Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 8042–8046, July 1997
Genetics

Retention of replication fidelity by a DNA polymerase functioning in a distantly related environment

(replication fidelity/DNA polymerase/bacteriophage T4/bacteriophage RB69)

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Communicated by Maurice S. Fox, Massachusetts Institute of Technology, Cambridge, MA, May 16, 1997 (received for review January 1, 1997)

ABSTRACT **The primary structures of the replicative DNA polymerases (gp43s) of bacteriophage T4 and its distant phylogenetic relative RB69 are diverged, retaining only 61% identity and 74% similarity. Nevertheless, RB69 gp43 substitutes effectively for T4 gp43 in T4 DNA replication *in vivo*. We show here that RB69 gp43 replicates T4 genomes *in vivo* with a fidelity similar to that achieved by T4 gp43. Furthermore, replication by RB69 gp43 in the distantly related environment does not enhance the mutator activities of mutations in T4 genes that encode other components of the multienzyme DNA replicase. We also show that the fidelities of RB69 gp43 and T4 gp43 are both high *in vitro* and that they are similarly and sharply reduced *in vivo* by mutations that eliminate the 3′-exonucleolytic proofreading function. We conclude that gp43 interactions with the other replication proteins are probably nonessential for polymerase fidelity.**

Replicative DNA polymerases control base selection by discriminating against both nucleotide misinsertions and chain extension from misinsertions (1). This fidelity is often enhanced by a 3′-exonucleolytic (Exo) proofreading activity. Exo function may reside either in the same polypeptide that specifies the polymerase (Pol) function, as in bacteriophage T4 (2), or in a separate subunit of a heteromeric enzyme, as in *Escherichia coli* DNA polymerase III (3). T4 DNA polymerase (gp43), the product of phage gene *43*, is a monomeric 898 amino acid multifunctional enzyme that replicates the phage genome and plays a major role in the control of spontaneous mutation rates (4). Like other replicative DNA-dependent DNA polymerases, gp43 functions as part of a multiprotein complex (5–7), whose assembly and biological specificity depend on specific protein–protein interactions among its components. Although required for efficient replication, these interactions may or may not be important for the fidelity of DNA synthesis. T4-induced proteins that increase the processivity of T4 gp43 also sometimes increase the fidelity of DNA synthesis on homopolymeric templates (8, 9). In addition, missense mutations in T4 replication genes other than *43* sometimes display modest mutator activities (4); however, it is not clear if these mutator activities are mediated through protein–protein interactions, which normally synergize the intrinsic fidelity of gp43, or act instead to reduce normal gp43 fidelity, or are independent of these interactions. We reasoned that if gp43 fidelity depended on protein–protein interactions, then this fidelity should decrease when the enzyme functions in a distantly related environment.

Phage RB69 is a phylogenetic relative of T4 (10). RB69 gp43 amino acid sequence is 61% identical (74% similar) to that of T4 (11). Nevertheless, plasmid-encoded gp43 from either phage source can support replication of the other (11). This property allowed us to examine the fidelity of phage replication when the RB69 DNA polymerase operates in the T4 environment. We show here that (*i*) the fidelity of T4 DNA replication is not compromised when RB69 gp43 substitutes for T4 gp43; (*ii*) the intrinsic fidelities of RB69 gp43 and T4 gp43 are similar and high in the wild-type enzymes and similarly reduced in exonuclease-deficient derivatives, independently of the source of the other replication proteins; and (*iii*) the magnitudes of mutator activities produced by defects in other proteins of T4 replication are largely independent of the source of the replicative gp43. In addition to reaffirming the importance of DNA polymerase as the primary determinant of replication fidelity in this biological system (4), our studies suggest that the optimal mutation rate of phage T4 (12) has been strongly conserved during the course of evolution.

MATERIALS AND METHODS

T4, *E. coli*, and Plasmid Strains. Multiple T4 mutants were constructed by recombination. The T4 gene-*43* double-amber mutant *amE4332 amE4322* (hereafter abbreviated *43am*) bears UAG chain-terminating codons at amino acid positions 202 and 386. This pair of amber mutations was chosen to minimize the production of nonsense-codon read-through in nonpermissive (*sup*^{0}) bacterial hosts and because the 201-residue N-terminal gp43 fragment encoded by *43am* exhibits no detectable biological activity. T4 *rII-43am* mutants, with or without a *ts* mutation in another phage gene, were grown either in amber-suppressing *E. coli* B40*su*$^+$II cells (which insert glutamine at UAG codons) or in *sup*^{0} BB cells carrying a plasmid-borne T4 or RB69 gene *43* (11) (abbreviated pT4.43 or pRB.43, respectively). The plasmid-bearing cells produce sufficient gp43 without induction to support the growth of gene-*43* mutants.

Wild-type T4 produces slightly larger plaques than does *43am* on either of these hosts. When *43am* is grown in BB cells carrying pT4.43, *43*$^+$ recombinants are produced by plasmid–phage recombination (marker rescue) and are distinguishable from *43am* by their larger plaque size and ability to plate on nonsuppressing host cells. When cells carrying pT4.43 are used as the host, a single passage of *43am* yields 0.1–1% *43*$^+$ phages, a typical plaque ($\approx 10^7$ phage) contains roughly 7% *43*$^+$, and stocks grown to about 10^{11} phage contain roughly 15% *43*$^+$. In contrast, because of the extensive divergence between the T4

Abbreviations: T4 *43am*, the T4 *amE4332 amE4322* double amber mutant; pT4.43, T4 gene *43* cloned in the vector pSP72; pRB 43, gene *43* cloned in pSP72; Exo, 3′-exonuclease activity; Pol, DNA template-dependent nucleotidyl transferase (DNA polymerase) activity; gp43, the polypeptide encoded by gene *43* and bearing both Pol and Exo activities.

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and RB69 genes *43* (11), $<10^{-7}$ *43*$^+$ are detected when *43am* is grown in cells carrying pRB.43. Because *43*$^+$ frequencies increase faster than predicted from recombination alone, *43*$^+$ recombinants must enjoy a modest selective advantage. However, these recombinants do not perturb our results because they appear only in the control population (T4 replication driven by T4 gp43); they do not interfere with the visual screening of r plaques in forward-mutation tests, and they are indistinguishable from *43am* in QA1 cells in the reversion tests described below.

Unlike *E. coli* B40*su*$^+$II, *E. coli* BB does not reveal the rapid-lysis (r) phenotype of T4 *rII* mutants. Thus, growing *rII* mutants on BB cells provides no selective advantage to *rII*$^+$ revertants (4). *E. coli* K12 strain CR63(λ*h*), which carries *su*$^+$I but restricts non-amber *rII* mutants, was used to measure frequencies of *rII*$^+$ revertants in both *43am* and *43*$^+$ stocks.

The Exo$^-$ derivatives of pT4.43 (a D219A–D324A double replacement) and pRB.43 (a D222A–D327A double replacement) were constructed from the wild-type clones by site-directed mutagenesis (13). These sites were chosen for alteration because they occupy conserved Exo motifs (11, 14, 15). The mutant gp43s display <0.1% of the wild-type 3′-exonuclease activity (16) in an oligonucleotide digestion assay (17).

Unless otherwise indicated, cells were grown at 37°C in modified Luria–Bertani broth and plates contained Drake agar (18).

Mutation Frequencies *in Vivo*. In phage stocks of similar titer, relative mutation rates are proportional to relative mutant frequencies (12). For simplicity, we report only frequencies.

Variation in mutant frequencies between replicate cultures is strongly clonal in nature and is frequently skewed by "jackpots" (19). Therefore, we use medians because they are more reproducible than means. For ratios based on sets of five stocks grown in parallel, reproducibility is usually within 2-fold (20). Most of our medians are based on measurements of five stocks grown on different days and, if homogeneous, pooled; the underlying data are available upon request.

We measured the frequencies of two kinds of mutations: all forward mutations in the T4 genome that produce the r phenotype, and reversions of specific *rII* mutations. Stocks were initiated from single particles.

In T4, the r phenotype results from mutations in any of several genes (including *rI*, *rII*, *rIII*, and *rV*) that together account for 2–3% of the genome or 4–5 kb of DNA (12, 21). In the forward-mutation assay, individual T4 *43am* plaques ($\approx 10^7$ phage) were recovered from lawns of BB cells bearing pT4.43 or pRB.43, resuspended in buffer, and plated on *E. coli* B40*su*$^+$II to yield about 600 plaques per plate.

To measure *rII*$^+$ revertant frequencies, stocks were grown on BB cells bearing pT4.43 or pRB.43 and assayed on CR63(λ*h*) cells to score revertants and on B40*su*$^+$II cells to score total phage.

Mutation Frequencies *in Vitro*. We used the Kunkel M13mp2 assay system (22, 23), in which the mutational target is a 250-bp *E. coli lacZ*α sequence. The forward-mutation assay scores reduced or abolished α complementation due to diverse kinds of mutations at many sites. The reversion assay measures mutation from nonsense to sense from TGA (UGA) at *lacZ* codon 89; eight of the nine possible single-base substitutions can be detected (24). Here, sampling variation is expected to produce a Poisson distribution because all mutations arise as "clones" of size 1 and there is no variation in clone size.

The *in vitro* DNA synthesis reaction mixtures (25 μl) consisted of 40 mM Tris·HCl (pH 7.5), 2 mM dithiothreitol, 10 mM $MgCl_2$, equimolar concentrations of dATP, dCTP, dTTP, and dGTP, 30 fmol of gapped M13mp2 DNA, and 0.9 pmol of T4 or RB69 DNA polymerase. Forward-mutation assays included equimolar concentrations of dNTPs at 50, 100, or 1,000 μM. Reversion assays included dGTP at 20–1,000 μM and the other three dNTPs at 20 μM each. Incubation was at 37°C for 30 min and reactions were terminated by adding EDTA to 15 mM. A portion of each reaction was analyzed by agarose gel electrophoresis to monitor the extent of synthesis, and the remainder was used to transform *E. coli* MC1061 cells and assay for α-complementation (24). In the forward-mutation assay, wild-type plaques are blue and mutant plaques are colorless to light blue. In the reversion assay, wild-type plaques are colorless and mutant plaques are blue.

RESULTS

Fidelity *in Vivo*. In $r^+ \rightarrow r$ forward-mutation tests, the T4 and RB69 gp43s displayed very similar fidelities (Table 1). With wild-type gp43s, mutant frequencies were identical and were close to historical values (4). With the Exo$^-$ derivatives, mutant frequencies increased about 400-fold, compared with previous estimates of about 700-fold using the somewhat smaller and less studied *ac* gene as a mutational target (14, 15). The RB69 Exo$^-$ enzyme displayed a roughly 1.6-fold lower fidelity than the T4 Exo$^-$ enzyme. This difference is likely to be real because it occurred both times in two 7-stock tests (the results of which were pooled) and because the 4 central values of the two 14-value sets (581, 623, 635, and 657 for RB69 gp43; 978, 985, 1071, and 1075 for T4 gp43) did not overlap.

Six different *rII*$^-$ → r$^+$ reversion tests revealed only small differences between the wild-type versions of the two polymerases (Table 2); no effect was more than 3-fold and the average over all six sites was 0.9-fold. The Exo$^-$ polymerases increased mutation frequencies by about 300- to 9,000-fold (Table 2). RB69 Exo$^-$ gp43 displayed an average 2.4-fold lower fidelity than T4 Exo$^-$ gp43.

Taken together, the results in Tables 1 and 2 indicate that T4 DNA replication exhibits the same average error frequency whether driven by the T4 or the RB69 DNA polymerase. However, in the Exo$^-$ enzymes, the RB69 enzyme is about one-half as accurate as the T4 enzyme.

Effects of Other Replication Proteins on Fidelity. Missense (*ts*) mutations in genes encoding several other T4 DNA replication proteins produce small mutator effects (4). We asked whether these effects are altered when T4 gp43 is replaced with RB69 gp43. Specifically, we used *rII* reversion tests to measure mutator ratios (revertant frequency in *ts* background divided by that in *ts*$^+$ background) in both gp43 backgrounds. In these tests, we used the *rII* mutations originally used to detect the mutator effects, instead of the *rII* mutations listed in Table 2. Because T4 mutation rates sometimes decrease at higher temperatures (ref. 25; L. A. Smith and J.W.D., unpublished results), we conducted our tests at both 32°C and 37°C; these *ts* mutants grow similarly well at both temperatures.

The results of these tests appear in Table 3 and reveal the following. First, although several revertant frequencies were lower at the higher temperature, there was no trend in mutator ratios between 32°C and 37°C. Second, when the T4 and RB69 mutator ratios are compared, there is no clear tendency for them to increase in the trans context: in two cases, they increased slightly (line 1 by 3.9-fold, line 8 by 2.6-fold), in two cases they probably decreased slightly (lines 5 and 6, by

Table 1. Forward mutation tests *in vivo* comparing the T4 and RB69 DNA polymerases

Type of DNA polymerase	No. of stocks	Median *r* mutants per 10^4 progeny	Exo$^-$/Exo$^+$
T4	7	2.1	
RB69	7	2.1	
T4 Exo$^-$	14	630	310
RB69 Exo$^-$	14	1,030	490

Table 2. Reversion tests *in vivo* comparing the T4 and RB69 DNA polymerases

		Revertants per 10^8 progeny*			Exo$^-$/Exo$^+$	
rII	Type†	RB69	T4	RB69/T4‡	RB69	T4
		Exo$^+$ polymerases				
FC11	FS	14	18	0.8		
131	FS	123	54	2.3		
UV256	G·C	17	18	1.0		
UV48	G·C	6.2	14	0.4		
UV357	Ochre	2.3	3.6	0.6		
UV375	Ochre	1.3	4.3	0.3		
		Exo$^-$ polymerases				
FC11	FS	2,100	1,000	2.1	550	1,490
131	FS	10,400	1,500	7.0	280	840
UV256	G·C	12,300	16,300	0.8	9,300	7,000
UV48	G·C	4,300	1,500	3.0	1,060	7,000
UV357	Ochre	400	400	1.0	970	1,350
Uv375	Ochre	700	1,300	0.6	3,000	5,400

*For Exo$^+$ entries, revertant frequencies are medians of 20 stocks grown in 4 sets of 5 each, with each set of 5 grown in parallel on the 2 polymerase sources. The first two sets used cells that had carried the plasmids over several passages, whereas the last two sets used freshly transformed cells. The results from older and newly transformed cells were indistinguishable and were therefore pooled. For Exo$^-$ entries, revertant frequencies are medians of 10 stocks grown in freshly transformed cells.

†The FS (frameshift) mutants revert by small additions and deletions; *FC11* reverts by diverse additions and deletions within an *rIIB* sequence of about 120 bp, whereas *131* reverts by single-base pair additions within a run of five A·T base pairs. The G·C mutants revert by transitions and perhaps also by transversions. The ochre mutants revert exclusively within the UAA codon by both transitions and transversions.

‡The ratios of the RB69 and T4 medians are expected, on the basis of experience, to be reproducible to within less than about 1.5-fold, so that ratios that are <0.6 or >1.5 are likely to reflect small but real differences.

2.3-fold), and in the remaining cases there was no significant difference. Thus, we observed either small or no enhancement of the mutator activity of mutations affecting other T4 replication proteins with RB69-driven replication.

Fidelity Tests *in Vitro*. We also examined T4 and RB69 gp43s *in vitro* to determine if they display similar fidelities in their combined base-selection and proofreading functions in the absence of any other proteins. A phage M13mp2 double-stranded DNA template carrying a single-stranded gap in the *lacZα* region was converted to fully double-stranded DNA using either T4 or RB69 gp43. The product was then used to transform *E. coli* cells and the resulting plaques were screened for *lacZα* complementation mutants.

Table 4. Mutation tests *in vitro* comparing T4 and RB69 DNA polymerases

Type of DNA polymerase	Total plaques	Mutant plaques	Mutants per 10^4 progeny
	Forward mutation		
T4	68,200	34	5.0
RB69	127,200	39	3.1
	Reversion		
T4	1,900,000	30	0.16
RB69	2,300,000	15	0.064

In the forward-mutation assay, we used equimolar dNTP concentrations at 50–1,000 μM. The results with this range of substrate concentrations were indistinguishable and were therefore pooled. Table 4 shows that RB69 gp43 yielded a slightly lower mutant frequency than did T4 gp43 (χ^2 test, $P = 0.049$), although mutation frequencies with both enzymes were at or below the lower boundary of the historical background of 5–7 $\times$ 10^{-4} for uncopied template DNA (T. A. Kunkel, personal communication). The phage forward-mutation frequencies of 3–5 $\times$ 10^{-4} are substantially lower than the corresponding values for three other Exo$^+$ DNA polymerases, the Klenow fragment of *E. coli* polymerase I (11–40 $\times$ 10^{-4}) (29), eukaryotic polymerases γ (30–50 $\times$ 10^{-4}) (30), and the *E. coli* polymerase III core enzyme (10–30 $\times$ 10^{-4}) (R. M. Schaaper and P. Pham, personal communication); note, however, that the last measurement employed a different mutational target, albeit of about the same size.

The reversion assay monitors the reversion of an opal (TGA) codon embedded in the template sequence 5′-C**TGA**-3′. Excess dGTP is used to increase the frequency of the most common misinsertion, G_{primer}·$\mathbf{T}_{template}$, and to promote extension from the mispair. Because mutant frequencies obtained with different dGTP concentrations were indistinguishable from one another, they were pooled. Table 4 again shows that RB69 gp43 yielded a slightly lower mutant frequency than did T4 gp43 (χ^2 test, $P = 0.0055$), but neither value fell markedly above the historical background of 0.02–0.05 $\times$ 10^{-4} for uncopied template DNA (T. A. Kunkel, personal communication).

The measurements summarized in Table 4 reveal that both gp43s have fidelities too high to generate mutant frequencies convincingly above historical backgrounds, even when dNTP concentrations are adjusted to favor error production. Nevertheless, our measurements clearly suggest that mutant frequencies *in vitro* are lower with RB69 gp43 than with T4 gp43. This apparent contradiction with results obtained *in vivo* and with the historical background is discussed below.

Table 3. Fidelity interactions between T4 mutator mutations and replicative polymerase

Mutator gene and allele*	Gene product†	*rII* tester mutation*	Growth temp. (°C)	No. of stocks	T4 polymerase‡			RB69 polymerase‡		
					wt	*ts*	Ratio	wt	*ts*	Ratio
32tsG26	Ssb	*UV183*	32	10	19	40	2.1	5.8	47	8.1
			37	5	3.8	11	3.0	2.0	6.5	3.3
41tsA14	DNA helicase	*375*	32	5	4.3	64	15	1.4	28	19
			37	20	6.3	26	4.1	3.9	21	5.4
44tsB110	Clamp loader	*Ed144*	32	5	9.7	33	3.4	26	39	1.5
			37	10	5.8	25	4.3	8.2	15	1.9
45tsCB129	Clamp	*375*	32	5	4.3	60	14	1.4	28	20
			37	10	5.3	28	5.3	1.2	17	14

*The gene *32* mutator (26), the gene *41* and *45* mutators (27), and the gene *44* mutator (28) were described previously. *rUV183* is an amber mutation, *r375* is an ochre, and *rED144* is a frameshift.

†For a review of the functions of these proteins see Nossal (5).

‡Values in the wt (wild-type) and *ts* columns are median revertant frequencies per 10^8 particles. The ratios are the *ts*/wt values.

DISCUSSION

T4 DNA replication displays similar accuracy whether driven by T4 DNA polymerase or by the distantly related enzyme of phage RB69. The overall accuracies of the two enzymes appear to be identical (Table 1), although reversion tests detect small site-to-site differences (Table 2) that simply average out. The Exo$^-$ phage polymerases are about 1/400th as accurate than their Exo$^+$ counterparts, in agreement with previous measurements using the T4 enzyme (14, 15), whereas eukaryotic Exo$^-$ DNA polymerases α (30, 31) and a *Thermus aquaticus* DNA polymerase (32) are only about one-fourth as accurate *in vitro* than cellular Exo$^+$ polymerases. Exo$^-$ RB69 gp43 displays slightly lower base insertion accuracy than does Exo$^-$ T4 gp43 (Tables 1 and 2, Exo$^-$ entries), but the RB69 3′-exonuclease appears to be slightly more processive than that of T4 (C.-C.W. and J.D.K., unpublished results) and may compensate for a small deficit in the accuracy of base selection. Kinetic partitioning of the Exo and Pol activities has been studied in the T4 enzyme (33), and similar studies are needed for RB69 gp43.

In contrast to the identical mutation rates observed *in vivo*, RB69 gp43 yielded slightly lower mutant frequencies than did T4 gp43 *in vitro* (Table 4). The difference between the results *in vivo* and *in vitro* may reflect small differences in how the two gp43s process lesions introduced during template preparation (see below). The *in vitro* mutant frequencies fell from slightly below to slightly above the historical backgrounds and are at least an order of magnitude smaller than values for Exo$^+$ DNA polymerases from cellular systems. To achieve the observed optimal microbial genomic mutation rate, cellular genomes must be replicated with higher accuracy per base pair than required for viral genomes (12). Cellular microbes achieve part of their accuracy from DNA mismatch repair. DNA mismatch repair seems not to operate in the phage T4 system (4, 20), but the high fidelity of the T4 Pol/Exo function provides the requisite accuracy.

Despite not rising much or at all above historical backgrounds for these assays, we suspect that the T4/RB69 *in vitro* fidelity differences are informative. In T4, certain gene-*43* mutations reduce spontaneous mutation rates at some but not all sites (34, 35). Such "antimutator" mutations also reduce mutagenesis induced by 5-bromouracil, 2-aminopurine, ethyl methanesulfonate, hydroxylamine, nitrous acid, and ultraviolet irradiation (36, 37). In the M13mp2 *lacZ*α assays, background mutant frequencies vary among template preparations and increase with age and handling (T. A. Kunkel, personal communication), suggesting that the background is caused by accumulated DNA damage in addition to replication errors generated during the growth of M13mp2 stocks. Because some replicases can suppress damage-induced mutagenesis and the T4 and RB69 replicases are among the most accurate replicases known, we suspect that the differing T4 and RB69 mutant frequencies described in Table 4 are real.

Because RB69 gp43 operates with high fidelity in the T4 replication complex, its fidelity *in vivo* is likely to be largely independent of its interactions with other DNA replication proteins, although the latter are essential and contribute markedly to the processivity and velocity of phage DNA replication (5). This conclusion has been anticipated at least twice previously, in fidelity studies (38) and in kinetic studies (31). However, the extent of direct interactions between gp43 and other replication proteins remains poorly understood, and it is not possible to reject the possibility that at least some interactions critical for fidelity were randomly conserved as T4 and RB69 diverged. A number of other studies have monitored DNA polymerase fidelity *in vitro* as a function of additional proteins. The results of some are difficult to interpret because of the use of homopolymeric templates or the analysis of only a single responding site (8, 9, 39). In a study similar to that described in Table 4, the fidelity of yeast Pol α was reported to be largely unaffected by the presence of the β subunit, the DNA primase subunits, or RF-A protein (40). On the other hand, human Ssb protein clearly increased the fidelity of human Pol α (41). Phage T7 DNA polymerase requires host thioredoxin as a processivity subunit; in an Exo$^-$ background, thioredoxin enhances T7 DNA polymerase frameshift fidelity and simultaneously decreases its base substitution fidelity, probably by promoting mispair extension (42). Similar observations have been made with bovine Pol δ, whose fidelity was also reduced by its processivity factor (proliferating cell nuclear antigen), again perhaps by promoting extension from mispairs (43). In summary, other studies on the effects of additional proteins on the fidelity of DNA polymerases are largely consistent with our observations.

Our conclusion concerning the autonomy of gp43 fidelity is strengthened by the failure of RB69 gp43 acting in *trans* to enhance mutator activities caused by missense mutations in T4 genes encoding proteins that support the polymerase in phage replication (Table 3). Because the primary structures of the RB69 and T4 gp43s diverged in separate lineages of coevolving replication proteins, the fidelity of each gp43 was optimized in its respective lineage. If optimization required specific interactions between gp43 and other phage-induced proteins, then the RB69 and T4 enzymes would have responded differently to the same set of mutagenic replication proteins. We therefore believe that fidelity is intrinsic to gp43 and is not strongly modulated by interactions with other proteins of the multienzyme DNA replicase. We suspect that the originally observed mutator activities represent qualitative new interactions between the mutant protein and gp43, rather than the failure of a function that supported fidelity.

It is important to consider the interplay of the Exo and Pol functions of gp43 in terms of the three-dimensional structure of the protein. The RB69 enzyme was recently crystallized and its structure has been determined (44). Although many details remain unknown, the amino acid determinants of the Exo and Pol catalytic activities clearly reside in distinct domains and may function independently to some extent. For example, Exo$^-$ gp43 mutants are viable and replicate well (Tables 2 and 3; refs. 14 and 15), and amino-terminal T4 gp43 polypeptide fragments have been described that exhibit no Pol activity but retain at least partial Exo activity (17, 45). The Pol and Exo sites have been highly conserved in gp43 as well as in a number of other DNA-dependent DNA polymerases (11, 46) and may have experienced coevolution. We should also point out that amino acid substitutions that alter the fidelity of gp43 need not map within the Pol or Exo sites, but may affect these activities via other effects on overall protein structure and function. In fact, many T4 gene *43 ts* mutations belong to this category of fidelity variants, as do numerous mutator and antimutator mutations (e.g., ref. 47). In this context, the conservation of high fidelity in the two diverged forms of gp43 may be viewed as an indication that gp43 is the primary determinant of spontaneous mutation throughout the T4 family of phages and that this enzyme has driven the divergence that has occurred between the T4 and RB69 genomes, including divergence of the polymerase gene itself. Other replicative polymerases in other organisms may have served similar roles in the evolution of their cognate genomes.

We thank Tom Kunkel for his numerous suggestions concerning the enzymological studies and Tom Kunkel and Roel Schaaper for critical assessments of the nascent manuscript. We thank W. H. Konigsberg, T. A. Steitz, and their coworkers for communicating unpublished information about the structure of RB69 gp43. J.D.K. and C.-C.W. were supported by Grants GM18842 and GM54627 from the National Institute of General Medical Sciences and by funds from Tulane University School of Medicine.

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