DNA Double-Strand Breaks Relieve USF-Mediated Repression of D β2 Germline Transcription in Developing Thymocytes

Jennifer L. Stone, Ruth E. McMillan, David A. Skaar, Justin M. Bradshaw, Randy L. Jirtle and Michael L. Sikes

*J Immunol* 2012; 188:2266-2275; Prepublished online 27 January 2012;
doi: 10.4049/jimmunol.1002931

http://www.jimmunol.org/content/188/5/2266

Supplementary Material  
http://www.jimmunol.org/content/suppl/2012/01/27/jimmunol.1002931.DC1

References  
This article cites 55 articles, 28 of which you can access for free at:
http://www.jimmunol.org/content/188/5/2266.full#ref-list-1

Subscription  
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions  
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
DNA Double-Strand Breaks Relieve USF-Mediated Repression of Dβ2 Germline Transcription in Developing Thymocytes

Jennifer L. Stone,* Ruth E. McMillan,* David A. Skaar,† Justin M. Bradshaw,* Randy L. Jirtle,‡ and Michael L. Sikes*

Activation of germline promoters is central to V(D)J recombinational accessibility, driving chromatin remodeling, nucleosome repositioning, and transcriptional read-through of associated DNA. We have previously shown that of the two TCRB locus (Tcrb) D segments, Dβ1 is flanked by an upstream promoter that directs its transcription and recombinational accessibility. In contrast, transcription within the Dβ2 segment cluster is initially restricted to the J segments and only redirected upstream of Dβ2 after D-to-J joining. The repression of upstream promoter activity prior to Tcrb assembly correlates with evidence that suggests Dβ2 recombination is less efficient than that of Dβ1. Because inefficient Dβ2 assembly offers the potential for V-to-DJβ2 recombination to rescue frameshifted V-to-DJβ1 joints, we wished to determine how Dβ2 promoter activity is modulated upon Tcrb recombination. In this study, we show that repression of the otherwise transcriptionally primed 5’Dβ2 promoter requires binding of upstream stimulatory factor (USF)-1 to a noncanonical E-box within the Dβ2 12-recombination signal sequence spacer prior to Tcrb recombination. USF binding is lost from both rearranged and germline Dβ2 sites in DNA-dependent protein kinase, catalytic subunit–competent thymocytes. Finally, genotoxic dsDNA breaks lead to rapid loss of USF binding and gain of transcriptionally primed 5’Dβ2 promoter activity in a DNA-dependent protein kinase, catalytic subunit–dependent manner. Together, these data suggest a mechanism by which V(D)J recombination may feed back to regulate local Dβ2 recombinational accessibility during thymocyte development. The Journal of Immunology, 2012, 188: 2266–2275.

Lympocytes express a diverse array of Ag-specific receptors. The genes that encode these receptors are uniquely assembled in developing lymphocytes through a series of somatic rearrangements termed V(D)J recombination after the variable, diversity, and joining gene segments that are recombined (1, 2). B and T cell Ag receptor genes are each assembled by a single enzymatic complex centered on the lymphocyte-specific recombination activating gene (RAG) 1/2 proteins that target conserved recombination signal sequences (RSSs) flanking each V, D, and J segment. Despite the singular nature of enzyme and substrate, proper lymphocyte development and function requires that V(D)J recombination follow a precise program of ordered gene assembly imposed in part by RSS genetic variation (3–5) and in part by epigenetic regulation of promoters that populate each Ag receptor gene (3, 6, 7). Activation of promoters associated with D or J segments (so-called germline transcription, reflecting the unrearranged nature of the transcribed template) augments the accessibility of transcribed segments to recombinase. Chromatin remodeling (8), nucleosome repositioning (9), and transcriptional elongation associated with germline promoter activation (10) facilitate the recombinational accessibility of individual gene segments. However, the mechanism by which promoter-mediated accessibility is modulated during lymphocyte development is unclear.

T cell development begins when early thymocyte progenitors emigrate from the bone marrow to the subcapsular region of the thymus cortex. Progression of early thymocytes from early thymocyte progenitors through CD4−CD8− double-negative (DN) development is coincident with rearrangement of TCRβ locus (Tcrb), Tcra, and Tcrg genes. If cells assemble functional Tcra, Tcrg, and Tcrb genes before completing Tcrb assembly, they commit to the γδ lineage (11). Conversely, expression of a rearranged Tcrb gene triggers the silencing of additional Tcrb recombination and drives the cell forward in development to the CD4+CD8− double-positive (DP) stage in which Tcra rearrangement occurs (12).

Tcrb assembly proceeds in a stepwise manner that involves independent D-to-J recombination at two Dβ1/Dβ2/Jβ1/Jβ2 gene segment clusters, followed by V rearrangement to a newly formed DJβ joint. Though DJβ1 RSS sequence strongly influences the order of gene segment assembly (5, 13, 14), the recombinational accessibility of individual RSSs is dependent on their chromosomal location (15) and the activity of associated germline promoters. Deletion of the Dβ1-associated promoter, PDβ1, alters nucleosomal phasing across the Dβ1 5’ RSS (9) and specifically impairs Tcrb Dβ1-to-Jβ recombination (16, 17) without affecting recombination at the downstream DJβ2 gene segment cluster (17).
Though the mechanism of PDβ1’s influence over DJβ1 assembly is unclear, the promoter’s position immediately upstream of DJβ1 (18) and its recruitment of switch/sucrose nonfermentable chromatin remodelers complexes are critical for efficient DJβ1 assembly (8). Indeed, moving PDβ1 progressively downstream of DJβ1 increasingly impairs its ability to direct DJβ1 assembly of chromosomal Tcrb transgenes (19).

Although both DJCβ clusters are transcriptionally active at the start of thymopoiesis (20), unarranged DJβ2 clusters persist in the endogenous loci of thymocytes from Tcrb transgenic mice, as well as from wild-type (wt) fetal thymocytes (21–24). Unlike germline transcription at DJβ1, transcription in the germline DJβ2 cluster predominantly initiates 400–600 bp downstream of DJβ2 (20). However, DJβ2 rearrangement, which deletes the germline promoter, results in the activation of a second promoter upstream of DJβ2. The role of promoter activity in DJβ2 recombination is unknown. Based on our understanding of DJβ1 assembly and the conserved role of promoter activity in driving recombination accessibility at other Ag receptor loci (7), it is likely that the downstream location of the germline DJβ2 promoter may contribute to the persistence of unarranged DJβ2 clusters during thymocyte development (20). By extension, transcription from the upstream transcriptionally primed 5’DJβ2 promoter (5’PDβ2), which passes through the DJβ2 coding sequence and flanking RSSs, would then be predicted to enforce DJβ2 accessibility during V-to-DJ recombination. Separate DJβ2 cassettes offer each Tcrb allele the potential for two attempts at assembling an in-frame V(D)J rearrangement, provided V elements initially target DJβ1. Repression of 5’PDβ2 until after DJβ2 recombination might offer a potential mechanism to limit the initial accessibility of DJβ2 RSSs and thereby increase the frequency with which VB elements target DJβ1. However, the process by which 5’PDβ2 repression is first imposed and then relieved in a timely manner after DJβ2 recombination is unknown.

Upstream stimulatory factor (USF)-1 and -2 are ubiquitously expressed stress-response regulators that belong to the E protein family of basic helix-loop-helix leucine zipper transcription factors (25). USF-1 and -2 bind as either homo- or heterodimers to E-box expressed stress-response regulators that belong to the E family of basic helix-loop-helix leucine zipper transcription factors (25). USF-1 and -2 bind as either homo- or heterodimers to E-box elements target DJβ2 repression is first imposed and then relieved in a timely manner after DJβ2 recombination is unknown.

Upstream stimulatory factor (USF)-1 and -2 are ubiquitously expressed stress-response regulators that belong to the E protein family of basic helix-loop-helix leucine zipper transcription factors (25). USF-1 and -2 bind as either homo- or heterodimers to E-box elements target DJβ2 repression is first imposed and then relieved in a timely manner after DJβ2 recombination is unknown.

Upstream stimulatory factor (USF)-1 and -2 are ubiquitously expressed stress-response regulators that belong to the E protein family of basic helix-loop-helix leucine zipper transcription factors (25). USF-1 and -2 bind as either homo- or heterodimers to E-box elements target DJβ2 repression is first imposed and then relieved in a timely manner after DJβ2 recombination is unknown.

Upstream stimulatory factor (USF)-1 and -2 are ubiquitously expressed stress-response regulators that belong to the E protein family of basic helix-loop-helix leucine zipper transcription factors (25). USF-1 and -2 bind as either homo- or heterodimers to E-box elements target DJβ2 repression is first imposed and then relieved in a timely manner after DJβ2 recombination is unknown.

Upstream stimulatory factor (USF)-1 and -2 are ubiquitously expressed stress-response regulators that belong to the E protein family of basic helix-loop-helix leucine zipper transcription factors (25). USF-1 and -2 bind as either homo- or heterodimers to E-box elements target DJβ2 repression is first imposed and then relieved in a timely manner after DJβ2 recombination is unknown.

Upstream stimulatory factor (USF)-1 and -2 are ubiquitously expressed stress-response regulators that belong to the E protein family of basic helix-loop-helix leucine zipper transcription factors (25). USF-1 and -2 bind as either homo- or heterodimers to E-box elements target DJβ2 repression is first imposed and then relieved in a timely manner after DJβ2 recombination is unknown.

Upstream stimulatory factor (USF)-1 and -2 are ubiquitously expressed stress-response regulators that belong to the E protein family of basic helix-loop-helix leucine zipper transcription factors (25). USF-1 and -2 bind as either homo- or heterodimers to E-box elements target DJβ2 repression is first imposed and then relieved in a timely manner after DJβ2 recombination is unknown.

Upstream stimulatory factor (USF)-1 and -2 are ubiquitously expressed stress-response regulators that belong to the E protein family of basic helix-loop-helix leucine zipper transcription factors (25). USF-1 and -2 bind as either homo- or heterodimers to E-box elements target DJβ2 repression is first imposed and then relieved in a timely manner after DJβ2 recombination is unknown.

Upstream stimulatory factor (USF)-1 and -2 are ubiquitously expressed stress-response regulators that belong to the E protein family of basic helix-loop-helix leucine zipper transcription factors (25). USF-1 and -2 bind as either homo- or heterodimers to E-box elements target DJβ2 repression is first imposed and then relieved in a timely manner after DJβ2 recombination is unknown.
pared as described (16), and DJB rearrangements were assessed using primers and conditions as shown (Supplemental Table I).

**CpG methylation**

Sodium bisulfite modification of DNA was performed using Epitect Bisulfite Kits (Qiagen) according to the manufacturer’s instructions. Methylation was quantitated by using different MassARRAY platforms and the Epityper analysis software (Sequenom). DNAseI Digestion Software (Sequenom) was used to design T7-tagged and matched primers to CpG-deficient targets across DJB (Supplemental Table III). PCR was performed using HotStarTaq (Qiagen), and products were processed using MassCLEAVE as per the manufacturer’s protocol (Sequenom). Resulting fragmented transcripts were spotted onto SpectroCHIPs for mass spectrometry analysis on a MassARRAY instrument (Sequenom) to quantify the methylated fraction in each amplicon.

**Chromatin immunoprecipitation**

Chromatin was prepared from formaldehyde-cross-linked P5424 or the indicated thymocytes and subjected to chromatin immunoprecipitation (ChIP) as described (33). Bound and input samples (4 μl) were subjected to qPCR with 1× SensiMix Plus (Quanta) in triplicate reactions. Primers and annealing temperatures for chromatin immunoprecipitation are shown (Supplemental Table I). Cycling parameters for 20 μl reactions were 95°C for 10 min, followed by 50 cycles of 95°C for 20 s; appropriate annealing temp for 30 s; and 72°C for 30 s. Average fold enrichment in bound fractions was calculated for triplicate amplifications as previously described (34). Where indicated, enrichment signals were further normalized to that obtained for isotype-matched control antisera.

**EMSA**

P5424 nuclear extracts and radioactive probes were prepared and EMSA reactions performed as previously described (20). Sequences of wt and mutant oligonucleotide EMSA primers are as shown (Supplemental Table II).

**Results**

**Repressed 5’PDβ2 remains accessible in DN thymocytes**

Transcriptional promoters embedded in the germline sequences of Ag receptor genes drive localized recombinational accessibility of proximal gene segments (7). We have previously shown that both of the DJB gene segment clusters in Tcrb contain germline promoters immediately upstream of their respective D segments (18, 20). However, DJB2 germline transcription differs significantly from DJB1 in that transcription of the unarranged DJB2 cluster initiates from a promoter positioned downstream of DJB2 and proximal to Jβ2.1 (20). Following DJB2-to-JB2 recombination, transcription is redirected to a promoter that sits upstream of DJB2 (20, 35), suggesting that the upstream promoter is initially repressed prior to DJB2 recombination.

To test the possibility that the 5’PDβ2 repressor is located downstream of DJB2 and is deleted upon DJB2 recombination, we sought to determine if transcription in DP thymocyte populations is restricted to rearranged DJB2 sequences (Fig. 1). Using quantitative RT-PCR (qRT-PCR) primer pairs specific for either unarranged DJB2 (Fig. 1A, primers a and b) or total germline Jβ2-Cβ2 spliced message (Fig. 1A, primers c and e), we assessed levels of DJB2 transcription in thymocytes from wt C57BL/6 mice (primarily DP cells) or mice deficient for Rag1 (DN cells). As expected, germline transcription was readily detected in both DN and DP thymocytes, as well as in the Rag1−/− and Rag2−/− DN cell line P5424, and significant levels of transcription through DJB2 were only apparent in DP cells (Fig. 1B, black bars). Because RT-PCR measures steady-state transcription levels, it remains possible if unlikely, that DJB2 transcripts are inherently less stable in DN cells than those initiating further downstream. Regardless, the abundance of germline DJB2 transcription in DP cells strongly suggests that activation of 5’PDβ2 does not require DJB2 recombination in cis.

To more directly address the role of recombination in 5’PDβ2 activity, we transiently transfected P5424 via repeated rounds of PGK–RAG1 electroporation and identified multiple subclones that harbored biallelic DJB1 and monoallelic DJB2 rearrangements, as well as rearrangements in their Tcra and Tcrb loci. DJB sequences of one such clone (Fig. 1C, c22), as well as a representative control that maintained unarranged DJB2 segments on both alleles (Fig. 1C, c20), are shown in Table I. Both c20 and c22, as well as parental P5424, contained germline transcripts downstream of DJB2 that spliced from Jβ2 segments to CB2 (Fig. 1D, middle panel). However, germline transcripts across DJB2 was limited to c22 (Fig. 1D, top panel), which also expressed the rearranged (DJ2)Jβ2.25 segment (data not shown). Similar results were obtained for three additional subclones that carried monoallelic DJB2 rearrangements with Jβ2.1, -2.3, and -2.5, respectively (data not shown). Together with the analysis of thymocyte transcription, these data suggest that relief of 5’PDβ2 repression during DJB2 recombination occurs at both rearranged and germline DJB2 gene segment clusters.

We have previously shown that repressed 5’PDβ2 is bound by a variety of transcription factors, including E47, Runx1, and GATA-3 (35), suggesting that 5’PDβ2 chromatin remains accessible prior to promoter activation. To directly measure chromatin accessibility in the P5424 subclones, we next used bisulfite conversion to map the methylation state of CpG dinucleotides near DJβ2 (Table II). Of the eight CpG dinucleotides found within 500 bp upstream and downstream of DJB2, all were strongly demethylated in P5424, c20, and c22, correlating with the general hypomethylation of the DJβ2 cluster in DN and DP thymocytes (36). In contrast, CpGs at −453, −373, +422, and +464 (relative to the first coding base of DJB2) were methylated in >30–80% of screened ampiclons from the Balb3T3 fibroblast cell line. Methylation in Balb3T3 was not universal, however, declining markedly proximal to DJB2. Indeed, methylation was essentially undeleted at −15 and +178 CpGs in fibroblasts, suggesting that DJB2 is protected from methylation irrespective of Tcrb accessibility. ChIP analyses of Rag2−/− thymocytes also found that sites across Tcrb are marked by histone modifications consistent with accessible promoter regions, including histone H3 lysine 9 acetylation (Fig. 2A) and H3 lysine 4 di- and trimethylation (Fig. 2B, 2C), and lacked H3 lysine 27 trimethylation found at silent promoters (Fig. 2D), corroborating previous analyses of DJB2 accessibility (37–39). Moreover, qPCR primers that selectively amplified either the germline or (DJB2)Jβ2.25 gene segment of c22 found equivalent levels of histone marking, again suggesting that 5’PDβ2 repression does not involve epigenetic silencing of the germline DJB2 chromatin.

**5’PDβ2 repression requires an E-box in the DJB2 12-RSS**

A mechanistic understanding of 5’PDβ2 repression necessitated the identification and characterization of the repressor element. Promoter activation in our rearranged subclones was restricted to cell lines that harbored DJβ2 joints. However, our transcriptional analyses (Fig. 1) excluded the possibility that 5’PDβ2 activity requires deletion of a downstream repressor by DJB2 recombination. Rather, the data suggest that either deletion of the repressor on one allele leads to loss of repression on the second allele or the repressor is not deleted by DJB recombination. To define the repressor’s location, we used luciferase reporter analyses (Fig. 3). Serial deletion of all downstream sequence, the DJB2 coding sequence, and the 12-RSS heptamer failed to relieve 5’PDβ2 repression (Fig. 3A, compare −1104/+230 through −1104/−7). In sharp contrast, 3’ deletion of an additional 21 bp fully restored promoter activity (Fig. 3A, compare −1104/−28 to the full-length 5’PDβ2 −1104/+230 construct), suggesting that repressor activity was localized to the DJβ2 12-RSS nonamer (−28 to −20) and/or spacer (−19 to −8).
To identify potential repressor elements, we screened a panel of 5'PDβ2 repression reporters (Fig. 3B) into which we had introduced tiled five-base TTCCA substitutions. Whereas promoter activity was repressed in the wt construct and mutants that harbored substitutions in either the nonamer or DB2 coding sequence, two contiguous mutations that spanned the spacer sequence induced promoter activity 2.5- and 3.5-fold over wt, respectively. These two mutations altered a noncanonical heptameric E-box (CACGATG) that included the strongly demethylated CpG at -15, suggesting that 5'PDβ2 repression may be localized to a single cis-acting element that is upstream of DB2.

To identify potential repressor elements, we screened a panel of 5'PDβ2 repression reporters (−1104/+13) into which we had introduced tiled five-base TTCCA substitutions (Fig. 3B). Whereas promoter activity was repressed in the wt construct and mutants that harbored substitutions in either the nonamer or DB2 coding sequence, two contiguous mutations that spanned the spacer sequence induced promoter activity 2.5- and 3.5-fold over wt, respectively. These two mutations altered a noncanonical heptameric E-box (CACGATG) that included the strongly demethylated CpG at -15, suggesting that 5'PDβ2 repression may be localized to a single cis-acting element that is upstream of DB2.

To identify potential repressor elements, we screened a panel of 5'PDβ2 repression reporters (−1104/+13) into which we had introduced tiled five-base TTCCA substitutions (Fig. 3B). Whereas promoter activity was repressed in the wt construct and mutants that harbored substitutions in either the nonamer or DB2 coding sequence, two contiguous mutations that spanned the spacer sequence induced promoter activity 2.5- and 3.5-fold over wt, respectively. These two mutations altered a noncanonical heptameric E-box (CACGATG) that included the strongly demethylated CpG at -15, suggesting that 5'PDβ2 repression may be localized to a single cis-acting element that is upstream of DB2.

To identify potential repressor elements, we screened a panel of 5'PDβ2 repression reporters (−1104/+13) into which we had introduced tiled five-base TTCCA substitutions (Fig. 3B). Whereas promoter activity was repressed in the wt construct and mutants that harbored substitutions in either the nonamer or DB2 coding sequence, two contiguous mutations that spanned the spacer sequence induced promoter activity 2.5- and 3.5-fold over wt, respectively. These two mutations altered a noncanonical heptameric E-box (CACGATG) that included the strongly demethylated CpG at -15, suggesting that 5'PDβ2 repression may be localized to a single cis-acting element that is upstream of DB2.

USF-I binds the 5'PDβ2 repressor element in DN thymocytes

We next used EMSA to determine if the element identified in our reporter assays could function as a bona fide E-box (Fig. 4). Indeed, a radiolabeled probe spanning the putative E-box strongly bound a single specific protein complex in nuclear extracts from the P5424 cell line (Fig. 4, lanes 1 and 8). Excess unlabeled probe readily competed for protein binding (Fig. 4, lane 2), whereas an oligonucleotide that carried the 10-bp repressor substitutions (−17 to −8) identified in our reporter assay failed to compete for protein binding (Fig. 4, lane 3). Mutation of the upstream −17CACGA−13 sequence (to ttCcA, in which lowercase indicates

FIGURE 1. DJβ2 rearrangements relieve repression at both germline and rearranged DJβ2 clusters. (A) Schematic representation of the DJCβ2 cluster and spliced transcripts from 5'PDβ2 and 3'PDβ2 (Jβ2.1), respectively. The positions of oligonucleotide primers used for expression and recombination assays are indicated (black arrows). (B) QRT-PCR of spliced Jβ2CB2 germline transcripts (gray bars, primers c and e), versus transcripts of unrearranged DB2 (black bars, primers a and b). Means (± SD; n = 3) are shown for DB2 signals relative to signals obtained in the absence of reverse transcriptase and normalized to β-actin loading controls. (C) PCR of DB2 (upper panel) and DJβ2 rearrangements (lower panel, primers a and d) in the P5424-c20 and c22 subclones. (D) RT-PCR of germline transcription across DB2 (upper panel, primers b and y) versus total Cβ2 mRNA spliced from Jβ1 or Jβ2.2 (middle panel, primers c and e) in the P5424 subclones. PCR of the unrelated β-actin message (lower panel) served as a loading control. (E) QRT-PCR of spliced DB2Jβ2CB2 germline transcripts (primers a and e) in the P5424-c20 and c22 subclones. Means (± SD; n = 3) for each subclone relative to P5424 parental cells were calculated by ΔΔCt and normalized to β-actin loading control signals. B cell, M12 B cell line; c20 and c22, RAG-transfected P5424 subclones; P5424, Rag1−/− p53−/− DN thymocyte cell line; R1-Thy, unsorted Rag2−/− thymocytes; wt Thy, unsorted C57BL/6 thymocytes.

To identify potential repressor elements, we screened a panel of 5'PDβ2 repression reporters (−1104/+13) into which we had introduced tiled five-base TTCCA substitutions (Fig. 3B). Whereas promoter activity was repressed in the wt construct and mutants that harbored substitutions in either the nonamer or DB2 coding sequence, two contiguous mutations that spanned the spacer sequence induced promoter activity 2.5- and 3.5-fold over wt, respectively. These two mutations altered a noncanonical heptameric E-box (CACGATG) that included the strongly demethylated CpG at −15, suggesting that 5'PDβ2 repression may be localized to a single cis-acting element that is upstream of DB2.

USF-I binds the 5'PDβ2 repressor element in DN thymocytes

We next used EMSA to determine if the element identified in our reporter assays could function as a bona fide E-box (Fig. 4). Indeed, a radiolabeled probe spanning the putative E-box strongly bound a single specific protein complex in nuclear extracts from the P5424 cell line (Fig. 4, lanes 1 and 8). Excess unlabeled probe readily competed for protein binding (Fig. 4, lane 2), whereas an oligonucleotide that carried the 10-bp repressor substitutions (−17 to −8) identified in our reporter assay failed to compete for protein binding (Fig. 4, lane 3). Mutation of the upstream −17CACGA−13 sequence (to ttCcA, in which lowercase indicates

Table 1. D-to-J recombinant sequence in clones c20 and c22

<table>
<thead>
<tr>
<th>Cloning Location</th>
<th>DJβ1 cluster</th>
<th>DJβ2 cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>P5424</td>
<td>DB1−GGGACAGGGG**</td>
<td>DB2−GGGACTGGGGG**</td>
</tr>
<tr>
<td>P5424-c20</td>
<td>DB1−GGGACAGGGG**</td>
<td>DB2−GGGACTGGGGG**</td>
</tr>
<tr>
<td>P5424-c22</td>
<td>DB1−GGGACAGGGG**</td>
<td>DB2−GGGACTGGGGG**</td>
</tr>
</tbody>
</table>

*DB coding sequence in parental P5424 is underlined.
substituted bases) was sufficient to abolish competition by the unlabeled primer (Fig. 4, lane 4), whereas primers carrying the $^{15}TGTAA$–$^8$TcCA mutation remained efficient competitors (Fig. 4, lane 5). Mutation of the critical CpG dinucleotide in the center of the putative E-box also abolished competition (Fig. 4, lane 6), though its methylation on unlabeled primers had little impact on their ability to compete for protein binding (Fig. 4, lane 7). Finally, the specificity of the protein complex was confirmed by its supershift in the presence of Abs to USF-1 and USF-2 (Fig. 4, lane 5), whereas Abs to other E proteins including E47, HEB, Myc, and Max all failed to alter binding activity (Fig. 4, lanes 11–14).

We had previously shown that although the more distal E-boxes within 5’PDβ2 specifically bound E47, USF-1 binding upstream of Dβ2 was nonetheless detected in vivo when either Rag2–/– thymocyte or P5424 cell line chromatin was assayed by ChIP, suggesting the presence of nearby USF-binding elements (35). Our EMSA findings now suggested that our previous ChIP assays were detecting USF-1 bound to the repressor E-box. Specifically, USF-1 but not USF-2 was strongly enriched at Dβ2 in chromatin from either Rag2–/– DN thymocytes or Rag2–/– thymocytes that express a rearranged Tcrb transgene and progress to the DP stage of development (Rxβ), but was absent in DP thymocytes from recombination-competent C57BL/6 mice (Fig. 5A).

Because both Rxβ and wt C57BL/6 thymocytes are predominantly DP, USF-1 binding in the Rxβ mice suggested that loss of USF-1 binding at Dβ2 is not strictly dependent on DN to DP development. However, it remained possible that USF-1 was retained in Rxβ DP cells because of accelerated DN development in the presence of the Tcrb transgene (15, 40). To exclude this possibility, we assessed USF-1 binding (Fig. 5B) in sorted DN1 and DN3 subpopulations of C57BL/6 thymocytes, as well as in the DN thymocytes from mice that lack DNA-PKcs or the pre-TCR signaling molecule linker for activation of T cells and are consequently prevented from maturing to DP cells (41). Although USF-1 binding was modestly reduced in wt DN3 cells relative to DN1, it was abolished in linker for activation of T cell-deficient cells that support normal Tcrb assembly but cannot complete β-selection. In sharp contrast, USF-1 was strongly enriched at Dβ2 in the recombinationally impaired DN cells of DNA-PKcs-deficient SCID mice (Fig. 5B), and this enrichment correlated with the absence of 5’Dβ2 transcription (Fig. 5C). USF-1 was similarly enriched in P5424, but was lost from both the germline and re-arranged Dβ2 clusters of c22 (Fig. 5B and data not shown).

Despite the loss of USF-1 from Dβ2 in wt DP cells, USF-1 RNA levels were equivalent among Rag2–/–, Rxβ, and wt thymocytes (Fig. 5D). Steady-state USF-1 RNA levels were also similar between P5424 and the c20 and c22 subclones, though <100-fold lower than USF-1 levels in primary thymocytes. The persistent expression of USF-1 in DN and DP thymocytes is consistent with its ubiquitous distribution in mammalian tissues (42) and argues against a mechanism in which loss of USF-1 from the 5’PDβ2 repressor in DP cells is due to downregulation of USF-1 expression. Indeed, we also found that USF-1 was absent from Dβ2 sequences in chromatin isolated from either the Balb3T3 fibroblast or M12 B cell lines despite USF-1 expression in both (data not shown). Taken together with our luciferase and EMSA findings, our in vivo analyses strongly suggest that USF-1 binding at the Dβ2 12-RSS is sufficient to repress 5’PDβ2 activity and that loss

**Table II.** Percent methylation of CpG dinucleotides surrounding Dβ2

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>CpG Positions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-453</td>
</tr>
<tr>
<td>B3T3</td>
<td>0.33</td>
</tr>
<tr>
<td>P5424</td>
<td>0.07</td>
</tr>
<tr>
<td>P5424-c20</td>
<td>0.09</td>
</tr>
<tr>
<td>P5424-c22</td>
<td>0.14</td>
</tr>
</tbody>
</table>

*Numbers relative to the first coding nucleotide of Dβ2.

**FIGURE 2.** Repression does not alter histone modifications at Dβ2. Chromatin from Rag2-deficient C57BL/6 thymocytes or the c22 subclone of P5424 was immunoprecipitated with Abs to: H3K9ac (A), H3K4me2 (B), H3K4me3 (C), and H3K27me3 (D). Resultant DNAs were analyzed by QPCR for histone modifications at the indicated Tcrb and control cad promoter (CAD) amplicons. Enrichment was calculated relative to preimmunoprecipitation input control levels and was normalized against signals obtained with nonspecific IgG control Abs. Bars indicate means (± SD; n = 3) and are representative of two experiments with independent chromatin preparations.
of USF-1 binding is triggered by DJβ2 recombination rather than by developmental progression.

**Genotoxic-induced DSBs lead to loss of USF-1 binding and relieve 5'PDβ2 repression**

Unlike RAG2 deficiency, lymphocytes that lack DNA-PKcs accumulate DSB intermediates of V(D)J recombination, leading to the activation of a variety of transcriptional programs via the related PI3K, Atm (31). However, DJβ2 remains bound by USF-1 in DNA-PKcs–deficient thymocytes, despite intact Atm signaling (Fig. 5). Given that DNA-PKcs directly regulates USF-1–dependent expression of fatty acid synthase in response to insulin signaling (29), we wished to determine if USF-1 binding at DJβ2 repression of fatty acid synthase in response to insulin signaling is similarly regulated by DNA-PKcs. Treatment of Rxβ thymocytes with either ionizing radiation or etoposide, both of which induce DSBs, unlike RAG2 deficiency, lymphocytes that lack DNA-PKcs accumulate DSB intermediates of V(D)J recombination, leading to the activation of a variety of transcriptional programs via the related PI3K, Atm (31). However, DJβ2 remains bound by USF-1 in DNA-PKcs–deficient thymocytes, despite intact Atm signaling (Fig. 5). Given that DNA-PKcs directly regulates USF-1–dependent expression of fatty acid synthase in response to insulin signaling (29), we wished to determine if USF-1 binding at DJβ2 is similarly regulated by DNA-PKcs. Treatment of Rxβ thymocytes with either ionizing radiation or etoposide, both of which induce DSBs, led to loss of USF-1 and a reciprocal increase in 5'PDβ2 expression (Fig. 6A, 6B). However, this genotoxin-induced derepression was blocked in cells pretreated with the DNA-PKcs inhibitor Nu7026 (Fig. 6C, 6D).

USF-1 is a pleiotropic stress response transcription factor that has been implicated in the activation or repression of many genes across a broad spectrum of tissues (43). Previous studies have shown that activation of the carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (cad) gene promoter in cycling cells is dependent on displacement of USF-1 by Myc/Max heterodimers (44). As predicted, USF-1 was absent from the transcriptionally active cad promoter in P5424 cells (Fig. 7). Genotoxic insult led to an enrichment of USF-1 and inhibition of cad expression. However, USF-1 binding and cad expression were not altered by addition of the DNA-PKcs inhibitor Nu7026. These data suggest that USF-1 binding is regulated by different mechanisms at the cad and 5'PDβ2 promoters. Additionally, the loss of USF-1 from both promoters suggests that DNA damage may alter the regulation of multiple USF-1 target genes.

Our finding that genotoxic DSBs can lead to activation of 5'PDβ2 suggests that development-dependent promoter activation is mediated by DNA-PKcs in response to physiologic RAG DSBs. Such a mechanism would account for derepression at either germine or rearranged DJβ2 clusters. However, 5'PDβ2 activity in the rearranged subclones was restricted to those that harbored DJβ2 joints. Consequently, it remained unclear whether loss of 5'PDβ2 repression during V(D)J recombination requires specific DJβ2 DSBs or results from the general accumulation of RAG DSBs. If loss of repression is regulated in trans by DSB-induced activation of DNA-PKcs, then perhaps the constitutive activation of 5'PDβ2 in c22 reflected the presence of extensive and/or persistent DSBs generated during the repeated transfections of pS3-deficient P5424 cells. To test this possibility, we assessed the impact on promoter activity of inhibiting DNA-PKcs. In contrast to controls exposed to DMSO carrier or inhibitors of p38 MAPK or Atm, when c22 cells were cultured with Nu7026, both USF-1 binding and 5'PDβ2 repression were induced (Fig. 8). The ability to restore repression in c22 suggests that 5'PDβ2 activity is sensitive to DSB signals and may not specifically require breaks in the DJβ2 cluster. More generally, our data suggest a model in which promoter contributions to DJβ2 recombinational accessibility are in turn regulated by the DSB-sensitive repressive actions of USF-1.

**FIGURE 3.** Repression of 5'DJβ2 promoter activity requires cis targets in the DJβ2 5' RSS. (A) The indicated PCR fragments were inserted upstream of the luciferase cassette in pGL3-Eβ. Numbering is relative to the first base of the DJβ2 coding sequence (+1). Protein extracts were assayed for luciferase activity 24 h after transfection with each plasmid and normalized to cotransfected renilla. Bars represent mean normalized luciferase activity ± SEM of at least six transfections and expressed as fold activity over the fully repressed −1104/+230 5'PDβ2 fragment (cite). (B) pGL3-Eβ constructs containing the wt −1104/+13 fragment (right panel) or the indicated TTCCA substitutions were assayed for luciferase activity 24 h after cotransfection with renilla plasmid. Bars represent mean normalized luciferase activity ± SEM and are expressed as percent activity of wt −1104/+13.

**FIGURE 4.** USF-1/2 bind the repressor site in the DJβ2 5’RSS spacer. Nuclear extracts from the P5424 cell line were incubated with a radio-labeled double-stranded oligonucleotide probe to the putative repressor site. Probes were incubated with nuclear extract alone (lanes 1 and 8), in the presence of 100-fold molar excess of unlabeled wt (lane 2), mutant (lanes 3–6), or methylated competitors (lane 7), or in the presence of the indicated Abs (lanes 9–15). Specific nucleoprotein (filled arrows) and Ab-supershifted complexes (empty arrows) are indicated.
Discussion

Recruitment of chromatin-modifying proteins to germline promoters and the subsequent transcriptional read-through of downstream RSSs contribute to gene segment recombinational accessibility (7). How such promoter-mediated accessibility shifts during lymphocyte development to target individual gene segments or clusters remains unclear. The Tcrg recombination bias toward Vγ3 and Vγ4 that is observed in fetal thymocytes is overcome in adult thymocytes by E2A-dependent repression of the Vγ3 and Vγ4 promoters (45, 46). Similarly, repression of distal germline promoters allows initial Vα-to-Jα joints assembled during Tcra recombination to target proximal Js (47). We have similarly shown that repression of germline promoter activity upstream of Dβ2 redirects germline transcription downstream of Dβ2 RSSs (20), which may account for the persistence of unrearranged DJβ2 sequences relative to DJβ1 in fetal thymocytes (21–23).

PDβ1 is required to displace histones from the Dβ1 23-RSS, augmenting its accessibility for RAG protein binding. Conversely, 5′PDβ2 repression may account for nucleosome occlusion of the Dβ2 23-RSS (9). We now show that this repression of 5′PDβ2 activity is mediated by binding of USF-1 to a noncanonical E-box within the Dβ2 12-RSS spacer sequence and that DSBs can induce a DNA-PKcs–dependent loss of USF-1 that relieves repression.

Based on the relative inefficiency with which PDβ1 directs DJβ1

FIGURE 5. USF-1 binds the repressor in the absence of recombination. (A) Unsorted thymocyte chromatin from Rag22/2, RxB, and wt C57BL/6 mice was immunoprecipitated with Ab to USF-1 (black bars) or USF-2 (gray bars) and analyzed by QPCR for binding proximal to Dβ2. (B) USF-1 binding at Dβ2 (see Fig. 1A, primers a and b) was analyzed by ChIP of chromatin from sorted DN1 and DN3 subsets of wt C57BL/6 thymocytes, as well as unsorted thymocytes from wt, Lat2/2, and Prkdc2/2 (SCID) mice and from the P5424 and P5424-c22 cell lines. Ab-dependent enrichment over input control is expressed relative to nonspecific IgG as mean – SD (n = 3) and is representative of two independent experiments. (C) QRT-PCR of 5′Dβ2 mRNA (see Fig. 1A, primers a and e) in thymocytes from Rag22/2, wt C57BL/6, RxB, and Prkdc2/2 (SCID) mice and in the P5424 subclones. Ab-dependent enrichment over input control is expressed relative to nonspecific IgG as mean – SD (n = 3) and is representative of two independent experiments. (D) QRT-PCR of USF-1 mRNA in Rag22/2, wt C57BL/6, and RxB thymocytes and in the P5424 subclones. Bars represent means (± SD; n = 3). Relative signals were calculated by ΔΔCT and normalized to β-actin controls.

FIGURE 6. Genotoxic DSBs relieve 5′PDβ2 repression. (A and C) ChIP QPCR analysis of USF-1 enrichment at Dβ2. (B and D) QRT-PCR of 5′Dβ2 mRNA. (A and B) Chromatin and mRNA signals in untreated RxB thymocytes (–) or in RxB thymocytes 1 d after treatment with DMSO carrier, increasing doses of ionizing radiation, or 3 μM etoposide. (C and D) Chromatin and mRNA signals in RxB (black bars) and P5424 (gray bars) 1 d after treatment with etoposide alone or after pretreatment with Nu7026. In each case, bars represent means ± SD (n = 3) for each sample. Fold enrichment of USF-1 and relative gene expression were calculated as described in Fig. 5.
recombinational accessibility when repositioned downstream of Dβ1 (19). 5’PDβ2 repression prior to recombination would be expected to limit DJβ2 accessibility. DSBs generated during recombination would be expected to subsequently increase accessibility by inducing a DNA-PKcs–dependent loss of 5’PDβ2 repression.

RAG DSBs impact the regulation of a wide range of genes in developing lymphocytes, principally through activation of the PI3 kinase, Atm (31). By extension, initial steps in V(D)J recombination could induce signals that feedback signals to regulate subsequent steps in Ag receptor assembly, perhaps acting to influence the order of Tcr gene assembly beyond an otherwise stochastic process in DN thymocytes. Indeed, the transcriptional regulation of DJβ2 suggests separate pathways by which recombination could feed back to regulate Tcrb assembly. Prior to recombination, 3’PDβ2 activity is dependent on low levels of constitutively nuclear NF-κB (48). However, given that NF-κB is activated by Atm (31), we speculate that RAG DSBs may simultaneously induce an Atm-dependent increase in activity of 3’PDβ2 and a DNA-PKcs–dependent activation of 5’PDβ2. As such, the downstream promoter may ensure β2 accessibility until the onset of V(D)J recombination extends accessibility to the DJβ2 RSSs. However, the location of the repressor box in the DJβ2 12-RSS spacer suggests an alternate model in which USF-1 could allosterically limit RAG access to the DJβ2 12-RSS prior to DJβ2 recombination and thereby contribute to beyond 12–23 regulation (5, 13, 49). The presence of transcription factor binding sites within an RSS is not unprecedented. The AP-1 protein c-Fos binds sites present in the 23-RSSs of both Dβ1 and Dβ2 and may enhance RAG deposition at the Dβ1 23-RSS while impeding RAG deposition at the 12-RSS (14). RAG1 and RAG2 are strongly enriched at both Dβ gene segments (39). However, a more detailed examination that distinguishes RAG occupancy between the closely spaced 12- and 23-RSSs of each Dβ will be necessary to test the potential of USF-1 to specifically limit RAG access to the DJβ2 12-RSS. Although regulation of DJβ2 promoter activity may impact the usage of individual Dβ2 segments in Vβ-to-DJβ recombination, DSB-inducing signaling could similarly impact ongoing rearrangements of other Tcr loci. For example, if USF-1 is similarly lost from the Dβ2 promoter where it is critical for Dβ2 transcription (50), RAG DSBs could theoretically impact Tcrd assembly.

USF-1 is a ubiquitously expressed stress-response protein that plays a critical role in lipid metabolism, cell cycle regulation, proliferation control, tumor suppression, and response to UV damage (25). It has also been linked to immune system development and function, regulating genes such as CIITA, β2-microglobulin, Igλ, and Igα (25) and germline Dβ2 promoter activity in Tcrd (50). In each of the latter cases, USF-1 acts as a transcriptional activator, whereas it appears to function as a repressor of the 5’PDβ2 and cad promoters. In addition, USF-1 is critical to the chromatin barrier function of the chicken β-globin insulator (51). We found no evidence that USF-1 regulates Dβ2 chromatin accessibility. Indeed, multiple studies have shown that the Dβ2 sequence is accessible in DN and DP thymocytes (36–39). Rather, our data are consistent with a narrower role for USF-1 in limiting germline transcription across Dβ2, which may in turn be necessary to enhance the recombinational accessibility of the Dβ2 RSSs.

Despite considerable overlap in the DNA-binding properties of various E proteins, our data suggest that 5’PDβ2 repression is uniquely mediated by USF-1. EMSA data (Fig. 4) suggest that the repressor site can be bound by USF-1 and/or USF-2. Nonetheless, we did not detect significant USF-2 binding in vivo. This absence, together with the DNA-PKcs sensitivity of 5’PDβ2 repression, suggests that USF-2, which lacks the phosphorylation sites that regulate USF-1 function (43), does not regulate the 5’PDβ2 repressor. The apparent inability of other E proteins to bind

**FIGURE 7.** Genotoxic DSBs induce USF-1 binding and loss of cad expression in P5424 cells. Shown are representative ChIP QPCR of USF-1 binding at the cad promoter (A) and cad mRNA levels (B) in P5424 1 d after treatment with ionizing radiation or etoposide ± Nu7026. (B) Bars represent means ± SD (n = 3) for each sample. Fold enrichment of USF-1 and relative gene expression were calculated as described in Fig. 5.

**FIGURE 8.** 5’PDβ2 repression is restored in c22 by inhibition of DNA-PKcs. Shown are representative ChIP QPCR of USF-1 binding at Dβ2 (A) and Dβ2 mRNA levels (B) in P5424-c22 1 d after treatment with the indicated kinase inhibitors. Bars represent means ± SD (n = 3) for each sample. Fold enrichment of USF-1 and relative gene expression were calculated as described in Fig. 5.
the repressor may owe to its noncanonical histepatic structure. Though myc-Max heterodimers can bind synthetic heptameric sites, they display a clear preference for canonical hexameric E-boxes (52). Conversely, the UV-responsive regulation of laminin-5 expression in epithelial cells is mediated by USF-1 binding to a heptameric E-box in the lamama3 promoter (53). Although USF-1/2 double-knockout mutations are lethal to embryonic development, no defects in thymocyte development or TCR repertoire diversity were reported for USF-1/2 mice (54). Indeed, our findings would predict that loss of USF-1 would manifest in a TCRβ repertoire subtly skewed toward inclusion of DJβ2 joints at the expense of DJβ1. USF-1–deficient mice are not currently available to confirm this prediction. However, it should be noted that thymocyte development and Tcrb recombination are essentially normal even in the complete absence of either the DJβ1 or DJβ2 gene segment clusters of mutant (17, 55) or New Zealand White mice.

Given P5424s p53 deficiency and relatively poor transfectability, it is unclear whether initial USF-1 loss from the P5424 subclones was triggered by RAG DSBs in general, but it is clear that DNA-PKcs repressor function is needed for efficient USF-1 loss and 5βPDB2 activation during repeated transfection. We speculate that DNA-PKcs–dependent modulation of USF-1 proximity to the Dβ2 joint is recruited for the repair of new DJβ2 joints during repeated transfections with PGK–RAG-1. Indeed, similar derepression of DJβ2 expression in epithelial cells is mediated by USF-1 binding to a heptameric E-box in the lama3 promoter: the beyond 12/23 restriction. Immunity 184: 6970–6977.

Acknowledgments

Thymocytes were harvested from Rag2−/−, Lat−/−, Rbxβ, and C57BL/6 mice kindly provided by Dr. Michael Krangel and Pkdac−/− mice kindly provided by Dr. Jorge Piedrahita. We thank Akinobade Oyegunwa, Erin Frankenfeld, Susan Gardner, and Jacob Lippincott for invaluable assistance in the preparation of this manuscript.

Disclosures

The authors have no financial conflicts of interest.

References


