Biochemical and Functional Analyses of Chromatin Changes at the TCR-β Gene Locus During CD4−CD8− to CD4+CD8+ Thymocyte Differentiation

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Allelic exclusion is the process wherein lymphocytes express Ag receptors from only one of two possible alleles, and is effected through a feedback inhibition of further rearrangement of the second allele. The feedback signal is thought to cause chromatin changes that block accessibility of the second allele to the recombinase. To identify the putative chromatin changes associated with allelic exclusion, we assayed for DNase I hypersensitivity, DNA methylation, and transcription in 100 kb of the TCR-β locus. Contrary to current models, we identified chromatin changes indicative of an active and accessible locus associated with the occurrence of allelic exclusion. Of 11 DNase I hypersensitive sites identified, 3 were induced during CD4−CD8− to CD4+CD8+ thymocyte differentiation, and demethylation and increased germline transcription of the locus were evident. We further examined the role of the most prominently induced site near the TCR-β enhancer (Eβ) in allelic exclusion by targeted mutagenesis. Two other sites were also examined in New Zealand White (NZW) mice that have a natural deletion in the TCR-β locus. TCR-β gene recombination and allelic exclusion were normal in both mutant mice, negating dominant roles for the three hypersensitive sites in the control of allelic exclusion. The data suggest that alternative cis-regulatory elements, perhaps contained in the Eβ enhancer and/or in the upstream Vβ region, are involved in the control of TCR-β allelic exclusion. The Journal of Immunology, 1998, 160: 1256–1267.

Immunglobulins and TCRs are heterodimers, consisting of heavy and light chains or α- and β-chains, respectively. The Ag-binding domains of Ig and TCR chains are encoded by variable gene segments, V, D, and J, for IgH and TCR-β-chains, and V and J for IgL and TCR-α-chains. Assembly of the variable gene segments through V(D)J recombination is required before the Ag receptor genes can be expressed (1–3). Because vertebrates are diploid and one-third of V(D)J recombination events result in the expression of functional protein chains (1, 4, 5), expression of functional protein chains (1, 4, 5), one-fifth of lymphocytes are expected to express any given Ag receptor chain from both alleles. However, the majority of lymphocytes express Ag receptors from only one of the two alleles. This phenomenon is known as allelic exclusion (reviewed in Refs. 6 and 7). Allelic exclusion was first observed in rabbits using allotypic markers for Ig heavy chains. Individual B cells were found to express Ig heavy chain from only one of the two alleles (8, 9). Similar studies with human and mouse B cells yielded the same results. Using Vβ-specific Abs, 99% of T cells were also found to express TCR-β chain from only one of the two alleles (10, 11). Compared with the IgH, IgL, and TCR-β genes, allelic exclusion at the TCR-α gene locus is less stringent (12, 13).

Over the years, many studies were performed in an attempt to elucidate the mechanisms that underlie the control of allelic exclusion. The presence of DJ rearrangements at the second excluded IgH locus in mature B cells and at the TCR-β locus in mature T cells strongly suggests that allelic exclusion is achieved through a mechanism regulating V(D)J recombination (5, 14). According to current understanding, each differentiating lymphocyte has two chances of assembling a productive VDJ rearrangement. Because DJ rearrangements occur on both alleles, the regulated step is at the V to DJ rearrangement step. If the first VDJ rearrangement is productive, then V to DJ joining on the other allele is inhibited. If the initial VDJ rearrangement is nonproductive, then V to DJ rearrangement can proceed on the second allele. Feedback inhibition of further rearrangement by a prior productive recombination event has been demonstrated by studies with transgenic and gene-targeted mice. Expression of the membrane-bound, but not the secreted μ heavy chain inhibits VH to DJH rearrangements at the endogenous IgH loci (15–18). Targeted deletion of μexons also abolishes IgH gene allelic exclusion (19). Similarly, expression of a TCR-β transgene blocks rearrangements of the endogenous loci at the DJβ stage (20, 21). Complete rearrangement and expression of the endogenous alleles occur only when the TCR-β transgene is deleted (22). In pro-B cells, the μ heavy chains form pre-B cell receptors by associating with surrogate light chains, λ, and VpreB, and Igα/Igβ heterodimers (pre-BCR) (23, 24). Signals initiated from the pre-BCR mediate not only IgH gene allelic exclusion, but also pro-B to pre-B cell differentiation and expansion of pre-B cells (6, 7, 25, 26). In an analogous manner, TCR-β chains associate with the pre-TCR-α chains (pTα) and CD3 components to form pre-TCRs (pre-TCR) (27, 28), which signals for

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1 The first two authors contributed equally to the work.

4 Abbreviations used in this paper: BCR, B cell receptor; DN, double negative; DP, double positive; Eβ, T cell receptor-β gene enhancer; ES, embryonic stem; HS, DNase I hypersensitive site; neo, neomycin-resistance gene; NZB, New Zealand Black; NZW, New Zealand White; RAG, recombination activating gene; RSB, reticulocyte standard buffer; SP, single positive.
TCR-β gene allelic exclusion as well as DN to DP thymocyte differentiation and the expansion of DP thymocytes (29–31). Thus, allelic exclusion at IgH and TCR-β loci is mediated through a feedback inhibition of V to DJ rearrangement, occurring during pro-B to pre-B cell and DN and DP thymocyte differentiation, respectively.

The feedback signal initiated from pre-BCR and pre-TCR could effect allelic exclusion by regulating recombinase activity as well as modulating substrate gene segment accessibility. It is known that pro-B to pre-B cell and DN to DP thymocyte differentiation is accompanied by a transient down-regulation of RAG-1 and RAG-2 gene transcription (32, 33). Furthermore, RAG-2 protein is degraded before cells enter the S phase, as occurring in rapid proliferating cells during pre-B cell and DP thymocyte expansion (34–36). Although the down-regulation of RAG expression following the feedback signaling has been postulated to prevent further V to DJ rearrangement on the second allele, thereby enabling the establishment of allelic exclusion (37), coupling of V(D)J recombination to the cell cycle is not essential for allelic exclusion (36, 38). RAG genes are re-expressed in pre-B cells and DP thymocytes for rearranging IgL and TCR-κ genes, respectively (39–41). To prevent V to DJ rearrangement on the second IgH and TCR-β allele in pre-B cells and DP thymocytes, respectively, an additional mechanism is required to specifically maintain their allelic exclusion. Because V(D)J recombinations at all Ig and TCR loci are mediated by conserved recombination signal sequences (1, 3), it seems unlikely that the control is achieved solely by modifying the recombinase so that it no longer utilizes IgH or TCR-β alleles as substrates. Rather, it seems more likely that allelic exclusion is maintained by controlling the accessibility of the involved gene segments to the recombinase (6, 7). In this model, signals from pre-BCR and pre-TCR induce the differentiation and proliferation of pre-B cells and DP thymocytes as well as stimulate chromatin structural remodeling at the IgH and TCR-β loci, resulting in inaccessibility to the recombinase. Supporting this model, exogenously added RAG-1 and RAG-2 core proteins can introduce dsDNA breaks at the recombination signal sequences flanking DγH and Vγ gene segments in nuclei of pro-B cells, but not pre-B cells (42). However, chromatin changes associated with allelic exclusion, their molecular nature, and the underlying cis-regulatory elements involved still need to be defined to prove this model.

Changes in DNase I hypersensitive sites, DNA methylation, and transcription have traditionally been used as indicators of changes in chromatin structure (43–48). V(D)J recombination activity is associated with increased DNase I hypersensitivity, hypomethylation, and transcription, before recombination, IgH and κ locus becomes hypersensitive to DNase I treatment, hypomethylated, and actively transcribed (49–52). Hypomethylation of recombination substrates also promotes their rearrangements in both cell lines and transgenic mice (53, 54). In addition, transcription enhancers such as Eμ, Ex, Eβ, and Eα are all known to promote recombination of their respective genomic loci and minilocus recombination substrates, although their mechanism of action remains elusive (41, 55–64). According to current understanding, allelic exclusion should be associated with a decrease in HS, hypomethylation, and diminished transcription at the IgH and TCR-β locus, all indicators of an inaccessible locus.

To characterize the chromatin changes associated with allelic exclusion at the DP stage of thymocyte development and to identify the cis-regulatory elements involved, we assayed for DNase I hypersensitive sites, DNA methylation, and transcription in DN and DP thymocytes in a 100-kb region of the TCR-β locus starting 20 kb upstream of DJβ1 and ending 50 kb downstream of Vβ14, Contrasting the current understanding of the control of V(D)J recombination, our data suggest that DNase I hypersensitivity, DNA methylation, and transcription in general may not be reliable molecular indicators of inaccessibility to the recombinase during allelic exclusion. In addition, we demonstrate that three of the DNase I hypersensitive sites identified are not critical for TCR-β gene allelic exclusion. These findings suggest that alternative cis-regulatory elements within the assayed region such as Eβ enhancer and/or in the upstream Vβ region are likely to play a dominant role in allelic exclusion control.

Materials and Methods

Mice

RAG-1- and RAG-2-deficient mice were obtained from Drs. Susumu Tonegawa (Massachusetts Institute of Technology, MA) and Fred Alt (Harvard Medical School, MA), respectively (65, 66). Activated lck transgenic mice were from Dr. Roger Perlmuter (University of Washington, WA) (67). Two different TCR-β transgenic mouse strains, one from Dr. Mark Davis (Stanford University School of Medicine, CA) and one from Dr. Eugenia Spanopoulou (Mount Sinai School of Medicine, NY), were used in the present studies. The former was constructed from a genomic TCR-β transgene, and the latter from a cDNA TCR-β transgene (68, 69). The two strains of TCR-β transgenic mice are essentially the same in our assays, except that Jβ2-Cβ2 intronic probes did not hybridize to the cDNA TCR-β transgene, and thus simplified Southern blot hybridization patterns in this assay. The same pattern of DNase I hypersensitive sites was detected in DN thymocytes from either RAG-1- or RAG-2-deficient mice (data not shown). Similarly, the same pattern of DNase I hypersensitive sites was detected in DP thymocytes from anti-CD3ε Ab-treated RAG-1- or RAG-2-deficient mice (data not shown), using a protocol developed by Shinkai and Alt (70), and later experiments were conducted with RAG-2-deficient mice only. TCR-β and lck transgenes were also on the RAG-2-deficient background. Cre transgenic deleter mouse was from Klaus Rajewsky (University of Cologne, Germany) (71). NZW mice were from The Jackson Laboratory (Bar Harbor, ME). The genomic TCR-β transgene was also introduced into the NZW mice and mice harboring a targeted deletion of Hs1 (see below). Mice were maintained under specific pathogen-free condition in the animal facilities at Massachusetts Institute of Technology (Cambridge, MA).

Probes and genomic clones

Two cosmid clones containing DJβ1, Jβ1, CB1, DJβ2, Jβ2, CB2, VB14, and 30 kb downstream of Vβ14 were kindly provided by Dr. Marie Malissen (Centre d’Immunologie, INSERM-CNRS de Marseille-Luminy, France) (72). A genomic clone extending 20 kb further downstream of the existing clones was identified by PCR from a Stratagene (La Jolla, CA) AKR mouse SuperCos library and by filter hybridization using probe H (Table I and Fig. 2A). Probes isolated from the cosmid clones are summarized in Table I and in Figure 2A. The probes used to assay for homologous recombination in

<table>
<thead>
<tr>
<th>Name</th>
<th>Size (kb)</th>
<th>Digest</th>
<th>Hybridizing Fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.6</td>
<td>HindIII-NcoI</td>
<td>9.0</td>
</tr>
<tr>
<td>B</td>
<td>0.9</td>
<td>ClaI-EcoRI</td>
<td>3.3</td>
</tr>
<tr>
<td>C</td>
<td>1.9</td>
<td>NcoI-HindIII</td>
<td>6.0</td>
</tr>
<tr>
<td>D</td>
<td>1.2</td>
<td>BamHI-SpeI</td>
<td>4.0</td>
</tr>
<tr>
<td>E</td>
<td>1.6</td>
<td>HindIII-BglII</td>
<td>6.7</td>
</tr>
<tr>
<td>F</td>
<td>1.2</td>
<td>BglII-BglII</td>
<td>6.0</td>
</tr>
<tr>
<td>G</td>
<td>1.1</td>
<td>BgIII-EcoRI</td>
<td>9.3</td>
</tr>
<tr>
<td>H</td>
<td>0.8</td>
<td>PstI-XbaI</td>
<td>9.0</td>
</tr>
<tr>
<td>I</td>
<td>0.8</td>
<td>ApaI-EcoRI</td>
<td>10.5</td>
</tr>
<tr>
<td>J</td>
<td>0.7</td>
<td>ApaI-EcoRI</td>
<td>16.0</td>
</tr>
<tr>
<td>K</td>
<td>0.7</td>
<td>ApaI-EcoRI</td>
<td>10.0</td>
</tr>
<tr>
<td>L</td>
<td>0.9</td>
<td>EcoRV-PstI</td>
<td>9.4</td>
</tr>
<tr>
<td>M</td>
<td>0.9</td>
<td>EcoRV-PstI</td>
<td>6.0</td>
</tr>
<tr>
<td>N</td>
<td>2.4</td>
<td>HindIII-HindIII</td>
<td>10.0</td>
</tr>
<tr>
<td>O</td>
<td>1.3</td>
<td>EcoRI-EcoRV</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Summary of the probes used to identify DNase I hypersensitive sites and their respective hybridizing fragments

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ES cells and for allelic exclusion in thymocytes are as follows: probe 1, 0.6-kb HindIII-PstI fragment; probe 2, 0.9-kb EcoRV-PstI fragment; and probe 3 is the same as probe H. Probes used for Northern hybridization were provided by Dr. Susumu Tonegawa and are as follows: Cβ2, a 430-bp cDNA fragment; Vβ8, a 190-bp EcoRI-PstI fragment; and Vβ14, a 668-bp Acel genomic fragment.

**Isolation of thymocytes**

Single cell suspensions of thymocytes were prepared for the isolation of DNA and RNA. Because RAG-deficient thymus contains only a few million cells compared with a hundred million cells in normal thymus (Table II), larger numbers of thymus were pooled to obtain enough cells for DNA and RNA isolation. Special caution was taken to remove thymic stromal cells by filtering through a nylon mesh. Thymocyte preparations were assayed for purity by FACS staining for CD4 and CD8. In general, thymocytes at least 95% DN cells from RAG mice and 95% DP cells from TCR-β/RAG, lck/RAG, and CD3/RAG mice. Thymocytes of TCR-β mice contained 80% DP cells.

**DNase I treatment**

Nuclei from DN and DP thymocytes were prepared according to Forrester et al. (73). Briefly, 1 × 10^9 cells were washed in cold PBS, centrifuged at 1000 rpm for 5 min, and resuspended into 10 ml reticulocyte standard buffer (RSB) containing 10 mM Tris-Cl, pH 8, 10 mM NaCl, and 10 mM MgCl₂. After slowly adding an equal volume of cold RSB containing 0.2% Nonidet P-40, the mixture was kept on ice for 10 min and centrifuged at 900 rpm for 5 min, and the resulting nuclei were resuspended in 2 ml of RSB. Two-hundred-microliter aliquots of nuclei were then treated with a range of DNase I (0.125–15 μg/ml) at 37°C, and the digestion was stopped by adding 200 μl of 2× lysis buffer containing 1.2 M NaCl, 20 mM Tris-Cl, pH 8, 10 mM EDTA, and 1% SDS. The lysates were treated with proteinase K overnight at 55°C, and DNA isolated by phenol-chloroform extraction.

**Southern and Northern hybridization**

For Southern analysis, 10 to 15 μg of DNA from DN and DP thymocytes were digested with specific enzymes and were fractionated on a 0.9% agarose gel. After denaturation and neutralization, DNA was transferred to Z-probe filters and hybridized with specific probes labeled with [α-32P]dCTP. For Northern analysis, 10 or 20 μg of RNA from DN and DP thymocytes were fractionated on a 0.9% agarose gel. RNA was transferred onto Z-probe filters and hybridized with specific probes labeled with [α-32P]dCTP. Filters were washed twice for 30 min in 2× SSC and 0.1% SDS at 65°C. Hybridization signals were detected by phosphor imaging and autoradiography.

**Targeted mutagenesis of HS1**

Mice harboring a targeted deletion of HS1 were constructed by targeted mutation in embryonic stem (ES) cells and then deriving a mouse strain from the mutant ES cells. The targeting vector was constructed by replacing a 780-bp XcmI-BglII fragment containing HS1 with a phosphoglycerate kinase promoter-driven neomycin-resistance (neo) gene flanked by loxP sites (Fig. 5). The targeting vector contained 5.9-kb and 8.2-kb homologous sequences at the 5’ and 3’ ends, respectively, and the thymidine kinase gene was inserted just outside of the 3’ homologous sequence. The targeting vector was transfected into J1 ES cells, and doubly resistant clones were picked from three independent transfections. Homologous recombinants were identified by EcoRI digestion of DNA and hybridization with a 0.6-kb HindIII-PstI fragment that does not hybridize to randomly integrated constructs. Among 258 clones analyzed, 31 yielded the expected hybridizing fragments at 10 and 7.4 kb from the normal and the targeted alleles, respectively. The correct targeting of the 31 positive ES cell clones was confirmed by Southern blotting using the deleted 780-bp fragment as a probe, and then a 9.9-kb BglII-Acel fragment containing the Efl as a probe. ES cells from 5 of the 31 clones were injected into C57BL/6 blastocysts. Chimeras with more than 95% agouti coat color were generated from all five clones and were bred with cre transgenic deleter mice for germine transmission and deletion of the introduced neo gene at the same time (71). Heterozygous mutant mice were interbred to obtain homozygous mutant mice, and the genomic TCR-β transgene was bred onto the mutant background. Data shown are from littersmates and/or age-matched mice.

### Results

**Experimental design**

To identify chromatin changes associated with allelic exclusion, we chose the TCR-β locus for study because it fulfilled two requirements (Fig. 1). First, the DNA region in which we sought to characterize differences in DNase I hypersensitive sites, DNA methylation, and transcription could be narrowed down to a manageable size. The IgH locus, which spans several megabases (74), is too large for such a study. In contrast, the entire TCR-β locus is within 600 kb (75, 76). Although it is still not feasible to analyze the entire TCR-β locus, we postulated that the chromatin changes associated with TCR-β allelic exclusion would most likely occur in a 100-kb region, starting 20 kb upstream of Dβ1 and ending 50 kb downstream of Vβ14 (Fig. 1, see Discussion). Second, DN and DP thymocytes representing the two developmental stages before and after allelic exclusion were readily available (77). Although the normal thymocyte population is a mixture containing approximately 5% DN, 80% DP, and 15% CD4 or CD8 single-positive (SP) thymocytes, a relatively pure population of DN and DP thymocytes can be obtained easily from several mutant/transgenic mouse strains (Table II). In RAG-2-deficient mice (referred to as RAG mice), thymocytes are all DN due to a block in V(D)J recombination nor-mally occurs (78, 79). Thus, the status of DNase I hypersensitivity,

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**Table II. List of transgenic/knockout mice and their thymocyte phenotypes and numbers**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Description</th>
<th>Phenotype</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAG</td>
<td>RAG-2-deficient mice</td>
<td>DN</td>
<td>10⁶</td>
</tr>
<tr>
<td>CD3/RAG</td>
<td>RAG-2-deficient mice treated</td>
<td>DP</td>
<td>10⁶</td>
</tr>
<tr>
<td>lck/RAG</td>
<td>with anti-CD3 Abs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCR-β/RAG</td>
<td>RAG-2-deficient mice</td>
<td>DP</td>
<td>10⁶</td>
</tr>
<tr>
<td>TCR-β</td>
<td>TCR-β transgenic mice on</td>
<td>DP (80%)</td>
<td>10⁵</td>
</tr>
</tbody>
</table>

---

**FIGURE 1.** Genomic organization of the TCR-β locus. Vertical lines and boxes indicate Vβ, Dβ, Jβ, and Cβ gene segments, and horizontal arrows indicate transcriptional orientations of the respective gene segments. A trypsinogen gene (Try) is located 20 kb upstream of Dβ1.
DNA methylation, and transcription in the region most likely represents the chromatin structure that is accessible to the V(D)J recombinase. On the other hand, introduction of a functionally assembled TCR-β transgene or an activated form of protein tyrosine kinase lck transgene onto the RAG-2-deficient background (referred to as TCR-β/RAG and lck/RAG mice, respectively), or injection of anti-CD3ε Abs into RAG-2-deficient mice (referred to as CD3/RAG mice) restores the differentiation of DP thymocytes to normal levels (Table II) (70, 80–82). Thymocytes in TCR-β/RAG, lck/RAG, and CD3/RAG mice do not undergo further differentiation into SP T cells due to their inability to rearrange the TCR-α locus and express a functional TCR, and thus are more than 95% DP. As a result of signaling through the pre-TCR pathway (28), the endogenous TCR-β loci in these DP thymocytes are likely to have undergone chromatin changes associated with allelic exclusion, even though they are still in the germline configuration. Nevertheless, to more closely mimic the normal situation, we also analyzed DNase I hypersensitive sites in thymocytes (80% DP) from TCR-β transgenic mice (80% DP) from TCR-β/RAG mice (RAG+), (referred to as TCR-β mice) in which the endogenous TCR-β loci undergo DβJβ rearrangement (Table II) (20, 21). If the same pattern of DNase I hypersensitive sites, methylation, and transcription is observed in DP thymocytes from TCR-β, TCR-β/RAG, lck/RAG, and CD3/RAG mice, these changes most likely represent the chromatin structure that is inaccessible to the V(D)J recombinase.

**Analysis for DNase I hypersensitive sites**

To begin our investigation, DNase I hypersensitive sites at the TCR-β locus were assayed in DN and DP thymocytes. Thymocytes were harvested from all five mutant/transgenic mice (Table II). Aliquots were analyzed by flow cytometry for CD4 and CD8 expression. Thymocytes were 98% DN from RAG mice; 97% DP from TCR-β/RAG, lck/RAG, and CD3/RAG mice; and 3% DN, 80% DP, and 17% SP from TCR-β mice (data not shown). Nuclei were prepared from the remaining thymocytes and treated with different amounts of DNase I (see Materials and Methods). DNA isolated was assayed for DNase I concentration was doubled for each successive treatment. For example, DNase I concentrations used to treat nuclei of DN thymocytes were 0.125, 0.25, 0.5, and 2 μg/ml. DNase I concentrations used to treat nuclei of DP thymocytes from lck/RAG mice were 0.125, 0.25, 0.5, and 1 μg/ml. DNase I concentrations used to treat nuclei of DP thymocytes from lck/RAG mice were 0.125, 0.25, 0.5, and 1 μg/ml.

**FIGURE 2.** Analysis of DNase I hypersensitive sites at the TCR-β locus in DN and DP thymocytes. A, Summary of DNase I hypersensitive sites (HS) identified in the 100-kb region of the TCR-β locus. Probes used in the study are labeled A to M, and their corresponding hybridizing fragments are shown as the lines below (see Table I). Arrows indicate the positions of the DNase I hypersensitive sites 1 through 11. Major and minor hypersensitive sites are indicated by larger and smaller arrows, respectively. Open circles are sites that did not change between DN and DP thymocytes, and filled circles are sites that were induced in DP thymocytes. B, Autoradiograms of Southern hybridization experiments showing HS1 through HS4. DNA was isolated from DNase I-treated nuclei of DN (RAG) and DP thymocytes (CD3/RAG, lck/RAG, TCR-β/RAG, and TCR-β), digested with BamHI plus BglII, and hybridized with probe E. In all five panels, DNA in the first lane was isolated from nuclei that were not treated with DNase I. For DNase I treatment, the lowest concentration was 0.125 μg/ml. DNase I concentration was doubled for each successive treatment. For example, DNase I concentrations used to treat nuclei of DN thymocytes were 0.125, 0.25, 0.5, and 1 μg/ml. DNase I concentrations used to treat nuclei of DP thymocytes from lck/RAG mice were 0.125, 0.25, 0.5, 0.1, and 2 μg/ml.

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DNA was digested by BamHI plus BglII, and the blots were hybridized with probe E, a 1.6-kb HindIII-BglII fragment from 5′ of the Vβ14 gene segment (Table I and Fig. 2A). In addition to the expected 6.7-kb fragment, with increased DNase I concentrations, four smaller hybridizing fragments representing HS1, HS2, HS3, and HS4 were detected at 4.4, 3, 1.2, and 0.8 kb, respectively (Fig. 2B). The minor fragments (HS3 and HS4) were not hybridization to sequences between HS1 and HS2, because probe E does not extend to the region (Fig. 2A). Furthermore, they were detected by probe E when NcoI that cuts in between HS2 and HS3 was used to digest DNAs (data not shown). HS2 and HS3 were detected in all five DNA samples, and therefore are present in both DN and DP thymocytes. In contrast, HS1 was only faintly detectable in DNA from thymocytes and was strongly induced in DNAs from DP thymocytes. Similarly, HS4 appeared to be present only in DP thymocytes from CD3/RAG, lck/RAG, TCR-β/RAG, and TCR-β mice (Fig. 2B).

Based on the fragment sizes, HS1 corresponds to a previously identified DNase I hypersensitive site found in pre-T cell lines, and HS2 corresponds to Eβ (83–86). This was further confirmed by digesting DNA with the same enzymes and hybridization with the same probes as in previous studies (data not shown) (85). In addition, DNA was digested with BamHI plus one of the following enzymes, BglII, EcoRI, NcoI, PstI, XbaI, or Hpal, which cut at different positions successively downstream of HS1. Hybridization of the filter with probe D from the BamHI (5′) end of the fragment gave rise to different sizes of the endogenous fragments (data not shown). Comparing the positions of HS1 and HS2 with the endogenous fragments enabled a more precise determination of the locations of HS1 and HS2 and showed that HS1 and HS2 colocalize with the previously identified hypersensitive sites.

In summary, we have assayed for DNase I hypersensitive sites in a 100-kb region, starting 20 kb upstream of DJβ1 and ending 50 kb downstream of Vβ14, and have found a total of 11 DNase I hypersensitive sites (Fig. 2). All of the identified sites are located within a 30-kb region, from 3 kb upstream of DJβ1 to 3 kb downstream of Vβ14. Eight of the sites (HS2, HS3, HS5–8, HS10, and HS11) are present in both DN and DP thymocytes; three (HS1, HS4, and HS9) are strongly induced in DP thymocytes. These data show that in DP thymocytes, the region of the TCR-β locus that we have analyzed remains accessible, and in some regions there are increases in accessibility to factor-binding activities.

**Analysis for DNA methylation**

To further characterize chromatin changes at the TCR-β locus, DNA methylation status was assayed in DN and DP thymocytes. Within the 100-kb region in which DNase I hypersensitive sites were assayed, 46 kb containing all of the identified DNase I hypersensitive sites have been sequenced (L. Rowen and L. Hood, personal communication). Within this 46-kb region, there are 23 MspI/HpaII sites and 19 HhaI sites, most of them clustered in two subregions, one surrounding 162 and the other downstream of the Vβ14 gene segment (Fig. 3C). The methylation status of these sites in DN and DP thymocytes was determined by Southern blotting using methylation-sensitive enzymes.

For example, DNA from DN and DP thymocytes was digested with EcoRI plus MspI, EcoRI plus HpaII, or EcoRI plus HhaI, and the blot was probed with the 5.1-kb EcoRI fragment covering the Vβ14 gene segment (Fig. 3B). Sequence data showed the presence of one MspI/HpaII site and three HhaI sites in the 5.1-kb region. EcoRI and MspI digestion yielded the two expected size fragments, one at 3.8 kb and the other at 1.3 kb in all of the samples (Fig. 3A, lanes 1, 4, 7, and 10). EcoRI and HpaII digestion of DNA from DN thymocytes of RAG mice yielded only the 5.1-kb fragment (Fig. 3A, lane 2), indicating that HpaII site is methylated in these cells. In contrast, DNA from DP thymocytes of CD3/RAG, lck/RAG, and to a lesser extent, TCR-β/RAG mice yielded the 5.1-kb as well as the 3.8- and 1.3-kb fragments (Fig. 3A, lanes 5, 8, and 11). Quantitation of the fragments showed that 5 to 20% of the DNAs are demethylated at the HpaII site in DP thymocytes. EcoRI and HhaI digestion of DNA from DN thymocytes generated 2.6- and 2.4-kb hybridizing fragments at equal intensities (Fig. 3A, lane 3). Since there are three HhaI sites in the fragment, the only possibility for this hybridization pattern is that the first HhaI site is unmethylated and the other two sites are completely methylated (Fig. 3B). Similarly, this HhaI site is also unmethylated in DP thymocytes from TCR-β/RAG, CD3/RAG, and lck/RAG mice.
RAG mice because the largest and dominant hybridizing fragments are at 2.6 and 2.5 kb (Fig. 3A, lanes 6, 9, and 12). In addition, two smaller hybridizing fragments of 1.7 and 1.1 kb were detected at submolar levels in DNA from DP thymocytes. (A 0.6-kb fragment was also visible on the original autoradiogram. The 0.6-kb fragment was expected to hybridize least because the entire 5.1-kb fragment was used as probe.) These patterns were generated when the other two HhaI sites were partially digested due to their partial demethylation. Quantitation of the fragments suggests 5% of the DNA is demethylated at the other two sites. Together, these data suggest that among the four detectable methylation sites in the 5.1-kb EcoRI fragment, one is unmethylated in both DN and DP thymocytes, and the other three sites are methylated in DN thymocytes and partially demethylated in DP thymocytes.

We have determined the methylation status of most of the MspI HpaII and HhaI sites in the 46-kb region. The results are summarized in Figure 3C. First, most of the sites in and around Jb2 gene segments are unmethylated in both DN and DP thymocytes. Three of the sites in the region become completely unmethylated in DP thymocytes. Second, among the four sites immediately downstream of the Vβ14 gene segment, one site is unmethylated in both DN and DP thymocytes, and the other three become partially demethylated during DN to DP thymocyte differentiation. Third, most sites farther downstream of the Vβ14 gene segment, including the cluster of nine HhaI sites, are methylated in both DN and DP thymocytes. These findings suggest that DN to DP thymocyte differentiation is associated with demethylation in the TCR-β locus.

Analysis for germline transcription

Chromatin changes at the TCR-β locus were also assayed by comparing germline transcripts from the locus in DN and DP thymocytes. Northern blot hybridization was performed with total RNA (10 μg) from thymocytes of RAG, CD3/RAG, lck/RAG, and TCR-β/RAG mice as controls. RNA from the EL4 T cell line and a thymoma derived from a DN thymocyte of a p53 and RAG-1 double-mutant mouse were used. Hybridization with a 430-bp Cβ2 cDNA probe revealed the presence of 1- and 1.6-kb germline transcripts from DN thymocytes that comigrated with those from the control thymoma (Fig. 4A). These germline transcripts were the same sizes as those identified previously from the TCR-β locus in DN thymocytes from normal and mutant mice (30, 78, 87). The unique 1.3-kb transcript present in RNA from TCR-β/RAG thymocytes comigrated with the mature TCR-β transcript from the EL4 T cell line and was most likely derived from the transgene in these mice. The hybridization signal larger than 28S was not informative, as it may have resulted from trace genomic DNA contamination in the RNA samples.

A duplicate filter was hybridized with a 680-bp Vβ14 probe. Two weakly hybridizing species, one at 1 kb and the other at 0.8 kb, were detected in RNA from DP thymocytes of CD3/RAG, lck/RAG, and TCR-β/RAG mice, but not from DN thymocytes of RAG mice, thymoma, or EL4 (Fig. 4B). These transcripts represent germline transcript from the endogenous Vβ14 gene segment, as the TCR-β transgene in the TCR-β/RAG mice utilizes the Vβ3 gene segment. To determine the transcription status of 5′ Vβ gene segments in DN and DP thymocytes, the same two filters were stripped and hybridized with a 190-bp Vβ8 probe or other Vβ probes, including Vβ6, 7, 9, and 13. In contrast to Vβ14, the level of Vβ8 germline transcript (including Vβ8.1, 8.2, and 8.3) was much higher in DN thymocytes from RAG mice than in DP thymocytes from CD3/RAG, lck/RAG, and TCR-β/RAG mice (Fig. 4C). No RNA transcripts were detected when Vβ6, 7, 9, or 13 probes were used individually (data not shown). Together, these data suggest that germline transcription in Cβ-Vβ14 region is increased in DP thymocytes, indicative of increased accessibility in this region, whereas germline transcription in the upstream Vβ region is nonexistent or decreased in DP thymocytes, suggesting a region with decreased accessibility.

Analysis of the role of HS1 in TCR-β gene allelic exclusion

We next focused our investigation on determining whether cis-regulatory elements contained within HS1 control TCR-β gene allelic exclusion. HS1 was interesting because it was the most prominent chromatin structural change identified in the TCR-β locus between DN and DP thymocytes, and because it was only 400 bp upstream of Eβ. Considering that cis elements within Eβ have a dominant role in promoting TCR-β VDJ recombination (41, 55, 63, 64), it was possible that factors binding to cis elements in HS1 might interact with and modulate those binding to Eβ and effectively suppress recombination at the entire locus, thereby mediating allelic exclusion. To test this possibility, a 780-bp Xcm1-BglII fragment containing HS1 was replaced with a floxed neo gene by homologous recombination in ES cells, and chimeric mice were produced for germline transmission of the alteration (Fig. 5; Materials and Methods). To minimize effects from the introduced neo gene, it was deleted by Cre/loxP-mediated recombination by breeding mutant mice with deleter mice, in which Cre is expressed at the two-cell stage of embryonic development (71). After Cre-mediated recombination, a single 34-nucleotide loxP site remains that is unlikely to influence the phenotype resulting from the HS1

**FIGURE 4.** Analysis of transcripts from the TCR-β locus in DN and DP thymocytes. A, Autoradiogram of a Northern hybridization with a Cβ2 probe to detect all Cβ-containing transcripts in total RNA from DN (RAG) and DP (CD3/RAG, lck/RAG, TCR-β/RAG) thymocytes. EL4 represents a mature CD4-positive thymoma, and expresses the mature 1.3-kb TCR-β transcript from a fully assembled allele as a control. Thymoma was from a p53 and RAG-1 double-mutant mouse, and expresses germline transcripts of 1 and 1.6 kb from unarranged alleles as a control. B, Autoradiogram of a duplicate filter hybridized with a Vβ14 probe. C, Autoradiogram of the same filter, as in A, hybridized with a Vβ8 probe. D, Ethidium bromide staining of 28S RNA on blot A to show the amount of RNA loaded in each lane.
deletion. The resulting mutant allele is detected as a 7.4-kb EcoRI fragment by Southern hybridization using probe 1 (Fig. 5B). Homozygous mutant mice with or without the neo gene deleted yielded normal numbers of thymocytes and peripheral T cells in spleen and lymph nodes (see below and data not shown). A comparison of DNase I hypersensitivity of thymocyte nuclei from normal and homozygous mutant mice, in which the neo gene was deleted, confirmed that HS1 was deleted successfully in mutant mice (Fig. 5C). HS2, HS3, and HS4 remained in the mutant mice despite the deletion of HS1, indicating that these DNase I hypersensitive sites form independently of HS1.

Phenotypic analyses of cells from spleen, lymph node, and thymus by FACS showed that T cell development and TCR-β expression in the mutant mice were indistinguishable from that of normal mice (Fig. 6). Additional FACS analyses of lymph node T cells stained with a panel of Abs specific for individual Vβs revealed no significant difference in Vβ repertoire usage between mutant and normal mice (data not shown). In addition, amplification of thymocyte DNA from normal and mutant mice by PCR to detect Dβ1 to Jβ1.1 through Jβ1.5, and Dβ1 to Jβ2.1 through Jβ2.5 rearrangements revealed no differences between the normal and mutant mice in D to J rearrangement (data not shown). Thus, the deletion of HS1 does not grossly affect TCR-β gene rearrangement, expression, and T cell development.

The effect of HS1 deletion on TCR-β gene allelic exclusion was next investigated by introducing a functionally assembled TCR-β transgene onto the homozygous mutant background and determining the extent of allelic exclusion by two different assays. First, lymph node T cells from normal and mutant mice with and without the TCR-β transgene were assayed by FACS for endogenous Vβ gene expression, including Vβ4, 5.1 and 2, 6, 7, 8.1 and 2, 10b, 11, 13, and 14. In the presence of the TCR-β transgene, the percentages of T cells expressing the endogenous Vβ were reduced to the same extent in both normal and mutant mice (data not shown). Simultaneous staining for Vβ3 of the TCR-β transgene and a panel of endogenous Vβs did not detect any dual TCR-β-expressing T cells in the mutant mice (data not shown), indicating a normal TCR-β gene allelic exclusion after HS1 deletion. Second, the extent of allelic exclusion was measured by Southern blot hybridization for the retention of a 2.2-kb PstI-fragment located upstream of Dβ1 (Fig. 7A). In normal mice, the level of hybridization to the

**FIGURE 5.** Deletion of HS1 from the TCR-β locus by targeted mutagenesis and Cre/loxP-mediated recombination. A, Schematic diagrams of the targeting vector and targeted alleles. B, Autoradiogram of a representative Southern hybridization for genotyping mutant mice. Tail DNA was digested with EcoRI and hybridized with the probe 1. Wild-type allele, 10 kb, and mutant allele, 7.4 kb. C, Autoradiograms of Southern hybridization showing the successful deletion of HS1 in mutant mice. DNA was isolated from DNase I-treated thymocyte nuclei of normal (+/+ ) and homozygous mutant (−/− ) mice, digested with BamHI plus BglII, and hybridized with probe E (see Fig. 2).

**FIGURE 6.** Phenotypic analysis of the effect of HS1 deletion on T cell development. A and B, Dot plots from FACS analysis of thymocytes (A) and lymph node cells (B) from normal and homozygous mutant mice stained with anti-CD4 and anti-CD8 Abs. The number indicates the percentage of cells in each quadrant. C, Dot plots of lymph node cells from normal and homozygous mutant mice stained with anti-TCR-αβ and anti-CD3ε Abs. The number indicates the percentage of cells in each quadrant. D, Histograms showing the levels of TCR-αβ expression on T cells from normal and homozygous mutant mice (gated on R1).
exclusion because these sites are maintained after Dβ to Iβ recombination (Fig. 2). In NZW mice, a natural deletion of 8.8 kb of DNA in the TCR-β locus has removed Cβ1, Dβ2, and Iβ2 gene segments, as well as HS7 and HS8 (88). Therefore, to assess the requirement of cis-regulatory elements associated with HS7 and HS8 for TCR-β gene allelic exclusion, we bred a functional TCR-β transgene onto the NZW mice, isolated thymocyte DNA, and then measured for the extent of allelic exclusion by Southern hybridization of probe 2 to the 2.2-kb Psfl fragment located upstream of Dβ1. As shown in Figure 7C, Vβ to DββIβ rearrangement of TCR-β in NZW mice was inhibited effectively by expression of the TCR-β transgene, as indicated by the increased hybridization signal to the 2.2-kb Psfl fragment compared with normal NZW mice. Therefore, deletion of HS7 and HS8 does not grossly affect TCR-β gene allelic exclusion. FACS analyses of lymph node T cells from NZW mice stained with a panel of Abs to endogenous Vβs to detect dual TCR-β expression confirmed that TCR-β allelic exclusion was intact in the NZW mice (data not shown). These data demonstrate that cis-regulatory elements within HS7 and HS8 are not required for TCR-β gene allelic exclusion.

FIGURE 7. Analyses of the effect of HS1 deletion, and HS7 and HS8 deletion on TCR-β gene allelic exclusion. A. A schematic diagram of TCR-β locus showing the position of probes 2 and 3 and their hybridizing fragments. B. Autoradiogram of Southern hybridization for retention of DNA upstream of Dβ1 in normal and homozygous mutant mice with and without the TCR-β transgene. DNA from kidney (K) and thymocyte (T) was digested with Psfl. Blot was hybridized with probe 2 to the 2.2-kb Psfl fragment upstream of Dβ1 and with probe 3 to a 10-kb Psfl fragment located 20 kb downstream of Vβ14 that serves as a normalization control for the amount of DNA loaded per lane. C. Autoradiogram of Southern hybridization for retention of DNA upstream of Dβ1 in DNA from kidney (K) and thymocytes (T) of NZW mice and NZW mice with a TCR-β transgene. DNA was digested with Psfl and hybridized with probes 2 and 3 simultaneously, as in B.

2.2-kb Psfl fragment was almost undetectable in thymocyte DNA (Fig. 7B). In the presence of the TCR-β transgene, the levels of hybridization were the same in DNA from thymocytes and kidney, indicating the occurrence of allelic exclusion. The retention of the signal in mice expressing the TCR-β transgene is not due to differences in DNA loading, as indicated by the similar level of simultaneous hybridization of probe 3 to a 10-kb Psfl control fragment located downstream of Vβ14 (Fig. 7, A and B). If the deletion of HS1 abolished allelic exclusion, we expected that Vβ to DββIβ recombination would persist in the presence of the TCR-β transgene and result in a lower level of retention of the 2.2-kb Psfl fragment located upstream of Dβ1. However, the level of hybridization was the same in the mutant samples as in the normal mice (Fig. 7B). The same results were also obtained when DNA isolated from purified lymph node T cells of mutant and normal mice were used for the assay (data not shown). In addition, ligation-mediated PCR measuring the levels of dsDNA break at the 5′ of Dβ1 and at the 3′ of Vβ14 gene segment in thymocytes failed to detect any consistent difference between the normal and homozygous mutant mice (data not shown). Therefore, these combined data show that cis elements within HS1 are not required for TCR-β gene allelic exclusion.

Analysis of the role of HS7 and HS8 in allelic exclusion

We next considered the possibility that cis-regulatory elements within HS7 and/or HS8 might play a role in controlling allelic exclusion because these sites are maintained after Dβ to Iβ recombination (Fig. 2). In NZW mice, a natural deletion of 8.8 kb of DNA in the TCR-β locus has removed Cβ1, Dβ2, and Iβ2 gene segments, as well as HS7 and HS8 (88). Therefore, to assess the requirement of cis-regulatory elements associated with HS7 and HS8 for TCR-β gene allelic exclusion, we bred a functional TCR-β transgene onto the NZW mice, isolated thymocyte DNA, and then measured for the extent of allelic exclusion by Southern hybridization of probe 2 to the 2.2-kb Psfl fragment located upstream of Dβ1. As shown in Figure 7C, Vβ to DββIβ rearrangement of TCR-β in NZW mice was inhibited effectively by expression of the TCR-β transgene, as indicated by the increased hybridization signal to the 2.2-kb Psfl fragment compared with normal NZW mice. Therefore, deletion of HS7 and HS8 does not grossly affect TCR-β gene allelic exclusion. FACS analyses of lymph node T cells from NZW mice stained with a panel of Abs to endogenous Vβs to detect dual TCR-β expression confirmed that TCR-β allelic exclusion was intact in the NZW mice (data not shown). These data demonstrate that cis-regulatory elements within HS7 and HS8 are not required for TCR-β gene allelic exclusion.

Discussion

Chromatin structure at the TCR-β locus in DN and DP thymocytes

Studies to identify all potential chromatin changes of the TCR-β locus are inherently difficult due to its immense size of 600 kb (Fig. 1) (75, 76). In our study, we first considered the following facts about the TCR-β locus to narrow down the region of analysis. Because allelic exclusion is regulated at the V to DJ rearrangement step, it is unlikely that cis elements involved in maintaining allelic exclusion reside between DJβ1 and Iβ2. Therefore, we focused our effort on identifying chromatin changes occurring in a 100-kb region from 20 kb upstream of Dβ1 to nearly 70 kb downstream of Iβ2. This represents the most comprehensive study ever undertaken for an Ag receptor locus. Second, we did not study beyond 20 kb upstream of Dβ1 because of the presence of a trypsinogen gene that, in our thinking, may separate upstream Vβs from the downstream gene segments and associated regulatory elements. Third, it was impractical to study chromatin changes in the entire upstream Vβ region due to its immense size. In addition, previous studies using TCR-β minilocus substrates have suggested that a functional Vβ promoter is not required for allelic exclusion and that the signal for allelic exclusion may be mediated by sequences 3′ of the Iβ2.2 gene segment (89). Therefore, we focused our study on the DNA flanking the unique Vβ14 gene segment, which is located downstream of the Dβ, Iβ, and Cβ gene segments and seems to be subjected to the same allelic exclusion control as all other Vβ gene segments. The Vβ14 gene segment is also interesting because of its inverted transcriptional orientation relative to all other TCR-β gene segments and the conservation of this configuration in human, mouse, and chicken. Because Vβ14 rearranges to the DββIβ complex by inversions joining, this configuration ensures that DNA between Vβ14 and the DββIβ complex is retained after rearrangement, suggesting that the Iβ2 to Vβ14 intervening region may contain critical cis-regulatory elements required for TCR-β gene recombination and/or transcription.

We identified a total of 11 DNase I hypersensitive sites in the 100-kb region. Three of the sites, HS1, HS4, and HS9, are unique or strongly induced in DP thymocytes, whereas the other eight sites identified are present in both DN and DP thymocytes (Fig. 2). Six of the sites are located between the Iβ2-Vβ14 intervening region, consistent with the importance of this region for TCR-β
gene regulation. A general correlation between transcriptional activity of the TCR-β locus and the presence of the DNase I hypersensitive sites is apparent. For example, HS2 maps to the previously identified Eβ enhancer (83, 84, 86), and is present in both DN and DP thymocytes (Fig. 2), consistent with the enhancer being active at both stages of development. Indeed, we detected germline transcripts (1 and 1.6 kb) containing Cβ sequences in both DN and DP thymocytes (Fig. 4), and mostMspI/HpaII and HhaI sites in the Cβ region are unmethylated (Fig. 3), a state usually associated with active transcription. In DP thymocytes, demethylation of three MspI/HpaII sites in the Jβ2 region correlated with increased levels of Cβ-containing transcripts and the induction of HS1 and HS9. HS9 is located immediately upstream of Dβ1, and analogous to Ig and TCR-α loci (51, 52, 90), may serve as the promoter for germline transcription through the Dβ, Jβ, and Cβ regions. Consistent with this notion, a germline transcript initiated immediately upstream of Dβ1 was identified recently in human fetal intestine (91). HS1 is 400 bp upstream of Eβ and has been implicated to influence transcription, perhaps by modulating Eβ activity (see below). Another site, HS5, maps to the Vβ14 transcriptional promoter. Although HS5 is present in both DN and DP thymocytes, Vβ14 germline transcripts are only detectable in DP thymocytes (Fig. 4). Vβ14 transcripts correlate with the partial demethylation of three MspI/HpaII sites located upstream of the Vβ14 gene segment in DP thymocytes. These observations suggest that if the factors that cause HS5 are involved in transcriptional activity, their binding alone is not sufficient for the induction of Vβ14 germline transcription. Additional factors or signals induced during the DP thymocyte stage are also most likely required for the induction of Vβ14 transcription. In summary, our findings suggest that chromatin structure in the 100-kb region of the TCR-β locus is dynamic and accessible in both DN and DP thymocytes, as reflected by the presence of multiple DNase I hypersensitive sites, hypomethylation, and active transcription. We did not find evidence supporting a model of allelic exclusion, wherein accessibility of the TCR-β locus is decreased dramatically during the DP stage of thymocyte development.

Chromatin changes and TCR-β gene allelic exclusion

Our studies reveal that none of the chromatin changes at the TCR-β locus in DN and DP thymocytes is indicative of a typical inaccessible locus. In DP thymocytes, 3 of 11 identified DNase I hypersensitive sites are either unique or strongly induced, 6 of 33 HpaII/HhaI sites show partial to complete demethylation, and increased germline transcription initiated from the Dβ-Cβ region and Vβ14 is evident. Moreover, all DNase I hypersensitive sites, hypomethylation, and transcription observed in DN thymocytes are also maintained in DP thymocytes. The observed changes most likely represent genuine chromatin alterations that are induced by pre-TCR signaling because the same changes are observed in DP thymocytes by either the expression of a TCR-β or an activated lck transgene, or by anti-CD3ε treatment. Because pre-TCR also signals for TCR-β gene allelic exclusion (28), the observed chromatin changes are either coincident or associated with allelic exclusion (38). Our data show that the TCR-β locus is accessible in DP thymocytes to the various enzymes and factors that bind DNA sequences and mediate the transcription and demethylation of the locus. However, in spite of this, the locus remains inaccessible to the V(D)J recombinase because the TCR-β gene is excluded from further rearrangement in DP thymocytes (28). This observation is significant for several reasons. First, although chromatin changes have been postulated to block recombinase accessibility or to re-target the recombinase for achieving allelic exclusion, the underlying mechanism is unknown (92). Our findings have eliminated some possibilities by showing that the mechanism does not involve the general shutdown of the locus, which would be reflected by an absence of transcription and active hypermethylation of the locus, and perhaps the disappearance of DNase I hypersensitive sites. Second, and contrary to our expectations, the locus becomes hypermethylated and more actively transcribed. This suggests that while transcription and hypomethylation may be required for the initiation of V(D)J recombination (6, 7), and may be used to measure general chromatin changes and accessibility, the status of transcription and methylation may not be indicative of the recombination inaccessibility associated with the excluded TCR-β allele. Third, new DNase I hypersensitive sites are induced at the excluded TCR-β locus. Therefore, if cis-regulatory elements associated with these new DNase I hypersensitive sites are involved in the control of allelic exclusion, it seems likely that the associated factors would function as repressors of V to DJ recombination.

HS1, HS7, and HS8 are not required for TCR-β gene allelic exclusion

HS1 is 400 bp upstream of Eβ and maps to a previously identified DNase I hypersensitive site found in precursor T cell lines (Fig. 2) (85). Although HS1 was shown to correlate with TCR-β expression in cell lines (85), findings from our more physiologic experimental system demonstrate that HS1 is primarily present in DP thymocytes induced in RAG-deficient mice by expression of a TCR-β or activated lck transgene or by anti-CD3ε Ab treatment. Importantly, HS1 was also detected in DP thymocytes from normal TCR-β transgenic mice, in which the endogenous TCR-β loci underwent Dββ rearrangement and were excluded from Vβ to Dββ rearrangement. Therefore, the data provide strong support that HS1 is induced by pre-TCR engagement during DN to DP thymocyte differentiation and could represent a chromatin change associated with TCR-β gene allelic exclusion.

Based on the close proximity of HS1 to Eβ, we hypothesized that factors binding to HS1 during the DP thymocyte stage might suppress Eβ function specifically in recombination, but not transcription, and consequently would promote allelic exclusion. To test this hypothesis, we deleted a 780-bp DNA fragment encompassing HS1 from the endogenous TCR-β locus by targeted mutagenesis. We found that while HS1 was deleted by this mutation, HS2, HS3, and HS4 remained (Fig. 5), suggesting that these sites may function independently of HS1. Compared with normal mice, homozygous mutant mice displayed normal TCR-β gene recombination, expression, and allelic exclusion either with or without a TCR-β transgene (Figs. 6 and 7). Therefore, our data show that HS1 is not required for the control of allelic exclusion. This finding suggests the possibility that other cis-regulatory elements in the locus may have a primary role in allelic exclusion control. If HS1 does participate in the process, it may do so in cooperation with other more essential and dominant elements. Although we do not currently know the function of HS1, its role in transcription has been suggested both by its proximity to Eβ and by the presence of several conserved sequence motifs homologous to known transcriptional elements (85). Octamer, c-myb, AP-1, and enhancer core sequences of SV40 and polyoma virus are present within two subregions that are most conserved between human and mouse (85 and 95% homology). Nevertheless, HS1 has no enhancer activity in both transient transfection assays and transgenic mice (83, 84) (Erik Selsing and Jim Miller, personal communication). Furthermore, our current biochemical evidence suggests that in DP thymocytes, factor binding to HS1 occurs in a region different from that described by the above consensus motifs and may function to repress TCR-β transcription (unpublished data). Additional studies
are required to understand the function of the nuclear factors binding to HS1.

HS7 and HS8, located within the Jβ2-Cβ2 intron, were detected previously in T cell lines, and nuclear factor-κB and additional factors were found to interact with specific sequences in the region (93, 94). We found that HS7 and HS8 were present in both DN and DP thymocytes, suggesting a role in transcription and/or recombination of the TCR-β locus at both stages of development. Interestingly, in mice harboring a targeted replacement of 15 kb of the locus encompassing Jβ1.3 to Cβ2, recombination of the remaining Dβ1, Jβ1, and Vβ gene segments did not occur. Although the recombination defect could be caused by the removal of other unknown elements within the region important for recombination, or due to the insertion of a neomycin gene cassette that has been shown to perturb recombination in other loci (56, 57, 61, 62, 95), we considered the possibility that the deletion of HS7 and HS8 might be responsible for the observed defect. In addition, HS7 and HS8 might also function in allelic exclusion control. NZW mice have naturally deleted 8.8-kb region encompassing Cβ1, Dβ2, and Jβ2 gene segments as well as HS7 and HS8 (88). From the literature, there was no evidence of a defect in the surface expression of TCR-β on NZW T lymphocytes, suggesting that transcription and recombination of the locus are normal. However, whether allelic exclusion was normal in these mice remained to be tested, especially considering that (NZW × NZB)F1 mice have a high incidence of autoimmune disease characterized by high levels of autoantibodies in the circulation (96–100), and that this disease state might be influenced indirectly by a loss of TCR-β gene allelic exclusion control. However, our analyses from Southern blotting and FACS demonstrated no apparent defect in the control of TCR-β gene allelic exclusion in NZW mice after introduction of a TCR-β transgene (Fig. 7 and data not shown), indicating that cis-regulatory elements within HS7 and HS8 are not required for the process. Therefore, it seems that the loss of HS7 and HS8 in NZW mice is unlikely to be a contributing factor to the development of autoimmune disease in (NZB × NZW)F1 mice.

Cis-regulatory elements involved in TCR-β gene allelic exclusion

DNase I hypersensitive sites are generated when nucleosomes are replaced by specific trans-acting factors that competitively bind to their cognate sequences. The presence of hypersensitive sites usually implies the presence of cis-regulatory elements and their associated factors. Although targeted deletion of HS1 or natural deletion of HS7 and HS8 has no apparent effect on TCR-β gene recombination, expression, or allelic exclusion, it remains possible that in normal circumstances these sites may contribute to any of these processes in cooperation with other more dominant regulatory elements such as Eβ in the assayed region. The Eβ enhancer was identified initially as a transcription enhancer (83, 84); it is also critical for the recombination of the TCR-β gene. Targeted deletion of a 0.5-kb region containing Eβ from the endogenous locus in mice results in a complete absence of Dβ to Jβ rearrangement and therefore a block of αβ T cell development (63, 64). Inclusion of Eβ in a minilocus recombination substrate readily promotes Vβ to DβJβ rearrangement in transgenic mice (41, 58). Thus, an effective mechanism of suppressing recombination of the entire TCR-β locus may be through the specific suppression of Eβ function in recombination. It should be emphasized that our data suggest that such a mechanism could not operate through decreasing transcription, as we observed increased transcriptional activity and associated demethylation of the TCR-β gene in DP thymocytes under conditions of allelic exclusion. Our data are consistent with a mechanism wherein Eβ may contain independent and separable cis elements that function to regulate either transcription or recombination/allelic exclusion of the TCR-β gene (101). If the mechanism of TCR-β gene allelic exclusion does involve a shutdown in chromatin accessibility to the recombinase, our data are also consistent with the possibility that the mechanism may involve cis elements residing completely outside of the 100-kb region of DNA that we have analyzed, perhaps in the upstream Vβ region. Indeed, we did observe a significant down-regulation of Vββ1 germline transcription in DP thymocytes compared with DN thymocytes, suggesting decreased accessibility to the transcriptional machinery. In this scenario, allelic exclusion of the downstream Vβ14 gene segment may involve a different mechanism since we observed increased germline transcription of this gene segment and demethylation near its promoter in DP thymocytes. However, it remains possible that germline transcription of Vβ gene segments may not be a reliable indicator of accessibility to the recombinase. This is consistent with previous studies utilizing minilocus recombination substrates, in which a correlation between transcription and recombination has not always been found (58, 101, 102). Interestingly, Alvarez et al. showed that while deletion of the decamer motif of a Vβ gene segment abolished transcription to below detectable levels, the rearrangement and allelic exclusion of the Vβ gene segment remained unaffected (89). Therefore, allelic exclusion of each Vβ gene segment may not be controlled individually, but by dominant cis-regulatory elements that control allelic exclusion of all Vβ gene segments (except Vβ14). If such dominant cis elements are present, they do not reside in the 100-kb region encompassing Vβ5.2 to Vβ9, because this 100 kb is deleted in SJL mice, and these mice have no apparent defect in TCR-β gene allelic exclusion (75, 76). Alternatively, allelic exclusion could be mediated by multiple local cis-regulatory elements, analogous to the T early α element for the rearrangement of the 5′ Jα gene segments (90).

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