DNA Polymerase Fidelity: From Genetics Toward a Biochemical Understanding

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ABSTRACT

This review summarizes mutagenesis studies, emphasizing the use of bacteriophage T4 mutator and antimutator strains. Early genetic studies on T4 identified mutator and antimutator variants of DNA polymerase that, in turn, stimulated the development of model systems for the study of DNA polymerase fidelity *in vitro*. Later enzymatic studies using purified T4 mutator and antimutator polymerases were essential in elucidating mechanisms of base selection and exonuclease proofreading. In both cases, the base analogue 2-aminopurine (2AP) proved tremendously useful—first as a mutagen *in vivo* and then as a probe of DNA polymerase fidelity *in vitro*. Investigations into mechanisms of DNA polymerase fidelity inspired theoretical models that, in turn, called for kinetic and thermodynamic analyses. Thus, the field of DNA synthesis fidelity has grown from many directions: genetics, enzymology, kinetics, physical biochemistry, and thermodynamics, and today the interplay continues. The relative contributions of hydrogen bonding and base stacking to the accuracy of DNA synthesis are beginning to be deciphered. For the future, the main challenges lie in understanding the origins of mutational hot and cold spots.

THE development of molecular biology has been profoundly influenced by genetic and biochemical studies using the bacteriophage T4. In particular, T4 has served as an invaluable tool for testing new ideas and refining concepts of mutagenesis and DNA polymerase fidelity. Through his studies on T4 mutagenesis, Jan Drake, to whom this issue of Genetics is dedicated, played a central role in initiating the remarkably fertile area of research into the biochemistry of fidelity.

In 1968, Drake reported the surprising discovery of antimutagenic T4 polymerase mutants (Drake and Allen 1968). Until then, mutations in the structural gene coding for the T4 polymerase, gene *43* (de Waard *et al.* 1965), had only been reported to generate mutator phenotypes (Speyer 1965; Speyer *et al.* 1966; Freese and Freese 1967). The notion that a "defective" (*i.e.*, mutant) polymerase might replicate DNA with higher fidelity than the wild-type was revolutionary.

Reversion frequencies in the nonessential *rII* region of T4, used by Seymour Benzer in his classic studies on genetic fine structure (Benzer 1961), were the phenotype of choice for determining the effects of mutations in the T4 pol gene. The various T4 mutant polymerases exhibited very different mutation rates. While the effect depended somewhat on which *rII* reversion was investigated, for several of the *rII* alleles reversion frequencies in the *tsL56* mutator and *tsCB120* antimutator backgrounds differed by as much as 10^3–10^4-fold (Drake and Allen 1968; Drake *et al.* 1969; Speyer 1965). The mutations in *tsL56* are A89T+D363N, and the mutation in *tsCB120*, also known as *tsL141*, is A737V (Reha-Krantz 1988, 1989).

Such large variation in error frequencies suggested that the polymerase may play an active role in base selection during DNA synthesis. To quote Speyer's paper "Mutagenic DNA Polymerase" (Speyer 1965)

> . . . the replicating enzyme is involved more directly in the selection of the base . . . [such that] the information of the parental DNA strand is transmitted sequentially by the enzyme to an allosteric site where selection of the nucleotide . . . occurs. Such an enzymic mechanism may permit selection by criteria other than the relatively weak hydrogen bonds postulated in the template hypothesis and account for the high accuracy of DNA replication.

However, as the mechanisms of exonuclease editing and mismatch repair emerged, the contribution of the polymerase active site to fidelity was deemphasized. But, recently, Speyer's conclusion is regaining prominence. For example, Eric Kool has constructed a base analogue of T that is geometrically similar to T but cannot form H-bonds with A (Figure 1), and has shown that it is nevertheless incorporated opposite A almost as well as T by DNA polymerase I Klenow exo$^-$ (Moran *et al.* 1997).

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Pioneers in genetic fidelity, such as Speyer, Drake, Freese and, of course, Watson and Crick, set the stage for three decades of ongoing research into the question of how DNA polymerases synthesize DNA with such exquisitely high accuracy. What follows is a review of key results from those decades and a personal assessment of how the fidelity field evolved from the early genetic experiments.

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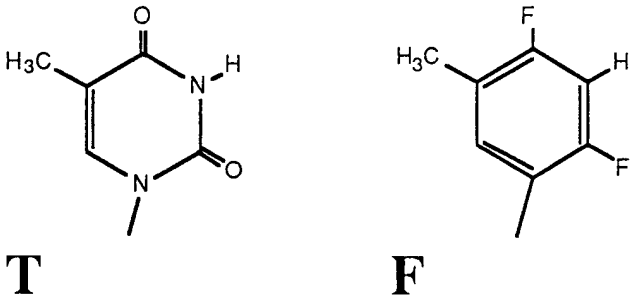

Figure 1.—Difluorotoluene, a non-hydrogen bonding base analogue of T. Chemical structures of thymine and difluorotoluene, an isosteric analog for thymine, used to demonstrate the relatively small influence of hydrogen bonding in DNA polymerase base selection (Goodman 1997; Moran *et al.* 1997).

Studies on the biochemical basis of mutation

The role of 3′-exonuclease proofreading in reducing polymerase errors: Two important papers published in 1972 suggested the existence of a polymerase-associated 3′→5′ exonuclease, which could increase fidelity by excising misincorporated nucleotides at their point of origin. Brutlag and Kornberg showed that *Escherichia coli* Pol I excised mispaired nucleotides in preference to correctly paired nucleotides from primer-3′-termini (Brutlag and Kornberg 1972). Bessman and co-workers, building on the work of Speyer and Drake, purified mutant and wild-type T4 polymerases and showed that the nuclease-to-polymerase (N/P) activity ratio was high for antimutator (*L141*), intermediate for wild type (43^+), and extremely low for mutator (*L56*) strains (Muzyczka *et al.* 1972).

In the latter experiments, polymerase and 3′-exonuclease activities were measured on an oligo dT-polydA primer-template, using saturating dTTP substrate concentrations. Individual phosphocellulose column fractions of the three T4 pols showed N/P ratios that were constant across each chromatographic peak but varied between peaks. Wild-type T4 pol excised 1 molecule dTMP per 25 molecules inserted. In contrast, the *L141* antimutator T4 pol excised 10 out of 11 dTMPs inserted, while the *L56* mutator polymerase excised only one out of 200. The apparent correlation between N/P ratio and polymerase fidelity was very suggestive and demanded further substantiation.

In 1972, Linda J. Reha-Krantz joined Bessman's laboratory as a graduate student and embarked on a thesis project of heroic proportions. She grew T4 gene *43* amber mutants in *E. coli* suppressor strains and measured their mutation frequencies. She then purified the mutant polymerases and determined their N/P ratios. She observed a near-perfect correlation between antimutator and mutator behavior *in vivo* and correspondingly high and low N/P ratios (Reha-Krantz and Bessman 1977). These results were solid evidence that the balance between the polymerase and 3′-exonuclease reactions was fundamentally linked to the overall accuracy of DNA synthesis.

But was the N/P ratio actually determining the accuracy of DNA synthesis or was it merely correlated with increased accuracy in the individual polymerization and excision reactions? Bessman and co-workers addressed this question by measuring the specificity of the individual nuclease and polymerase reactions (Bessman *et al.* 1974). They showed that mutator, antimutator, and wild-type T4 pols (*L56*, *L141*, and 43^+) inserted the mutagenic base analogue 2-aminopurine (2AP) opposite T with similar frequencies. What's more, the three polymerases were also similarly specific in removing 2AP: excising one correctly inserted A for every two to three "misinserted" 2AP molecules. The difference was in the overall activity of two reactions. The *L141* antimutator pol excised about 91% of the misinserted 2AP, resulting in a "low" net misincorporation frequency of about 3%, whereas the *L56* mutator excised only 20% of the 2APs, resulting a "high" error frequency of 10%.

The relevance of data using 2AP *in vitro* to the bacteriophage T4 system *in vivo* was documented in experiments showing that 2AP incorporation into T4 DNA *in vivo* was highest for *tsL56* mutator and very low for *tsL141* antimutator relative to 43^+ (Goodman *et al.* 1977), and that the mutant and wild-type strains converted 2AP-free-base to 2AP-triphosphate with roughly similar efficiencies, giving rise to similar d(2AP)TP/dATP pool ratios for the three strains infecting *E. coli in vivo* (Hopkins and Goodman 1985).

Concurrently with experiments from Bessman's group, Nancy Nossal and her students at NIH were also using the T4 system to study polymerase fidelity (Hershfield 1973; Hershfield and Nossal 1972). Gillen and Nossal (1976) found that *L141* (CB120) polymerase had difficulty carrying out strand displacement, suggesting that an impediment to forward translocation may enable the enzyme to proofread more effectively. Indeed, it has been shown that the A737V mutation in *L141* causes an increase in exonuclease processivity at the expense of polymerase processivity (Spacciapoli and Nossal 1994). These results provide a mechanistic explanation for the increase in nuclease/polymerase ratio for the *L141* antimutator relative to wild-type polymerase.

To test and refine this mechanistic link between N/P ratio and polymerase fidelity, we carried out a kinetic analysis of the fidelity of *L141*, wild-type, and *L56* polymerases, comparing the incorporation of 2AP in direct competition with A opposite a template T (Clayton *et al.* 1979). We found that although 2AP misinsertion frequencies were the same for each enzyme at all dNTP concentrations, 2AP misincorporation frequencies were highly dependent on substrate concentration. At saturating dNTP concentrations, 2AP misincorporation frequencies were higher for mutator (*L56*) and lower for

antimutator (*L141*) compared to wild type but all three converged to the same value at low-dNTP concentrations. The effect of dNTP concentration was most pronounced for the relatively inactive *L56* exonuclease. The relatively active *L141* exonuclease was only marginally affected. Thus, we concluded that when low dNTP concentrations limit polymerase activity, even inactive exonucleases are able to edit out the majority of polymerase errors.

The logic can be seen by analogy to quality control along an assembly line. A polymerase is like a machine that makes widgets and sends them down the line at a certain rate. An exonuclease is like a worker responsible for removing defective widgets that come down the line. The worker sometimes removes perfect widgets by mistake. (The fewer such mistakes, the more "specific" the worker.) However, the number of defective widgets that get past the worker depends primarily on how many widgets the worker checks as the assembly line rolls by. If the assembly line slows down (*i.e.*, there arises an impediment to forward translocation), the worker will be able to check more widgets and therefore let fewer defective ones go by.

It should be noted, however, that N/P ratio is not a fail-safe indicator of a mutator phenotype. As Jan Drake has pointed out, it was fortunate that A·T→G·C mutations were investigated early on for the *tsL141* allele, otherwise it may not have been identified as an antimutator (Drake 1992).

> From the beginning (Drake and Allen 1968; Drake *et al.* 1969), it was clear that [antimutators] consistently reduce A·T→G·C transition rates (sometimes by more than 100-fold), reduce some but not all base-addition and base-deletion rates, but tend either not to affect or else to increase G·C→A·T transition rates.

Of course, antimutators will always exhibit some mutational specificity in the sense that they will only be found for alleles that are not well corrected in the wild type (Reha-Krantz 1995). And N/P ratio may not reflect on the ability to correct mutations templated by unusual (*e.g.*, slipped out) primer/template structures. *L141*, for example, exhibits an *increased* mutagenicity for simple frameshifts (Ripley and Shoemaker 1983), perhaps due to the altered processivity of its polymerase. Despite this lack of universality (Drake 1993), the N/P ratio continues to serve as an important enzymatic "marker" of polymerase fidelity.

Studies on the biophysical basis of mutation

Models of DNA polymerase fidelity: The discovery of proofreading spurred the development of theoretical models to account for polymerase fidelity. John Hopfield proposed that polymerases might rely on "kinetic proofreading" to edit out miscreant base pairs (Hopfield 1974). The key idea was that, after binding a dNTP in the polymerase active site, the enzyme might irreversibly enter an activated state, perhaps driven by hydrolysis of ATP. Discrimination between correct and incorrect nucleotides could then occur twice: first, upon entering the active site, where difference in the free energy of binding of right vs. wrong dNTPs would favor the correct nucleotide, and again upon leaving the activated state, where the reaction rates of hydrolysis or unbinding might also distinguish between correct and incorrectly bound nucleotides. Jacques Ninio proposed a similar model, invoking a "time delay" that facilitated nonproductive hydrolysis of a wrongly bound nucleotide (Ninio 1975). These models offered a means for reducing the number of nucleotides misinserted by a DNA polymerase without resorting to "brute force" excision by a dedicated proofreading exonuclease (Hopfield 1974).

We now know that Nature has found "brute force" acceptable, however, and a model which explicitly invokes a 3′→5′ exonuclease to excise polymerase insertion errors has proven most useful. The model was proposed by Galas and Branscomb (1978) in the context of analyzing the data of Bessman and co-workers (Bessman *et al.* 1974) for the incorporation and proofreading of 2AP using T4 *L56* mutator, *43*$^{+}$, and *L141* antimutator polymerases. A simplified sketch of this polymerase-proofreading model is presented in Figure 2.

The model treats polymerization and proofreading as two possible outcomes of a series of random events, which take place after a dNTP (right or wrong) binds to the enzyme. In the sketch, polymerization occurs in the lower reaction pathway and proofreading takes place in the upper pathway. Connecting the two pathways are the states (A) and (M), referring to annealed and melted primer-3′-termini, respectively. No distinction is made between right and wrong base pairs, except to recognize that Watson-Crick (WC) pairs favor the annealed state, $k_A > k_M$, while non-WC pairs tend to be melted out, $k_M > k_A$. However, a non-WC pair may, with low probability, be in the annealed state and get incorporated into a growing DNA chain, while a proper WC pair may be melted out and get excised. The model therefore suggests that it is the equilibrium between melted and annealed primer-3′-termini, rather than any intrinsic/geometric difference between WC and non-WC base pairs, that determines whether proofreading is likely to occur.

It was originally assumed that following either an incorporation or excision the system was constrained to begin a new polymerization-proofreading cycle starting from the annealed state (A). This assumption led to the prediction that saturating concentrations of a next-correct dNTP (complementary to the template base immediately downstream from the initial dNMP incorporation site) would completely suppress proofreading. However, the experimental data clearly showed that, although the excision of dNMP by the proofreading exonuclease diminished at saturating next-nucleotide

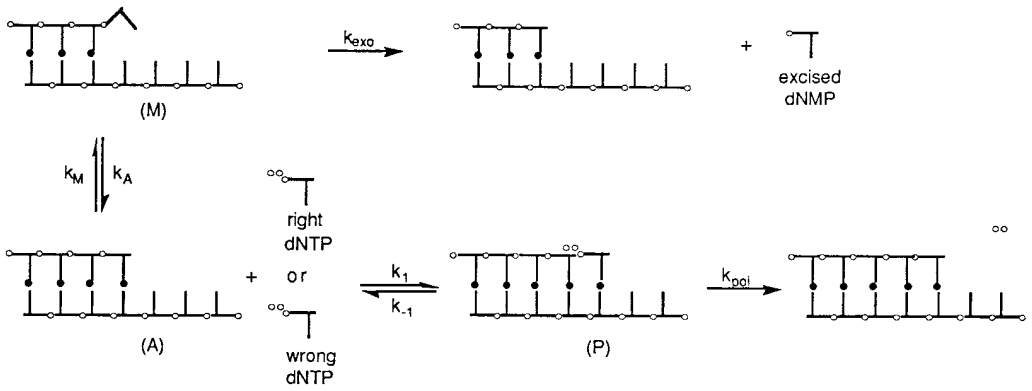

Figure 2.—Polymerase-proofreading model. Sketch of a simple model illustrating insertion and 3′-exonuclease proofreading of right and wrong nucleotides. State (M) refers to a melted primer terminus from which exonucleolytic excision takes place; state (A) refers to an annealed primer terminus along the polymerization pathway. Selective hydrolysis of misincorporated nucleotides results from the ratio, k_M/k_A, being much larger for mismatches than for correct matches. Polymerization from state (P) is favored over proofreading from state (M) as the concentration of rescue dNTP is increased. Following either excision or insertion, a shift occurs one base backward or forward to allow the cycle to repeat. When cycling occurs, the terminal base is assumed to reach an equilibrium distribution between states (A) and (M), explaining why proofreading is not entirely suppressed even at saturating concentrations of rescue dNTP (Clayton *et al.* 1979).

concentrations, it was nevertheless present to a significant extent (Clayton *et al.* 1979). A refinement of the model, allowing the system to reach an equilibrium distribution of melted and annealed primer termini following nucleotide incorporation and excision, resolved the problem. Partial suppression of proofreading in the presence of high dNTP concentrations is referred to as the "next-nucleotide effect" (Clayton *et al.* 1979; Fersht 1979), and has come to be recognized as a basic hallmark of proofreading (Echols and Goodman 1991; Goodman *et al.* 1993).

The Galas-Branscomb model highlights the importance of the interactions between polymerases, proofreading exonucleases, and primer-template DNA. It has served as a starting point for investigations into why mutational spectra and error rates differ substantially among polymerases in different sequence contexts.

Sequence context effects on DNA polymerase fidelity: One of the most general and important sequence context effects can be understood by examining the influence of local DNA stability on N/P ratios. Simply stated, stable regions are less frequently melted out, and so less available to exonuclease. Consequently, base substitution mutations tend to occur more frequently in more stable (*e.g.*, G·C-rich) sequences and less frequently in less stable (A·T-rich) regions. For example, it has been shown that T4 mutation frequencies *in vivo* and misincorporation of 2AP by T4 pol *in vitro* decrease with increasing temperature (Bessman and Reha-Krantz 1977). Were it not that higher temperatures made stable regions more accessible to exonuclease proofreading, one might expect mutations to increase because of higher rates of deamination and depurination reactions. The same study also showed that sites on DNA which are relatively insensitive to temperature also did not show an appreciable difference in mutation comparing *43*$^+$ and antimutator *L141* alleles.

The ambiguous base pairing properties of 2AP make it a useful compound for studying fidelity *in vitro* and mutagenesis *in vivo* (Echols and Goodman 1991; Ronen 1979). However, 2AP has another extremely useful property; it is moderately fluorescent and can therefore be used to study polymerase mechanisms by observing its insertion by polymerase and excision by exonuclease on a pre-steady-state time scale (Bloom *et al.* 1993; Bloom *et al.* 1994; Frey *et al.* 1995).

Further evidence for the effect of local DNA stability on mutagenesis came from such pre-steady-state measurements. Excision of 2AP was measured on a millisecond time scale by its increase in fluorescence upon excision from a primer-3′-terminus and a concomitant increase in rotation, as measured by fluorescence depolarization (Bloom *et al.* 1994). 2AP was placed at a primer-3′-terminus opposite template T, C, A or G, while maintaining a constant surrounding sequence context. The observed excision rate correlated inversely with the stability of the base pair. Thus, removal of 2AP was slowest when paired opposite T, with the order of excision being 2AP·T < 2AP·A < 2AP·C < 2AP·G. Measurements were then made of the hydrolysis of 2AP·N base pairs placed proximal to either A·T- or G·C-rich neighboring sequences. It was found that a proper Watson-Crick 2AP·T base pair in an A·T-rich environment was actually excised faster than a wobble 2AP·C mispair in a G·C-rich environment (Bloom *et al.* 1994).

Another important sequence context effect on fidelity comes from the influence of base-stacking interactions. Ronen and Rahat (1976) first showed that neighboring base pairs influenced 2AP-induced base substitution mutation rates. Later, Pless and Bessman (1983) cre-

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