

***In vivo* transposition mediated by V(D)J recombinase in human T lymphocytes**

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The rearrangement of immunoglobulin (Ig) and T-cell receptor (TCR) genes in lymphocytes by V(D)J recombinase is essential for immunological diversity in humans. These DNA rearrangements involve cleavage by the RAG1 and RAG2 (RAG1/2) recombinase enzymes at recombination signal sequences (RSS). This reaction generates two products, cleaved signal ends and coding ends. Coding ends are ligated by non-homologous end-joining proteins to form a functional Ig or TCR gene product, while the signal ends form a signal joint. *In vitro* studies have demonstrated that RAG1/2 are capable of mediating the transposition of cleaved signal ends into non-specific sites of a target DNA molecule. However, to date, *in vivo* transposition of signal ends has not been demonstrated. We present evidence of *in vivo* inter-chromosomal transposition in humans mediated by V(D)J recombinase. T-cell isolates were shown to contain TCR α signal ends from chromosome 14 inserted into the X-linked hypoxanthine–guanine phosphoribosyl transferase locus, resulting in gene inactivation. These findings implicate V(D)J recombinase-mediated transposition as a mutagenic mechanism capable of deleterious genetic rearrangements in humans.

Keywords: HPRT/human T-cell receptor/RAG1/2/transposition/V(D)J recombination

Introduction

The genes responsible for the development of a mature and diverse immune system in vertebrates are unique, consisting of multiple germ-line gene sequences that undergo site-specific somatic DNA rearrangements. These recombination events rearrange variable (V), diversity (D) and joining (J) gene sequences to form the immunoglobulin (Ig) and T-cell receptor (TCR) gene products responsible for antigen and cell-specific immune diversity (Tonegawa, 1983). Specific V(D)J recombination signal sequences (RSS) are located at the borders of each rearranging gene segment. The RSS contains both a conserved heptamer (consensus 5'-CACAGTG-3') and nonamer (consensus 5'-ACAAAACC-3'), separated by 12 or 23 base pairs

(Sakano *et al.*, 1979). Normal V(D)J recombination occurs between an RSS with a 12 bp spacer and an RSS with a 23 bp spacer (Hiom and Gellert, 1998). RAG1/2 and high mobility DNA binding proteins HMG1 and HMG2 bind to and bring the two RSS gene segments into close proximity (Sawchuk *et al.*, 1997; reviewed in van Gent *et al.*, 1997; Fugmann *et al.*, 2000). The DNA is subsequently cleaved, generating four free ends, which include the blunt 5' phosphorylated signal ends containing the complete RSS, and the covalently joined hairpin coding ends (McBlane *et al.*, 1995) (Figure 1). Ligation of the coding segments involves several non-homologous end-joining proteins that result in nucleotide nibbling (loss of bases) and insertions (bases added) at the coding junction (reviewed in Fugmann *et al.*, 2000). This newly formed coding joint is retained in the chromosome to form a new gene product. The signal ends either form extra-chromosomal circularized DNA molecules that are lost from the cell during cell division, or become inverted and are retained in the chromosome, depending on their orientation (reviewed in Lewis, 1994).

Several similarities have been identified between the mechanism of V(D)J recombination and the movements of transposable elements (reviewed in Thompson, 1995). RAG1 and RAG2 (RAG1/2) genes have a compact genomic organization and are convergently transcribed. This is consistent with components of a transposable element (Oettinger *et al.*, 1990). RSS resemble inverted repeats found at the ends of the Tc1 family of invertebrate transposons (Dreyfus, 1992). V(D)J cleavage, retroviral integration and transposition also proceed via a common pathway that involves exposure of a 3' hydroxyl group and a subsequent attack on a target phosphodiester bond (Mizuuchi, 1992; Craig, 1995; van Gent *et al.*, 1996). In addition, the RAG proteins remain bound to the RSS sites after cleavage, another common feature seen in transposition events (Agrawal and Schatz, 1997; Hiom *et al.*, 1998; Jones and Gellert, 2001). *In vitro* experiments have subsequently provided specific evidence that RAG1/2 can act as a transposase, mediating the transposition of cleaved signal ends into non-specific sites of a target DNA molecule (Agrawal *et al.*, 1998; Hiom *et al.*, 1998). However, there has been no evidence of RAG1/2-mediated transposition *in vivo*. In this report, we present evidence for transposition of cleaved TCR α signal ends into intron 1 of the hypoxanthine–guanine phosphoribosyl transferase (*HPRT*) gene in human peripheral T cells.

Results

Identification of mutants at the HPRT locus in human T cells

Analysis of mutations at the *HPRT* locus in peripheral T lymphocytes has been used to study *in vivo* somatic

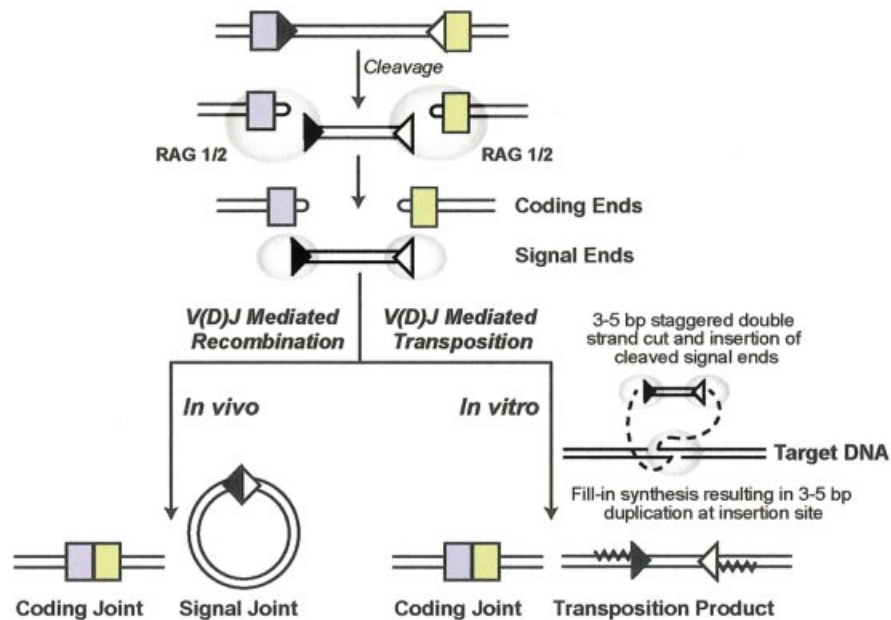


Fig. 1. V(D)J recombinase-mediated rearrangements. The steps involved in V(D)J recombination include site-specific recognition and cleavage at 12 bp RSS (black triangles) and 23 bp RSS (white triangles) sites by RAG1/2 (gray ovals), generating the hairpin coding ends (colored boxes) and the signal ends. Non-homologous end joining proteins process the coding ends. The result is a ligated coding joint that forms the functional gene product and a circularized signal joint. RAG1/2 are also capable of mediating transposition *in vitro* by strand transfer of the signal ends into a non-RSS target site. Transposition involves a staggered double-strand cut by RAG1/2, insertion of the signal ends, and fill-in synthesis resulting in a 3–5 bp target site duplication. This figure is adapted from Lewis and Wu (2000).

Table I. Summary of *HPRT* cDNA containing transposed TCR α signal ends

	<i>HPRT</i> mutant	
	F1	MFS6 M2
Subject		
Sex	Male	Female
Age	45 years	Newborn
Insert length (bp)	369	347
TCR α V	TCR V23	TCR α V35
Nibbled nucleotides ^a	AGCA (91 267–91 270)	AG (226 402–226 403)
TCR α J	TCR α J48	TCR α J46
Nibbled nucleotides ^a	TATCTAACTT (30 737–30 746)	AGAAGA (33 647–33 652)
N nucleotide insertions	TTATTGAG	GAAGAGG
TCR reading frame	TGA in V-J junction	TGA in J
<i>HPRT</i> reading frame	TGA at new codon 11	TGA at new codon 16

^aNucleotides in the TCR α region are numbered based on sequence data submitted to the DDBJ/EMBL/GenBank database under accession No. AE000660 for TCR α V21–35, and accession No. M94081 for TCR α J.

mutational events in humans (O'Neill *et al.*, 1987). Selection of mutations that have occurred *in vivo* is accomplished by direct plating of mononuclear cells by limiting dilution in the presence of the cytotoxic purine analog, 6-thioguanine. T lymphocytes with a functional *HPRT* enzyme utilize 6-thioguanine and die, whereas *HPRT* mutant T lymphocytes proliferate. The assay thereby selects for individual T lymphocytes that have acquired *in vivo* mutations which result in a loss of *HPRT* function. *HPRT* mutant MFS6 M2 was isolated from the cord blood of a full-term healthy newborn female (Yoshioka *et al.*, 2001), while mutant F1 was isolated from a healthy 45-year-old male (Hou, 1994). As previously reported, RT-PCR analysis of each mutant revealed cDNA products ~350 bp larger than expected

(Hou, 1994; Yoshioka *et al.*, 2001). Sequence analysis revealed insertion of a 347 bp (MFS6 M2) or a 369 bp (F1) TCR α coding gene segment, consisting of V35 joined to J46, and V23 joined to J48 (Hou, 1994), respectively, between exons 1 and 2 in the *HPRT* cDNA. These TCR α rearrangements are non-functional due to a stop codon at the V-J junction (F1) or in the J region (MFS6 M2) (Table I).

Breakpoint analysis reveals transposition of TCR α signal ends

In light of these observations and current evidence of V(D)J-mediated transposition *in vitro*, we hypothesized that the non-functional TCR α V-J coding segments were excised via RAG1/2, generating signal ends that inserted

Table II. PCR and breakpoint analysis of *HPRT* transposition mutants^a

<i>HPRT</i> mutant	PCR primer pair		PCR product	Sequence results (5'→3')
	Sense	Antisense		
F1				
3' breakpoint				
1st round	TCRα J ₄₆ (33 679–33 700)	<i>HPRT</i> 5821 (5844–5821)	5 kbp	TCRα J ₄₆ seq. and <i>HPRT</i> (3127–5821)
2nd round	TCRα J ₄₆ (33 679–33 700)	<i>HPRT</i> 3127 (3148–3127)	2 kbp	TCRα J ₄₅ RSS→ <i>HPRT</i> (1871–3127)
5' breakpoint				
1st round	<i>HPRT</i> A106 (1835–1851)	TCRα V ₂₁ (58 044–58 024) V ₂₂ (75 717–75 697)	250 bp	<i>HPRT</i> (1851–1874)→TCRα V ₂₂ RSS
MFS6 M2				
3' breakpoint				
1st round	TCRα J ₄₆ (33 679–33 700)	<i>HPRT</i> 5821 (5844–5821)	3.7 kbp	TCRα J ₄₆ seq. and <i>HPRT</i> (4671–5821)
2nd round	TCRα J ₄₆ (33 679–33 700)	<i>HPRT</i> 4001 (4021–4001)	1.8 kbp	TCRα J ₄₄ RSS→ <i>HPRT</i> (2183–4000)
5' breakpoint				
1st round	<i>HPRT</i> A106 (1835–1851)	TCRα V ₃₄ (212 419–212 400) V _{26S2} (207 707–207 690) V ₃₃ (194 753–194 734) V ₃₂ (190 295–190 273) V ₃₁ (181 913–181 894) V ₃₀ (173 342–173 322)	2.5 kbp	<i>HPRT</i> (1851–1944) and TCRα V ₃₄ seq.
2nd round	<i>HPRT</i> 2089 (2089–2112)	TCRα V ₃₄ (212 419–212 400)	400 bp 700 bp	<i>HPRT</i> (2052–2042)→TCRα V ₃₄ RSS <i>HPRT</i> (2089–2760)

^aNucleotides in the TCRα region are numbered based on sequence data submitted to the DDBJ/EMBL/GenBank database under accession No. AE000660 for TCRα V21–35; accession No. M94081 for TCRα J; and accession No. M26434 for *HPRT*.

into intron 1 of the *HPRT* gene. The non-functional V-J coding joints were detected due to splicing of the V-J coding joint as an exon into the *HPRT* mRNA. In addition to the non-functional coding joint in the cDNA, the proposed signal end insertions could include other V and J gene segments, and any intervening sequence flanking the non-functional coding joint. These segments would be detected by genomic analysis but would not be spliced into the *HPRT* message, and therefore would not be observed in the cDNA.

Primary PCR analysis focused on the 3' breakpoint for each mutant, since a fragment containing multiple J regions could be more easily amplified given the smaller intervening regions between J coding sequences when compared with intervening regions of the V coding sequences. Initial PCR analysis of the 3' breakpoint for mutant MFS6 M2 utilized a sense primer designed for the TCRα J46 region present in the cDNA product and an antisense *HPRT* primer located at position 5821 in intron 1 of *HPRT* (Table II). Each end of a unique 3.7 kbp PCR product was sequenced verifying capture of the breakpoint within the fragment. Sequence analysis of a nested 1.8 kbp PCR product generated using a sense primer for the TCRα J45 region and an antisense *HPRT* primer located at 4001 revealed TCRα sequence J44 RSS (nonamer–12 bp spacer–heptamer) GGTTCGT–12 bp–CACAGTG inserted at position 2183 in intron 1 of *HPRT* (Figure 2). Complete sequence analysis of the 3' breakpoint fragment showed the expected *HPRT* sequence from position 2183 to 5821.

Analysis of the 3' breakpoint for mutant F1 utilized the same sense primer (J46) located 3' of the J48 coding region present in the cDNA product and the *HPRT* antisense

primer located at position 5821 in intron 1 (Table II). Each end of a unique 5 kbp PCR product was sequenced, verifying capture of the breakpoint within the fragment. Sequence analysis of a nested 2 kbp PCR product, generated utilizing a sense TCRα primer for J46 and an antisense *HPRT* primer located at position 3127 in intron 1, revealed TCRα J45 RSS (nonamer–12 bp spacer–heptamer) AGTTTATGT–12 bp–CAGAGTG inserted at position 1871 in intron 1 of *HPRT* (Figure 2). Identification of the 3' genomic breakpoint of both mutants revealed characteristic RSS signal ends of the TCRα sequence inserted at different sites within intron 1 of *HPRT*.

The 5' breakpoint for mutant F1 was determined utilizing previously published Southern data (Hou, 1994) in conjunction with identification of the *HPRT* insertion site at the 3' breakpoint. These data suggested that the breakpoint sequence would contain an RSS from either TCRα V21 or V22. A multiplex PCR was carried out using a sense *HPRT* primer located at position 1835 and two antisense TCRα V sequence primers located near the RSS for V21 and V22 (Table II). The PCR resulted in a unique 250 bp fragment containing the 5' breakpoint that showed the V22 RSS sequence (heptamer–23 bp spacer–nonamer) CACAGTG–23 bp–ACACAAACC inserted at position 1874 of *HPRT*. These results support the transposition of TCRα signal ends containing ~16.8 kbp of intervening sequence, consisting of TCRα V22RSS, V23 joined to J48, J47, J46, and J45RSS into intron 1 of the *HPRT* gene (Figure 2). The sequence GGCA (bases 1871–1874) was present at both the 5' and 3' insertion site of the TCRα transposed fragment, indicative of a staggered cut at the *HPRT* insertion site (Figure 2). These observations are in

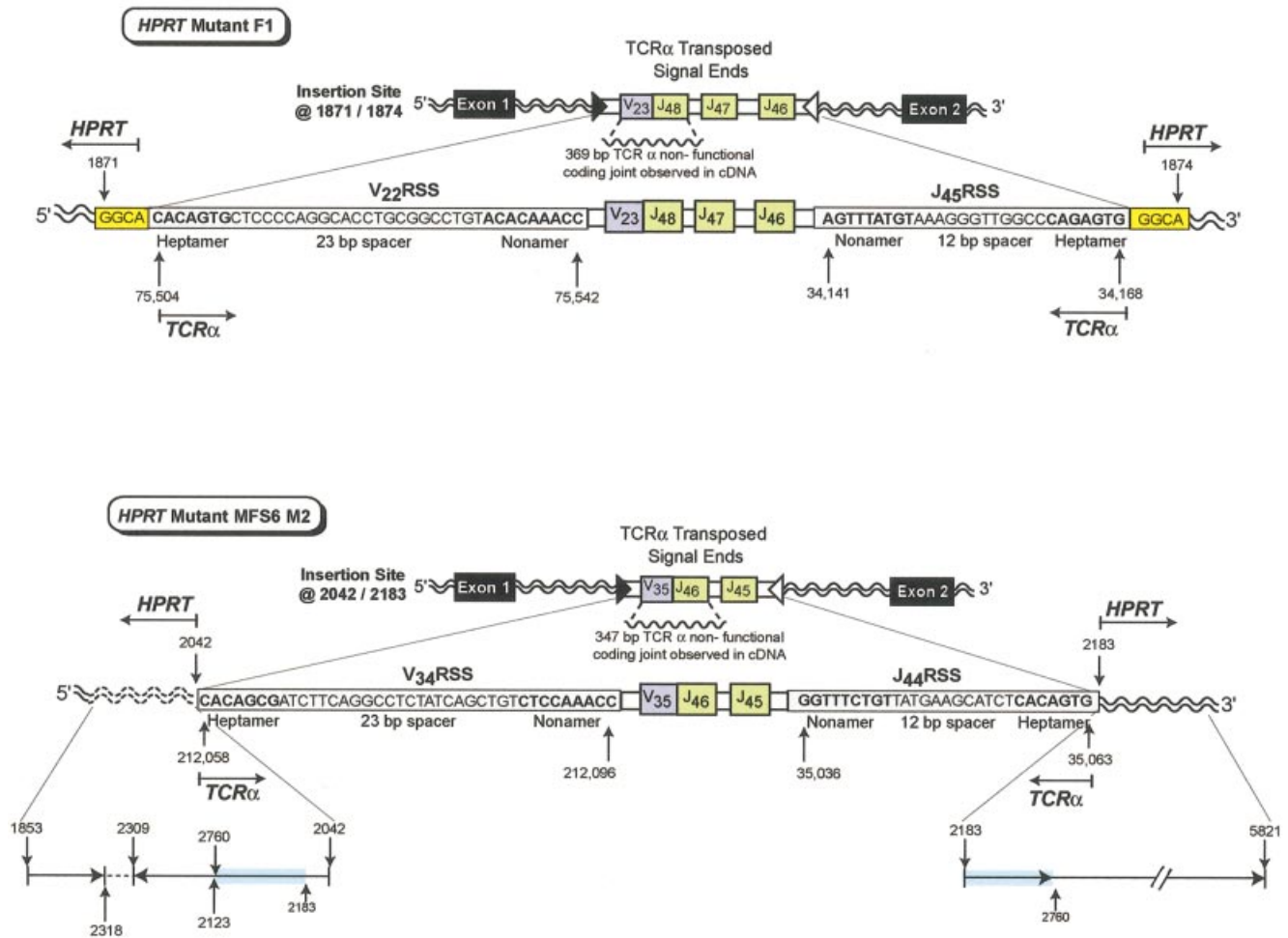


Fig. 2. *In vivo* transposition of TCR α V-J signal ends from chromosome 14 into the *HPRT* gene on the X chromosome. *HPRT* mutant F1 contains transposed TCR α signal ends containing ~16.8 kbp of intervening sequence that includes the V22 RSS (heptamer–23 bp spacer–nonamer), a V23 joined to J48 non-functional coding joint, J47, J46, and the J45 RSS (nonamer–12 bp spacer–heptamer) inserted into *HPRT* at bases 1871/1874. The insertion site is located in intron 1 of the *HPRT* gene. A 4 bp target site duplication, GGCA (1871–1874), is shown at both sides of the insertion. *HPRT* mutant MFS6 M2 contains transposed TCR α signal ends containing ~16.1 kbp of intervening sequence that includes the V34 RSS (heptamer–23 bp spacer–nonamer), a V35 joined to J46 non-functional coding joint, J45, and the J44 RSS (nonamer–12 bp spacer–heptamer) inserted into *HPRT* at 2042/2183. The insertion site is located in intron 1 of the *HPRT* gene. Reading 5' to 3', the 5' insertion site includes bases 1853–2318 followed by the inverted sequence 2309–2123 and 2760–2042. There is normal sequence at the 3' *HPRT* insertion site from 2183 to 5821 that includes the same duplication of bases from 2183 to 2760 observed at the 5' insertion site. *HPRT* bases that are duplicated are highlighted in blue. The inverted segment is shown by an arrow in the 3' to 5' direction.

agreement with the 3–5 bp target site duplication observed in previous *in vitro* transposition studies (Agrawal *et al.*, 1998; Hiom *et al.*, 1998).

Analysis of the 5' breakpoint for mutant MFS6 M2 used a similar multiplex approach. The PCR utilized a sense *HPRT* primer located at position 1835 and antisense TCR α primers designed for the next six V regions (V34, V33, V26s2, V32, V31, V30) 5' of the V35 coding region contained in the *HPRT* cDNA (Table II). This reaction was followed by a semi-nested PCR using a sense *HPRT* primer located at position 2089 and each individual antisense V primer. The PCR that included the V34 primer resulted in a 400 bp fragment containing the 5' breakpoint. Sequence analysis revealed V34 RSS sequence (heptamer–23 bp spacer–nonamer) CACAGCG–23 bp–CTCCAAACC inserted at position 2042 of *HPRT*. These results support the transposition of TCR α signal ends containing ~16.1 kbp of intervening sequence, consisting of TCR α

V34RSS, V35 joined to J46, J45, and J44RSS into intron 1 of *HPRT* at position 2042 at the 5' breakpoint and position 2183 at the 3' breakpoint (Figure 2). The PCR discussed above also generated additional products. Sequence analysis of these fragments confirmed the presence of an inverted duplication of the *HPRT* gene (Figure 2). Further analysis of intron 1 sequence 5' of the insertion confirmed *HPRT* bases 1853–2318 following *HPRT* exon 1. This region is followed by a gap in confirmed sequence, then the inverted *HPRT* bases from position 2309 to 2123. This inverted sequence appears to be directly followed by an additional inverted region that includes *HPRT* bases from position 2760 to 2042, located directly adjacent to the inserted TCR α V34RSS at the 5' insertion site (Figure 2). In addition, bases from position 2183 to 2760 are located at the 3' end of the TCR α J44RSS insertion. This transposition event shows a more complex sequence surrounding the insertion site than did the first transposition event

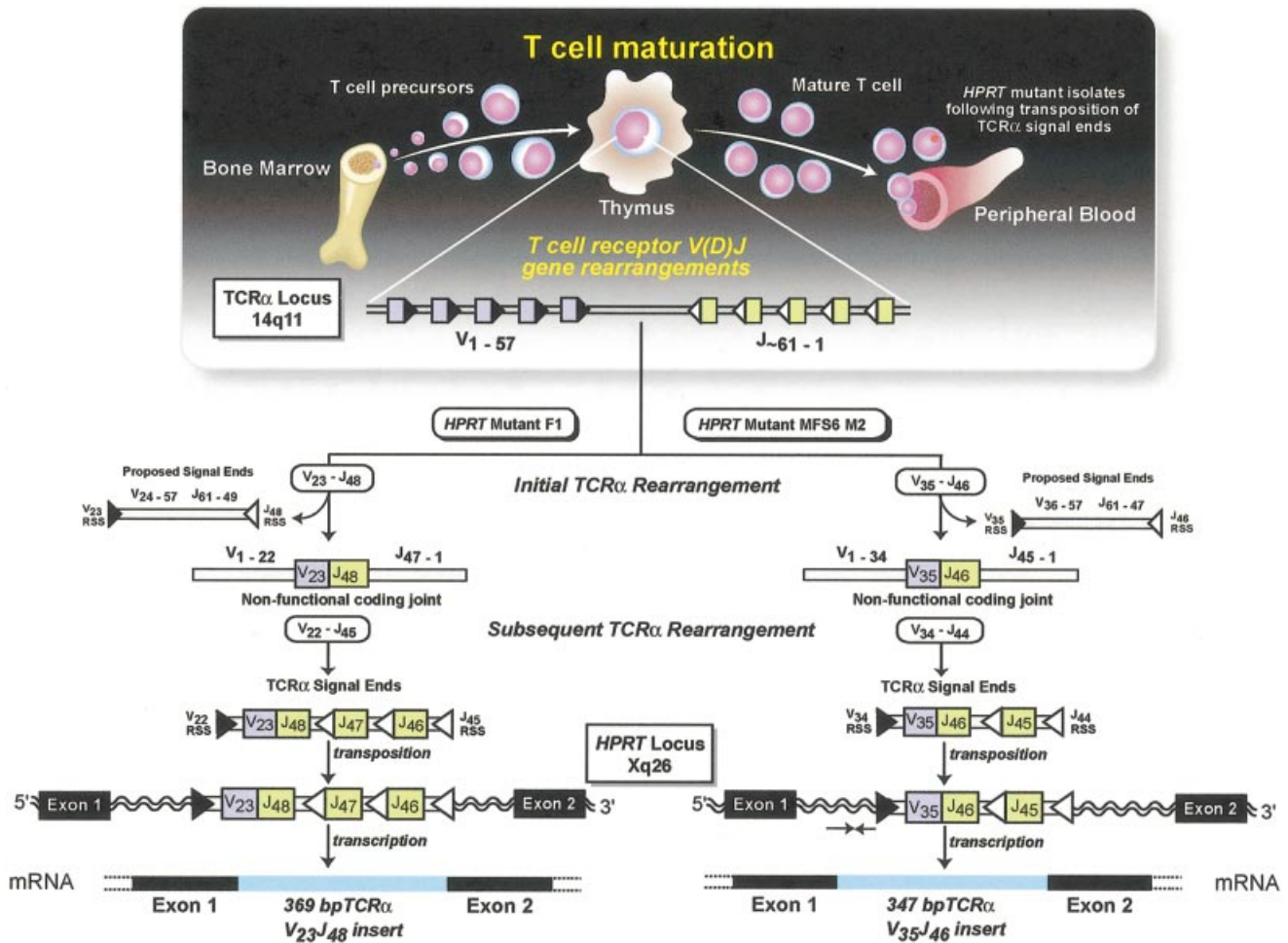


Fig. 3. *In vivo* TCR events during maturation of T-cell clones F1 and MFS6 M2. During T-cell maturation, V(D)J-mediated rearrangements occur at the TCR α locus, generating V-J coding joints and cleaved signal ends. Both mutant clones underwent a rearrangement that resulted in the formation of a non-functional V-J coding joint V23 joined to J48 (F1) or V35 joined to J46 (MFS6 M2) (colored boxes), and their respective cleaved signal ends. A subsequent rearrangement occurred in an attempt to form a functional TCR α , generating cleaved signal ends that included the observed non-functional V-J coding joint. These TCR α signal ends were inserted via a transposition reaction into intron 1 of the *HPRT* gene. The non-functional V-J coding joints were observed in the cDNA due to correct splicing as an exon into the mRNA.

described in the male subject. Since we are observing a resolved *in vivo* transposition product, we can only speculate whether the inverted sequence was created during the transposition event, or occurred prior to the transposition, thereby targeting the signal ends to this site. Inverted repeats resulting in DNA hairpins have been shown to be preferential sites for transposition *in vitro* (Lee *et al.*, 2002). Even though we have provided clear evidence of insertion of TCR α signal ends into *HPRT* for this mutant, an *in vivo* system is complicated by the fact that there are several pathways that could be involved in repair of double strand breaks (DSB) at the target site, due to nucleophilic attack by the signal ends. The observed inverted duplication at the 5' insertion site with a loss of contiguous bases at the 3' site suggests that other mechanisms were involved in the resolution of this event. It is possible that homologous recombination utilizing the additional X allele as template in the female subject was used in the resolution of this event. Alternatively, the exposed single-strand DNA at a DSB could be involved in snap-back replication (Morrical *et al.*, 1991; Jackson, 2002). If this scenario took place at the time

of insertion, the inverted duplication could be observed while the expected fill-in synthesis that creates the 3–5 bp duplication at the insertion site might not be. Lastly, if the inverted duplication formed prior to the transposition and was followed by insertion of the signal ends at two opposite hairpin ends, then one would observe the inverted duplication but might not observe a 3–5 bp duplication, as the two strands would be attacked in an uncoupled manner (Hiom *et al.*, 1998; Lee *et al.*, 2002). Inverted duplications were not observed in the products of the initial *in vitro* studies using RAG1/2 and HMG1/2 only (Agrawal *et al.*, 1998; Hiom *et al.*, 1998). However, inverted segments and duplications of DNA surrounding the insertion sites have been observed and described for other cut-and-paste type transposases, including Tn10, Mu and Tn7 (Foster *et al.*, 1981; Mizuuchi *et al.*, 1992; Kennedy *et al.*, 1998).

Discussion

These data provide the first description of V(D)J recombinase-mediated inter-chromosomal transposition in humans. The presence of TCR α signal ends in both

mutants is consistent with previous observations that the TCR α receptor locus can undergo several rearrangements, resulting in the generation of multiple signal ends (Livak and Schatz, 1996; McMahan and Fink, 1998). This would provide signal end substrates for transposition as a normal by-product of T-cell maturation. We propose that at least two TCR α V-J rearrangements occurred with the mutants described (Figure 3): the rearrangement that generated a non-functional coding joint and a subsequent rearrangement that formed a functional TCR α allowing for positive selection and release into the periphery. It was this second rearrangement that generated the signal ends that were inserted into the *HPRT* gene of these mutants. The fact that both insertions are located in intron 1 of the *HPRT* gene is of interest. The V(D)J enzyme complex cleaves at a cryptic RSS located at 2197 in intron 1 of *HPRT* (Fusco *et al.*, 1991), providing evidence that this area of the intron is accessible to the enzyme system. In addition, it is possible that this intron provides some structure that is favorable for targeting of a transposition event.

The previous lack of evidence supporting *in vivo* transposition has led to speculation that the V(D)J recombinase enzyme system once acted as a transposon capable of excision and reintegration of DNA, but has since diverged to provide only the site-specific reactions necessary to generate immunological diversity. Others have speculated that this enzyme system may be capable of transposition, but is closely regulated by additional elements that favor signal joint formation, leaving fewer signal ends for transposition (reviewed in Thompson, 1995; Lewis and Wu, 2000; Fugmann, 2001). Recent *in vitro* data have shown that signal joint formation does not prevent transposition because signal joints can be cleaved by a nick–nick mechanism, and are then capable of transposition (Neiditch *et al.*, 2002).

Our data provide the first evidence that the V(D)J enzyme system is capable of *in vivo* transposition of cleaved signal ends. However, the frequency at which transposition occurs *in vivo* remains unclear. Even though it is not possible to determine the frequency of these events in the general population or address the frequency at loci other than *HPRT*, we can determine the frequency of these events at the *HPRT* locus in the groups that were studied. Subject MFS6 was one of 53 newborns studied where the mean *HPRT* mutant frequency (Mf) for the group was $\sim 1 \times 10^{-6}$. A total of 138 mutants were analyzed in this study. Therefore, the frequency of the transposition event in this group was 7×10^{-9} . In comparison, the frequency of the two most common mutations found in newborns, V(D)J recombinase mediated *HPRT* exon 2–3 deletions and transitions (C \rightarrow T or G \rightarrow A) at CpG dinucleotide sequences are 3.9×10^{-7} and 6.5×10^{-8} , respectively (Yoshioka *et al.*, 2001). Subject Hou F was one of 23 adults studied with a total of 109 mutants. The estimated Mf for the group is 15×10^{-6} . Therefore, the frequency of the transposition event in this adult population is estimated to be 1×10^{-7} . These frequency calculations are only for events that would result in a phenotypic loss of *HPRT* gene function allowing for mutant selection with the T-cell assay. There may be transposed signal ends that result in innocuous somatic mutations that are not accounted for in our frequency calculations. This suggests that trans-

position occurs more frequently than our calculations indicate.

It is of interest that in addition to the RSS sites located at the Ig and TCR loci, it has been estimated that there may be as many as 10 million cryptic RSS sites dispersed throughout the human genome (Lewis *et al.*, 1997). Cleavage at these cryptic sites could generate additional signal ends, outside of the Ig and TCR loci, that might be transposed by RAG1/2. V(D)J recombinase-mediated cleavage at cryptic RSS sites does generate signal ends that result in large deletions at the *HPRT* locus in healthy individuals and at the *sil/scl* loci in individuals with T-cell leukemia (Aplan *et al.*, 1990; Bernard *et al.*, 1990; Macintyre *et al.*, 1992; Breit *et al.*, 1993; Finette *et al.*, 1996; Yoshioka *et al.*, 2001). These data suggest there may be large numbers of signal ends available for transposition. It is clear that additional observations which include other loci are needed to determine the overall frequency of transposition events in humans. However, our observation of *in vivo* inter-chromosomal transposition of signal ends demonstrates that RAG1/2-mediated transposition can occur in humans. In addition, there is increasing evidence that V(D)J recombinase mediates chromosomal translocations observed in neoplastic lymphoid cells (Tycko and Sklar, 1990; Rabbitts, 1994; Davila *et al.*, 2001). Our observation of *in vivo* V(D)J transposition of signal ends to another chromosome supports the models that have been proposed for chromosomal translocation as a consequence of RAG1/2 processing of transposition intermediates (Hiom *et al.*, 1998; Gellert *et al.*, 1999; Lee *et al.*, 2002). These data provide the first *in vivo* evidence that an essential process in lymphocyte development can result in mutagenic inter-chromosomal transposition, further supporting V(D)J-mediated transposition in the development of lymphoid malignancies.

Materials and methods

Isolation of *HPRT* mutant T-cell clones

HPRT mutant clones were isolated using a T lymphocyte cloning assay utilizing 6-thioguanine as the selective agent as described previously (O'Neill *et al.*, 1987). Mutant clones were expanded *in vitro*, pelleted and then frozen at -80°C for future analysis.

cDNA analysis

Cell pellets were used to generate cDNA by reverse transcription using a RNA PCR core kit (Perkin Elmer) utilizing M-MuLV reverse transcriptase with oligoDT(18) primers. RT and subsequent PCRs were performed using a Perkin Elmer 2400 thermocycler. The synthesized cDNA was amplified using primers flanking the coding region of *HPRT*. The first round reaction used sense primer 5'-CCTCTGCTCCGCCA-CCG-3' and antisense primer 5'-CGCCAAAGGGAACTGATACTC-TATAGGC-3', with a PCR profile of 94°C for 5 min followed by 30 cycles of 94°C (1 min), 65°C (1 min), 72°C (2 min) and a final extension at 72°C for 7 min. A second round nested PCR used the internal sense primer 5'-CCTGAGCAGTCAGCCCGCGC-3' and antisense primer 5'-GCAAAAAGCTCTACTAAGCAGATGGCCACAG-3' using a similar profile, with the exception of an annealing temperature of 55°C . Primers were purchased from Gibco-BRL. PCR products were purified using the QIAquick gel extraction kit (Qiagen), followed by cycle sequencing on an ABI 373 sequencer (Applied Biosystems).

Genomic analysis

Mutant F1: PCR amplification of the 3' breakpoint utilized a sense primer TCR α J46, 5'-GGGACCGGACTCGTTTAGCAG-3'; and antisense *HPRT* primer 5821, 5'-GGAATGGGCAGAAATTGCTAGTT-3'. This reaction was followed by a semi-nested PCR using sense primer TCR α J46; and antisense *HPRT* primer 3127, 5'-GTATGTCTGTTAGCCT-

CTCTGA-3'. PCR amplification of the 5' breakpoint utilized a sense *HPRT* primer A106, 5'-CAGTTTCCCGGGTTCGG-3'; and antisense primers *TCRα* V21, 5'-AAGATAGGCAGAGGAGTAGGG-3' and *TCRα* V22, 5'-GGAGGTGTGTATTGAAAAGGG-3'.

Mutant MFS6 M2: PCR amplification of the 3' breakpoint utilized a sense primer *TCRα* J46 and antisense *HPRT* primer 5821. This reaction was followed by a semi-nested PCR using sense primer *TCRα* J46; and antisense *HPRT* primer 4001, 5'-GGCAGGCATCACACCCCAAAG-3'. PCR amplification of the 5' breakpoint utilized sense *HPRT* primer A106; and antisense primers *TCRα* V34, 5'-GGTGGGTTAAATAGCAAAGGG-3', V26s2, 5'-GGGGCTAGATGAAGAAAATG-3', V33, 5'-GTGCCT-TCTTCCTGTCTGTG-3', V32, 5'-GGATCTCAATGAAACAACCT-CAG3', V31, 5'-GCTGCTAACAAATGCCTGGG-3' and V30, 5'-GTCCTATTGTCAAGCTGTGGG-3'. This reaction was followed by a semi-nested reaction using *HPRT* sense primer 2089, 5'-TTGGGG-TGCGATGGTGAGGTTCTC-3'; and the antisense primer *TCRα* V34. PCRs were performed using a Perkin Elmer 480 thermocycler with the Expand Long Template System (Roche Biosciences) 1× buffer system 2 (2.25 mM MgCl₂, 500 μM each dNTP, 300 nM primer, 100–200 ng genomic DNA). When multiple V primers were used, primer concentrations were adjusted to 150 nM each with two V primers, or 100 nM each with six V primers. PCRs were carried out in a final volume of 25 μl. First round reactions were performed at 94°C for 5 min followed by the addition of 2 U of Expand Long Template enzyme mixture (Taq DNA and Pwo DNA polymerase) with 40 cycles of 94°C (20 s), annealing and elongation at 63°C (8 min), with a final elongation at 68°C (8 min). Semi-nested PCRs were performed at 94°C (20 s), annealing at 63°C (1 min), elongation at 68°C (2 min) for 30 cycles, with a final elongation at 68°C (5 min). PCR products were purified using QIAquick-spin columns (Qiagen) and sequenced using Taq DyeDeoxy terminator cycle sequencing (ABI). For sequencing we used one round of 25 cycles at 96°C (30 s), 50°C (15 s) and 60°C (4 min). The products were purified with Sephadex G-50 (Pharmacia) using micro-spin columns (Promega) and then electrophoresed and read on an ABI automated sequencer model 373A (Applied Biosystems). Nucleotides in the *TCRα* region are numbered based on *TCRα* V21–35 sequence submitted to the DDBJ/EMBL/GenBank database under accession No. AE000660; *TCRα* J sequence submitted to the DDBJ/EMBL/GenBank database under accession No. M94081; and *HPRT* sequence submitted to the DDBJ/EMBL/GenBank database under accession No. M26434. Primers were obtained from Operon.

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