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Out-of-Frame T Cell Receptor Beta Transcripts Are Eliminated by Multiple Pathways In Vivo

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Abstract

Non-productive antigen receptor genes with frame shifts generated during the assembly of these genes are found in many mature lymphocytes. Transcripts from these genes have premature termination codons (PTCs) and could encode truncated proteins if they are not either inactivated or destroyed by nonsense-mediated decay (NMD). In mammalian cells, NMD can be activated by pathways that rely on the presence of an intron downstream of the PTC; however, NMD can also be activated by pathways that do not rely on these downstream introns, and pathways independent of NMD can inactivate PTC-containing transcripts. Here, through the generation and analysis of mice with gene-targeted modifications of the endogenous T cell receptor beta (Tcrb) locus, we demonstrate that in T cells in vivo, optimal clearance of PTC-containing Tcrb transcripts depends on the presence of an intron downstream of the PTC.

Introduction

Lymphocyte antigen receptor chains are composed of N-terminal variable regions encoded by the first two exons of the antigen receptor gene and C-terminal constant regions encoded by the remaining exons [1]. The second exon of all lymphocyte antigen receptor genes is assembled by the V(D)J recombination reaction, which is initiated when the RAG endonuclease introduces DNA double strand breaks at the border of recombining variable (V), diversity (D) and joining (J) gene segments and their flanking RAG recognition sequences (recombination signals, RSs) [2]. These DNA double strand breaks are repaired by the non-homologous end-joining (NHEJ) pathway, joining the V, D and J gene segments [1]. NHEJ is imprecise and the random gain and loss of nucleotides that accompanies this joining process is essential for antigen receptor gene diversification and adaptive immunity. However, this diversification also leads to reading frame shifts and premature termination codons (PTGs) in two out of the three reading frames. These out-of-frame antigen receptor alleles are actively transcribed in lymphocytes, providing a rich source of PTC-containing transcripts that if not inactivated or destroyed by nonsense-mediated decay (NMD) could lead to the production of truncated antigen receptor peptides that could be deleterious to developing lymphocytes. Consistent with this notion the selective ablation of Upf2, a central mediator of NMD, in thymocytes leads to defects in T cell development, which could be due, in part, to the accumulation of T cell receptor gene transcripts with premature termination codons [3].

In lower eukaryotes, NMD is activated when PTCs are sensed in incompletely processed transcripts that are then released into the cytoplasm and the cytoplasmic PTC-dependent decay complex (PDC) binds to the PTC to initiate nonsense-mediated mRNA decay (NMD) [4]. In contrast, mammalian cells can activate NMD through pathways that recognize termination codons as premature if they lie upstream of an excised intron, irrespective of their distance from the poly(A) tract [5,6,7,8]. Activation of this pathway, termed 5′-3′ decay, requires that exon junction complexes (EJC) exist downstream of ribosomes stalled at PTCs during the first round of translation [9,10]. EJCs are deposited ~20–24 nt upstream of an exon-exon border, and in order for a PTC to activate NMD by EJC-dependent pathways, it must lie at a distance of greater than 50–55 bp upstream of the exon-exon junction [7,9,10,11,12]. Transcripts that have PTCs lying less than 50 bp upstream of an exon-exon junction or that have PTCs in the final exon are still able to activate NMD in mammalian cells [3,11,12,13,14]. Activation of NMD by this fail-safe pathway is thought to require sensing the distance of the PTC from the poly-A tract, as is the case in lower eukaryotes [15]. Although activation of fail-safe NMD does not rely on the presence of an intron downstream of the PTC, it requires the presence of an intron elsewhere in the gene [11,14,16,17,18]. In addition, mechanisms that, for example, alter splicing can inactivate PTC-containing transcripts without leading to their degradation [19,20,21,22,23,24,25,26]. In this regard, pre-mRNAs with PTCs can accumulate un-spliced, or incompletely spliced, in the nucleus. Moreover, PTC-containing transcripts can be alternatively spliced removing the exon with the PTC, a process termed nonsense-associated altered splicing. How PTCs are sensed in these incompletely processed transcripts is not completely understood. Together, these mechanisms prevent transcripts with PTCs from encoding truncated proteins that could have detrimental effects.

T cell receptor beta (Tcrb) chain locus transcripts containing PTCs are readily destroyed by NMD in vivo [27]. Here, we develop an approach to directly determine the requirement for an intron downstream of the PTC in efficiently clearing transcripts...
templated by the endogenous Tcβ locus in thymocytes. Using multi-step gene targeting, we generated two minimally modified versions of the mouse Tcβ locus, TcβA and TcβF, that undergo normal rearrangement and expression in developing thymocytes. These two alleles are identical except that TcβF allele PTCs will have downstream introns whereas TcβA allele PTCs will not. By comparing the stability of PTC-containing TcβA and TcβF transcripts in thymocytes, we demonstrate that the normal clearance of these transcripts relies on the presence of introns downstream of the PTC.

Results and Discussion

Generation of the TcβA and TcβF alleles

The mouse Tcβ locus spans 0.7 Mb, with 34 Vb gene segments lying in a 0.4 Mb region upstream of two Db-Jb clusters, each with a single Db and 6 or 7 Jb gene segments (Fig. 1a) [28]. The second exon is completed when a Vb gene segment rearranges to a DJb rearrangement at either of the Db-Jb clusters. Four constant region exons lie downstream of each Db-Jb cluster (Cb1 and Cb2) (Fig. 1a). VDJb rearrangements to DJb1 are transcribed with the four Cb2 exons; likewise, VDJb rearrangements to DJb2 are transcribed with the four Cb1 exons.

Multi-step gene targeting was used to generate mice with two modified endogenous Tcβ loci (TcβA and TcβF, Fig. 1b). Initially, the Db1 gene segment was deleted, limiting rearrangement of Vb gene segments to the Db2-Jb2 gene segment cluster (Step A, Fig. 1a, see Jb11M3 allele in ref. [28]). The Db2, Jb2.1, Jb2.2 and Jb2.3 gene segments were then replaced with a DJb2.3 rearrangement in its native configuration to generate the TcβA allele (Step B, Figs. 1a and S1). The TcβA allele is identical to the TcβF allele except that the four Cb2 exons have been replaced with a DNA fragment containing a fusion of these exons without any introns (Step C, Figs. 1a and S2). Importantly, as the TcβA and TcβF alleles were generated through modest gene targeted modifications of the wild type endogenous Tcβ locus, the TcβA and TcβF alleles remain under the same cis-acting regulation (endogenous Tcβ promoters and enhancers) as the wild type Tcβ locus.

T cell development in TcβA/A and TcβF/F mice

Efficient assembly and expression of a productive Tcβ chain gene is required for the normal development of T cells. Several lines of evidence demonstrate that rearrangement of both the TcβA and TcβF alleles occurs with near normal efficiency. First, in lymph node TcβA/A and TcβF/F T cell hybridomas, complete rearrangements occurred at a high frequency on both the TcβA and TcβF alleles (Fig. 2a and Table S1). Moreover, analysis of TcβA/A and TcβF/F T cell hybridomas revealed that 32% and 33%, respectively, have complete VDJb rearrangements on both alleles (Fig. 2b and Table S2). This is close to the maximum 40% expected due to allelic exclusion [29,30]. Finally, analysis of TcβA/F T cell hybridomas with single VDJb rearrangements revealed approximately equal frequencies of VDJb rearrangements on the TcβA and TcβF alleles (Fig. 2c and Table S3).

Thymocyte development in TcβA/A and TcβF/F mice was indistinguishable from wild type mice (Fig. 2d and e). In this regard, wild type (Tcβ+/−), TcβA/A and TcβF/F mice had similar numbers of CD4+CD8− (double negative, DN), CD4+CD8− (double positive, DP), and CD4+CD8− or CD4−CD8− (single positive, SP) thymocytes (Fig. 2d and e). Flow cytometric analysis of Tcβ chain expression revealed no significant differences between TcβA/A, TcβF/F and Tcβ+/− thymocytes (data not shown). Finally, TcβA/A, TcβF/F and Tcβ+/− mice have similar numbers of mature CD4+ and CD8+ splenic T cells (Fig. 2f). Taken together these data demonstrate that the TcβA and TcβF alleles are efficiently rearranged and expressed and can support normal T cell development.

Differential stability of PTC-containing TcβA and TcβF transcripts

Like the wild type Tcβ locus, TcβF PTCs lie in the third of six exons while TcβA PTCs lie in the third and final exon; thus, TcβA PTCs have downstream introns whereas TcβF PTCs do not.

Figure 1. TcβA and TcβF alleles. (a) Schematic of the mouse Tcβ locus. Vb, Db and Jb gene segments (open boxes), RSS (open triangles), Cb1 and Cb2 exons (blue boxes), enhancer (Eb and promoters (open circles) and loxP sequences (shaded ovals) are shown. (b) Schematics of the TcβA and TcβF alleles.

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Fig. 3a. Notably, TcrbA and TcrbF transcript PTCs lie at the same distance (0.7 kbp) from the poly(A) tract and have two upstream introns in the same locations (Fig. 3a). Thus, comparing the stability of PTC-containing TcrbA and TcrbF transcripts in TcrbA/A and TcrbF/F thymocytes allows us to determine the relative contribution of mechanisms that rely on introns downstream of the PTC in mediating degradation of PTC-containing Tcrb transcripts in vivo.

To this end, TcrbA/A and TcrbF/F CD25+ DN thymocytes were purified by flow cytometric cell sorting and VDJb rearrangements utilizing five different Vb gene segments were amplified and sequenced from genomic DNA and cDNAs generated from both Tcrb pre-mRNAs and mature transcripts. A total of 1592 sequences were analyzed to identify those that are in-frame (no PTC, PTC+) and those that are out-of-frame (PTC-containing, PTC+). (Fig. 3b, red bar and Table S4). Thus, PTC-containing TcrbA mRNAs are eliminated more efficiently than PTC-containing TcrbF mRNAs.

Concluding Remarks

Here, we show that PTC-containing TcrbA and TcrbF transcripts have differing abilities to be eliminated in mammalian thymocytes in vivo. The only difference between the TcrbA and TcrbF alleles is the presence of introns downstream of the PTC. Thus, these findings demonstrate that these downstream introns are mechanistically important components in the efficient elimination of PTC-containing Tcrb transcripts in vivo, consistent with the notion that they are required to activate EJC-dependent NMD. Nevertheless, PTC-containing transcripts templated by the TcrbF allele are reduced three-fold in their abundance. Thus, mechanisms that do not rely on downstream introns are also capable of eliminating PTC-containing transcripts, although not to levels achieved when downstream introns are present. As transcripts templated by the TcrbF allele will have two introns it is conceivable that PTC-containing TcrbF transcripts can also be inactivated by nonsense-associated altered splicing or other mechanisms that alter the splicing of PCT-containing transcripts. Notably, PTCs in...
the endogenous immunoglobulin light chain kappa gene frequently reside in the last exon, and like Tcrb\textsuperscript{b} transcripts in thymocytes, the abundance of PTC-containing immunoglobulin light chain kappa transcripts is also reduced by about three-fold in developing lymphocytes versus in vivo, maximal elimination of these transcripts depends on mechanisms that rely on the presence of introns downstream of the PTC.

**Materials and Methods**

**Ethics statement**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Washington University Animal Studies Committee (#20070189).

**Generation of Tcrb\textsuperscript{A/A} and Tcrb\textsuperscript{F/F} mice**

The 5’ homology arm of pLNTK-DJ (Fig. S1) contains a Db\textsuperscript{2}Jb\textsuperscript{2.3} rearrangement amplified with 5’HDJ and 3’Jb\textsuperscript{2.3} and the Jb\textsuperscript{2.4}-Jb\textsuperscript{2.7} gene segments amplified with 5’Jb\textsuperscript{2.4US} and 3’Jb\textsuperscript{2.7DS} (oligonucleotide sequences are listed in Table S3). The Jb\textsuperscript{2.3} gene segment has a single G to C change to eliminate a PTC generated using the 5’Jb\textsuperscript{2.3}, Jb\textsuperscript{2.3M1}, Jb\textsuperscript{2.3M2} and 3’Jb\textsuperscript{2.3X}, oligonucleotides. The 3’ homology arm is a 3.2 kbp Clal/Spld fragment downstream of Jb\textsuperscript{2.7}. The 5’ homology arm of pLNTK-Chb\textsuperscript{2F} (Fig. S2) is a 2 kbp Clal/PstI Tcrb fragment. The 3’ homology arm was generated by amplifying the constant region of a Tcrb cDNA with oligonucleotides A through F, 5’Ch\textsuperscript{2.3}-S’ and 3’Ch\textsuperscript{2.3}-S’ as shown in figure S2. Deletion of the Db\textsuperscript{1} gene segment is described elsewhere [32]. Embryonic stem cells (ES) were transfected, selected and injected into C57BL/6 blastocysts as previously described [32]. Intercrossing of Tcrb\textsuperscript{A/A} and Tcrb\textsuperscript{F/F} mice led to the expected Mendelian ratios of Tcrb\textsuperscript{A/A} or Tcrb\textsuperscript{F/F} mice, respectively.

**Southern blotting**

Southern blot analysis of ES cells targeted with pLNTK-DJ was performed on Sac\textsuperscript{I}-digested genomic DNA with probe A as previously described [33]. For ES cells targeted with pLNTK-Chb\textsuperscript{2F}, Pst\textsuperscript{I}-digested genomic DNA was probed with a 400 bp Hind\textsuperscript{II}/Bam\textsuperscript{HI} genomic fragment 3’ of the 3’ homology arm, and Sac\textsuperscript{I}-digested genomic DNA probed with probe A.

**Hybridomas**

Hybridomas were generated and Tcrb gene rearrangements analyzed as previously described [23].

**Flow cytometric analyses and cell purification**

Flow cytometric analyses were performed on a FACSCalibur (BD Biosciences) using FITC-conjugated anti-CD25, PE-Cy5-conjugated anti-CD4 and FITC-conjugated anti-CD8. CD25\textsuperscript{+} DN thymocytes were purified from 4–5 mice for each genotype by flow cytometric cell sorting (FACSVantage BD Biosciences).

**Sequence analyses**

Genomic DNA and RNA were isolated as previously described from CD25\textsuperscript{+} DN thymocytes purified by flow cytometric cell sorting [33,34]. The SuperScriptII Reverse Transcriptase kit (Invitrogen) was used to synthesize cDNA using oligo-dT or the intronic 3’Jb\textsuperscript{2.2}-11 oligonucleotide for mature mRNA or pre-mRNA, respectively. VDJb rearrangements were amplified from DNA using Vb\textsuperscript{2}, Vb\textsuperscript{6}, Vb\textsuperscript{8.1}, Vb\textsuperscript{14} or Vb\textsuperscript{16} and 3’Jb\textsuperscript{2.6}. PCR conditions were 200 ng genomic DNA in 50 ul with 1 mM MgCl\textsubscript{2}, 100 mM dNTPs and 10 picomoles of each oligonucleotide. The same Vb oligonucleotide. cDNA was amplified as above, except using Cb\textsuperscript{2.1} and Cb\textsuperscript{2.2} oligonucleotides in the primary and secondary reactions, respectively. The significance of the fold decrease of pre-mRNA or mRNA relative to DNA was calculated using a binomial test. The p-value for fold decrease of mRNA for Tcrb\textsuperscript{A} versus Tcrb\textsuperscript{b} was calculated using a Monte Carlo simulation (script available on request).

**Supporting Information**

Figure S1  Targeting strategy for generating the Tcrb\textsuperscript{A} allele. Generation of the Tcrb\textsuperscript{b} allele. Shown is a schematic of

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**Figure 3. PTC-containing Tcrb\textsuperscript{b} transcripts show resistance to NMD.** (a) Tcrb\textsuperscript{A} and Tcrb\textsuperscript{b} alleles with completely assembled second exons (VDJb), with four Cb\textsuperscript{2} exons (blue rectangles 3, 4 and 6, Tcrb\textsuperscript{b}) or the fusion of these exons (blue rectangle 3, Tcrb\textsuperscript{b}). The positions of the two potential PTCs (red octagons) and the normal termination codon (TC, open octagon) are shown. Pre-mRNAs with exons (green bars) and introns (connecting black lines) are shown, as are completely processed PTC-containing Tcrb\textsuperscript{A} and Tcrb\textsuperscript{b} transcripts (green bars) with exon junctions (EJ, purple dots). (b) Fold reduction (relative to PTC-containing alleles) in PTC-containing Tcrb pre-mRNA (open bars) and completely processed mRNA (red bars) in Tcrb\textsuperscript{A/A} and Tcrb\textsuperscript{F/F} DN thymocytes. P-values were calculated by a binomial test for pre-mRNA versus mRNA, and by Monte Carlo simulation for pre-mRNA versus Tcrb\textsuperscript{b}.

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part of the Tcrb allele in which the Db1 gene segment has been deleted (top, Jb1 in ref. [29]). The Db and Jb gene segments are shown as open rectangles (except for Jb2.3, shown as a shaded rectangle) and the RsJs as open triangles. The Cb1 and Cb2 exons (blue rectangles) are also shown, as is the pLNTK-DJ targeting vector used to generate the Tcrb^loxP^ allele, which has a targeted replacement of the four Cb2 exons with a DNA fragment containing a fusion of these exons and the loxP-flanked neomycin resistance gene (Neo^K^). The Tcrb^A^ allele generated after Cre-mediated deletion of the neomycin resistance gene, leaving a single loxP site (filled oval), is shown. The relative positions of the different restriction sites are shown, as is probe A, which was used for Southern blot analysis of the different targeted alleles. Also shown is a Southern blot of genomic DNA from targeted ES cell lines digested with SacI and hybridized to probe A. The molecular weight markers and relative positions of the bands generated by the different Tcrb alleles are indicated.

(TIF)

Figure S2 Targeting strategy for generating the Tcrb^F^ allele. a) Generation of the Cb2 fusion. Shown are schematics of the four Cb2 exons (labeled 1 through 4) in genomic DNA and in cDNA generated from completely processed mRNA, and oligonucleotides A through F (labeled arrows). b) Generation of the Tcrb^F^ allele. Shown is a schematic of the Cb2 region of the Tcrb^A^ allele (top) and the pLNTK-Cb2F targeting vector used to generate the Tcrb^loxP^ allele, which has a targeted replacement of the four Cb2 exons with a DNA fragment containing a fusion of these exons and the loxP-flanked neomycin resistance gene. Also shown is the Tcrb^F^ allele generated after Cre-mediated deletion of the neomycin resistance gene, leaving a single loxP site. The relative positions of the different restriction sites are shown, as are probes A and B, which were used for Southern blot analyses. Southern blots of genomic DNA from targeted ES cell lines that were digested with PstI and hybridized to probe B, or digested with SacI and hybridized to probe A are shown. The molecular weight markers and relative position of the bands generated by the different Tcrb alleles are indicated.

(TIF)

Table S1 Number of Tcrb^A^, Tcrb^F^ and Tcrb^B^ alleles in the VDJb configuration in the Tcrb^loxP^ and Tcrb^loxP^+ T cell hybridomas analyzed. The total number (n) of hybridomas analyzed is indicated.

(TIF)

Table S2 Number of Tcrb^loxP^ and Tcrb^loxP^+ T cell hybridomas with Tcrb alleles in the VDJb/DJb and VDJb/VDJb configuration.

(TIF)

Table S3 Number of Tcrb^loxP^+ T cell hybridomas with Tcrb alleles in the VDJb^V^/DJb^V^ and DJb^V^/VDJb^V^ configuration.

(TIF)

Table S4 Total number of sequences (n) and the number with (PTC+) or without (PTC−) PTCs from genomic DNA, pre-mRNA and mRNA from Tcrb^loxP^ and Tcrb^loxP^+ DN thymocytes.

(TIF)

Table S5 Oligonucleotide sequences.

(TIF)

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Author Contributions

Conceived and designed the experiments: BPS GKM BK. Performed the experiments: GKM MAM CM. Analyzed the data: GKM MAM BPS. Wrote the paper: BPS GKM.

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