ble, because it is secreted in significant amounts from the infected cells (*4*) and can also easily be demonstrated in the sera of infected animals (*12*). Secreted members of the RNase superfamily are the focus of growing attention as a result of their involvement in diverse biological processes, including organogenesis, cancer, and immunomodulation (*13–15*). Cytotoxic action of E0 against the host immune system would be in agreement with known properties of other RNases, such as pancreatic-type bovine seminal RNase (*13, 16, 17*) and the S-RNases of plants (*10*). In vivo studies on the biological functions of E0 will require either defined E0 mutants of CSFV or large amounts of active protein from heterologous expression systems.

The structural E0 protein is apparently important for virus growth for several reasons: (i) E0 represents a well-conserved structural glycoprotein within the *Pestivirus* genus; (ii) immunization with E0 alone mediates a protective immune response against lethal CSF (*18*); and (iii) E0 has retained its intrinsic catalytic activity during evolution despite its large divergence from classical RNases. Because RNases are among the best studied proteins with respect to their structure and catalytic mechanistics, the E0 RNase activity should serve as a convenient target for the development of antiviral drugs. The unexpected finding that a viral glycoprotein is a potent RNase will thus not only further our understanding on the pathogenesis of diseases caused by mammalian viruses but may also provide us with the means to combat them.

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Lack of N Regions in Antigen Receptor Variable Region Genes of TdT-Deficient Lymphocytes

Toshihisa Komori, Ami Okada, Valerie Stewart, Frederick W. Alt

During the assembly of immunoglobulin and T cell receptor variable region genes from variable (V), diversity (D), and joining (J) segments, the germline-encoded repertoire is further diversified by processes that include the template-independent addition of nucleotides (N regions) at gene segment junctions. Terminal deoxynucleotidyl transferase (TdT)–deficient lymphocytes had no N regions in their variable region genes, which shows that TdT is responsible for N region addition. In addition, certain variable region genes appeared at increased frequency in TdT-deficient thymocytes, which indicates that N region addition also influences repertoire development by alleviating sequence-specific constraints imposed on the joining of particular V, D, and J segments.

Immunoglobulin (Ig) and T cell receptor (TCR) variable region genes are created by the assembly of V, D, and J segments [V(D)J recombination] in developing lymphocytes. V(D)J recombinase activity is targeted by conserved recognition sequences (RS's) that flank each germline gene segment. The reaction involves recognition of RS's, introduction of double-strand breaks at RS-coding junctions; potential loss, addition, or both of nucleotides at coding junctions; and polymerization-ligation activities to complete joining (*1, 2*). The initiation of the reaction is probably mediated by one or two tissue-specific components, whereas most other events are carried out by more generally expressed activities (*2*).

Variable region diversity is created both by combinatorial assortment of V, D, and J segments as well as by the loss or addition of nucleotides at their junctions. Additions fall into two categories: template-dependent (P nucleotides) (*3, 4*) and template-independent (N regions) (*5*). N region addition has been hypothesized to be effected by TdT (*5*), which can add deoxynucleotides to available 3′ ends (*6*). In support of this notion, TdT is found in immature lymphocytes (*7*) and leukemic cells (*8*), but not in nonlymphoid cells. Likewise, the abundance of N regions in V(D)J junctions in adult as compared to fetal lymphocytes also correlates with substantial TdT expression in precursors of the former but not the latter populations (*4, 9–11*). N region addition to V(D)J junctions in cell lines also has been correlated with TdT expression (*12–14*). However, some cell lines that lacked readily detectable TdT activity were found to add N regions (*13, 14*), leading to speculation that N regions also may be added by other mechanisms (*15*).

To unequivocally evaluate the role of TdT in V(D)J recombination and repertoire development, we used gene-targeted mutation to generate chimeric mice in which all mature lymphocytes develop from precursors lacking TdT expression. We prepared a targeting vector that eliminated the TdT promoter and first exon and allowed for both positive and negative selection (*16*) (Fig. 1A). We generated 43 independent TdT knockout ($TdT^{+/-}$) clones of the CCE line of embryonic stem (ES) cells (Fig. 1B; representative data are shown). We then selected with increased G418 (*17*) and obtained independent homozygous TdT knockout clones ($TdT^{-/-}$) from four different $TdT^{+/-}$ clones (Fig. 1B; representative clones are shown).

All mature lymphocytes in chimeras formed by injection of ES cells into RAG-2–deficient blastocysts derive from the injected ES cells [RAG-2–deficient blastocyst complementation (*18*)]. Complementation of RAG-2–deficient blastocysts with either $TdT^{-/-}$ ES cells or $TdT^{+/-}$ ES cells generated chimeras with substantial numbers of ES cell–derived B and T cells in primary and peripheral lymphoid organs (Fig. 1, B and C). However, we did not detect either

T. Komori and V. Stewart, Howard Hughes Medical Institute, Children's Hospital, Boston, MA 02115.
A. Okada, Department of Genetics and Center for Blood Research, Harvard University Medical School, Boston, MA 02115.
F. W. Alt, Howard Hughes Medical Institute, Children's Hospital, Boston, MA 02115, and Department of Genetics and Center for Blood Research, Harvard University Medical School, Boston, MA 02115.

TdT enzymatic activity or transcripts from the mutated TdT gene (by RNA blotting analyses) in $TdT^{-/-}$ thymocytes (*19*). Therefore, inability to express TdT has no gross effect on lymphocyte differentiation.

To characterize V(D)J junctions in $TdT^{-/-}$ and $TdT^{+/-}$ lymphocytes, we used various specific primers (*20*) to amplify and sequence multiple TCR β, γ, and δ chain V-D-J or V-J junctions from RNA or genomic DNA of 2.5- to 6-week-old mouse thymuses (Fig. 2) and Ig heavy chain (IgH) V-D-J junctions from genomic DNA of 2.5-week-old mouse spleens (Fig. 3). In contrast to junctions from $TdT^{+/-}$ lymphocytes, those from $TdT^{-/-}$ lymphocytes contained virtually no N regions (Figs. 2 and 3 and Table 1). Thus, TdT is the only major activity involved in physiological N region addition. Other activities speculated to be involved in this process, primarily based on V(D)J recombination substrate studies and illegitimate recombination studies in lymphoid and nonlymphoid cell lines (*15*), are not a significant source of N regions in normal developing lymphocytes. Also, P nucleotides occurred at similar frequencies in $TdT^{+/-}$ and $TdT^{-/-}$ junctions (Table 1), confirming that P nucleotide addition is independent of N nucleotide addition and TdT activity.

Short homologies at or near coding sequence breakpoints have been proposed to mediate V(D)J joining and lead to the appearance of certain variable regions at increased frequency in fetal repertoires (*5, 10, 21*). The low TdT expression in developing fetal lymphocytes has been speculated to promote this phenomenon (*10*). However, the overall role of such homologies in the V(D)J joining process (leading to "overlapping" junctional nucleotides) has been difficult to evaluate because N regions may have provided occult homology in junctions where none was evident (for example, in most junctions of adult repertoires). In this context, 45% (286/583) of $TdT^{-/-}$ junctions (V-D, D-D, D-J, or V-J) had homologies of two or more nucleotides as compared with 18% (56/306) of $TdT^{+/-}$ junctions (Figs. 2 and 3 and Table 1) (*19*). Therefore, the occurrence of homology-mediated joins is enhanced in $TdT^{-/-}$ adult lymphocytes—confirming the proposed role of TdT expression on the relative occurrence of such joins in fetal as compared to adult repertoires. However, over 25% of $TdT^{-/-}$ junctions lacked even one base pair of overlap (Table 1), indicating the existence of a V(D)J joining pathway that is homology-independent.

The γδ T cells of epidermis mostly have a single in-frame $V_\gamma3J_\gamma1$ junction that also is the predominant ("canonical") $V_\gamma3J_\gamma1$ join in fetal thymocytes; these repertoires also have two predominant out-of-frame $V_\gamma3J_\gamma1$ joins as well (*4, 22–24*). The frequency of these joins in particular repertoires was suggested to result from cellular selection (*25*). However, experiments with transgenic TCRγ recombination substrates (*23*) and $TCR\delta^{-/-}$ mice (*24*), coupled with observations on fetal Ig and TCR repertoires (*9–11*), indicated that canonical joins are favored as a result of biased joining by the recombination machinery in fetal thymic progenitors (*26*).

In $TdT^{-/-}$ thymuses, 30 of 40 in-frame $V_\gamma3J_\gamma1$ joins were canonical, and 42 of 65 out-of-frame joins were one of the two joins that predominate in fetal thymocytes (Fig. 2). Taken together, these three junctions represented 70% of all $V_\gamma3J_\gamma1$ junctions in $TdT^{-/-}$ thymocytes, a number comparable to that observed in fetal repertoires (*4, 22*). In contrast these same three junctions represented only 32% of the $V_\gamma3J_\gamma1$ joins in the $TdT^{+/-}$ thymocytes, in agreement with the percentage previously observed in adult thymocytes (*4, 11*). Therefore, both productive and nonproductive $V_\gamma3J_\gamma1$ predominant joins occurred at increased frequency in $TdT^{-/-}$ thymocytes. Our data strongly support the biased recombination model and indicate that lack of TdT, as opposed to either some other property of the V(D)J recombinase or to cellular selection, promotes appearance of predominant junctions in fetal thymocytes. Homology-mediated joining has been proposed to promote the frequent occurrence of particular junctions (*10, 26*); canonical $V_\gamma3J_\gamma1$ joins may be promoted by two short overlaps (AT, TAG) that appear in the majority of $V_\gamma3J_\gamma1$ junctions (*26*) (Fig. 2). If so, the lack of use of other nearby potential overlaps (TC, CT, TA) in $TdT^{-/-}$ thymocytes (Fig. 2) suggests that other factors, such as

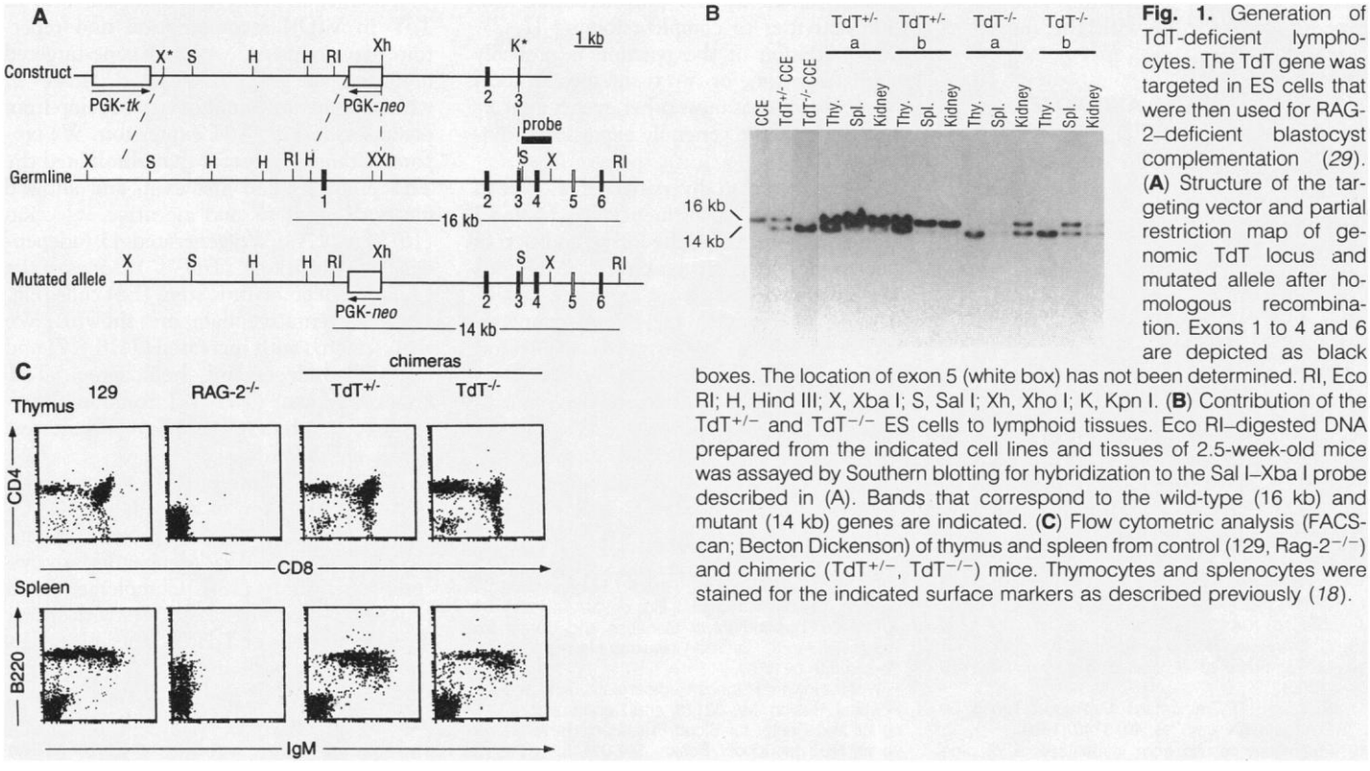

Fig. 1. Generation of TdT-deficient lymphocytes. The TdT gene was targeted in ES cells that were then used for RAG-2–deficient blastocyst complementation (*29*). (**A**) Structure of the targeting vector and partial restriction map of genomic TdT locus and mutated allele after homologous recombination. Exons 1 to 4 and 6 are depicted as black boxes. The location of exon 5 (white box) has not been determined. RI, Eco RI; H, Hind III; X, Xba I; S, Sal I; Xh, Xho I; K, Kpn I. (**B**) Contribution of the $TdT^{+/-}$ and $TdT^{-/-}$ ES cells to lymphoid tissues. Eco RI–digested DNA prepared from the indicated cell lines and tissues of 2.5-week-old mice was assayed by Southern blotting for hybridization to the Sal I–Xba I probe described in (A). Bands that correspond to the wild-type (16 kb) and mutant (14 kb) genes are indicated. (**C**) Flow cytometric analysis (FACScan; Becton Dickenson) of thymus and spleen from control (129, Rag-$2^{-/-}$) and chimeric ($TdT^{+/-}$, $TdT^{-/-}$) mice. Thymocytes and splenocytes were stained for the indicated surface markers as described previously (*18*).

TdT$^{+/-}$

Total	a	b	c	$V_\gamma3$	P	N	P	$J_\gamma1$	
				Germline TGT GCC TGC TGG GAT CT				AT AGC TCA GGT TTT	
8/63	2/28	4/17	2/18	* TGT GCC TGC TGG GAT				AGC TCA GGT TTT	+
1/63			1/18	TGT GCC TGC TGG GAT				AT AGC TCA GGT TTT	+
1/63	1/28			TGT GCC TGC TGG GA			AT	AT AGC TCA GGT TTT	+
4/63	1/28	3/17		* TGT GCC TGC TGG GAT CT	AG			C TCA GGT TTT	-
8/63	3/28	2/17	3/18	* TGT GCC TGC TGG G			AT	AT AGC TCA GGT TTT	-
1/63	1/28			TGT GCC TGC TGG GAT C				TCA GGT TTT	-
1/63	1/28			TGT GCC TGC TGG G			T	AT AGC TCA GGT TTT	-
1/63	1/28			TGT GCC TGC TGG			AT	AT AGC TCA GGT TTT	-
1/63	1/28			TGT GCC TGC TGG G				AGC TCA GGT TTT	-
1/63	1/28			TGT GCC TGC TGG G			T	AT AGC TCA GGT TTT	-
1/63		1/17		TGT GCC TGC TGG G		TGGGGT		GC TCA GGT TTT	+
1/63	1/28			TGT GCC TGC TGG G		G		T AGC TCA GGT TTT	+
1/63		1/17		TGT GCC TGC TG		ATATAA		C TCA GGT TTT	+
1/63	1/28			TGT GCC TGC TGG GAT CT	AG	TTGGGGG		T AGC TCA GGT TTT	+
1/63	1/28			TGT GCC TGC TGG GAT C		AG	T	AT AGC TCA GGT TTT	+
1/63	1/28			TGT GCC TGC TGG GAT		GGAGGAA		AT AGC TCA GGT TTT	+
1/63			1/18	TGT GCC TGC TGG GAT CT	AG	ATGCAGC		T AGC TCA GGT TTT	+
1/63			1/18	TGT GCC TGC TGG GAT		GGTG		GC TCA GGT TTT	+
1/63			1/18	TGT GCC TGC TGG GA		ACAC		AGC TCA GGT TTT	+
1/63			1/18	TGT GCC TGC TG		CGCC	T	AT AGC TCA GGT TTT	+
1/63		1/17		TGT GCC TGC TGG GAT CT		TGAA		GC TCA GGT TTT	-
1/63		1/17		TGT GCC TGC TGG G		GAGG	T	AT AGC TCA GGT TTT	-
1/63		1/17		TGT GCC TGC TGG GAT CT	AG	TATGT		T AGC TCA GGT TTT	-
1/63		1/17		TGT GCC TGC TGG GAT		TGGG		AGC TCA GGT TTT	-
1/63	1/28			TGT GCC TGC TGG GA		CCTA		AT AGC TCA GGT TTT	-
1/63		1/17		TGT GCC TGC TGG GAT C		A		AT AGC TCA GGT TTT	-
1/63	1/28			TGT GCC TGC TGG GA		ATG	T	AT AGC TCA GGT TTT	-
1/63		1/17		TGT GCC TGC TGG GA		GGCGC	AT	AT AGC TCA GGT TTT	-
1/63	1/28			TGT GCC TGC TGG G		G		AT AGC TCA GGT TTT	-
1/63	1/28			TGT GCC TGC TGG GAT CT		T	AT	AT AGC TCA GGT TTT	-
1/63	1/28			TGT GCC TGC TGG GA		ACGT	AT	AT AGC TCA GGT TTT	-
1/63	1/28			TGT GCC TGC TGG G		T	T	AT AGC TCA GGT TTT	-
1/63	1/28			TGT GCC TGC TGG G		GGG	AT	AT AGC TCA GGT TTT	-
1/63	1/28			TGT GCC TGC TG		AGA		AT AGC TCA GGT TTT	-
1/63	1/28			TGT GCC TGC TGG GAT C		CT		GC TCA GGT TTT	-
1/63	1/28			TGT GCC TGC TGG		AAA	AT	AT AGC TCA GGT TTT	-
1/63	1/28			TGT GCC TGC TGG GAT CT	A	AA		AGC TCA GGT TTT	-
1/63	1/28			TGT GCC TGC TGG GAT C		AAATTGG		AGC TCA GGT TTT	-
1/63			1/18	TGT GCC TGC TGG GAT CT	A	CG		AGC TCA GGT TTT	-
1/63			1/18	TGT GCC TGC TGG GA		G		T AGC TCA GGT TTT	-
1/63			1/18	TGT GCC TGC TGG G		GGA	AT	AT AGC TCA GGT TTT	-
1/63			1/18	TGT GCC TGC TGG G		CG		GC TCA GGT TTT	-
2/63			2/18	TGT GCC TGC TGG		AC		AT AGC TCA GGT TTT	-
1/63			1/18	TGT G		ACTG	AT	AT AGC TCA GGT TTT	-
1/63			1/18	TG		G	T	AT AGC TCA GGT TTT	-

TdT$^{-/-}$

Total	d	e	f	g1	g2	$V_\gamma3$	P	N	P	$J_\gamma1$	
						Germline TGT GCC TGC TGG GAT CT				AT AGC TCA GGT TTT	
30/105	10/31	4/23	4/15	5/21	7/15	* TGT GCC TGC TGG GAT				AGC TCA GGT TTT	+
1/105		1/23				TGT GCC TGC TGG GAT CT				AT AGC TCA GGT TTT	+
2/105		1/23		1/21		TGT GCC TGC TGG GAT C				GC TCA GGT TTT	+
1/105	1/31					TGT GCC TGC TGG G				GC TCA GGT TTT	+
1/105	1/31					TGT GCC TGC TGG GAT C				AT AGC TCA GGT TTT	+
1/105			1/15			TGT GCC TGC TGG GAT CT	AG			GT TTT	+
2/105				2/21		TGT GCC TGC TGG GAT CT				C TCA GGT TTT	+
2/105				2/21		TGT GCC TGC				T AT AGC TCA GGT TTT	+
28/105	8/31	6/23	4/15	5/21	5/15	* TGT GCC TGC TGG GAT CT	AG			C TCA GGT TTT	-
14/105	4/31	6/23	2/15	2/21		* TGT GCC TGC TGG G				AT AGC TCA GGT TTT	-
1/105		1/23				TGT GCC TGC TGG				AT AGC TCA GGT TTT	-
6/105	3/31			2/21	1/15	TGT GCC TGC TGG				AT AGC TCA GGT TTT	-
1/105		1/23				TGT GCC TGC TGG GAT				AT AGC TCA GGT TTT	-
1/105		1/23				TGT GCC TGC TGG G				C TCA GGT TTT	-
1/105		1/23				TGT GCC TGC TG				TCA GGT TTT	-
1/105	1/31					TGT GCC T				TTT	-
2/105	1/31	1/23				TGT GCC TGC T				T AGC TCA GGT TTT	-
1/105	1/31					TGT GCC TGC T				AGC TCA GGT TTT	-
1/105	1/31					TGT GCC TGC TGG GA				GC TCA GGT TTT	-
2/105			1/15	1/21		TGT GCC TGC TGG GAT CT	AG			T AGC TCA GGT TTT	-
1/105					1/15	TGT GCC TGC TGG GAT CT				T AT AGC TCA GGT TTT	-
1/105			1/15			TGT GCC TGC TGG GAT CT				TTT	-
1/105				1/21		TGT GCC TGC TGG GAT C				T AT AGC TCA GGT TTT	-
1/105					1/15	TGT GCC TGC TGG G				T AT AGC TCA GGT TTT	-
2/105			2/15			TGT GCC TGC TGG G				T TTT	-

Fig. 2. Lack of N regions in V(D)J junctions from TdT$^{-/-}$ T lymphocytes. Polymerase chain reaction (PCR)–amplified products (*20*) from thymic genomic DNA of two 4-week-old mice (a and b), two 6-week-old mice (d and e) and three 2.5-week-old mice (c, f, and g) are shown. Two independent PCR amplification reactions were analyzed from one 2.5-week-old chimera (g1 and g2). The frequency of each junction is listed to the left of the sequence. Overlapping nucleotides that could be encoded by either germline segment (including P nucleotides) are underlined. The reading frame [(+) or (−)] and canonical in-frame and predominant out-of-frame sequences (*4, 22–24*) are indicated by an asterisk.

TdT$^{+/-}$

	V_H81X	P	N	P	D	P	N	P	J_H2	
Germline	TGT GCA AGA CA								ACTACTTT GACTACTGG	
22a2-4	TGT GCA AGA		GCCCCCA		TAACTGG		T		ACTACTTT GACTACTGG	-
22a2-10	TGT GCA AGA				TGATTACGAC				TACTTT GACTACTGG	-
22a2-12	TGT GCA AGA C		GG		TACTATGATTACGAC	G	G		TTT GACTACTGG	-
22a2-13	TGT GCA AGA C				GATTACGAC	G			ACTACTGG	-
22a2-3	TGT GCA AGA CA	T	AG		AGTATGGTAAC		CC	T	ACTACTAT GACTACTGG	-
22a2-11	TGT GCA AGA CA	T	AG		AGTATGGTAAC		CC	T	ACTACTTT GACTACTGG	-
22b2-4	TGT GCA AG		GC		CTATGGTAACT		GAGG	GT	ACTACTTT GACTACTGG	-
22b2-1	TGT GCA AGA C				GGTTACT				GG	-
22b2-7	TGT GCA AGA CA	T	A		CTATGATGGT			T	ACTACTTT GACTACTGG	-
22a2-6	TGT GCA AGA CA		GGG		ACTATAGGTACGAC	G	AAGGGC		T GACTACTGG	-
22a2-9	TGT GCA AGA C				TATAGGTACGAC				TTT GACTACTGG	-
22b2-2	TGT GCA AGA CA		GG		CTACTATAGGTACG		CG		GACTACTGG	-
22b2-3	TGT GCA AGA CA		GG		CTATAGGTACGAC	GT			C TACTGG	-
22b2-11	TGT GCA A				GG CCTACTATAGGTACGAC	GT			ACTTT GACTACTGG	+
22b2-13	TGT GCA AGA		GGGG		AGGTACG		TG		CTACTTT GACTACTGG	-
22b2-5	TGT GCA AGA CA	T			TACTACGGTAGTAGCT				C TACTGG	-
22b2-12	TGT GCA A		TAT		TACGGC			T	ACTACTTT GACTACTGG	-
22a2-1	TGT GCA AGA CA		G		GGGCTAC				TGG	-
22a2-5	TGT GCA AGA C								C TTT GACTACTGG	-

TdT$^{-/-}$

	V_H81X	P	N	P	D	P	N	P	J_H2	
Germline	TGT GCA AGA CA								ACTACTTT GACTACTGG	
84a2-3	TGT GCA AGA CA	TG			CTGGGAC				TACTTT GACTACTGG	-
84b2-4	TGT GCA AG				GAC				TACTGG	-
84b2-11	TGT GCA AGA C				TGGGAC				TTT GACTACTGG	-
84b2-16	TGT GCA AGA C				TAACTGGGAC				TACTTT GACTACTGG	-
84b2-19	TGT GCA A				ACTGGGAC	GT			ACTTT GACTACTGG	-
84a2-12	TGT GCA AGA C				TATGATTACGAC				TACTGG	-
84a2-17	TGT GCA AGA CA	TG			TGATTACGAC				TTT GACTACTGG	-
84b2-7	TGT GCA AGA CA	TG			ATTACGAC	GT			ACTACTTT GACTACTGG	-
84a2-6	TGT GCA AG				GTAAC				T ACTACTTT GACTACTGG	-
84a2-13	TGT GCA AGA CA	TG			TGGTAACTAC				TTT GACTACTGG	-
84b2-1	TGT GCA AGA C				TGGTAAC				T ACTACTTT GACTACTGG	-
84b2-2	TGT GCA AGA C				GGTAACT				TT GACTACTGG	-
84b2-3	TGT GCA AGA C				GGTAACTA				ACTACTGG	-
84b2-13	TGT GCA AGA CA	TG			TGGTAACTAC				TTT GACTACTGG	-
84b2-17	TGT GCA AGA				TGGTAAC				T ACTACTTT GACTACTGG	-
84b2-18	TGT GCA AGA CA	T			ATGGTAACTAC				TTT GACTACTGG	-
84b2-8	TGT GCA AGA C				CTACTATGGT				GACTACTGG	-
84a2-4	TGT GCA AGA CA				G CCTAGTATGGTAACTAC				TGG	-
84a2-7	TGT GCA AGA CA	TG			TATGGTAACTAC				TTT GACTACTGG	-
84a2-8	TGT GCA AGA				TAGTATGGTAACTAC	G			ACTACTGG	-
84b2-14	TGT GCA AG				TATGG				GACTACTGG	-
84b2-20	TGT GCA AGA C				TAGTATGGTAACTAC				TTT GACTACTGG	-
84b2-21	TGT GCA				GTATGGTAAC				T ACTACTTT GACTACTGG	-
84a2-21	TGT GCA AGA CA	TG			GTATGGTAACT				TT GACTACTGG	-
84a2-1	TGT GCA AGA CA	T			ACTATAGGTACGAC				TACTGG	-
84a2-5	TGT GCA AGA				TAGGTACGAC				TACTTT GACTACTGG	-
84a2-9	TGT GCA AG				TACTATAGGTACGAC				TACTGG	-
84a2-11	TGT GCA AGA C				TATAGGTA				ACTACTGG	-
84a2-14	TGT GCA AGA CA				CTATAGGTACGAC	GT			ACTTT GACTACTGG	-
84b2-6	TGT GCA AGA CA	T			AGGTA				GACTACTGG	-
84a2-10	TGT GC				AATTTATTACTACGGTAGTAGC				T ACTACTTT GACTACTGG	+
84a2-18	TGT GCA AGA C				TACTACGGTAGTAG				ACTACTTT GACTACTGG	-
84a2-2	TGT GCA AG				A TAGACAGCTCGGGCTAC				TACTGG	-
84a2-15	TGT GCA AGA				C				T ACTACTTT GACTACTGG	-
84b2-9	TGT GCA AGA C				T				TT GACTACTGG	-
84b2-10	TGT GCA AGA C				GG				GT ACTACTTT GACTACTGG	-

Fig. 3. Lack of N regions in V(D)J junctions from TdT$^{-/-}$ B cells. PCR products of genomic DNA from spleens of 2.5-week-old mice (two independent spleens each) are shown. Nucleotide sequences are aligned with the germline sequence (*22, 30, 31*). Overlapping nucleotides and reading frame are as in Fig. 2.

Table 1. V(D)J junctions of TCR and Ig genes. Thymocyte cDNA (V_β), thymocyte genomic DNA (V_γ and V_δ), and spleen genomic DNA (V_H) were used as templates to amplify junctions of the indicated rearrangements by PCR. Two 2.5-week-old mice were analyzed in each set (except for $V_\gamma3$-$J_\gamma1$ sequences, where older mice were also used to further evaluate the frequency of canonical joins; Fig. 2). Identical sequences detected in products from the same PCR reaction are counted only once, except when they were found multiple times in independent amplifications (which only occurred for $V_\gamma3$-$J_\gamma1$ reactions).

	Number of sequences analyzed	Number of N+ sequences (%)*	Average number of N†	Number of overlapping junctions (%)‡			Number of P+ junctions (%)§	Number of overlapping P (%)¶
				V-D	D-J	Total		
				TdT$^{+/-}$				
$V_\beta8$-D-J_β	25	23 (92)	3.0	2 (8)	4 (16)	6 (12)	15 (30)	3 (20)
$V_\beta14$-D-J_β	19	18 (95)	3.3	1 (5)	0 (0)	1 (3)	10 (26)	1 (10)
$V_\gamma3$-$J_\gamma1$	63	36 (57)	3.4			22 (35)	35 (56)	13 (37)
$V_\delta1$-D-$J_\delta1$	6	3 (50)	2.7	4 (67)	2 (33)	7 (54)#	6 (46)	5 (83)
$V_\delta1$-D-$J_\delta2$	3	2 (67)	3.5	2 (67)	1 (33)	3 (50)	1 (17)	1 (100)
V_HS107-D-J_H2	14	7 (50)	3.9	7 (50)	7 (50)	14 (50)	9 (32)	5 (56)
V_HS107-D-J_H3	21	15 (71)	5.0	6 (29)	8 (38)	14 (33)	13 (31)	6 (46)
V_H81X-D-J_H2	19	12 (63)	4.3	5 (26)	9 (47)	14 (37)	15 (39)	6 (40)
V_H81X-D-J_H3	14	7 (50)	5.1	7 (50)	7 (50)	14 (50)	10 (36)	5 (50)
Total	184	123 (67)	3.7	34 (28)	38 (31)	95 (31)	114 (37)	45 (39)
				TdT$^{-/-}$				
$V_\beta8$-D-J_β	59	2 (3)	1	42 (71)	31 (53)	73 (62)	32 (27)	16 (50)
$V_\beta14$-D-J_β	25	1 (4)	1	22 (88)	13 (52)	35 (70)	14 (30)	12 (80)
$V_\gamma3$-$J_\gamma1$	105	0	0			84 (80)	58 (55)	47 (81)
$V_\delta1$-D-$J_\delta1$	16	0	0	8 (50)	11 (69)	26 (63)#	21 (51)	17 (81)
$V_\delta1$-D-$J_\delta2$	7	0	0	7 (100)	6 (86)	15 (94)#	10 (67)	9 (90)
V_HS107-D-J_H2	29	0	0	26 (90)	26 (90)	52 (90)	13 (22)	13 (100)
V_HS107-D-J_H3	40	1 (3)	1	32 (80)	28 (70)	60 (75)	16 (20)	13 (81)
V_H81X-D-J_H2	36	0	0	24 (67)	30 (83)	54 (75)	24 (33)	17 (71)
V_H81X-D-J_H3	27	0	0	20 (74)	21 (78)	41 (76)	20 (37)	17 (85)
Total	344	4 (1)	1	181 (76)	166 (69)	440 (74)	208 (35)	161 (77)

*Percentage of complete junctions (V-D-J or V-J) with N regions. †The number was calculated based on the sequences that have N regions. ‡Percentage of individual overlapping junctions (V-D, D-D, and D-J); potential P nucleotides that overlap with germline sequences are included. §Percentage of individual junctions (V-D, D-D, and D-J) with P nucleotides. ¶Percentage of P nucleotides with overlap. #D-D junctions are included in the total.

sequence context, must contribute to this process.

Thus, TdT is a tissue-specific component of V(D)J recombinase. This enzyme is not required for the reaction, but if expressed it qualitatively modifies the outcome by adding N regions to V(D)J junctions. The presence or absence of TdT expression during variable region gene assembly can affect the resulting variable region repertoire in at least two ways. First, N region addition generates a substantial amount of diversity in the portion of antigen receptor variable regions that have a major role in antigen recognition and TCR–major histocompatibility complex protein interactions (*27*). In addition, absence of TdT leads to the appearance of particular variable region genes at increased frequency, probably because N region addition reduces the probability of the homology-mediated joining of certain variable region gene segments. In the accompanying report, another group has reached nearly identical conclusions based on the analysis of a germline TdT mutation in which a different TdT exon was targeted (*28*). Finally, we note that TdT$^{-/-}$ chimeras as old as 6 months have no marked abnormalities. However, more detailed analyses may reveal important immunological consequences of the limited variable region repertoire in TdT$^{-/-}$ mice.

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20. Complementary DNA was made with the use of C_β primer and cDNA, and DNA was amplified at the total volume of 100 μl by Vent polymerase (New England Biolabs). Nested primers were as follows: C_β, 5′-GGTGAATTCACATTTCTCAGATCCT-3′ and 5′-GCAATCTCTGCTTTGATGG-3′; $V_\beta8$, 5′-ATGTACTGGTATCGGCAGGA-3′ and 5′-ACTCGGGATCCGCTGAGGCTGATCCATT-3′; $V_\beta14$, 5′-ACCTCTAAGCTTCCAGGCCGAAGGACG-3′ and 5′-AAATCAAGCCCTAACCTCTAC-3′; $V_\gamma3$, 5′-CT-

CAAGCTTGGAAATTGATGAG-3′ and 5′-CTGG-TACCAACTGAAAGAAG-3′; $J_\delta1$, 5′-GGAATTCCT-TCTGCAAATACCTTG-3′ and 5′-AGAGGGAAT-TCCTATGAGCT-3′; $V_\delta1$, 5′-AATAGGAATTCTAC-TGATGGTGG-3′ and 5′-ATGGATCCAATGCTCT-GTTTTTAG-3′; $J_\delta1$, 5′GTGGGATCCTTGTCCAAA-GAC-3′ and 5′-GTGGATCCACAGTCACTTG-3′; $J_\delta2$, 5′-GCCGGATCCAAAAAACATCTG-3′ and 5′-GGGATCCACAAAGAGCTC-3′; V_HS107, 5′-CTG-GAATTCGAAACAAAGCTAATG-3′ and 5′-TCTG-GAGGAGGCTTGGTACA-3′; V_H81X, 5′-CCTGT-GAATCCAATGAATACG-3′ and 5′-GTGGAGTCTG-GGGGAGGCTTA-3′; and J_H3, 5′-TGCAGAGAATC-TTGGTCCTG-3′ and 5′-ACTTCAAGCTTCAGTTC-TGG-3′. Twenty-five to 30 cycles of amplification were done with a programmable thermal controller (MJ Research, Inc.) (1 min at 94°C, 1 min at 52°C to 60°C, and 30 s to 1 min at 72°C). A second round of amplification was done starting with 2 to 15 μl of the first-round mixture [M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White. Eds., *PCR Protocols: A Guide to Methods and Applications* (Academic Press, San Diego, CA, 1989), pp. 21–27].
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29. To construct the targeting vector, we cloned a 0.7-kb genomic Eco RI–Hind III fragment 5′ of the TdT gene into pBluescript SK (Stratagene) containing a 2.7-kb Eco RI–Hind III PGK–HSV-*tk* fragment in the Sst I site. A 1.6-kb Eco RI–Bgl II PGK-*neo* fragment was blunt end–ligated in the opposite transcriptional orientation into the Hind III site. To obtain the final targeting vector, we inserted an 8-kb genomic Xba I*–Eco RI and 7-kb genomic Xho I–Kpn I* fragment respectively, into the Eco RI site and Xho I site of this construct (asterisk denotes sites in cloning vector). ES cells (CCE, 2 × 10^7) were transfected with Kpn I–linearized targeting vector (20 μg) and selected with G418 (0.4 mg/ml) and gancyclovir (1 μM) (*16*). Three hundred and sixty-two selected clones were screened by Southern (DNA) blot analysis of Eco R I–digested genomic DNA probed with the Sal I–Xba I fragment 3′ of the region included in the targeting construct to identify 43 $TdT^{+/-}$ clones. Four $TdT^{+/-}$ clones were selected in increased G418 concentration (1.6 to 4 mg/ml) to obtain $TdT^{-/-}$ clones (*17*). Subclones of single ($TdT^{+/-}$) or double ($TdT^{-/-}$) mutated ES cells were injected into blastocysts of RAG-2–deficient blastocysts and transferred into $B6CBAF_1/J$ (Jackson Laboratory) females (*18*).
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Mice Lacking TdT: Mature Animals with an Immature Lymphocyte Repertoire

Susan Gilfillan, Andrée Dierich, Marianne Lemeur, Christophe Benoist, Diane Mathis

In adult animals, template-independent (or N) nucleotides are frequently added during the rearrangement of variable (V), diversity (D), and joining (J) segments of lymphocyte receptor genes, greatly enhancing junctional diversity. Receptor genes from adult mice carrying a mutation in the terminal deoxynucleotidyl transferase (TdT) gene have few N nucleotides, providing proof that this enzyme is essential for creating diversity. Unlike those from normal adults, receptor genes from adult mutant mice show extensive evidence of homology-directed recombination, suggesting that TdT blocks this process. Thus, switch-on of the TdT gene during the first week after birth provokes an even greater expansion of lymphocyte receptor diversity than had previously been thought.

The repertoire of B and T cell antigen receptors expressed in adult animals is more diverse than that in perinates (*1*). One major difference is the amount of N region diversity at the junctions of rearranged immunoglobulin (Ig) and T cell receptor (TCR) gene segments. N nucleotides are rare in V(D)J junctions from fetal or newborn animals, but constitute a major component of the diversity of Igs and TCRs from adults (*2–7*). This dissimilarity may be due to differential expression of TdT. Terminal deoxynucleotidyl transferase catalyzes template-independent addition of nucleotides in vitro (*8*), and the amount expressed in vivo correlates with the degree of N region diversity in antigen receptors (*9*, *10*). Another difference between adult and perinatal repertoires lies in the diversity of V-J, V-D, and D-J junctional sequences. Examination of large sets of fetal and newborn Ig and γδ TCR sequences revealed overrepresentation of some junctions, coincident with short stretches of homology between abutted gene segments (*3–5*, *11–13*). In the case of γδ TCRs, certain dominant junctions (termed "canonical") are functionally significant because they give rise to the quasi-monoclonal receptors in specific anatomical locations such as the skin. Overrepresented joints were not generally observed in adult sequences, only in some of those lacking N nucleotides. Initially, the presence of dominant junctions of γδ TCRs was attributed to cellular selection (*5*, *14*), but a preference for rearranging at short stretches of homology is more probable (*15*, *16*). Why such homology-directed recombination is pronounced in perinates but rare in adults is an open question.

One approach to better understanding the adult-perinate dichotomy is to artificially produce mature animals with repertoires having immature features. Thus, we generated, through homologous recombination in embryonic stem cells, a strain of mice lacking TdT (*17*). The mutation of TdT we obtained was an insertion of the neomycin gene into exon 4, as illustrated in Fig. 1 and confirmed by extensive Southern (DNA) blot analysis. Given the predicted location of the TdT active site and its presumed globular nature (*18*), exons 4 to 7 are probably critical for TdT function. No mRNA corresponding to regions 3′ of the neomycin insert was detected in thymus RNA from homozygous mutant mice after polymerase chain reaction (PCR) amplification, for which we used a primer pair on the 3′ side of the insertion; nor was any revealed by in situ hybridization of the appropriate probe to thymic sections (*19*). Abrogation of protein expression was confirmed by staining of thymocytes with a polyclonal antiserum to TdT (*19*).

Homozygous mutant $TdT^{-/-}$ mice breed well and appear healthy in a conventional animal facility, are of normal size, and do not have increased susceptibility to infection, as is common for immunodeficient animals in our colony. The mutants show no marked abnormalities in the major T or B cell compartments and are capable of mounting T and B cell responses to complex antigens like keyhole limpet hemocyanin and ovalbumin (*20*).

To evaluate the effect of a TdT deficiency on the lymphocyte repertoires of adult mice, we sequenced the V(D)J junctions of more than 300 rearranged Ig and TCR genes from adult animals (most from 6 to 8 weeks of age) (*21*). Representative sets of $V_\gamma3$ DNA sequences from total thymocytes (Fig. 2), V_H7183 DNA sequences from splenocytes (Fig. 3), and $V_\beta8$ RNA sequences from $CD4^+$-$CD8^+CD3^{lo}$ thymocytes (Fig. 3) are shown. The enzyme TdT was responsible for the bulk of N region diversity because

Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS et Unité 184 de Biologie Moléculaire de l'INSERM, Institut de Chimie Biologique, 11 rue Humann, 67085 Strasbourg Cédex, France.