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Promoter activity 5′ of Dbeta2 is coordinated by E47, Runx1, and GATA-3

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Abstract

V(D)J recombination involves the stepwise assembly of B and T cell receptor genes as lymphocytes progress through the early stages of development. While the mechanisms that restrict each step in recombination to its appropriate developmental stage are largely unknown, they share many of the components that regulate transcription. For example, enhancer-dependent modifications in histone acetylation and methylation are essential for both germline transcription and rearrangement of antigen receptor genes. Promoters positioned proximal to individual D and J gene segments in *Tcra*, *Tcrb*, *Tcrd*, *IgH*, and *Igk* also contribute to antigen receptor gene assembly, though their effects appear more localized than those of enhancers. *Tcrb* assembly initiates with D-to-J joining at each of two D-J-C gene segment clusters in DN1/2 thymocytes. DJ joints are fused with Vβ elements to complete *Tcrb* recombination in DN3 cells. We have previously shown that Dβ2 is flanked by upstream and downstream promoters, with the 5′ promoter being held inactive until D-to-J recombination deletes the NFκB-dependent 3′ promoter. We now report that activity of the 5′ promoter reflects a complex interplay between Runx1, GATA-3, and E47 transcription factors. In particular, while multiple E47 and Runx1 binding sites clustered near the Dβ2 5′RS and overlapping *inr* elements define the core 5′PDβ2, they act in concert with an array of upstream GATA-3 sites to overcome the inhibitory effects of a 110 bp distal polypurine·polypyrimidine (R·Y) tract. The dependence of 5′PDβ2 on E47 is consistent with the reported role of E proteins in post-DN1 thymocyte development and V-to-DJβ recombination.

Keywords

T cell receptor; transcription; V(D)J recombination; thymocytes; double negative; E47; GATA-3; Runx1

1. Introduction

The ability of the immune system to respond to virtually any pathogen depends on developing B and T cells generating a diverse repertoire of antigen receptors. During lymphocyte development, each cell assembles unique antigen receptor genes through stepwise recombination of arrays of variable (V), diversity (D), and joining (J) gene segments (Schatz, 2004). In the thymus, T cell receptor β (*Tcrb*) gene assembly is initiated by Dβ1-Jβ and Dβ2-Jβ rearrangements in $CD4^{-}CD8^{-}$ double negative (DN) thymocyte subsets including $CD44^{+}$

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$CD25^-$(DN1) and $CD44^+$ $CD25^+$ (DN2) cells, followed by Vβ-DJβ rearrangement in $CD44^-CD25^+$ (DN3) thymocytes (Oltz, 2001). Productive Vβ-DJβ rearrangement leads to the cell surface expression of the TCRβ protein, together with preTCRα (pTa) and the CD3 complex as a preTCR. Signaling through the preTCR allows developmental progression to the $CD4^+CD8^+$ double positive (DP) stage, rearrangement of *Tcra*, and exclusion of further Vβ-DJβ rearrangement (Schatz, 2004).

Each V, D, and J gene segment is flanked by recombination signal sequences (RSS), which serve as recognition and cleavage sites for Rag-1 and –2, the lymphocyte-specific components of recombinase (Fugmann et al., 2000). The stepwise nature of antigen receptor gene assembly and its cell type and developmental specificities have long argued that changes in the accessibility of individual RSS targets to RAG proteins must ultimately govern V(D)J recombination. Consistent with the proposition of recombinational accessibility, transcription of unrearranged gene segments, which requires epigenetic chromatin opening (McMurry and Krangel, 2000), correlates with the onset of rearrangement at the same gene segments (Abarrategui and Krangel, 2006). In fact, transcriptional enhancers have been shown to directly influence the recombinational accessibility of each antigen receptor locus (Cobb et al, 2006), coordinating chromatin opening along large segments of the variable region, and initiating germline transcription from promoters positioned proximal to individual D or J segments. The potential impact of promoter activity on recombination appears to be subtler and more localized. Targeted deletion of the T early α promoter (TEA) selectively impairs Vα rearrangement to the most proximal Jα gene segments (Villey et al., 1996), while more distal promoters regulate accessibility of their associated Jα coding segments (Hawwari et al., 2005). Similarly, deletion of the Dβ1-associated promoter, PDβ1, impairs *Tcrb* Dβ1-to-Jβ recombination (Sikes et al., 1999; Whitehurst et al., 1999) without effecting rearrangement or transcription of the downstream DJβ2 gene segment cluster (Whitehurst et al., 1999).

Whereas both DJβ cassettes possess recombinational accessibility in DN1 cells (McMillan and Sikes, 2008), DJβ2 rearrangements have long been shown to accumulate more slowly than DJβ1 joints (Born et al., 1985; Haars et al., 1986; Lindsten et al., 1987; Uematsu et al., 1988). We have previously shown Dβ2 is flanked by two independently regulated promoters positioned 5′ and 3′ of Dβ2 (McMillan and Sikes, 2008). The 3′Dβ2 promoter is located 400-600 bp downstream of the Dβ2 gene segment and proximal to the Jβ2.1 RSS. Germline DJβ2 transcription during DN thymocyte development is restricted to 3′PDβ2, is dependent on constitutively nuclear P65 RelA-containing NFκB complexes (Sen et al., 1995; Weih et al., 1994), and initiates downstream of the Dβ2 RSS (McMillan and Sikes, 2008). We previously showed that moving PDβ1 to a similar position between Dβ1 and Jβ1.1 impairs its ability to direct recombinational accessibility of Dβ1 transgenes (Sikes et al., 2002).

Transcription from the upstream Dβ2 promoter (5′PDβ2), which passes through the D RSS, was only detected in *Tcrb* alleles upon Dβ2Jβ2 rearrangement, which deletes 3′PDβ2 and relieves 5′PDβ2 repression (McMillan and Sikes, 2008). Given the coordinated regulation of promoter activity and recombinational accessibility, we wished to define the elements that coordinate 5′PDβ2 activity. In this study, we characterize the regulation of 5′PDβ2 by Runx1, GATA-3, and the E protein, E47. We have previously shown that Dβ1 and Dβ2 are both flanked by multiple GATA-3 binding sites (McMillan and Sikes, 2008; Sikes et al., 1998). We now show that 5′PDβ2 contains 4 distinct GATA-3 binding sites, though GATA-3 binding at endogenous 5′PDβ2 sequences in the *Rag1*$^{-/-}$*P53*$^{-/-}$ DN thymocyte cell line P5424 is modest relative to that at PDβ1. In contrast, endogenous 5′PDβ2 is strongly and preferentially enriched for E47, which has previously been shown to play a critical role in *Tcrb* assembly (Agata et al., 2007). The minimal sequence necessary for promoter activity localized to a 220 bp region immediately 5′ of Dβ2 that contains both E boxes, as well as a binding site for Runx1 and overlapping RNA polymerase II (RNAP2) initiator (*inr*) elements. Finally, we show that a 110

bp polypurine·polypyrimidine stretch approximately 700 bp upstream of the minimal promoter acts to antagonize 5′PDβ2 reporter activity. Our data suggest that 5′PDβ2-dependent transcription immediately upstream of the Dβ2 coding sequence is coordinated by a dynamic interplay between positive and negative elements in DN T cell precursors.

2. Materials and Methods

2.1. Cells and antibodies

The *Rag1*$^{-/-}$, *P53*$^{-/-}$ P5424 pro-T cell line has been previously described (Mombaerts et al., 1995). Cells were cultured at 37°C/5% CO2 in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.01% penicillin/streptomycin, and 50 μM β-mercaptoethanol. Antibodies to Runx-1 (sc-28679x), GATA-3 (sc-268x), E47 (sc-763x), Sp1 (sc-59x) and USF-1 (sc-229x) were all purchased from Santa Cruz Biotechnology. Control rabbit IgG (10-4102) was purchased from Rockland Immunochemicals.

2.2. EMSA

Double-stranded oligonucleotides (Table 2S) were radioactively labeled using Klenow (New England Biolabs) by filling in 3-5 base overhangs with dNTP mixtures containing [α-32P] dCTP and [α-32P]dATP. Nuclear protein extracts were prepared as previously described (Sikes et al., 1998) from either P5424 or thymocytes isolated from 4-8 wk old *Rag2*$^{-/-}$ mice. Mouse thymus harvests were reviewed and approved by the institutional animal care and use committee at North Carolina State University. Binding reactions (20 μl) were performed at room temperature (30 min.) in a mixture containing nuclear protein extract (20 μg), radiolabeled probe (1 ng), poly(dI-dC) (1 μg), and BSA (10 μg) buffered in 20 mM HEPES (pH 7.9), 5% glycerol, 1 mM EDTA, 1% Nonidet P-40, and 5 mM DTT. Where indicated, unlabeled double-stranded competitor oligonucleotide (100-fold molar excess) or antibody (1 μg) was incubated with the nuclear extract for 30 min. on ice prior to addition of probes. Nucleoprotein complexes were resolved by electrophoresis on a 5% nondenaturing polyacrylamide gel, and visualized by autoradiography.

2.3. Chromatin immunoprecipitation

Chromatin was prepared from formaldehyde-crosslinked P5424 cells as described (Sikes et al., 2009). Prior to ChIP, each antibody (1 μg) was incubated (1 hr) with paramagnetic Dynabeads (Invitrogen) separately coupled to Protein A (10 μl/IP) and Protein G (10 μl/IP) in 50 μl RIPA buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% NaDeoxycholate) supplemented with BSA (50 mg/ml), sheared salmon sperm DNA (0.5 mg/ml), Halt protease inhibitor cocktail (Thermo Scientific), and 1 mM PMSF. Conjugated antibody:bead complexes were washed 2X in supplemented RIPA buffer.

For ChIP, each antibody:bead complex was mixed with chromatin (10^6 P5424 cell equivalents) in 100 μl supplemented RIPA buffer. Protein-DNA complexes were immunoprecipitated for 2 hours at 4°C with rotation, and then sequentially washed 3X in RIPA buffer and 2X in TE, pH 8.0. Protein-DNA complexes were eluted 15 min. in $NaHCO_3$ (100 mM), and crosslinks were reversed for 15 min. at 95°C after addition of NaCl (200 mM). Samples and matched inputs were incubated with Proteinase K (10 μg/ml) 1 hr @ 45°C, and DNA was purified using Qiaquick nucleotide removal columns (Qiagen) according to the manufacturer's instructions.

For realtime PCR, bound (3 μl) and input (3 μl of 1:100 dilution) samples were amplified using 1X SensiMix *Plus* (Quantace) and primers specific for PDβ1 (forward: 5′-TCACCTTCCTTATCTTCAACTCCC-3′; reverse: 5′-TCCCATAGAATTGAATCACCGTGG-3′), and 5′PDβ2 (forward: 5′-

GTTTCTGAGGCATGTGTCTCTGCG-3′; reverse: 5′ TCCTCTTTGTCACAGTGCCCACC-3′). Cycling parameters for 20 μl reactions were 95°C 10 min., followed by 40 cycles of 95°C, 20 sec; 63°C, 30 sec; 72°C, 30 sec. Fold enrichment in the bound fractions relative to input was calculated as previously described (Ciccone et al., 2004), and the average enrichment for triplicate amplifications was reported.

2.4. Plasmids

All luciferase reporter plasmids were generated by cloning individual restriction fragments or PCR amplification products of p5′D2JJ-BS (McMillan and Sikes, 2008) into the *Sma*I site of pGL3-Eβ (McMillan and Sikes, 2008), in which luciferase expression is driven in a T lineage-dependent manner by the mouse *Tcrb* enhancer, Eβ. Site-specific mutations were introduced into individual reporter constructs using the Quickchange II site-directed mutagenesis kit (Stratagene) according to the manufacturer's recommendations. Oligonucleotide primers used to introduce each mutation are as shown (Table 1S). The integrity of all promoter constructs was confirmed by restriction digest and sequencing. Plasmid DNA for luciferase reporter assays was prepared using the PureYield Endotoxin-free Midiprep kit (Promega) according to the manufacturer's instructions.

2.5. Luciferase assays

Luciferase reporter assays were performed as previously described (McMillan and Sikes, 2008). Briefly, 10^7 P5424 cells in log-phase growth were electroporated using a Bio-RAD gene pulser II (260 V, 950 μF, 200 Ω) in 0.4 cm cuvettes containing minimal RPMI 1640 medium (300 μl) 5′PDβ2 reporter construct (10 μg) and pSV-RL (250 ng, Promega). Cells were allowed to recover 10 min. on ice before transfer to complete culture medium. After 24 hr culture, cells were harvested, total protein extracted, and luciferase and renilla luminescence were sequentially determined for 50 μg total protein using the Dual-Luciferase Reporter Plasmid System (Promega) and a Centro LB960 luminometer (Berthold Technologies). All transfections were performed 3 or more times using independent plasmid preparations.

3. Results

3.1. Runx1, GATA-3, and USF1 bind multiple sites 5′ of Dβ2

We previously described two regions of promoter activity flanking the Dβ2 gene segment (McMillan and Sikes, 2008). Before Dβ2-Jβ2 rearrangements, germline transcription in $CD44^+25^-$ DN1 thymocytes is initiated at multiple sites within a 400 bp region downstream of Dβ2. Upon D-to-J rearrangement, 3′PDβ2 is deleted and transcript initiation is redirected predominantly to overlapping initiator (*inr*) elements positioned immediately upstream of Dβ2. Consistent with transcription patterns before and after D-to-J joining, we showed in reporter studies that a second Dβ2 promoter is located upstream of the D coding sequence in a position analogous to the Dβ1 promoter, PDβ1 (Sikes et al., 1998), and contains putative binding sites for a number of transcription factors central to hematopoietic development (Fig. 1). Moreover, activity of this promoter in our reporter experiments was only revealed when germline sequence 3′ of Dβ2 was deleted (McMillan and Sikes, 2008), suggesting that 5′ PDβ2 activity may play a role in Tcrb assembly after DJβ2 joints are formed.

The Runx family of transcription factors (including Runx1, Runx2, and Runx3) are critical for multiple stages of T cell development (Anderson, 2006). Canonical RACCRCA motifs exist in the enhancers of all four TCR loci. Moreover, chromatin immunoprecipitation studies have shown that Runx1 recognizes unidentified sites flanking both Dβ1 and Dβ2 (Oestreich et al., 2006). We identified 2 consensus Runx binding sites in 5′PDβ2 (Fig. 1, *R-I and R-II*). Radiolabeled probes to both sites detected a complex pattern of binding activities when mixed with nuclear proteins from P5424 cells (Fig. 2, *lanes 1 and 8*). Unlabeled Runx1-A or Runx1-

B oligonucleotides were equally effective at inhibiting all of the binding complexes at both sites (*lanes 2-3, 9-10*), whereas mutation of core ACC trinucleotide completely abolished each probe's competitive ability (*lanes 4-5, 11-12*). Nucleoprotein complexes formed between R-I or R-II and proteins from the DN3-staged P5424 cells were supershifted by Runx1 Ab (*lanes 6 and 13, respectively*), but not by isotype-matched controls (*lanes 7 and 14*) or by antibodies to either Runx2 or Runx3 (*data not shown*). Given that expression of the three Runx proteins varies during thymopoiesis (Taghon et al., 2006), we assessed binding site occupancy using nuclear extracts from mature T (BW5147) and B (M12) cell lines. Whereas R-I and R-II were bound by Runx2 in BW5147, both sites were occupied by Runx3 in M12 cells (data not shown).

We previously identified 3 canonical GATA binding sites 3′ of the Dβ2 coding sequence, two of which are bound by the T cell specific GATA-3 transcription factor (McMillan and Sikes, 2008). Although these GATA binding sites were dispensable for 3′PDβ2 activity, GATA-3 is essential for activation of the PDβ1 promoter (Sikes et al., 1998) and Eβ (Porritt et al., 2004). The four GATA binding sites upstream of Dβ2 are positioned within a 415 bp stretch. Three sites conform to the canonical GATA motif, WGATAR (Merika and Orkin, 1993), while the fourth (G-II; TGATTA) is mismatched at a single nucleotide. Despite the mismatch in G-II, radiolabeled probes to each of the 4 sites generated a series of complexes with nuclear protein from the P5424 cell line, though binding patterns differed between the separate sites (Fig. 3). Antibodies to GATA-3 either attenuated (*G-I, G-II, and G-IV*) or supershifted the primary complexes generated with each probe (*G-I, G-III, and G-IV*). In contrast, GATA-3 Abs had no effect on nucleoprotein complexes assembled with control NF-Y probes (Boothby et al., 1989).

Core 5′PDβ2 activity is confined to a 120 bp region that contains the R-II site and 2 potential E-boxes, E-I and E-II (Fig. 1). Radiolabeled probes of the two E boxes formed at least three nucleoprotein complexes with P5424 extracts that were abolished upon competition with unlabeled probes to either site (Fig. 4A, *lanes 1-3 and 6-8*). Mutation of the E box 3′ half-site in either probe eliminated its ability to compete for binding (*lanes 4-5 and 9-10*). ChIP-on-chip analyses showed a strong enrichment of E47 binding across Dβ2 (Agata et al., 2007). Likewise, E47 Abs supershifted complexes formed between P5424 or *Rag2*$^{-/-}$ thymocyte nuclear extracts and either E-I (Fig. 4B) or E-II probes (data not shown). Abs against upstream stimulatory factor-1 (USF1), a bHLH protein has been shown to bind the Dδ2 promoter (Carabana et al., 2005), failed to supershift E-I complexes in P5424 (*lane 2*) or *Rag2*$^{-/-}$ thymocyte nuclear extracts (*lane 6*). When we assayed nuclear extracts from E47-deficient Jurkat T cells, E-I complexes were now attenuated with USF1 Abs (*lanes 9-12*), suggesting that loss of E47 during thymocyte maturation may open the 5′PDβ2 E boxes for at least partial USF1 occupancy. At the same time, Abs to other bHLH proteins including c-MYC and MAX failed to alter E-I binding (data not shown).

3.2. 5′PDβ2 E boxes are bound by E47

Our EMSA results demonstrate that 5′PDβ2 contains multiple binding sites for a number of different transcription factors. We next used chromatin IP to determine whether GATA-3, Runx1, and E47 bind the promoter region *in vivo*. To that end, we enzymatically sheared P5424 chromatin that had been crosslinked with formaldehyde, and immunoprecipitated protein-DNA complexes using Abs specific for Runx1, GATA-3, E47, and for Sp1, which binds sites immediately 3′ of Dβ2. As a control, we compared antibody-dependent enrichment of 5′ PDβ2 sequence to that of PDβ1. Consistent with the dependence of Dβ1 transcription on GATA-3 and Sp1 (Sikes et al., 1998), both proteins were strongly enriched at PDβ1, relative to a nonspecific IgG control (Fig. 5). Antibodies to Runx1 failed to enrich PDβ1 sequences above isotype control levels. E47 Abs enriched PDβ1 sequences 3.25 fold above IgG levels, though they enriched 5′PDβ2 sequences more than 80 fold over IgG. The HEB and USF1

protiens were also enriched at 5′PDβ2 in P5424, despite their lack of detection by EMSA (Fig. 4). Given the relative levels of enrichment, our ChIP analyses suggest that E47 is the predominant protein at the 5′PDβ2 E boxes in P5424. HEB enrichment may reflect the presence of E47:HEB heterodimers in thymocytes (Sawada and Littman, 1993). There are no reports of E47 heterodimerizing with USF1. Rather, E47 may simply outcompete USF1 for occupancy of the 5′PDβ2 E boxes.

Consistent with EMSA results, Runx1 and GATA-3 were detected at the Dβ2 upstream promoter (7 and 2 fold over IgG, respectively). Additionally, antibodies to Sp1 enriched 5′ PDβ2 sequence approx. 3 fold over IgG. Sequence analysis predicts one potential Sp1 site between R-II and E-II, though it is poorly conserved and failed to bind protein in EMSA studies (data not shown). Although cryptic Sp1 sites may exist in 5′PDβ2, our ChIP results most likely reflect antibody binding to the Sp1 site downstream of Dβ2 (360 bp 3′ of the 5′PDβ2 ChIP amplicon). Similarly, the modest levels of GATA-3 enrichment we detect at 5′PDβ2 may derive from binding to the 2 GATA-3 sites 3′ of Dβ2 (400 and 480 bp 3′ of the 5′PDβ2 amplicon), though EMSA clearly implicated GATA-3 binding 5′ of Dβ2. Alternatively, relative differences between GATA-3 binding at PDβ1 and 5′PDβ2 could reflect the transcriptional activity of the former and inactivity of the latter prior to DJ recombination.

3.3. Promoter activity upstream of Dβ2

To localize the core 5′PDβ 2 promoter, we generated a series of luciferase reporter constructs in which the *Tcrb* enhancer, Eβ, was paired with restriction fragments that spanned the 1 kb stretch of DNA immediately upstream of Dβ2. Each construct was transiently transfected into the *Rag1*$^{-/-}$*P53*$^{-/-}$ DN thymocyte cell line P5424, along with control pSV-RL (Promega), and transfectants were assayed for luciferase expression (Fig. 6). Consistent with our previous findings, promoter activity was primarily localized to a 220 bp region immediately 5′ of the Dβ2 5′RS containing the R-II binding site, both E boxes, and 2 overlapping transcription initiator (*inr*) elements. A 140 bp deletion of the 3′-most portion of this core region that eliminated the *inr* elements and proximal E box (E-II) reduced promoter activity to 44% of the full-length promoter fragment (Fig. 6A, *3′Δ1 vs flP*). Promoter activity was further reduced with subsequent 3′ deletions that eliminated the 4 putative binding sites for the GATA family of transcription factors (3′Δ2 and 3′Δ3). However, 5′ deletions that removed the first 2 (*5′Δ1*) or all 4 GATA sites (*5′Δ2*) actually led to modest increases in promoter activity.

Progressive 5′ deletion of E-I and R-II reduced promoter activity to 38% of flP (Fig. 6A, *5′ Δ3*), suggesting that while GATA binding may or may not enhance 5′PDβ2, E-I and R-II appear to be critical for activity of the core promoter. When normalized to the intact core promoter (*5′Δ2*), specific deletion of E-I led to a 26% reduction in luciferase activity (Fig. 6B), suggesting that proteins recruited to E-I are required for full activity of the core 5′PDβ2. Similar results were obtained for the 5′ and 3′ deletion studies when constructs were transfected into Jurkat cells or when Eβ was replaced with the SV40 enhancer (data not shown).

3.4. Mutational analysis of 5′PDβ2

Deletion assays demonstrate that the bulk of promoter activity is retained in the –280/-61 5′ Δ2 fragment (Fig. 6A), and suggest only minimal roles for the upstream GATA and R-I sites. To assess individual contributions of transcription factor binding sites to 5′PDβ2 activity, each site in the full-length reporter was selectively mutated (Fig. 7). Consistent with 5′ deletion, disruption of the R-I site had no impact on promoter activity. Likewise, the GATA site at -401 (G-III) appeared dispensable. In contrast, mutation of either G-I or G-II showed a modest reduction in 5′PDβ2 function (16% and 9% below flP, respectively). Our deletion studies suggested that all of the distal sites (R-I and the 4 GATA sites) are dispensable for promoter activity. However, we found that mutation of the most proximal GATA site (G-IV) yielded a

31% reduction in promoter activity, similar to mutation of either the E-I or R-II sites within the core 5′PDβ2 (81% and 75% of flP, respectively). Whereas E-I mutation impaired 5′PDβ2, mutation of E-II failed to reduce promoter activity. This unexpected result for E-II suggests that reduced promoter activity in our minimal 3′ deletion construct, 3′Δ1 (Fig. 6A), may solely derive from loss of the overlapping *inr* elements.

We observed little effect from mutation of the canonical TATAA sequence that lies between G-IV and E-I, 193-197 bp upstream of the *inr* elements (Fig. 7). Introduction of 4-bp mutation into the paired *inr* elements resulted in an 88% decrease in promoter activity, consistent with their predominant role in 5′PDβ2 transcript initiation (McMillan and Sikes, 2008). 3′ deletions that removed a 142 bp *Ban*I/*Alw*NI fragment containing the *inr* and E-II elements proved somewhat less destructive than selective *inr* mutation, reducing promoter activity by 56% (Fig. 6A, *compare 3′Δ1 to flP*). Although the *inr* elements are not closely flanked by canonical TATA or downstream promoter elements (DPE, RGWCGTG), we did note the presence of a partial DPE sequence (GGTCTCC) 27 bp 3′ of the first *inr*. Since motif searches and EMSA failed to detect alternative binding moities in the *Ban*I/*Alw*NI fragment, the specific sensitivity of 5′ PDβ2 reporter constructs to targeted *inr* mutation may indicate activities that normally restrict transcription initiation to the *inr* sites, and are lost in 3′Δ1.

3.5. Distal elements modulate 5′PDβ2

Our deletion analyses suggested that, while the GATA sites appear dispensable for core 5′ PDβ2 activity, fragments that span the R-I and GATA sites nonetheless maintain minimal promoter activity. We previously found that a minority of 5′PDβ2 transcripts initiated upstream of the consensus *inr* elements (McMillan and Sikes, 2008). Indeed, one start site was positioned 26 nucleotides downstream of a consensus TATA box that sits between G-IV and E-I. To more directly test the potential of the GATA sites in contributing to 5′PDβ2 activity, we generated a second panel of pGL3-Eβ luciferase vectors that contained PCR fragments that selectively removed core 5′PDβ2 components (Fig. 8). Relative to the PCR-generated full-length promoter control (*flP′*), a fragment truncated immediately 3′ of the E-I site (*ΔR/E-II*) retained 70% promoter activity, while mutagenesis of ΔR/E-II to remove E-I reduced activity to promoterless control levels (*ΔcP*).

Polypurine·polypyrimidine sequences have the potential to assemble as triplex or quadruplex DNA (Frank-Kamenetskii and Mirkin, 1995), and have been associated with a diverse array of functions. R·Y stretches are enriched in the upstream and intronic regions of genes (Gaddis et al., 2006). Moreover, R-Y tracts have been linked to mutagenesis, ds breaks, and transcriptional repression (Jain et al., 2008). 5′ deletions that removed the relatively long R-Y tract upstream of the core 5′PDβ2, along with 1 or more GATA-3 sites appeared to enhance promoter activity (Fig. 6A). Given the presence of a canonical TATA box downstream of G-IV, we wished to determine if R·Y deletion might restore promoter activity to the GATA-rich AcP fragment. Indeed, R·Y deletion essentially restored ΔcP activity to parental ΔR/E-II levels (Fig. 8). The upstream Runx binding site, R-I, is located 114 bp 5′ of the polypurine sequence. When we destroyed R-I by site-directed mutagenesis (*ΔR-I ΔR·Y*), we observed a 36% reduction in promoter activity relative to ΔR·Y, suggesting that R-I has modest activating potential that is masked by the R·Y tract. Taken together, these studies suggest that the R-I and GATA sites may work with downstream Runx and E box elements of the core promoter to overcome the inhibitory effects of the RY tract and drive 5′PDβ2.

4. Discussion

In contrast to the regulatory simplicity of 3′PDβ2, a number of factors appear to contribute to overall 5′PDβ2 activity. The core promoter is localized to a 220 bp sequence that contains binding sites for E47 and Runx1, as well as the predominant transcription start sites positioned

over paired *inr* elements. An array of 4 GATA-3 sites and an additional Runx1 site upstream of the core promoter appear dispensable for core promoter activity, acting instead to counterbalance the inhibitory effects of a 110 bp polypurine repeat. Multiple GATA-3 sites have similarly been found in the other 2 *Tcrb* germline promoters: PDβ1 and 3′PDβ2. While paired GATA-3 sites upstream of Dβ1 are critical for PDβ1 activity (Sikes et al., 1998), similarly paired sites between Dβ2 and Jβ2.1 failed to augment 3′PDβ2 reporter activity (McMillan and Sikes, 2008). Alternatively, the 6 GATA-3 sites flanking Dβ2 may act to promote a general state of chromatin opening across the Dβ2 regulatory region, rather than directly activating transcription as in PDβ1.

Transcriptional promoters positioned proximal to D and J gene segments play pivotal roles in antigen receptor gene assembly; serving as synaptic targets for downstream enhancers (Oestreich et al., 2006), recruiting chromatin modifying proteins (Osipovich et al., 2007), and driving transcription through germline sequences. We have previously shown that promoter activity 5′ of the Dβ1 element is essential for Dβ1 recombination (Sikes et al., 1999). In contrast, germline transcription of the DJβ2 cassette is dependent on the actions of a promoter positioned between Dβ2 and Jβ2.1 (McMillan and Sikes, 2008), likely leading to the less efficient assembly of DJβ2 joints in early DN thymocytes (Born et al., 1985; Haars et al., 1986; Lindsten et al., 1987; Uematsu et al., 1988). Mutations that diminish germline transcription also reduce V assembly with D reporter targets in *Tcrb* (Khor et al., 2009) and J segments in *Tcra* (Abarrategui and Krangel, 2006; Hawwari et al., 2005), indicating a conserved role for germline promoters at each stage of recombination. Whereas downstream positioning of 3′ PDβ2 may enhance overall *Tcrb* diversity by limiting initial Vβ recombination with DJβ2 joints, activation of 5′PDβ2 upon 3′PDβ2 deletion may be necessary to ensure that DJβ2 joints are recombinationally accessible once formed. In that regard, the close proximity of the 5′ promoter to the Dβ2 recombination signal sequences mirrors that of Dβ1 and Dδ2 promoters. Such proximity may be essential for recombinational accessibility, targeting nucleosomal disruption at the D RSS elements that have recently been suggested to initiate both D-to-J and V-to-DJ stages of *Tcrb* assembly (Franchini et al., 2009).

The class I bHLH proteins (E proteins) have significant and diverse roles in antigen receptor gene rearrangement and lymphocyte development (Murre, 2005). In particular, E47 is required for expression of RAG1 and RAG2 in pro-B cells (Hsu et al., 2003), as well as for regulating *Igk* recombination (Romanow et al., 2000). Likewise, E47 enforces developmental arrest of DN3 thymocytes until assembly of a functional *Tcrb* gene (Engel et al., 2001). More recently, Murre and colleagues have shown that E47 binding augments Vβ chromatin accessibility, and that E47 is also strongly localized over DJβ2 and downstream Eβ and Vβ14 elements (Agata et al., 2007). Additionally, histone H3 acetylation and Dβ-to-Jβ recombination are both reduced in DN cells of E47-deficient mice. Our ChIP studies in P5424 suggest that multiple factors including E47 are bound at 5′PDβ2 prior to DJ recombination and promoter activation. As such, E47 binding upstream of Dβ2 may be involved in the recruitment of histone acetyltransferase (HAT) activity to germline D and J sequences, facilitating recombinational accessibility and activation of the 3′PDβ2, before it contributes to 5′PDβ2 activity.

Separate studies confirm that H3Ac levels at the DJβ cassettes remain elevated in DP cells (Morshead et al., 2003; Tripathi, 2002), despite the obligate reduction of E47 DNA binding activity during β selection (Engel et al., 2001). Together, these data suggest a critical but temporally restricted role for E47 in regulating Dβ accessibility during DN development. Since transcripts that initiate at 5′PDβ2 are readily detected in DP cells (McMillan and Sikes, 2008), it is possible that E47 is replaced at the 5′PDβ2 E boxes in DP cells. E47 and E12 can recognize the same sequence, albeit with differing affinities, and HEB binding at 5′PDβ2 is consistent with E47:HEB heterodimers in developing thymocytes (Sawada and Littman, 1993). Alternatively, as suggested by EMSA (Fig. 4), the 5′PDβ2 E boxes could be occupied

by other bHLH proteins like USF1 in more mature cells after E47 expression declines, though the significance of DJβ histone acetylation in DP thymocytes remains unclear. Irrespective of function later in development, our data are consistent with a model in which E47 facilitates 5′ PDβ2 activation in DN2/DN3 cells.

The RY tract located between the R-I and G-I binding sites was found to inhibit 5′PDβ2 activity. RY elements are disproportionately enriched in the upstream portions of genes, can form stable triplex DNA structures (Jain et al., 2008), and have been shown to repress transcription (Grabczyk and Fishman, 1995; Grabczyk and Usdin, 2000). In particular, the S1 nuclease-sensitive R-Y palindrome upstream of the human c-*Myc* P1 and P2 promoters has been shown to assemble a short triplex hairpin that partially arrests c-*Myc* transcription and may underlie *c-Myc* mutagenicity (Belotserkovskii et al., 2007). The ability of R·Y triplexes to pause RNAP2 has been predicted to have a more significant repressive effect when positioned in the vicinity of other transcription termination elements. Indeed, the Dβ2 R·Y element begins 265 bp downstream of the Cβ1 polyadenylation sequence. Since transcriptional elongation from upstream *Tcra* promoters has been shown to alter the recombinational accessibility of downstream Jα elements (Abarrategui and Krangel, 2006), we suggest that one role of the Dβ2 R·Y tract may be to isolate the Dβ2 coding and RSS sequences from the effects of readthrough Dβ1 germline transcription.

The partially denatured structure of triplex DNA leaves it as a frequent hotspot for mutagenesis and recombination. The RAG-mediated t(14;18) translocation that juxtaposes *bcl*-2 with the intronic IgH enhancer and underlies follicular lymphoma has been linked to a triplex-forming RY element within the *bcl-2* major breakpoint (Raghavan et al., 2005). If the *bcl*-2 R·Y repeat can augment RAG recruitment to *bcl*-2, the Dβ2 R·Y might similarly enhance RAG recruitment to the Eβ regulatory region of *Tcrb*. In this regard, we note that 2 additional 100 bp R·Y tracts are also found in the DJβ region, one approximately 3.5 kb upstream of Dβ1, and one between Jβ1.4 and Jβ1.5. Future studies will be necessary to determine how these R·Y tracts may affect *Tcrb* assembly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

RSS
: recombination signal sequence

Tcrb
: T cell receptor β locus

Tcra
: T cell receptor α locus

DN double negative
: DP, double positive, Eβ, *Tcrb* enhancer, PDβ1, Dβ1 promoter

bHLH
: basic helix-loop-helix

USF1

upstream stimulatory factor 1, ChIP, chomatin immunoprecipitation

Rag

Recombination activating gene

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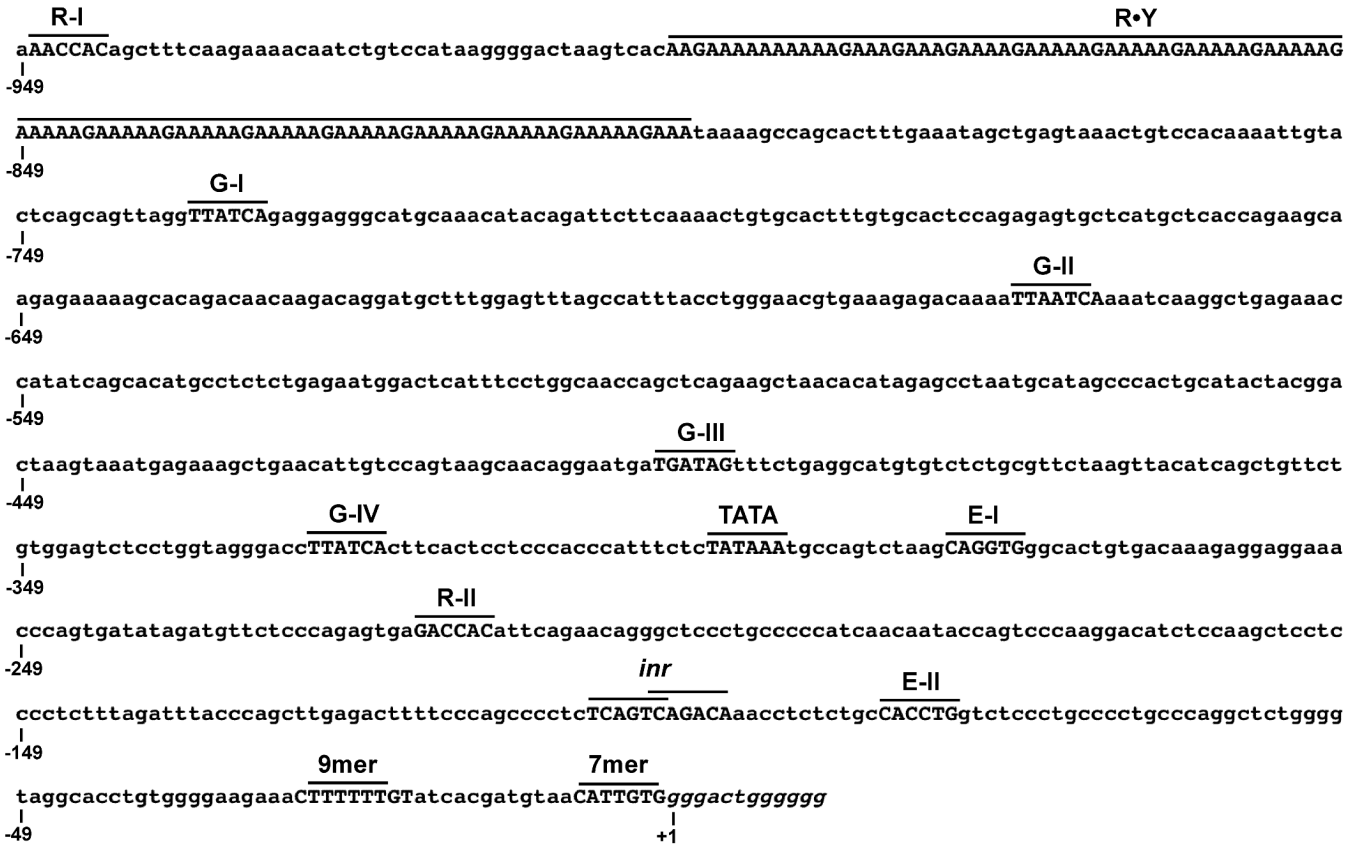

Figure 1.

Organization of the Dβ2 sequence analyzed for 5′ promoter activity. The positions of putative binding sites for Runx (*R*), GATA (*G*), and E proteins (*E*) are shown, as well as consensus TATA and initiator elements and polypurine·polypyrimidine DNA (R·Y). Dβ2 coding segments (*italics*) and 5′RSS sequences are also indicated. Numbering is relative to the first base of the Dβ2 coding sequence (+1).

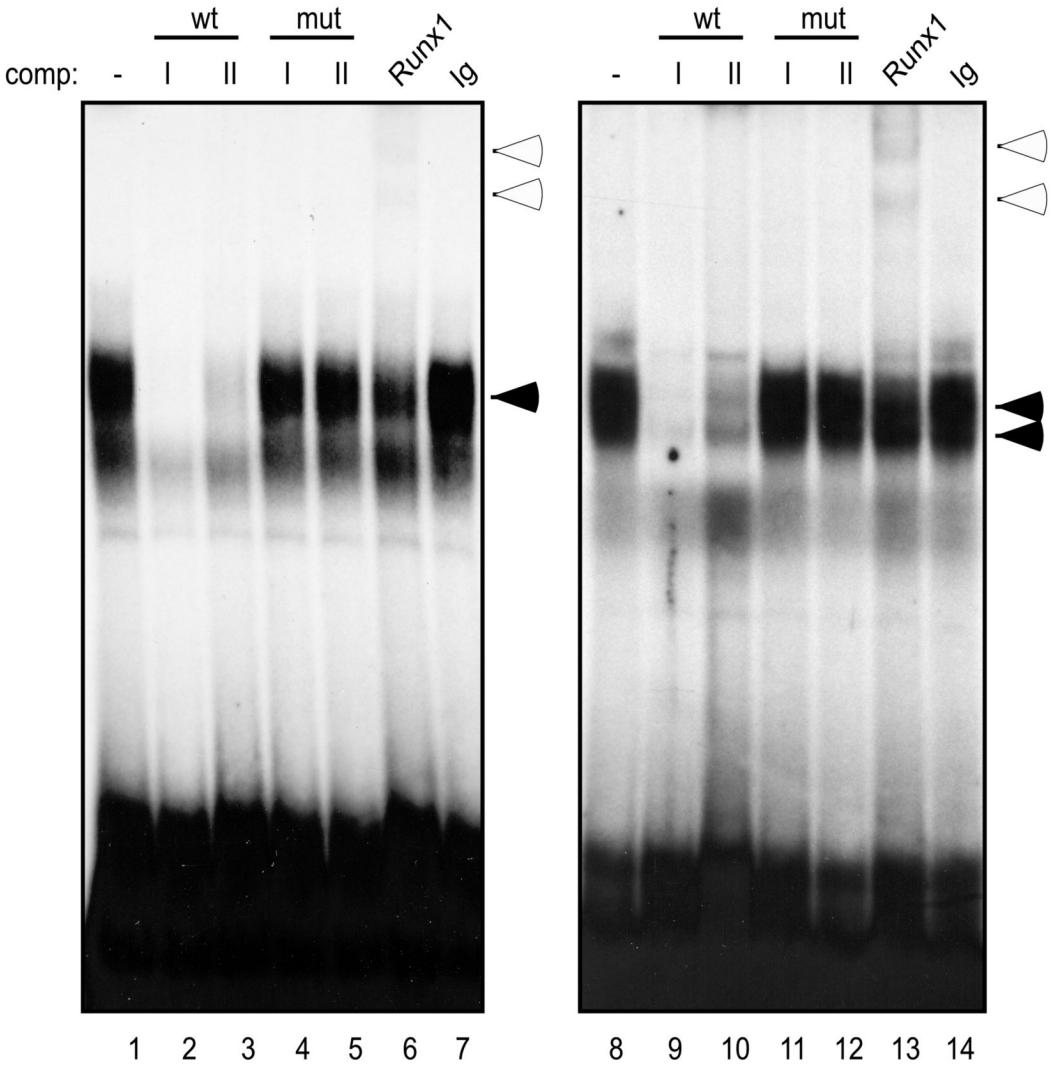

Figure 2.

Runx1 binds separate sites upstream of Dβ2. Nuclear extracts from the P5424 cell line were incubated with radiolabeled ds oligonucleotide probes to the putative Runx binding sites: R-I (*lanes 1-7*) and R-II (*lanes 8-14*). Probes were incubated with nuclear extract alone (*lanes 1 and 8*), in the presence of 100-fold molar excess of the indicated unlabeled competitors (*lanes 2-5 and 9-12*), or in the presence of the indicated Abs (*lanes 6-7 and 13-14*). Specific nucleoprotein (*filled arrows*) and Ab-supershifted complexes (*empty arrows*) are indicated.

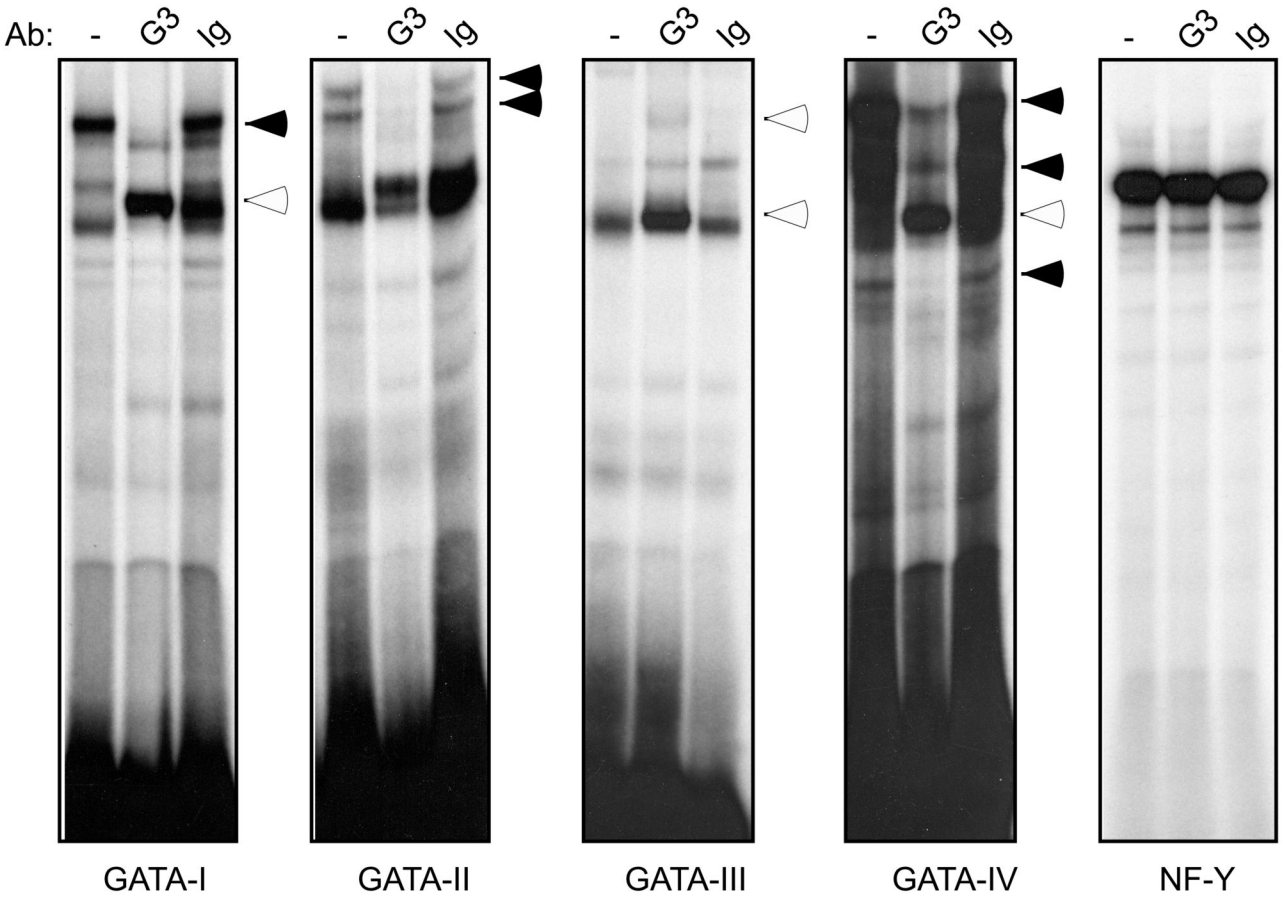

Figure 3.

GATA-3 binds multiple sites within 5′PDβ2. Nuclear extracts from the P5424 cell line were incubated with radiolabeled probes to the 4 putative GATA binding sites in 5′PDβ2 as well as an NF-Y control. In each case, reaction mixtures included probe and nuclear extract alone (-), or in the presence of the indicated Abs. Antibody-attenuated (*filled arrows*) and supershifted complexes (*empty arrows*) are indicated.

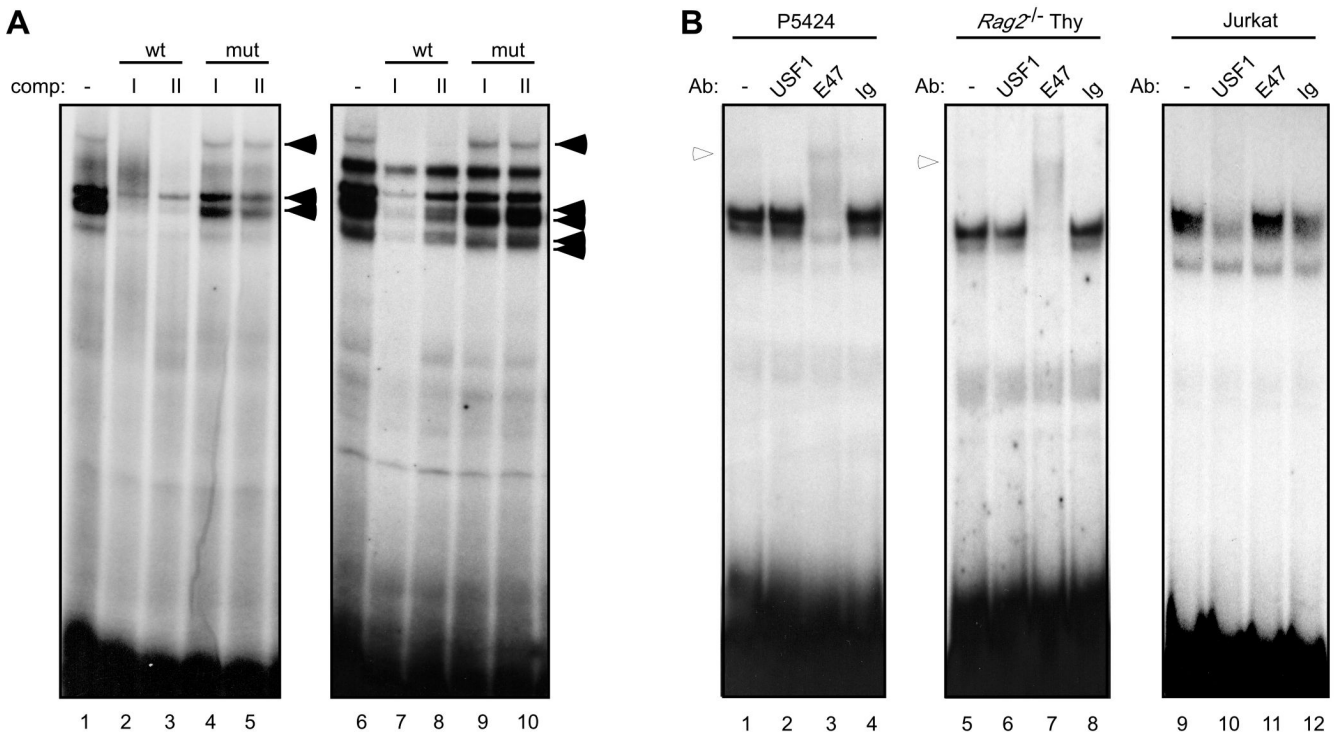

Figure 4.

Separate E boxes within the minimal 5′PDβ2 bind E47. (A) Nuclear extracts from the P5424 cell line were incubated with radiolabeled probes to the putative E boxes: E-I (*lanes 1-5*) and E-II (*lanes 6-10*) alone (*lanes 1 and 6*), or in the presence of 100-fold molar excess of the indicated unlabeled competitors (*lanes 2-5 and 7-10*). Specific nucleoprotein complexes are indicated (*filled arrows*). (B) Probes to the E-I site were incubated with nuclear extracts from P5424 (*lanes 1-4*), *Rag2*$^{-/-}$ thymocytes (*lanes 5-8*), or Jurkat (*lanes 9-12*) alone (*lanes 1, 5, and 9*) or in the presence of the indicated Abs (*lanes 2-4, 6-8, and 10-12*). Ab-supershifted complexes are indicated (*empty arrows*).

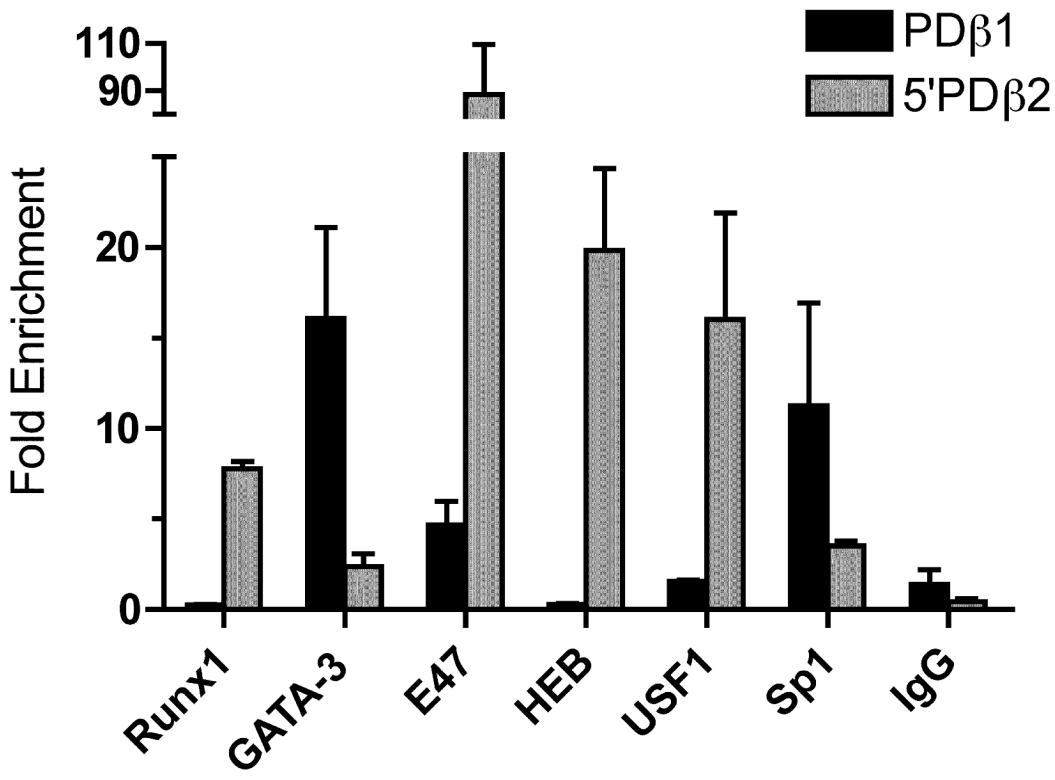

Figure 5.

Transcription factor *in vivo* association with 5′PDβ2. Chromatin from the P5424 cell line was immunoprecipitated with the indicated antibodies, and analyzed by qPCR for transcription factor association with the 5′PDβ2 (*grey*) and PDβ1 (*black*) promoters. Fold enrichment of each promoter sequence following IP with each antibody is shown relative to an untreated input sample. Bars indicate means (± SD) of triplicate qPCRs, and are representative of 2 experiments with independent chromatin preparations.

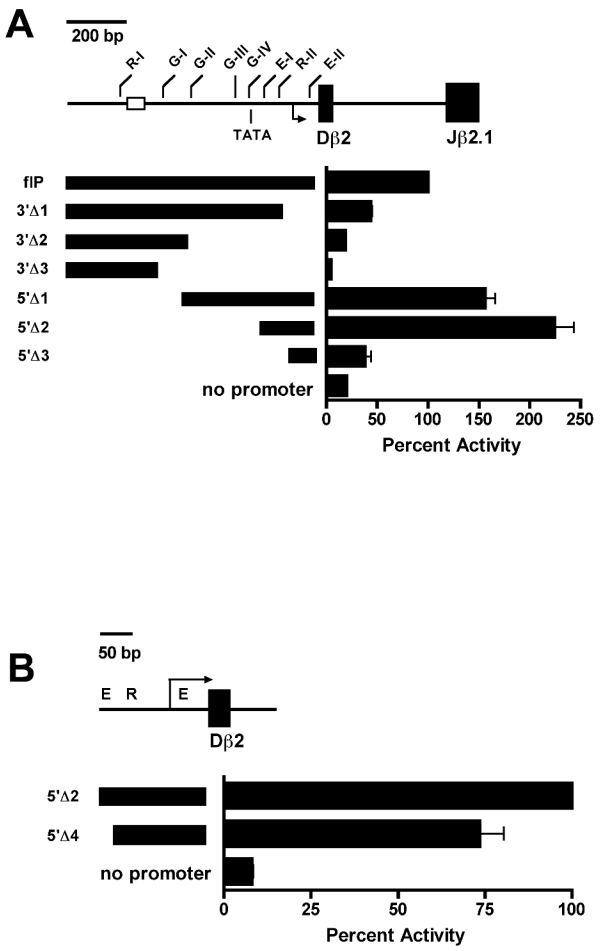

Figure 6.

Promoter activity upstream of Dβ2. (A) The indicated DNA fragments were inserted upstream of the luciferase cassette in pGL3-Eβ. Each plasmid was co-transfected with pSV-RL into P5424 cells, and protein extracts were assayed for luciferase activity 24 hours after transfection. Values from 3 or more independent transfections were normalized to renilla controls. Bars represent mean normalized luciferase activity ± SEM, and are expressed as percent activity of a 1 kb *Stu*I/*Alw*NI full length promoter fragment (flP). (B) pGL3-Eβ constructs containing the indicated DNA fragments were assayed for luciferase activity 24 hrs after co-transfection with pSV-RL into P5424 cells. Bars represent mean normalized luciferase activity ± SEM, and are expressed as percent activity of the 219 bp 5′Δ2 minimal promoter fragment.

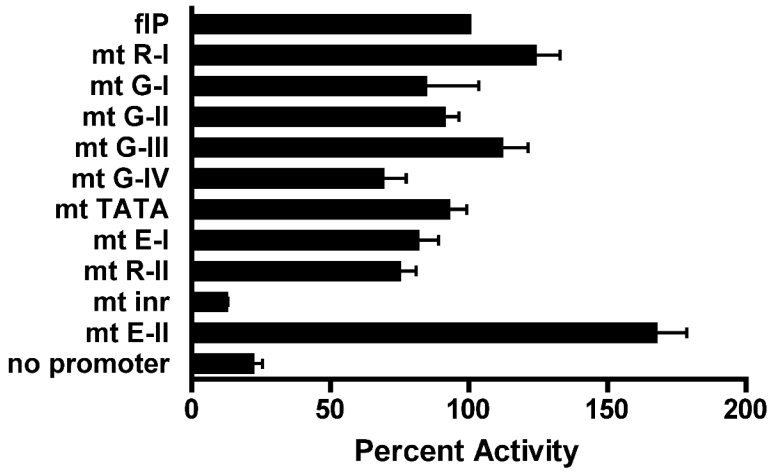

Figure 7.

Promoter activity of pGL3-Eβ containing the indicated PCR fragments is expressed as percent of PCR-generated full-length promoter control (flP′). Bars represent mean normalized luciferase activity ± SEM of 4 independent transfections. Targeted binding site mutations are indicated as white boxes.

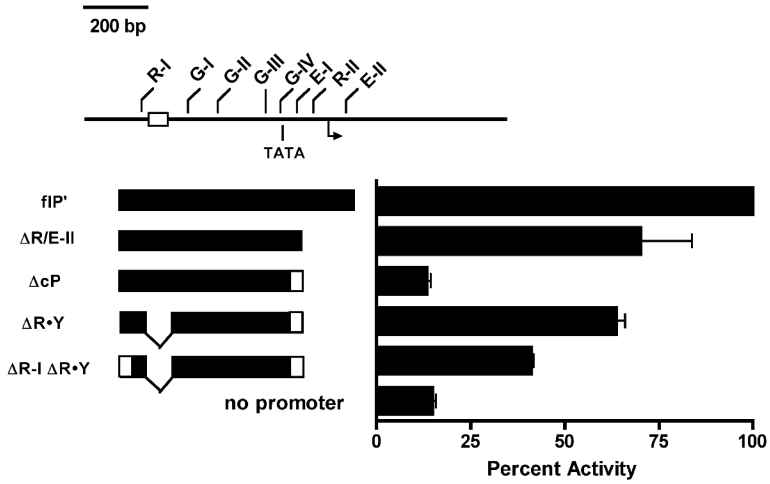

Figure 8.

Mutational analysis of 5′PDβ2 regulation. Individual transcription factor binding sites within the full-length promoter fragment were selectively mutated to assess their contribution to 5′ PDβ2 activity. P5424 transfectants of the indicated mutant flP pGL3-Eβ reporters were assayed for promoter activity. Results from 7 independent co-transfections with pSV-RL were normalized to *Renilla* levels, and expressed as percent wildtype flP.