1 mM DTT, 1 mM PMSF, 5% glycerol, and 0.5% Triton X-100 with 1 μg of poly(dI:dC) (double stranded). Incubations were on ice for 45 min. For binding site competition assays, a 50-fold molar excess of unlabeled oligonucleotide was added to the binding reaction before the addition of labeled probe. The sequences (5′–3′) of the oligonucleotide probes used are as follows: 5′-CTCF, TGGGAGTCCACCACACTTGTGATGCTCCT; 3′-CTCF, GGTAGACCCACCGGGGCGACCAGTACTCC; β-globin FII, CCCAGGGATGTAATTACGTCCCTCCCCCGCTAGGGGGCAGCA.

Transgenic mice

These transgenic animal studies have been reviewed and approved by the Hunter College Institutional Animal Care and Use Committee. DNA fragments for microinjection were twice purified on low-melting point agarose gels (SeaPlaque) and isolated using β-agarose (New England Biolabs). The purified DNA was microinjected into the male pronucleus of (C57BL/6 x 129/Sv) F1 fertilized mouse eggs (Sloan-Kettering Transgensics Facility) that were then transferred to pseudopregnant female mice. Transgenic founders were identified by Southern blot analyses of tail DNA digested with HaeIII (New England Biolabs) and probed with a 900-bp Smal fragment from the LCR that detected the transgene and the endogenous locus on different size restriction fragments. Relative copy number was determined for each line by analysis of at least two Southern blots by PhosphorImager quantitation (Molecular Dynamics). All lines directly compared here were analyzed for relative copy number on the same Southern blots using the same probe and enzyme digestion. The signal from the endogenous TCRα locus was used as a normalizing control.

DNA constructs

The β-1 transgene construct containing a 4.9-kb human β-globin gene fragment and a 7.4-kb fragment of the TCRα LCR containing HS1 through HS6 has been previously described (15). β1-6ΔCTCF is a deletion mutant version of β1-6 in which a 94-bp Avrl to Scal fragment within HS1 containing tandem CTCF binding sites was removed. The β1-6ΔHS6-316 construct was designed to delete an ApoLI to DraI fragment containing 1.1 kb of HS4 region DNA from the β1-6 wild-type LCR-linked transgene. β1-6ΔHS6-316 is a construct that removes a 316-bp PstI to BglII fragment in
the HS6 region from the β:1-6 transgene. Transgene inserts for microinjection were liberated from vector DNA by using the XhoI and CiaI sites of the parent cloning vector pSP72 (Promega).

RNA analyses
RNA was prepared as previously described using a single-step isolation protocol (29) from mouse tissues that were dissected of fat and washed with PBS to minimize contamination with blood. Five micrograms of RNA per sample was run on a 1% agarose gel and transferred onto a noncharged nylon membrane (Genescreen) for Northern blot hybridization analyses using Quickhyb solution (Stratagene). A 428-bp BamHI-NcoI genomic fragment of human β-globin gene coding region was used to probe for the mRNA product of the reporter transgene. To normalize for loading variation, blots were stripped and reprobed with a 500-bp Sau3A1 fragment of the TCRα constant region (Cα) cDNA or a probe to 18S rRNA (Ambion). All probes were labeled with [α-32P]dCTP using a random primer labeling kit (Invitrogen Life Technologies). Transgene signals were quantified and normalized to the relevant loading control signal by PhosphorImager analysis.

FIGURE 1. DNase I-mediated in vivo footprint reveals occupation of tandem CTCF-like sites in the endogenous TCRα LCR. A, Ligation-mediated PCR on plain genomic DNA or DNase I-treated nuclei (mouse thymus, NIH3T3 mouse fibroblasts). Triangle indicates increasing DNase I concentration. Brackets indicate two strong footprint regions named 5’ CTCF-like and 3’ CTCF/TAD1 (25). B, Comparison of occupied sequences in HS1’ to CTCF consensus binding sequences of Ig2/H19 (50) and the FII site of the chicken β-globin insulator (18). Shaded areas indicate conservation of sequence identity among the four CTCF sites. C, The 3’ and 5’ CTCF-like sites cross-compete for each others’ binding complexes (indicated by arrow). EMSA using 3’-CTCF/TAD1 and 5’-CTCF-like site-labeled oligonucleotides (top) and thymic nuclear extract. Unlabeled competitor oligonucleotides used are indicated (in vertical type). FII indicates an oligonucleotide of the chicken β-globin insulator CTCF binding site, which also efficiently competes for the complexes formed on both 3’ and 5’ CTCF-like sites. Probe lane indicates labeled probe incubated without nuclear extract. The position of the unbound “free probe” is indicated with a bracket.

Results
In vivo footprinting reveals two CTCF-like binding sites in the HS1’ region
The HS1’ element is a critical component of the TCRα LCR affecting long-range chromatin structure and epigenetic modification at transgene loci (15, 27). To identify DNA sequences within HS1’ interacting with nuclear factors, we performed DNase I-mediated in vivo footprinting on this region of the endogenous TCRα LCR. Nuclei of mouse thymocytes (which express both TCRα and Dad1) and NIH3T3 mouse fibroblasts (which express Dad1 but not TCRα) were treated with limiting amounts of DNase I. The cleavage products were amplified by ligation-mediated PCR using locus-specific primers. In both types of nuclei, the data revealed two adjacent occupied sequences that have homology to the consensus recognition site for the CTCF protein (Fig. 1). The 3’ site (labeled 3’ CTCF/TAD1) was previously confirmed as a bona fide CTCF binding site by EMSA. Ab “supershift” assays, and chromatin immunoprecipitation (25). In contrast, the 5’ CTCF-like site located 14 bp upstream had not been previously recognized. EMSA experiments were performed using separate oligonucleotides representing either the 5’ or 3’ CTCF site homolog and nuclear extracts from thymocytes or fibroblasts. These experiments showed that both CTCF sites formed protein-DNA complexes of similar mobility in both thymocyte and fibroblast extracts (data not shown) and could cross-compete for each other’s complexes in unlabeled oligonucleotide competitor inhibition assays (Fig. 1C). Furthermore, excess unlabeled CTCF site DNA from the chicken β-globin insulator (18) also effectively competed for the complexes bound to both the 5’ CTCF-like and 3’ CTCF/TAD1 sites (Fig. 1C). Based on these experiments in combination with the data reported
The inset percent thymic expression levels for each organ among the six transgenic lines. Error bars, The SD in the as 100%. Reporter expression from other organs within the same line was normalized transgene expression in the indicated organs (T, thymus; S, spleen; K, kidney; Lg, lung; Li, liver; H, heart) among the various lines. Note the low variation in percentage of thymic expression from independent transgenic lines bearing the /H9252

A reporter transgene model for assessing the function of TCRα LCR subelements

Much of our understanding of the components of TCRα LCR function has come from analyses of reporter transgene loci in mice. The present study was conducted using a 4.9-kb human β-globin reporter gene fragment linked to either the wild-type TCRα LCR (a construct named β-1-6) or internal deletion mutants thereof (Fig. 2B). The β-globin reporter fragment contains the human β-globin promoter, exons, introns, and 3′ enhancer sequence. It is highly subject to position-effect silencing in the absence of additional elements (30, 31) and has long been used as a reporter of LCR activity in multiple systems (11, 32–34). Including an intact LCR in the transgene eliminates these position effects. Linking the β-globin reporter gene to HS1-6 of the TCRα LCR yields the high-level, lymphoid organ-specific and relatively consistent reporter expression levels per copy (within a narrow 2- to 3-fold range) indicative of integration-site independence and full TCRα LCR activity in vivo (15).

As mentioned earlier, our previous analyses led us to focus on elements within the three prominent HS (HS1′, HS4, and HS6) in the insulator-like/position-effect suppressing region of the LCR downstream of Eor (Fig. 2A) (15, 24, 25). We created three different LCR internal deletion mutants to assess the contribution of the deleted sequences to LCR activity (Fig. 2B). These mutant LCRs are missing either the region of HS1′ containing the tandem CTCF-like binding sites identified above, the region of HS4 containing the target of tissue- and site-specific DNA demethylation (27) or the HS6-316 position-effect suppressing region previously described in cell culture (26). Each of these mutant LCRs was linked separately to the human β-globin fragment to create the transgenes named β-1-6ΔCTCF, β-1-6ΔHS4, and β-1-6ΔHS6-316, respectively. Multiple transgenic lines bearing these constructs were generated. The activity of these mutant LCR-driven transgenes was compared with that of the wild-type β-1-6 construct using a new set of β-1-6-transgenic lines. In all, 20 new, independent transgenic mouse lines are analyzed here.

Inconsistent tissue distribution of mutant TCRα LCR-driven transgene expression

Reporter gene expression levels under the control of the TCRα LCR activity in vivo (15). As mentioned earlier, our previous analyses led us to focus on elements within the three prominent HS (HS1′, HS4, and HS6) in the insulator-like/position-effect suppressing region of the LCR downstream of Eor (Fig. 2A) (15, 24, 25). We created three different LCR internal deletion mutants to assess the contribution of the deleted sequences to LCR activity (Fig. 2B). These mutant LCRs are missing either the region of HS1′ containing the tandem CTCF-like binding sites identified above, the region of HS4 containing the target of tissue- and site-specific DNA demethylation (27) or the HS6-316 position-effect suppressing region previously described in cell culture (26). Each of these mutant LCRs was linked separately to the human β-globin fragment to create the transgenes named β-1-6ΔCTCF, β-1-6ΔHS4, and β-1-6ΔHS6-316, respectively. Multiple transgenic lines bearing these constructs were generated. The activity of these mutant LCR-driven transgenes was compared with that of the wild-type β-1-6 construct using a new set of β-1-6-transgenic lines. In all, 20 new, independent transgenic mouse lines are analyzed here.

Inconsistent tissue distribution of mutant TCRα LCR-driven transgene expression

Reporter gene expression levels under the control of the TCRα LCR consist of highest in the thymus, next highest in the spleen and very low (<10% of thymic expression) in nonlymphoid organs (9, 12, 15). The six new independent β-1-6-transgenic lines

FIGURE 3. Line-to-line consistency in relative tissue distribution of transgene expression from wild-type and mutant LCR-driven constructs. A, PhosphorImager analyses of Northern blots of organ RNA from six independent transgenic lines bearing the β-1-6 (wild-type LCR-driven) reporter construct. The inset is a representative Northern blot on β-1-6 line 42 showing the reporter β-globin signal and 18S rRNA signal used as a normalizing control. The graph represents the average relative tissue distribution of normalized transgene expression in the indicated organs (T, thymus; S, spleen; K, kidney; Lg, lung; Li, liver; H, heart) among the various lines. Within each line examined, thymic transgene expression was designated as 100%. Reporter expression from other organs within the same line was plotted as a percentage of thymic expression. Error bars, The SD in the percent thymic expression levels for each organ among the six β-1-6-transgenic lines. Note the low variation in percentage of thymic expression from line-to-line and that nonlymphoid expression is well below the 10% of maximum level marked by the horizontal bar. B, Similar analyses of four independent lines of transgenic mice bearing the β-1-6ΔCTCF construct with the Northern blot from β-1-6ΔCTCF line 16 shown in the inset. C, Similar analyses of six independent transgenic lines bearing the β-1-6ΔHS6-316 construct with the Northern blot from β-1-6ΔHS6-316 line 20 shown in the inset. D, Similar analyses of four independent transgenic lines bearing the β-1-6ΔHS4 construct with the Northern blot from β-1-6ΔHS4 line 40 shown in the inset. Note the greater variation in relative tissue distribution of transgene expression seen in C and D as compared with A and B.
analyzed here consistently displayed this expression pattern with very little variation (Fig. 3A). We previously observed that mutant TCRα LCR-driven β-globin transgenes (26), as well as non-LCR-containing β-globin reporter transgenes (Ref. 28 and B. D. Ortiz, unpublished observations), do not consistently maintain this tissue distribution. This is likely due to the effects of interference by native regulatory sequences at the varying sites of transgene integration. Therefore, we interpret significant deviation from the wild-type expression pattern (Fig. 3A) as indicative of position effects and, thus, impaired LCR activity. The effect of deleting the CTCF sites from the LCR on the normal transgene expression pattern was minor with mRNA levels only barely crossing the 10% “threshold” (relative to thymic mRNA levels) in lung and heart (Fig. 3B). Tissue distribution of transgene expression was somewhat more perturbed in the absence of the 316-bp region of HS6 with average relative expression levels in some nonlymphoid organs exceeding 20% of that seen in thymus (Fig. 3C). However, the relative tissue distribution of multiple lines bearing the β1-6ΔHS4 mutant transgene was plainly abnormal, leading to large variations in organ expression levels relative to thymus among the four lines analyzed (Fig. 3D). These data indicate that the ΔHS6-316 and ΔHS4 mutant LCRs are less able to protect the transgene from position effects.

Minor reduction in LCR-driven transgene expression levels upon tandem CTCF-like site deletion

Because LCRs confer copy number-related expression upon a linked transgene, we examined whether transgene expression levels were altered when driven by the various mutant LCRs. Using Northern blots and PhosphorImager analyses, we compared the expression levels (per transgene copy) of the wild-type β1-6 and mutant β1-6ΔCTCF transgenes in lymphoid tissues. Detected β-globin mRNA levels were normalized to those of endogenous TCRα in thymus. The products were then divided by the relative copy number estimates among the various lines. A representative Northern blot is shown in Fig. 4A and quantified expression levels per copy are shown in Fig. 4B. The data show that the average transgene expression levels per copy from the ΔCTCF mutant construct are slightly reduced from the average of wild-type β1-6 transgene expression levels, with 77% of wild-type expression in thymus. Coupled with the data in Fig. 3B, it appears that the tandem CTCF sites are minor contributors, at best, to the TCRα LCR’s activity in this system.

The ΔHS4 and ΔHS6-316 mutations impair TCRα LCR activity

As mentioned previously, the region of HS4 and a 316-bp region within HS6 were identified as candidate participants in TCRα LCR function, and Fig. 3, C and D, suggest that these sequences may be necessary for the LCR to fully protect transgenes from position effects. Using the same methods described for Fig. 4, we quantified the contribution of the HS4 and HS6-316 elements to TCRα LCR-driven transgene expression in thymus. We compared transgene mRNA levels (per copy) produced by the β1-6ΔHS4 and β1-6ΔHS6-316 transgenes to those of the wild-type LCR-driven β1-6 construct. In the representative experiment shown in Fig. 5, average transgene mRNA levels per copy are reduced over 4-fold from wild-type levels when HS4 DNA is absent from the LCR. Similarly, average expression levels of the ΔHS6-316 mutant are 3.3-fold lower per transgene copy, compared with that driven by the wild-type LCR. Along with the results in Fig. 3, C and D, these data help confirm that the HS4 and HS6-316 regions of the TCRα LCR harbor in vivo functional elements.
Discussion

Identifying the cis-acting gene regulatory elements of the complex TCRα/TCRβ/Dad1 gene locus that are active in vivo is an important endeavor. Among other things, these elements collectively help achieve the carefully orchestrated gene rearrangements that ensure generation of both αβ and γδ T cells with a wide repertoire of TCR specificities. In addition, it is important to understand the regulation, and possible coordination, of TCRα and Dad1 gene expression. TCRα gene assembly and expression are the final step before thymic selection processes that entail sorting through the randomly generated TCR repertoire. These processes promote further development of desirable T cells bearing TCR specificities that are able to use self-MHC without being overly self-reactive (35). Those T cells bearing TCR specificities that do not meet these criteria die by apoptosis. In this light, the evolutionarily conserved linkage of the TCRα gene to the antia apoptosis gene Dad1 is particularly intriguing. The expression patterns of these two genes are different from each other, both in terms of tissue distribution and developmental timing (6, 8). The TCRα LCR is flanked by the TCRα gene on its 5′ end and the Dad1 gene on its 3′ end. The roughly 6-kb of LCR DNA in between the HS1/Eα element and the most proximal end of the Dad1 locus is critical for LCR activity. It would also be expected to play an important role in coordinating and/or separating the regulation of the TCRα and Dad1 genes during thymocyte development (as well as throughout the embryo), perhaps in a manner analogous to insulator elements.

Although a connection between LCR activity and insulator elements has been proposed (17), in vivo directly linking the two are sparse. With the rare exception of imprinted gene loci (36), the activity of the various types of insulators is typically studied in vitro before thymic selection processes that entail sorting through the randomly generated TCR repertoire. These processes promote further development of desirable T cells bearing TCR specificities that are able to use self-MHC without being overly self-reactive (35). Those T cells bearing TCR specificities that do not meet these criteria die by apoptosis. In this light, the evolutionarily conserved linkage of the TCRα gene to the antia apoptosis gene Dad1 is particularly intriguing. The expression patterns of these two genes are different from each other, both in terms of tissue distribution and developmental timing (6, 8). The TCRα LCR is flanked by the TCRα gene on its 5′ end and the Dad1 gene on its 3′ end. The roughly 6-kb of LCR DNA in between the HS1/Eα element and the most proximal end of the Dad1 locus is critical for LCR activity. It would also be expected to play an important role in coordinating and/or separating the regulation of the TCRα and Dad1 genes during thymocyte development (as well as throughout the embryo), perhaps in a manner analogous to insulator elements.

Seemingly conflicting data have been reported regarding the role of CTCF and its cognate binding site in position-effect suppression in chromatin. Work on the chicken β-globin insulator has produced clear evidence that its barrier capacity is independent of its CTCF binding sequences (38, 39). However, reports using other experimental systems have asserted that CTCF can block the spreading of repressive chromatin (40), regulate the balance between activating and silencing histone modifications (41), and protect from position effects (42). We found that a deletion of the tandem CTCF-like binding sites of the TCRα LCR did not have a significant impact on its position-effect suppressing activity in vivo. These data indicate that the enhancer-blocking activity demonstrated for this CTCF-binding region does not meaningfully contribute to overcoming heterochromatin-induced position effects in our system. Overcoming these effects is a hallmark of LCR activity at ectopically integrated transgene loci (43, 44). Nonetheless, the enhancer-blocking data reported previously (25) suggest that these sites do bear some activity. It is possible that the CTCF sites act as an enhancer blocker that is only active elsewhere in the embryo and/or in restricted situations arising during thymocyte development that would not be detected in analyses of bulk thymocytes.

The unchecked spreading of condensed chromatin is one likely cause of the position-effect gene silencing often observed from randomly integrated transgenes in mice (13, 43, 44). TCRα LCR activity prevents these effects. We find that the HS4 and HS6-316 regions of the LCR are required for complete LCR activity in vivo and may well be two examples of CTCF-independent barrier-type insulator elements. Although we do not formally test these elements as self-contained barrier insulators here, these elements do share some important characteristics with known barrier insulators. For example, in T cell, the endogenous HS4 region DNA shows a higher level of histone acetylation than its neighboring sequences (25), a trait of the chicken β-globin insulator (45, 46). Also, the HS6-316 region protects stable-transfected reporter constructs from silencing in chromatin (26), the hallmark property of barrier insulators (47). The discovery of multiple functional elements in the position-effect suppressing region of the TCRα LCR provides evidence in support of a proposed model of barrier insulator activity (17), where barrier function depends on the local balance between negative and positive regulatory activity in chromatin. In this model, the three functional elements in the 3′ end of the TCRα LCR described to date (Ref. 26 and the present study) would synergize to create a strong positive regulatory influence to prevent position-effect silencing. We have shown that removal of any one of these elements reduces the strength of LCR activity (Fig. 6). This model may also help explain the cell-type specificity of TCRα LCR activity. In nonlymphoid tissue, HS4 region DNA is selectively and heavily methylated (27) and the TF123 region of HS6, described previously (26), is not occupied in vivo by the factors that do bind it in T cells (28). According to the above barrier model, inactivation of HS4 and TF123 elements in this way could render the LCR too weak to fully protect from position effects in nonlymphoid organs.

Our data are also consistent with a model in which the HS4 and HS6-316 elements are not insulators, per se, but rather are positive regulators of gene expression in chromatin. However, in this model, the mechanism of action of these elements would be distinct from those used by classic transcriptional enhancers. The HS4 and HS6-316 regions are devoid of such classic enhancer activity (14). Another reason to consider this alternative activity at ectopically integrated transgene loci (43, 44). Nonetheless, the enhancer-blocking data reported previously (25) suggest that these sites do bear some activity. It is possible that the CTCF sites act as an enhancer blocker that is only active elsewhere in the embryo and/or in restricted situations arising during thymocyte development that would not be detected in analyses of bulk thymocytes.

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Our data are also consistent with a model in which the HS4 and HS6-316 elements are not insulators, per se, but rather are positive regulators of gene expression in chromatin. However, in this model, the mechanism of action of these elements would be distinct from those used by classic transcriptional enhancers. The HS4 and HS6-316 regions are devoid of such classic enhancer activity (14). Another reason to consider this alternative
hypothesis is that insulator elements have typically been found in between genes that are not destined to be coexpressed. There are stages of T cell development where both of these genes become coexpressed (6). Therefore, the TCRα/Dad1 gene locus would present an interesting challenge to an insulator element.

In either model (insulator vs positive regulator), these elements may play a role in coordinating the expression of the TCRα and Dad1 genes, which display diverging kinetics during thymocyte development. TCRα gene rearrangement and transcription begin at the CD4+ CD8+ CD25- CD44+ (DN4) stage of thymocyte development (48, 49). In contrast, high levels of Dad1 expression are not seen until the later, single positive stage (6). Therefore, it is likely that a strong separation of the TCRα and Dad1 gene regulatory influences would be warranted at early thymocyte stages. However, at later stages, it might be desirable to selectively reduce that regulatory separation so that Dad1 and TCRα genes may be coordinately up-regulated in T cells that have successfully survived thymic selection. In principle, having multiple cooperating elements working collectively in the TCRα/Dad1 intergenic region would make it possible to dynamically modulate this region’s regulatory activity via selective inactivation of its component elements. Having localized and confirmed the function of these TCRα LCR subelements, these hypotheses, along with the important question of what gene regulatory “machinery” these elements interact with, can now be explored.

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