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Fidelity of DNA Replication

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The six billion nucleotides of the diploid human genome are replicated in only a few hours while generating so few errors that the spontaneous mutation rate may be less than 1 mutation per genome per cell division (Loeb 1991). This incredible accuracy results from three major error-avoidance processes: the high selectivity of DNA polymerases, exonucleolytic proofreading, and postreplication mismatch repair. In this chapter, we review our current understanding of the first two of these processes. Readers interested in eukaryotic mismatch repair are referred to a recent review (Modrich 1994).

We begin by describing the steps in the polymerization reaction cycle that discriminate against base substitution errors, then review studies of the substitution fidelity of the five classes of eukaryotic DNA polymerases. We then consider several ways to make errors by template-primer slippage and review what is known about eukaryotic DNA polymerase frameshift error rates. Finally, we present information on the fidelity with which the multiprotein replication machinery replicates undamaged DNA and DNA containing adducts of known carcinogens.

BASE SUBSTITUTION FIDELITY

Discrimination Steps in a Polymerization Cycle

The error discrimination steps that operate during incorporation of a single nucleotide have been worked out primarily with prokaryotic and viral DNA polymerases. The steps (Fig. 1) include binding of the polymerase to the DNA, formation of a ternary complex with the incoming deoxyribonucleoside triphosphate (dNTP), a conformational change in this complex to position the substrates for phosphodiester bond formation, the chemical reaction step to form the bond, a second conformational change following the chemical reaction, and release of pyrophos-

DNA Replication in Eukaryotic Cells

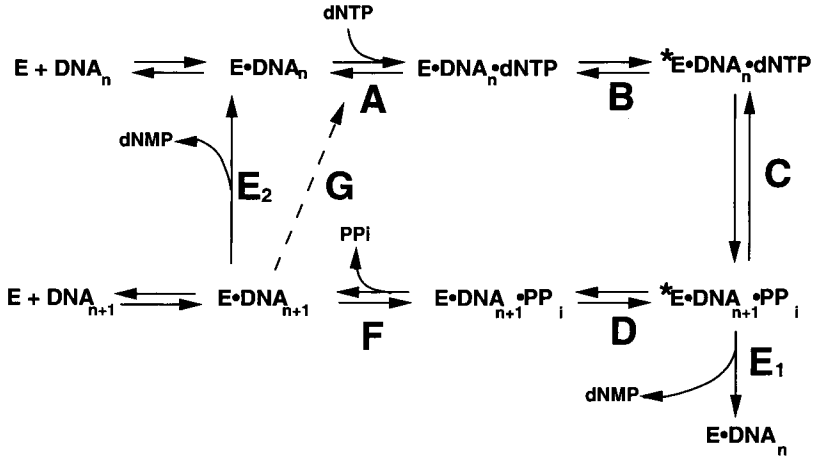

Figure 1 Reaction pathway for exonuclease-proficient DNA polymerases. Asterisks represent enzyme (E) in a different conformation. The enzyme conformations in the ternary E•DNA$_n$•dNTP and E•DNA$_{n+1}$•PPi complexes are unknown and not necessarily the same. Entry into the next cycle of polymerization is indicated by G. Translocation has not been assigned to a particular step in the reaction. Steps A–F are discussed in the text. (Reprinted, with permission, from Kunkel 1992.)

phate (PPi). After the chemical step, the polymerase translocates to commence the next cycle of polymerization, extending the nascent strand in the 5′ to 3′ direction.

DNA Polymerase Selectivity

There are several points in this cycle where discrimination against errors by the DNA polymerase occurs. The first is during binding of the dNTP to the complex formed between the polymerase and a correctly paired template primer (Fig. 1, step A). Incorrect dNTPs bind much less avidly than do correct dNTPs. At this step, discrimination against several mispairs, quantitated by kinetic analyses includes: Klenow polymerase, the large fragment of *Escherichia coli* DNA polymerase I, 0 to 23-fold (Eger and Benkovic 1992); T7 DNA polymerase, 200- to 400-fold (Wong et al. 1991; for review, see Johnson 1993); T4 DNA polymerase, ≥250-fold (Capson et al. 1992); and human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT), 25- to 400-fold (Johnson 1993; Zinnen et al. 1994). The amount of discrimination varies over a considerable range, depending on the composition of the mispair and the surrounding sequence context. This reflects differences in hydrogen-bonding poten-

tials for various combinations of dNTPs and template bases, sequence-dependent differences in base stacking, and a demand for equivalent base-pair geometry (for review, see Echols and Goodman 1991).

For the three viral DNA polymerases mentioned above, the amount of binding discrimination exceeds that predicted from the free-energy differences between correct and incorrect base pairs in aqueous solution. Within a DNA polymerase active site, hydrogen-bonded water molecules are displaced from the transition state for complementary base pairs (Fersht 1985), and this exclusion of water from the active site may amplify base-pair free-energy differences, thus enhancing fidelity (Petruska et al. 1986; Abbotts et al. 1991; Johnson 1993). A differential ability to exclude water from the active site might partly explain DNA polymerase-dependent differences in dNTP-binding discrimination. Klenow polymerase utilizes dNTP-binding discrimination to a lesser extent than do the other three polymerases examined. This has led to the suggestion that in the "ground state" (i.e., prior to the inferred conformational change at step B), initial binding of the dNTP to Klenow polymerase may not involve base-pairing (Johnson 1993). It has also been suggested (Capson et al. 1992; Johnson 1993) that any reduction in enzymatic efficiency of *E. coli* pol I resulting from competitive inhibition by the three incorrect dNTPs might be tolerated by the cell because the primary role for this polymerase in vivo is in DNA repair or replacement of RNA primers during replication. In contrast, reduced efficiency might be less acceptable for polymerases that replicate entire genomes. All three viral polymerases mentioned above, whose role is genomic replication, discriminate more strongly against incorrect dNTP binding than does Klenow polymerase.

The next selectivity step in the polymerization cycle, inferred from several lines of evidence (Johnson 1993; Zinnen et al. 1994), involves a conformational change in the ternary complex to position the dNTP for subsequent phosphodiester bond formation (Fig. 1, step B). For T7 DNA polymerase, this change to a "closed" structure in which the polymerase is thought to lock onto the template-primer·dNTP complex (Johnson 1993) is much more rapid for correct base pairs that can adopt Watson-Crick geometry than for incorrect base pairs that cannot. This leads to a 2000- to 4000-fold faster rate of incorporation of correct nucleotides (Johnson 1993). Similarly, a difference in the rate of change in protein conformation with correctly versus incorrectly bound dNTPs has been suggested to enhance the selectivity of HIV-1 RT by 7- to 17,000-fold (Johnson 1993; Zinnen et al. 1994), depending on the mispair considered.

The situation is somewhat different with Klenow polymerase. Although a change in polymerase conformation has been inferred to limit the catalytic rate for correct incorporation by this enzyme, this step is not rate-limiting for incorrect incorporation. Rather, discrimination against incorrect incorporation is mostly due to a strong reduction in the rate of phosphodiester bond formation (Fig. 1, step C; Kuchta et al. 1988).

Steps A, B, and C in Figure 1 all contribute to polymerase selectivity against insertion of incorrect nucleotides, with the relative importance of each step dependent on the polymerase examined. The contributions of these individual steps to the fidelity of eukaryotic DNA polymerase remain to be established. However, steady-state kinetic analyses have established misinsertion and mispair extension rates of some eukaryotic DNA polymerases (see below).

Exonucleolytic Proofreading

With the Klenow polymerase, a slow step has been detected after chemistry and prior to pyrophosphate release (Carroll and Benkovic 1990; Polesky et al. 1992). This delay, suggested to result from a second conformational change in the enzyme-template·primer complex, provides an opportunity for its intrinsic $3' \rightarrow 5'$ exonuclease to remove misinserted nucleotides (step E_1). Once a nucleotide is incorporated, the $E \cdot DNA_{n+1}$ complex can enter the next cycle of polymerization (Fig. 1, step G). However, the rate of correct incorporation onto a terminal mispair is much slower than onto a correctly paired terminus. This slow step provides another opportunity for excision (Fig. 1, step E_2), after transferring the misinserted nucleotide from the polymerase active site to the exonuclease active site. These sites are on separate domains of Klenow polymerase and are spatially separated in other DNA polymerases. Movement into the exonuclease active site can occur with or without enzyme dissociation from the template primer, depending on the polymerase (Joyce 1989; Donlin et al. 1991; Capson et al. 1992; Reddy et al. 1992). If the enzyme does dissociate, it may rebind to the exonuclease active site and edit the misinsertion. It is also formally possible that a terminal misinsertion may be bound and proofread by another exonuclease, one associated with a different polymerase or one not associated with a polymerase at all.

Prokaryotic polymerases containing associated $3' \rightarrow 5'$ exonuclease activities have average base substitution error rates of about 10^{-6} to 10^{-7}. The proofreading contribution to these rates has been assessed by selectively stimulating the polymerase activity relative to the exonuclease ac-

tivity by increasing the concentration of the next correct nucleotide to be incorporated after a misinsertion. Alternatively, exonuclease activity can be inhibited by adding to the reaction a nucleotide monophosphate (dNMP), the end product of exonuclease action. Proofreading can also be eliminated by changing amino acid residues essential for exonuclease activity. These residues are found in three conserved DNA sequence motifs common to the coding sequences of DNA polymerases containing intrinsic 3′→5′ exonucleases (Ito and Braithwaite 1990; Blanco et al. 1991; Chung et al. 1991; Morrison et al. 1991; Simon et al. 1991; Zhang et al. 1991). The results obtained from these three approaches suggest that proofreading contributes on average about 100-fold to fidelity. This value is consistent with estimates from in vivo studies using *E. coli* strains deficient in proofreading activity (Schaaper 1993) and with calculations suggesting that the energetic cost of improving fidelity by more than this amount using exonucleolytic activity could be unacceptably high for an organism due to too much excision of correctly paired bases (Fersht et al. 1982).

The contribution of exonucleolytic proofreading to base substitution fidelity can vary over a wide range, from only a few-fold (Bebenek et al. 1990) to almost 1000-fold (West Frey et al. 1993). This results partly from the different rate constants for polymerization from the 12 possible mispairs and also reflects enzyme- and sequence-specific influences. These are expected on the basis of the idea originally proposed by Brutlag and Kornberg (1972) and subsequently supported by extensive data (see, e.g., Bloom et al. 1994; Carver et al. 1994 and references therein) that a terminus containing a terminal mispair has a higher probability of being single-stranded ("frayed") than does a correctly paired terminus. Such a frayed end will preferentially bind to the exonuclease active site, which prefers single-stranded DNA. Similarly, a matched and, therefore, double-stranded terminus will preferentially bind to the polymerase active site, which prefers double-stranded DNA (for review, see Joyce and Steitz 1994). Because the stability of the duplex region of the template-primer will depend on its DNA sequence, proofreading efficiency is expected to differ in different sequence contexts having differing stabilities. Moreover, the degree of fraying needed to allow single-stranded DNA to bind to the exonuclease active site may vary, depending on the distance between the polymerase and exonuclease active sites. This distance, estimated to be 25–30 Å for the Klenow polymerase, could be greater for some enzymes than others (e.g., compare data in Cowart et al. [1989] to data in Capson et al. [1992]; for review, see Joyce and Steitz 1994), leading to enzyme-mediated differences in proofreading efficiency. It has

also been proposed that the Klenow polymerase active site promotes movement of DNA into the exonuclease active site by rejecting aberrant primer termini (Carver et al. 1994).

Base Substitution Error Rates of Eukaryotic DNA Polymerases

The above concepts provide a framework for considering what is known about the substitution fidelity of eukaryotic DNA polymerases. Error rates during catalysis in vitro by the five template-dependent eukaryotic DNA polymerases (see Wang, this volume) are shown in Table 1. These were obtained using fidelity assays that require both misinsertion and mispair extension to score an error. The "average" values are from copying a 250-base single-stranded template sequence of the *lacZ* α-complementation gene in bacteriophage M13mp2 DNA (Kunkel 1985a). This assay scores all stable misincorporations that yield an M13 plaque

Table 1 Error rates of eukaryotic DNA polymerases

DNA polymerase	Average error rate ($\times 10^{-6}$)	Refs.	Range of error rates ($\times 10^{-6}$)	Refs.
Substitution errors				
pol-α	160[a]	1–3	1.2–380	1–13
pol-β	670	2	45–1000	2, 9
pol-δ	~10[b]	4	≤2.3–29	4
pol-ε	≤6.7	4	≤1–19[c]	4, 14
pol-γ	1.8[d]	15, 16	1.8–1200[e]	2, 9, 15, 16
One-base frameshift errors				
pol-α	50[a]	1, 4, 17		
pol-β	900	17		
pol-δ	18[b]	4		
pol-ε	5[c]	4		
pol-γ	2.4	24		

References: (1) Kunkel et al. 1989; (2) Kunkel and Alexander 1986; (3) Roberts and Kunkel 1988; (4) Thomas et al. 1991b; (5) Copeland and Wang 1991; (6) Reyland and Loeb 1987; (7) Grosse et al. 1983; (8) Kaguni et al. 1984; (9) Kunkel and Loeb 1981; (10) Brooke et al. 1991; (11) Copeland et al. 1993; (12) Dong et al. 1993a; (13) Perrino and Loeb 1989b; (14) Kunkel et al. 1987; (15) Wernette et al. 1988; (16) Kunkel and Mosbaugh 1989; (17) Kunkel 1986.

[a]Measurements have been made with pol-α preparations from several sources; the values given are averages.

[b]Reactions contained PCNA to stimulate gap-filling synthesis by pol-δ.

[c]Higher values obtained with reactions in which proofreading was compromised; thus, they probably represent a minimal estimate of the accuracy of the enzyme.

[d]Data are from reversion assays.

[e]See comment in Reference 16 regarding the higher error rate values.

with reduced blue-color intensity. The type of polymerase error is defined by sequencing the identified M13 mutants. The substitution error rates are thus average values per detectable nucleotide polymerized, for all 12 possible single-base mispairs in a variety of sequence contexts (Bebenek and Kunkel 1995). The wide range of error rates reported in the literature (Table 1) illustrates that substitution fidelity depends on the DNA polymerase, the composition of the mispair, and the local template-primer sequence. The influence of these parameters can be examined at specific template positions using steady-state kinetic approaches (Mendelman et al. 1989, 1990). This allows the amount of discrimination at both the misinsertion and mispair extension steps to be estimated separately. The fidelity of the eukaryotic DNA polymerases is considered in more detail below.

DNA Polymerase-α

This is the most extensively studied eukaryotic polymerase. DNA polymerase-α (pol-α) isolated from several sources has an average base substitution error rate of 160×10^{-6} when a variety of errors in numerous sequence contexts are considered collectively (Table 1). Similar values have been obtained when the fidelity of the yeast or human p180 catalytic subunit alone is compared to that of the four-subunit DNA polymerase-α:RNA primase complex (Kunkel et al. 1989; Copeland et al. 1993). Similar values for misinsertion fidelity, representing the product of steps A, B, and C in Figure 1, have also been observed by kinetic analyses of the *Drosophila melanogaster* DNA polymerase-α:DNA primase (Mendelman et al. 1989). Thus, highly purified DNA pol-α is not particularly accurate relative to the high fidelity required to replicate eukaryotic genomes (see below). However, a 10^{-4} error rate may more than suffice if pol-α is only responsible for synthesis of a small number of nucleotides from an RNA primer. Mistakes made here could also be removed during the RNA primer excision-replacement synthesis reaction. Moreover, pol-α fidelity estimates are thus far limited to synthesis initiated from exogenously supplied DNA primers. Since several recent observations suggest that pol-α synthesis coupled to primase activity may differ in some respects (Sheaff et al. 1994), it is possible that the fidelity of RNA-primed DNA synthesis could be higher (or lower) than present data suggest.

An average error rate of about 10^{-4} is consistent with the fact that many preparations of purified pol-α lack $3' \rightarrow 5'$ exonuclease activity and that pol-α genes lack the three conserved sequence motifs character-

istic of such exonucleases. Nonetheless, evidence exists for pol-α-associated proofreading. A 3′→5′ exonuclease activity is present in preparations of *D. melanogaster* pol-α from which the associated 70-kD subunit has been removed (Cotterill et al. 1987), and the resulting polymerase is more accurate than the pol-α:primase complex (Cotterill et al. 1987; Reyland et al. 1988). Proofreading activity may be removed during some purification schemes but retained during others. For example, mouse and human pol-α preparations have been reported that contain 3′→5′ exonuclease activity (Chen et al. 1979; Bialek et al. 1989), and the latter catalyzes high-fidelity synthesis. Precedent for proofreading by exonuclease activity of a separate gene product comes from studies in *E. coli*, where the polymerase (α subunit) and exonuclease (ε subunit) activities of the replicative Pol III holoenzyme are encoded by two different genes, designated *dnaE* and *dnaQ*, respectively (for review, see Echols and Goodman 1991). Thus, the difficulty that pol-α has in extending certain mispairs (Perrino and Loeb 1989a; Mendelman et al. 1990) could provide the opportunity for a separate exonuclease activity to proofread misinserted nucleotides, especially if pol-α dissociates from the template primer. This possibility is supported by the observation of high-fidelity DNA synthesis by pol-α in the presence of the *E. coli* ε subunit (Perrino and Loeb 1989b) or pol-δ (Perrino and Loeb 1990), which contains an intrinsic 3′→5′ exonuclease activity. The extraordinary range of error rates reported in the literature for synthesis by pol-α (Table 1) thus reflects the large number of studies performed and the use of different assay methods and pol-α preparations. It also clearly reflects differences in discrimination for mispairs of varying composition and in different local sequence contexts.

There has been much recent progress in defining the structure of DNA polymerases (for review, see Joyce and Steitz 1994). Moreover, several conserved amino acid motifs have been identified by sequence alignments of DNA polymerase genes (Delarue et al. 1990; Braithwaite and Ito 1993 and references therein). These data are the starting points to identify amino acid residues that, when changed, alter replication fidelity. Among the eukaryotic polymerases, this approach has been applied first to human pol-α. Several mutant enzymes have been constructed containing single amino acid differences in the most conserved motifs, designated I and II (for review, see Wang 1991). The proteins were overproduced, and their biochemical properties were examined. Two of the motif I mutants, having single-amino-acid differences in residues important for binding the divalent metal ion required for catalysis, have higher discrimination than the wild-type pol-α for nucleotide misinsertion and

for mispair extension in Mn^{++}-activated kinetic assays (Copeland et al. 1993). Likewise, two of the motif II mutants, having single-amino-acid differences in residues suggested to be important for binding the incoming dNTP and for interacting with the primer, have improved insertion fidelity (Dong et al. 1993a), whereas another has reduced discrimination against mispair extension (Dong et al. 1993b). A continuation of this approach with pol-α should increase our understanding of the fidelity of this polymerase. Polymerases with reduced or enhanced fidelity should also be useful "biomarkers" for defining their roles in replication and repair.

DNA Polymerase-β

This smallest of the eukaryotic DNA polymerases is also the least accurate. The single subunit polymerases purified from rat hepatoma cells or chicken embryos have average substitution error rates of 670×10^{-6} (Kunkel and Alexander 1986). For individual mispairs, error rate values range from 45×10^{-6} to 1000×10^{-6}. Similar, and in a few instances even higher, error rates have been obtained for direct misinsertion by the rat enzyme, using steady-state kinetic analyses of rat pol-β (Boosalis et al. 1989). These rates are consistent with the fact that purified pol-β lacks associated 3′→5′ exonuclease activity (and the three exonuclease motifs conserved in proofreading polymerases). However, although pol-β shares this property with pol-α, it is even less accurate, demonstrating that, independent of proofreading, selectivity against substitution errors depends on the DNA polymerase.

Low-fidelity synthesis by pol-β may be consistent with a modest catalytic role in vivo, i.e., filling gaps of one or a few nucleotides during base excision repair. Alternatively, pol-β may have higher accuracy than current estimates suggest. Thus far, pol-β fidelity has been measured using template primers containing long single-stranded template regions, where synthesis is distributive rather than processive. Recently, pol-β has been shown to catalyze processive synthesis on a template adjacent to a 5′-phosphoryl end (Singhal and Wilson 1993). As this type of synthesis may more closely resemble that occurring during base excision repair, it will be interesting to determine pol-β fidelity using substrates containing single-stranded gaps of one or a few nucleotides. It is also possible that pol-β fidelity is influenced by accessory proteins. For example, mammalian DNase V, a 12-kD protein having both 3′→5′ and 5′→3′ exonuclease activity (Mosbaugh and Meyer 1980), associates with pol-β in vitro.

X-ray crystallographic structural information is now available for the ternary complex of pol-β-template•primer-dNTP (Pelletier et al. 1994). This structure provides an excellent opportunity for polymerase engineering to understand fidelity.

DNA Polymerase-δ

The fidelity of DNA synthesis in vitro by this polymerase is remarkably understudied, given its central role in eukaryotic replication. In one study (Thomas et al. 1990), the average substitution error rate in vitro of pol-δ plus proliferating cell nuclear antigen (PCNA) is about 10×10^{-6} (Table 1). The error rate of pol-δ alone was not determined because pol-δ would not complete gap-filling synthesis in the absence of PCNA. The fact that pol-δ (plus PCNA) is more accurate than pol-α or pol-β is consistent with proofreading of misinsertions by its associated $3' \rightarrow 5'$ exonuclease activity, which has properties expected of an editing exonuclease (for review, see Bambara and Jessee 1991). The coding sequences of the yeast and human genes have the three conserved exonuclease motifs characteristic of proofreading enzymes (Chung et al. 1991; Zhang et al. 1991).

DNA Polymerase-ε

This polymerase also has an associated exonuclease activity, and the coding sequence of the gene has the three conserved exonuclease motifs characteristic of proofreading enzymes (Morrison et al. 1991). Pol-ε is highly accurate, having a very low rate for substitutions that revert a termination codon in the *lacZ* gene (Kunkel et al. 1987)[1] and an average error rate of $\leq 6.7 \times 10^{-6}$ in the forward mutation assay. In fact, substitution errors by pol-ε are only detected when dGMP, a putative inhibitor of exonucleolytic proofreading, is included in the synthesis reaction. This is consistent with the idea that the $3' \rightarrow 5'$ exonuclease of pol-ε removes misinsertions during synthesis in vitro.

DNA Polymerase-γ

The mitochondrial replicative polymerase from several sources has high base-substitution fidelity (Table 1). The polymerase also has an associated $3' \rightarrow 5'$ exonuclease activity (Kunkel and Soni 1988a; Insdorf and Bogenhagen 1989; Kaguni and Olsen 1989; Kunkel and Mosbaugh 1989;

[1]At the time this study was performed, what is now known to be DNA polymerase-ε was then designated DNA polymerase-δII, hence the title of the article.

Foury and Vanderstraeten 1992; Gray and Wong 1992), and the coding sequence of the yeast gene has the three conserved exonuclease motifs characteristic of proofreading enzymes (Foury and Vanderstraeten 1992). The catalytic properties of the exonuclease and the fact that fidelity is reduced in reactions containing a high dNTP concentration or dGMP (Kunkel and Soni 1988a; Kunkel and Mosbaugh 1989) are consistent with a proofreading role in vitro.

ERRORS INVOLVING TEMPLATE-PRIMER MISALIGNMENT

In addition to direct misincorporation of noncomplementary nucleotides, base-addition, -deletion, and even -substitution errors can be generated by processes involving template-primer misalignments during replication.

Frameshifts[2] Initiated by Template-primer Slippage

Strand slippage during replication of iterated[3] sequences results in misaligned intermediates stabilized by correct base pairs (Fig. 2A). Subsequent polymerization from the misaligned intermediate leads to deletion if the unpaired nucleotide(s) is in the template strand (Fig. 2A) or to addition if the unpaired nucleotide(s) is in the primer strand (not shown). This mechanism predicts (Streisinger et al. 1967) that the error rate should increase as the length of the run increases, because the potential number of correct base pairs that could stabilize the misaligned intermediates increases, as does the number of potential misaligned intermediates that can form (Fig. 2). Furthermore, the longer the run, the greater the distance between the extra nucleotide and the 3′-OH primer terminus, potentially reducing interference by the extra base during phosphodiester bond formation within the enzyme active site.

In support of this logic, frameshift error rates, expressed per nucleotide polymerized to correct for differences in the number of nucleotides in runs of different lengths, do indeed increase as the length of a homopolymeric run increases, for DNA pol-α (Kunkel 1990) and T7 DNA polymerase (Kunkel et al. 1994). In addition, error rates for one-base deletions in homopolymeric runs by pol-β (Kunkel 1986) and HIV-

[2]Although the term frameshift mutation usually refers to changes in the number of base pairs in a protein-coding sequence that are not multiples of three, for convenience it refers here to mutations resulting from any difference in the number of base pairs, regardless of location.

[3]A non-iterated nucleotide is one having 5′ and 3′ neighbors that are not identical to the nucleotide considered. An iterated nucleotide has at least one identical neighbor. Iterated means repeated; reiterated means re-repeated.

1 RT (Bebenek et al. 1993) decrease when the template sequence is altered to either shorten or eliminate the repetitive sequence. That the slippage mechanism operates in vivo is suggested by the instability in repetitive sequences associated with cancer (for review, see Loeb 1994) and neurodegenerative diseases (see, e.g., Willems 1994).

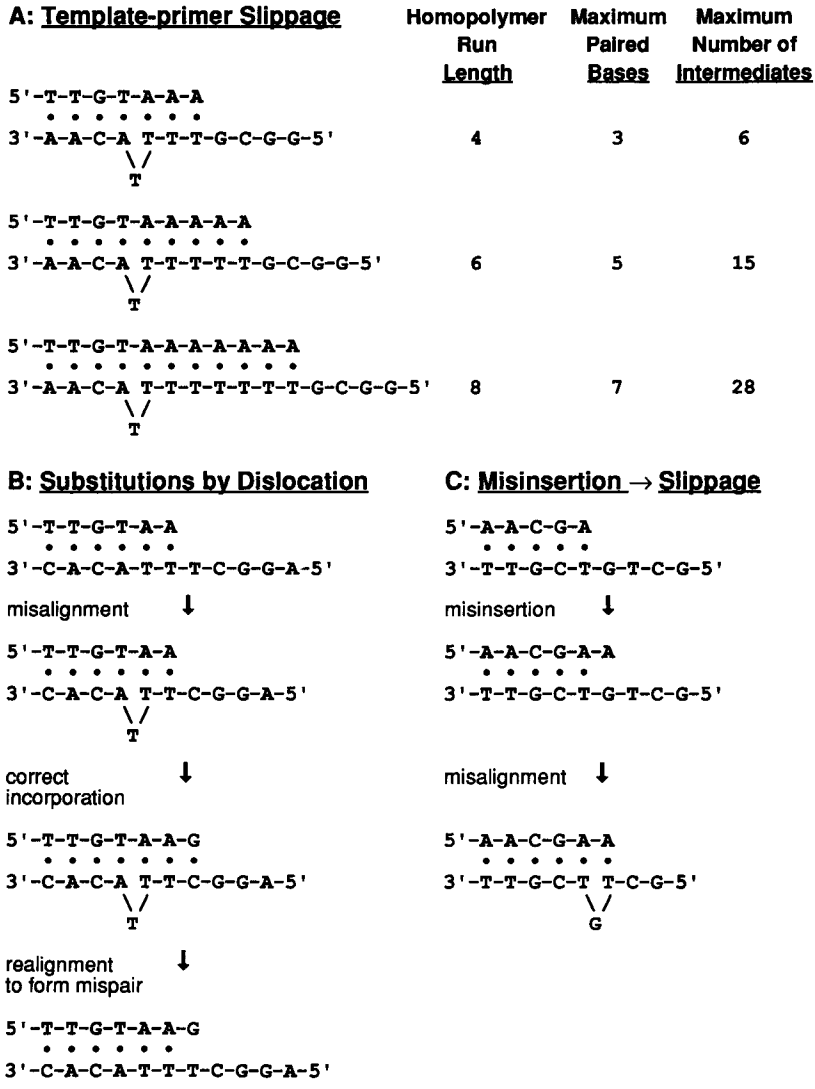

Figure 2 Pathways for errors involving misaligned template primers. See text for description.

Base Substitutions Initiated by Strand Slippage

Following slippage, correct incorporation of another nucleotide followed by realignment before continued incorporation generates a terminal mispair (Kunkel 1985a). This can yield a base substitution, but in this case, initiated by slippage rather than by misinsertion. This process has been termed dislocation mutagenesis (Kunkel and Alexander 1986) by analogy with a dislocated shoulder joint that pops out of alignment but ultimately resumes a normal position. Strong support for the model comes from fidelity studies with pol-β (Kunkel and Soni 1988b; Boosalis et al. 1989) and HIV-1 RT (Bebenek et al. 1993) in vitro. With both polymerases, strong base substitution hot spots are observed at the ends of several different homopolymeric runs, and the substitution specificity depends on the immediate template neighbor. The dislocation concept is not limited to the situation shown in Figure 2, but can involve an extra nucleotide in the primer strand (Fig. 5A in Bebenek et al. 1993), two unpaired template nucleotides (Fig. 5C in Bebenek et al. 1993), or many nucleotides (Fig. 3C in Kunkel and Soni 1988a; for review, see also Ripley 1990).

Frameshifts Initiated by Misinsertion

A distinctly different way to generate a misaligned substrate is misincorporation followed by template-primer rearrangement to provide a correct terminal base pair for continued polymerization (Fig. 2C). The resulting misalignment ultimately leads to a frameshift error, but in this case it is initiated by misinsertion rather than strand slippage. This model was suggested by several observations (for review, see Kunkel 1990) indicating that "difficult-to-extend" mispairs may realign such that synthesis proceeds from a substrate containing an extra nucleotide in the template strand but a correct base pair at the terminus.

In principle, this mechanism is possible at any template position and is not limited to the production of minus-one-base errors. Thus, frameshift errors at template runs, as well as plus and minus errors of varying numbers of nucleotides, may initiate by misincorporation. The model has been supported by fidelity studies with yeast pol-α (Kunkel et al. 1989), Klenow DNA polymerase (Bebenek et al. 1990), and HIV-1 RT (Bebenek et al. 1992) in vitro. The concept of difficult-to-extend termini led to the suggestion (Kunkel and Soni 1988b) that incorporation opposite damaged templates might also yield frameshifts by this mechanism. Studies involving replication of DNA containing several different lesions (Wang and Taylor 1992; Shibutani and Grollman 1993; Lindsley and Fuchs 1994; Napolitano et al. 1994) strongly support this suggestion.

Frameshift Error Rates of Eukaryotic DNA Polymerases

The average frameshift error rates of the five template-dependent eukaryotic DNA polymerases are shown in Table 1. These rates are errors per detectable nucleotide polymerized with the *lacZ* α-complementation gene target and are for single-base frameshift errors. Similar to the situation with substitution errors, DNA pol-α and pol-β, which lack associated proofreading activity, are less accurate than are pol-δ, pol-ε, and pol-γ, which have intrinsic exonucleases. Direct comparison of the frameshift fidelity of wild-type versus exonuclease-deficient derivatives of Klenow polymerase (Bebenek et al. 1990) and T7 DNA polymerase (Kunkel et al. 1994) strongly suggests that frameshift intermediates at both non-run sequences and in homopolymeric runs of up to 5 bp are subject to exonucleolytic proofreading. Moreover, the frameshift fidelity of pol-ε is reduced in reactions containing a high concentration of dNTPs and dGMP (Thomas et al. 1991b), suggesting that frameshift errors by this enzyme are also proofread.

Polymerase rates for minus-one-base errors are higher than for plus-one-base errors. Just as for substitution errors, frameshift error rates are sequence-dependent and polymerase-dependent, with error rates for the same mistake varying over 1000-fold (for detailed discussion, see Kunkel 1990). Polymerases can also delete or add more than a single nucleotide. Some of these errors can be explained by strand slippage between perfectly repeated DNA sequences separated by a variable number of intervening nucleotides (for review, see Kunkel 1990; Ripley 1990). More complicated models involving strand-switching, primer loop-back, and palindromic DNA sequences have also been invoked to explain complex frameshift mutations generated by DNA polymerases.

Processivity and Frameshift Fidelity

One property of polymerization relevant to polymerase frameshift fidelity at homopolymeric runs is processivity, the number of nucleotides incorporated per polymerase association/dissociation with the template primer. This was suggested by the observation that pol-α is both more accurate and more processive than pol-β (Kunkel 1985b). This correlation has been examined in greater detail with HIV-1 RT. This polymerase is inaccurate for one-base frameshifts within some but not all template runs. These hot spots for frameshift errors are also template positions where the probability of termination of processive synthesis is high (Bebenek et al. 1989). Moreover, when changes were introduced into the sequences flanking the runs, increases or decreases in frameshift

error rates were observed, and these often correlated with concomitant increases or decreases in termination of processive synthesis within the run (Bebenek et al. 1993). The data thus reveal a consistent pattern wherein low processivity correlates with low frameshift fidelity, consistent with the idea that the formation and/or utilization of misaligned template primers is increased during the dissociation-reinitiation phase of a polymerization reaction.

Effect of Accessory Proteins on DNA Polymerase Fidelity

Further support for a relationship between processivity and fidelity comes from a study of the frameshift fidelity of T7 DNA polymerase with and without its processivity protein, thioredoxin (Kunkel et al. 1994). T7 DNA polymerase alone has low processivity, adding only 1–50 nucleotides before dissociating. However, when it is complexed with its accessory subunit thioredoxin, polymerization proceeds for thousands of nucleotides without dissociation. Fidelity measurements with an exonuclease-deficient mutant of T7 pol showed that the rate for one-base addition frameshifts at homopolymeric runs was 46-fold higher in the absence of thioredoxin than in its presence. This may have general significance, given that accessory proteins that enhance processivity are a general feature of multiprotein replication complexes (see Stillman, this volume). Frameshift fidelity conferred by the polymerase clamp protein is particularly interesting inasmuch as replication infidelity is one possible explanation for the instability of repetitive genomic sequences reported for several diseases (Loeb 1994; Willems 1994).

In the absence of thioredoxin, the exonuclease-deficient T7 DNA polymerase was found to be more accurate during DNA synthesis in vitro for substitutions and 1- and 2-nucleotide deletions (Kunkel et al. 1994). One possible explanation is that the premutational intermediates formed during polymerization are not successfully extended unless the polymerase is complexed with thioredoxin. The biological implication is that, under some circumstances, an accessory protein-mediated alteration in the extension rate from an unusual template primer, e.g., following incorporation opposite a damaged base or slippage at a damaged site, could actually serve a mutator function by enhancing extension synthesis to seal the error before transfer to the exonuclease active site for removal.

Another accessory protein that logically could influence fidelity is single-stranded DNA-binding protein (SSB). The substitution fidelity of synthesis by several polymerases is increased a few fold in reactions containing *E. coli* SSB (Kunkel et al. 1979, 1983). Similarly, the rate of

deletions between direct repeats generated by yeast pol-α is reduced in reactions containing a yeast SSB (Roberts et al. 1990). Human replication protein A (RP-A) is a 3-subunit SSB required for replication of SV40-origin-containing DNA in HeLa cell extracts. This protein increased the fidelity of pol-α-catalyzed gap-filling synthesis by 4-fold in one study (Carty et al. 1992) but had little effect on the frameshift fidelity of yeast pol-α:primase complex in a different study (Roberts et al. 1990). Results to date thus suggest that SSB does affect fidelity, but the effects are small relative to the high degree of discrimination imposed by the polymerase and exonuclease.

FIDELITY OF MULTIPROTEIN REPLICATION COMPLEXES

A full appreciation of how genomes are stably replicated, and how instability may arise to generate disease, requires a better understanding of the fidelity of the multiprotein replication machinery. An important step toward achieving this understanding has been the development of systems that replicate double-stranded DNA in vitro (numerous chapters in this volume). One system for studying human genomic replication depends on the SV40 origin of replication (Hassell and Brinton, this volume). Circular, double-stranded DNA substrates containing the SV40 origin can be fully replicated by the proteins present in primate cells, with only the addition of SV40 T antigen needed to initiate replication at the origin. For studies in vitro, replication can be performed in extracts of cells grown in culture or by reconstitution with purified proteins (see, e.g., Waga and Stillman 1994). The latter approach has defined two polymerases (pol-α and pol-δ) and several additional proteins required for complete replication.

High Replication Fidelity with Undamaged DNA

Using forward mutation assays with either the *lacZ* (Roberts and Kunkel 1988, 1993) or *supF* (Hauser et al. 1988) reporter gene, DNA replicated in human HeLa and simian CV-1 cell extracts was found to have a mutant frequency that was not increased significantly above the background mutant frequency of unreplicated DNA. Sequence analysis of *lacZ* mutants recovered from the unreplicated as well as replicated DNA showed no significant differences, yielding error rates varying from $\leq 6.2 \times 10^{-6}$ to $\leq 0.1 \times 10^{-6}$, depending on the substitution or frameshift error considered (Thomas et al. 1991b). Inasmuch as these HeLa cell extracts

have mismatch repair activity (Thomas et al. 1991a), these rates represent the sum of both replication fidelity and any heteroduplex repair occurring in the extract. Two studies (see below) suggest that mismatch repair in the extract only affects error rate determinations by 2- to 3-fold, suggesting that replication fidelity itself is high. Because no replication errors are detected with undamaged DNA and equimolar dNTP concentrations, further understanding of how high replication fidelity is achieved and how it can be perturbed requires manipulation of reaction components in order to obtain replication errors. Several approaches have been used to address specific questions.

Proofreading and the Fidelity of Leading- and Lagging-strand Replication

One obvious question is whether proofreading is partly responsible for high replication fidelity. To answer this question, reactions were performed with unequal dNTP concentrations to force specific misinsertions. For example, misincorporation of dGTP to revert a TGA opal codon can be obtained by increasing the concentration of dGTP relative to dATP (Fig. 3). The contribution of proofreading to replication fidelity can then be examined either by increasing absolute dNTP concentrations to stimulate polymerization at the expense of proofreading, or by inhibiting proofreading by adding deoxynucleoside monophosphate to the replication reaction. Results from both approaches suggest that proofreading

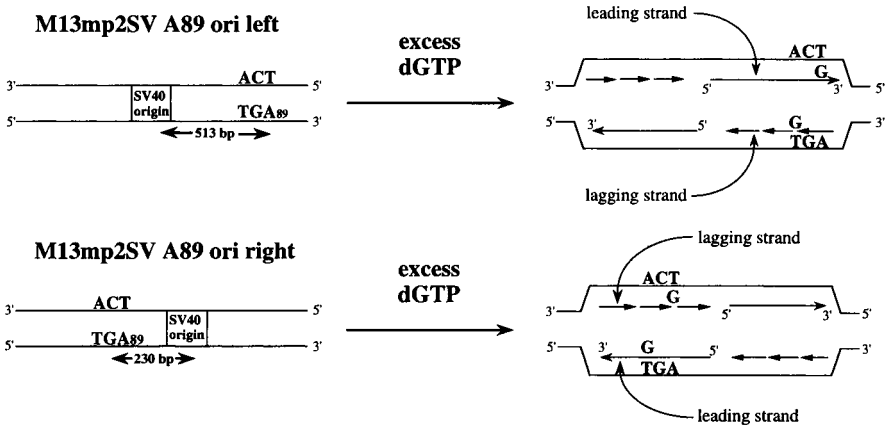

Figure 3 Representation of leading- and lagging-strand synthesis across an opal codon. See text for description.

contributes substantially to replication fidelity for base substitution (Roberts et al. 1991) and frameshift errors (Roberts et al. 1993).

The approach has been extended to examine the fidelity of the leading- and lagging-strand replication machinery. This requires comparing results with two vectors. The first (ori left, Fig. 3) contains the origin a few hundred nucleotides to the left of the reporter gene. This distance is small relative to the size of the vector (7398 bp). Previous studies (Edenberg and Huberman 1975; Li and Kelly 1985) have indicated that the rate of replication fork movement is similar in both directions from the origin. Thus, with this vector the (+) viral strand within the *lacZ* target is likely to be replicated by the lagging-strand replication apparatus. The second vector contains the origin to the right of the target, again only a few hundred nucleotides from the reporter gene (Fig. 3). In this vector, the (+) viral strand in the *lacZ* target is assumed to be replicated by leading-strand replication proteins. Fidelity measurements with these two vectors allow determination of the fidelity of replication of the same sequence by either the leading- or lagging-strand apparatus, provided that the strand on which the error was made can be assigned. This is done with dNTP substrate pool imbalances (Fig. 3), where the dNTP provided in excess is assumed to be responsible for the substitution error observed, or with template DNA damage (see below).

When this approach was combined with the reaction conditions that diminish proofreading, the data suggested that proofreading contributes to substitution fidelity during both leading- and lagging-strand replication (Roberts et al. 1991). The strategy has also been used to describe average leading- and lagging-strand replication rates for errors resulting from misinsertion of either TTP (Roberts et al. 1994) or dGTP (Izuta et al. 1995). Average error rates for replication of the *lacZ* template in a HeLa cell extract suggest that leading- and lagging-strand replication fidelity is similar for several errors but different for others (Table 2) (for additional data, see Izuta et al. 1995). There are several possible explanations for leading- and lagging-strand error rate differences. One is that mismatch repair in the extract is responsible for the asymmetry. A second is that replication of the two strands is highly asymmetric, providing unequal opportunities to make mistakes. Replication of the two strands may be performed by different DNA polymerases or perhaps the same polymerase but with a different complement of accessory proteins (for models, see Waga and Stillman 1994; Stillman; Hassell and Brinton; both this volume). This could yield differences in misinsertion rates and/or ability to extend rather than proofread mispaired or misaligned template primers. Replication on the leading strand is highly processive

Table 2 Comparative leading- and lagging-strand replication error rates

Error considered	Strand	Mispair frequency x 10^{-6} leading	lagging	Ratio lagging:leading
Average from extract reaction				
G•dTTP	–	18	39	2:1
C•dTTP	–	28	32	1:1
G•dTTP	+	2.5	82	33:1
C•dTTP	+	2.5	21	8:1
Average from reconstituted reaction				
G•dTTP	–	43	60	1:1
C•dTTP	–	≤7.1	43	≥6:1
G•dTTP	+	≤5.4	200	≥37:1
C•dTTP	+	11	43	4:1
Error rates in extract at specific sites				
T•dGTP	+ (121)	1.6	19	12:1
G•dTTP	+ (145)	≤4.8	71	≥15:1

as compared to discontinuous synthesis of Okazaki fragments on the lagging strand, which involves more than one DNA polymerase and/or one or more switches between enzymes as well as the synthesis and eventual replacement of RNA primers.

The possible influence of mismatch repair on replication fidelity in extracts has been examined by separating a HeLa cell extract into two fractions, neither of which has replication activity. When combined, these fractions reconstitute replication activity that is devoid of mismatch repair activity (Roberts et al. 1994). When the fidelity of this reaction was examined using excess dTTP (Roberts et al. 1994) or excess dGTP (Izuta et al. 1995), error rates were increased by only 2- to 3-fold over those observed in extracts having mismatch repair activity, confirming that replication is indeed highly accurate. Moreover, the error specificity was similar to that in extracts (Table 2) (Roberts et al. 1994; Izuta et al. 1995), including unequal leading- and lagging-strand rates for G•dTTP and C•dTTP errors on the plus strand (Table 2). Thus, at least these error rate asymmetries are not due to differential mismatch repair. The availability of human cell extracts having high replication activity but defective in mismatch repair due to mutant mismatch repair genes (Umar et al. 1994) will facilitate future studies of the fidelity of the human replication machinery in the absence of mismatch repair.

In another study of replication reconstituted from individual components, reactions were performed with pol-α as the only DNA polymerase (Carty et al. 1990). Fidelity was found to be intermediate be-

tween that of the unfractionated replication system and gap-filling synthesis by purified HeLa cell pol-α. This suggests that pol-α can replicate DNA with high fidelity when carrying out semiconservative DNA replication, but that additional cellular factors not present in the reconstituted system may be contributing to the higher replication fidelity of the unfractionated system. Even unfractionated extracts may be missing fidelity components that are active in vivo or simply not functioning in the extract.

Leading- and lagging-strand error rates differ at template positions 121 and 145 on the plus strand of the *lacZ* gene in M13mp2SV (Table 2). Two observations suggest that these differences may be due to differential proofreading. First, at both positions, the next correct dNTP to be incorporated following the misinsertion is the nucleotide present in excess (and, therefore, at high concentration) during replication. As explained above (Fig. 1), this situation would favor polymerization at the expense of excision of the terminal mispair. Thus, both hot spots for lagging-strand replication errors are sites where proofreading might be partially suppressed. Second, when replication reactions were repeated with added dGMP to inhibit proofreading, the error rates at both positions increased with both ori left and ori right substrates (Izuta et al. 1995). If one assumes that the addition of dGMP does not affect the inherent base selectivity of the insertion step, then the dGMP-dependent increase in error rate suggests that misinsertions are indeed occurring that, in the absence of monophosphate, are removed by the exonuclease. Inasmuch as fidelity in the absence of dGMP is higher during leading-strand replication, this suggests that leading-strand misinsertions are more effectively excised than are the lagging-strand errors that are readily detected even when dGMP is absent. Differential proofreading thus provides one mechanism to explain differences in leading- and lagging-strand replication fidelity. Since the assignment of the leading- and lagging-strand DNA polymerases during eukaryotic replication is not yet definitive, proofreading on the two strands could be carried out by any of several exonucleases.

Replication Fidelity with Damaged Substrates

A large number of genes in eukaryotic cells either control or catalyze the repair of a wide variety of physical and chemical insults, some resulting from normal cellular processes (e.g., deamination, depurination, oxidative stress, alkylation) and others from exposure to the external environment (for recent reviews, see Hanawalt 1994; Hartwell and Kastan 1994; Sancar 1994 and numerous references therein). When these repair sys-

tems fail, lesions may persist in DNA or in dNTP precursor pools. A plethora of lesions have been described over the years that have different structures and thus potentially affect replication fidelity by altering different discrimination steps. For example, an alkylated base may have altered hydrogen-bonding potential and lead to direct misinsertion errors, whereas an abasic site has lost base hydrogen-bonding potential altogether. Bulky lesions may perturb base-pair geometry or base-stacking interactions leading to misinsertion, or their structures may be inconsistent with continued replication, leading to termination or template-primer rearrangement and frameshift errors. The presence of lesions in the template strand could also affect communication between the polymerase and exonuclease active sites that is critical for proofreading. Lesion-induced replication infidelity has been examined in a number of studies with purified DNA polymerases (for review, see Echols and Goodman 1991). Discussing all this information is beyond the scope of this chapter; we briefly review only a few recent studies of the fidelity of SV40 origin-dependent replication using damaged substrates.

Replication Infidelity with 8-O-dGTP

Oxidative metabolism is known to generate mutagenic compounds within cells, one of which is 8-oxo-deoxyguanosine. The presence of several lines of defense against mutations resulting from this base analog (for review, see Michaels and Miller 1992; Grollman and Moriya 1993) suggests that it is biologically important. A variety of DNA polymerases, as well as the replication complex in HeLa cell extracts, misincorporate the triphosphate form of this base analog, 8-O-dGTP, opposite template adenines, yielding A→C transversions (Cheng et al. 1992; Pavlov et al. 1994; Minnick et al. 1995). The data suggest that 8-O-dGTP could be highly mutagenic during genomic replication in eukaryotes. This may be the case for other modified dNTPs as well (see, e.g., Feig et al. 1994). The amount of 8-O-dGTP in human cells may be modulated by hydrolysis by the human homolog (Sakumi et al. 1993) of the *E. coli mutT* protein. If so, inactivating mutations in this gene (or other enzymes that sanitize dNTP pools) might result in a mutator phenotype in human cells.

Mutagenic Translesion Replication of DNA Containing Cyclobutane Pyrimidine Dimers

Among the insults that generate lesions in DNA, ultraviolet radiation has received perhaps the greatest attention, partly due to its established role as a skin carcinogen (Brash et al. 1991). The mutagenic potential of UV

photoproducts during replication by eukaryotic proteins has been examined in several studies. Pol-α is unable to synthesize past UV lesions (Moore et al. 1981), whereas DNA pol-δ in the presence of PCNA is able to replicate past *cis*-syn and *trans*-syn TT dimers (O'Day et al. 1992). Three studies have demonstrated translesion replication of cyclobutane pyrimidine dimers in cell extracts using SV40 origin-containing vectors (Carty et al. 1993; Thomas and Kunkel 1993; Thomas et al. 1993). This replication is mutagenic, with an error specificity similar to that observed in vivo (Keyse et al. 1988; Armstrong and Kunz 1990).

Mutagenic Translesion Replication of DNA Containing AAF Adducts

The ability of the replication complex in a HeLa cell extract to bypass site-specific N-2-acetylaminofluorene (AAF) adducts has also been examined (Thomas et al. 1994). The major effect was inhibition of replication, with termination occurring immediately before incorporation opposite the adduct. Among the replicated products was a higher proportion of those representing replication of the undamaged strand, leaving open the possibility that the first fork to encounter the lesion became uncoupled, i.e., replication of the damaged strand ceased while replication of the undamaged strand continued. Product analysis suggested that translesion bypass had occurred and that frameshift errors had been generated by the mechanism involving correct incorporation opposite the lesion followed by slippage (Fig. 2C).

FIDELITY OF DNA REPLICATION IN VIVO

How accurate is replication in vivo? This question has been elegantly addressed in *E. coli* by measuring mutation rates for a variety of sequence changes, using strains selectively disabled in key fidelity processes. The wild-type spontaneous mutation rate in the *lacI* reporter gene is 10^{-10} mutations per base pair replicated per generation (Table 3) (see, e.g., Schaaper 1993). A *mutL* mutant lacking methyl-directed postreplication mismatch repair has a 20- to 400-fold higher spontaneous mutation rate, depending on the type of mutation considered. With some simplifying assumptions (Schaaper 1993), the resulting mutation rate of about 10^{-7} can be considered as the fidelity of chromosomal replication. Analysis of a double mutant lacking mismatch repair and defective in proofreading by the ε subunit of the replicative DNA polymerase III holoenzyme (which replicates both the leading and lagging strands) suggests that

Table 3 Estimated contributions of the three major discrimination steps to replication fidelity

	Contribution		
	E. coli	eukaryotic	
Discrimination step	in vivo	in vitro	in vivo
Nucleotide selectivity	2×10^{-5}–2×10^{-6}	10^{-3}–10^{-6}	?
Exonucleolytic proofreading	40–200	0–200	10–≥200
Mismatch repair	20–400	–	10–≥700
Mutation rate	10^{-10}		$\leq 10^{-10}$

The values listed depend on simplifying assumptions and often involve caveats that are discussed in the text and in the references cited therein.

proofreading contributes between 40- and 200-fold to this rate, with the balance (factors of 200,000 to 2,000,000) representing the base selectivity of the replication machinery (Table 3).

Estimating replication fidelity in eukaryotes is complicated by the possibility of several types of mismatch repair, the influence of spontaneous damage, and the likelihood of multiple damage-repair pathways. Moreover, estimates are based on a few reporter genes, providing a limited view of replication fidelity for large eukaryotic genomes. Despite these qualifications, existing mutation rate data in mutant cells reveal a similar picture to that seen in *E. coli*. Mutation rates in eukaryotic cells are generally $\leq 10^{-10}$ mutations per base pair replicated per generation (see Loeb 1991 and references therein). The mutation rate in mismatch-repair-defective yeast (see, e.g., Strand et al. 1993; Prolla et al. 1994 and references therein) and human tumor cells (Kat et al. 1993; Bhattacharyya et al. 1994; Farber et al. 1994; Eshleman et al. 1995) are elevated up to several hundred-fold. Moreover, yeast mutants of pol-δ, pol-ε, and pol-γ containing substitutions for conserved exonuclease residues also have spontaneous mutation rates that are increased by up to several hundred-fold (Morrison et al. 1991, 1993; Foury and Vanderstraeten 1992), emphasizing the importance of proofreading in vivo during both nuclear and mitochondrial DNA replication.

CONCLUDING REMARKS

In many ways, our current view of eukaryotic replication fidelity is quite limited. Eukaryotic genomes are huge compared to the few hundred nucleotides scanned by current reporter genes. For example, the human genome contains a wide variety of repetitive sequence elements (see,

e.g., Beckman and Weber 1992) whose instability is associated with cancer (for review, see Loeb 1994) and neurodegenerative diseases (see, e.g., Willems 1994). Despite this association, we know very little about replication error rates in repetitive sequences except for short homopolymeric runs. The fact that eukaryotic cells devote an enormous amount of energy to checkpoints in the cell cycle and a multiplicity of DNA repair processes clearly indicates the biological risk associated with unrepaired lesions. Appropriately, a great deal of attention has been paid to studies of these processes, as exemplified by the fact that DNA repair was the "molecule of the year" in 1994 (Culotta and Koshland 1994). To fully appreciate the effects of unrepaired lesions, more studies are needed to define the consequences of an encounter between an unrepaired lesion and a eukaryotic replication fork, and, possibly, the fidelity of DNA synthesis associated with the repair processes themselves. Our understanding should increase as studies are performed using a variety of systems, eventually including those that mimic replication of highly organized nuclear and mitochondrial DNA.

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