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# Rearrangements of DNA mediated by terminal transferase

**(immunoglobulin diversity/fidelity/DNA replication/DNA polymerase/antibodies)**

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ABSTRACT **To assess the involvement of terminal transferase in generating immunoglobulin diversity, the mutagenic potential of this enzyme has been measured. The frequency of single base substitutions during copying of $\phi$X174 DNA by DNA polymerase $\beta$ is increased by, at most, 3-fold upon the addition of terminal transferase. However, terminal transferase is highly mutagenic, either alone or with DNA polymerase $\beta$, in a forward mutation assay using M13mp2 DNA. The frequency of complex mutants, as determined by DNA sequence, is increased by greater than 100-fold. These mutants involve the deletion of a variable number of bases initially present in the template sequence and the addition of a sequence of nucleotides rich in guanine residues. Analysis of these mutants suggests an antibody diversity model implicating terminal transferase in the imprecise linkage of variable, joining, and diversity segments during the formation of functional immunoglobulin genes.**

Terminal deoxynucleotidyltransferase (EC 2.7.7.31, TdT) catalyzes the polymerization of deoxynucleoside triphosphates onto the 3'-OH terminus of a polydeoxynucleotide without template instruction. The involvement of TdT in generating immunological diversity has been suggested from consideration of both its catalytic properties and its restricted tissue distribution (1, 2). TdT is found predominantly, if not exclusively, in cortical thymocytes and primitive bone marrow cells and in peripheral blood lymphocytes in certain forms of acute leukemia (3). Baltimore (1) formulated a model for the generation of somatic mutations in the variable (V) region of immunoglobulin genes by TdT. A single-strand scission in the V region is made by a site-specific endonuclease and further hydrolyzed by exonuclease, then TdT inserts noncomplementary nucleotides during repair synthesis by DNA polymerase. This model accounts for the introduction of somatic mutations only during the early course of B-cell differentiation since only at that time is TdT expressed (4).

The V regions of both the heavy and light immunoglobulin chains are encoded in multiple germ-line DNA segments that are rearranged combinatorially to form functional V region genes (5–7). Alt and Baltimore (8) proposed that, during antibody gene rearrangement, TdT incorporates additional nucleotides, the N region, at the V-D and D-J junctions in the heavy chain (where D stands for diversity and J stands for joining). These N regions consist of one to seven contiguous nucleotides, rich in guanine residues, which do not appear to be present in germ-line gene segments (4, 9). In different transformed lymphoblastoid cell lines, the presence of these short nucleotide insertions correlates with the presence of TdT (4).

We have examined the mutagenic potential of TdT in different *in vitro* DNA synthesizing systems. TdT is highly mutagenic, producing a striking increase in the frequency of complex mutations. From an analysis of the DNA sequences in a collection of these complex mutants we have postulated a mechanism for their formation and have extended this model to account for the role of TdT in generating immunological diversity.

## MATERIALS AND METHODS

**Enzymes.** Homogeneous TdT was purified (10) and generously provided by M. Modak (Memorial Sloan–Kettering Cancer Center, New York) and by F. Bollum (Uniformed Services University of the Health Sciences, Bethesda, MD). DNA polymerase $\beta$ (pol $\beta$) was provided from the following sources: Novikoff rat hepatoma, homogeneous fraction VI (11) by D. Mosbaugh (University of Texas, Austin); chicken embryo, homogeneous fraction VIII (12) by A. Matsukage (Aichi Cancer Center, Nagoya, Japan).

**$\phi$X174 Assay.** The fidelity assay used $\phi$X174 *am3* DNA (13–15) and an oligonucleotide primer (16). The template was a single strand circular $\phi$X174 DNA containing a TAG amber codon (amber 3 in gene E) in place of the TGG wild-type codon. Synthesis was initiated at the 3'-OH primer terminus, which was three nucleotides away from the *am3* site. The accuracy of *in vitro* DNA synthesis was quantitated by transfecting the copied DNA into *Escherichia coli* spheroplasts and measuring the titer of the resultant progeny phage on permissive and nonpermissive indicator bacteria.

**M13mp2 Assay.** The fidelity assay using M13mp2 DNA was that of Kunkel (17). The general outline was to construct a gapped double-stranded circular molecule in which the gap contains a *lacZ* $\alpha$ gene target sequence that was filled *in vitro* by DNA synthesis using DNA polymerase. A portion of the product was then analyzed to monitor synthesis, and the remainder was used to infect cells to score $\alpha$-complementation mutants. The errors presented in each mutant were determined by DNA sequence.

## RESULTS

**Measurements of Single-Base Substitutions in $\phi$X174 DNA.** To assess the ability of TdT to promote single-base substitutions in natural DNA templates, we first utilized the $\phi$X174 fidelity system (13). $\phi$X174 single-stranded circular DNA containing an amber mutation was primed with a synthetic oligonucleotide and copied by DNA polymerase *in vitro* (16). Each time the amber mutation was copied, there was a possibility of inserting a noncomplementary nucleotide that could revert the amber mutant to wild type. The copied DNA was used to infect *E. coli* spheroplasts, and these spheroplasts were then plated with indicator bacteria either permissive or nonpermissive for the amber mutation. The reversion frequency of the amber mutation thus reflected the fidelity of DNA synthesis *in vitro*. The $\phi$X fidelity system is an exceptionally sensitive assay for single-base substitution errors. When using the amber 3 mutation in the gene *D/E* overlap, the assay measures only single-base errors at posi-

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Abbreviations: TdT, terminal deoxynucleotidyltransferase; pol, polymerase; V, variable; J, joining; D, diversity.

tion 587. DNA polymerase β was chosen for the following reasons. The mechanisms of catalysis by both pol β and TdT are distributive (18); i.e., they release from the template after each nucleotide addition step. Thus, TdT should be able to effectively compete with pol β for adding nucleotides onto the free 3′-OH termini. Once nontemplate-directed incorporation by TdT has occurred, pol β, having no associated 3′ exonuclease (19) with which to excise errors, has a reasonable probability of continuing synthesis due to its relaxed primer requirements (20, 21).

Consistent with reports (22), pol β alone is error prone, producing single-base substitution errors at high frequency. Most importantly, in a large number of experiments, only a slight enhancement in reversion frequency was observed upon addition of TdT at any concentration examined; and, in fact, the reversion frequency was decreased in the presence of a large excess of TdT (Table 1). This decrease in reversion frequency occurred when synthesis proceeded past the amber mutation. Thus, TdT could interfere with the expression of the phenotype of the newly synthesized strand, perhaps by producing errors other than single-base changes.

**Enhanced Mutagenesis by TdT.** To assess the spectrum of mutations produced by TdT, we used a forward mutation assay (17). *In vitro* errors produced during synthesis within a single-stranded gap containing the *lacZα* gene carried in M13mp2 DNA are detected by loss of α-complementation, when the product of the *in vitro* reaction is used to transfect competent cells. Mutant plaques are detected as colorless or light blue. This assay is capable of detecting a wide variety of base substitutions, frameshift, deletion, and complex errors, since errors are produced in a gene that is nonessential for phage production. Four to six percent of the *lacZα* sequences copied by pol β alone result in decreased α-complementation. Thirty to fifty percent of these are single-base substitutions; one noncomplementary base substitution error is incorporated for every 1500 bases polymerized (23). The second major class of pol β mutations are single-base frameshifts. A small number of deletions, mostly between direct repeats, is also observed. This spectrum of errors is reflected in the mutation frequencies for DNA copied by pol β from rat hepatoma (5.7%) and chicken embryo (3.3%) (Table 2).

The addition of TdT to the pol β copying reactions increases the frequency of mutant production in a concentration-dependent manner to as high as 44%. This enhancement in mutagenesis is eliminated if the TdT is heat inactivated. Unexpectedly, nucleotide incorporation by TdT in the absence of pol β also results in a 50-fold increase in mutant frequency over that observed with uncopied gapped DNA. The majority of mutants (>90%) produced by TdT alone or together with pol β, were colorless rather than the light blue phenotype characteristic of most base substitutions. This implies that other types of errors predominate.

The 5-fold increase in mutant frequency (from 5.7 to 26%, Table 2), due to the addition of TdT, suggests that the majority of the mutants analyzed resulted from TdT-dependent errors. DNA sequence analysis was performed on the DNA of 22 independent mutants in reactions catalyzed by rat hepatoma pol β in the presence of 7.5 units of TdT. Among the light blue mutants sequenced, five were single-base substitutions, and five were single-base frameshifts. These limited sequence data suggest, at most, a 2-fold increase in single-base substitutions and frameshifts by TdT (see legend to Table 3). The remaining 12 mutants were complex and different (Table 3); however, common features were apparent. In each mutant, a number of bases were deleted, and new bases were added presumably by TdT. No mutations of this type were observed among the collection of 296 rat pol β mutants described (17), which calculates to a 100-fold increase in complex mutations, clearly suggesting that they are indeed produced by TdT. In the 12 complex mutants, the sequence of new additions begins at 10 different nucleotide positions, which are dispersed over a more than 100 nucleotide distance (Table 3), suggesting that TdT competes effectively with pol β at many different positions. In 9 of the 12 instances, the new sequences added were shorter, resulting in a net loss of from 1 to 233 bases in each mutant. The added sequences were 1 to 26 bases in length and rich in guanine (in the newly synthesized strand). Of the total 84 new bases identified in these 12 complex mutants, the frequency of bases incorporated was 55% guanine, 23% thymine, 12% adenine, and 11% cytosine. In 11 of the 12 mutants, an adenine or thymine was the last base of known sequence in the newly synthesized minus strand. In all of these mutants, no other base substitutions were observed. Thus, the major

Table 1. Reversion frequency of φX174 *am3* DNA copied by DNA pol β with and without TdT

| Addition | | Nucleotides per template | Reversion frequency ($\times 10^{-6}$) |
|---|---|---|---|
| None | | 2 | 5 |
| Pol β only | | 37 | 78 |
| Pol β + TdT | (2 units) | 39 | 138 |
| | (10 units) | 62 | 39 |
| | (20 units) | 46 | 32 |
| | (50 units) | 58 | 18 |
| | (100 units) | 38 | 7 |

Reactions and transfections were performed as described (21) using DNA pol β from rat hepatoma. φX174 *am3* DNA was hybridized with a 5-fold molar excess of an oligonucleotide primer whose 3′-OH terminus was three nucleotides from the amber site. After removal of the unhybridized primer by gel filtration on Sephadex G-100, the primer-template complex was copied by DNA pol β in the presence of various amounts of TdT. Each reaction in a final volume of 0.025 ml contained 20 mM Tris·HCl (pH 8.4), 2 mM dithiothreitol, 0.1 M NaCl, 1.5 mM $MnCl_2$, 100 μM each of dATP, dCTP, dGTP, and [α-$^{32}$P]dGTP (800 cpm/pmol), 0.04 unit DNA pol β, and the indicated amounts of TdT. Incubation was for 15 min at 30°C. Nucleotides per template are the average number of nucleotides added to each primer terminus based on incorporation data.

Table 2. Mutagenicity of TdT during gap-filling DNA synthesis of M13mp2 DNA *in vitro* by DNA pol β

| DNA polymerase | TdT, units | Total pfu | Mutant pfu | Mutant frequency, % |
|---|---|---|---|---|
| Rat hepatoma | | | | |
| DNA pol β | 0 | 3074 | 176 | 5.7 |
| | 3 | 1903 | 305 | 16.0 |
| | 7.5 | 1713 | 437 | 26.0 |
| | 15 | 2353 | 595 | 25.0 |
| Chicken embryo | | | | |
| DNA pol β | 0 | 3304 | 110 | 3.3 |
| | 3 | 2476 | 209 | 8.4 |
| | 7.5 | 930 | 410 | 44.0 |
| | 7.5* | 2374 | 72 | 3.0 |
| None | 0 | 1592 | 3 | 0.19 |
| | 7.5 | 974 | 100 | 10.0 |

DNA synthesis was carried out in a final volume of 0.05 ml containing 20 mM Hepes (pH 7.8), 2 mM dithiothreitol, 10 mM $MgCl_2$, 500 μM each of dATP, dGTP, dCTP, and [α-$^{32}$P]dTTP (500 to 1000 cpm/pmol), 300 ng gapped circular M13mp2 DNA, 0.4 unit of rat hepatoma or 0.8 unit chicken embryo DNA pol β and the indicated amount of TdT. Incubation was at 37°C for 60 min, and transfections were performed as described (17). pfu, plaque forming units.
*TdT was heat inactivated at 100°C for 5 min before addition to reaction.

Table 3. DNA sequence analysis of complex mutations generated by TdT and DNA pol β

| Mutant | Normal sequence deleted: Bases lost, no. | Normal sequence deleted: Position in target* | Random bases incorporated: Bases, no. | Random bases incorporated: Base composition† (minus strand, 5′→3′) |
|---|---|---|---|---|
| 1 | 11 | 128–138 | 1 | (A) G |
| 2 | 6 | 98–103 | 1 | (A) G |
| 3 | 4 | 82– 85 | 2 | (G) GG |
| 4 | 7 | 131–137 | 3 | (A) GAG |
| 5 | 4 | 46– 49 | 4 | (A) GCGC |
| 6 | 5 | 82– 86 | 4 | (A) TGGG |
| 7 | 238 | −154– 84 | 5 | (T) GTGGG |
| 8 | 27 | 85–111 | 6 | (A) TGGAGG |
| 9 | 7 | 131–137 | 10 | (A) GGAGCTTGGG |
| 10 | 14 | 125–138 | 10 | (A) GGGTCGGTGG |
| 11‡ | 12 | 55– 66 | 12 | (A) TAGTTTGGGATC |
| 12 | 61 | −36– 25 | 26 | (T) CTAGGATGGATAGATCTG-TCTGCGGG |

The less than 2-fold increase in base substitution and frameshift errors by TdT was calculated from the mutation frequency and the proportion of light blue and colorless mutants.

*The positions within the mutational target for the bases known to be missing are numbered relative to the first transcribed base, which is position +1.

†The base composition of the newly incorporated bases (in the minus strand) is shown. The base in parentheses is the last base of known correct sequence and is, therefore, presumably the primer for random addition by TdT.

‡This mutant also contained a −1 base frameshift, the loss of a single thymine in a run.

mutagenic effect resulting from the addition of TdT to reactions catalyzed by pol β is the production of complex mutants; these involve deletions of variable segments of the template and the concomitant incorporation of noncomplementary nucleotides into the newly synthesized DNA strand.

**TdT Produces Complex Mutations in the Absence of pol β.** The DNA sequences of mutants produced by the *in vitro* action of TdT alone are tabulated in Table 4. Further chain elongation is presumably catalyzed by a DNA polymerase in the *E. coli* spheroplast. In all cases, the mutants are complex, containing deletions of from 5 to 349 bases, which are replaced by new sequences, 5 to 31 bases in length. With only one exception (mutant 4), the new bases begin immediately after the 3′-OH terminus provided in the gapped molecule (a thymine residue at position 175). Again, the new bases added are rich in deoxyguanosine residues (68% guanine, 12% thymine, 14% adenine, and 6% cytosine). In all the mutants, the template sequences copied after the deletion of known bases are rich in cytosine residues. This would allow a stable primer to be formed between guanine (new strand) and cytosine (template) residues, for further synthesis *in vivo* to fix the complex mutation. If this is the case, the number of nucleotides added by TdT is actually greater than that depicted in Table 4. If we arbitrarily designate the resumption of known sequence with two or more consecutive G·C base pairs and then consider only the next 10 base pairs, five single-base mutations are present in the four mutants. In contrast, no such errors are seen in these mutants at sites farther from the junction, or in any of the mutants shown in Table 3, totaling over 2000 nucleotides.

Table 4. DNA sequence analysis of mutants produced by TdT during *in vitro* DNA synthesis in the absence of DNA polymerase

| Mutant | Normal bases deleted: Bases lost, no. | Normal bases deleted: Position in target* | Random bases incorporated: Bases, no. | Random bases incorporated: Base composition† (minus strand, 5′→3′) |
|---|---|---|---|---|
| 1 | 6 | 169–174 | 5 | (T) GGGGT |
| 2 | 6 | 169–174 | 5 | (T) GGGGT |
| 3 | 6 | 169–174 | 5 | (T) GGTGT |
| 4 | 5 | 169–173 | 6 | (C) GGGAGG |
| 5 | 6 | 169–174 | 7 | (T) TGGGAGG |
| 6 | 323 | −149–174 | 8 | (T) GGTGTATG |
| 7 | 31 | 144–174 | 21 | (T) GTGGAGCGCGGGG-CATCGAGA |
| 8 | 323 | −149–174 | 27 | (T) GGGGGAGGGGAGGG-GGCGGGATGGGGA |
| 9 | 349 | −175–174 | 31 | (T) GTCAGGGTGGAGAGGG-GGGGTCGGAGGGGAG |

The sequence indicates that TdT inserted a complementary deoxyguanosine as the first nucleotide inserted, except in mutant 5. Mutants 1 and 2 have identical sequences, but are of independent origin. Additional substitutions in the newly synthesized minus strand were observed in mutants 6 (A → G at −156), 7 (A → G at 137), 8 (A → G at −154), and 9 (A → G at −179).

*The positions within the mutational target for the bases known to be missing are numbered relative to the first transcribed base, which is position +1.

†The base composition of the newly incorporated bases (in the minus strand) is shown. The base in parentheses is the last base of known correct sequence and is, therefore, presumably the primer for random addition by TdT.

## DISCUSSION

The simplest concept for mutagenesis by TdT, and the one that initiated these studies, would involve the nontemplate-directed incorporation of single base substitutions each time TdT substituted for DNA polymerase at the growing primer terminus. Our results do not provide strong support for this concept. With the $\phi$X system, in a large number of experiments with different DNA polymerases the largest increase in reversion frequency observed by the addition of TdT was at most 3-fold. Similarly, in the M13mp2 assay, at most a 2-fold increase in single base substitutions was observed. In these model *in vitro* systems, we have varied the ratio of TdT to pol $\beta$ to maximize the addition of single noncomplementary nucleotides by TdT. Thus, the small increase in single-base substitutions we observed may be of little significance in cells where the ratio of TdT to polymerase is not optimized. Furthermore, with respect to immunoglobulin genes, TdT appears to be expressed in assayable amounts only early in the course of B-cell differentiation (4) and single-base mutations are likely to also accumulate at later times (24, 25).

A mechanism, for the rearrangement of DNA mediated by TdT by using M13 as a template, is suggested by the nucleotide sequence of the complex mutants (Fig. 1). This mechanism accounts for (*i*) large deletions resulting from the joining of two distant nucleotide sequences, (*ii*) the insertion of new sequences that are deoxyguanosine-rich, as well as (*iii*) additional neighboring single-base substitutions. Step 1 involves dissociation of the DNA polymerase from the primer-template DNA. This provides a free 3′-OH primer terminus for nontemplate instructed additions by TdT. The competition between pol $\beta$ and TdT is apparent from the multiple positions within the mutational target where complex mutants are observed. Nucleotides at 10 different positions were used to initiate the additions by TdT (Table 3); in 11 of 12 mutants, these occur at A·T or T·A base pairs. This suggests that TdT has a greater probability of effectively competing with a DNA polymerase when the 3′-OH primer terminus can "breathe." The predominance of deoxyguanosine residues in the added nucleotide segment in the mutants is in accord with the catalytic properties of TdT (27) (Fig. 1, step 2). Among the mutants generated, the number of new nucleotides added varied from 1 to 31. To provide a functional primer stem for subsequent elongation following addition of noncomplementary nucleotides by TdT, we postulate reformation of the primer-template complex by imperfect hybridization (Fig. 1, step 3); the added nucleotides search for and hydrogen bond to complementary sequences on a template strand. The preference for guanine incorporation by TdT predicts that the deletion end points would occur at cytosine-rich template sequences, as observed. The length of deletions suggests that this process can occur *in vitro* over a distance of at least several hundred nucleotides. The fact that such a primer is functional in the *E. coli* cells in the transfection experiments with the product of the TdT-only reactions (Table 4) was unexpected. Furthermore, whichever enzyme in *E. coli* is performing the elongation steps apparently has more stringent (i.e., longer) primer stem requirements than does pol $\beta$, where none of the complex mutants show additional base substitutions. After extensive template-

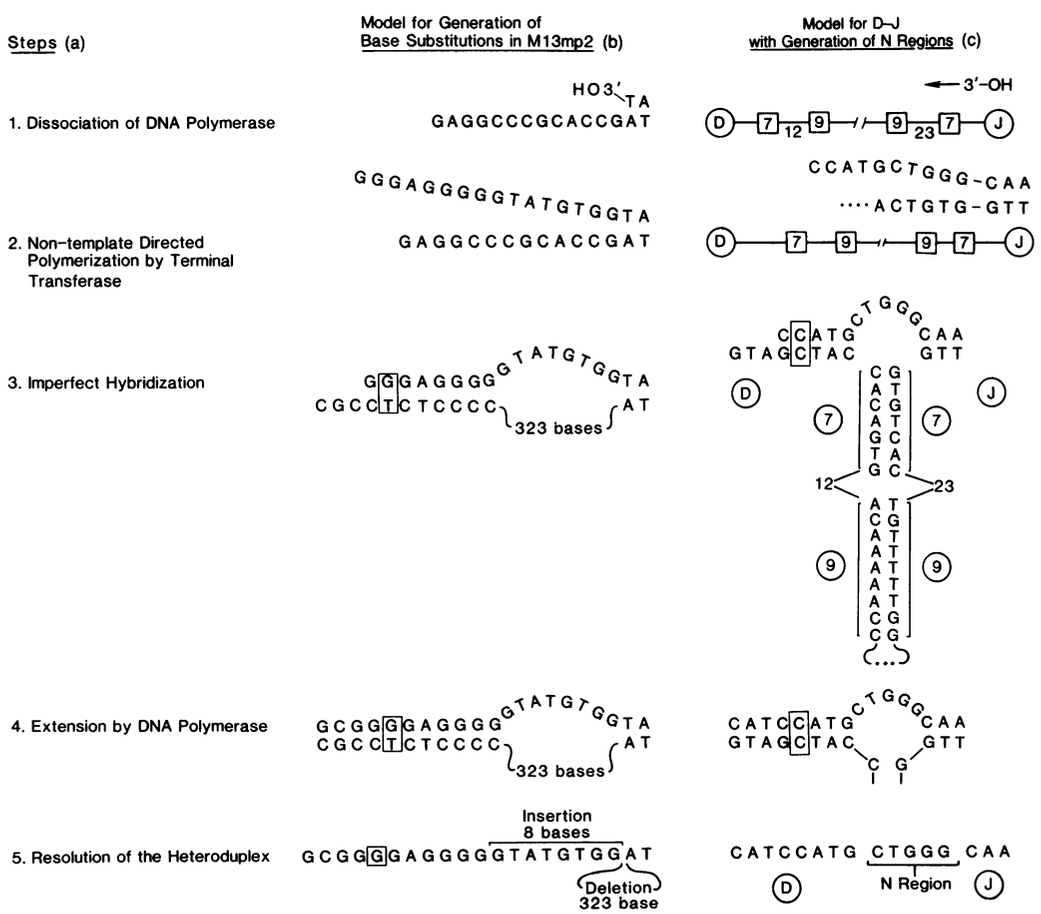


FIG. 1. Scheme for role of TdT in the generation of N regions and DNA rearrangements. The M13mp2 is mutant 6 in Table 4. The sequence for D region and J region in *c* is MC101, which corresponds to the germline D sequence DFL16.1 and is that determined by Kurosawa and Tonegawa (9). The J region segment is that given in Sakano *et al.* (7) and figure 4 in Honjo *et al.* (26). Synthesis was considered to be from J to D based on the possibility that imperfect hybridization might be a significant contributor to the high frequency of single-base substitutions in the D region. An analogous mechanism can be formulated for V-J joining, including the addition of N regions.

directed synthesis by DNA polymerase (Fig. 1, step 4), the heteroduplex is resolved into the newly synthesized and parental strands (Fig. 1, step 5). The newly synthesized DNA strand contains both a deletion of the template strand and the addition of a segment of nucleotides not complementary to those in the template. The parental strand contains the nonrearranged original sequence.

We speculate that this mechanism for generating complex rearrangements could be central to the mechanism for joining heavy chain gene segments, and could be relevant to recombinational processes involving the assembly of the $\beta$ polypeptide of the T-cell antigen receptor (28), as well as immunoglobulin light chains (29). In Fig. 1*c*, we diagrammed the steps that might be responsible for the joining of the D-J segments of heavy chain genes. The position for joining the segments would be determined by the inverted complementary structures of the heptamer and nonamer sequences, presumably stabilized by specific DNA binding proteins (30). A role for palindromic sequences in determining the end points of large deletions has been demonstrated (31). The nucleotides added by TdT would constitute the N region as originally proposed by Alt and Baltimore (8). Although pol $\beta$ was used in our *in vitro* experiments, it is equally possible that pol $\alpha$ could be involved in this process *in vivo*. The fact that TdT incorporates nontemplate-directed nucleotides rich in guanine residues argues against the concept that N regions are coded for by multiple undescribed D segments (32). In preliminary experiments using M13mp7 as a template, we have observed that TdT facilitates DNA polymerization across the base of a stem-loop region by inserting nontemplate coded nucleotides (B. Zelus and L.A.L., unpublished results). Reformation of a template·primer complex prior to continued polymerization could occur by imperfect hybridization. The length of the N region would be governed by statistical considerations with respect to formation of a functional primer·template. In the simplest form, resolution of the heteroduplex would generate one daughter strand containing the N region and a large deletion; the parental strand could remain intact or be processed to delete the germ-line genes. If synthesis is from J to D, as shown in Fig. 1, then imperfect hybridization could account for the high frequency of single-base substitutions observed in D regions (9, 33). Imperfect hybridization as a source of multiple base mutations can explain why silent mutations are linked with selectable missense single-base changes (34) and why base changes are clustered (35). Such a mechanism could also account for the hypermutation observed in a pre-B cell line (36). It is important to note that this model does not require crossing-over or reciprocal recombination; indeed, reciprocal structures have rarely been found in the case of the $\lambda$ or heavy chain genes (8). In its strictest interpretation, this model is not applicable to rejoining of $\kappa$ genes, since the latter may involve reciprocal structures (29).

The model for producing gene rearrangements and somatic mutations on the basis of imperfect hybridization was formulated in consideration of the involvement of TdT in the generation of antibody diversity. In a sense, we have postulated that TdT functions as a "recombinase." However, similar mechanisms for gene rearrangements can be postulated even in the absence of nontemplate-directed polymerization by TdT. Any mechanism that generates breathing of DNA with extensive single-stranded regions would permit imperfect hybridization during DNA replication, leading to extensive deletions. A key identification for such a mechanism is gene rearrangements in the absence of duplications and reciprocal structures.

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