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Distinct requirements for Ku in N nucleotide addition at V(D)J- and non-V(D)J-generated double-strand breaks

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ABSTRACT

Loss or addition of nucleotides at junctions generated by V(D)J recombination significantly expands the antigen-receptor repertoire. Addition of nontemplated (N) nucleotides is carried out by terminal deoxynucleotidyl transferase (TdT), whose only known physiological role is to create diversity at V(D)J junctions during lymphocyte development. Although purified TdT can act at free DNA ends, its ability to add nucleotides (i.e. form N regions) at coding joints appears to depend on the nonhomologous end-joining factor Ku80. Because the DNA ends generated during V(D)J rearrangements remain associated with the RAG proteins after cleavage, TdT might be targeted for N region addition through interactions with RAG proteins or with Ku80 during remodeling of the post-cleavage complex. Such regulated access would help to prevent TdT from acting at other types of broken ends and degrading the fidelity of end joining. To test this hypothesis, we measured TdT's ability to add nucleotides to endonuclease-induced chromosomal and extrachromosomal breaks. In both cases TdT added nucleotides efficiently to the cleaved DNA ends. Strikingly, the frequency of N regions at non-V(D)J-generated ends was not dependent on Ku80. Thus our results suggest that Ku80 is required to allow TdT access to RAG post-cleavage complexes, providing support for the hypothesis that Ku is involved in disassembling or remodeling the post-cleavage complex. We also found that N regions were abnormally long in the absence of Ku80, indicating that Ku80 may regulate TdT's activity at DNA ends *in vivo*.

INTRODUCTION

During their development, B and T lymphocytes generate an astonishing variety of antigen receptors by employing a combinatorial gene assembly strategy called V(D)J recombination. This site-specific DNA recombination reaction brings together gene segments (termed V, D and J) to form a rearranged antigen receptor variable region gene (1). The V, D and J coding segments are flanked by recombination signal sequences (RSSs) that are recognized and bound by the recombinase, which consists of the RAG-1 and RAG-2 proteins. RAG-1 and RAG-2 act in concert to assemble an appropriate pair of RSS into a synaptic complex and introduce double-strand breaks (DSBs) into the DNA to form two types of DNA ends: blunt signal ends and covalently sealed (hairpin) coding ends. The RAG proteins remain associated with the four ends in the form of a post-cleavage complex *in vitro* (2–4) and *in vivo* (5–9), but end joining, i.e. formation of coding joints (which encode the rearranged antigen receptor gene) and signal joints, is accomplished with the help of nonhomologous end-joining (NHEJ) proteins (Ku70, Ku80, DNA-PK, XRCC4, DNA Ligase IV, Artemis and at least one other unidentified factor) (1).

Although the combinatorial diversity created by the rearrangement process contributes substantially to the size of the antigen receptor repertoire, the lion's share of the heterogeneity in antigen-binding sites is actually generated by imprecision in the joining of coding segments (10). This so-called junctional diversity is produced by loss and/or addition of nucleotides to the broken DNA ends generated during V(D)J recombination. One of the major mechanisms responsible for addition of nucleotides to V(D)J junctions involves the enzyme terminal deoxynucleotidyl transferase (TdT). TdT is expressed specifically in developing lymphocytes at the time of V(D)J recombination and adds nontemplated (N) nucleotides to free 3′ ends (11). By this mechanism, TdT increases diversity in T-cell receptor and immunoglobulin molecules and reduces the formation of repetitive homology-driven junctions (12–14). More than 70% of coding joints from adult mice contain N nucleotide additions, with an

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average insert size of 3–5 nt (15). A similar situation is observed in Chinese hamster ovary (CHO) cell lines transfected with plasmid substrates and RAG and TdT expression vectors; in this system, efficient N nucleotide addition is observed at both coding and signal joints (16).

Yet the mechanism by which TdT adds N nucleotides to broken DNA ends remains murky, and even the form of the substrate with which TdT interacts is undefined. Early observations that TdT efficiently adds nucleotides to free DNA ends *in vitro* in the absence of other proteins initially suggested that TdT might act independently of the complexes involved in V(D)J recombination (such as the RAG post-cleavage complex), perhaps by direct interaction with exposed DNA ends. This view was thoroughly undermined by the surprising observation that N nucleotides are virtually absent from V(D)J joints that form in Ku80-deficient mice, which have a defect comparable to that seen in TdT-deficient mice (17). Analysis of N nucleotide addition in Ku80-deficient fibroblasts transfected with TdT expression vectors confirmed that Ku80 is required for N nucleotide addition at V(D)J joints (16). Indeed, Ku and TdT can be coimmunoprecipitated from cell extracts (18). These data suggest that Ku might normally recruit TdT to the broken DNA ends during V(D)J recombination (16).

What is the mechanistic basis for the requirement of Ku in N nucleotide addition? We considered two possibilities. We have previously proposed that Ku might help disassemble or remodel the highly stable RAG post-cleavage complex (19), much like the chaperone ClpX removes the bacteriophage Mu transposase (20). In this case, we would predict that the requirement for Ku in N nucleotide addition would be specific to V(D)J recombination-generated DSBs. Alternatively, Ku could help recruit TdT to broken DNA ends, a function that need not be restricted to V(D)J recombination intermediates. To test these possibilities, we set out to determine whether TdT can efficiently add N nucleotides to ends generated by non-V(D)J means and, if so, whether this activity is Ku dependent. We find that Ku is not necessary for TdT to add nucleotides to non-V(D)J-generated ends, but that N regions are abnormally long in its absence. These data support the notion that Ku involvement in N region addition in V(D)J recombination involves remodeling of the post-cleavage complex to make the DNA ends accessible to the NHEJ machinery. Furthermore, the abnormal length of junctional inserts formed in the absence of Ku80 indicates that Ku regulates the activity of TdT at DNA ends *in vivo*.

MATERIALS AND METHODS

Vectors for chromosomal experiments

The vector pMHAd is a derivative of pHPRTminigene (21) in which exons 1–2 and 3–9 of the human HPRT gene are fused together to form a 3.5 kb gene that contains two exons with the 1.7 kb intron 2 of the HPRT gene between them (Fig. 1). Into the XbaI site near the middle of the intron, we cloned a 181 bp fragment containing an exon and the surrounding splice regions of the major late mRNA leader sequence of adenovirus type 2 (Genbank locus: ADRCG, region 7026–7188). The original adenovirus exon was extended by 22 bp to form a 94 bp exon (22) and the pyrimidine run of the splice

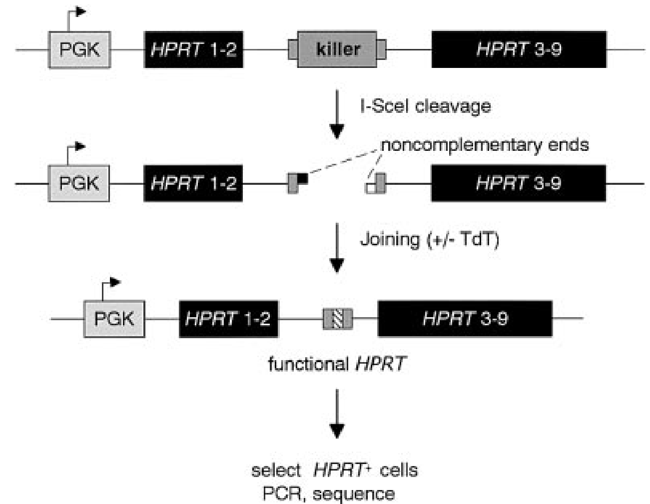

Figure 1. Structure of the killer exon construct. The phosphoglycerol kinase (PGK) promoter is shown upstream of the two exons of the HPRT minigene (fused together from original exons 1–2 and 3–9 of the human HPRT gene). The shaded box indicates the adenovirus killer exon with the surrounding adenovirus intron sequences. The killer exon is flanked by I-*Sce*I endonuclease sites (small rectangles), which are in opposite orientations. Cleavage by I-*Sce*I thus generates ends with noncomplementary 3′ overhangs. The final junction is shown as a hatched box, which may contain both deletion and addition of nucleotides to the ends. Rejoining without excessive deletion generates a functional HPRT gene, which confers the ability to grow in selective media.

acceptor region was replaced with a triple-helix-forming sequence for the sake of experiments not described here. These modifications do not impair the ability of the adenovirus exon to disrupt function of the HPRT minigene, presumably by efficient incorporation into the mRNA (22).

On either side of the adenovirus fragment we placed recognition sites for the rare cutting endonuclease I-SceI (5′-TAGGGATAACAGGGTAAT) in opposing orientations, so that the 3′ single-stranded extensions produced by I-SceI cleavage are noncomplementary. The vector also contains a hygromycin-resistance gene cloned from pInd-Hygro (Invitrogen, Carlsbad, CA), which allows selection for the integrated vector in mammalian cells. The expression vector pCMVISceI (23) was used to express the I-SceI endonuclease transiently in CHO cells, and pSV40TdTs (16) was used to transiently express the short form of TdT.

Chromosomal end-joining experiments

GS22–23 CHO cells, which carry a large deletion at the APRT locus, were derived from HPRT$^-$ GS19–43 cells (24). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, nonessential amino acids and antibiotics. Stable cell lines with the integrated pMHAd vector were constructed by transfecting 1 µg of linearized plasmid into 10^6 cells with Fugene 6 transfection reagent (Roche) according to the supplier's protocol. Forty-eight individual colonies, obtained by selection with 200 µg/ml hygromycin, were checked for spontaneous HPRT$^+$ background, good induction of HPRT$^+$ colonies with I-SceI treatment and low copy number by Southern analysis. Clone DSB1/20, which carries a single

integrated copy of the construct, was used for the experiments described here.

HPRT+ colonies were obtained by cotransfecting cell line DSB1/20 with 2 µg of the I-SceI expression vector pCMVISceI and 4 µg of the TdT expression vector pSV40TdTs. A similar sized vector that lacks TdT was used in control experiments. Cells were replated the following day at 10^4–10^5 cells per plate and selection for the HPRT+ phenotype was applied 1 day later using Hat medium (100 µM hypoxanthine, 0.4 µM aminopterine, 16 µM thymidine). After 14 days of selection individual clones were isolated and grown up from six independent transfections.

Analysis of chromosomal end-joining junctions

Chromosomal DNA was isolated from HPRT+ colonies according to standard protocols, and a 962 bp region around the adenovirus exon was amplified by PCR. Oligonucleotides 5′-CAG TCC TAA ACA GGG TAA TGGA and 5′-GCA ACA GAG ACC TTG TCT CAAA were used as sense and antisense primers with binding sites at 381 bp upstream and 318 bp downstream, respectively, of the I-SceI cut sites. The PCR amplified DNA from each HPRT+ colony was cloned using the TOPO-TA PCR cloning kit (Invitrogen), transformed into *Escherichia coli* and sequenced. About 10% of HPRT+ colonies initially yielded no detectable PCR product, suggesting that one of the primer sites may have been deleted; those colonies were not analyzed further. HPRT+ colonies with identical intron sequences that arose from different transfections were counted as independent events, whereas identical clones from the same transfection were considered siblings and only one of them was counted.

Plasmid end-joining experiments

The plasmid pDR3 was created from the parent plasmid pJH452 (25) by removal of a ClaI fragment containing the bacteriophage λ oop transcription terminator, allowing expression of the chloramphenicol acetyl transferase gene. Thus pDR3, which also bears a β-lactamase (*bla*) gene, confers resistance to both chloramphenicol and ampicillin. To create a substrate for end joining that contains a pair of 3′ extensions, pDR3 was linearized by digestion with PvuI (which cuts twice within the *bla* gene). After digestion, the DNA was gel purified and quantified prior to transfection.

Cells [RMP41, a derivative of CHO cells (26), or xrs-6, a CHO derivative deficient for Ku80 (27)] were transiently transfected with 1.5 µg of linear pDR3 with or without the addition of 1.5 µg of TdT expression vector. For complementation experiments, xrs-6 cells were transfected with 1.5 µg hamster Ku86 cDNA in pcDNA3 (16) (a kind gift from Dr Penny Jeggo). Transfections were performed using Fugene 6 (Boehringer Mannheim, USA) according to the manufacturer's instructions. DNA was harvested after 48 h according to the method of Hirt (28). Transfection efficiency was determined to be >90% using a plasmid that encodes β-galactosidase followed by a β-galactosidase assay (29).

Assay for N-nucleotide addition

N-nucleotide addition was measured by transforming the DNA recovered from CHO cell transfections into competent *E.coli* DH5α cells. Transformants arising from rejoined circular plasmids were selected on LB agar plates containing chloramphenicol (11 µg/ml). Since PvuI cuts twice within the *bla* gene, the rare transformants resulting from undigested parental plasmid DNA were readily excluded by screening for the presence of the intervening segment.

PCR products were obtained from bacterial colonies using either MLC2 (5′-GGC GTT ACC CAA CTT AAT CGC C-3′) and MLC3 (5′-GTT CCC AAC GAT CAA GGC GAG-3′) or MLC4 (5′-CGG TCA ATT CAC TGG CCG TCG-3′) and MLC5 (5′-CAT CGT GGT GTC ACG CTC GTC-3′) as primers. Products were sequenced using ABI Prism BigDye Terminator Cycle Sequencing Kit (PE Biosystems, USA). Sequencing primers were bla23 (5′-TGC GCA ACG TTG TTG CCA-3′), MLC2, MLC4 or MLC5.

Statistical analysis

Means were compared by the two-tailed t test. Distributions were compared by the chi-square test. For all comparisons a P value of 0.05 was used to accept or reject the null hypothesis, i.e. that the means or distributions were the same. All calculations for the statistical tests were performed by using the PHStat add-in for Excel.

RESULTS

The 'killer exon' system

To generate a system that would allow us to induce chromosomal DSBs and analyze their repair, we inserted an adenovirus exon with the surrounding RNA splicing signals into the middle of the intron in the human *HPRT* minigene (Fig. 1). This 'killer exon' blocks the function of the *HPRT* minigene, presumably by incorporation into the spliced mRNA (22), which would shift the translation reading frame for the protein. For the experiments described here, we used the cell line DSB1/20, which carries a single integrated copy of the disabled *HPRT* minigene.

The killer exon in the *HPRT* minigene in DSB1/20 is flanked by recognition sites for the rare cutting endonuclease I-SceI, which has no other identified sites in the CHO genome. Cleavage by I-SceI removes the killer exon, and subsequent joining of the broken ends allows HPRT to be expressed. The I-SceI sites were placed in opposite orientations to ensure that the two 4 nt single-stranded extensions produced by cleavage are noncomplementary and cannot be directly ligated. Rejoining of the broken ends restores the function of the *HPRT* gene, allowing clones of cells that have undergone repair of the site-specific DSBs to be identified by growth in HAT medium, which selects for HPRT function. As shown in Table 1, expression of I-SceI in DSB1/20 cells increased the frequency of HPRT+ colonies by more than three orders of magnitude.

TdT efficiently adds N nucleotides to chromosomal DSBs

The killer exon system was used to determine whether TdT can add nucleotides at the site of a chromosomal DSB. Cell line DSB1/20 was cotransfected with an I-SceI expression vector and either a TdT expression vector or an equivalent amount of a control plasmid. HPRT+ colonies was selected in HAT medium and counted (Table 1). The frequencies of I-SceI-induced HPRT+ colonies were similar in the presence

Table 1. Summary of analysis of end-joining junctions arising in the presence and absence of TdT

Cells	TdT	I-SceI	Ku80	HPRT+ ($\times 10^{-6}$)	Total junctions	N regions Present (%)	Absent	P[a]
GS22–23	–	–		<1				
GS22–23	–	+		2400 ± 230	28	1 (4)	27	
GS22–23	+	+		1300 ± 92	24	17 (71)	7	4×10^{-7}
RMP41	–				41	5 (12)	36	
RMP41	+				27	9 (33)	18	0.03
xrs-6	–		–		15	0 (<7)	15	
xrs-6	+		–		28	8[b] (29)	20	0.02
xrs-6	+		+		13	6 (46)	7	0.003

[a]*P* values refer to a comparison of junctions derived from a TdT-positive transfection versus the corresponding TdT-negative experiment in the same cell line.
[b]Six of these eight junctions had abnormally long N regions.

and absence of TdT expression, indicating that TdT expression does not have a dramatic effect on the efficiency of end joining.

To examine the characteristics of the repaired DSBs, DNA from individual HPRT+ colonies was amplified by PCR and sequenced (Table 2). As expected, the killer exon was deleted from the intron in the minigene in the HPRT+ clones. Only one of the 28 junctions (4%) analyzed from HPRT+ colonies generated in the absence of TdT treatment had N nucleotides, whereas 17 of 24 HPRT+ colonies (71%) generated in the presence of TdT had N nucleotides at the junction. The difference in frequencies of N nucleotides in the presence and absence of TdT is highly significant ($P = 4 \times 10^{-7}$). Moreover, the frequency of N nucleotides observed here in the presence of TdT is similar to the frequency of N nucleotides at junctions produced by V(D)J recombination in CHO cells transfected with the same TdT expression vector (16). In addition, the GC content of the extra nucleotides in these experiments was 68% (26/38), which is in agreement with previous observations that TdT preferentially adds G nucleotides to ends *in vitro* (30) and also with the high GC content of N additions observed at V(D)J junctions formed in lymphocytes (31,32) and in CHO cells transfected with the same TdT expression vector (16).

Variable numbers of nucleotides were lost from the I-*Sce*I-digested ends before the breaks were repaired, but the losses were not extensive (Table 2). Indeed, 19/27 junctions in the absence of TdT and 17/24 in its presence had retained nucleotides from at least one of the original four base overhangs. On average, the number of nucleotides removed from an end in the absence of TdT was 11.9 ± 13.9, and in the presence of TdT it was 8.0 ± 8.6. These means are not significantly different ($P = 0.24$). Thus the presence of TdT apparently does not affect the processes responsible for removal of nucleotides from broken DNA ends. It should be noted that the short form of TdT was used in these experiments. In contrast to the long form of TdT, which is a 3′ to 5′ exonuclease (33), the short form lacks nuclease activity and was not expected to contribute to nucleotide removal at I-SceI-induced DNA breaks.

In the absence of TdT, 23 of 28 junctions exhibited a 1 to 4 nucleotide microhomology at the junction (Table 2), in agreement with previous observations on end joining in mammalian cells [reviewed by Roth and Wilson (34)]. Microhomology usage at the five of seven junctions that lacked N nucleotides in the TdT treatment group was not significantly different ($P = 0.85$). Assuming that TdT was present in the cells where junctions without N nucleotides were generated, these results suggest that the mere presence of TdT does not affect the processes responsible for microhomology usage.

Addition of normal-length N regions during end joining requires Ku

To determine whether N nucleotide addition to broken DNA ends not created by V(D)J recombination depends on Ku, we turned to a plasmid system that has been used previously for the study of DNA end joining in cells (25). Wild-type or Ku80-deficient CHO fibroblasts were transfected with a linear substrate plasmid (pDR3) digested with PvuI, which generates 3′ overhangs. In experiments involving TdT addition, the TdT expression vector was transfected first and the linearized plasmid substrate was added 24 h later, by which time TdT expression was at its peak as determined by western blotting (data not shown). DNA was harvested 48 h after the last transfection, and circular plasmids produced by end joining were identified by bacterial transformation with selection on media containing the appropriate antibiotics. We then determined junctional sequences.

As expected, the frequency of N nucleotides at junctions produced in the absence of TdT was low (5/41 junctions; 12%) (Tables 1 and 3). Extra nucleotides are observed in ~10% of junctions produced by recircularization of transfected DNA molecules (34,35), and several TdT-independent mechanisms have been proposed to account for them (35–37). In the presence of TdT, however, N regions were seen at 9/27 junctions (33%), which is significantly higher ($P = 0.03$) (Table 1).

To examine the dependence of N nucleotide addition on Ku80, we employed the Ku80-deficient CHO cell line xrs-6, which lacks the ability to add N regions to junctions produced by V(D)J recombination (16). None of the 15 junctions (<7%) contained extra nucleotides in the absence of TdT (Tables 1 and 3). In the presence of TdT, 8/28 junctions (29%) contained inserted nucleotides (Table 1), but six of these eight inserts were unusually long (11–27 nt) (Table 3). These abnormally long inserts contrast sharply with those seen in wild-type cells: 17 of 17 inserts at chromosomal breaks were ⩽10 nt in wild-type cells (Table 2), and the longest N addition observed in plasmid end-joining experiments in wild-type cells in this study was 7 nt (Table 3).

Short N regions are consistent with observations at endogenous V(D)J rearrangements. The average length of N

Table 2. Junctions in GS22–23 CHO cells with and without transfected TdT

(A) GS22-23 CHO cells without TdT

Clone	Δl[a]	Left[b]	Insert	Right	Δr[a]	ΔT[a]
611	0	AGGGATA**A**		TCCCTAGC	3	3
731	0	AGGGATA**A**		TCCCTAGC	3	3
243	0	AGGGATA**A**		TCCCTAGC	3	3
722	2	CTAGGGA**T**		TATCCCTA	1	3
641	1	TAGGGA**TA**		TCCCTAGC	3	4
171	4	AGCTAGGG		TTATCCCT	0	4
311	2	CTAGGG**AT**		CCCTAGCT	4	6
671	2	CTAGGG**AT**		CCCTAGCT	4	6
231	5	TAGCTAGG		TCCCTAGC	3	8
334	0	AGGGATA**A**		GCTAGAGC	9	9
221	7	TCTAGC**TA**		TCCCTAGC	3	10
140	12	GTGTGTC**T**		TATCCCTA	1	13
661	1	TAGGGA**TA**		GAGCTATC	13	14
642	0	AGGGATA**A**		GCTATCTA	15	15
681	3	GCTAGGG**A**		GAGCTATC	13	16
361	1	TAGGGATA		CTAATATA	20	21
121	6	CTAGCT**AG**		CTATCTAA	16	22
721	10	GTGT**CTAG**		AGCTATCT	14	24
741	1	TAGGG**ATA**		TTTTAAAG	28	29
712	7	TCTAGC**TA**		TATTTTAA	26	33
321	24	CTTTTAAT		GCTAGAGC	9	33
651	11	TGTG**TCTA**		ATATATTT	23	34
631	0	AGGGAT**AA**		AGGTTGCA	34	34
762	3	GCTAGGGA	A	TGCATAGC	38	41
771	42	TTTAAAT**G**		CTATCTAA	16	58
222	54	TATGAG**TA**		GAGCTATC	13	67
150	34	TTATTGG**A**		GGTTGCAT	35	69
711	30	TGGACAC**T**		CTTATGGA	53	83

(B) GS22–23 CHO cells with TdT

Clone	Δl[a]	Left[b]	Insert	Right	Δr[a]	ΔT[a]
421	4	AGCTAGGG	TC	TTATCCCT	0	4
450	4	AGCTAGGG	CG	TTATCCCT	0	4
611	0	AGGGATAA	AA	CCCTAGCT	4	4
331	0	AGGGATAA	CCCTCCTTGG	CCCTAGCT	4	4
411	2	CTAGGG**AT**		CCCTAGCT	4	6
721	2	CTAGGGAT	TGC	CCCTAGCT	4	6
333	4	AGCTAGGG	CA	CCCTAGCT	4	8
621	0	AGGGATAA	GGG	AGCTAGAG	8	8
513	0	AGGGATA**A**		GCTAGAGC	9	9
422	0	AGGGATA**A**		GCTAGAGC	9	9
320	11	TGTGTCTA	T	TTATCCCT	0	11
412	11	TGTGTCTA	T	TTATCCCT	0	11
221	10	GTGTCTAG	G	TATCCCTA	1	11
351	0	AGGGATAA	GGG	GCTATCTA	15	15
631	6	CTAGCTAG	T	TAGAGCTA	11	17
512	1	TAGGGA**TA**		TCTAATAT	19	20
124	3	GCTAGGGA	GG	TATCTAAT	17	20
460	11	TGTGTCTA		AGAGCTAT	12	23
210	10	GTGT**CTAG**		AGCTATCT	14	24
431	0	AGGGATAA	G	ATATTTTA	25	25
511	24	CTTTTAAT		GCTAGAGC	9	33
711	0	AGGGATAA	GG	GGTTGCAT	35	35
222	11	TGTGTCTA	A	ATATTTTA	25	36
121	28	GACACTTT	C	GAGCTATC	13	41

[a]The numbers of nucleotides deleted from the left side of the break, the right side of the break and the total number of deleted nucleotides are indicated by Δl, Δr and ΔT, respectively.
[b]Microhomologies present at junctions without N regions are indicated in bold.

regions at endogenous antigen receptor gene rearrangements in mice is <5 nt (15); N regions >10 nt in length are quite rare (17,38,39), even in the absence of selection for a productive receptor (40). Likewise, N regions >10 nt are very rarely observed in junctions formed by V(D)J rearrangement of artificial substrates (41), even when TdT is ectopically expressed at high levels (42). Indeed, analysis of a large number of N regions formed by V(D)J recombination in wild-type CHO cell lines transfected with the TdT expression vector used in this study failed to detect N regions >10 nt (16). These data strongly suggest that N regions >10 nt are abnormal.

Table 3. Junctions with inserted nucleotides derived from plasmid end-joining experiments

Line	Clone	Δl[a]	Left	Insert	Right	Δr[a]	ΔT[a]
RMP41 – TdT	2	0	GCACCGAT	T	GAGGACCG	1	1
	12	2	CCGCACCG	T	GGAGGACC	0	2
	7	0	GCACCGAT	CGGTGGC	GGAGCTAA	11	11
	29	14	TAGCGAAG	T	CTTTTTTG	22	36
	33	17	TAATAGCG	CG	TGCACAAC	28	45
RMP41 + TdT	2	0	GCACCGAT	AG	GGACCGAA	3	3
	8	3	CCCGCACC	CC	GGAGGACC	0	3
	9	4	GCCCGCAC	C	AGGACCGA	2	6
	11	6	AGGCCCGC	C	GGAGGACC	0	6
	5	0	GCACCGAT	GGG	GAAGGAGC	8	8
	15	8	AGAGGCCC	C	GGAGGACC	0	8
	16	8	AGAGGCCC	CC	GGAGGACC	0	8
	23	27	CCAGCTGG	GG	CGCTTTTT	20	47
xrs-6 + TdT	20	2	CCGCACCG	TC	AACCGCTT	17	19
	8	7	GAGGCCCG	TTTCTCCATGTCTGTCATGGCATGAAA	CTAACCGC	15	22
	24	13	AGCGAAGA	AACC	GAGCTAAC	12	25
	25	14	TAGCGAAG	TTCGCCAGCTG	ACCGCTTT	18	32
	3	2	CCGCACCG	TGGGCTGTTACTCCACA	ACAACATG	31	33
	27	20	GCGTAATA	GTAGCTCGGGCGGGGCTCTTTGT	CGCTTTTT	20	40
	15	32	TTTCGCCA	GATGTTCGGCA	ACCGCTTT	18	50
	14	30	TCGCCAGC	AGGCAACTGTTGTTCA	TTTTTTGC	23	53
xrs-6 + TdT + Ku80	1	0	GCACCGAT	AAC	AGGACCGA	2	2
	8	10	GAAGAGGC	GGG	GGACCGAA	3	13
	3	1	CGCACCGA	CC	TTGCACAA	27	28
	11	38	TCCCCCTT	TGTT	TTGGGAAC	65	103
	12	64	ACCCAACT	C	TTGGGAAC	65	129
	13	78	AAAACCCT	TGTGA	AATGAAGC	81	159

[a]The numbers of nucleotides deleted from the left side of the break, the right side of the break and the total number of deleted nucleotides are indicated by Δl, Δr and ΔT, respectively. The mean for the number of nucleotides deleted from an end for RMP41 cells in the absence of TdT (14.6 ± 16.0) was not significantly different (P = 0.3) from the mean in the presence of TdT (11.8 ± 15.2). The mean for the number of nucleotides deleted from an end for xrs-6 cells in the absence of TdT (25.2 ± 23.6) was not significantly different from the mean in the presence of TdT (18.5 ± 16.9) (P = 0.1) or from the mean in the presence of TdT and Ku80 (25.1 ± 25.7) (P = 0.99).

If these junctions with abnormal inserts are removed from the dataset obtained in the presence of TdT in xrs-6 cells, only 2/28 junctions (7%) contain normal N regions, which is not significantly different from the results in the absence of TdT (P = 0.3). In other words, addition of normal-length N regions is Ku80-dependent. The presence of extraordinarily long N regions at junctions produced in Ku80-deficient cells is reminiscent of previous *in vitro* experiments which found that the DNA-dependent protein kinase, of which Ku is a component, modulates TdT activity by limiting the length of N nucleotide insertions (43).

To verify that the abnormal-length N regions in xrs-6 cells indeed resulted from a lack of Ku80, we performed a complementation experiment in which 1.5 µg of a Ku80 expression vector, an amount sufficient to normalize V(D)J junctions formed in xrs-6 cells (16), was transiently transfected into xrs-6 cells. Six of 13 junctions (46%) isolated from the complemented cells displayed N regions, and all were of normal length. These data demonstrate that addition of normal-length N regions by TdT to free DNA ends requires Ku80.

DISCUSSION

Both chromosomal and plasmid NHEJ pathways are accessible to TdT

The observation that TdT is present only in cells actively undergoing V(D)J recombination, along with its extremely restricted physiologic function (diversification of rearranging antigen receptor genes), suggested that N nucleotide addition might be specific to V(D)J recombination junctions and targeted through interactions with the RAG proteins themselves or with Ku80 during remodeling of the RAG post-cleavage complex (16,17). In view of the potential deleterious effects of N nucleotide addition to non-V(D)J-related ends, one might imagine that cells would not allow TdT free access to chromosomal DSBs. We found, however, that TdT efficiently adds N nucleotides to chromosomal and extra-chromosomal DSBs. Indeed, joining of I-SceI-induced chromosomal DSB in the presence of TdT generated a proportion of junctions with N regions (71%) similar to that typically produced by V(D)J recombination in CHO cells transfected with the same TdT expression vector (16) and in lymphoid cells expressing endogenous TdT (17,43–45). These data provide conclusive evidence that TdT has ready access to broken DNA intermediates joined by NHEJ and is not specifically targeted to V(D)J recombination intermediates.

Regulation of TdT activity by DNA-PK

Our observation that most of the N regions formed in the absence of Ku80 are abnormally long (six out of eight are >10 nt) agrees with our previous discovery of rare extremely long N inserts formed during V(D)J recombination in Ku80-deficient mice and cell lines (16,17). Our findings indicate that, although TdT can access broken DNA ends in cells on its own, it behaves differently in the presence of Ku80. It is

interesting to note that DNA-PK modulates the activity of purified TdT in the test tube, limiting the length of N additions to broken DNA ends (43). Because Ku is a component of the DNA-PK holoenzyme and is thought to help recruit the DNA-PK complex to DNA ends (46), our data converge nicely with this previous finding. Thus, although Ku is not absolutely required for N addition to non-V(D)J-mediated breaks, it nevertheless plays some role in modulating N nucleotide addition. This is consistent with the observation that Ku and TdT colocalize to etoposide-induced nuclear foci, which presumably represent DSBs (18).

Ku80 is not required for addition of N regions during plasmid end joining: implications for end processing of V(D)J recombination intermediates

We previously suggested that the requirement for Ku80 for the addition of N nucleotides during V(D)J recombination might reflect a need for Ku-mediated disassembly or remodeling of stable RAG-DNA end complexes to make ends available for TdT (16,17). That possibility is supported by our discovery that non-V(D)J-generated ends are accessible to TdT even in the absence of Ku80. This result suggests that Ku80 either increases the accessibility of the coding and signal ends (e.g. by disassembling the complex) or that it recruits TdT to the complex (but is not required for targeting TdT to other broken DNA ends).

Our data raise an interesting question. At what point during the V(D)J joining reaction does TdT access the ends? Biochemical experiments provide strong evidence that the signal ends remain associated with the RAG proteins in an extremely stable post-cleavage complex whose disruption requires harsh treatments (high temperatures, high salt, phenol extraction) (4,9,47,48). In addition, experiments in cells with a number of joining-deficient RAG mutants provide the best evidence to date that a RAG post-cleavage complex is essential for both signal and coding joint formation (5–7,9). We have suggested that the end processing and joining steps, including N nucleotide addition, might occur in the context of this post-cleavage complex (16,19). Our observation that N nucleotides are added with similar efficiency to both I-SceI-generated DSB and to ends generated by the V(D)J recombinase supports an alternative possibility: end processing (and presumably the subsequent joining step) might occur after the coding and signal ends have been 'handed off' to a general (NHEJ) end-joining complex. Indeed, we have recently obtained evidence that one function of the post-cleavage complex is to shepherd the broken ends to the classical NHEJ pathway (9). Understanding precisely how the NHEJ apparatus coordinates end processing (including addition of N nucleotides by TdT) and the subsequent joining steps will require further investigation.

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