DNA polymerases replicate DNA with high fidelity because of accurate nucleotide incorporation coupled with exonucleolytic proofreading to remove misincorporated nucleotides. This statement is taken for granted today, in large part, because of groundbreaking discoveries made 30 years ago that mutations in the DNA polymerase gene of bacteriophage T4 can have dramatic effects on the fidelity of DNA replication. “Mutator” DNA polymerases (Speyer et al. 1966) are mutant DNA polymerases that replicate DNA with less accuracy than the wild-type enzyme, and “antimutator” DNA polymerases (Drake and Allen 1968; Drake et al. 1969) are mutant DNA polymerases that replicate DNA with more accuracy, at least for certain types of DNA replication errors. Biochemical characterization revealed that the balance between the two DNA polymerase functions, nucleotide incorporation and the opposing 3′→5′ exonuclease activity, was altered in the mutants compared to the wild type enzyme. Decreased 3′→5′ exonuclease activity was detected for many of the mutant DNA polymerases (Muzyczka et al. 1972) while increased 3′→5′ exonuclease activity, relative to polymerase activity, was observed for the antimutator DNA polymerases (Muzyczka et al. 1972; Gillin and Nossal 1976a,b). The correlation of decreased 3′→5′ exonuclease activity measured in vitro assays with decreased accuracy of DNA replication in vivo, and increased 3′→5′ exonuclease activity with increased DNA replication fidelity indicates that the 3′→5′ exonuclease activity of T4 DNA polymerase is an exonucleolytic proofreading activity. 

These observations were made at an exciting time in DNA polymerase research. I was fortunate then to be a graduate student in Maurice Bessman’s lab at Johns Hopkins University and to see first hand the biochemical characterization of T4 mutator and antimutator DNA polymerases. But of equal importance was the opportunity to meet Jan Drake and to learn about the use of genetics to study the fidelity of DNA replication. The juxtaposition of biochemistry and genetic analyses to elucidate the proofreading role for the 3′→5′ exonuclease activity of T4 DNA polymerase made a strong impact on my graduate studies and continues to do so today. I am still intrigued by the genetic analyses from the Drake lab, which demonstrated that single point mutations in the T4 DNA polymerase gene can increase or decrease mutation rates by 100-fold or more (Drake et al. 1969).

Mutational analysis is a powerful method to probe enzyme function. Even today with the ready availability of molecular techniques, genetic screens and selections are useful because informative mutant enzymes can be identified without structural information or assumptions about function. Most importantly, since classical genetic methods rely on detection and characterization of mutant phenotypes in vivo, alterations in function are observed within the context of the living organism. In the case of antimutator DNA polymerases, these mutants provide a handle to probe DNA polymerase function in vivo.

Genetic and biochemical techniques have continued to be used in studies of antimutator DNA polymerases. This research has led in a number of different directions including studies of the evolution of spontaneous mutation rates (Drake 1990, 1991a, 1991b), the effect of antimutator DNA polymerases on frameshift mutagenesis (Kaiser and Ripley 1995), the identification of antimutator DNA polymerases in other organisms (Fijsal-kowska et al. 1993), and additional studies of DNA polymerase function (Clayton et al. 1979; Spaccipoli and Nossal 1994a,b; Reha-Krantz and Nonay 1994; Reha-Krantz and Wong 1996). The focus of this short review is the regulation of DNA polymerase proofreading activity (Stöckl et al. 1995). Other aspects of T4 antimutator DNA polymerases are presented in this volume (e.g., Nossal, Wang and Ripley).

T4 antimutator DNA polymerases: Mutations within genes result typically in mutant enzymes with reduced activity and even loss of function; however, in the case of antimutator DNA polymerases, the mutant enzymes appear to be working better, at least with respect to the accuracy of DNA replication. Since single point mutations can give rise to antimutator DNA polymerases, why have more accurate DNA polymerases not evolved? Part of the answer must be that there are negative consequences of the antimutator phenotype that reduce the efficiency of the DNA polymerase and the overall fitness of the organism. There is a cost associated with DNA polymerase proofreading (Ferst et al. 1982). For example, if T4 antimutator DNA polymerases replicate DNA more slowly than the wild-type enzyme, then fewer phage progeny may be produced. Higher concentrations of deoxynucleoside triphosphates (dNTPs) are required to support DNA replication by T4 antimutator
DNA polymerases (Gauss et al. 1983; Beauchamp and Richardson 1988) due to increased exonucleolytic proofreading, which removes correct nucleotides in addition to incorrect nucleotides (Muzyczka et al. 1972; Gillin and Nossal, 1976a; reviewed in Goodman et al. 1993). Another potential disadvantage of increased DNA replication accuracy is the possible necessity of a certain minimal mutation rate that is sufficient to allow an organism to adapt to fluctuations in the environment (Drake 1990, 1991a, 1991b). Another possible problem with exonucleolytic proofreading stems from the observation by Drake et al. (1969) that the antimutator activity of the mutant T4 DNA polymerases appears not to be a general antimutator activity, but is directed to decreasing AT—GC transitions and base analogue induced mutations. Other mutational pathways such as GC—AT transitions, transversions, and frameshifts are not reduced at some sites by T4 antimutator DNA polymerases, and may even be increased (Drake and Greening 1970; Ripl ey 1975; Kaiser and Ripl ey 1995). Thus, any gain in fitness obtained by a decrease in AT—GC transition mutations may be offset by increases in other types of DNA replication errors (Drake 1993).

T4 DNA polymerase has a potent 3′—5′ exonuclease activity, about 1000-fold more active than the 3′—5′ exonuclease activity of Escherichia coli DNA polymerase I (Capson et al. 1992); hence, a mechanism must be in place to prevent indiscriminate degradation of the newly synthesized DNA and the other disadvantages of excessive proofreading discussed above. This mechanism fails or regulation is reduced in antimutator DNA polymerases. If the molecular basis of the antimutator phenotype can be learned, then this information can be applied to determining how the normal balance between polymerase and exonuclease activities is maintained. A first step in understanding the regulation of exonucleolytic proofreading is to determine the location of mutations that confer the antimutator phenotype.

**Locations of mutations within the T4 DNA polymerase gene that confer antimutator and mutator phenotypes:** Drake (Drake and Allen 1968; Drake and Greening 1970) mapped mutations that confer the antimutator phenotype by recombination. Two of the mutations that produce a strong antimutator phenotype encode Val to Ala substitutions at codons 737 and 777 (Reha-Krantz 1989). The locations of these mutations in the primary structure of T4 DNA polymerase are illustrated in Figure 1. Additional mutations that confer the antimutator phenotype have also been located in this region at codons 730, 731, 827, and 844 (Reha-Krantz 1988, 1994, 1995a). Mutations in the T4 DNA polymerase that confer the antimutator phenotype have also been identified by genetic selection for second-site mutations that suppress sensitivity to the pyrophosphate analogue, phosphonoacetic acid (Reha-Krantz and Nonay 1994). These mutations are located near the center of the gene (Figure 1) and encode the conservative amino acid substitutions Thr for Ser411, Ile for Leu412, and Val for Ile417 in the highly conserved DNA polymerase motif, DXXS_x_L_yYPSxL_427, that forms part of the polymerase active center. Another mutation that encodes a Cys substitution for Arg335 was also identified by this selection strategy (Reha-Krantz and Wong 1996). Thus two regions of the DNA polymerase have been identified in which single amino acid substitutions can produce a strong antimutator phenotype.

Most mutations that produce a strong mutator phenotype encode amino acid substitutions in the N-terminal region of T4 DNA polymerase (Reha-Krantz 1988, 1989). Alanine substitutions for aspartate residues D112, D219, and D324 (Figure 1) that are required for the hydrolysis reaction increase the mutation rate up to several hundredfold, as expected if exonucleolytic proofreading activity is prevented (Reha-Krantz et al. 1991; Frey et al. 1993; Reha-Krantz and Nonay 1993).

The recent determination of the structure of the RB69 DNA polymerase (Wang et al. 1997), a T4-like DNA polymerase, allows placement of residues that are important for DNA replication fidelity in a three-dimensional, structural context. Residues in the C-terminal domain, as well as residues in the exonuclease domain, make contacts with single-stranded DNA bound in the
exonuclease catalytic center (Wang et al. 1997). Thus, some residues in the C-terminal domain, the location of residues A737 and A777 (Figure 1), are in close contact with the exonuclease domain (see Nossal 1998). Amino acid substitutions that produce the antimutator phenotype were also identified in the polymerase active center, but the polymerase and exonuclease active centers are separated by a relatively great distance, about 30 Å (Wang et al. 1997). Proofreading requires that the primer strand be moved from the polymerase to the exonuclease active center. The locations of mutations that confer the antimutator phenotype suggest the possibility that residues in the polymerase active center and in the C-terminal domain affect how the DNA is transferred between the two active centers.

**Antimutator DNA polymerases have more opportunity to proofread:** Biochemical characterizations of the antimutator DNA polymerases revealed that the balance between nucleotide incorporation and exonuclease activities was shifted toward increased exonuclease activity in the mutant enzymes by providing more opportunity to proofread (Gillin and Nossal 1976a). The relative opportunities to proofread or to extend a primer-terminus can be demonstrated by preforming Enz-DNA complexes in the presence of dNTPs but in the absence of Mg2+, which is required for both nucleotide incorporation and exonuclease proofreading activities. The addition of Mg2+ then allows preformed Enz-DNA complexes in which the primer-terminus resides in the polymerase active center (EnzpolDNA-dNTP complex) to incorporate nucleotides and Enz-DNA complexes in which the primer-terminus resides in the exonuclease active center (EnzexoDNA complex) to remove the terminal nucleotide. The antimutator I417V-DNA polymerase, Val substitution for Ile417 within the polymerase active center, forms active exonuclease complexes almost twofold more frequently than the wild-type enzyme (Reh-Krantz and Nonay 1994).

These results were interpreted to indicate that the I417V substitution destabilizes interactions with the primer-terminus within the polymerase active center and thus increases the opportunity of the enzyme to form complexes in which the primer-terminus resides in the exonuclease active center. Reduced dNTP concentrations can also enhance exonuclease activity by decreasing the formation of EnzpolDNA-dNTP complexes which then favors increased formation of active EnzexoDNA complexes (Clayton et al. 1979).

**Kinetic partitioning between polymerase and exonuclease activities:** The studies above and numerous additional studies of T4 DNA polymerase and other DNA polymerases (Hopfield 1974; Fersht et al. 1982; Capson et al. 1992; Johnson 1993; Goodman et al. 1993) have resulted in formulation of a kinetic scheme that describes the regulation between nucleotide incorporation and exonuclease proofreading activities. A primer-terminus is normally extended by T4 DNA polymerase at the rapid rate of about 400 sec⁻¹ (Capson et al. 1992), but elongation of a mismatched primer-terminus is extremely slow (reviewed by Goodman et al. 1993). The greatly reduced ability of a DNA polymerase to extend a mismatched primer-terminus provides a window of opportunity to transfer the primer-terminus from the polymerase to the exonuclease active center, a slower reaction measured at the apparent rate of 4 sec⁻¹ (Capson et al. 1992; Marquez and Reh-Krantz 1996). The relatively slow transfer of DNA from the polymerase to the exonuclease active center serves as a kinetic barrier to the exonuclease proofreading pathway that protects correct primer-termini from indiscriminate degradation.

The antimutator phenotype can be explained in the context of the kinetic scheme by proposing that the antimutator DNA polymerases have even less ability than the wild-type DNA polymerase to extend a mismatched primer-terminus. One prediction of this proposal is that antimutator DNA polymerases would also have less ability to extend a correctly matched primer-terminus. Both the I417V-DNA polymerase, which has decreased ability to form the EnzpolDNA-dNTP complex (Reh-Krantz and Nonay 1994), and the A737V-DNA polymerase, which is defective in translocation (Gillin and Nossal 1976a; Spacciapoli and Nossal 1994a), have high rates of turnover of correct nucleotides (Muzyczka et al. 1972; Gillin and Nossal 1976a; reviewed in Goodman et al. 1993). The high turnover of correct nucleotides by T4 antimutator DNA polymerases was used as the basis of a genetic selection strategy to isolate second-site mutations that suppress the excessive proofreading of antimutator DNA polymerases (Stocki et al. 1995).

**Suppressors of excessive DNA polymerase proofreading decrease transfer of the primer-terminus from the polymerase to the exonuclease active center:** Excessive proofreading by T4 antimutator DNA polymerases is detected in vivo in infections of a host bacterial strain, optA1, which has increased ability to degrade dGTP (Gauss et al. 1983; Beauchamp and Richardson 1988). The reduced dGTP concentration in the restrictive host is not sufficient to support DNA replication by antimutator DNA polymerases and prevents replication of the phage genome. We have used this conditional lethal phenotype to identify second-site mutations that suppress the excessive exonuclease proofreading activity of four mutant T4 DNA polymerases (Stocki et al. 1995). Two antimutator DNA polymerases identified by Drake, the A737V- and A777V-DNA polymerases, were part of the study. Nineteen second-site suppressor mutations were found. Although the suppressor mutations are located in four different regions of the DNA polymerase, the mechanism of suppression appears to be the same—to raise the kinetic barrier to the proofreading pathway. One of the suppressor mutations will be discussed here to illustrate this point.
Figure 2.—Dynamics of DNA polymerase proofreading. For nucleotide incorporation, DNA is bound in the polymerase active center in a base-paired configuration. For exonucleolytic proofreading, the primer-terminus is partially separated from the template strand and transferred to the exonuclease active center. The exonuclease active center is illustrated as an inverted "V" in order to mimic the single-stranded binding groove observed in crystallographic structures of the T4 DNA polymerase proofreading complex (Wang et al. 1996, 1997). Amino acid residue Gly255 resides in a novel protein loop involved in transfer of the primer-terminus from the polymerase to the exonuclease active center (Marquez and Reha-Krantz 1996).

The most frequently isolated second-site suppressor mutation of excessive proofreading activity encodes a Ser substitution for residue Gly255, which resides in the exonuclease domain (Figure 1). The G255S-DNA polymerase in vivo displays a strong mutator phenotype compared with some of the exonuclease-deficient DNA polymerases. The purified G255S-DNA polymerase, however, has near wild-type levels of 3′→5′ exonuclease activity on single-stranded DNA substrates, but reduced activity on duplex DNA substrates. This observation indicates that the G255S-DNA polymerase retains the ability to catalyze hydrolysis of the phosphodiester bond, but the mutant is deficient in converting duplex DNA to the partially strand-separated DNA substrate that is required for exonuclease activity (Figure 2).

Kinetic studies using the fluorescence of the base analogue 2-aminopurine were used to demonstrate that the rate of movement of the primer-terminus from the polymerase to the exonuclease active center was reduced by about 10-fold for the G255S-DNA polymerase compared to the wild-type enzyme (Marquez and Reha-Krantz 1996). The slow transfer rate was linked to reduced ability of the mutant DNA polymerase to separate the primer strand from the template strand. The defect in moving the primer-terminus from the polymerase to the exonuclease active center, called "active-site-switching" (Stocki et al. 1995), is correlated with structure. Residue G255 is located in a novel protein loop structure (Figure 3) that resides away from the exonuclease active center (Wang et al. 1996). The
reduced ability of the G2555-DNA polymerase to transfer the primer-terminus to the exonuclease active center indicates that the protein loop is involved in some aspect of this process (Figure 2).

If the excessive proofreading activity of antimutator DNA polymerases can be corrected by alterations in the enzyme that raise the kinetic barrier to the proofreading pathway, then alterations that produce antimutator DNA polymerases may lower the kinetic barrier by increasing the rate of transfer of the primer-terminus from the polymerase to the exonuclease active center. The antimutator 1417V-DNA polymerase, which has a conservative amino acid substitution in the polymerase active center, appears to directly affect transfer of DNA from the polymerase to the exonuclease active center, because an increased rate of transfer was detected with the 2-aminopurine fluorescence assay that was used to study the G2555-DNA polymerase (L. A. Marquez and L. J. Reha-Krantz, unpublished observations).

An important future experiment is to determine if amino acid substitutions in the C-terminal region, for example A737V or A777V, also increase the rate of transfer of DNA from the polymerase to the exonuclease active center or if these substitutions affect another aspect of proofreading. For example, translocation, the ability of the DNA polymerase to move along the DNA template, is reduced for the A737V-DNA polymerase (Gillin and Nossal 1976a; Spacciapoli and Nossal 1994a). A delay in translocation is predicted to provide more opportunity to initiate the proofreading pathway, since reduction in the rate of nucleotide incorporation would make initiation of the proofreading pathway more competitive with primer extension. Yet, the A737V-DNA polymerase also has a more processive exonuclease activity, which means that sometimes additional nucleotides besides the terminal 3'-nucleotide are excised (Spacciapoli and Nossal 1994b). The T4 DNA polymerase proofreading activity normally removes just the terminal nucleotide, but sometimes the penultimate nucleotide is also excised (Reddy et al. 1992). If removal of multiple nucleotides is increased by the A737V substitution, this may account for the antimutator phenotype in cases where the mismatch is not at the primer-terminus.

These observations indicate that there is a delicate balance between primer extension and movement of the primer-terminus between the polymerase and exonuclease active centers, which means that there is also a delicate balance between more or less accurate DNA replication. This model, however, does not explain the apparent AT → GC mutational specificity of T4 antimutator DNA polymerases (Drake et al. 1969). How can antimutator DNA polymerases reduce AT → GC transition mutations 100-fold, but not spontaneous GC → AT transitions, transversions or other types of DNA replication errors?

The AT → GC mutational specificity of T4 antimutator DNA polymerases: One way to answer this question is to propose that the in vivo AT → GC mutational specificity of T4 antimutator DNA polymerases identifies a class or classes of mismatches that more frequently escape proofreading by the wild-type level of exonucleolytic proofreading activity (Reha-Krantz 1995b). Mutations at sites that are not reduced by antimutator DNA polymerases may then identify mutational pathways that are not sensitive to increased exonucleolytic proofreading. Drake et al. (1969) pointed out that while the GC → AT mutational pathway at certain sites appears unaffected by antimutator DNA polymerases, antimutator DNA polymerases do strongly reduce base-analogue-induced GC → AT mutations. These observations suggest that base-analogue-induced GC → AT mutations arise by a proofreading-sensitive mechanism, but that spontaneous GC → AT mutations at certain sites arise by a proofreading-insensitive mechanism.

An example of a proofreading-insensitive type of pathway is one in which mutations arise from slippage between the primer and template strands to form a transient misaligned primer-terminus. Misalignment of the primer and template DNA strands was originally proposed by Stelisinger et al. (1966) as a mechanism to generate frameshift mutations in DNA sequences with monotonic runs of nucleotides or simple repeats. Today, misalignment mutagenesis is recognized as a significant contributor not just to insertions and deletions of nucleotides but also to transitions and transversions (note reviews by Ripley 1990; Drake 1991b; Kunkel 1990, 1993). Thus, base substitution DNA replication errors can arise not only from direct misinsertion of nucleotides, for example to produce G-T or A-C mismatches, but also from insertion of correct nucleotides within incorrect alignments of the primer-template. Since the base pairing is correct in the misaligned DNA strands, these matched primer-termini are not expected to be substrates for DNA polymerase exonucleolytic proofreading.

If reduction in AT → GC mutations by antimutator DNA polymerases is due to increased exonucleolytic proofreading without a change in nuclease specificity, then any mechanism that increases exonucleolytic proofreading should parallel the mutational specificity observed for the antimutator DNA polymerases. An increase in temperature from 20° to 42° enhances exonucleolytic proofreading to a greater extent than nucleotide incorporation (Büntag and Kornberg 1972; Bessman and Reha-Krantz 1977). Increased temperature is proposed to favor proofreading by destabilizing the primer-terminus and in assisting in strand separation. The temperature effect on exonucleolytic proofreading was observed for the wild-type T4 DNA polymerase in vitro and in vivo. In vivo, the largest reductions in mutation rates produced by high temperature were detected for AT → GC mutations at sites that also showed the largest reductions in reversion rates by T4 antimuta-
ator DNA polymerases (Bessman and Reha-Krantz 1977). Sites that were not sensitive to antimonial DNA polymerases were also not affected by temperature. Since the same mutational specificity was detected for antimonial DNA polymerases and for the wild-type DNA polymerase at high temperature, the AT→GC mutational specificity can be attributed to a general effect on the level of proofreading rather than to a specific effect by a mutant DNA polymerase on a specific mutational pathway.

Errors in DNA replication at one antimonial- and temperature-sensitive site have been sequenced (Reha-Krantz 1995b). This site is an ochre codon and any of the three base pairs in the codon can be mutated to provide protein function. Yet, about 90% of the revertants are AT→GC mutations at the first base pair position of the codon, which means that this position is a "hotspot" for AT→GC mutations. For the wild-type DNA polymerase, hotspot AT→GC transitions were detected at a frequency of about 1 per 10^6 while transversion mutations at this position and transition and transversion mutations at the other two base pairs of the codon were detected 10- to >100-fold less frequently. The antimonial A737V-DNA polymerase reduced the hotspot AT→GC transitions about 100-fold, but this site was still a relative hotspot since other mutations within the three base pair codon were also reduced about 100-fold. Thus, the antimonial DNA polymerase reduces the hotspot AT→GC mutation as well as other less frequent replication errors equivalently.

Together, these observations suggest that the increased exounucleolytic proofreading of antimonial DNA polymerases reduces both transition and transversion DNA replication errors, but there are sites in which certain types of DNA replication errors are refractory to proofreading. Mutations at proofreading-refractory sites may be produced by mechanisms in which "right bases are in the wrong places" due to misalignment of the primer and template DNA strands (Drake 1991b). A goal of future experimentation will be to determine why certain sites are hotspots for AT→GC mutations and why these sites escape proofreading by the wild-type DNA polymerase. Another goal is to understand how the antimonial A737V-DNA polymerase increases the frequency of other types of mutations (see Wang and Ripley 1998).

Summary: Only a relatively few discoveries or observations in science have great impact in advancing knowledge and in stimulating new lines of thought. Such was the discovery of T4 antimonial DNA polymerases (Drake and Allen 1968; Drake et al. 1969). The availability of antimonial DNA polymerases was critical in the elucidation of the role played by the DNA polymerase 3′→5′ exounuclease activity in proofreading newly synthesized DNA. Continued biochemical characterization of antimonial DNA polymerases is providing further insights into the regulation of exounucleolytic proofreading activity and the mechanism of "active-site-switching." Perhaps an even more important benefit of studies of antimonial DNA polymerases is an awareness that errors in DNA replication can arise by a variety of mechanisms in addition to nucleotide misinsertion. Although the level of exounucleolytic proofreading detected for wild-type T4 DNA polymerase reduces DNA replication errors by 100-fold or more, increased proofreading as detected for antimonial DNA polymerases cannot further reduce the frequency of all DNA replication errors. Some types of replication errors are insensitive to exounucleolytic proofreading. The cost and limitations of DNA polymerase proofreading may be linked to the development of mismatch-repair systems that correct replication errors that are missed by proofreading.

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