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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 TCRβ 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: 000–000.

Lymphocytes 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 unarranged 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), Tcrg, and Tcra genes. If cells assemble functional Tcrg and Tcra joints 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 rearrangement at two Dβ1β3βγ gene segment clusters, followed by V rearrangement to a newly formed Dβ1 joint. Though Dβ2 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-Jβ2 recombination (16, 17) without affecting recombination at the downstream Dβ2 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 remodeling 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 Tcρ transgenes (19).

Although both DJβ1 clusters are transcriptionally active at the start of thymopoiesis (20), unarranged DJβ2 clusters persist in the endogenous loci of thymocytes from Tcρ 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 target DJ (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 Tcρ 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 Vβ 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 targets (CANN TG) (26) at promoters across the mammalian genome (27). USF proteins serve as master transcriptional regulators capable of interacting with a variety of transcription factors and chromatin modifiers to regulate such stress responses as UV-induced melanin production and insulin-dependent lipogenesis (25). DNA damage following UV treatment of keratinocytes and melanocytes induces phosphorylation of USF-1 by the MAPK p38, which in turn alters USF’s gene regulatory properties (28). During periods of fasting, the fatty acid synthase promoter is repressed by USF-1 associated with histone deacetylase 9. Upon feeding, USF-1 is phosphorylated by the DNA-dependent protein kinase, catalytic subunit (DNA-PKcs), leading to disassociation of histone deacetylase 9 and activation of the fatty acid synthase promoter (29). V(D)J recombination also critically depends on DNA-PKcs, as well as the related kinase Ataxia telangiectasia mutated (Atm), which are activated as part of a broader response to the dsDNA break (DSB) intermediates of recombination (30). Indeed, DSBs generated during V(D)J recombination alter the expression of a wide array of genes including cell type-specific genes not directly linked to the canonical DNA damage response (31).

In this study, we show that 5′PDβ2 repression in DN thymocytes is mediated by USF-1 bound to a noncanonical E-box within in the spacer sequence of the DJβ2 12-RSS. Developmental activation of 5′PDβ2 correlates with loss of USF-1 from the repressor element of both rearranged and germline DJβ2 clusters in DNA-PKcs–competent thymocytes. Finally, we show that 5′PDβ2 activity and loss of USF-1 can be induced in RAG2-deficient thymocytes and cell lines following treatment with the chemical genotoxin, etoposide, or with sublethal doses of ionizing radiation to generate DSBs. Downregulation of USF-1 binding and 5′PDβ2 repression is blocked by the DNA-PKcs inhibitor Nu7026. Given the general role of promoter activity in regulating recombinational accessibility, our data suggest a model in which DJβ2 promoter activity both inhibits and is instructed by V(D)J recombination.

Materials and Methods

Cells and Abs

The Rag1−/−, P53−/− P5424 pro-T cell line was cultured as previously described (20). CD44−/CD25− (DN1) and CD44−/CD25+ (DN3) C57BL/6 cell populations were isolated from DP-depleted thymocytes using a three-laser MoFlo cell sorter (DakoCytomation) as previously described (20). P5424 subclones harboring rearrangements of their DJβ1 and DJβ2 gene segment clusters were isolated after repeated transient transfection of P5424 parental cells with p-phosphorylase kinase (PKG)-RAG1 as described below. Transfected cells were subsequently screened for transceptor recombinations by PCR, and specific DJβ2 recombinations were confirmed by sequencing. Thymii were isolated from 4- to 5-wk-old mice including: wt C57BL/6, Rag2−/−, Prkd−/−, Lat−/−, and Rsβ (Rag2−/− mice that express a functionally rearranged TCRβ transgene (32)). Harvest procedures were reviewed and approved by the institutional animal care and use committee at North Carolina State University.

To induce dsDNA breaks in P5424 conventional or transgenic thymocytes, cells were plated in fresh RPMI and exposed to increasing doses of ionizing radiation using a GammaCell 220 cobalt-60 irradiator (MDS Nordion) or plated in RPMI supplemented with 3 μM etoposide for 4 h. After genotoxic insult, cells were replated in normal RPMI and allowed to recover for 18–24 h prior to chromatin and RNA extraction. For kinase inhibition studies, cells were pretreated with 0.1% DMSO, SB203580 (10 μM; Calbiochem), Nu7026 (10 μM; Cayman Chemical), or Ku55933 (15 μM; Selleck Chemicals) for 40 min at 37˚C before genotoxic insult.

Abs to CD44 (PE-labeled IM7; BD Pharmingen), CD25 (FITC-labeled 7D4; BD Pharmingen), CD117 (allopophycin-cyanin-labeled 2B8; BD Pharmingen), H3K9ac (Ab10812; Abcam), H3K4me2 (07-030; Millipore), H3K4me3 (39159; Active Motif), H3K27me3 (Abcam; ab66002), as well as the following Abs from Santa Cruz Biotechnology: USF-1 (sc-229), USF-2 (sc-862), E47 (sc-763), HEB (sc-357), Myc (sc-764), and Max (sc-197). Rabbit IgG (10-4102) was from Rockland Immunocchemicals.

Plasmids and transient transfection

For all transfections, 105 P5424 cells in log-phase growth were electrooporated, and luciferase reporter assays were conducted as previously described (20). Luciferase transfections were performed four or more times using independent plasmid preparations. For repeated PGK–RAG1 transfections, 2 × 106 cells were electroporated at 400 V in 0.2 μL V/950 μL with 10 μg pAG plasmid and 5 μg pMACS4.1 (Milenyi Biotech) and allowed to recover overnight. Transfectants were enriched using Dynabeads FlowCount Mouse CD4 magnetic beads (Invitrogen) and retransfected as above prior to subcloning. Luciferase reporter plasmids were generated by cloning individual restriction fragments or PCR amplification products of p5′DJβ1-BS (20) into the Smal site of pGL3-BE (20). Tiled site-specific mutations (TTCCA) were introduced into individual reporter constructs using QuicChange II (Stratagene) according to the manufacturer’s recommendations. The integrity of all reporter constructs was confirmed by sequencing.

Germline transcription and recombination

RNA was extracted using TRI Reagent (Sigma-Aldrich), according to the manufacturer’s instructions. DNA contaminants were removed using DNase-free DNase I (Fermentas) according to the manufacturer’s instructions, and 1–3 μg DNA-free RNA was reverse transcribed as previously described (20). The resultant cDNAs were amplified using standard (30–35 cycles) or quantitative PCR (QPCR; 50 cycles) reaction mixes (10 mM Tris-Cl [pH 9], 50 mM KCl, 2 mM MgCl2, 200 mM 2′-deoxy-nucleoside 5′-triphosphates, and 1 U Taq or 1 U SensiMix Plus [Quantace], respectively) as noted. Primer sequences are as shown (Supplemental Table I). The relative abundance of each rearranged DNA was quantified following QPCR by ΔΔ threshold cycle (CΔ) normalization to matched untreated controls and standardized for loading variations by comparison with values obtained for β-actin. Genomic DNA PCR extracted were pre-
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 the Sequenom MassARRAY platform with Epityper analysis software (Sequenom). EpiDesigner 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 assayed by 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 Tcρb contain germline promoters immediately upstream of their respective D segments (18, 20). However, DJb 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 unrearranged DJb2 (Fig. 1A, primers a and b) or total germline Jb2-Cb2 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−/− p53−/− 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 Tcρb and Tcρg loci. DJb sequences of one such clone (Fig. 1C, c22), as well as a representative control that maintained unrearranged 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 Jb2 segments to Cb2 (Fig. 1D, middle panel). However, germline transcription across DJb2 was limited to c22 (Fig. 1D, top panel), which also expressed the rearranged (DJb2JCb2.5 segment (data not shown). Similar results were obtained for three additional subclones that carried monoallelic DJb2 rearrangements with Jb2.1, -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, Runx-1, 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 DJb2 (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 hypo-methylation of the DJb2 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 amplicons from the Balb/T3T fibroblast cell line. Methylation in Balb/T3T was not universal, however, declining markedly proximal to DJb2. Indeed, methylation was essentially undetected at −15 and +178 CpGs in fibroblasts, suggesting that DJb2 is protected from methylation irrespective of Tcρb accessibility. ChIP analyses of Rag2−/− thymocytes also found that sites across Tcρb 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 (DJb2JCb2.5 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 DJb2 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 suppression. To define the repressor’s location, we used luciferase reporter analyses (Fig. 3). Serial 3′ 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 DJb2 12-RSS nonamer (−28 to −20) and/or spacer (−19 to −8).

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To identify potential repressor elements, we screened a panel of 5'PDβ2 repression reporters (21104/+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-1 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 (217 to 218) identified in our reporter assay failed to compete for protein binding (Fig. 4, lane 3). Mutation of the upstream 217CACGA213 sequence (to ttCcA, in which lowercase indicates

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

<table>
<thead>
<tr>
<th>D/Jβ1 cluster</th>
<th>P5424</th>
<th>P5424-c20</th>
<th>P5424-c22</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5'Db1—GGGACAGGGGGGCCACGGTGATTCAATTCTATGG</td>
<td>5'Db1—GGGACAGGGGGGCCACGGTGATTCAATTCTATGG</td>
<td>5'Db1—GGGACAGGGGGGCCACGGTGATTCAATTCTATGG</td>
</tr>
<tr>
<td></td>
<td>a) 5'Db1—GGGACAGGGGGGCCACGGTGATTCAATTCTATGG</td>
<td>a) 5'Db1—GGGACAGGGGGGCCACGGTGATTCAATTCTATGG</td>
<td>a) 5'Db1—GGGACAGGGGGGCCACGGTGATTCAATTCTATGG</td>
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<tr>
<td></td>
<td>b) 5'Db1—GGGACAGGGGGGCCACGGTGATTCAATTCTATGG</td>
<td>b) 5'Db1—GGGACAGGGGGGCCACGGTGATTCAATTCTATGG</td>
<td>b) 5'Db1—GGGACAGGGGGGCCACGGTGATTCAATTCTATGG</td>
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<tr>
<td></td>
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<td>a) 5'Db2—GGGACAGGGGGGCCACGGTGATTCAATTCTATGG</td>
</tr>
<tr>
<td></td>
<td>b) 5'Db2—GGGACAGGGGGGCCACGGTGATTCAATTCTATGG</td>
<td>b) 5'Db2—GGGACAGGGGGGCCACGGTGATTCAATTCTATGG</td>
<td>b) 5'Db2—GGGACAGGGGGGCCACGGTGATTCAATTCTATGG</td>
</tr>
</tbody>
</table>

*Dbβ 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 $^{\text{12}}$TGTAA$^{-8}$ to 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 8). Despite the loss of USF-1 from D$\beta_2$ in the recombinationally impaired DN cells of DNA-PKcs-deficient SCID mice (Fig. 5B), and this enrichment correlated with the absence of 5$'$D$\beta_2$ transcription (Fig. 5C), USF-1 was similarly enriched in P5424, but was lost from both the germline and re-arranged D$\beta_2$ clusters of c22 (Fig. 5B and data not shown).

Because both RxB and wt C57BL/6 thymocytes are predominantly DP, USF-1 binding in the RxB mice suggested that loss of USF-1 binding at D$\beta_2$ is not strictly dependent on DN to DP development. However, it remained possible that USF-1 was retained in RxB 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 D$\beta_2$ in the recombinationally impaired DN cells of DNA-PKcs-deficient SCID mice (Fig. 5B), and this enrichment correlated with the absence of 5$'$D$\beta_2$ transcription (Fig. 5C). USF-1 was similarly enriched in P5424, but was lost from both the germline and re-arranged D$\beta_2$ clusters of c22 (Fig. 5B and data not shown).

Despite the loss of USF-1 from D$\beta_2$ in wt DP cells, USF-1 RNA levels were equivalent among Ragu$^{2-/-}$, RxB, 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$'$D$\beta_2$ repressor in DP cells is due to downregulation of USF-1 expression. Indeed, we also found that USF-1 was absent from D$\beta_2$ 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 D$\beta_2$ 12-RSS is sufficient to repress 5$'$D$\beta_2$ activity and that loss

Table II. Percent methylation of CpG dinucleotides surrounding D$\beta_2$

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>CpG Positions$^{*}$</th>
</tr>
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<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>

$^{*}$Numbering relative to the first coding nucleotide of D$\beta_2$.
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 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 germline 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 p53-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.
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 $Vg_3$ and $Vg_4$ that is observed in fetal thymocytes is overcome in adult thymocytes by E2A-dependent repression of the $Vg_3$ and $Vg_4$ promoters (45, 46). Similarly, repression of distal germline promoters allows initial $V\alpha$-to-$J\alpha$ joints assembled during $Tcra$ recombination to target proximal Js (47). We have similarly shown that repression of germline promoter activity upstream of $D\beta_2$ redirects germline transcription downstream of $D\beta_2$ RSSs (20), which may account for the persistence of unrearranged $DJ\beta_2$ sequences relative to $DJ\beta_1$ in fetal thymocytes (21–23).

$PD\beta_1$ is required to displace histones from the $D\beta_1$23-RSS, augmenting its accessibility for RAG protein binding. Conversely, $5'PD\beta_2$ repression may account for nucleosome occlusion of the $D\beta_2$ 23-RSS (9). We now show that this repression of $5'PD\beta2$ activity is mediated by binding of USF-1 to a noncanonical E-box within the $D\beta_2$ 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 $PD\beta_1$ directs $DJ\beta_1$
recombinational accessibility when repositioned downstream of Dß1 (19), 5′PDß2 repression prior to recombination would be expected to limit Dß2 accessibility. DSBs generated during recombination would be expected to subsequently increase accessibility by inducing a DNA-PKcs–dependent loss of 5′PDß2 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 Dß2 suggests separate pathways by which recombination could feed back to regulate Tcrb assembly. Prior to recombination, 3′PDß2 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′PDß2 and a DNA-PKcs–dependent activation of 5′PDß2. As such, the downstream promoter may ensure Jß2 accessibility until the onset of V(D)J recombination extends accessibility to the Dß2 RSSs. However, the location of the repressor box in the Dß2 12-RSS spacer suggests an alternate model in which USF-1 could allosterically limit RAG access to the Dß2 12-RSS prior to Dß2 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 Dß1 and Dß2 and may enhance RAG deposition at the Dß1 23-RSS while impeding RAG deposition at the 12-RSS (14). RAG1 and RAG2 are strongly enriched at both Dß gene segments (39). However, a more detailed examination that distinguishes RAG occupancy between the closely spaced 12- and 23-RSSs of each Dß will be necessary to test the potential of USF-1 to specifically limit RAG access to the Dß2 12-RSS. Although regulation of Dß2 promoter activity may impact the usage of individual Dß segments in Vß-to-DßJß recombination, DSB-inducing signaling could similarly impact ongoing rearrangements of other Tcr loci. For example, if USF-1 is similarly lost from the Dß2 promoter where it is critical for Dß2 transcription (50), RAG DSBs could theoretically impact Tcrßd 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 CITA, β2-microglobulin, Igh, and Igα (25) and germline Dß2 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′PDß2 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 Dß2 chromatin accessibility. Indeed, multiple studies have shown that the Dß2 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 Dß2, which may in turn be necessary to enhance the recombinational accessibility of the Dß2 RSSs.

Despite considerable overlap in the DNA-binding properties of various E proteins, our data suggest that 5′PDß2 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′PDß2 repression, suggests that USF-2, which lacks the phosphorylation sites that regulate USF-1 function (43), does not regulate the 5′PDß2 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′PDß2 repression is restored in c22 by inhibition of DNA-PKcs. Shown are representative ChIP QPCR of USF-1 binding at Dß2 (A) and Dß2 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 hematopoietic structure. Though myc-Max heterodimers can bind synthetic hematopoietic 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 hematopoietic E-box in the lama3 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/−/− 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 Tcρb 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 5′PDβ2 repressor in the P5424 subclones was triggered by RAG DSBs in general, DJβ2 DSBs specifically, or DNA stress accumulated after multiple transfections with PGK–RAG-1. Indeed, similar derepression of 5′PDβ2 was observed in thymocytes treated with UVB radiation (data not shown), which induces pyrimidine dimer and DNA aduct formation and leads to USF-1 phosphorylation by p38 MAPK (28). The ability of Nűtø26e to restore USF-1–mediated repression in c22 suggests that sustained derepression in the P5424 subclones may derive from persistent DNA damage or RAG DSBs accrued during repeated transfection. We speculate that DNA-PKcs recruited for the repair of new DJβ2 joints would, by dint of its proximity to the DJβ2 12-RSS, be in position to ensure USF-1 removal and 5′PDβ2 activation for subsequent V-to-DJβ2 recombination. However, a DNA-PKcs–dependent modulation of USF-1 would not appear to require specific DJβ2 rearrangements per se to achieve 5′PDβ2 derepression.

In conclusion, our findings suggest that in addition to functional differences between the various DJ β and J β RSSs (5), differential promoter usage at DJβ2 and its attendant epigenetic modifications may account for the longstanding observations of enhanced DJβ1 recombination efficiency relative to DJβ2 (21–24). Future studies will be necessary to determine the precise mechanism by which USF-1 mediates repression of 5′PDβ2, how repression is resolved, and the extent to which this repression impacts DJβ2 recombinational efficiency. However, the remarkable detail to which DJβ2 transcriptional control has now been mapped renders Tcρb assemblment an ideal system to tease apart the relative contributions of RSS- and promoter-driven control to recombinational accessibility.

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Disclosures
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9

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