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Processivity Clamp gp45 and ssDNA-Binding-Protein gp32 Modulate the Fidelity of Bacteriophage RB69 DNA Polymerase in a Sequence-Specific Manner, Sometimes Enhancing and Sometimes Compromising Accuracy

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ABSTRACT

Numerous studies of the impact of accessory proteins upon the fidelity of DNA synthesis have provided a complex and sometimes discordant picture. We previously described such an analysis conducted *in vitro* using various bacteriophage RB69 gp43 mutator DNA polymerases with or without the accessory proteins gp32 (which binds single-stranded DNA) plus gp45/44/62 (processivity clamp and its loaders). Mutations were scored at many sites in the *lacZ*α mutation reporter sequence. Unexpectedly, the accessory proteins sometimes decreased and sometimes increased fidelity at a handful of specific sites. Here, we enlarge our analysis with one particular mutator polymerase compromised in both insertion accuracy and proofreading and also extend the analysis to reactions supplemented only with gp32 or only with gp45/44/62. An overall 1.56-fold increase in mutation frequencies was produced by adding single or multiple accessory proteins and was driven mainly by increased $T_{template}$•G_{primer} mispairs. Evidence was found for many additional sites where the accessory proteins influence fidelity, indicating the generality of the effect. Thus, accessory proteins contribute to the site-specific variability in mutation rates characteristically seen in mutational spectra.

THE first mutational spectrum (Benzer 1961) revealed strikingly different mutabilities of sites within a gene, and specific transition mutation rates were soon observed to vary vastly across sites (Salts and Ronen 1971). When a mutation-reporter sequence was copied by different DNA polymerases, site-specific mutabilities varied both *in vitro* (Kunkel 1985a,b; Kunkel and Alexander 1986) and *in vivo* (Dressman *et al.* 1997).

The contributions of polymerase accessory proteins to the accuracy of DNA synthesis have also been explored, both *in vivo* and *in vitro*. Because null mutations were almost always lethal, studies *in vivo* focused on conditional-lethal mutations using temperature-sensitive mutants at semipermissive temperatures or amber mutants suppressed by the insertion of non-wild-type amino acids. The bacteriophage T4 accessory proteins (Nossal 1994) are gp32 (the single-strand DNA-binding or SSB protein), gp45 (the canonical circular processivity clamp), and gp44/62 (the complex that loads gp45 onto DNA); encoded by genes *32*, *45*, *44*, and *62*, respectively. The mutation-reporter system was usually the reversion of *rII* mutations. Taken together (Drake and Ripley 1983, 1994; Sinha and Goodman 1983), these studies reported small mutator effects from mutations in genes *32*, *44*, *45*, and *62*, whereas mutations in gene *43* (which encodes gp43, the DNA polymerase/proofreading exonuclease itself) could produce huge mutator factors. It remains unclear whether the small mutator effects resulted simply from diminished protein function or from a modified function that would not have been seen had it been possible to test null mutations. Some *Escherichia coli* SSB mutants also displayed mutator activities, although these may have been mediated by the SOS system (Meyer and Laine 1990). In *Saccharomyces cerevisiae*, mutator mutations were found in *POL30* (which encodes a gp45-like clamp) and in *CDC44* (which encodes a gp44/62-like clamp loader). While many of these mutations seemed to act by impairing DNA mismatch repair rather than DNA synthesis itself, some *POL30* mutators may act more directly to enhance deletion or base substitution mutagenesis (Chen *et al.* 1999 and references therein).

Because unassisted DNA polymerases often suffice *in vitro* in a simple gap-closing mutation-reporter assay such as *lacZ*α (Bebenek and Kunkel 1995), it is possible to probe the specific contributions of accessory proteins to the accuracy of DNA synthesis. The fidelity of yeast

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Pol α copying a *lacZ*α reporter sequence was not improved by RF-A (a complex SSB analog; Kunkel *et al.* 1991). The fidelity of a *Thermus thermophilus* polymerase was increased slightly by a cognate SSB (Perales *et al.* 2003). The *E. coli* processivity clamp had little effect on Pol III in a single context in a primer-extension assay, increasing fidelity twofold for one mispair and decreasing it 1.5-fold and twofold for two other mispairs (Bloom *et al.* 1997), and had no effect on the average fidelity of the lesion-bypass polymerase Pol IV in a *lacZ*α assay (Kobayashi *et al.* 2002). The fidelity of yeast Pol δ for a T•G mispair in a single context in a primer-extension assay was unaffected by the processivity factor (Chen *et al.* 2000). The fidelity of a mammalian mitochondrial Pol γ was enhanced by an order of magnitude in a single context in a primer-extension assay (Johnson and Johnson 2001) but was reduced at many sites in a mutation-spectrum analysis (Longley *et al.* 2001 and see below). The fidelity of a herpes simplex virus polymerase was enhanced a few fold by its processivity factor UL42 in a single context in a primer-extension assay (Chaudhuri *et al.* 2003). In contrast to these results, the frequency with which mammalian Pol δ generated $G_{template}•T_{primer}$ mispairs was enhanced ~27-fold in a primer-extension assay by the processivity clamp (Mozzherin *et al.* 1996) and the frequencies of all three possible mispairs with G were enhanced from slightly to a few fold with both Pol δ and its proofreading-deficient mutant encoded by *pol3-01* (Hashimoto *et al.* 2003).

Just as forward-mutation assays coupled with sequencing often provide different insights than do reversion tests, the *lacZ*α system soon began to reveal a picture even more complicated than that sketched above. The phage T7 DNA polymerase uses a tightly bound host protein, thioredoxin, as a processivity factor. While thioredoxin strongly increased accuracy for base addition mutations, it *decreased* accuracy about threefold for base-substitution errors and severalfold for deletions of one or two bases, suggesting that certain mutational intermediates might be produced in the absence of thioredoxin but then poorly extended and eventually lost (Kunkel *et al.* 1994). On the other hand, when *E. coli* Pol III was examined *in vitro* using a *lac*I forward-mutation system, the mutation rate (with 1 mm dNTPs) was about threefold lower with the complex holoenzyme that also contains the processivity clamp than with the catalytic α-subunit alone (Mo and Schaaper 1996; Pham *et al.* 1998); including the SSB protein in the reaction did not affect accuracy. A combined kinetic and *lacZ*α mutational analysis of phage T4 proofreading-deficient gp43 (Kroutil *et al.* 1998) found that gp32 and gp45 had little effect on misincorporation or on the K_m to extend a T•T mispair but increased the k_{cat} for extension, thus promoting stable misincorporation. The accessory proteins may have increased the *lacZ*α mutation rate by up to twofold. In *lacZ*α reversion tests, the accessory proteins had little effect on some mispairings errors but clearly promoted others, particularly those arising as G•G. Rates of single-base deletions in short repeats were unaffected, but larger deletions were strongly prevented by the accessory proteins. In a similar study of mammalian mitochondrial Pol γ, adding its processivity subunit increased rates for most kinds of base mispairs and base additions and deletions but reduced rates for a few others (Longley *et al.* 2001).

At this point, the impacts of SSBs and processivity clamps on fidelity presented an inconsistent picture, with both increases and decreases and hints of base-specific differences. The hypothesis that processivity clamps might increase mutation rates by improving the extension of primer strands terminating in mispairs was provocative and was supported in some sequence contexts but not others. A possible determinant of such complexity then emerged in a study using the gp43 of phage RB69, a relative of phage T4. Several gp43 derivatives were used that displayed sharply increased mutation rates because of a defect in proofreading, an alteration of a residue at the polymerase site, or both. Spectra were obtained *in vitro* with the *lacZ*α reporter either using a gp43 alone or with gp32 and gp45 (plus the gp44/62 clamp loader complex; Bebenek *et al.* 2002). With roughly similar numbers of *lacZ*α mutations for each polymerase, roughly five sites per polymerase showed significantly different numbers of mutations in the presence *vs.* the absence of accessory proteins. This result suggested that these accessory proteins produce site-specific impacts on fidelity, but it remained unclear how many sites might be affected altogether and whether the impacts were driven by gp32, gp45, or both. To examine these issues, we have now generated much more extensive mutational spectra for one of these polymerases in four combinations of accessory proteins: none, gp32 alone, gp45/44/62, and both SSB and processivity clamp.

MATERIALS AND METHODS

RB69 DNA polymerases and accessory proteins: Starting from an overproducing plasmid (Bebenek *et al.* 2001), RB69 gp43 Y567A D222A D327A (Pol^M Exo^-) was purified as for T4 gp43 (Kadyrov and Drake 2001), estimating the gp43 concentration using an A_{280} extinction coefficient of 135,800. T4 gp32, which supports RB69 DNA replication (Borjac 1997), was prepared as described (Kadyrov and Drake 2001). RB69 gp45 and gp44/62 were a gift from Lu-Shu Yeh and Jim Karam (Tulane University). The gp44/62 preparation contained traces of 3′-exonuclease and DNA polymerase activities, perhaps from *E. coli* Pol I, but at levels below those able to influence the present experiments because even by 15 min, only 9% of the primer had been extended (*vs.* close to 100% in the presence of gp43 with or without accessory proteins) and no extensions >15 nt were detected, whereas the primer starts 63 nt before encountering the first *lacZ*α site where a detectable mutation can occur.

Mutation assays: The *lacZ*α mutation assays and subsequent sequencing were conducted as described previously (Bebenek and Kunkel 1995; Bebenek *et al.* 2002) with minor modifica-

tions: gp43 was at 40 nM alone or at 20 nM with accessory proteins [gp32 at 4 μM, gp45 at 80 nM (as a monomer), gp44/62 at 15.7 nM] and the DNA substrate was at 2 nM in a reaction volume of 30 μl. Mutation frequencies, kinds, and spectra are based exclusively on mutations that are detectable in the *lacZ*α assay, which means mutations that have arisen at least twice as single mutations in experiments over about two decades.

Statistical analyses: To determine the extent to which the distribution of mutations across the mutation-bearing sites was similar in the various reactions, a chi-square statistic was calculated on the basis of the observed distributions. However, because of the low expected mutation frequencies at each site, this statistic was not compared to standard chi-square tables. Instead, using a described approach (Piegorsch and Bailer 1994), a Monte Carlo simulation was performed, conditional upon the observed totals of mutations from the several reactions and the numbers of mutations at each site. The overall significance of the differences among reactions with regard to the observed pattern of mutation frequencies was determined by comparing the observed chi-square statistic to chi-square statistics from 10,000 simulated data sets. Comparisons of mutation frequencies at each site were made by two-sided Fisher's exact tests. The number of tests expected to be significant by sampling variation alone was calculated from the distribution of Fisher's exact test.

RESULTS

RB69 gp43 drives T4 DNA replication *in vivo* efficiently and with the same high fidelity as does T4 gp43 (Dressman *et al.* 1997). For generating mutational spectra, we used a double-mutator version of RB69 gp43 (Pol^M Exo^-) containing the replacement Y567A at the polymerase site, which sharply reduces the fidelity of incorporation, and the replacements D222A and D327A, which completely inactivate the proofreading exonuclease (Bebenek *et al.* 2001, 2002). This gp43 was chosen over either single-mutator version because it previously displayed somewhat more and somewhat stronger impacts on site-specific mutabilities by accessory proteins. The wild-type polymerase cannot be used with the *lacZ*α system because its fidelity is too high to produce a signal against the high background of spontaneous mutations in phage M13 (Drake 1991; Bebenek *et al.* 2002).

Frequencies and kinds of mutations: Our mutational analyses *in vitro* used the *lacZ*α system, in which gap-filling synthesis of a mutation-reporter sequence can be conducted with or without accessory proteins, in marked contrast to DNA replication *in vivo*. Mutants of independent origin were collected in the Pol^M Exo^- background in the presence of four different combinations of accessory proteins: none, gp32 (either alone or in the presence of gp44/62), gp45 plus gp44/62, or gp32/45/44/62. The mutation frequencies, rates, and kinds were obtained both from a previous study (Bebenek *et al.* 2002) and from subsequent reactions. (The gp32/44/62 permutation was performed at the same time as the previous study in the context of a now-discarded hypothesis, but was not described in that study.) Mutation frequencies are shown in Table 1. The historical background mutation frequency is $\sim 6 \times 10^{-4}$ (Bebenek and Kunkel 1995; Bebenek *et al.* 2002) and the frequencies in Table 1 are all sufficiently far above this value for background mutations not to have affected our analyses. Frequency variations in replicate experiments were ≤1.35-fold, typical of the *lacZ*α system. The summed frequencies for all three experiments in which some combination of accessory proteins was present are very close ($251–257 \times 10^{-4}$), and these values are ~1.56-fold higher than the frequency with no accessory proteins. Thus, both the SSB and the processivity clamp modestly decrease fidelity *in vitro*, but in a nonadditive manner.

Table 2 shows the kinds of mutations produced under each combination of accessory proteins and in replicate experiments and their frequencies after pooling the old and new data. Historical experience with the *lacZ*α system indicates that stored DNA substrates tend to accumulate mutations of two types, C → T (presumably by cytosine deamination) and G → T (presumably by guanine oxidation) (Bebenek and Kunkel 1995). The impact of any such mutations must be small or nil in the present case because of the high mutation frequencies produced by the double-mutator gp43, and because C → T mutations are ≤10% of all mutations (average 5.5%) and G → T mutations are ≤3% of all mutations (average 1.1%). Statistical testing for differences between "old" and "new" entries revealed several instances in which replicate experiments produced somewhat different fractions of particular kinds of mutations at $P < 0.05$. For mutants from reactions with no accessory proteins, they were the entries for G → A (old *vs.* new rates of 58 *vs.* 38 per 10^4, respectively), T → C (39 *vs.* 85), and G → T (2 *vs.* 13); from reactions with gp32, they were the entries for G → A (33 *vs.* 62) and T → C (171 *vs.* 108); and from reactions with gp45, they were the entries for C → T (30 *vs.* 9) and −G (≤1.4 and 11). In each case, the number of such differences expected from sampling variations alone was ~0.4. Thus, of the seven differences, roughly six may represent unrecognized differences in either purification procedures or reaction conditions. Because the numbers of differences with no accessory proteins, with gp32 ± gp44/62, and with all four accessory proteins were similar (3, 2, and 2, respectively), it is possible that the presence of gp44/62 in the old reactions with gp32 reflects unrecognized variables. However, gp44/62 binds better to single-stranded DNA coated with gp32, thus increasing the ability of gp44/62 to compete with gp43 in binding to the 3′ end of the primer strand (*e.g.*, Capson *et al.* 1991), although this effect seems unlikely to affect fidelity in the present reactions.

In all reactions summarized in Table 2, the large majority of mutations were transitions (predominantly G → A and T → C), transversions were usually dominated by T → A and T → G, and single-base deletions outnumbered single-base additions. Because this mutator poly-

TABLE 1

Mutation frequencies

Accessory proteins	Total plaques[a]	Mutants	Sequenced mutants	Correction factor[b]	MF[c] per 10^4
None	57,952	883	143	1.06	162
	3,983	71	71	1.04	186
	61,935	954	214	1.06	163
gp32/44/62	13,478	335	201	1.12	279
gp32	12,658	281	281	1.06	236
	26,136	616	482	1.09	257
gp45/44/62	13,806	314	314	1.11	251
gp32/45/44/62	71,394	1,556	152	1.13	245
	10,034	297	192	1.11	330
	81,428	1,853	344	1.12	255

[a] Triple entries record two experiments followed by their total. The first values for no and for all four accessory proteins are from the previous study (Bebenek *et al.* 2002).

[b] Correction factor for mutants with no mutation in *lacZ*α and for mutants with more than one detectable mutation.

[c] Mutation frequency (MF) = (mutants)(correction factor)/(total plaques). The values from the previous collection (Bebenek *et al.* 2002) for no and for all four accessory proteins have changed minutely (from 164 and 247, respectively) due to small corrections.

merase produces few deletions larger than a single base, it is impossible to ascertain whether the accessory proteins restrained deletion mutagenesis, as was observed in a study using entirely T4 proteins (Kroutil *et al.* 1998). A statistical analysis was then performed to detect variations in the frequencies of kinds of mutations across accessory-protein configurations. Of the 20 kinds of mutations in Table 2, 7 varied across accessory-protein configuration with $P < 0.05$, whereas only 0.8 were expected to vary by sampling variations alone. The ratios of (maximum frequency)/(minimum frequency) across all accessory-protein combinations for 2 of these (G → A and T → C) were only 1.6-fold and were also associated with mutations at sites for which there was variation between replicate experiments. In 5 infrequent kinds of mutations (G → T, A → T, T → G, −G, and +A) the ratios were 3.6- to 8-fold (with two ≥7-fold); with reference to the no-accessory-protein value, 3 of these represented reductions, 1 represented an increase, and 1 represented a mixed response to accessory proteins.

The impact of accessory proteins on rates of specific kinds of mutations can be ascertained from the mutation-frequency (MF) columns of Table 2. Compared to the rates with no accessory proteins, the G → A transition rate is ~46% higher with gp45 and the T → C transition rate is more than doubled by any combination of accessory proteins. The only other marked change is the T → G transversion rate, which is increased on average 8.5-fold (range 5.5- to 13-fold) by the three combinations of accessory proteins. Despite the large numbers of mutants sequenced, the numbers of mutants in most other classes are small and their rate variations may represent only sampling variations.

Mutational spectra: All of the mutations are listed in Table 3 except those classified as undetectable (Bebenek and Kunkel 1995; Bebenek *et al.* 2002), which are typically observed as hitchhikers in mutants that also contain a detectable mutation.

In this study, we were interested in the impacts of accessory proteins on frequencies of specific kinds of mutations at specific sites. We observed nine site-specific differences (at $P < 0.10$) between the gp32 spectra with (32^+) and without (32^-) gp44/62: T → G at position −7; T → C at 61, 73, and 87; G → A at 88 and 89; T → C at 139; and G → A at 148 and 149, with the difference at position 88 being especially strong. Sampling alone could have generated 2.5 differences at $P \leq 0.10$, so ~6.5 of the differences were likely to have been the result either of an unexpected impact of the gp44/62 clamp loader or of undefined experimental differences. However, the impact of gp44/62 in the absence of gp32 and gp45 was not investigated. To concentrate on the central issue of the extent of influence of gp32 and gp45 on fidelity, we then merged the two gp32 spectra for subsequent analyses.

When the total number of mutations at a site is small, even a substantial accessory-protein impact may be impossible to validate statistically. Therefore, we gathered all sites from Table 3 at which *either* at least five mutations appeared in at least one of the four spectra *or* the distribution of mutations was nonrandom with $P < 0.10$ across the four accessory-protein configurations. These

TABLE 2

Kinds of mutations produced *in vitro* depending on accessory proteins

	None				gp32 ± gp44/62				gp45/44/62		gp32/45/44/62			
Change	Old	New	Total	MF	Old	New	Total	MF	New	MF	Old	New	Total	MF
Mutations	152	74	226		226	299	525		347		171	214	385	
Small	146	74	220	158	225	299	524	256	338	245	170	209	379	251
Other	6	0	6		1	0	1		9		1	5	6	
G → A	54	15	69	50	27	79	106	52	82	59	56	55	111	73
C → T	12	3	15	11	8	13	21	10	19	14	21	6	27	18
A → G	0	0	0	≤0.7	5	1	6	3	1	0.7	0	0	0	≤0.7
T → C	37	34	71	51	138	137	275	135	147	106	68	87	155	103
Transitions	*103*	*52*	*155*	*112*	*178*	*230*	*408*	*200*	*249*	*180*	*145*	*148*	*293*	*194*
G → T	2	5	7	5	1	1	2	1	4	3	0	4	4	3
G → C	4	0	4	3	1	3	4	2	7	5	0	2	2	1
C → A	0	1	1	0.7	0	0	0	≤0.5	3	2	2	2	4	3
C → G	0	0	0	≤0.7	0	1	1	0.5	0	≤0.7	0	0	0	≤0.7
A → T	2	1	3	2	0	0	0	≤0.5	2	1	1	2	3	2
A → C	1	0	1	0.7	0	0	0	≤0.5	0	≤0.7	0	1	1	0.7
T → A	8	3	11	8	8	10	18	9	7	5	3	11	14	9
T → G	0	1	1	0.7	12	7	19	9	7	5	1	5	6	4
Transversions	*17*	*11*	*28*	*20*	*22*	*22*	*44*	*22*	*30*	*22*	*7*	*27*	*34*	*22*
−G	5	2	7	5	1	5	6	3	14	10	0	7	7	5
−C	4	3	7	5	4	8	12	6	11	8	3	10	13	9
−A	0	0	0	≤0.7	0	1	1	0.5	0	≤0.7	0	3	3	2
−T	6	2	8	6	11	14	25	12	20	14	11	7	18	12
−1	*15*	*7*	*22*	*16*	*16*	*28*	*44*	*22*	*45*	*33*	*14*	*27*	*41*	*27*
+G	0	0	0	≤0.7	1	1	2	1	1	0.7	0	0	0	≤0.7
+C	1	1	2	1	0	3	3	1	0	≤0.7	0	0	0	≤0.7
+A	4	0	4	3	1	4	5	2	0	≤0.7	0	0	0	≤0.7
+T	6	3	9	6	7	11	18	9	13	9	4	7	11	7
+1	*11*	*4*	*15*	*11*	*9*	*19*	*28*	*14*	*14*	*10*	*4*	*7*	*11*	*7*

Most Old entries are from Bebenek *et al.* (2002). The New entries are from more recent reaction experiments, Total is the sum of the Old and New values, and MF is the mutation frequency × 10^4 for the Total column (the product of the pooled MF values from Table 1 times the fraction of mutations of each type). Note, however, that the Old gp32 entries are from reactions performed in the presence of gp44/62 while the New gp32 entries are from reactions with gp32 alone. The Other mutations include complex mutations and indels (mostly deletions) of ≥2 nucleotides and were not included in the spectral analyses. "≤" values are as if 1 mutation had been observed. Numbers in italics are subtotals.

are displayed in Table 4 together with indicators for both $0.05 < P < 0.10$ and $P \leq 0.05$.

In Table 4, $P < 0.10$ for 25 entries ($P \leq 0.05$ for 15 entries). The number expected from sampling variations alone was 11/25. In the previous study (Bebenek *et al.* 2002), $P \leq 0.10$ in seven instances, of which only two were expected from sampling variations. Thus, the expanded collection of mutations revealed 25 − 11 = 14 valid entries *vs.* the previous 7 − 2 = 5. However, the increased resolving power came at a price: the predicted proportions of valid identifications fell from 5/7 to 14/25. (The expanded study reidentified five out of the seven previously identified candidate sites, namely −68, 87, 89, 118, and 183.) Table 4 also illustrates a similar number of well-populated sites at which no statistically significant variation across accessory-protein configurations could be observed.

The 11 of 25 entries probably generated by sampling variations alone render more detailed analysis difficult. Putting this uncertainty aside, the largest class of responses comprises the 13 instances in which all three combinations of accessory proteins resulted in fewer mutations than predicted by the number of mutations in the no-accessory-protein reactions (G → A at −68, T → A at −58, +T at −(29–27), −G at 69, G → A at 84, T → A at 87, G → C and G → T at 88, G → A at 90, G → T at 102, C → T at 106, C → T at 108, and +C at 132–136). In contrast, there were only two entries (T → C at 87 and T → C at 183) in which all accessory-protein combinations increased the mutation frequency

TABLE 3

Mutations arising in reactions depending on accessory proteins

Site	Mutation	Accessory Protein(s) 0	32^+	32^-	45	4
−69	T → A	0	0	0	1	0
	T → C	0	1	1	0	3
	T → G	0	1	1	0	0
−68	G → A	5	0	0	1	1
	G → C	0	0	1	0	0
	−G	1	0	0	0	1
−67	T → C	1	0	0	3	1
−66	G → A	5	2	2	6	6
	G → C	1	0	1	0	0
	+G	0	1	0	0	0
−62	T → C	0	0	0	1	1
−61	−A	0	0	0	0	1
−60	−G	0	0	0	1	0
−58	T → A	2	0	0	1	0
	T → C	1	4	2	2	6
−57	C → T	1	0	0	0	1
	−C	0	0	0	0	1
	+C	0	0	2	0	0
−55	C → T	0	1	2	0	0
−(51–50)	−T	1	1	0	0	0
−(48–47)	−G	0	0	0	1	0
−45	−A	0	0	1	0	0
−(44–41)	−C	1	1	3	3	2
−37	C → T	2	0	1	2	0
−36	T → C	6	5	8	12	9
	T → A	0	0	2	0	3
	T → G	0	0	1	1	0
−(36–34)	+T	0	1	3	3	3
	−T	0	1	0	0	0
−35	T → C	9	15	18	15	15
−34	T → C	1	0	4	3	2
−32	C → T	2	0	2	0	1
	C → A	1	0	0	0	1
	C → G	0	0	1	0	0
−(29–27)	+T	4	3	2	0	2
−25	T → C	0	0	0	0	1
−(22–21)	+T	1	0	3	0	2
−13	G → A	0	0	1	0	2
−12	T → C	2	3	1	6	1
−11	A → T	1	0	0	1	0
	A → G	0	2	0	0	0
−10	T → C	1	0	0	0	0
	T → G	0	0	0	1	0
−8	T → C	0	0	1	1	1
−7	T → C	7	16	17	13	16
	T → G	0	5	1	1	3
	T → A	0	2	0	0	2
20	T → G	0	0	1	0	0
21	T → C	0	0	1	1	1
29	G → A	1	1	2	1	0
30	G → A	1	0	0	0	0
35	A → C	0	0	0	0	1
38	T → C	0	0	0	1	0
	T → G	0	0	0	0	2
40	T → C	2	3	1	4	2
	T → G	1	0	0	0	1
41	G → A	0	1	0	1	0
42	A → G	0	1	0	1	0
43	C → T	0	0	0	1	0
49	T → C	2	2	1	1	2
52	C → T	0	0	0	1	1
56–57	−T	0	0	0	0	2
57	T → C	0	5	2	1	2
58	C → A	0	0	0	1	0
	C → T	0	1	0	1	0
	−C	0	0	0	0	1

(*continued*)

TABLE 3

(Continued)

Site	Mutation	Accessory Protein(s) 0	32^+	32^-	45	4
61	T → C	2	5	1	4	7
	T → A	2	1	0	1	1
	T → G	0	1	0	0	0
62	T → C	0	0	0	0	1
63	G → A	0	1	0	1	1
64	C → T	1	0	0	2	1
64–65	−C	0	0	0	0	1
66	G → A	0	0	0	0	1
	+G	0	0	0	1	0
69	−G	2	0	0	0	0
70	T → C	2	1	0	1	1
	T → A	0	0	0	0	1
70–73	−T	2	1	0	1	2
	+T	1	0	0	0	0
72	T → C	0	1	0	0	3
73	T → C	0	5	0	1	2
74	−A	0	0	0	0	1
75	C → T	0	0	1	1	1
76–77	+A	1	0	0	0	0
78	−C	1	0	0	1	0
79	G → A	1	1	0	0	0
81	C → T	1	0	0	1	4
	C → A	1	0	0	1	0
82	G → A	1	1	2	2	0
	G → T	1	0	0	0	0
	G → C	0	0	0	2	0
	−G	0	0	0	1	0
83	−T	0	1	0	1	1
84	G → A	5	0	0	1	4
	−G	0	0	0	1	0
85	A → T	1	0	0	0	0
	A → G	0	1	0	0	0
87	T → A	5	3	5	2	0
	T → C	5	20	16	18	12
	T → G	0	2	1	2	0
88	G → A	4	1	12	10	13
	G → C	2	0	0	1	0
	G → T	2	0	0	0	0
88–90	−G	0	0	0	1	0
	+G	0	0	1	0	0
89	G → A	8	2	11	9	4
	G → C	0	0	0	3	1
90	G → A	2	0	1	3	0
91	A → G	0	0	1	0	0
94	A → T	0	0	0	1	0
91–94	+A	1	0	1	0	0
95–97	−C	0	0	0	1	1
96	C → T	0	1	0	0	0
97/98	+A	1	0	0	0	0
98	+T	0	0	0	2	0
	−T	0	1	3	0	2
99–100	−G	0	0	0	2	1
102	G → T	2	0	0	1	1
	−G	1	0	0	3	0
103	T → C	1	3	1	3	2
	T → G	0	1	1	0	0
	T → A	0	0	1	0	0
103–104	−T	0	0	1	3	0
106	C → T	2	0	0	0	1
106–108	−C	1	0	0	1	0
108	C → T	4	1	1	0	2
109	A → T	0	0	0	0	1
109	A → G	0	1	0	0	0
112	T → C	8	10	21	17	20
112–113	+T	0	2	0	1	0
115	A → T	0	0	0	0	1

(*continued*)

TABLE 3

(Continued)

Site	Mutation	Accessory Protein(s) 0	32^+	32^-	45	4
118	G → A	1	1	4	1	11
	G → C	0	1	0	0	1
	−G	1	0	0	0	0
121	T → C	11	9	16	10	9
121–122	−T	2	2	2	7	5
	+T	3	1	2	6	2
127	C → T	0	0	1	0	0
129	C → T	0	1	2	3	5
	C → A	0	0	0	0	2
130	A → T	1	0	0	0	0
131	T → A	1	0	1	1	4
	T → G	0	1	1	2	0
	−T	1	0	1	0	1
132–136	+C	2	0	0	0	0
	−C	3	3	5	4	1
133	C → T	2	2	1	1	4
136	C → T	0	0	0	1	0
136/137	+A	1	1	3	0	0
137–139	−T	2	4	7	7	4
	+T	0	0	1	1	2
138	T → A	1	0	0	0	1
139	T → C	8	25	19	23	24
	T → A	0	0	0	0	1
140	−C	1	0	0	1	5
	+C	0	0	1	0	0
141	G → A	1	0	0	0	3
	−G	0	0	1	0	0
142	C → T	0	0	1	1	1
144	A → T	0	0	0	0	1
145	G → A	11	3	9	0	17
	G → T	1	1	0	3	1
146	C → A	0	0	0	1	0
	−C	0	0	0	0	1
147	T → C	2	4	6	5	3
	T → A	0	2	1	1	1
	T → G	0	1	0	0	0
148	G → C	1	0	0	0	0
	G → A	3	3	13	2	6
	G → T	0	0	0	0	2
148–149	−G	1	0	0	1	3
149	G → A	7	1	9	8	13
	G → T	0	0	1	0	0
150	C → T	0	1	0	3	3
151	G → T	1	0	0	0	0
	G → C	0	0	0	1	0
	G → A	0	0	1	2	2
	−G	1	0	3	1	1
163	−A	0	0	0	0	1
165	G → A	11	9	11	20	19
166	C → T	0	0	1	1	0
168	C → T	0	0	0	0	1
169	G → A	2	0	1	4	8
	G → C	0	0	1	0	0
	−G	0	1	1	1	0
182–183	−T	0	0	0	1	1
183	T → C	0	1	0	1	8
184	C → A	0	0	0	0	1
184–186	−C	1	0	0	0	0
186	C → T	0	0	0	0	1
191	−G	0	0	0	1	1

The sites are those of the standard *lacZα* sequence. Only mutations detectable as single mutations are shown (Bebenek *et al.* 2002). The accessory proteins are none (0); gp32 either in the presence of gp44/62 (32^+) or alone (32^-); gp45 plus gp44/62 (45); or gp32, pg45, and gp44/62 (4). The total numbers of mutants in each category are 220, 524, 338, and 379, respectively.

TABLE 4

Impacts of accessory proteins on fidelity

Site	Mutation	Accessory Proteins 0	32	45	All	*P*
All		220	524	338	379	
−68	G → A	5	0	1	1	**
−66	G → A	5	4	6	6	
−58	T → A	2	0	1	0	**
	T → C	1	6	2	6	
−36	T → C	6	13	12	9	
−35	T → C	9	33	15	15	
−(29–27)	+T	4	5	0	2	*
−12	T → C	2	4	6	1	
−7	T → C	7	33	13	16	
	T → G	0	6	1	3	
57	T → C	0	7	1	2	
61	T → C	2	6	4	7	
69	−G	2	0	0	0	**
73	T → C	0	5	1	2	
81	C → T	1	0	1	4	*
82	G → C	0	0	2	0	*
84	G → A	5	0	1	4	**
87	T → A	5	8	2	0	**
	T → C	5	36	18	12	**
88	G → A	4	13	10	13	
	G → C	2	0	1	0	**
	G → T	2	0	0	0	**
89	G → A	8	13	9	4	
	G → C	0	0	3	1	*
90	G → A	2	1	3	0	*
98	+T	0	0	2	0	*
102	G → T	2	0	1	1	*
	−G	1	0	3	0	**
106	C → T	2	0	0	1	*
108	C → T	4	2	0	2	**
112	T → C	8	31	17	20	
118	G → A	1	5	1	11	**
121	T → C	11	25	10	9	
121–122	−T	2	4	7	5	
	+T	3	3	6	2	
132–136	+C	2	0	0	0	**
	−C	3	8	4	1	
137–139	−T	2	11	7	4	
139	T → C	8	44	23	24	
140	−C	1	0	1	5	**
141	G → A	1	0	0	3	*
145	G → A	11	12	10	17	
147	T → C	2	10	5	3	
148	G → A	3	16	2	6	*
149	G → A	7	10	8	13	
165	G → A	11	20	20	19	
169	G → A	2	1	4	8	**
183	T → C	0	1	1	8	**

Those entries from Table 3 are shown for which the probability of a nonrandom distribution was $P < 0.10$ or for which $P > 0.10$ and at least five mutants arose in at least one category. *$0.05 < P < 0.10$; **$P \leq 0.05$.

and four entries (at sites 82, 89, 98, and 118) in which at least one accessory protein increased the mutation frequency but none decreased it. There was no easily discernible correlation between the accessory-protein impact pattern and the kind of mutation.

One goal of these experiments was to determine the

TABLE 5

Sequence context of sites displaying mutation rates subject to modulation by accessory proteins TA

Mutation	Site	Context
G → A	−68	ATTAATGTGAGTT
	84	CGTCGTGACTGGG
	90	GACTGGGAAAACC
	118	TTAATCGCCTTGC
	141	CCTTTCGCCAGCT
	148	CAGCTGGCGTAAT
	169	AGGCCCGCACCGA
G → T	88	GTGACTGGGAAAA
	102	CCTGGCGTTACCC
G → C	82	AACGTCGTGACTG
	88	GTGACTGGGAAAA
	89	TGACTGGGAAAAC
−G	69	GCCGTCGTTTTAC
	102	CCTGGCGTTACCC
T → C	87	CGTGACTGGGAAA
	183	CGCCCTTCCCAAC
T → A	−58	GTTAGCTCACTCA
	87	CGTGACTGGGAAA
+T	−(29–27)	TACACTTTATGCT
	98	AAACCCTGGCGTT
C → T	81	CAACGTCGTGACT
	106	GCGTTACCCAACT
	108	GTTACCCAACTTA
−C	140	CCCTTTCGCCAGC
+C	132–136	ACATCCCCCTTTC

The sites are those from Table 4 at which effects were observed at $P \leq 0.1$ and include ~11 sites detected from sampling variation alone and also 5 sites (−68, 87, 89, 118, and 183) detected previously (Bebenek *et al.* 2002) and confirmed here.

extent to which only gp32 or only gp45 (plus gp44/62) affected mutation frequencies. Patterns consistent with the former were infrequent (at sites 90 and 118) or missing. The pattern at site 183 was that expected if both proteins were needed for any effect. The patterns at 11 other sites were mixed in a manner that suggested antagonistic impacts. However, these assignments are blurred by the fact that ~44% of the statistically highlighted entries were probably simple sampling accidents.

Finally, we attempted to determine whether the sites at which fidelity was differentially affected by accessory proteins exhibited common sequence contexts. The 25 sites highlighted in Table 4, probably comprising 14 real sites plus 11 false positives, are gathered together in Table 5 by kind of mutation and with the six flanking residues on either side. Visual screening reveals no sequence characteristics among these sites. Further analysis was not attempted because of the small and uncertain sample.

DISCUSSION

Impacts of accessory proteins on fidelity: Overall, each of the three combinations of accessory proteins increased mutation frequencies by close to 1.56-fold (Table 1). (In the *lacZ*α system, frequencies can be converted to rates by the factor 1/0.6 if desired.) Because G → A and T → C transitions together compose 63–72% of the mutations produced by this mutator gp43, most of the 1.56-fold increase must have affected these mutations. However, G → A mutations (modulated by $G_{template}$•T_{primer} mispairs) were only slightly affected (Table 2), their frequencies increasing from 50 per 10^4 without accessory proteins to 52, 59, and 73 per 10^4 in the presence of accessory proteins. In contrast, T → C mutations (modulated by $T_{template}$•G_{primer} mispairs) increased from 51 per 10^4 without accessory proteins to 135, 106, and 103 per 10^4 in the presence of accessory proteins. (In addition, the only two T → C sites highlighted in Table 4 both show increased mutation frequencies in the presence of any combination of accessory proteins.) Both T•G and G•T are usually among the most easily extended mispairs (Kunkel and Bebenek 2000), so it is perhaps surprising that only the former is affected by accessory proteins in the present system, an observation deserving of structural analysis. Frequencies of T → G and −T were also increased, by ~9-fold and ~2-fold, respectively. The impact on total frequencies from increases in these mutations was negligible in our spectra because of their low initial proportions, but could be large in other spectra if they were more common.

The joint impact of gp32 and gp45 on average mutation frequencies was determined previously for the two single-mutator polymerases and was either negligible or small (Bebenek *et al.* 2002). The ratios (with accessory proteins)/(without accessory proteins) for total mutation frequencies and for frequencies adjusted for the (somewhat uncertain) *lacZ*α backgrounds were 0.89 and 1.07 for Pol^+ Exo^- and were 1.27 and 1.41 for Pol^M Exo^+. Thus, the impacts of the accessory proteins on average mutation rates depend not only on specific sites but also on the polymerase itself.

We have confirmed our earlier observation that the accessory proteins produce discernible effects in both directions on mutation frequencies at specific sites, and we have substantially enlarged the number of sites at which the impact was detected (from 5 to ~14). However, the cost of enlargement was an increase in the fraction of highlighted sites, from 2/7 to 11/25, at which the effect could be attributed to sampling variations alone. At many of the highlighted sites, changes were produced by either gp32 or gp45 alone to about the same extent as by both together, but sampling uncertainties prevented us from specifying sites at which only gp32 or only gp45 affected fidelity or at which they exhibited additive or antagonistic effects. The major conclusion to be drawn from the present enlargement of the spectra is that continued sampling would produce still more net sites at which the accessory proteins affect fidelity. For instance, T → C mutation frequencies were by far the main component in the general increase

of 1.56-fold in total mutation frequencies by all three combinations of accessory proteins, yet only 2 sites were highlighted statistically (both showing increases). However, of the 29 sites at which T → C mutations were observed, 21 showed increases for the average of the three accessory-protein combinations compared to the value with no accessory proteins, while 3 showed no change and only 5 showed a decrease. Although only 2 of the 21 were highlighted statistically, probably most of the other 19 would be highlighted in a sufficiently massive study. In contrast, several of the sites in Table 4 that were not statistically highlighted contain numerous mutations in proportions closely similar to those of total mutants. Thus, there are sites at which the impact of accessory proteins on fidelity is at most quite small.

A qualifier to these conclusions is that the RB69 Pol^M Exo^- gp43 differs from the cognate wild-type enzyme in being deficient both in the accuracy of base insertion and in proofreading. The mutant gp43 is strongly biased toward the production of transitions *in vitro* via G•T and T•G mispairs (Bebenek *et al.* 2002), whereas the wild-type enzyme produces twice as many transversions as transitions *in vivo* (Bebenek *et al.* 2001). Within the limits imposed by sample sizes, the kinds of mutations arising at sites affected by accessory proteins tended to reflect these specificities for all three mutant enzymes, and the Pol^M Exo^- gp43 affected transitions almost exclusively (Bebenek *et al.* 2002 and this report). Therefore, using this gp43 alone, it is impossible to learn much more about the site specificity of the impact of accessory proteins on transversions and insertions/deletions (indels) except for the already noted increases in frequencies of T → G and −T. In addition, because the high background of the assay system prevents the detection of mutations produced by the wild-type enzyme, it is a formal possibility that impacts occur only with enzymes with the Y567A polymerase or the D222A D327A proofreading exonuclease defects.

Mechanisms: The mechanistic basis of these results can be considered at two levels, the enzymological and the evolutionary. At the enzymological level, the modest overall decrease in fidelity (seen with gp43 Pol^M Exo^- and perhaps Pol^M Exo^+ but not with Pol^+ Exo^-) might result from an increase in the formation of mispairs and misalignments, a decrease in the efficiency of proofreading, or an increase in the efficiency of extensions from mispairs and misalignments. In the present case the proofreading function is disabled and cannot contribute catalytically, but kinetic partitioning between the Pol and Exo sites may nevertheless be modulated by the accessory proteins in a manner affecting extension from mispairs. As summarized in the Introduction, the available information about insertion fidelities as a function of gp43 accessory proteins is limited, directing attention instead to their ability to promote the extension of mispairs. In limited kinetic analyses, the Pol^M Exo^- gp43 mutator (with no accessory proteins) increased the rate of formation of $G_{template}$•T_{primer} mispairs by 180-fold (the reverse configuration being untested) but *decreased* extension from the $T_{template}$•G_{primer} mispair by >30-fold, at least in a single sequence context (Bebenek *et al.* 2001). The balance between such kinetic factors is one measure of fidelity and is worthy of investigation using more extensive and more complex kinetic methods in a variety of sequence contexts.

At the evolutionary level, one should consider the selective forces acting on gp43. Fidelity is one component, but the universal genomic mutation rate independent of genome size for DNA-based microbes (Drake 1991; Dressman *et al.* 1997; Grogan *et al.* 2001) sets limits on the extent to which organisms will invest resources to evolve ever-lower mutation rates, and mutation rates in T4 are determined primarily by gp43 (Drake and Ripley 1994). Velocity of replication is another component, and multiple origins of replication and the processivity clamp both contribute to rapid DNA replication. Other DNA transactions, such as the resolution of recombinational intermediates and interactions between replication and transcription machineries, must also lay constraints on the evolution of gp43 function. Finally, mutation rates vary hugely from site to site in this as in all mutational spectra, necessarily as a function of local DNA sequences. Thus, evolution will tend to optimize the impacts of accessory proteins on fidelity while at the same time tending to optimize at least the factors just noted, including a vast number of local DNA sequences. It is impossible that the point-to-point balance that we measure as fidelity should be the same for all the sequences. Thus, it was likely from the start that different impacts, extending to both increases and decreases in fidelity, would be observed.

In this context, two interesting questions arise. First, does a replicase coevolve with its cognate genome to minimize such variation? Because the *lacZ*α template is not a cognate sequence for RB69 gp43, *lacZ*α may display more site-to-site variation of the kind studied here than would be observed with a template such as the T4 *rI* gene (Bebenek *et al.* 2001). Second, is there a counterpart to the accessory-protein impacts *in vivo*? There are clear-cut differences (as well as similarities) between the kinds of mutations produced *in vivo* (Bebenek *et al.* 2001) and *in vitro* (Bebenek *et al.* 2002), implying either the operation *in vivo* of fidelity factors yet to be discovered or important physiological differences such as molecular crowding.

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