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Error-prone replication of repeated DNA sequences by T7 DNA polymerase in the absence of its processivity subunit

(fidelity/strand slippage/DNA replication/accessory proteins)

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ABSTRACT **We have examined the effect of thioredoxin, an accessory protein that confers high processivity to bacteriophage T7 DNA polymerase, on the fidelity of DNA synthesis. In the presence of thioredoxin, exonuclease-proficient T7 DNA polymerase is highly accurate. In fidelity assays that score errors that revert M13mp2 *lacZ* α-complementation mutants, error rates are $\leq 2.2 \times 10^{-6}$ for base substitution and $\leq 3.7 \times 10^{-7}$ and $\leq 4.5 \times 10^{-7}$ for frameshifts that revert mutations in the +1 and −1 reading frames, respectively. Rates are more than 10-fold higher during synthesis by polymerase·thioredoxin complex lacking 3′ → 5′ exonuclease activity, demonstrating that frameshift as well as substitution errors are subject to proofreading. The contribution of thioredoxin to accuracy has been examined by comparing the fidelity of the exonuclease-deficient polymerase in the presence or absence of the accessory protein. Thioredoxin either enhances or reduces fidelity, depending on the type of error considered. In the absence of thioredoxin, T7 DNA polymerase is 3-fold more accurate for base substitutions and ≥27-fold and 9-fold more accurate, respectively, for 1- and 2-nt deletion errors at nonreiterated nucleotide sequences. Higher fidelity for all three errors may reflect the inability of the polymerase to continue synthesis from the premutational intermediates in the absence of the accessory protein. In marked contrast, the rate for frameshift errors wherein one or more nucleotides has been added to a repeated DNA sequence increases 46-fold when thioredoxin is absent from the polymerization reaction. The error rate increases as the length of the repeated sequence increases, consistent with a model where strand slippage creates misaligned template–primers. Thus, replicative expansion of repetitive sequences occurs in the absence of a replication accessory protein.**

Studies of the enzymology of DNA replication have demonstrated that DNA polymerases act in concert with a number of additional proteins to accomplish genomic replication (1). It is of interest to determine what effects these "accessory" proteins may have on the properties of the polymerase catalytic subunit. One system for such studies that is attractive for its simplicity is the replication apparatus of bacteriophage T7 (2). T7 gene *5* encodes a single 80-kDa polypeptide having both 5′ → 3′ DNA polymerization activity and 3′ → 5′ exonuclease activity (3–7). Within T7-infected *Escherichia coli* cells, this protein forms a tight, 1:1 complex with thioredoxin (3, 4, 8–10), a 12-kDa host protein. Working in coordination with the T7 gene *4* helicase–primase, this two-subunit complex can replicate the entire T7 genome (for review, see refs. 2 and 11).

One property of polymerization by the gene *5* protein alone that is not ideal for rapid genomic replication is its low processivity. Upon association with a template–primer, T7 DNA polymerase typically adds only 1–50 nt before dissociating (12). However, when the polymerase is complexed with thioredoxin, the stability of the gene *5* protein·template–primer complex is greatly increased (13) and the two-subunit complex can polymerize thousands of nucleotides without dissociating (12).

The ability of thioredoxin to confer high processivity on the polymerase catalytic subunit is of particular interest given observations obtained with other DNA polymerases on a possible relationship between the processivity and fidelity of polymerization. For example, a study with three eukaryotic DNA polymerases revealed a correlation between processivity and frameshift fidelity (14). The least processive enzyme, DNA polymerase β, had very low fidelity for 1-nt deletion errors; moderately processive DNA polymerase α had intermediate frameshift accuracy, and highly processive DNA polymerase γ was highly accurate. In another study, the reverse transcriptase of human immunodeficiency virus type 1 showed a strong tendency to terminate processive synthesis at certain nucleotide positions (15, 16). When these positions were homopolymeric runs of three or more of the same nucleotide, they were found to be hot spots for 1-nt frameshift mutations (15, 17). Furthermore, introducing single nucleotide changes into the template sequence at certain positions concomitantly changed both the processivity and the fidelity of the reverse transcriptase. When processivity increased, so did frameshift fidelity. When the base change resulted in a higher probability of termination of processive synthesis, frameshift fidelity at that site decreased (17). Together, these correlations between processivity and fidelity suggest that the formation and/or utilization of misaligned template–primers is increased during the dissociation–reinitiation phase of a polymerization reaction.

Given the effect of thioredoxin on the processivity of T7 DNA polymerase, the present study was undertaken primarily to determine whether the fidelity of the nonprocessive T7 DNA polymerase catalytic subunit alone is different from that of the highly processive polymerase·thioredoxin complex. To examine this, we took advantage of progress from earlier studies of the fidelity of the T7 DNA polymerase·thioredoxin complex (18–20). Those studies showed that the wild-type complex is highly accurate for base substitutions due to the high selectivity of the polymerase against both misinsertion and mispair extension, the latter providing for efficient editing by the 3′ → 5′ exonuclease activity. In those studies, the role of the exonuclease in enhancing base substitution fidelity was established by comparing results with the wild-type complex to those obtained with an exonuclease-deficient polymerase·thioredoxin complex. Correspondingly, here we first compare wild-type versus exonuclease-deficient polymerase·thioredoxin complex, in order to assess whether

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the exonuclease can also edit nucleotide addition and deletion errors. The results indicate that frameshift errors are indeed proofread. We then examine the contribution of thioredoxin to fidelity by comparing exonuclease-deficient polymerase·thioredoxin complex to exonuclease-deficient polymerase alone. The data indicate that thioredoxin can enhance or diminish fidelity, depending on the error considered. The most striking observation is that the rate of addition errors within repetitive sequences is greatly increased in T7 DNA polymerase reactions catalyzed in the absence of thioredoxin.

MATERIALS AND METHODS

Strains and Reagents. *E. coli* strains, the sources of reagents, and preparation of substrates were as described (21, 22). T7 DNA polymerase, its exonuclease-deficient D5A E7A derivative, and *E. coli* thioredoxin were purified as described (18). The polymerase–thioredoxin complex was reconstituted as described (18).

DNA Synthesis Reactions. Reaction mixtures (25 μl) contained 40 mM Tris·HCl (pH 7.5), 2 mM dithiothreitol, 10 mM $MgCl_2$, equimolar concentrations of dATP, dGTP, dCTP, and dTTP, 30 fmol of the designated gapped M13mp2 DNA, and either 300 fmol of T7 DNA polymerase·thioredoxin complex or 600 fmol T7 DNA polymerase alone. Reactions were performed first with 1000 μM dNTPs in order to maximize the probability that if a nucleotide was misinserted it would be sealed into double-stranded DNA by continued synthesis and then scored as an error upon transformation in *E. coli*. Subsequent reactions were also performed with 50 μM dNTPs, a concentration more closely approximating that found *in vivo*. (As shown below, mutant frequencies are similar for the two reaction conditions.) Incubation was at 37°C for 15 min (T7 DNA polymerase·thioredoxin complex) or 30 min (T7 DNA polymerase alone) and reactions were terminated by adding EDTA to 15 mM. A portion of each reaction was analyzed by agarose gel electrophoresis (23) to monitor the extent of synthesis. In all cases, polymerization yielded reaction products (not shown) that migrated as a discrete band coincident with a double-stranded nicked (replicative form II, RFII) DNA standard. No unfilled or partially filled gapped molecules were observed. Under the conditions of electrophoresis used, resolution of a gapped DNA molecule from the RFII standard can be made to within ≈50 nt. Note that, for the forward mutation assay (described below), synthesis need proceed for only 250 of the 390 nt in the gap in order to completely copy the target (23). Similarly, for the reversion assays, synthesis need proceed for only 130 of the 361 nt in the gap to copy the reversion target sequences (21, 24).

Fidelity Assays. All fidelity assays used here score errors in the *lacZ* sequence in M13mp2 DNA, generated during polymerization to fill a short gap in an otherwise double-stranded molecule. For the forward mutation assay (23), correct polymerization produces DNA that yields dark blue M13 plaques upon transfection of an α-complementation *E. coli* host strain followed by plating on indicator plates. Errors are scored as lighter blue or colorless plaques. With this assay, a large number of different types of mutations at many different sites can be monitored (see below).

For reversion assays (21, 24), the gapped substrates (gap lengths of 361, 362, or 363 nt, depending on the assay; see below) contain a mutant sequence that encodes a colorless plaque phenotype. Incorrect polymerization produces DNA that yields blue revertant plaques upon transfection and plating. The three reversion assays employed in this study are described below. The procedures for transfection of copied DNA, plating infected cells, scoring plaques colors, confirmation of mutant phenotypes, and sequence analysis of independent mutants have been published (21, 22).

RESULTS

Fidelity of Wild-Type T7 DNA Polymerase. The fidelity of the exonuclease-proficient T7 DNA polymerase·thioredoxin complex was examined by using reversion assays that score several different errors. The first DNA substrate contained a TGA termination codon in the *lacZ* gene (at positions 87–89), and monitors eight substitution errors that restore α-complementation—i.e., blue plaque color (21). Synthesis by the wild-type polymerase generates products that yield reversion frequencies (Table 1, top line) that are not significantly different from the background frequency of the assay (typically $0.2–0.5 \times 10^{-5}$). This limits the estimation of fidelity to a "less than or equal to" error rate, which can be calculated from the reversion frequency by correcting for the expression of errors in the newly synthesized strand upon transfection into the *E. coli* host cell (for calculation, see legend to Table 1). The average substitution rate per detectable nucleotide incorporated at the three nucleotides of the opal codon is $\leq 2.2 \times 10^{-6}$.

The second assay (24) uses a DNA substrate containing an extra T in a run of template T residues at positions 70–73 in the *lacZ* coding sequence and scores errors that restore the correct reading frame—i.e., −1, +2, −4, +5, etc. This includes −1 nucleotide frameshifts at the TTTTT run (dark-blue phenotype) as well as −1 errors at 36 other template nucleotides (light-blue phenotypes). Again, the reversion frequency of DNA copied by the wild-type T7 DNA polymerase (Table 1, top line of second experiment) is not significantly different from the background (typically $1–2.5 \times 10^{-5}$). Careful inspection of the plaque colors (as described in ref. 24) revealed that both dark- and light-blue plaque phenotypes occurred at the background frequency. The average frameshift error rate for the 41-nt target is thus $\leq 3.7 \times 10^{-7}$.

The third assay uses a DNA substrate containing two extra template nucleotides, a T and a C, at the run of T residues at positions 70–73 in the *lacZ* coding sequence. The substrate is thus in the −1 reading frame and scores errors such as +1, −2, +4, −5, etc. (In this assay, all revertants are lighter blue.)

Table 1. Fidelity of three forms of T7 DNA polymerase in reversion assays

Exonuclease activity	Thioredoxin	No. of plaques scored: Total	No. of plaques scored: Blue	Revertant frequency × 10^5
Base substitutions at TGA codon				
+	+	770,000	3	0.4 (0.9)
−	+	660,000	46	7.0 (6.7)
−	−	140,000	4	2.9
Frameshifts reverting a TTTTT run in +1 reading frame				
+	+	1,500,000	13	0.9 (1.8)
−	+	490,000	190	39 (26)
−	−	430,000	826	190
Frameshifts reverting a TTTTT run in −1 reading frame				
+	+	270,000	3	1.1 (≤0.5)
−	+	140,000	16	11 (11)
−	−	78,000	691	890

Reaction mixtures contained 1 mM dNTPs. Values in parentheses are from reactions performed with 50 μM dNTPs, with plaque counts (revertants/total) as follows, in order of presentation from top to bottom in the table: 7/790,000; 46/690,000; 12/680,000; 139/540,000; 0/210,000; and 11/98,000. Error rates were calculated by dividing revertant frequencies by 60% (to correct for expression of errors upon transfection; ref. 21) and then dividing by the number of detectable sites [3 for base substitutions (21), 41 for −1 frameshifts (24), and 42 for +1 frameshifts (this study)].

As before, the reversion frequency of DNA copied by the wild-type T7 DNA polymerase (Table 1, top line of third experiment) is not significantly different from the background ($0.5–1.5 \times 10^{-5}$), yielding an average frameshift error rate of $\leq 4.5 \times 10^{-7}$. Thus, the exonuclease-proficient T7 DNA polymerase·thioredoxin complex is highly accurate for a variety of substitution and frameshift errors scored in the three assays.

Fidelity of the Exonuclease-Deficient T7 DNA Polymerase·Thioredoxin Complex. Similar fidelity measurements with the polymerase·thioredoxin complex but devoid of 3′ → 5′ exonuclease activity yield reversion frequencies (Table 1, second line of each experiment) well above the background of each assay and well above the values obtained with the wild-type polymerase·thioredoxin complex. Therefore, the calculated polymerization error rates are 39×10^{-6}, 16×10^{-6}, and 4.5×10^{-6}, respectively, for the three assays. The differences in error rates between the wild-type and exonuclease-deficient complexes are ≥18-, ≥43-, and ≥10-fold, respectively for the substitution and two frameshift assays. These data confirm earlier studies (18–20) demonstrating that substitution errors are subject to proofreading and demonstrate that frameshift errors are also edited by the exonuclease. That both dark- and light-blue plaque revertants occurred at higher frequency in the absence of exonuclease (for experiment 2 in Table 1) suggests that frameshifts at both the TTTTT run and at nonreiterated sites are corrected.

Fidelity of the Exonuclease-Deficient T7 DNA Polymerase in the Absence of Thioredoxin. The base-substitution fidelity of the exonuclease-deficient polymerase in the absence of thioredoxin was slightly lower than that of the exonuclease-deficient two-subunit complex (Table 1, first experiment, compare 2.9×10^{-5} with 7.0×10^{-5}). In contrast, frameshift error rates were substantially increased during polymerization in the absence of thioredoxin (Table 1, second and third experiments). The frameshift reversion rate for the substrate in the +1 reading frame increased 5-fold and reflected an increase in dark blue revertants—i.e., frameshifts at the TTTTT run. The frameshift reversion rate for the substrate in the −1 reading frame increased >80-fold.

Table 2. Fidelity of exonuclease-deficient T7 DNA polymerase in the presence and absence of thioredoxin

Thioredoxin	No. of plaques scored: Total	No. of plaques scored: Mutant	Mutant frequency × 10^4
Experiment 1 (50 μM dNTPs)			
+	13,214	140	110
−	17,698	148	84
Experiment 2 (1 mM dNTPs)			
+	6,334	104	160
−	5,274	111	210

Error Specificity of the Exonuclease-Deficient T7 DNA Polymerase in the Presence or Absence of Thioredoxin. The selective effects of thioredoxin on substitution and frameshift fidelity shown in Table 1 prompted an examination of error specificity with a forward mutation assay. The assay scores a variety of changes in a 250-nt template sequence, with errors defined by DNA sequence analysis of collections of independent mutants. Transfection of the DNA products of polymerization reactions catalyzed by the exonuclease-deficient polymerase with and without thioredoxin yielded similar overall mutant frequencies (Table 2). However, DNA sequence analysis of mutants revealed substantial differences in polymerase error specificity depending on whether thioredoxin was present in the reaction. The predominant errors observed with the two-subunit complex were single-base

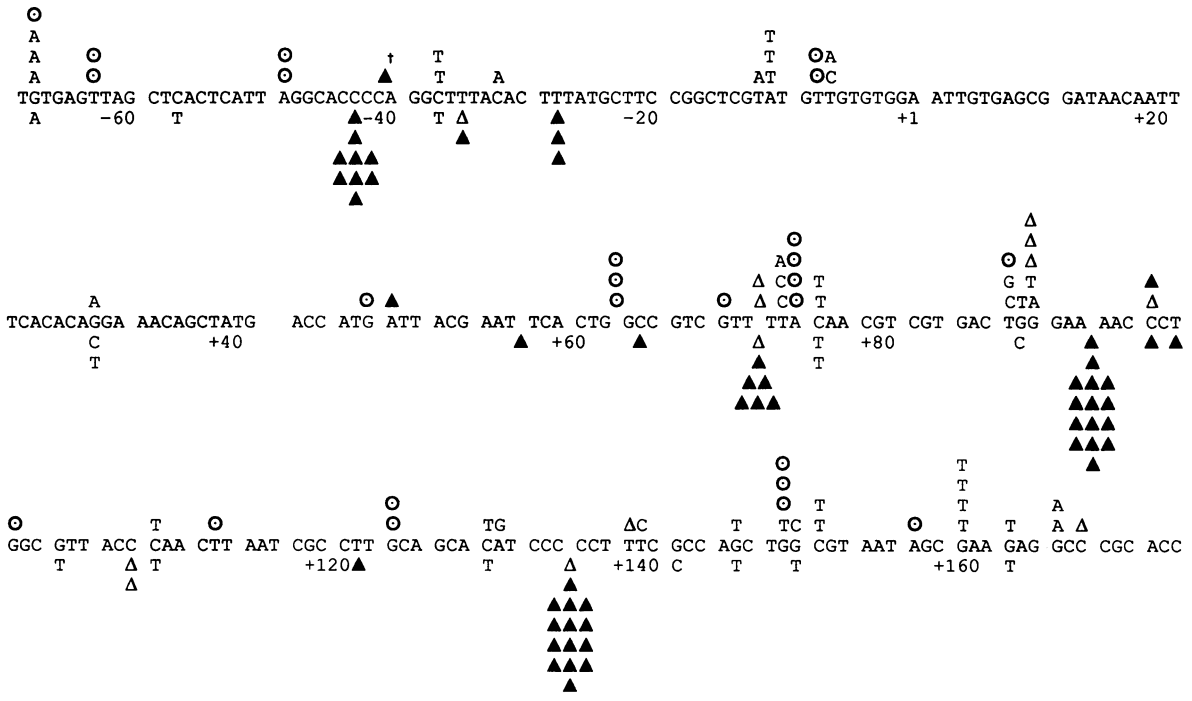

FIG. 1. Point mutations by exonuclease-deficient T7 DNA polymerase with and without thioredoxin. Three lines of *lacZ* α-complementation sequence are shown. Position +1 is the first transcribed nucleotide. Substitutions are indicated by letters above or below the sequence. An open triangle (Δ) represents the loss of one nucleotide in a reiterated sequence (i.e., a homopolymeric run of two or more of the same nucleotide); a filled triangle (▲) represents the addition of one nucleotide; a dotted circle (⊙) represents the loss of a nucleotide in a nonreiterated sequence. When frameshifts occur in homopolymeric runs, the nucleotide lost or added is not known. Errors above the sequence are those by exonuclease-deficient T7 DNA polymerase in the presence of thioredoxin; errors below the sequence are generated by exonuclease-deficient T7 DNA polymerase in the absence of thioredoxin. The filled triangle between positions −41 and −40 (†) indicates the addition of a T between these two nucleotides.

substitutions and single-base deletions at nonreiterated nucleotide positions; only one single-base addition at a homopolymeric sequence was observed (Fig. 1, changes shown above the lines of *lacZ* sequence). In contrast, in the absence of thioredoxin, substitutions and deletions were less prevalent, while 49 of 81 mutants contained single-base additions within homopolymeric runs (Fig. 1, changes shown below the lines of *lacZ* sequence).

For each class of error, the data can be expressed as mutant frequencies (Table 3) or as error rates (Fig. 2). The results indicate that, in the absence of thioredoxin, the polymerase is ≥28-fold more accurate for 1-nt deletions at nonreiterated sequences, 10-fold more accurate for 2-nt deletions, 3-fold more accurate for substitutions, and 1.8-fold more accurate for 1-nt deletions at homopolymeric sites. However, in the absence of thioredoxin the polymerase is 46-fold less accurate for 1-nt additions in homopolymeric runs.

DISCUSSION

This study provides information on three aspects of replication fidelity in this model system. First, the data in Table 1 with the wild-type T7 polymerase·thioredoxin complex extend previous observations on base substitution fidelity (18–20) to also reveal high accuracy for frameshift errors. Second, comparison of the fidelity of the wild-type complex with that of the exonuclease-deficient complex (Table 1) suggests that, as for substitutions, more than 9 of 10 frameshift intermediates are edited by the exonuclease activity of the wild-type complex. Extensive evidence suggests that these intermediates are likely to be misaligned template–primers containing one or more unpaired nucleotides (refs. 17, 26, and 27; Fig. 3 and text below). When such misalignments occur in repetitive sequences, the unpaired nucleotide may be several base pairs removed from the 3′-hydroxyl terminus. The effect of the exonuclease on frameshift fidelity (Table 1) includes a substantial reduction in the frequency of dark blue revertants known to result from loss or gain of a nucleotide within the TTTTT run in the target sequence. Correction of such errors within the run implies that several correctly paired nucleotides can be excised from a misaligned intermediate. This is consistent with the high activity of the 3′ → 5′ exonuclease with double-stranded DNA when the T7 DNA polymerase is complexed with thioredoxin (6, 7, 12).

The third aspect and major objective of this study was to examine the effects of a replication accessory protein on the fidelity of its polymerase catalytic subunit. The results in Fig. 2 clearly demonstrate that thioredoxin can strongly influence the fidelity of the polymerase catalytic subunit. In the absence of thioredoxin, the exonuclease-deficient T7 DNA polymerase is actually more accurate for substitutions and 1- and 2-nt deletions (Fig. 2). One possible explanation is that the premutational intermediates formed during polymerization are not successfully extended unless the polymerase is

Table 3. Mutant frequencies by class

Type of mutation	+ thioredoxin		− thioredoxin	
	Mutants	Mutant frequency × 10^4	Mutants	Mutant frequency × 10^4
Substitution	39	44	15	16
−1, in run	8	9.1	5	5.2
−1, non-run	25	28	0	≤1.0
−2	9	10	1	1.0
+1, in run	1	1.1	49	51

The total number of mutants sequenced were 97 (+ thioredoxin) and 81 (− thioredoxin), obtained from reaction mixtures containing 50 μM dNTPs. In addition to the mutants listed, several others were sequenced that did not fit any of the above categories. These included mutants with deletions of >2 nt, mutants with more than one change, or "mutants" having no change within the target sequenced (positions −84 through 174).

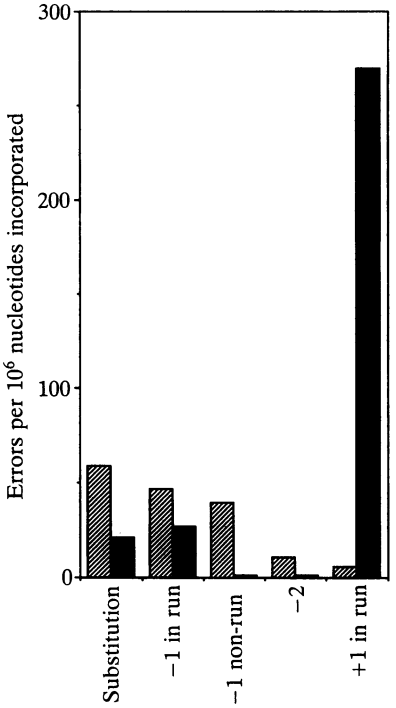

FIG. 2. Error rates for exonuclease-deficient T7 DNA polymerase with (hatched bars) and without (filled bars) thioredoxin. Error rates were calculated as described in the legend to Table 1, using the mutant frequencies from Table 2, the error specificity shown in Fig. 1 and Table 2, a value of 60% for expression of errors upon transfection, and the following number of known detectable sites: base substitutions, 125 sites (22); −1 and +1 frameshifts in runs, 32 sites (25); −1 frameshifts at nonreiterated positions, 118 sites (25); −2 frameshifts, 150 sites (25).

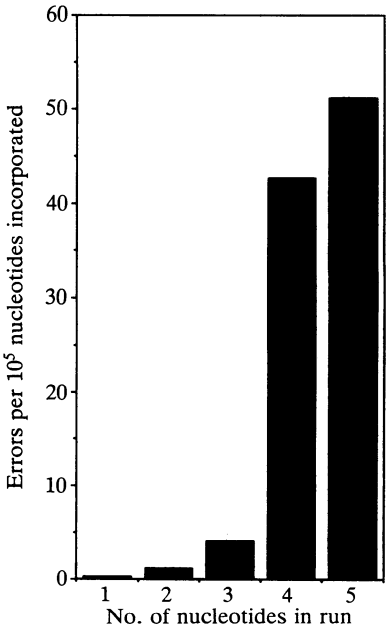

FIG. 3. Relationship between the length of runs and +1-nt error rate for exonuclease-deficient T7 DNA polymerase in the absence of thioredoxin. Error rates were calculated by using a mutant frequency of 84 × 10^{-4}, the error specificity shown in Fig. 1, a value of 60% for expression of errors upon transfection, and the number of known detectable sites for each length run (25).

complexed with thioredoxin. From studies of the base substitution fidelity of the T7 DNA polymerase·thioredoxin complex, a change to a closed protein conformation has been proposed to provide steric checks for proper Watson–Crick base-pair geometry (28). This conformational change is inhibited by mismatches between the bound dNTP and the template base or in any of the three terminal base pairs of the template-primer (28). It may also be inhibited by unpaired nucleotides in the template–primer. The polymerase·thioredoxin complex thus extends mispaired termini much more slowly than correctly paired termini (19). It is reasonable to expect that the extension rate for mispaired and misaligned template–primers might be even slower in the absence of thioredoxin. If unextended, these errors would remain at the template–primer terminus and be excised when the DNA is introduced into the host cell to express the mutant plaque phenotype. Such an accessory protein-mediated alteration in extension rate from unusual template–primers could well have biological implications. Under certain circumstances—e.g., following "incorrect" incorporation opposite a damaged base or slippage at a damaged site—an accessory protein may actually serve as a mutator protein by enhancing extension synthesis to seal the error before transfer to the exonuclease active site for removal.

Quantitatively, the greatest effect of thioredoxin on fidelity is for addition errors within homopolymeric sequences (Fig. 2). For these errors, the two-subunit complex is 46-fold more accurate than the polymerase alone. To our knowledge, this is the most substantial effect of an accessory protein on polymerase fidelity observed to date. That these addition errors likely result from template–primer slippage is suggested by the observation that the error rate, expressed per nucleotide polymerized to correct for differences in the number of nucleotides in a run, increases as the length of the run increases (Fig. 3). This is expected for misaligned template–primers because as the length of the run increases, both the number of potential misaligned intermediates and the potential number of correct base pairs that could stabilize the misaligned intermediates increase (e.g., see figure 2 in ref. 27). Furthermore, the longer the run, the greater the distance can be between the extra nucleotide and the 3′-hydroxyl terminus, reducing potential interference by the extra base in phosphodiester bond formation in the active site. For example, mismatches embedded in the double-stranded template–primer as far as 3 bp from the 3′-hydroxyl terminus still slow the rate of polymerization (S. Edwards and K.A.J., unpublished work).

Thioredoxin may enhance fidelity for addition errors by altering any of several possible steps in the reaction. It greatly enhances T7 DNA polymerase processivity (12) and the stability of the gene *5* protein·template–primer complex (13). Thus, in the presence of thioredoxin, the polymerase likely copies homopolymeric runs without dissociation–reassociation, perhaps providing less opportunity for template–primer slippage. This idea is consistent with earlier observations on the relationship between processivity and frameshift fidelity with the human immunodeficiency virus type 1 reverse transcriptase (15–17) that led to the suggestion that the association–dissociation phase of a polymerase reaction may be a critical determinant of frameshift fidelity. Alternatively, as pointed out by Richardson and colleagues (12, 13), thioredoxin could affect other critical steps in the reaction—e.g., a change in enzyme conformation in the ternary enzyme·dNTP·template–primer complex. The contributions to frameshift fidelity of individual steps in the reaction pathway remain to be determined, perhaps by using the strategies previously employed for base substitution fidelity (18–20) and mutant polymerase and thioredoxin (29).

Whether this study has implications for the fidelity of other replication systems remains to be established. It is worth noting that most replication complexes do contain accessory proteins that enhance DNA polymerase processivity (1). That the absence of an accessory protein known to confer high processivity on a DNA polymerase affects the fidelity of replication of repetitive DNA is also noteworthy, given that replication infidelity is one possible explanation for the instability of repetitive genomic sequences recently reported for several diseases (30–32).

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