Published in final edited form as:
J Immunol. 2012 March 1; 188(5): 2266–2275. doi:10.4049/jimmunol.1002931.

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**[*,1]

[*]Department of Microbiology, North Carolina State University, Raleigh, NC 27695, USA

[†]Department of Radiation Oncology, Duke University, Durham, NC 27710, USA

Abstract

Activation of germline promoters is central to V(D)J recombinational accessibility, driving chromatin remodeling, nucleosome repositioning and transcriptional readthrough of associated DNA. We have previously shown that of the 2 *Tcrb* D segments, Dβ1 is flanked by an upstream promoter that directs its transcription and recombinational accessibility. In contrast, transcription within the DJβ2 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 DJβ2 recombination is less efficient than that of DJβ1. Since inefficient DJβ2 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. Here, we show that repression of the otherwise transcriptionally primed 5'Dβ2 promoter (5'PDβ2) requires binding of USF-1 to a non-canonical E-box within the Dβ2 12-RSS spacer prior to *Tcrb* recombination. USF binding is lost from both rearranged and germline Dβ2 sites in DNA-PKcs-competent thymocytes. Finally, genotoxic double-stranded DNA breaks lead to rapid loss of USF binding and gain of 5'PDβ2 activity in a DNA-PKcs-dependent manner. Together, these data suggest a mechanism by which V(D)J recombination may feedback to regulate local Dβ2 recombinational accessibility during thymocyte development.

Keywords

T cells; T cell receptor; Transcription factors; Gene rearrangement; Gene regulation; repressor; promoter; USF-1; DNA-PKcs

1. Introduction

Lymphocytes express a diverse array of antigen-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 antigen receptor genes are each assembled by a single enzymatic complex centered around the lymphocyte-specific RAG1/2 proteins that target conserved Recombination Signal Sequences (RSS) 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

[1]*Corresponding author at*: Department of Microbiology, 100 Derieux Place, Campus Box 7615, North Carolina State University, Raleigh, NC 27695, USA. Tel.: +1 919 513 0528; fax: +1 919 515 7867. mlsikes@ncsu.edu..

part by epigenetic regulation of promoters that populate each antigen 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 (ETPs) emigrate from the bone marrow to the subcapsular region of the thymus cortex. Progression of early thymocytes from ETP through $CD4^{-}CD8^{-}$ double negative (DN) development is coincident with rearrangement of *Tcrb*, *Tcrg* and *Tcrd* genes. If cells assemble functional *Tcrg* and *Tcrd* 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 where *Tcra* rearrangement occurs (12).

Tcrb assembly proceeds in a stepwise manner that involves independent D-to-J recombination at two DβJβCβ gene segment clusters, followed by V rearrangement to a newly formed DJβ joint. Though Dβ 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 Dβ1(18), and its recruitment of SWI/SNF chromatin remodeling complexes are critical for efficient DJβ1 assembly (8). Indeed, moving PDβ1 progressively downstream of Dβ1 increasingly impairs its ability to direct DJβ1 assembly of chromosomal *Tcrb* transgenes (19).

While both DJCβ clusters are transcriptionally active at the start of thymopoiesis (20), unrearranged DJβ2 clusters persist in the endogenous loci of thymocytes from *Tcrb* transgenic mice, as well as from wildtype fetal thymocytes (21–24). Unlike germline transcription at Dβ1, transcription in the germline DJβ2 cluster predominantly initiates 400–600 bp downstream of Dβ2 (20). However, DJβ2 rearrangement, which deletes the germline promoter, results in the activation of a second promoter upstream of Dβ2. 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 antigen receptor loci (7), it is likely that the downstream location of the germline Dβ2 promoter may contribute to the persistence of unrearranged DJβ2 clusters during thymocyte development (20). By extension, transcription from the upstream promoter (5'PDβ2), which passes through the Dβ2 coding sequence and flanking RSSs, would then be predicted to enforce DJβ2 accessibility during V-to-DJ recombination. Separate DJβ 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 Dβ2 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 factors-1 and -2 (USF-1/2) are ubiquitously expressed stress-response regulators that belong to the E protein family of bHLH-zip transcription factors (25). USF-1 and -2 bind as either homo- or heterodimers to E-box targets (*CANNTG*) (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 prodution and insulin-dependent lipogenesis (25). DNA damage following UV treatment of keratinocytes and melanocytes induces phosphorylation of USF-1 by the mitogen-activated protein kinase (MAPK) p38, which in turn alters USF's gene regulatory properties (28). During periods of fasting, the fatty acid synthase (FAS) promoter is repressed by USF-1 associated with HDAC9. 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 FAS 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 double-stranded DNA 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 non-canonical E box within in the spacer sequence of the Dβ2 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 Dβ2 promoter activity both instructs and is instructed by V(D)J recombination.

2. Materials and Methods

2.1. Cells and antibodies

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 3 laser MoFlo cell sorter (Cytomation) 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 pPGK-RAG1 as described below (section 2.2). Transfectants were subcloned and screened for antigen receptor recombinations by PCR, and specific Dβ recombinations were confirmed by sequencing. Thymii were isolated from 4–5 wk old mice including: wt C57BL/6, *Rag2*$^{-/-}$, *Prkdc*^{scid}, *Lat*$^{-/-}$, and *Rx*β (*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 double-stranded DNA breaks in P5424 cells or primary 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 hours. After genotoxic insult, cells were replated in normal RPMI and allowed to recover for 18-24 hrs prior to chromatin and RNA extraction. For kinase inhibition studies, cells were pretreated with 0.1% DMSO, SB203508 (10 μM, Calbiochem),

Nu7026 (10 μM, Caymen Chemical), or Ku55933 (15 μM, Selleck Chemicals) for 40 min at 37°C before genotoxic insult.

Antibodies to CD44 (PE-labeled IM7, BD-Pharmingen), CD25 (FITC-labeled 7D4, BD-Pharmingen), CD117 (APC-labeled 2B8, BD-Pharmingen),H3K9ac (Abcam, Ab10812), H3K4me2 (Millipore, 07-030), H3K4me3 (Active Motif, 39159), H3K27me3 (Abcam, mAb6002), 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 Immunochemicals.

2.2. Plasmids and transient transfection

For all transfections, 10^7 P5424 cells in log-phase growth were electroporated and luciferase reporter assays were conducted as previously described (20). Luciferase transfections were performed 4 or more times using independent plasmid preparations. For repeated PGK-RAG1 transfections, 7×10^6 cells were electroporated (300 μl serum free RPMI, 260 V/950 μF), with 10 μg RAG plasmid and 5 μg pMACS4.1 (Miltenyi Biotech), and allowed to recover overnight. Transfectants were enriched using Dynabeads FlowComp 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'D2JJ-BS (20) into the *Sma*I site of pGL3-Eβ (20). Tiled site-specific mutations (*TTCCA*) were introduced into individual reporter constructs using Quickchange II (Stratagene) according to the manufacturer's recommendations. The integrity of all reporter constructs was confirmed by sequencing.

2.3 Germline Transcription and recombination

RNA was extracted using TRI Reagent (Sigma), according to the manufacturer's instructions. DNA contaminants were removed using RNase-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 QPCR (50 cycles) reaction mixes (10 mM Tris-Cl (pH 9), 50 mM KCl, 2 mM $MgCl_2$, 200 mM dNTPs and 1 U Taq, or 1× SensiMix Plus (Quantace), respectively) as noted. Primer sequences are as shown (Table S1). The relative abundance of Dβ or USF-1 cDNAs was quantified following QPCR by $\Delta\Delta C_T$ normalization to matched untreated controls, and standardized for loading variations by comparison to values obtained for β actin. Genomic DNA PCR extracts were prepared as described (16), and DJβ rearrangements were assessed using primers and conditions as shown (Table S1).

2.4 CpG methylation

Sodium bisulfite modification of DNA was performed using EpiTect Bisulfite Kits (Qiagen) according to 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 Dβ2 (Table S3). 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.

2.5. Chromatin immunoprecipitation

Chromatin was prepared from formaldehyde-crosslinked P5424 or the indicated thymocytes and assayed by ChIP as described (33). Bound and input samples (4 μl) were subjected to

QPCR with 1× SensiMix *Plus* (Quantace) in triplicate reactions. Primers and annealing temperatures for chromatin immunoprecipitation are shown (Table S1). Cycling parameters for 20 μl reactions were 95°C 10 min., followed by 50 cycles of 95°C, 20 sec.; appropriate annealing temp, 30 sec.; 72°C, 30 sec. 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.

2.6. EMSA

P5424 nuclear extracts and radioactive probes were prepared and EMSA reactions performed as previously described (20). Sequences of wildtype and mutant oligonucleotide EMSA primers are as shown (Table S2).

3. Results

3.1. Repressed 5'PDβ2 remains accessible in DN thymocytes

Transcriptional promoters embedded in the germline sequences of antigen receptor genes drive localized recombinational accessibility of proximal gene segments (7). We have previously shown that both of the DJβ gene segment clusters in *Tcrb* contain germline promoters immediately upstream of their respective D segments (18, 20). However, Dβ2 germline transcription was differs significantly from Dβ1 in that transcription of the unrearranged DJβ2 cluster initiates from a promoter positioned downstream of Dβ2 and proximal to Jβ2.1 (20). Following Dβ2-to-Jβ2 recombination, transcription is redirected to a promoter that sits upstream of Dβ2 (20, 35), suggesting that the upstream promoter is initially repressed prior to DJβ2 recombination.

To test the possibility that the 5'PDβ2 repressor is located downstream of Dβ2, and is deleted upon DJβ2 recombination, we sought to determine if transcription in DP thymocyte populations is restricted to rearranged DJβ2 sequences (Fig. 1). Using QRT-PCR primer pairs specific for either unrearranged Dβ2 (Fig. 1A, primers *a* and *b*) or total germline Jβ2-Cβ2 spliced message (primers *c* and *e*), we assessed levels of DJβ2 transcription in thymocytes from wildtype C57BL/6 mice (primarily DP cells) or mice deficient for RAG-1 (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, significant levels of transcription through Dβ2 was only apparent in DP cells (Fig. 1B, black bars). Since RT-PCR measures steady state transcription levels, it remains possible if unlikely, that Dβ2 transcripts are inherently less stable in DN cells than those initiating further downstream. Regardless, the abundance of germline Dβ2 transcription in DP cells strongly suggests that activation of 5'PDβ2 does not require DJβ2 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 DJβ1 and monoallelic DJβ2 rearrangements, as well as rearrangements in their *Tcrd* and *Tcrg* loci. Dβ sequences of one such clone (Fig. 1B, c22), as well as a representative control that maintained unrearranged DJβ2 segments on both alleles (c20) are shown in Table 1. Both c20 and c22, as well as parental P5424 contained germline transcripts downstream of Dβ2 that spliced from Jβ2 segments to Cβ2 (Fig. 1D, middle panel). However, germline transcription across Dβ2 was limited to c22 (top panel), which also expressed the rearranged (Dβ2)Jβ2.5 segment (data not shown). Similar results were obtained for 3 additional subclones that carried monoallelic DJβ2 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 DJβ2 recombination occurs at both rearranged and germline DJβ2 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 Dβ2 (Table 2). Of the 8 CpG dinucleotides found within 500 bp upstream and downstream of Dβ2, 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 Dβ2) were methylated in >30–80% of screened amplicons from the Balb3T3 fibroblast cell line. Methylation in Balb3T3 was not universal however, declining markedly proximal to Dβ2. Indeed, methylation was essentially undetected at −15 and +178 CpGs in fibroblasts, suggesting that Dβ2 is protected from methylation irrespective of *Tcrb* accessibility. Chromatin immunoprecipitation (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 (Figs. 2B and 2C), and lacked H3 lysine 27 trimethylation found at silent promoters (Fig. 2D), corroborating prevous analyses of Dβ2 accessibility (37–39). Moreover, QPCR primers that selectively amplified either the germline or (Dβ2)Jβ2.5 gene segment of c22 found equivalent levels of histone marking, again suggesting that 5'PDβ2 repression does not involve epigenetic silencing of the germline Dβ2 chromatin.

3.2. 5'PDβ2 repression requires an E box in the Dβ2 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 downstream repressor by DJβ2 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 Dβ recombination. To define the repressor's location, we used luciferase reporter analyses (Fig. 3). Serial 3' deletion of all downstream sequence, the Dβ2 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 (compare −1104/−28 to the full-length 5'PDβ2 −1104/+230 construct), suggesting that repressor activity was localized to the Dβ2 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 (−1104/+13) into which we had introduced tiled five-base *TTCCA* substitutions (Fig. 3B). Whereas promoter activity was repressed in the wildtype construct and mutants that harbored substitutions in either the nonamer or Dβ2 coding sequence, two contiguous mutations that spanned the spacer sequence induced promoter activity 2.5 and 3.5-fold over wildtype, 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 Dβ2.

3.3. 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 (lanes 1 and 8). Excess unlabeled probe readily competed for protein binding (lane 2), while an oligonucleotide that carried the 10-bp repressor substitutions (−17 to −8) identified in our reporter assay failed to compete for protein binding (lane 3). Mutation of the upstream $^{-17}$*CACGA*$^{-13}$ sequence (to *ttCcA*, where lower case indicate substituted bases) was sufficient to abolish competition by the unlabeled primer (lane 4), while primers carrying the $^{-12}$*TGTAA*$^{-8}$ to *TtccA* mutation remained efficient competitors (lane 5). Mutation of the critical CpG dinucleotide in the center of the putative E box also abolished competition (lane 6), though its methylation on unlabeled primers had little impact on their ability to compete for protein binding (lane 7). Finally, the specificity of the protein complex was confirmed by its supershift in the presence of antibodies to USF-1 and USF-2 (lanes 9–10), whereas antibodies to other E proteins including E47, HEB, Myc and Max all failed to alter binding activity (lanes 11–14).

We had previously shown that while 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 (LAT), and are consequently prevented from maturing to DP cells (41). While USF-1 binding was modestly reduced in wildtype DN3 cells relative to DN1, it was abolished in LAT-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 5'Dβ2 transcription (Fig. 5C). USF-1 was similarly enriched in P5424, but was lost from both the germline and rearranged DJβ2 clusters of c22 (Fig. 5B and data not shown).

Despite the loss of USF-1 from Dβ2 in wildtype DP cells, USF-1 RNA levels were equivalent between *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 of USF-1 binding is triggered by DJβ2 recombination rather than by developmental progression.

3.4. Genotoxin-induced DSBs lead to loss of USF-1 binding and relieve 5'PDβ2 repression

Unlike RAG-2 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, Dβ2 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 Dβ2 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 (Figs. 6A–B). However, this genotoxin-induced derepression was blocked in cells pretreated with the DNA-PKcs inhibitor, Nu7026 (Figs. 6C–D).

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 synthatase 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. 6E–F). 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 suggest 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. 7A–B). 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 Dβ2 recombinational accessibility are in turn regulated by the DSB-sensitive repressive actions of USF-1.

4. Discussion

Recruitment of chromatin-modifying proteins to germline promoters and the subsequent transcriptional readthrough 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 non-canonical 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 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 antigen 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 Dβ2 suggests separate pathways by which recombination could feedback to regulate *Tcrb* assembly. Prior to recombination, 3'PDβ2 activity is dependent on low levels of constitutively nuclear NFκB (48). However, given that NFκB is activated by Atm (31), we speculate that RAG DSBs may simultaneously induce and Atm-dependent increase in activity of 3'PDβ2 and a DNA-PKcs-dependent activation of 5'PDβ2. As such, the downstream promoter may ensure Jβ2 accessibility until the onset of V(D)J recombination extends accessibility to the Dβ2 RSSs. However, the location of the repressor box in the Dβ2 12-RSS spacer suggests an alternate model in which USF-1 could allosterically limit RAG access to the Dβ2 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). RAG-1 and RAG-2 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 Dβ2 12-RSS. While regulation of Dβ2 promoter activity may impact the usage of individual DJβ 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 Dδ2 promoter where it is critical for Dδ2 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*, *Igh* and Iλ (25) and germline Dδ2 promoter activity in *Tcrd* (50). In each of the latter cases, USF-1 acts as a transcriptional activator, while 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 the repressor may owe to its non-canonical hepatmeric 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 *lama3* promoter (53). While 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 *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 P5424's 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-RAG1. Indeed, similar de-repression of 5'PDβ2 was observed in thymocytes treated with UVB radiation (data not shown), which induces pyrimidine dimer and DNA adduct formation, and leads to USF-1 phosphorylation by p38 MAPK (28). The ability of Nu7026 to restore USF-1-mediated repression in c22 suggests that sustained de-repression 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 dent of its proximity to the Dβ2 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 de-repression.

In conclusion, our findings suggest that in addition to functional differences between the various Dβ and Jβ RSSs (5), differential promoter usage at Dβ2 and its attendant epigenetic modulations 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 DJβ transcriptional control has now been mapped renders *Tcrb* assembly an ideal system to tease apart the relative contributions of RSS- and promoter-driven control to recombinational accessibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Thymocytes were harvested from *Rag2*$^{-/-}$, *Lat*$^{-/-}$, Rxβ and C57BL/6 mice kindly provided by Dr. Michael Krangel, and *Prkdc*^{scid} mice kindly provided by Dr. Jorge Piedrahita. We thank Akinbolade Oyegunwa, Erin Frankenfield, Susan Gardner and Jacob Lippincott for their invaluable assistance in the preparation of this manuscript.

This work was partially supported by a grant to MLS from the National Institute of Allergy and Infectious Diseases (R56AI070848-01A1).

Abbreviations used in this paper

RSS	recombination signal sequence
Tcrb	T cell receptor β locus
DN	double negative
DP	double positive
Eβ	*Tcrb* enhancer
bHLH-zip	basic helix-loop-helix leucine zipper
USF	upstream stimulatory factor
ChIP	chomatin immunoprecipitation
Rag	Recombination activating gene
DNA-PKcs	DNA-dependent protein kinase, catalytic subunit

References

1. Arstila TP, Casrouge A, Baron V, Even J, Kanellopoulos J, Kourilsky P. A direct estimate of the human alphabeta T cell receptor diversity. Science. 1999; 286:958–961. [PubMed: 10542151]
2. Rajewsky K. Clonal selection and learning in the antibody system. Nature. 1996; 381:751–758. [PubMed: 8657279]
3. Feeney AJ. Genetic and epigenetic control of V gene rearrangement frequency. Adv Exp Med Biol. 2009; 650:73–81. [PubMed: 19731802]
4. Cowell LG, Davila M, Ramsden D, Kelsoe G. Computational tools for understanding sequence variability in recombination signals. Immunol Rev. 2004; 200:57–69. [PubMed: 15242396]
5. Franchini DM, Benoukraf T, Jaeger S, Ferrier P, Payet-Bornet D. Initiation of V(D)J recombination by Dbeta-associated recombination signal sequences: a critical control point in TCRbeta gene assembly. PLoS ONE. 2009; 4:e4575. [PubMed: 19238214]
6. Abarrategui I, Krangel MS. Germline transcription: a key regulator of accessibility and recombination. Adv Exp Med Biol. 2009; 650:93–102. [PubMed: 19731804]
7. Osipovich O, Oltz EM. Regulation of antigen receptor gene assembly by genetic-epigenetic crosstalk. Seminars in immunology. 2010; 22:313–322. [PubMed: 20829065]
8. Osipovich O, Cobb RM, Oestreich KJ, Pierce S, Ferrier P, Oltz EM. Essential function for SWI-SNF chromatin-remodeling complexes in the promoter-directed assembly of Tcrb genes. Nat Immunol. 2007; 8:809–816. [PubMed: 17589511]
9. Kondilis-Mangum HD, Cobb RM, Osipovich O, Srivatsan S, Oltz EM, Krangel MS. Transcription-Dependent Mobilization of Nucleosomes at Accessible TCR Gene Segments In Vivo. J Immunol. 2010
10. Abarrategui I, Krangel MS. Regulation of T cell receptor-alpha gene recombination by transcription. Nat Immunol. 2006; 7:1109–1115. [PubMed: 16936730]
11. von Boehmer H. Selection of the T-cell repertoire: receptor-controlled checkpoints in T-cell development. Adv Immunol. 2004; 84:201–238. [PubMed: 15246254]
12. Hesslein DG, Schatz DG. Factors and forces controlling V(D)J recombination. Adv Immunol. 2001; 78:169–232. [PubMed: 11432204]
13. Tillman RE, Wooley AL, Hughes MM, Khor B, Sleckman BP. Regulation of T-cell receptor beta-chain gene assembly by recombination signals: the beyond 12/23 restriction. Immunol Rev. 2004; 200:36–43. [PubMed: 15242394]

14. Wang X, Xiao G, Zhang Y, Wen X, Gao X, Okada S, Liu X. Regulation of Tcrb recombination ordering by c-Fos-dependent RAG deposition. Nature immunology. 2008; 9:794–801. [PubMed: 18500346]
15. Yang-Iott KS, Carpenter AC, Rowh MA, Steinel N, Brady BL, Hochedlinger K, Jaenisch R, Bassing CH. TCR beta feedback signals inhibit the coupling of recombinationally accessible V beta 14 segments with DJ beta complexes. J Immunol. 2010; 184:1369–1378. [PubMed: 20042591]
16. Sikes ML, Suarez CC, Oltz EM. Regulation of V(D)J recombination by transcriptional promoters. Mol Cell Biol. 1999; 19:2773–2781. [PubMed: 10082543]
17. Whitehurst CE, Chattopadhyay S, Chen J. Control of V(D)J recombinational accessibility of the D beta 1 gene segment at the TCR beta locus by a germline promoter. Immunity. 1999; 10:313–322. [PubMed: 10204487]
18. Sikes ML, Gomez RJ, Song J, Oltz EM. A developmental stage-specific promoter directs germline transcription of D beta J beta gene segments in precursor T lymphocytes. J Immunol. 1998; 161:1399–1405. [PubMed: 9686603]
19. Sikes ML, Meade A, Tripathi R, Krangel MS, Oltz EM. Regulation of V(D)J recombination: A dominant role for promoter positioning in gene segment accessibility. Proc Natl Acad Sci U S A. 2002; 99:12309–12314. [PubMed: 12196630]
20. McMillan RE, Sikes ML. Differential activation of dual promoters alters Dbeta2 germline transcription during thymocyte development. J Immunol. 2008; 180:3218–3228. [PubMed: 18292546]
21. Lindsten T, Fowlkes BJ, Samelson LE, Davis MM, Chien YH. Transient rearrangements of the T cell antigen receptor alpha locus in early thymocytes. J Exp Med. 1987; 166:761–775. [PubMed: 3040885]
22. Haars R, Kronenberg M, Gallatin WM, Weissman IL, Owen FL, Hood L. Rearrangement and expression of T cell antigen receptor and gamma genes during thymic development. J Exp Med. 1986; 164:1–24. [PubMed: 3487610]
23. Born W, Yague J, Palmer E, Kappler J, Marrack P. Rearrangement of T-cell receptor beta-chain genes during T-cell development. Proc Natl Acad Sci U S A. 1985; 82:2925–2929. [PubMed: 3873070]
24. Uematsu Y, Ryser S, Dembic Z, Borgulya P, Krimpenfort P, Berns A, von Boehmer H, Steinmetz M. In transgenic mice the introduced functional T cell receptor beta gene prevents expression of endogenous beta genes. Cell. 1988; 52:831–841. [PubMed: 3258191]
25. Corre S, Galibert MD. Upstream stimulating factors: highly versatile stress-responsive transcription factors. Pigment Cell Res. 2005; 18:337–348. [PubMed: 16162174]
26. Solomon DL, Amati B, Land H. Distinct DNA binding preferences for the c-Myc/Max and Max/Max dimers. Nucleic Acids Res. 1993; 21:5372–5376. [PubMed: 8265351]
27. Rada-Iglesias A, Ameur A, Kapranov P, Enroth S, Komorowski J, Gingeras TR, Wadelius C. Whole-genome maps of USF1 and USF2 binding and histone H3 acetylation reveal new aspects of promoter structure and candidate genes for common human disorders. Genome Res. 2008; 18:380–392. [PubMed: 18230803]
28. Corre S, Primot A, Baron Y, Le Seyec J, Goding C, Galibert MD. Target gene specificity of USF-1 is directed via p38-mediated phosphorylation-dependent acetylation. J Biol Chem. 2009; 284:18851–18862. [PubMed: 19389701]
29. Wong RH, Chang I, Hudak CS, Hyun S, Kwan HY, Sul HS. A role of DNA-PK for the metabolic gene regulation in response to insulin. Cell. 2009; 136:1056–1072. [PubMed: 19303849]
30. Gapud EJ, Sleckman BP. Unique and redundant functions of ATM and DNA-PKcs during V(D)J recombination. Cell Cycle. 2011; 10:1928–1935. [PubMed: 21673501]
31. Bredemeyer AL, Helmink BA, Innes CL, Calderon B, McGinnis LM, Mahowald GK, Gapud EJ, Walker LM, Collins JB, Weaver BK, Mandik-Nayak L, Schreiber RD, Allen PM, May MJ, Paules RS, Bassing CH, Sleckman BP. DNA double-strand breaks activate a multi-functional genetic program in developing lymphocytes. Nature. 2008; 456:819–823. [PubMed: 18849970]

32. Shinkai Y, Koyasu S, Nakayama K, Murphy KM, Loh DY, Reinherz EL, Alt FW. Restoration of T cell development in RAG-2-deficient mice by functional TCR transgenes. Science. 1993; 259:822–825. [PubMed: 8430336]
33. Sikes ML, Bradshaw JM, Ivory WT, Lunsford JL, McMillan RE, Morrison CR. A streamlined method for rapid and sensitive chromatin immunoprecipitation. J Immunol Methods. 2009
34. Ciccone DN, Morshead KB, Oettinger MA. Chromatin immunoprecipitation in the analysis of large chromatin domains across murine antigen receptor loci. Methods Enzymol. 2004; 376:334–348. [PubMed: 14975316]
35. McMillan RE, Sikes ML. Promoter activity 5' of Dbeta2 is coordinated by E47, Runx1, and GATA-3. Mol Immunol. 2009; 46:3009–3017. [PubMed: 19592096]
36. Chattopadhyay S, Whitehurst CE, Schwenk F, Chen J. Biochemical and functional analyses of chromatin changes at the TCR-beta gene locus during CD4−CD8− to CD4+CD8+ thymocyte differentiation. Journal of Immunology. 1998; 160:1256–1267.
37. Morshead KB, Ciccone DN, Taverna SD, Allis CD, Oettinger MA. Antigen receptor loci poised for V(D)J rearrangement are broadly associated with BRG1 and flanked by peaks of histone H3 dimethylated at lysine 4. Proc Natl Acad Sci U S A. 2003; 100:11577–11582. [PubMed: 14500909]
38. Ji Y, Little AJ, Banerjee JK, Hao B, Oltz EM, Krangel MS, Schatz DG. Promoters, enhancers, and transcription target RAG1 binding during V(D)J recombination. The Journal of experimental medicine. 2010; 207:2809–2816. [PubMed: 21115692]
39. Ji Y, Resch W, Corbett E, Yamane A, Casellas R, Schatz DG. The in vivo pattern of binding of RAG1 and RAG2 to antigen receptor loci. Cell. 2010; 141:419–431. [PubMed: 20398922]
40. Brady BL, Oropallo MA, Yang-Iott KS, Serwold T, Hochedlinger K, Jaenisch R, Weissman IL, Bassing CH. Position-dependent silencing of germline Vss segments on TCRss alleles containing preassembled VssDJssCss1 genes. Journal of Immunology. 2010; 185:3564–3573.
41. Zhang W, Sommers CL, Burshtyn DN, Stebbins CC, DeJarnette JB, Trible RP, Grinberg A, Tsay HC, Jacobs HM, Kessler CM, Long EO, Love PE, Samelson LE. Essential role of LAT in T cell development. Immunity. 1999; 10:323–332. [PubMed: 10204488]
42. Sirito M, Lin Q, Maity T, Sawadogo M. Ubiquitous expression of the 43-and 44-kDa forms of transcription factor USF in mammalian cells. Nucleic acids research. 1994; 22:427–433. [PubMed: 8127680]
43. Corre S, Galibert MD. Upstream stimulating factors: highly versatile stress-responsive transcription factors. Pigment cell research / sponsored by the European Society for Pigment Cell Research and the International Pigment Cell Society. 2005; 18:337–348. [PubMed: 16162174]
44. Boyd KE, Farnham PJ. Myc versus USF: discrimination at the cad gene is determined by core promoter elements. Mol Cell Biol. 1997; 17:2529–2537. [PubMed: 9111322]
45. Xu CR, Schaffer L, Head SR, Feeney AJ. Reciprocal patterns of methylation of H3K36 and H3K27 on proximal vs. distal IgVH genes are modulated by IL-7 and Pax5. Proc Natl Acad Sci U S A. 2008; 105:8685–8690. [PubMed: 18562282]
46. Su IH, Basavaraj A, Krutchinsky AN, Hobert O, Ullrich A, Chait BT, Tarakhovsky A. Ezh2 controls B cell development through histone H3 methylation and Igh rearrangement. Nat Immunol. 2003; 4:124–131. [PubMed: 12496962]
47. Abarrategui I, Krangel MS. Noncoding transcription controls downstream promoters to regulate T-cell receptor alpha recombination. EMBO J. 2007; 26:4380–4390. [PubMed: 17882258]
48. Sen J, Venkataraman L, Shinkai Y, Pierce JW, Alt FW, Burakoff SJ, Sen R. Expression and induction of nuclear factor-kappa B-related proteins in thymocytes. Journal of Immunology. 1995; 154:3213–3221.
49. Drejer-Teel AH, Fugmann SD, Schatz DG. The beyond 12/23 restriction is imposed at the nicking and pairing steps of DNA cleavage during V(D)J recombination. Mol Cell Biol. 2007; 27:6288–6299. [PubMed: 17636023]
50. Carabana J, Ortigoza E, Krangel MS. Regulation of the murine Ddelta2 promoter by upstream stimulatory factor 1, Runx1, and c-Myb. J Immunol. 2005; 174:4144–4152. [PubMed: 15778374]

51. Huang S, Li X, Yusufzai TM, Qiu Y, Felsenfeld G. USF1 recruits histone modification complexes and is critical for maintenance of a chromatin barrier. Molecular and cellular biology. 2007; 27:7991–8002. [PubMed: 17846119]

52. Blackwell TK, Huang J, Ma A, Kretzner L, Alt FW, Eisenman RN, Weintraub H. Binding of myc proteins to canonical and noncanonical DNA sequences. Molecular and cellular biology. 1993; 13:5216–5224. [PubMed: 8395000]

53. Virolle T, Coraux C, Ferrigno O, Cailleteau L, Ortonne JP, Pognonec P, Aberdam D. Binding of USF to a non-canonical E-box following stress results in a cell-specific derepression of the lama3 gene. Nucleic acids research. 2002; 30:1789–1798. [PubMed: 11937633]

54. Sirito M, Lin Q, Deng JM, Behringer RR, Sawadogo M. Overlapping roles and asymmetrical cross-regulation of the USF proteins in mice. Proceedings of the National Academy of Sciences of the United States of America. 1998; 95:3758–3763. [PubMed: 9520440]

55. Bassing CH, Alt FW, Hughes MM, D'Auteuil M, Wehrly TD, Woodman BB, Gartner F, White JM, Davidson L, Sleckman BP. Recombination signal sequences restrict chromosomal V(D)J recombination beyond the 12/23 rule. Nature. 2000; 405:583–586. [PubMed: 10850719]

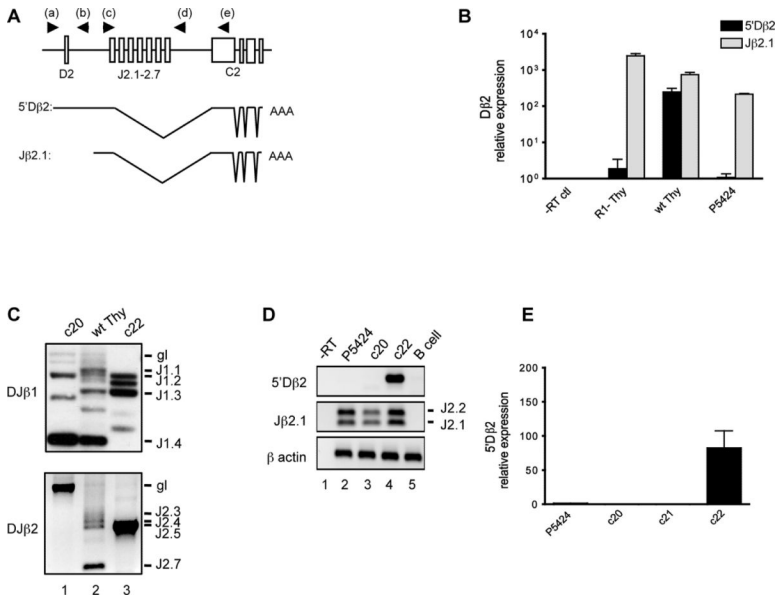

Figure 1.

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 Jβ2Cβ2 germline transcripts (grey bars, primers *c* and *e*), versus transcripts of unrearranged Dβ2 (black bars, primers *a* and *b*). Means (± SD, n = 3) are shown for DJβ2 signals relative to signals obtained in the absence of reverse transcriptase, and normalized to β-actin loading controls. (C) PCR of DJβ1(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 Dβ2 (upper panel, primers *a* and *b*), 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 Dβ2Jβ2Cβ2 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 $\Delta\Delta C_T$, and normalized to β actin loading control signals. Abbreviations include: R2- Thy (unsorted *Rag2*$^{-/-}$ thymocytes), wt Thy (unsorted C57BL/6 thymocytes), P5424 (*Rag1*$^{-/-}$p53$^{-/-}$ DN thymocyte cell line), c20 and c22 (RAG-transfected P5424 subclones), and B cell (M12 B cell line).

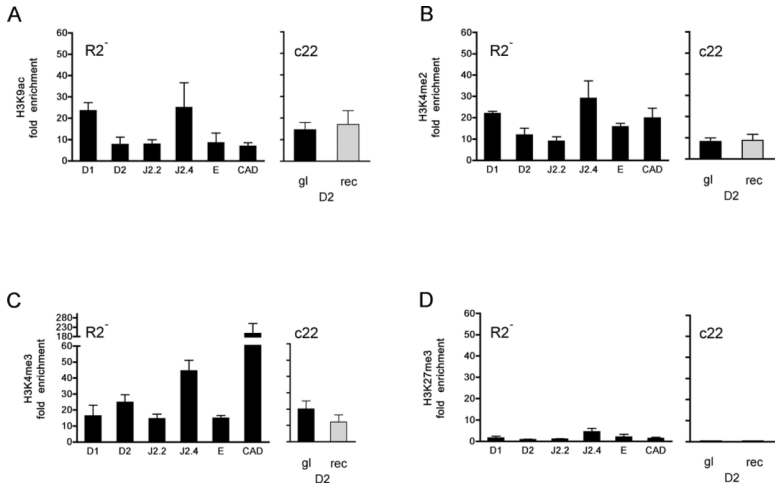

Figure 2.

Repression does not alter histone modifications at Dβ2. Chromatin from RAG2-deficient C57BL/6 thymocytes or the c22 subclone of P5424 was immunoprecipitated with the indicated antibodies. Resultant DNAs were analyzed by QPCR for histone modifications at the indicated *Tcrb* and control *cad* promoter (CAD) amplicons. Enrichment was calculated relative to pre-IP input control levels and was normalized against signals obtained with non-specific IgG control antibodies. Bars indicate means (± SD, n = 3), and are representative of 2 experiments with independent chromatin preparations.

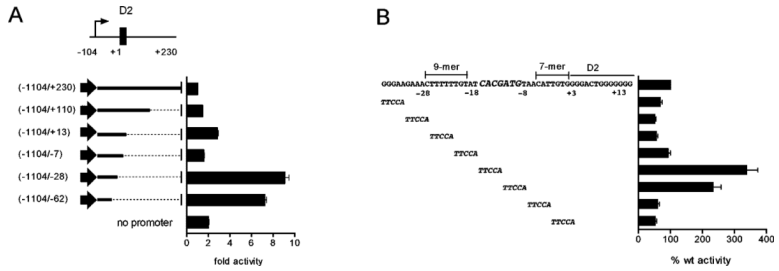

Figure 3.

Repression of 5'Dβ2 promoter activity requires *cis* targets in the Dβ2 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 Dβ2 coding sequence (+1). Protein extracts were assayed for luciferase activity 24 hours after transfection with each plasmid and normalized to co-transfected renilla. Bars represent mean normalized luciferase activity ± SEM of at least 6 transfections, and expressed as fold activity over the fully repressed −1104/+230 5'PDβ2 fragment (cite). (B) pGL3-Eβ constructs containing the wildtype −1104/+13 fragment (top) or the indicated *TTCCA* substitutions were assayed for luciferase activity 24 hrs after co-transfection with renilla plasmid. Bars represent mean normalized luciferase activity ± SEM, and are expressed as percent activity of wildtype −1104/+13.

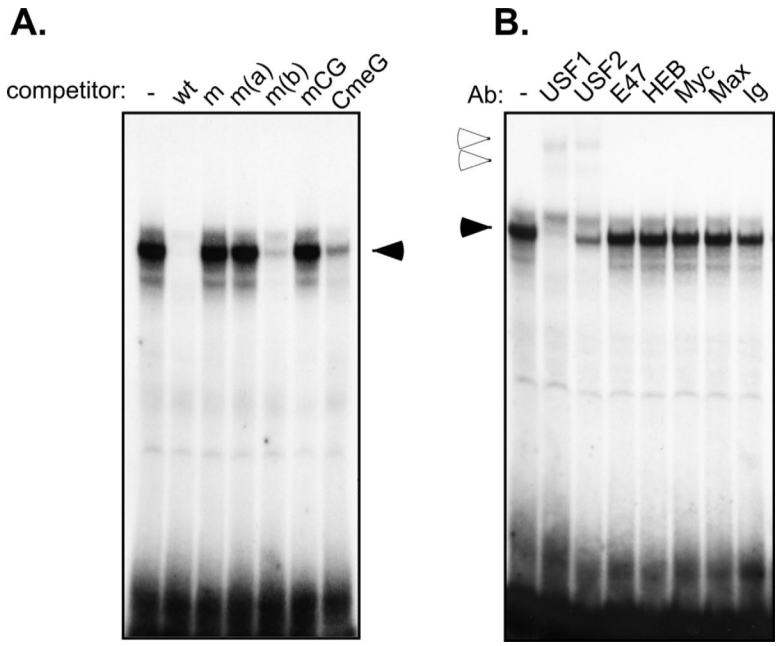

Figure 4.

USF-1/2 bind the repressor site in the Dβ2 5'RSS spacer. Nuclear extracts from the P5424 cell line were incubated with a radiolabeled ds 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.

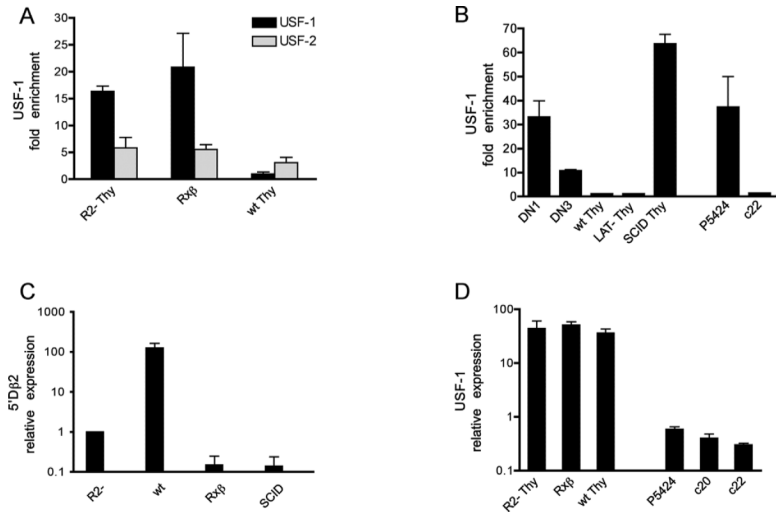

Figure 5.

USF-1 binds the repressor in the absence of recombination. (A) Unsorted thymocyte chromatin from *Rag2*$^{-/-}$, Rxβ, and wildtype C57BL/6 mice was immunoprecipitated with antibody to USF-1 (black bars) or USF-2 (grey 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 wildtype C57BL/6 thymocytes, as well as unsorted thymocytes from wildtype, *Lat*$^{-/-}$, and *Prkdc*$^{-/-}$ (SCID) mice and from the P5424 and P5424-c22 cell lines. Antibody-dependent enrichment over input control is expressed relative to nonspecific IgG as mean ± SD (n = 3), and is representative of 2 independent experiments. (C) QRT-PCR of 5'Dβ2 mRNA (see Fig. 1A, primers *a* and *e*) in thymocytes from *Rag2*$^{-/-}$, wildtype C57BL/6, Rxβ, and *Prkdc*$^{-/-}$ (SCID) mice. (D) QRT-PCR of USF-1 mRNA in *Rag2*$^{-/-}$, wildtype C57BL/6, and Rxβ thymocytes, and in the P5424 subclones. Bars represent means (± SD, n = 3). Relative signals were calculated by $\Delta\Delta C_T$ and normalized to β actin controls.

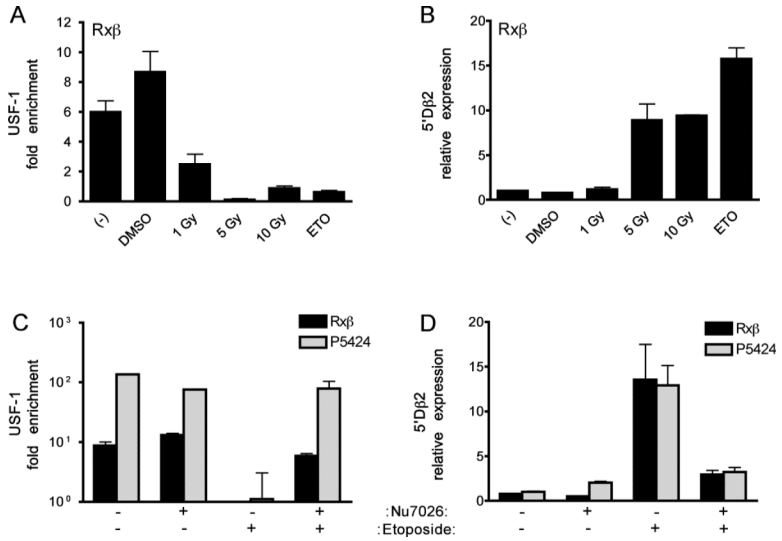

Figure 6.

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 Rxβ thymocytes (−) or in Rxβ thymocytes one day after treatment with DMSO carrier, increasing doses of ionizing radiation, or 3 μM etoposide. (C and D) Chromatin and mRNA signals in Rxβ (black bars) and P5424 (grey bars) one day 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.

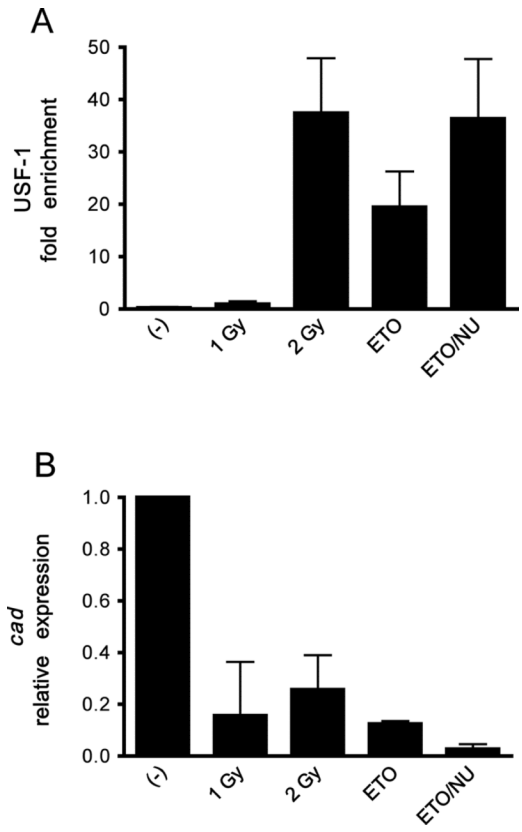

Figure 7.

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 one day 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.

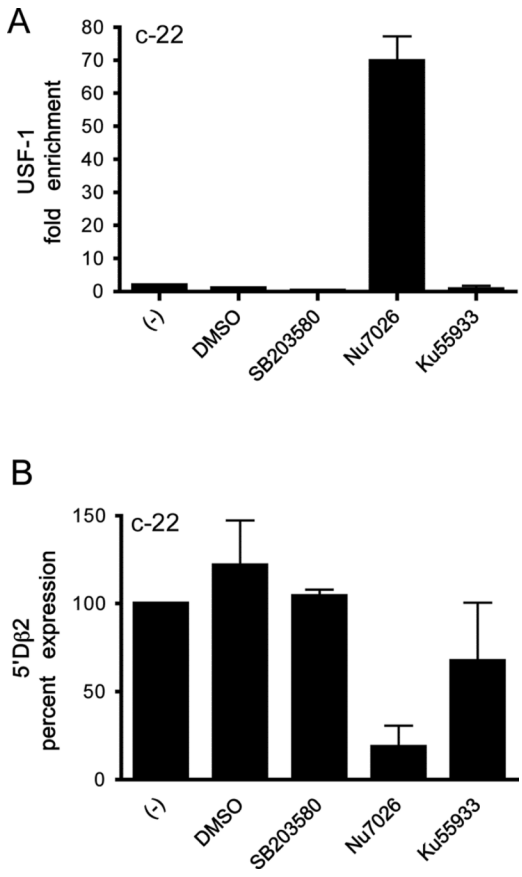

Figure 8.

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 one day 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.

Table 1

D-to-J recombinant sequence in clones c20 and c22

Dβ1Jβ1 cluster:	
P5424	Dβ1—**GGGACAGGGGGCCACGGTGATTCAATTCTATGG**
P5424-c20	a) Dβ1—GGGACAGGGGG* ---t--- TTTCCAACGAAA—Jβ1.4
	b) Dβ1—GG********** ------- **AACTCCGACT—Jβ1.2
P5424-c22	a) Dβ1— GGGACAGGG*** --agg-- ***ACTCCGACT—Jβ1.2
	b) Dβ1—GGGACAGGGG** ---tc-- TTCTGGAAATAC—Jβ1.3
Dβ2Jβ2 cluster:	
P5424	**Dβ2—GGGACTGGGGGGGCCACAATGATTCAACTGGAA**
P5424-c20	a) Dβ2—GGGACTGGGGGGGCCACAATGATTCAACTGGAA
	b) Dβ2—GGGACTGGGGGGGCCACAATGATTCAACTGGAA
P5424-c22	a) Dβ2—GGGACTGGGGGGGCCACAATGATTCAACTGGAA
	b) Dβ2—GGGACTGGGG** ---t--- AACCAAGACACC—Jβ2.5

Table 2

Percent methylation of CpG dinucleotides surrounding Dβ2

Cell line	CpG positions*							
	−453	−374	−15	+178	+251	+422	+464	+529
B3T3	0.33	0.48	0.07	0.05	0.18	0.44	0.80	0.21
P5424	0.07	0.04	0	0.02	0.04	0	0.08	0.04
P5424-c20	0.09	0.05	0.08	0.02	0.02	0.02	0.07	0.01
P5424-c22	0.14	0.05	0	0.01	0.05	0.05	0.13	0.05

* numbering relative to the first coding nucleotide of Dβ2