

The Interleukin 7 Receptor Is Required for T Cell Receptor γ Locus Accessibility to the V(D)J Recombinase

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Abstract

Defects in the interleukin (IL)-7 signal transduction pathway lead to severe immunodeficiency in humans and in mice. In IL-7 receptor-deficient (IL-7R^{-/-}) mice, lymphoid precursors show a reduced survival rate and variable/diversity/joining region V(D)J recombination is variously affected in different loci, being arrested in the T cell receptor (TCR)- γ locus, aberrant in the immunoglobulin heavy chain (IgH) locus, and delayed in the TCR- β locus. Here, we analyze the recombination defect of the TCR- γ locus. Using ligation-mediated polymerase chain reaction, we sought intermediates of the recombination process. In the absence of the IL-7 signal, no initiation of recombination of the TCR- γ locus was observed, whereas recombination intermediates at the TCR- β locus could be detected. Thus, the failure to rearrange the TCR- γ locus is due to a failure to initiate cleavage rather than a failure to religate broken DNA ends. V(D)J recombination was previously thought to begin at the pro-T2 stage of T cell development after the arrest of IL-7R^{-/-} thymocytes at the pro-T1 stage. However, here we show that both TCR- γ and - β recombination intermediates are readily detectable in normal T1 cells, but only TCR- β intermediates were detected in IL-7R^{-/-} T1 cells, supporting a mechanistic role for IL-7 in TCR- γ locus rearrangement. Since reduced recombination activating gene (*rag*) expression has been reported in the absence of the IL-7 signal, we directly tested whether the TCR- γ locus is accessible to cleavage by recombinant Rag proteins *in vitro*. We found a reduction in chromatin accessibility for Rag-mediated cleavage in IL-7R^{-/-} thymocytes compared with wild-type. Thus, IL-7 controls recombination at the TCR- γ locus by regulating locus accessibility.

Key words: recombination • T lymphocytes • interleukins • chromatin • immunology

Introduction

IL-7, a cytokine produced by stromal cells from the thymus or bone marrow, is essential for development of lymphoid cells (for reviews, see references 1, 2). IL-7 binds to the IL-7R α chain, inducing association with the γ c chain, followed by activation of the Janus kinases Jak1 and Jak3. Deletion of any of these components, IL-7, IL-7R α , γ c, Jak1, or Jak3, results in severe inhibition of T and B cell development in mice (for a review, see reference 3). In humans, severe T cell immunodeficiency results from reduced levels of the IL-7R α chain (4) or from mutations in the genes for

γ c or Jak3 (5, 6). The requirement for IL-7 *in vivo* is partly attributable to trophic effects (IL-7 contributes to the survival of lymphoid precursor cells [7–9]), and partly due to effects on V(D)J recombination of immune receptor genes. Mice with a deletion of the IL-7R α chain show a severe reduction of recombination at the TCR- γ locus (10–12) or the distal elements of the V IgH locus (13). Mice with a deletion of IL-7 show a delay in rearrangement of the TCR- β , - γ , and - δ loci during fetal development (2).

In this report, we study in detail the recombination defects at the TCR- γ and - β loci and analyze at which stage during recombination the IL-7R^{-/-} thymocytes are arrested by searching for V(D)J recombination reaction intermediates. To test whether the absence of IL-7 leads to a change in chromatin structure, we analyzed directly the ac-

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cessibility of the TCR- γ locus to recombination-activating gene (Rag)-mediated cleavage in vitro.

Materials and Methods

PCR Analysis. IL-7R^{-/-} mice (C57BL/6 [B6], 129; reference 14) and IL-7R^{-/-} B6 mice (bred onto C57BL/6J from The Jackson Laboratory) were bred as homozygotes in microisolators. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 86-23, 1985). For each experiment, 6–10 mice were killed at the age of 4–8 wk and their thymi were pooled for DNA preparation. Each IL-7R^{-/-} thymus (14) yielded $\sim 2 \times 10^5$ thymocytes, whereas each control C57BL/6J thymus yielded $>10^8$ thymocytes. The IL-7R^{-/-} thymocytes (14) used in this study are 99% negative for the cell surface markers CD4, CD8, or CD25. Fetal thymocytes (C57BL/6J day 14 of gestation) were $>98\%$ positive for CD44 and sorted into pro-T1 (95% negative for CD25) and pro-T2 populations (91% positive for CD25). To purify pro-T1 populations from adult animals, C57BL/6J thymocytes were partially predepleted of CD4⁺ and CD8⁺ cells using biotinylated CD4 and CD8 antibodies and streptavidin-coated magnetic beads (Dyna-beads; DYNAL). The predepleted cells were stained with the appropriate antibodies in the presence of the Fc receptor blocking antibody 2.4G2 and sorted into a population termed T1* that is negative for CD4, CD8, CD25, CD3, B220, and Mac-1 and $>95\%$ c-kit⁺ (CD117⁺) using a FACStar^{PLUS}™ (Becton Dickinson). Genomic DNA (200 ng) was amplified in 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 0.001% gelatin, 0.5 U Taq polymerase (Perkin-Elmer Corp.), 0.2 pmol of each primer, 0.1 mM dNTP, for 30 cycles. Each cycle consisted of 1 min denaturation at 94°C, 1 min annealing at 60°C, and 1 min elongation at 72°C. The last cycle was followed by a 10-min elongation at 72°C. The DNA fragments were separated by agarose gel electrophoresis, blotted, and hybridized using a specific radio-labeled oligonucleotide. The primers used for detection of V γ 2-J γ 1 and V γ 3-J γ 1 are as in reference 12. The following primers were used: V β 2 sense, 5'-GCTGGAGCAAACCCAAGGTGG-3'; V β 4 sense, 5'-CTGATATGCGAACAGTATC-TAG-3'; V β 8 sense, 5'-GATGACATCATCAGGTTTTGTC-3'; V β 14 sense, 5'-GCCCTAACCTCTACTGGTAC-3'; J β 2.1 antisense, 5'-GGACGGTGTGCTGTCCCTG-3'; J β 2.1 probe, 5'-CTATGCTGAGCAGTTCTTCGG-3'; V γ 5 sense, 5'-CAACTTGAAGAAAGAATAATG-3'; V γ 4 sense, 5'-GGA-ACGAGTCTCACGTACCTCTG-3'; V γ 1.1 sense, 5'-GAA-GTATCCGTCACCAGAGAG-3'; J γ 4 antisense, 5'-ACTACGA-GCTTTGTCCTTTG-3'; J γ 2 sense, 5'-GGAATTACTATGA-GCTTTG-3'; V γ 1.2 probe, 5'-GCCGGTACCAATGTAT-AGCTG-3'; and V γ 1.2 antisense, 5'-GTCACCAGAGAGACA-GATGAG-3'.

Ligation-mediated PCR Analysis of TCR- β and TCR- γ Recombination Signal Sequence Breaks. Genomic DNA (1 μ g) was subjected to linker ligation as described (15). Individual aliquots of the linker-ligated DNA were analyzed for V(D)J recombinase-generated double strand DNA breaks at recombination signal sequences (RSSs) in the TCR- β and TCR- γ loci in PCR assays using two nested locus-specific primers (J γ -C and J γ -D, or D β 2R2 and D β 2R1) and the linker primer BW-H (15, 16). PCR products were analyzed by gel electrophoresis and Southern blot hybridization using locus-specific oligonucleotide probes (J γ -E and D β 2R). Control reactions amplify the nonrearranging

CD14 locus as described (17). The following primers and oligonucleotides were used: D β 2R2, 5'-TGGCGTAATTTCCCATGCATGTACG-3'; D β 2R1, 5'-GCTGATGGTTAACATGATAAGGGC-3'; D β 2R, 5'-GAGTTGGTGTTTTTTTGGGTAG-3'; J γ -C, 5'-TCAATTATCACTAACTCCAGGGAGAACA-3'; J γ -D, 5'-GAAGATTGCAAGATATCAGATACCACA-3'; and J γ -E, 5'-GACTTTCTAAGGAACCCAGAAGATCCG-3'.

In Vitro RSS Cleavage Assay. Nuclear substrates were purified, and in vitro cleavage reactions were performed as described (17). In brief, cleavage reactions contained 100,000 nuclei and were incubated at 30°C for 1 h in the presence of 40 mM Hepes (pH 7.6), 2.5 mM Tris-HCl (pH 8.0), 110 mM KCl, 1 mM MnCl₂, 2.5 mM dithiothreitol, 0.2 mM MgCl₂, 0.05 mM PMSF, 0.025 mM EDTA, 7% glycerol (vol/vol), 0.6 μ g rRAG-1, and 2 μ g bovine thymus nuclear extract. Nucleic acid recovered from these reactions was subjected to ligation-mediated (LM)-PCR analysis as described above.

Results and Discussion

Suppressed Recombination at All TCR- γ Clusters in IL-7R α ^{-/-} Thymocytes. Targeting of the VDJ recombinase to the immune receptor genes is under cell type-specific, stage-specific, and locus-specific control (for reviews, see references 18–20). Thus, TCR genes only fully rearrange in T cells (Ig in B cells), and TCR- β locus rearrangement precedes that of the α locus. Furthermore, rearrangement of the TCR- γ gene is strictly regulated during ontogeny such that cluster 1 V regions 3 and 4 recombine first during fetal development followed by recombination of clusters 2 and 4 and the remaining elements in cluster 1 (Fig. 1 a). IL-7 is an extracellular signal that appears to induce certain loci to become accessible for recombination, as IL-7R α ^{-/-} B cells fail to rearrange some IgV genes but succeed with others (13), and IL-7R α ^{-/-} T cells fail to rearrange the TCR- γ locus (10–12) but recombine the TCR- β locus.

Recent evidence demonstrates that there is also specific control for recombination within one locus. Mice with a deletion of the T early α (TEA) element in the TCR- α locus are only able to perform recombination for the most proximal J α elements, but fail to do so at the distal J α regions (21). IL-7R α ^{-/-} precursor B cells successfully recombine proximal V regions of their IgH locus but not the distal V regions (13), and pax5^{-/-} B cells recombine D to J but not V to DJ regions in the IgH locus (22). Since control of TCR- β rearrangement has many similarities with control of the IgH locus (e.g., both loci undergo D–J joining before V–D and both display allelic exclusion), we analyzed whether IL-7R α ^{-/-} thymocytes might show deficient recombination of the distal V regions of TCR- β using the IL-7R α -deficient strain originated by Peschon's laboratory (14). As shown in Fig. 1 b, V β 8 and V β 4 genes (located ~ 520 and 620 kb upstream of the D region elements, respectively) were recombined normally in IL-7R α ^{-/-} thymocytes compared with wild-type thymus (fetal or adult). The most distal V region V β 2 (150 kb upstream of the bulk of V regions) also showed a normal recombination frequency. However, successful recombination involving V β 14, which is located downstream of the constant region

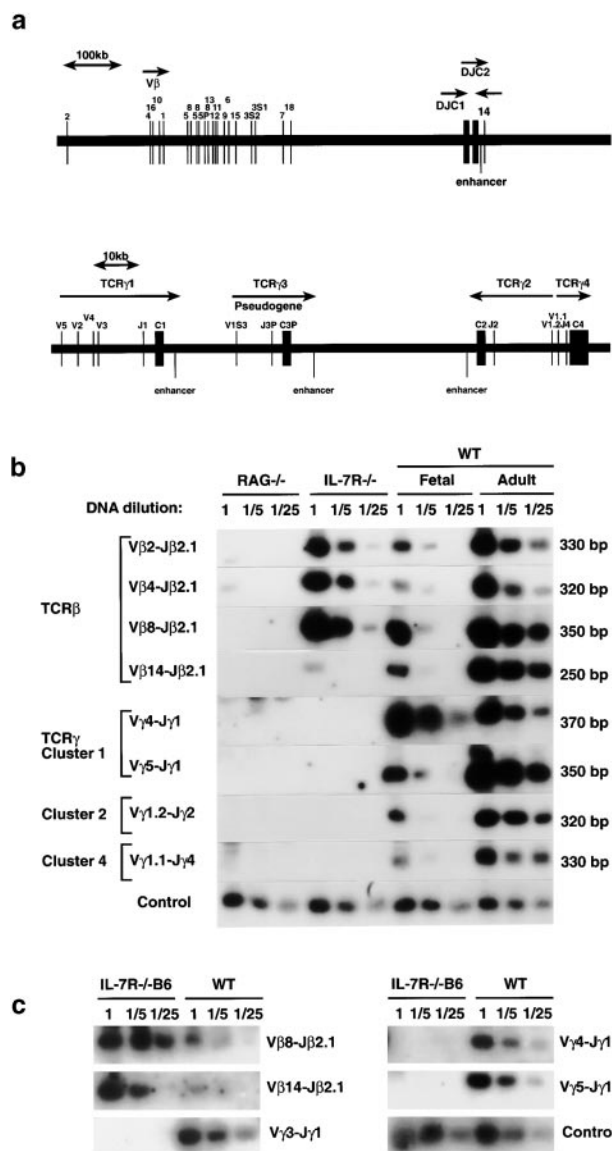


Figure 1. Suppression of recombination at all clusters of the TCR- γ locus. (a) Modified map of the murine TCR- β (top) and TCR- γ (bottom) loci (reference 32). (b) PCR analysis of genomic DNA from thymus of IL-7R $\alpha^{-/-}$ (reference 14), Rag-2 $^{-/-}$, or wild-type (WT) thymus from C57BL/6J mice derived at day 15 of gestation (Fetal) or at 4–8 wk of age (Adult). Control reaction amplifies V region of the TCR- γ locus (V2) independent of the recombination event. (c) PCR analysis of genomic DNA from thymus of IL-7R $\alpha^{-/-}$ B6 (IL-7R $\alpha^{-/-}$ strain cross-bred onto C57BL/6J for more than five generations) compared with wild-type (WT) thymus derived from C57BL/6J mice at day 15 of gestation. Control reaction amplifies V region of the TCR- γ locus (V2) independent of the recombination event.

C2 (and is rearranged by an inversion process), was less frequently detected in IL-7R $\alpha^{-/-}$ thymocytes.

In contrast to the relatively normal TCR- β pattern, the TCR- γ locus showed a severely reduced frequency of recombination at all three clusters (clusters 1, 2, and 4, as shown in Fig. 1 b). Clusters 1, 2, and 3 are 83–92% homologous in the regions consisting of J, C, and enhancer elements, and their enhancers are 98% identical (23, 24).

However, cluster 1 recombines before cluster 2 during fetal development (cluster 3 is a pseudogene). Thus, it is unlikely that the identified TCR- γ enhancer alone would be responsible for regulation of accessibility at the γ locus. Moreover, cluster 4 does not share the identified enhancer with the other clusters and cannot be recombined in the absence of IL-7 either. This suggests that the identified enhancer may be necessary but not sufficient for regulation of recombination and that as yet unidentified cis-acting control elements of the TCR- γ locus may be responsible for mediating the IL-7 effect for recombination.

The IL-7R α -deficient mice used in this study show a severe phenotype such that the thymocytes are arrested at the pro-T1 cell stage (14). However, backcrossing these mice onto C57BL/6J strain results in partial restoration of α/β T cell development although at greatly reduced numbers (for a review, see reference 2). Thus, it was important to examine the effect of IL-7R signaling on TCR- γ locus recombination in the backcrossed strain. As shown in Fig. 1 c, severe suppression of TCR- γ gene rearrangements was also found in IL-7R $\alpha^{-/-}$ B6 mice. In contrast, V β 14 rearrangement at the TCR- β locus was easily detectable in IL-7R $\alpha^{-/-}$ B6 thymocytes. Similarly, recombination intermediates at the V β 14 locus could be detected using thymocytes from the IL-7R $\alpha^{-/-}$ B6 backcross but were absent in the original IL-7R $\alpha^{-/-}$ strain (data not shown). Thus, the control of recombination at the V β 14 locus does not appear to depend solely on IL-7R signaling and can be substituted by as yet unidentified genetic factors. In contrast, control of recombination at the TCR- γ locus requires the IL-7R signal, and thus was chosen for further, more detailed studies.

Absence of γ Locus Recombination Intermediates in IL-7R $\alpha^{-/-}$ Thymocytes. Successful V(D)J recombination involves the targeting of the recombinase to the appropriate site, the cleavage of the signal sequence, the generation of hairpin coding ends and blunt signal ends, and the ligation of these ends. To understand which of these recombination processes is controlled by the IL-7R signal, we analyzed at which stage during recombination of the TCR- γ locus the arrest occurred.

Recombination intermediates (signal sequences that have been cleaved but not yet religated) for the TCR- γ locus were examined using a previously described LM-PCR approach (15). Fig. 2 b shows that IL-7R $\alpha^{-/-}$ T cells had no detectable broken DNA ends at the RSSs within the TCR- γ locus, whereas the β locus serving as a control did have such broken ends. This result was observed using either the IL-7R $\alpha^{-/-}$ mixed background strain (14) and the IL-7R $\alpha^{-/-}$ B6 backcross. This indicates that IL-7R $\alpha^{-/-}$ thymocytes fail to initiate cleavage at the signal sequences in the TCR- γ locus, rather than failing in subsequent steps of recombination such as end modification or ligation. This is in contrast to the recently reported TCR- β enhancer knockout mouse (25) that failed to rearrange the TCR- β locus, but for different reasons: cleavage was initiated, although at reduced levels, but ligation of coding ends could not be successfully completed (16).

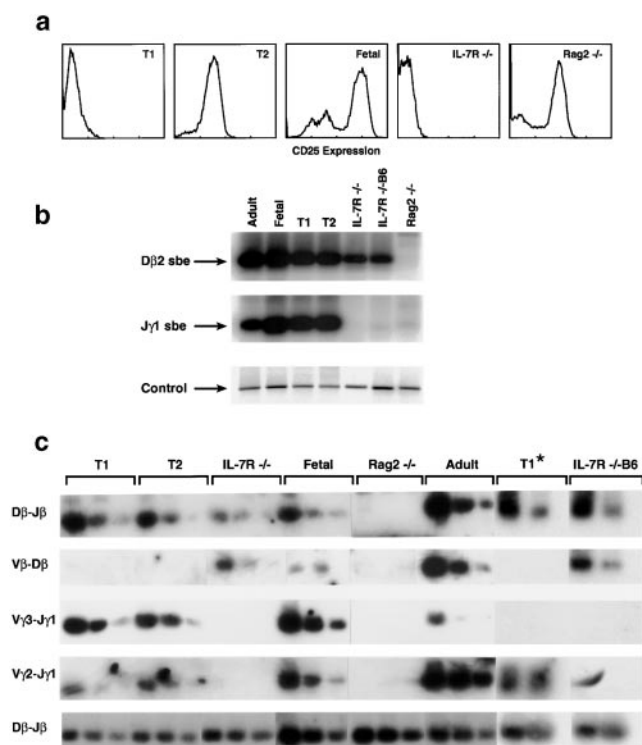


Figure 2. Failure to initiate recombination at the TCR- γ locus. (a) FACS[®] analysis for CD25 cell surface expression for thymocytes of IL-7R $\alpha^{-/-}$ mice (reference 14), Rag-2 $^{-/-}$ thymocytes, or wild-type thymocytes from C57BL/6 mice at day 15 of gestation (Fetal). Pro-T1 and -T2 thymocytes were sorted by CD44/CD25 cell surface expression from C57BL/6J mice at day 14 of gestation. (b) To detect broken DNA ends in the TCR- γ or - β loci, genomic DNA from IL-7R $\alpha^{-/-}$ thymocytes (14), thymocytes from IL-7R $\alpha^{-/-}$ B6 backcrossed mice, Rag-2 $^{-/-}$ thymocytes, or wild-type thymocytes from C57BL/6J mice at day 15 of gestation (Fetal) or 4–8 wk old (Adult) or pro-T1 or -T2 cells were linker ligated and analyzed by PCR. Control reactions amplify the CD14 gene that does not undergo recombination. sbe, signal broken end. (c) To detect complete rearrangements for the gene segments indicated to the right, PCR was performed using genomic DNA that was titrated for better comparison in 1:5 dilution steps. T1 and T2 populations were purified from day 14 fetal thymocytes. T1* populations were purified as a CD4 $^{-}$ CD8 $^{-}$ CD25 $^{-}$ c-kit $^{+}$ population from adult thymus and directly compared with IL-7R $\alpha^{-/-}$ B6 backcrossed thymocytes using consecutive PCR analysis (20, 40, and 60 cycles).

IL-7R $\alpha^{-/-}$ thymocytes appear to be arrested at the pro-T1 stage based on their CD44 $^{+}$ CD25 $^{-}$ surface phenotype; this is the stage of the earliest thymic immigrants from bone marrow or fetal liver. Early thymic development then proceeds through pro-T2 (CD44 $^{+}$ CD25 $^{+}$), pro-T3 (CD44 $^{-}$ CD25 $^{+}$), and pro-T4 (CD44 $^{-}$ CD25 $^{-}$) stages, followed by further intermediate and late stages. T cells with a deletion of either Rag-1 or Rag-2, which are unable to rearrange TCR genes, are arrested at the pro-T3 stage with high levels of CD25 expression (see Fig. 2 a). Thus, progression beyond pro-T3 requires gene rearrangement, and normal pro-T3 cells indeed show extensive rearrangements. It is not clear, however, at which stage rearrangement begins. As the TCR- β locus is rearranged in IL-7R $\alpha^{-/-}$, it implies that this locus can undergo rearrangement at the pro-T1 stage. However, a previous study

using Southern blot hybridization failed to detect TCR- β locus rearrangement at this stage (26). Determining the onset of TCR- γ rearrangement has relevance to the IL-7R mechanism, in that if it occurred later than the pro-T1 stage, IL-7R $\alpha^{-/-}$ thymocytes might fail to rearrange the γ locus because they fail to mature to the stage at which rearrangement normally occurs.

As shown in Fig. 2 b, recombination intermediates for both TCR- γ and - β loci were found in both pro-T1 and -T2 populations sorted from fetal thymocytes, indicating that the onset of rearrangement of both loci clearly begins in pro-T1 cells. The recombination is not only initiated at pro-T1 and -T2 but also successfully completed, since V-J joins can be detected in the γ locus and D-J (but not V-D) joins in the β locus in T1 as well as T2 populations sorted from fetal thymus (as shown in Fig. 2 c). For additional comparison, pro-T1 cells were purified from adult thymocytes as a CD4 $^{-}$ CD8 $^{-}$ CD25 $^{-}$ c-kit $^{+}$ population. Similarly to the fetal T1 populations, these T1 cells derived from adult mice also showed complete D-J joining (but not V-D joining) at the TCR- β locus as well as completed V-J joining at the TCR- γ locus. Another study failed to demonstrate D-J joining at the β locus in “adult” T1 populations using distinct purification techniques that may have depleted the recombined fraction we were able to detect (27). However, the T1 population we purified from fetal or adult thymocytes does not appear to contain mature T cell contaminants, since only D-J but not V-D joining could be detected at the TCR- β locus, indicating a discrete stage of T cell differentiation.

The finding that at least some rearrangements normally occur at the pro-T1 stage has two implications. First, it explains how IL-7R $\alpha^{-/-}$ thymocytes, whose surface markers correspond to pro-T1 cells (14), could initiate TCR- β rearrangements (which then become complete V-DJ joinings without expressing the usual T3 surface markers). Second, it shows that the TCR- γ locus can fully rearrange in pro-T1 cells; therefore, the failure of the TCR- γ locus to rearrange in IL-7R $\alpha^{-/-}$ cells is due to a selective failure to initiate cleavage of the TCR- γ locus, rather than to a failure to progress to later stages of development.

Reduced TCR- γ Locus Accessibility in IL-7R $\alpha^{-/-}$ Thymocytes. IL-7R $\alpha^{-/-}$ thymocytes are unable to initiate cleavage at the TCR- γ locus (this paper), and we previously showed a reduction of sterile transcripts from the V γ and C γ regions (12). Recently, it has been demonstrated that the transcription factor signal transducer and activator of transcription 5 (Stat5) can partially replace the IL-7 signal for both germline transcription and rearrangement from the TCR- γ locus (28). Transcription is thought to be an indicator of “open” chromatin, which would also be accessible to the VDJ recombinase (29). However, recent evidence suggests that transcription and recombination may not always strictly correlate. Deletion of the TCR- β enhancer suppresses transcription of the locus but cleavage by the VDJ recombinase can still be performed (albeit at a reduced rate [16, 25]). Thus, we tested directly whether the TCR- γ locus in IL-7R $\alpha^{-/-}$ thymocytes was accessible

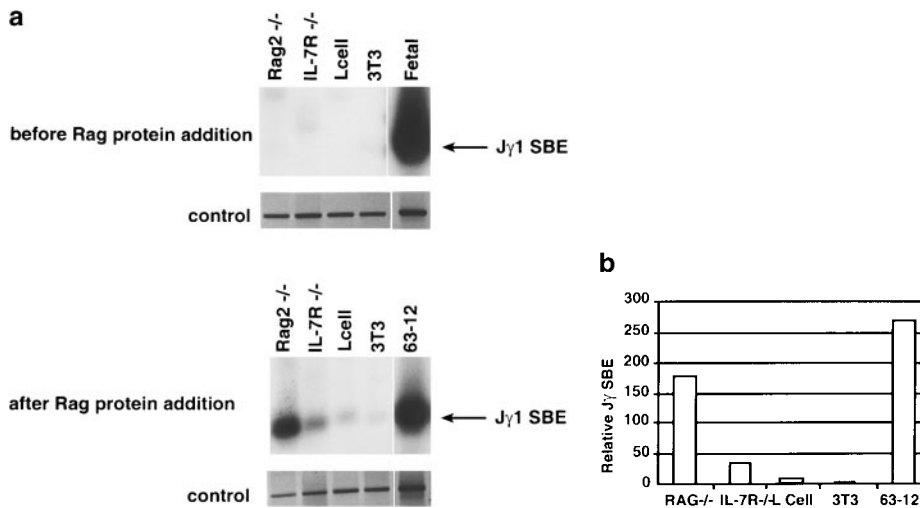


Figure 3. Reduced accessibility of the TCR- γ locus in IL-7R $\alpha^{-/-}$ thymocytes to Rag-mediated cleavage. (a) Nuclei were prepared from IL-7R $\alpha^{-/-}$ (14) or Rag-2 $^{-/-}$ thymus, from fibroblast cell lines (3T3, L cells), or from the pro-B cell line 63-12 and incubated with recombinant Rag-1 and fetal cow thymus nuclear extract for 60 min. Genomic DNA was extracted before or after the addition of Rag proteins, linker ligated, and the PCR reaction was performed for detection of broken DNA ends. Control reactions amplify the CD14 gene that does not undergo recombination. SBE, signal broken end. (b) The results of the Rag-mediated cleavage experiment were analyzed by PhosphorImager[®] (Molecular Dynamics) and expressed as relative breakage at the TCR- γ locus.

to Rag-mediated in vitro cleavage (17). Nuclei purified from IL-7R $\alpha^{-/-}$ thymocytes were incubated with exogenous Rag protein. Nuclei from Rag-2 $^{-/-}$ thymocytes were used as a control because they also fail to initiate cleavage at the γ locus, but have a normal accessibility to Rag cleavage at this site. Fig. 3 a shows that no broken DNA ends can be detected at the TCR- γ locus in the chromatin of IL-7R $\alpha^{-/-}$ or Rag-2 $^{-/-}$ thymocytes before addition of the Rag proteins. However, after incubation with the Rag proteins, only the chromatin from Rag-2 $^{-/-}$ thymocytes could be efficiently cleaved in contrast to chromatin from IL-7R $\alpha^{-/-}$ thymocytes. As shown in Fig. 3 b, the cleavage rate in vitro is greatly reduced for IL-7R $\alpha^{-/-}$ compared with Rag-2 $^{-/-}$ nuclei. A small degree of cleavage that was detectable in IL-7R $\alpha^{-/-}$ thymocytes may indicate a minor pathway independent of IL-7R signaling that controls chromatin accessibility at the TCR- γ locus. However, this minor pathway does not lead to substantial Rag-mediated cleavage rates in vivo (Fig. 2 b). Thus, IL-7 is the main effector in controlling the accessibility of the chromatin at the TCR- γ locus for the V(D)J recombinase.

Mononucleosomes, the smallest units of chromatin structure, have been shown in vitro to inhibit cleavage by the Rag proteins (30, 31) and can be modulated by high mobility group 1 (HMG1) proteins (31). Several histone modifications have been suggested to relieve the inhibitory effects of mononucleosomes on transcription, among them histone acetylation. Trichostatin A, a specific inhibitor of histone deacetylase, can overcome the block caused by the absence of the IL-7 signal, suggesting that histone acetylation can relieve the block caused by mononucleosomes (12). However, the acetylation of histone tails only minimally influences the mononucleosomal structure directly. Thus, it is thought that histone acetylation may rather attract other factors that can interrupt mononucleosomal arrays. The sucrose nonfermenting (SNF) or nucleosome-remodeling factor (NURF) complexes, which contain SNF2 factors thought to be the ATP-driven motors of chromatin

remodeling, are candidates. However, no recombination-specific mononucleosomal remodeling complex has yet been identified. Furthermore, no cis-acting DNA elements have been identified that are required for targeting the TCR- γ locus. The inaccessibility of the chromatin at the TCR- γ locus in the absence of the IL-7 signal may serve as a model to understand the mechanism by which chromatin is altered to allow for recombination.

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