Nucleotide pool imbalance and adenosine deaminase deficiency induce alterations of *N*-region insertions during V(D)J recombination

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Template-independent nucleotide additions (*N* regions) generated at sites of V(D)J recombination by terminal deoxynucleotidyl transferase (TdT) increase the diversity of antigen receptors. Two inborn errors of purine metabolism, deficiencies of adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP), result in defective lymphoid development and aberrant pools of 2′-deoxynucleotides that are substrates for TdT in lymphoid precursors. We have asked whether selective increases in dATP or dGTP pools result in altered *N* regions in an extrachromosomal substrate transfected into T-cell or pre–B-cell lines. Exposure of the transfected cells to 2′-deoxyadenosine and an ADA inhibitor increased the dATP pool and resulted in a marked increase in A–T insertions at recombination junctions, with an overall decreased frequency of V(D)J recombination. Sequence analysis of V_H-D_H-J_H junctions from the IgM locus in B-cell lines from ADA-deficient patients demonstrated an increase in A–T insertions equivalent to that found in the transfected cells. In contrast, elevation of dGTP pools, as would occur in PNP deficiency, did not alter the already rich G–C content of *N* regions. We conclude that the frequency of V(D)J recombination and the composition of *N*-insertions are influenced by increases in dATP levels, potentially leading to alterations in antigen receptors and aberrant lymphoid development. Alterations in *N*-region insertions may contribute to the B-cell dysfunction associated with ADA deficiency.

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Introduction

Immunoglobulin and T-cell receptor gene assembly occurs through site-specific recombination of V, D, and J segments in developing lymphocytes (reviewed in refs. 1 and 2). The V(D)J recombinase activity is targeted to conserved recombination signal sequences (RSSs), composed of a palindromic heptamer and an A–T-rich nonamer separated by either a 12- or a 23-bp spacer, which flank each germline gene segment. Two main types of junctions are formed as a result of V(D)J recombination: (*a*) coding joints are formed through splicing of the gene segments, and (*b*) reciprocal or signal joints are formed by the joining of contiguous RSS elements. Recombination is initiated with the introduction of a site-directed double-strand break at the RSS elements, after which the coding ends and signal ends are processed very distinctly. Coding ends are highly processed and rapidly joined after passing through a hairpin intermediate. The blunt signal ends are slowly joined, and efficient resolution may depend on downregulation of V(D)J recombination or reentry into the cell cycle (3). The combinatorial joining of the numerous V, D, and J elements and nucleotide loss or addition at the recombination junction together result in enormous potential for antigen receptor diversity.

Terminal deoxynucleotidyl transferase (TdT) is responsible for all template-independent nucleotide additions (*N* regions) during V(D)J recombination (4, 5). *N* regions are typically G–C rich (6, 7) and are found at coding joints and at a lesser frequency at reciprocal signal joints (8–10). This difference in TdT activity may be a result of the different substrate DNA ends generated at signal and coding recombination intermediates, or it may be the result of differential interactions of TdT with components of the V(D)J recombination machinery.

In addition to increasing antigen receptor diversity, TdT-mediated insertions have been shown to block the homology-directed recombination apparent in fetal or neonatal lymphocytes (11–13). The junctional repertoires of adult TdT knockout mice, which lack *N* regions, resemble those of a neonatal animal, and an analysis of coding joints lacking *N* regions from TdT-expressing cells reveals an absence of homology-directed recombination (4). These observations suggest that the interaction of TdT with free DNA ends may facilitate or modify the joining events of V(D)J recombination irrespective of actual nucleotide addition. An important role for TdT in the recombination complex is supported by its presence throughout vertebrate evolution (14–16).

Studies of the inherited diseases resulting from deficiencies of adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) activities have provided insight into the effects of intracellular deoxyribonucleoside triphos-

phate (dNTP) pool imbalances on lymphocyte development (17). Both diseases cause abnormalities in purine nucleoside metabolism that selectively interfere with either or both thymocyte viability and function and result in immunodeficiency. Lack of ADA activity leads to the accumulation of its substrate dAdo, selective increases in deoxyadenosine triphosphate (dATP) levels in thymocytes and pre–B cells, and severe combined immunodeficiency disease (SCID), and PNP deficiency leads to dGuo and deoxyguanosine triphosphate (dGTP) accumulation, with predominantly T-cell depletion. Studies of dATP toxicity in resting lymphocytes have shown that exposure to dAdo causes the accumulation of single-strand DNA breaks and depletion of ATP and NAD (18, 19). It has also been proposed that high levels of dATP interfere with both DNA synthesis and repair and deplete the levels of other dNTPs through inhibition of the enzyme ribonucleotide reductase (reviewed in refs. 17, 20, and 21). We have hypothesized that alterations of intracellular purine dNTP pools may affect TdT activity during V(D)J recombination. To test this hypothesis, we have analyzed the effects of nucleotide pool imbalances on V(D)J recombination and TdT activity on exogenous plasmid substrates for both signal and coding junctions. In addition, we have examined the rearranged V_H-D_H-J_H Ig-μ locus from ADA-deficient patients to determine whether increased intracellular levels of dATP affect recombination in these individuals by influencing the insertion of nucleotides by TdT into the *N* regions of differentiating B cells.

Methods

Plasmid constructs. The human TdT expression vector was constructed by inserting a 1944-bp blunt-ended cDNA into the *Eco*RV site of the pCDNA3 vector. The Rag-1 and Rag-2 expression vectors have been described previously (22) and were obtained from M. Oettinger (Harvard University, Cambridge, Massachusetts, USA). The human pCJ and pSJ V(D)J recombination substrates have also been described previously (23) and were obtained from M. Lieber (Washington University, St. Louis, Missouri, USA). An F1 origin of replication was cloned in front of the pTrp promoter of both pCJ and pSJ to facilitate single-strand template isolation for sequencing. pSJ undergoes deletion to retain a signal joint on the plasmid, and pCJ undergoes deletion to retain a coding joint.

Cell culture and transfections. Jurkat (mature T), Nalm-6 (pre B), Molt-4 (immature T), Raji (mature B), KT-1 (pre T), and KE-37 (mature T) cell lines were maintained in RPMI medium supplemented with 10% FBS. For DNA transfections, cells in exponential growth at a density of 1×10^6/ml were used. Five μg of recombination substrate, 6 μg of Rag-1 expression vector, and 4.8 μg of Rag-2 expression vector with or without 5 μg of TdT expression vector were transfected into cells by electroporation. After 48 h, cells were washed with PBS, pelleted, and stored at –70°C.

V(D)J recombination assay. The assay for V(D)J recombination with extrachromosomal plasmid substrates was performed as described previously (24). Plasmid DNA was harvested from transfected cell pellets by the rapid alkaline-SDS lysis method (24) and electroporated into Electromax DH12S competent *Escherichia coli* (GIBCO BRL, Bethesda, Maryland, USA). The transformed bacteria were grown on ampicillin (100 μg/ml) and ampicillin/chloramphenicol (100 μg/ml and 22 μg/ml, respectively) plates. Only plasmids that have undergone deletion will confer resistance to both ampicillin and chloramphenicol, and the ratio of the double-resistant colonies to single-resistant colonies reflects the proportion of DNA rearranged by recombination events in the lymphoid cell lines.

Sequencing of V(D)J recombinants. Clones resistant to both ampicillin and chloramphenicol were selected and grown overnight at 37°C in the presence of helper phage MK107. Single-strand templates were prepared from the supernatant of the overnight culture using an NaI method (25). Single-strand DNA was annealed with primers to the pTrp region of the recombination plasmid, and sequencing reactions were performed in microtiter trays using Sequenase 2.0 (United States Biochemical, Cleveland, Ohio, USA). Reaction products were separated on 6% polyacrylamide gels in TBE (0.9 M Tris, 0.9 M borate, 0.4 mM EDTA).

dNTP pool perturbations. Nalm-6 and Jurkat cells were electroporated as already described. After 24 h recovery at 37°C, cells were left untreated or exposed to either 100 μM dGuo or 3 μM deoxycoformycin (dCF), an ADA inhibitor, for 20 min, followed by 50 μM dAdo, and incubated for 18–24 h. Cells were harvested and assayed for V(D)J recombinants as already described, or extracted with 60% methanol and frozen at –70°C in a 1 ml total volume for dNTP quantitations. Treatment with dCF in the absence of dAdo did not significantly alter recombination frequencies: coding-joint recombination was increased twofold in Jurkat cells and decreased 1.5-fold in Nalm-6 cells; signal-joint formation decreased by 1.7-fold in Jurkat and increased by 1.7-fold in Nalm-6 cells.

Intracellular dNTP quantitations. The quantitation of intracellular dATP and dGTP pools was performed by enzymatic assay using Sequenase (United States Biochemical) enzyme as described previously (26). The reaction mixture contained 0.25 μM annealed template primer specific for dGTP or dATP, 50 mM Tris-HCL (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 2.5 mM [^{3}H]dATP (15 Ci/mmol for dGTP determinations), or 2.5 mM [^{3}H]dTTP (15 Ci/mmol for dATP determinations), and 0.05 units Sequenase (2.0). Extracts prepared from 10^7 cells (5 μl) were then added to 45 μl of the reaction mixture. The reaction was carried out in duplicate at room temperature for 20 min and dried onto Whatman DE81 paper. The filters were washed three times with 5% Na_2HPO_4, rinsed with ethanol (95%), dried, and counted in a scintillation counter.

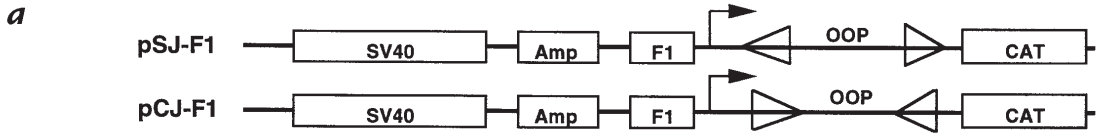
Statistical analysis. Tests for equality of ratios were done using 2 × 2 contingency tables on count data from nucleotides incorporated into the *N* regions of signal or coding joints of the recombination substrates. The Fisher's exact test was used under a two-sided binomial model computed using software developed and kindly provided by R. Berger (North Carolina State University; software available at http://www.stat.ncsu.edu/~berger/tables.html).

B-lymphoblast cell lines. An Epstein-Barr virus–transformed B-lymphoblast cell line derived from peripheral blood of a normal 2-year-old individual (control A) was maintained in RPMI medium supplemented with 10% FBS. Control B represents primary B cells isolated from the peripheral blood of a healthy adult donor. For mRNA isolation, ~10^7 cells were washed with PBS, pelleted, and stored at –70°C. Poly(A)$^+$ mRNA was prepared using RNeasy (QIAGEN Inc., Chatsworth, California, USA). Poly(A)$^+$ mRNA from five ADA-deficient patients (patient no. 1, T.A.; patient no. 2, J.B.; patient no. 3, B.J.; patient no. 4, L.B.; and patient no. 5, B.C.) was the gift of L. Markert (Duke University, Durham, North Carolina, USA). Each of these patients presented with severe ADA deficiency and manifestations of SCID.

5′ RACE and DNA sequence analysis of Ig-μ heavy chain. To analyze *N*-region insertions at the rearranged V_H-D_H-J_H junction of the Ig-μ locus in these cell lines, 5′ RACE was performed following the specifications of GIBCO BRL (Gaithersburg, Maryland, USA). Briefly, 500 ng of mRNA was annealed to 100 ng of gene-specific primer 1 (see later

here) for the human Ig-μ constant region, and first-strand cDNA was synthesized and then dC-tailed. First-strand dC-tailed cDNA was amplified by PCR in the presence of 200 ng of nested gene-specific primer 2 (see later) and 200 ng of 5′ RACE anchor primer (GIBCO BRL) for 30 cycles of hot start PCR using a programmable thermal cycler (Perkin-Elmer, Norwalk, Connecticut, USA): 1.5 min at 94°C, 1.5 min at 55°C, and 2.5 min at 70°C. PCR-amplified products were cloned using the CLONEAMP-pAMP1 system and transformed into Subcloning Efficiency DH5α frozen competent cells (GIBCO BRL). GSP1 5′: GTGATGGAGTCGGGAAGGAAGTC-3′; GSP2 5′: CAUCAUCAUCAUGTATCCGACGGGGAATTCTCACA-3.

The DNA sequences of individual clones were tested for homology to human immunoglobulin gene sequences using the database located at http://www.mrc-cpe.cam.ac.uk/imt-doc/restricted/VSEQ.html. The VSEQ database was separated into V_H, D_H, and J_H sequences, and regions of homology with the input Ig-μ sequences from ADA-deficient patients and controls were determined by comparison against each of the three databases using the Genetics Computer Group (Madison, Wisconsin, USA) program FASTA. Alignment to known Ig sequences is shown in Table 2. The V_H, D_H, or J_H sequence name illustrated in Table 2 corresponds to the first entry in the VSEQ database. Assignment of V, D, or J was listed only after positive alignment by FASTA analysis. Partial alignments were not shown in Table 2. The intervening sequences were listed as *N* regions. The presence of open reading frames in the analysis of Ig-μ sequences was determined by locating nucleotides coding for the conserved residues VYYC[92] in the V region adjacent to the D sequence shown in Table 2. Functional Ig-μ was ascertained by the presence of the same reading frame from V sequence Cys92 through the J region, with no stop codons in N or D sequences.



b

Signal Joints

	N Regions	
GCACTGTG	GTCC	CACAGTGG
GCACTGTG	GGGCCCC	CACAGTGG
GCACTGTG	CC	CACAGTGG
GCACTGTG	GGGGCC	CACAGTGG
GCACTGTG	GCCC	CACAGTGG
GCACTGTG	GGC	CACAGTGG
GCACTGTG	GGACC	CACAGTGG
GCACTGTG	GC	CACAGTGG
GCACTGTG	CCC	CACAGTGG
GCACTGTG	GGGGGCC	CACAGTGG
GCACTGTG	TCC	CACAGTGG
GCACTGTG	GGTC	CACAGTGG
GCACTGTG	GG	CACAGTGG
GCACTGTG	GGGC	CACAGTGG
GCACTGTG	GGTCCC	CACAGTGG

Coding Joints

	N Regions	
GGGTCGA	TCCCC	. . ATCCCC
GGG	CCT	GGATCCCC
GGGTC . . .	TCCAA	. GATCCCC
GGG	GGA	GGATCCCC
GG	CCCC	GGATCCCC
GGGTCGAC	CA	 CCCC
GGG	CTT	 C
GGGTCGAC	ACC	. . ATCCCC
GGGTCG . .	TC	. GATCCCC
GGGT	AG	 CCCC
GGGTCGAC	GTAAG	 CC
GGG	GGT	 CC
GGGTCGAC	CAAGAC	. . . TCCCC
GGG	CCCG	. GATCCCC
GGGT	CAA	 CCC

2X2 CONTINGENCY TABLE

ANALYSIS OF 40 *N* REGIONS

	LEFT HALF	RIGHT HALF
# OF G's	39	7
# OF C's	9	41

2X2 CONTINGENCY TABLE

ANALYSIS OF 37 *N* REGIONS

	LEFT HALF	RIGHT HALF
# OF G's	8	13
# OF C's	25	15

Figure 1

Analysis of TdT-mediated *N*-region insertions at signal and coding joints. (***a***) pSJ-F1 and pCJ-F1 plasmid substrate. (***b***) Fifteen illustrative *N*-region insertion sequences are shown for signal or coding junctions. Nucleotide insertions are shown between the adjacent sequences in the recombined joint. G + C = 75% and A + T = 25% for total number of *N*-region nucleotides analyzed at signal joints. G + C = 67% and A + T = 33% for total number of *N*-region nucleotides analyzed at coding joints. A 2 × 2 contingency table analysis of G–C composition of 40 independent recombinants containing *N*-region insertions is shown. For the purposes of G–C analysis, the *N* region was divided into left and right halves, with the central nucleotide omitted for odd numbers of nucleotides. The *P* value determined for equivalent *N*-region addition to each strand versus no effect, according to the Fisher's exact test, was $P < 0.0001$ for signal joints and $P = 0.105$ for coding joints.

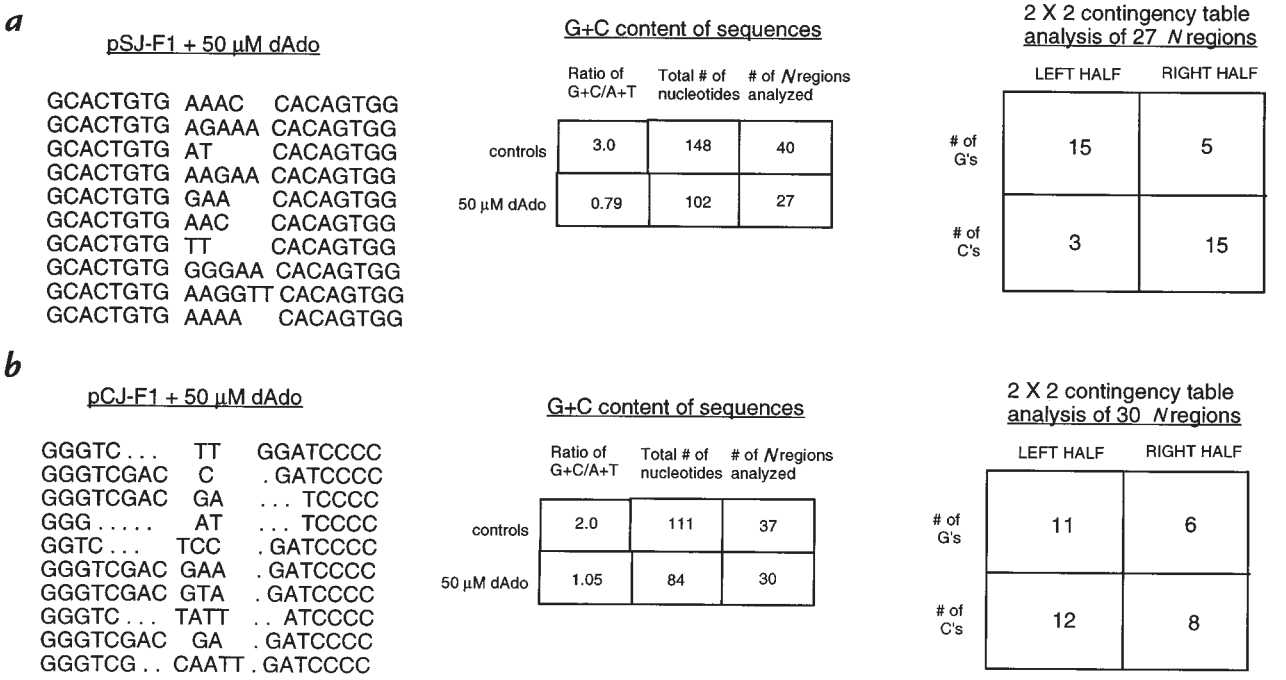


Figure 2

Analysis of V(D)J recombinants from Jurkat cells treated with 2′-deoxyadenosine. T cells transfected with either pSJ-F1 or pCJ-F1 were exposed to 3 μM dCF and 50 μM dAdo for 18 h. Sequences of *N*-region insertions from 10 illustrative recombinants are presented for both signal and coding joints. The G–C content of *N*-region insertions from controls and nucleoside-treated recombinants was determined by dividing the total number of G + C nucleotides by the total number of A + T nucleotides in each *N* region. Comparison of the G + C/A + T ratio from controls with the G + C/A + T ratio from dAdo-treated recombinants was significant at $P < 10^{-4}$ for pSJ-F1 and $P = 0.04$ for pCJ-F1. P nucleotides were present in 12% of the coding junctions. The omission of P nucleotides did not significantly alter the probability calculation. Subtraction of P nucleotides resulted in the following G + C/A + T ratios at *N*-region coding joints: control = 1.9; dAdo-treated = 1.02. A 2 × 2 contingency table analysis of G–C composition of 27 independent recombinants containing *N*-region insertions from signal joints and 30 independent recombinants containing *N*-region insertions from coding joints is presented. The *P* value determined for equivalent *N*-region addition to each strand versus no effect, according to the Fisher's exact test, was $P = 0.0004$ (signal joint) and $P = 1.0$ (coding joint).

Results

Sequence analysis of V(D)J recombinants. To examine the role of TdT in the resolution of the human V(D)J recombination reaction, *N* regions were analyzed at both signal and coding junctions. Jurkat (mature T) or Nalm-6 (pre-B) cell lines were cotransfected with pSJ-F1 (signal joint) or pCJ-F1 (coding joint) recombination substrates (4), RAG-1, RAG-2 (24), and human TdT expression vectors. The RAG expression vectors were not included in transfections of Nalm-6 because these cells have endogenous RAG activity. Two days after transfection, 111 coding and signal-joint recombinants from Jurkat and 102 from Nalm-6 cells were recovered and sequenced. In the presence of the TdT expression construct, *N* regions were observed in >60% of the signal joints and >73% of the coding joints examined from both Jurkat and Nalm-6 cells (Table 1). Nucleotide insertions at both the signal and coding joints were G–C rich and varied in length from one to eight nucleotides. Analysis of *N* regions from 116 total pSJ-F1 V(D)J recombinants revealed insertions that were often composed of homopolymer tracts of G nucleotides followed by tracts of C nucleotides. Analysis of the sequence composition of the *N* regions using a 2 × 2 contingency table revealed a strong bias at the signal joints for insertions of tracts of G nucleotides from the 3′-OH ends of both sides of the junction, suggesting that either end is an equivalent substrate for TdT. This nucleotide addition pattern produced *N* regions with tracts of G nucleotides followed by tracts of C nucleotides (Fig. 1).

In contrast, analysis of *N* regions from 97 coding joints (pCJ-F1 recombinants) demonstrated frequent homopolymer tracts of either G nucleotides or C nucleotides (Fig. 1). Upon resolution, the *N* regions were composed primarily of G nucleotides or C nucleotides. Thus, the results obtained at coding joints differ from those at signal joints, in that only one of two possible coding-joint intermediates appears to be available for TdT addition.

Nucleotide pool perturbations in V(D)J recombinants. To examine the effects of transient perturbations of purine dNTP pools on TdT activity and V(D)J recombination, the transfected cells were exposed to 100 μM dGuo or 50 μM dAdo in the presence of the ADA inhibitor 2′-deoxycoformycin (dCF) for 18–24 hours. The concentrations of dAdo and dGuo used in this study approximate the concentration used to inhibit T-lymphoblast DNA synthesis by 50% at 72 h, as determined previously (27). Incubation of Nalm-6 and Jurkat cells with 100 μM dGuo for 18 hours resulted in a five- to sixfold increase in intracellular dGTP levels (Table 1). Exposing the cells to 50 μM dAdo and an ADA inhibitor resulted in a sixfold increase in the intracellular dATP pools (Table 1).

The frequency of pCJ recombination was reduced an average of 15-fold in T-lineage cell lines exposed to dAdo and was decreased fourfold after exposure to dGuo,

compared with untreated controls (Table 1). The decrease in recombination frequency was apparently not due to generalized cell death, because the initiation of apoptosis, as measured by cleavage of poly(ADP)ribose polymerase, was absent under these conditions (data not shown). Signal-joint recombination in immature T cells was inhibited by an average of 10-fold in the presence of dAdo and twofold after exposure to dGuo (Table 1). Albeit to a lesser extent, CJ or SJ recombination frequency was also reduced in the Raji B-cell line under similar conditions, but not in Nalm-6 pre–B cells (Table 1).

Sequence analysis of signal-joint insertions in 102 recombinants from cells exposed to dGuo and 90 recombinants from cells exposed to dAdo was performed to determine the influence of nucleotide pool perturbations on *N*-region composition. Coding-joint insertions were also analyzed after transfected cells were exposed to dGuo or dAdo. The percentages of clones containing TdT-mediated *N*-region insertions were comparable to those in untreated controls (Table 1), as was the overall number of TdT insertions observed at the coding joints versus the signal joints. TdT-mediated insertions consequent to exposure to dGuo or dAdo were analyzed by 2×2 contingency tables and compared with the untreated controls. In all cases, *N* regions at the signal joints were composed of homopolymeric nucleotide tracts at both sides of the junctions, whereas homopolymeric strings of *N*-region nucleotides were observed at only one side of the coding joint.

The ratio of G + C/A + T in the *N* regions was decreased two- to fourfold after cells were exposed to dAdo (Fig. 2). The ratio was reduced from 3.0 to 0.79 in the signal joints and from 2.0 to 1.0 in the coding joints (Fig. 2). Treatment with dGuo did not significantly alter the ratio of G + C/A + T from control values (Fig. 3) and did not increase the already high percentage of G residues in either the signal or coding joints.

N-region analysis at V_H-D_H-J_H junctions of Ig-μ heavy chains from B cells. To examine the effects of increased intracellular levels of dATP occurring in lymphoid precursors from ADA-deficient children on V(D)J recombination and *N*-region addition in B cells, the rearranged V_H-D_H-J_H regions of Ig-μ heavy chains were amplified using a 5′ RACE protocol with primers in Cμ and the corresponding cDNAs were cloned and transformed into *E. coli.* Colonies were selected at random, and DNA was prepared and sequenced. Unique sequences were selected for further analysis. FASTA sequence alignments were used to determine the splice junctions of the V_H-D_H-J_H region in the amplified clones, and the nucleotide composition of *N* regions from the ADA-deficient patients was directly examined (Table 2).

Five different sequences were analyzed from each of five patients who presented with severe ADA deficiency and SCID and from two normal controls (Table 2). Forty-two of 46 total V_H-D_H and D_H-J_H junctions examined had undergone *N*-region additions. The length of the *N* regions varied from 1 to 11, nucleotides with an average insertion size of 6 bp (Table 2). As summarized in Table 2, analysis of the *N*-region nucleotide compositions revealed a highly significant increase in A–T content in the junctions of ADA-deficient patients, with the ratio of G + C/A + T nucleotides decreasing from 3.2 in controls to 1.1 in ADA-deficient cells ($P < 0.0001$).

V_H sequence was not definitively identified in 11 of 25 sequences in ADA-deficient patients and in nine of 10

Table 1
The effect of nucleoside treatment on the frequency of V(D)J recombination in lymphoblast cell lines

Cell line Plasmid substrate	DA[A]	DAC[B]	R[C]	Fold decrease in R after dAdo or dGuo	No. of sequences analyzed	Percent containing *N* regions	dATP pmol/10^5 cells	dGTP pmol/10^5 cells
Jurkat							7.3 ± 0.4[E]	6.7 ± 0.6[E]
pSJ-F1	547,000	943	0.0017		60	60		
pCJ-F1	85,600	240	0.0028		51	73		
Nalm-6							7.4 ± 1.6[E]	6.8 ± 2.6[E]
pSJ-F1	175,000	271	0.0015		56	61		
pCJ-F1	62,000	200	0.0032		46	76		
Jurkat + 50 μM dAdo[D]							39.6 ± 7.3[E]	7.9 ± 0.6[F]
pSJ-F1	186,000	33	0.00017	10	47	57		
pCJ-F1	169,300	30	0.00018	16	38	79		
Nalm-6 + 50 μM dAdo[D]							44.1 ± 9.2[E]	6.0 ± 1.4[F]
pSJ-F1	256,000	252	0.00098	1.5	43	63		
pCJ-F1	101,600	135	0.0013	2.5	23	74		
Jurkat + 100 μM dGuo[D]							7.7 ± 1.9[F]	36.0 ± 10.3[E]
pSJ-F1	212,000	144	0.00068	2.5	57	65		
pCJ-F1	350,600	129	0.00038	7.4	49	89		
Nalm-6 + 100 μM dGuo[D]							6.8 ± 0.1[F]	31.7 ± 10.2[E]
pSJ-F1	152,900	696	0.0045	0.33	45	82		
pCJ-F1	279,000	732	0.0026	0.9	49	82		

[A]Dpn-I ampicillin-resistant colonies. Values represents the mean of three separate determinations. [B]Dpn-I ampicillin/chloramphenicol–resistant colonies. [C]Recombination frequency measured by the ratio of DAC/DA. R values were also measured in Molt-4, Raji, KT-1, and KE-37. The R values were different for each cell line. The range of signal joint R values was 0.0012–0.0015, and the range for coding joint R was 0.0019–0.0035 in the absence of drug. After dAdo treatment, the range of coding joint R had decreased 8- to 21-fold, signal joint R had decreased 5- to 14-fold in T cells, and Raji B cells had decreased fivefold in coding joint R and sevenfold in signal joint R. In the presence of dGuo, the range of coding joint R had decreased two- to fourfold, and signal joint R had decreased twofold in the T-cell lines, while coding joint R was decreased fivefold and signal joint R was decreased fourfold in Raji B cells. Sequence analysis was not performed in transfection experiments with Molt-4, Raji, KT-1, or KE-37 cells. [D]Treatment with dCF in the absence of dAdo did not significantly alter recombination frequencies: pCJ recombination increased by twofold in Jurkat and decreased by 1.5-fold in Nalm-6, while pSJ recombination decreased 1.7-fold in Jurkat and increased by 1.7-fold in Nalm-6. [E]Intracellular dNTP values represent the mean ± SD from five separate experiments performed in duplicate. [F]Intracellular dNTP values represent the mean ± SD from two separate experiments performed in duplicate.

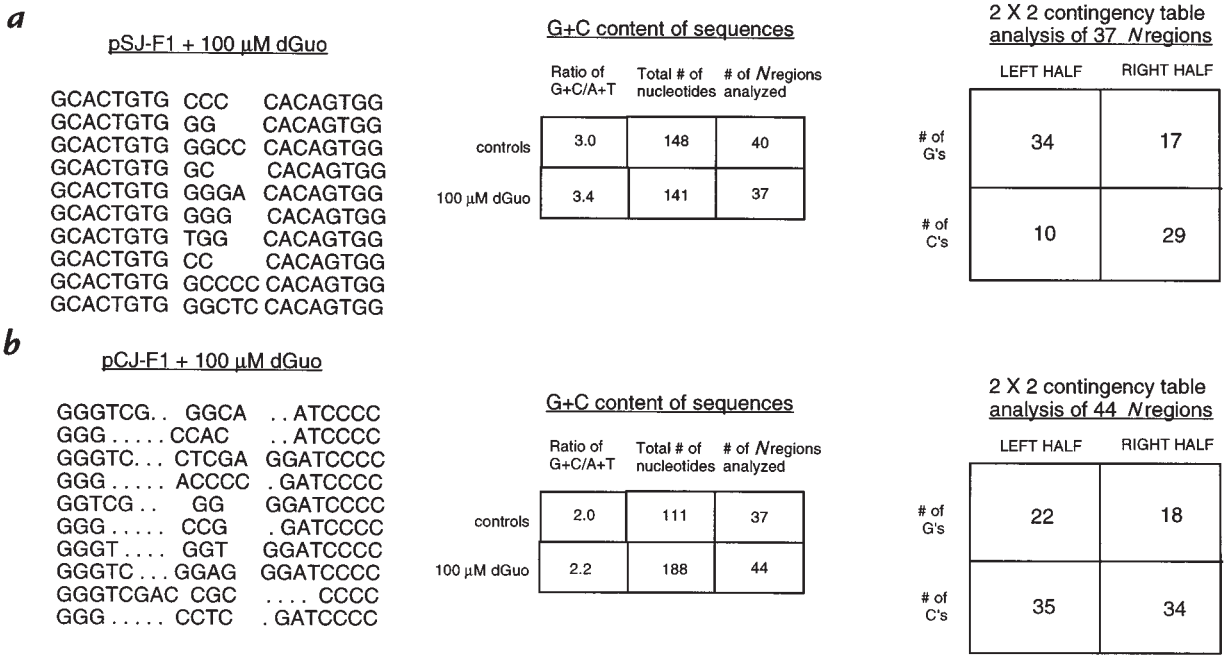


Figure 3

Analysis of V(D)J recombinants from Jurkat cells treated with 2′-deoxyguanosine. T cells transfected with either pSJ-F1 or pCJ-F1 were exposed to 100 μM dGuo for 18 h. Sequences of *N*-region insertions from 10 illustrative recombinants for each are shown. Comparison of the G + C/A + T ratio from controls with the G + C/A + T ratio from dGuo-treated recombinants was not significant for either construct ($P = 0.68$ for pSJ-F1; $P = 0.22$ for pCJ-F1). A 2 × 2 contingency table analysis of G–C composition of 37 independent recombinants containing *N*-region insertions from signal joints, and 44 independent recombinants containing *N*-region insertions from coding joints, is presented. As in Fig. 2, the omission of P nucleotides did not significantly alter the probability calculation. Subtraction of P nucleotides resulted in the following G + C/A + T ratios at *N*-region coding joints: control = 1.9; dGuo treated = 2.1. The *P* value determined for equivalent *N*-region addition to each strand versus no effect, according to the Fisher's exact test, was $P = 0.0001$ (signal joint) and $P = 0.70$ (coding joint).

control sequences either because of the inability to read sequence upstream of D without ambiguity or potentially because of the presence of non-coding Ig-μ transcripts in which the V_H sequence did not produce a good alignment to the sequences in the database. Functional Ig molecules containing open reading frames from V through D and J were conclusively identified in five of 14 sequences from patients and in the one control analyzed.

Discussion

We have examined the contribution of TdT activity to V(D)J recombination. The sequence compositions at both signal and coding junctions were analyzed in a total of 563 recombinant clones. A high frequency of *N*-region insertions was observed at both signal and coding joints after inclusion of the human TdT expression vector in the transfection experiments. The consistent presence of nucleotide insertions at signal joints is most likely due to high levels of TdT activity, as previous studies on murine cells have demonstrated a low frequency of *N*-region insertions at signal junctions in the absence of high levels of endogenous TdT (10). By comparing the sequence composition of the *N* regions inserted at the coding joint with those at the signal joint, we have observed an apparent difference in the polymerase activity of TdT at these two junctions. The nucleotide insertions at the coding joint often contained homopolymer tracts of Gs or Cs. These data strongly suggest the preferential insertion of dGTP by TdT at the 3′-OH DNA end of only one side of the coding junction and are similar to recent findings (28) that *N* regions are derived from a single polymerization product at coding joints during endogenous murine V(D)J recombination. Kepler *et al.* (28) reported that V(D)J joint formation and splice site selection are influenced by TdT-mediated *N*-region additions. The preferential addition of Gs by TdT to a single *N* strand resulted in an embedding of homologous motifs at coding ends that influenced recombination site usage and is hypothesized to account for the disruption of homology-directed rearrangements in normal adult mice (28).

In contrast, we observed that *N* regions at signal joints contained homopolymer-G tracts followed by homopolymer-C tracts. One hypothesis for this result is that extensions of dGTP occurred from both DNA ends. In this case, the activity of TdT at both sides of the junction may have caused a concatenation of nucleotides beginning at the 3′-OH of both strands that were ligated together to form a recombined signal joint. The apparent differential polymerization by TdT at the signal and coding joints may result from the distinct resolution mechanisms required for the formation of the two end products of V(D)J recombination. Recent studies (3, 29, 30) of V(D)J recombination *in vitro* have demonstrated that the earliest steps in the recognition and cleavage of the RSS elements result in the formation of coding-end hairpin intermediates and the release of a 5′-phosphorylated blunt-ended signal fragment. Our data are consistent with these studies in that the signal ends were all full length, indicating a blunt-end

double-strand break intermediate. Sequencing results of *N* regions derived from signal joints reflected the indiscriminant ability of TdT to incorporate dGTP preferentially at any free 3′-OH DNA end.

At coding ends, a greater complexity of proteins is required to resolve the hairpin intermediate, although all the components of this process have not yet been defined (2). Studies from x-ray–sensitive rodent cell lines have demonstrated a requirement for the Ku 70/86, p450, and XRCC4 proteins for the resolution of the coding-joint plasmid substrate (31–34). Our data suggest that only one 3′-OH DNA end is accessible to nucleotide incorporation by TdT and that the strand selection by TdT for *N* insertions is random. One hypothesis is that the hairpin ends are processed in a sequential manner and the presence of a required protein complex for coding junction resolution serves to restrict the access of TdT to the DNA ends at a defined step in the hairpin processing.

Although the frequency of coding-joint formation was similar to previous reports (23), recombination of the signal-joint substrate was five- to 10-fold lower in our experiments. This apparent decrease in the resolution of signal joining may be due to variability between investigators. An alternative explanation is that the signal-joint recombination complex may have been destabilized by high-level TdT expression. However, the same level of TdT activity did not result in a decreased recovery of coding joints. Perhaps the unusual hairpin structure and the rapid joining of coding intermediates (3) had a protective effect on coding-joint recombination.

Table 2
Sequences from V_H-D_H-J_H junctions of ADA-deficient patients and normal controls

	V_H	N	D	N	J_H
Patient					
1					
	1.		D4 TAGTACTACTTTCTATGCCT	TTGGGAAAGTA	TGATGCTTTTGATGTCTGGGGCC JH3a
	2.		D2-2 (inv) ACTACTACAATATCC	CCCCC	CCGTTTGCCC(15 bp)TAGTAGTAGTAGTAAT JH6c
	3.		D3-3 GATTTTTGGACTGGTTATTATACCG	ATA	TATGGACGTCTGGGGGCAAGGGACCAC JH6a
	4.		D3(inv) GGAATAGCTATCACCACCACAATATGCT	ATCCA	ACTGAGGAGACAGTGACCAGGGTG JH2
	5 V3-49(VH) TCTCAAGGAATGGTT	GTAAA	D2-15(inv) GGAGTAGCAGCTACCACCA	AAATCTAAAT	CCCTGGCCCCAGTAGTCAAAGT JH4b(inv)
2					
	1. DP-54(VH) AAGAACTCACTG(39 bp)GTGTATTACTGTGCG	GTTCC	D3-22 GTATTACTATGATAGTAG	CATAA	TTTGACTACTG(24 bp)CTCCTCAG JH4b
	2. DP-47(VH) AGGGGCTGGAGTGGTCTCAGCTATTAG		D2-15 TTGTAGTGGTGGTAGCTGCTACTCC	GTCAAAAC	TCTGGGG(24 bp)CTCCTCAG JH6b
	3. Cos-14(VH)(inv) AGCCCTTGTTAATGGAC	TTGGA	D3-3 (inv) GGTATAATAACCACTCCAAAA	CTTTGCA	AGGAGACGG(21 bp)AGTAGTCAAA JH4a(inv)
	4. DP-35 VH) GGG CTG GAG TGG GTT(138 bp)GCG AGA G		D5-18 TG GAT ACA GCT ATG GT	C	CTT TTG ATG(33 bp)CAG GGA G JH3a(ORF)
	5. DP-34 (VH) ATCTGCAAATGAACCA(24 bp)ATTACTGTGCGAG	A	D5-18 GTGGATACAGCTATGGT	CCAT	TTTTGATGTC(27 bp)TCTTCAG JH3a
3					
	1. 12M28(VH) TAC ATG GAG CTG AGC AGC(30 bp) TGT GCG	GAA AG	D6-13 G GGT ATA GCA GCA GCT GG	C CT	T ACT ACT ACT (43 bp)CTC AG JH6c(ORF)
	2. V3-36P(VH) AGGTCTGTGTCACTGTGGT		D3-3 ATTACGATTTTTGGAGT	TAAACGG	ACTGGTACTTCGATC(15 bp)CCCTGGTCACTG JH2
	3. 4.3(VH) GAGCTCTTGTGACCCGACGG(18 bp)GTCGGAGAGA	GGGTAGCGAT	D3-3 TTTTTGGAGTGGTTATTATACC	CCTT	ACTACTTTGACTACTGG(24 bp)TCCTCAG JH4a
	4.		D4-b(inv) GGCATAGCAGCTG	CATA	TACTACTACTACTACA(21 bp)ACCACGGTCA JH6c
	5.		D4-b GCTACAAGTGCTTGGAGCACTGGGGCAGGGC	CTT	GTACTACTACAA(9 bp)CCGACTAGTACGGGG JHy2
4					
	1.		D3-3 ATTTTTGGAGTGGT	AGCTA	TACTACTACTACGGTATGGACGTCT JH6a
	2.		D4(inv) CATAGCAGCTGGTACTAC	GTT	TACGGTATGGACGTC JH6a
	3.1-1(VH) TGTATTACTGTGCGAGAGAGA	CCCTACGG	D5-18 CAGCTATGGT	C	CTTTTGATATCTGG(18 bp)ACCGTCTCTTCAG JH3b
	4.Cos-19(VH) AGGTCTGTGTCACTGTGGT		D3-3 ATTACGATTTTTGGAGT	TCCAAAG	CTGGTACTTCGA(24 bp)CACTGTCTCCTCAG JH2
	5.1-1(VH) GTG TAT TAC TGT GGC GGA GGA GGA G	GG G	D-3 TA CGA TTT TTG GAG(6 bp) TTA TCC	CCC TTA G	CT ACT TTG (33 bp) CCT CAG G JH4b(ORF)
5					
	1.		D4 AGGATATTGTAGTAGTAC	AAAGG	TACTACTACTACTACAT(34 bp)GTCTCCTCAG JH6c
	2.12M28(VH) AGCCTACATGGAGCTGAGCA(36 bp)GAAAGA	AGAG	D4(inv) GGCATAGCAGCTGG	CCT	TACTACTACTACA(36 bp)CTCCTCAG JH6c(ORF)
	3.		D3-3 TTGGAGTGGTTATTATAC	GGGA	ACTACTACTACTCGTACATGGACGTCTGGTA JH6c
	4. 29II(VH) TCC AAG AAC CAG (48 bp)TAC TGT GCG AGA	GAG GGG	D1-26 TAC GAT TTT TGG (9 bp)TAT CC	C CCT T	AC TAC TTT GAC(33 bp)TCA GGG JH4b(ORF)
	5.		D3-3GTATTACGATTTTTGGAGT	TCCACGG	CTGGTACTTCGATCTCTGGGGCCGTGGCACCC JH2
	V_H	N	D	N	J_H
Control					
A					
	1. DP-35(VH) ACA ACG CCA AGA(57 bp)GTG CGA GAG AG	A C	D5-18 CG TGG ATA CAG CTA TGG T	CC C	TT TGA TAT CTG(27 bp) TTC AG JH3b(ORF)
	2.		D3-16 ATAACTCCCCCAAACGTAATCATAAT	CGAGCG	GATTACTACTACTACTACGGTATGGCAGTC JH6a
	3.		D4-b TAGTACTACTTTCTATGCCG	GGCC	TTACTACTACTACTACG JH6a
	4.		D5-12 GTGGATATAGTGTCTACGATTAC	GGGGCA	TACTGGTACTTCGGTCTCTGGGGC JH2
	5.		D3(inv) GGAATAGCTATCACCACCACAATATGCT	AGCC	CTGGTACTTCG(18 bp)CCCTGGTCACTGTCTCC JH2
B					
	1.		D3-10 GTATTACTATGTTCGGGGAGTTAT	GGGAGGAGT	TGATGCTTTTGAT(24 bp)ACCGTCTCTTCAG JH3a
	2.		D2-2 AGAATATTGTAATAGTACTA	CTGGAG	GGTATGGACGTC(21 bp)ACCGTCTCCTCAG JH6a
	3.		D4-b (inv) GGCATAGCAGCTGGTACTACTACAA	CCT	CGTATGGACGTC(21 bp)ACCGTCTCCTCAG JH6a
	4.		D3-10 GTATTACTATGGTTCGGGGAGTTATT	GGCTGGAGT	TGATGCTTTGATATC(21 bp)ACCGTCTCTTCA JH3b
	5.		D2-2 (inv) AGCTGGTACTACTACAA	CC	GTATGGACGTCTG(15 bp)GGTCACCGTCTC JH6a

Ig-μ V_H, D_H, and J_H sequences with TdT-mediated N nucleotides are shown for patients with ADA deficiency (patients) or controls. GC/AT ratio of controls (3.2) and patients (1.1) were significantly different at $P < 10^{-4}$. Subtraction of P nucleotides did not significantly alter the *N*-region GC/AT ratios. Omission of P nucleotides results in the following *N*-region GC/AT ratios: control: 3.6; patients: 1.0. Open reading frames from V through J were indicated (*ORF*). Sequences were omitted because of space limitations, and the number of bases left out was indicated in parentheses. Inversion of sequences during recombination was noted by (*inv*). Additional details are described in Methods.

In vitro studies (35, 36) of the TdT reaction mechanism have demonstrated that TdT is a nonprocessive polymerase and that there is no preferential incorporation of dGTP compared with dATP onto single-strand DNA substrates. In contrast, the initial rate of dGTP incorporation was much greater than that observed for dATP when a double-strand DNA template was used (37). This result may explain the G–C bias observed in *N* regions *in vivo*. Exposure of the Jurkat and Nalm-6 cells to 50 μM dAdo or 100 μM dGuo resulted in transient perturbations of the intracellular dNTP pools that have been demonstrated to include the reduction of dCTP, in addition to the elevations in dATP, as a consequence of inhibition of the enzyme ribonucleotide reductase (38, 39). Recombinant plasmids from treated cells were sequenced to analyze directly the aggregate effects of these alterations in dNTP pools on coding and signal-joint composition. The frequency of V(D)J recombination was substantially decreased in five lymphoid cell lines treated with dAdo but was considerably less affected in the Nalm-6 cells, despite similar degrees of intracellular dATP pool alterations. Whether these results reflect a differential sensitivity of Nalm-6 cells to changes in dNTP pools is unknown, but the consistent reduction in recombination frequency in other cell lines in the absence of evidence for incipient apoptosis may have implications for the immune dysfunctions associated with ADA and PNP deficiency. In these disorders, it has been proposed (17) that the perturbations in dNTP pools resulting from high levels of circulating 2′-deoxynucleosides that are substrates for these enzymes may trigger apoptosis in immature thymocytes, resulting in T-cell deficiency. In addition, the accumulation of deoxynucleoside metabolites in the immature lymphocytes of these patients is thought to interfere with DNA recombination and/or repair enzymes required for normal lymphoid differentiation (reviewed in refs. 17, 20, and 21). Whether a similar mechanism contributes to the prolonged immunodeficiency status of patients treated with ADA inhibitors such as 2′-deoxycoformycin remains a matter of speculation.

The *N* regions recovered from both Jurkat and Nalm-6 cells treated with dAdo were A–T rich. The implications of these results are twofold. First, TdT activity is influenced by the intracellular milieu of dNTP pools, and the effect of an increased concentration of dATP, possibly in combination with decreases in dCTP levels, is to increase the number of dAMP residues inserted. The effects of loss of G–C bias at *N*-region insertions are unknown but could potentially influence lymphoid development as well as the diversity of immunologic responses. Second, the decrease in the frequencies of V(D)J recombination in the lymphoblast cell lines after exposure to dAdo may reflect a destabilization of the resolution complex. Alternatively, the recombination substrates may have acquired a tightly packaged chromatin configuration inaccessible to the V(D)J recombinase (1) after DNA replication was inhibited by a high intracellular level of dATP (27).

Our results also indicate that elevations of dATP influence the composition of *N*-region insertions at sites of V(D)J recombination in ADA-deficient patients, with *N* regions from five patients containing 49% A–T nucleotides versus 24% in our controls. These data are given added meaning by the high G–C content of *N* regions generally acknowledged to exist in normal B cells (1). These results indicate that the widely recognized preference for the incorporation of dGTP by TdT at the recombination junction is altered by the increased dATP nucleotide pools during the development of B cells in ADA-deficient patients. The finding of a decrease in recombination frequency in established lymphoblast cell lines due to high levels of dATP suggests that a similar effect may occur in ADA-deficient patients and result in a decrease in the production of functional Ig.

Indeed, there is clinical evidence for defective antibody responses independent of the reduction in the B-cell number in ADA-deficient patients. Antibody responses to repetitive challenges with bacteriophage ϕX174 were measured in 10 ADA-deficient patients to assess B-cell function before and after polyethylene glycol (PEG)-ADA enzyme replacement therapy (40). Before replacement therapy, all patients had depressed responses to the bacteriophage as characterized by low titers of antibodies that failed to undergo normal isotype switching from IgM to IgG in response to secondary challenges. Patients who received the PEG-ADA treatment demonstrated a reduction in dAdo and dATP levels and had improved B-cell recovery compared with patients treated with bone marrow transplantation, indicating that antigen recognition and B-cell differentiation into Ig-secreting plasma cells were rescued with enzyme replacement therapy.

Nine of the 14 evaluable Ig sequences recovered from ADA-deficient individuals in our study were out of frame. Although these data are not sufficient to demonstrate that the dNTP pool alterations induce dysfunctional Ig transcripts, it is possible to speculate that the increases of intracellular dATP resulting from ADA deficiency may inhibit B-cell function by interfering at the molecular level with the V(D)J recombination event, resulting in decreased numbers of functional Ig receptors. This hypothesis will be further explored by analyzing the rearrangements at this locus in cells derived from ADA-deficient patients before and after enzyme replacement therapy.

Interestingly, TdT knockout mice have normal B- and T-cell development, indicating that an absence of TdT activity does not cause the SCID phenotype found in patients lacking ADA. However, the significant reduction in recombination frequency due to increased dATP pools that we have observed in lymphoblast cell lines, in conjunction with altered TdT-mediated *N* regions, may contribute to the immunodeficiency. In contrast to the effects of dATP, increases in the dGTP pool of a similar magnitude did not further increase the G–C content of *N* regions. PNP-deficient individuals have an increased incidence of autoimmune disorders (reviewed in ref. 17), but in general, they do not have the reduced level of B-cell function found in ADA deficiency. Although lymphoblast cell lines from PNP-deficient patients have not been examined, the presumed lack of alteration in *N*-region base composition is consistent with the normal B-cell maturation in the majority of these individuals.

In conclusion, our results are in agreement with data from Kepler *et al*. (28) and are consistent with the hypothesis of Gauss and Lieber (41), both of whom support a potential role for TdT in V(D)J recombination that is not random

and could constrain junctional diversity. The ability of alterations in intracellular dNTP pools to contribute to variability in *N*-region composition clearly could effect junctional diversity and may contribute to defects of lymphoid development in immunodeficiency disorders associated with altered dNTP pools.

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