# Templated Mutagenesis in Bacteriophage T4 Involving Imperfect Direct or Indirect Sequence Repeats

## Gary E. Schultz, Jr.,[1] and John W. Drake[2]

*Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709*

## ABSTRACT

Some mutations arise in association with a potential sequence donor that consists of an imperfect direct or reverse repeat. Many such mutations are complex; that is, they consist of multiple close sequence changes. Current models posit that the primer terminus of a replicating DNA molecule dissociates, reanneals with an ectopic template, extends briefly, and then returns to the cognate template, bringing with it a locally different sequence; alternatively, a hairpin structure may form the mutational intermediate when processed by mismatch repair. This process resembles replication repair, in which primer extension is blocked by a lesion in the template; in this case, the ectopic template is the other daughter strand, and the result is error-free bypass of the lesion. We previously showed that mutations that impair replication repair can enhance templated mutagenesis. We show here that the intensity of templated mutation can be exquisitely sensitive to its local sequence, that the donor and recipient arms of an imperfect inverse repeat can exchange roles, and that double mutants carrying two alleles, each affecting both templated mutagenesis and replication repair, can have unexpected phenotypes. We also record an instance in which the mutation rates at two particular sites change concordantly with a distant sequence change, but in a manner that appears unrelated to templated mutagenesis.

TEMPLATED mutations are initiated when a DNA primer strand dissociates from its cognate template and anneals with a fragment of complementary sequence in an ectopic template. The relocated primer may be extended, acquiring a short sequence that is not fully complementary to the original template, and then reanneal with its cognate template. If the acquired non-complementary bases escape correctly oriented proofreading and mismatch repair, mutations will result. Lynn RIPLEY (1982) described two models for templated mutagenesis based on imperfect palindromic repeats, in one of which the ectopic template was in the other parental strand and in the other of which the ectopic template was in the daughter strand itself. Examination of other mutations added imperfect direct repeats as mutagenic templates (RIPLEY *et al.* 1986; SHINEDLING *et al.* 1987). Templated mutations are usually invoked when they simultaneously acquire multiple changes for which a plausible donor template can be found. Despite the intrinsic fascination of templated complex mutations and their potential importance for certain evolutionary paths and some human genetic disorders, the enzymology that creates them has not been characterized.

Template switching also occurs in a mutation-avoiding mode called replication repair. In the canonical model for replication repair (FUJIWARA and TATSUMI 1976; HIGGINS *et al.* 1976), a primer strand whose extension has been blocked by DNA damage switches to the other daughter strand as a template, extends briefly, and then switches back to its cognate template, thus accurately bypassing the damaged site.

In 1987, a mechanism for surviving DNA damage was described in bacteriophage T4, which was distinct from and additive to the classical T4 DNA recombination-repair and excision-repair systems (WACHSMAN and DRAKE 1987). This system was defined by mutant alleles of two T4 genes, *32* (encoding gp32, the "SSB" protein that binds to single-stranded DNA) and *41* (encoding gp41, the main replicative DNA helicase). Subsequent enzymological analyses using an eight-protein T4 DNA replication system *in vitro* showed that strand switching could indeed be promoted by DNA damage in the template strand and that the resulting replication repair was severely compromised when the mutant gp32 and gp41 proteins replaced their wild-type counterparts (KADYROV and DRAKE 2003). Then, somewhat surprisingly, an alternative T4 system of replication repair was discovered *in vitro* in which gp32 and gp41 were replaced by the classical T4 recombinase UvsX and a different T4 DNA helicase, Dda (KADYROV and DRAKE 2004). To date, these are the only genetically and enzymologically well-defined replication-repair systems.

[1]*Present address:* Biology Department, Marshall University, 1 John Marshall Dr., Huntington, WV 25755.

[2]*Corresponding author:* Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, 111 South Alexander Dr., Research Triangle Park, NC 27709. E-mail: drake@niehs.nih.gov

## TABLE 1

### Primers

| Construct | Expected phenotype | Direction | Primers |
|---|---|---|---|
| AGC → TCA at 145–147 | $r^+$ | 5′ → 3′ | 127-ATGCTTTTTGAAAATAAA<u>TCA</u>GTAGAATCGTC-158 |
| | | 3′ → 5′ | 116-CCGTGCAAATATACGAAAAACTTTTATTT-144 |
| TAG → CGC at 171–173 | $r^+$ | 5′ → 3′ | 151-GAATCGTCTGAACAATTCTA<u>CGC</u>TTTTATGAGAAC-185 |
| | | 3′ → 5′ | 141-ATTTTCGCATCTTAGCAGACTTGTTAAGAT-170 |
| ATAG → CTGA at 171–174 | $r$ | 5′ → 3′ | 151-GAATCGTCTGAACAATTCTA<u>CTGA</u>TTTATGAGAAC-185 |
| | | 3′ → 5′ | 141-ATTTTCGCATCTTAGCAGACTTGTTAAGAT-170 |

The introduced sequence change is shown both in the first column and in the top of each pair of primers, the latter underlined.

Because both replication repair and the genesis of many complex mutations depend upon template switching, we tested whether the repair-defective alleles of genes *32*, *41*, and *uvsX* perturbed templated mutagenesis (Schultz *et al.* 2006). All three tested alleles displayed a general, nonspecific mutator activity that included templated mutagenesis, for which the mutator factor was particularly strong with the mutant alleles *32mms* or *41uvs79*. Thus, these defects in replication repair enhance templated mutagenesis.

These studies were facilitated by the availability of a hotspot of templated mutation in the T4 *rI* gene, although other more sporadic templated mutations were also observed. Here, we used this system to further explore templated mutagenesis in T4. We conducted a test to inquire whether the donor site for the hotspot was also a recipient site for the reciprocal templated mutation, and we examined rates of templated mutagenesis in two combinations of the three previously tested mutator mutations. Several surprising results ensued.

## MATERIALS AND METHODS

Most of our bacteriophage T4 and *Escherichia coli* strains and methods have been described (Schultz *et al.* 2006). The T4 double-mutant *32mms 41uvs79* was constructed in an earlier millennium (Wachsman and Drake 1987). The T4 double-mutant *gp32mms uvsXam* (where *uvsXam* = *uvsXam64am67*) was constructed by recombination using a 10:1 ratio of *uvsXam* to *32mms*. Progeny were screened first on *E. coli* Tab32, which does not support the growth of *32mms*, and the *uvsXam* allele was then scored by DNA sequencing, which was also used to confirm the further genotypes of both double mutants.

The pseudo-wild-type (PsWT) replacement AGC → TCA at 145–147 and the strains carrying TAG → CGC at 171–173 or TAGT → CTGA at 171–174 were constructed as follows. A PCR product was obtained containing the wild-type *rI* gene using the downstream primer 5′-AATCAAATCTGGCAACT-3′ and the upstream primer 5′-TTATGACAGCTCGATT-3′. The PCR consisted of a 1-min preheating step followed by 30 cycles of 1 min at 94°, 1 min at 46°, and 1 min at 72° followed by an extension time of 10 min at 72° using Taq DNA polymerase (Invitrogen, Carlsbad, CA). This product was cloned into the plasmid vector pCR2.1-TOPO using the TOPO TA cloning kit (Invitrogen). The plasmid with the *rI* insert was transformed into One Shot TOP10 competent *E. coli* cells (Invitrogen).

Plasmids were purified by miniprep (QIAGEN, Valencia, CA). Using the GeneTailor site-directed mutagenesis system (Invitrogen), the plasmids were methylated and then amplified in a reaction with the primers shown in Table 1 containing the mutant targets. The product of this reaction is linear double-stranded DNA containing the desired alteration. This DNA was then transformed into *E. coli* DH5α-TI cells (Invitrogen), which circularize the linear DNA. To rescue the introduced alleles from a plasmid into T4, log-phase DH5α-TI cells carrying the desired donor plasmid were infected with T4 at a phage/cell ratio of ∼10 and lysis was completed with chloroform at 40 min. The lysate was then plated on *E. coli* BB cells. For an expected *r* phenotype, the infecting T4 was *r⁺* and *r* plaques were isolated. For an expected *r⁺* phenotype, the infecting T4 carried an *rI* amber mutation (G → T at position 241) and *r⁺* plaques were isolated. The desired genotypes were confirmed by sequencing.

T4 *r* mutants produce large, sharp-edged plaques relative to the wild type (except for leaky mutants, which produce an intermediate phenotype). About three-fourths of the *r* mutants detected on *E. coli* BB cells or on K12 strains arise in the *rI* gene, which is a useful mutation reporter because it is not involved in DNA metabolism, displays a mutant phenotype with many missense mutations, and, at 294 bp, is well suited to DNA sequencing and for detecting mutational warm spots as well as hotspots. To grow stocks to be screened for *r* mutants, T4 strains were first plated on BB cells and individual plaques were recovered. These ministocks were then plated at ∼500 plaques/plate and the plates were screened for *r* plaques, plating wild type, *32mms*, *41uvs79*, and *32mms 41uvs79* on BB cells and *uvsXam* and *32mms uvsXam* on CR63 *suI⁺* cells. Subsequent DNA sequencing and calculation of mutation rates have been described (Schultz *et al.* 2006). Sequencing identifies which *r* mutants are *rI*, and the *rI/r* ratios for the seven genotypes wild type, PsWT, *32mms*, *41uvs79*, *uvsXam*, *32mms 41uvs79*, and *32mms uvsX* were 66/88, 118/146, 39/65, 37/47, 65/93, 66/83, and 59/71, respectively. All mutation rates are per genome replication under the geometrical model.

## RESULTS AND DISCUSSION

**A QP mutational hotspot:** The first use of the T4 *rI* gene as a mutation reporter (Bebenek *et al.* 2001) revealed a hotspot of complex mutations consisting of GCG → CTA replacements at positions 146–148 (Schultz *et al.* 2006). These mutations were associated with a quasi-palindrome (QP; an imperfect inverse repeat, usually with a central spacer between the arms of the palindrome). Extending the canonical models of

## Intermolecular Template Switching

```
3'··TTTATTTTCGCATCTTAGCAGACTTGTTAAGATATCAAAATACT··5'
5'··AAATAAAAGCGTAGAATCGTCTGAACAATTCTATAGTTTTATGA··3'
```

↓ Strand separation, then DNA synthesis from right to left:

```
3'··TTTATTTTCGCATCTTAGCAGACTTGTTAAGATATCAAAATACT··5'
                                +
           ←TCTTAGCAGACTTGTTAAGATATCAAAATACT··5'
5'··AAATAAAAGCGTAGAATCGTCTGAACAATTCTATAGTTTTATGA··3'
```

↓ Template switching:

```
3'··TTTATTTTCGCATCTTAGCAGACTTGTTAAGATATCAAAATACT··5'
                     AGACGATTCT
                    (CTTGTTAAGATATCAAAATACT··5'
5'··AAATAAAAGCGTAGAATCGTCTGAACAATTCTATAGTTTTATGA··3'
```

↓ Daughter-strand extension (in bold face):

```
3'··TTTATTTTCGCATCTTAGCAGACTTGTTAAGATATCAAAATACT··5'
                     AGACGATTCTATAGTTTT
                    (CTTGTTAAGATATCAAAATACT··5'
5'··AAATAAAAGCGTAGAATCGTCTGAACAATTCTATAGTTTTATGA··3'
```

↓ Return of daughter strand to cognate template:

```
        TTTTGATATCTTAGCAGACTTGTTAAGATATCAAAATACT··5'
5'··AAATAAAAGCGTAGAATCGTCTGAACAATTCTATAGTTTTATGA··3'
```

↓ Completion of top daughter strand *and* next round of replication:

```
··TTTATTTGATATCTTAGCAGACTTGTTAAGATATCAAAATACT··5'
5'··AAATAAAACTATAGAATCGTCTGAACAATTCTATAGTTTTATGA··3'
```

## Intramolecular Template Switching

DNA synthesis from right to left:

```
              TCTTAGCAGACTTGTTAAGATATCAAAATACT··5'
5'··AAATAAAAGCGTAGAATCGTCTGAACAATTCTATAGTTTTATGA··3'
```

↓ Melting and intramolecular reannealing of daughter strand:

```
        AGACGATTCT
       C       TTGTTAAGATATCAAAATACT··5'
                            +
5'··AAATAAAAGCGTAGAATCGTCTGAACAATTCTATAGTTTTATGA··3'
```

↓ Extension of daughter strand (in bold face):

```
        AGACGATTCTATAGTTTT
       C       TTGTTAAGATATCAAAATACT··5'
                            +
5'··AAATAAAAGCGTAGAATCGTCTGAACAATTCTATAGTTTTATGA··3'
```

↓ Return of daughter strand to its cognate template:

```
        TTTTGATATCTTAGCAGACTTGTTAAGATATCAAAATACT··5'
5'··AAATAAAAGCGTAGAATCGTCTGAACAATTCTATAGTTTTATGA··3'
```

↓ Completion of top daughter strand *and* next round of replication:

```
··TTTATTTGATATCTTAGCAGACTTGTTAAGATATCAAAATACT··5'
5'··AAATAAAACTATAGAATCGTCTGAACAATTCTATAGTTTTATGA··3'
```

## Mismatch Repair



```
 T C      TG A          T C      TG A          T C      TG A
  G   C              G   C              G   C
  C   A              C   A              C   A
  T•A                T•A                T•A
  A•T                A•T                A•T
  A•T                A•T                A•T
  G•C                G•C                G•C
  A•T                A•T                A•T
  T•A                T•A                T•A
  G•C          G   T         A•T
  C•G   ←      C   A    →     T•A
  G•C          G   G         C•G
  A•T    MMR          MMR    A•T
  A•T                A•T                A•T
  A•T                A•T                A•T
  A•T                A•T                A•T
  T•A                T•A                T•A
  A A A•T GA         A A A•T GA         A A A•T GA
```
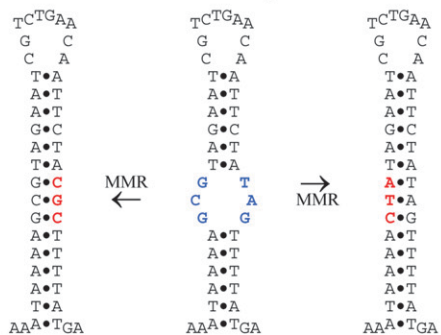
FIGURE 1.—Models for mutations templated by quasi-palindromes. In the top two pathways (intermolecular and intramolecular template switching), donor sequences are blue, GCG target sequences are boldface black letters, and resulting mutations are red letters. In the bottom pathways (mismatch repair), donor and target sequences are reciprocally related and are both blue letters, while mutant sequences are red letters.

RIPLEY (1982), QP-mediated mutations might arise in three different ways (Figure 1). The top two pathways in Figure 1 involve primer melting from the cognate template strand, annealing with an ectopic template strand (the other parental strand in the top pathway, an earlier region of the same primer strand in the middle pathway); primer extension; and, finally, a return to the cognate template strand, thus introducing a sequence mismatch. The resulting mismatch must fall sufficiently far from the primer terminus to escape proofreading.

The bottom pathway of Figure 1 involves primer-strand melting and self-annealing of the full arms of the QP followed by DNA mismatch repair, which excises the mismatch within the stem and fills the gap using the other half of the stem as a template. When the primer strand reverts to full annealing with the cognate template strand, either of two possible mismatches are formed. Whether this pathway occurs in T4 is unclear. Mismatches produced by T4 polymerase insertion errors and escaping proofreading are subject to subsequent DNA mismatch repair in most (but not all) cellular organisms, but phage T4 seems to lack such mismatch repair as judged mainly by failure to observe the corresponding mutator mutations that would result from its inactivation (DRAKE and RIPLEY 1994). However, mismatches in T4 DNA that are formed by recombination are subject to a different kind of mismatch repair, provided that the mismatch involves an indel and thus forms a looped structure very close to the recombinational junction, in which case the looped-out bases are preferentially removed (SHCHERBAKOV and PLUGINA 1991 and references therein; SHCHERBAKOV *et al.* 1995). Thus, the substrate in the bottom pathway of Figure 1 might be processed by this kind of mismatch repair or might not because it has a loop–loop configuration rather than a loop on only one strand. However, when the "repaired" (homogenized) QP reannealed with its cognate template (not shown in Figure 1), one or the other mismatch would be recreated, but might be efficiently rerepaired to the wild type.

The *rI* complex hotspot and its wild-type and mutated DNA and amino-acid sequences are shown in Figure 2. (In all experiments to date, the additional central CGT…ACA has not contributed to QP mutations.) The apparent donor sequence whose complement CTA appears at 146–148 is TAG at 171–173. Not all complex hotspot mutations at 146–148 are GCG → CTA. Instead, some are GCG → ACTA (8 of 57 to date). As speculated previously (SCHULTZ *et al.* 2006), the ACTA replacements could result if a primer strand that had elongated on the other parental strand then reannealed to the cognate template with its terminal TTTT out of register with the template AAAA, leaving one T unpaired. T4 A·T-runs are strongly prone to such

**A**
```
AAATAAAAGCGTAGAATCGTCTGAACAATTCTACGCTTTTATGA
                                  ↑ (not observed)
138-AAATAAAAGCGTAGAATCGTCTGAACAATTCTATAGTTTTATGA-181
         ↓ (observed)
AAATAAAACTATAGAATCGTCTGAACAATTCTATAGTTTTATGA
```

**B**
```
  ser  val                       tyr  ser
   ↑                              ↑
  AGC GTA        wild type       TAT AGT
   ↓                              ↓
  ACT ATA        mutated         TAC GCT
   ↓                              ↓
  thr ile                        tyr ala
```

**C**
```
AAATAAATCAGTAGAATCGTCTGAACAATTCTACTGATTTATGA

AAATAAATCAGTAGAATCGTCTGAACAATTCTATAGTTTTATGA
```

**D**
```
  ser  val                       tyr  ser
   ↑                              ↑
  TCA GTA        pseudowild      TAT AGT
   ↓                              ↓
  ACT ATA        mutated         TAC TGA
   ↓                              ↓
  thr ile                        tyr CT
```

FIGURE 2.—The *rI* QP hotspot region, its potential mutations, and their coded amino acids. (A) The middle sequence is that of the wild type with the two arms of the QP underlined and separated by a CGTCTGAACA spacer. The noncomplementary three bases in each arm (at 146–148 and 171–173) are in larger boldface letters. (B) The coding consequences of the mutations with replacements in boldface letters. (C) The sequences are the pseudo-wild type. The engineered sequence in the left site now directs the mutation that would be introduced in the right site. (D) Coding at the left site remains unchanged while the templated mutation at the right site introduces a chain-termination (CT) mutation.

single-base slippage mutations (STREISINGER and OWEN 1985).

**Testing bidirectionality of transfer:** Results obtained in *E. coli* suggest a bias in the preferred direction of mutagenic templating between the two potential donor/recipient elements of a QP (TRINH and SINDEN 1991; ROSCHE *et al.* 1997; VISWANATHAN *et al.* 2000; YOSHIYAMA *et al.* 2001). When the direction of DNA synthesis is fixed, as it is in *E. coli*, such polarity might reflect an underlying difference in the vulnerabilities of the leading and lagging strands or perhaps an association with the direction of transcription (YOSHIYAMA and MAKI 2003). In T4, however, the direction of DNA synthesis at any point is variable, first, because multiple origins are used early in replication and, second, because random origins are created by recombination for

most DNA replication (KREUZER and MORRICAL 1994). While ~80 GCG → CTA mutations have been observed to date at 146–148, the inverse mutation, TAG → CGC at 171–173, has never been observed. While the GCG → CTA mutation produces the protein change ser val → thr ile, the TAG → CGC mutation would produce only ser → ala (Figure 2B), which might not produce a detectable *r* phenotype. Thus, either of two hypotheses could explain the observed pattern of sequence transfer: (1) it is strongly nonreciprocal or (2) the TAG → CGC mutation goes undetected.

To explore this matter, the sequence at 145–148 was altered from the wild-type AGCG to the pseudo-wild-type (PsWT) TCAG (Figure 2C). As a result, the sequence at 145–148 encodes the same amino acids as does the wild type but would introduce a chain-termination mutation at 172–174 when its complement was inserted there (Figure 2D). The AGCG → TCAG replacement also reduces the length of the QP from 12 base complementarities between arms to 11 and increases the central region of noncomplementarity within the arms from 3 to 4 bp. Genotypes were constructed and tested to confirm our prior expectations about phenotypes: the replacement of TAG at 171–173 with the CGC expected to be introduced from 146 to 148 did indeed produce a wild phenotype, and the replacement of TAGT at 171–174 with the CTGA expected to be introduced from the PsWT TCAG at 145–148 did indeed present an *r* phenotype. Mutational spectra were then obtained using the wild-type and PsWT *rI* mutation-reporter genes. Unexpectedly, no complex mutations were detected in the PsWT spectrum at *either* 145–148 or 171–174 (Table 2). (Full descriptions of the two spectra are provided in Figures 4 and 5 and Table 4.) Because the wild-type and PsWT *rI* mutation rates are similar, the wild-type spectrum predicts about $(118/66)(6) = 10.7$ mutations at 145–148 in the PsWT spectrum, whereas the observed number was 0. If the reciprocal complex mutations arose at 171–174 at the same rate in the wild type but were invisible, then another 10.7 of detected complex mutations would be expected at 171–174 in the PsWT spectrum. These differences have $P = 0.001$ in a $\chi^2$ test in which genotypes were weighted in proportion to their mutation rates. We will return to this result later.

**Potential secondary structure in the *rI* gene:** Erlan Ramanculov kindly provided us with an estimate of secondary structure potential in the *rI* gene (Figure 3). The

TABLE 2

QP-associated complex mutations in the wild-type and PsWT spectra

| Sequence at 145–148 | Total *rI* mutations | $\mu(rI)$ | Complex mutations at 145–148 | $\mu$(complex) at 145–148 | Complex mutations at 171–174 | $\mu$(complex) at 171–174 |
|---|---|---|---|---|---|---|
| Wild type (AGCG) | 66 | $2.8 \times 10^{-5}$ | 6 | $2.6 \times 10^{-6}$ | 0 | $\leq 0.43 \times 10^{-6}$ |
| PsWT(TCAG) | 118 | $4.0 \times 10^{-5}$ | 0 | $\leq 0.34 \times 10^{-6}$ | 0 | $\leq 0.34 \times 10^{-6}$ |

$\mu$, mutation rate per genome replication. "≤" values are calculated on the basis of one mutation.
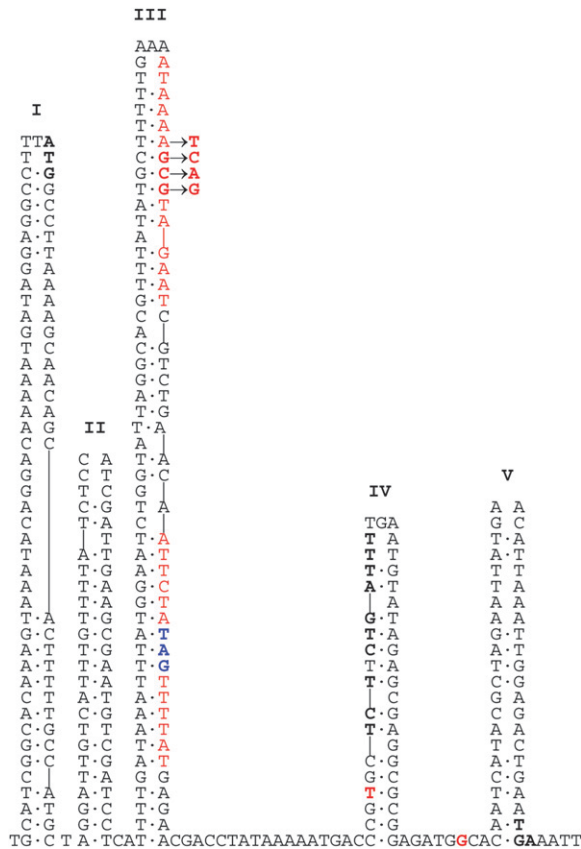
FIGURE 3.—Potential secondary structure in the *rI* gene, courtesy of Erlan Ramanculov. The sequence begins in the gene upstream of *rI* whose TAG terminus is separated from *rI* by 9 intervening bases. The *rI* gene starts with the boldface ATG at the top of region I and ends with the boldface TGA at the base of region V. Vertical lines indicate no base, implying that the opposite base(s) would be looped out. Potential base pairs in secondary structures, which may include G·T, are joined by dots. The palindrome associated with the GCG → CTA hotspot is shown in red letters with bases 146–148 in boldface red letters and 171–173 in boldface blue letters. The engineered PsWT change at 145–148 is indicated by arrows and might be capable of an alternative pairing with the boldface sequence on the left side of region IV. The hotspot bases 205 and 247 are in boldface red letters near the base of region IV and between IV and V, respectively.

amount of internal complementarity in *rI* is somewhat unusual for a protein-coding gene, and the QP associated with the GCG → CTA hotspot turns out to be particularly complicated because each arm of the QP is also largely complementary to yet a different patch of sequence, which appears in Figure 3 as the left half of region III. However, this alternative region cannot act as a donor for the CTA replacement because the corresponding region is TGC rather than TAG. On the other hand, when the 145–148 TCAG PsWT sequence is in place (Figure 3, arrows in region III), that arm of the QP has considerable complementarity with the sequence shown in boldface type on the left side of region IV. If this region were acting as a donor, it would produce the mutation 149T → A with a corresponding val → gln

replacement. This mutation has never been observed among ~700 *rI* mutants, so either it is rarely produced or it lacks a mutant phenotype.

The presence of a complement to part of the PsWT sequence might suggest that interactions between the two sequences affect the ability of the PsWT sequence to donate to its intended 171–174 target, but this possibility remains speculative.

**Modulation of site-specific mutation rates by a distant sequence change:** Base-pair substitution (BPS) mutation rates vary hugely but mostly inexplicably across virtually all mutational spectra (BENZER 1961; SALTS and RONEN 1971; RONEN and RAHAT 1976; RONEN *et al.* 1976). The experimental modification of site-specific mutation rates by changes in neighboring bases has been studied both *in vitro* (KUNKEL and SONI 1988; BEBENEK *et al.* 1993) and *in vivo* in T4, where KOCH (1971) was the first to demonstrate changes in site-specific mutation rates when their neighboring bases were altered. At least in the cases of T4 (which lacks conventional DNA mismatch repair) and of DNA synthesis *in vitro* by various polymerases, the rate of formation and/or disposition of a mismatch during synthesis can reasonably be expected to vary as a function of its close neighbors, but the number of template and primer bases contacted by the polymerase and/or proofreading exonuclease is small, so the effects of more distant bases might be expected to extinguish quickly. It was therefore surprising to observe that a particular BPS increased mutation rates at sites separated from it by 11 and 12 intervening bases (CONKLING *et al.* 1980; SUGINO and DRAKE 1984 as recently confirmed by unpublished results of G. T. CARVER and J. W. DRAKE).

Relative rates of mutation in the wild-type and PsWT spectra were mostly indistinguishable across classes of mutations, with three exceptions. One was the absence (described above) of either class of hotspot mutations in the PsWT spectrum. Two other differences were changes in site-specific BPS rates (Table 3). At 205, the number of T → G transversions was predicted from the wild-type spectrum to be $\leq 1$ in the PsWT spectrum, whereas 8 were observed. At 247, the number of G → A transitions predicted by the wild-type spectrum to appear in the PsWT spectrum was $(118/66)(7) = 12.5$, whereas 22 were observed. (Both of these mutations appeared in most of the other five spectra, sporadically in the case of T205G and frequently in the case of G247A.) Their different representations between the wild-type and PsWT spectra have $P = 0.021$ and $0.136$ for T205G and G247A, respectively, in $\chi^2$ tests in which genotypes were weighted in proportion to their mutation rates. The number of bases intervening between 148 and 205 is 56, and between 148 and 247 it is 92, both numbers far exceeding any simply conceived footprint of the T4 DNA polymerase. Thus, either these two apparent actions at a distance are merely sampling deviations, or they imply as-yet-unconceived aspects of replication fidelity. These

**TABLE 3**

**BPS hotspots in the wild-type and PsWT spectra**

| Sequence at 145–148 | Total $rI$ mutations | $\mu(rI)$ | No. of 205T $\rightarrow$ G | $\mu$(T $\rightarrow$ G) at 205 | No. of 247G $\rightarrow$ A | $\mu$(G $\rightarrow$ A) at 247 |
|---|---|---|---|---|---|---|
| Wild type (AGCG) | 66 | $2.8 \times 10^{-5}$ | 0 | $\leq 0.43 \times 10^{-6}$ | 7 | $3.0 \times 10^{-6}$ |
| PsWT(TCAG) | 118 | $4.0 \times 10^{-5}$ | 8 | $2.7 \times 10^{-6}$ | 22 | $7.5 \times 10^{-6}$ |

$\mu$, mutation rate per genome replication. The "$\leq$" value is calculated on the basis of one mutation.

two sites are in boldface red type in Figure 3 near the bottom of regions IV and V, and we could find no credible nearby templating donor in either the QP or the direct-repeat configuration, nor an inviting impact upon secondary-structure potential in the $rI$ gene. We searched the rest of the T4 genome for possible donor sites and found one QP of size 9 for the hotspot at 205 and two direct repeats of size 10 for the hotspot at 247, all three

| Site | B | M | WT | PsWT | 32 | 41 | X | 32/41 | 32/X |
|---|---|---|---|---|---|---|---|---|---|
| 001 | A | | | | | | | | |
| 002 | T | C | | | | | | | 1 |
| | | A | | | | | | 1 | |
| 003 | G | + | | 1 | | | | | |
| | | T | 1 | | | | | | |
| | | A | | | | | | | 1 |
| 004 | G | | | | | | | | |
| 005 | C | + | 1 | | | | 1 | | |
| 006 | C | | | | | | | | |
| 007 | T | | | | | | | | |
| 008 | T | | | | | | | | |
| 009 | A | – | | | | | | | 2 |
| 010 | A | | | | | | | | |
| 011 | A | | | | | | | | |
| 012 | A | | | | | | | | |
| 013 | G | T | | | | 1 | | | |
| 014 | C | | | | | | | | |
| 015 | A | | | | | | | | |
| 016 | A | | | | | | | | |
| 017 | C | | | | | | | | |
| 018 | A | | | | | | | | |
| 019 | G | T | | | | 1 | 1 | | |
| 020 | C | | | | | | | | |
| 021 | A | | | | | | | | |
| 022 | C | | | | | | | | |
| 023 | T | – | | | 2 | | 1 | | |
| | | + | | | 1 | | | 1 | |
| 024 | T | | | | | | | | |
| 025 | T | | | | | | | | |
| 026 | T | | | | | | | | |
| 027 | T | | | | | | | | |
| 028 | G | | | | | | | | |
| 029 | C | – | | | | | | | 2 |
| | | + | | | 1 | | | | |
| | | A | | | | | 1 | | |
| 030 | C | | | | | | | | |
| 031 | A | | | | | | | | |
| 032 | T | + | 1 | | | | | | |
| 033 | G | | | | | | | | |
| 034 | C | | | | | | | | |
| 035 | T | G | 1 | | | | | | |
| | | C | | | | | | | 1 |
| 036 | A | | | | | | | | |
| 037 | G | | | | | | | | |
| 038 | G | | | | | | | | |
| 039 | A | | | | | | | | |
| 040 | T | | | | | | | | |
| 041 | T | | | | | | | | |
| 042 | G | | | | | | | | |
| 043 | T | C | | | | | 1 | | |
| 044 | C | A | 1 | | | | 1 | | |
| 045 | A | | | | | | | | |
| 046 | T | + | | | 1 | | 1 | | |
| 047 | T | | | | | | | | |
| 048 | T | | | | | | | | |
| 049 | G | | | | | | | | |
| 050 | T | – | 1 | 1 | | | | | |
| | | A | | | 1 | | | | |
| 051 | T | | | | | | | | |

| Site | B | M | WT | PsWT | 32 | 41 | X | 32/41 | 32/X |
|---|---|---|---|---|---|---|---|---|---|
| 052 | T | | | | | | | | |
| 053 | T | | | | | | | | |
| 054 | A | | | | | | | | |
| 055 | T | | | | | | | | |
| 056 | C | + | 1 | | | | 1 | | |
| 057 | T | | | | | | | | |
| 058 | C | + | | 1 | | | | | |
| 059 | C | | | | | | | | |
| 060 | A | | | | | | | | |
| 061 | T | | | | | | | | |
| 062 | C | | | | | | | | |
| 063 | G | | | | | | | | |
| 064 | A | | | | | | | | |
| 065 | T | | | | | | | | |
| 066 | T | | | | | | | | |
| 067 | G | | | | | | | | |
| 068 | A | T | | | | 1 | | | |
| 069 | A | | | | | | | | |
| 070 | G | A | | | | 1 | | | |
| 071 | C | T | 1 | | | | | | |
| 072 | G | | | | | | | | |
| 073 | A | | | | | | | | |
| 074 | A | T | | | | 1 | | | |
| 075 | T | | | | | | | | |
| 076 | G | + | | 1 | | | | | |
| 077 | T | C | | | | 1 | 1 | | |
| 078 | C | | | | | | | | |
| 079 | G | | | | | | | | |
| 080 | A | G | 1 | 1 | 1 | | | 2 | 1 |
| | | T | | | | | 1 | 1 | 2 |
| 081 | T | | | | | | | | |
| 082 | C | T | 1 | | | | | | |
| 083 | C | T | 1 | | | | | | |
| 084 | T | | | | | | | | |
| 085 | C | | | | | | | | |
| 086 | A | – | | 1 | | | | | |
| 087 | T | – | | | | | 1 | | |
| 088 | T | | | | | | | | |
| 089 | T | | | | | | | | |
| 090 | T | | | | | | | | |
| 091 | G | | | | | | | | |
| 092 | A | | | | | | | | |
| 093 | T | | | | | | | | |
| 094 | A | – | | | 3 | | 2 | | |
| 095 | A | | | | | | | | |
| 096 | A | | | | | | | | |
| 097 | T | – | | | 2 | | | | |
| 098 | T | | | | | | | | |
| 099 | T | | | | | | | | |
| 100 | A | + | 2 | | | 1 | 1 | | |
| 101 | T | C | | | | | | | 1 |
| 102 | G | + | 2 | | | | | 1 | 2 |
| 103 | G | T | | 1 | | | | | |
| 104 | A | – | 3 | 1 | | 1 | | 1 | |
| 105 | A | | | | | | | | |
| 106 | T | C | | | | 1 | | | |
| | | – | | | 1 | | 2 | | |
| 107 | C | | | | | | | | |
| 108 | T | + | | 1 | | | | | |

| Site | B | M | WT | PsWT | 32 | 41 | X | 32/41 | 32/X |
|---|---|---|---|---|---|---|---|---|---|
| 109 | G | T | | 1 | | | | | |
| 110 | G | A | | 6 | 1 | 1 | 1 | | |
| | | T | | | | | | 1 | |
| 111 | T | | | | | | | | |
| 112 | A | | | | | | | | |
| 113 | T | | | | | | | | |
| 114 | T | | | | | | | | |
| 115 | A | | | | | | | | |
| 116 | G | | | | | | | | |
| 117 | G | | | | | | | | |
| 118 | C | | | | | | | | |
| 119 | A | C | | | | | 1 | | |
| 120 | C | | | | | | | | |
| 121 | G | | | | | | | | |
| 122 | T | – | | | | | | | 1 |
| 123 | T | | | | | | | | |
| 124 | T | C | | | | | | | 1 |
| 125 | A | – | | 1 | | | | | |
| | | G | | 3 | | | | | |
| 126 | T | | | | | | | | |
| 127 | A | | | | | | | | |
| 128 | T | + | | 1 | | | | | |
| 129 | G | | | | | | | | |
| 130 | C | | | | | | | | |
| 131 | T | + | 1 | 2 | 1 | | 1 | | 1 |
| | | – | | 1 | | 1 | 1 | | |
| 132 | T | | | | | | | | |
| 133 | T | | | | | | | | |
| 134 | T | | | | | | | | |
| 135 | T | | | | | | | | |
| 136 | G | T | 1 | | | | | | |
| 137 | A | – | | 2 | | 2 | | | |
| 138 | A | | | | | | | | |
| 139 | A | | | | | | | | |
| 140 | A | | | | | | | | |
| 141 | T | | | | | | | | |
| 142 | A | – | 2 | | | | | 1 | 1 |
| | | G | | | | | | 1 | |
| 143 | A | | | | | | | | |
| 144 | A | | | | | | | | |
| 145 | A | | | | | | | | |
| **146** | **G** | **C** | | | | | | | |
| | | T | | | | | | | |
| **147** | **C** | **T** | 5[1] | 0[0] | 14[1] | 7[0] | 3[2] | 20[1] | 0[3] |
| | | A | | | | | 1 | 1 | |
| **148** | **G** | **A** | | | | | | | |
| | | + | | | | | | | 1 |
| 149 | T | C | | 1 | | | | | |
| 150 | A | | | | | | | | |
| 151 | G | T | | 1 | | | | | |
| 152 | A | | | | | | | | |
| 153 | A | | | | | | | | |
| 154 | T | | | | | | | | |
| 155 | C | A | | | | 1 | | | |
| 156 | G | | | | | | | | |
| 157 | T | | | | | | | | |
| 158 | C | | | | | | | | |
| 159 | T | | | | | | | | |
| 160 | G | T | 1 | | | | | | |

FIGURE 4.—T4 $rI$ mutational spectra. Sites start with the first base of the initiation codon and end with the last base of the termination codon. B, base; M, mutation; "–," single-base deletion; "+," single-base duplication. Indels in a run are usually entered at the first (sometimes the last) base of the run. WT, wild type, PsWT, the silent modification at 145–148, *32, 32mms*; *41, 41uvs79*; *X, uvsXam*. CTA replacements at 146–148 are indicated at 147, where superscripts are the additional number of ACTA replacements (see text). Other mutations are described in Table 4 and Figure 5.

| Site | B | M | WT | Ps WT | 32 | 41 | X | 32 41 | 32 X |
|---|---|---|---|---|---|---|---|---|---|
| 161 | A | | | | | | | | |
| 162 | A | | | | | | | | |
| 163 | C | | | | | | | | |
| 164 | A | | | | | | | | |
| 165 | A | | | | | | | | |
| 166 | T | A | | | | 1 | | | |
| 167 | T | | | | | | | | |
| 168 | C | A | 1 | | | | | | |
| 169 | T | | | | | | | | |
| 170 | A | G | | | | | 1 | 2 | |
| 171 | T | G | | | | 1 | | | |
| 172 | A | | | | | | | | |
| 173 | G | | | | | | | | |
| 174 | T | - | | | | | | 1 | 1 |
| 175 | T | | | | | | | | |
| 176 | T | C | | | | 1 | | | |
| 177 | T | | | | | | | | |
| 178 | A | | | | | | | | |
| 179 | T | C | | 1 | | | | | |
| 180 | G | A | | | | | 1 | | |
| 181 | A | | | | | | | | |
| 182 | G | | | | | | | | |
| 183 | A | | | | | | | | |
| 184 | A | | | | | | | | |
| 185 | C | | | | | | | | |
| 186 | G | T | | 1 | | | | | |
| 187 | A | C | | | | | 1 | | 1 |
| 188 | C | | | | | | | | |
| 189 | C | | | | | | | | |
| 190 | T | | | | | | | | |
| 191 | A | - | 1 | | | | | | |
| 192 | T | | | | | | | | |
| 193 | A | - | 4 | 13 | 1 | 2 | 1 | 4 | 4 |
| | | + | | | | | 2 | 1 | |
| | | T | | | | | | | 1 |
| 194 | A | | | | | | | | |
| 195 | A | | | | | | | | |
| 196 | A | | | | | | | | |
| 197 | A | | | | | | | | |
| 198 | T | | | | | | | | |
| 199 | G | | | | | | | | |
| 200 | A | | | | | | | | |
| 201 | C | - | 1 | 1 | | | | | |
| 202 | C | T | 1 | | | | | | |
| 203 | C | T | 1 | 1 | | | 2 | 2 | 1 |
| | G | A | 1 | | | | | | |
| | | A | | 1 | | | | | |
| 204 | G | | | | | | | | |
| 205 | T | C | 2 | 3 | | | | 4 | 3 |
| | | G | | 8 | | | 3 | 3 | 2 |
| | | A | | | | | | 1 | |
| 206 | G | A | 1 | 2 | | | | 4 | 1 |
| 206 | | T | | 2 | | | | | |
| 207 | C | A | 1 | 1 | | | | | 1 |
| 208 | T | | | | | | | | |
| 209 | C | | | | | | | | |
| 210 | T | | | | | | | | |
| 211 | T | | | | | | | | |
| 212 | C | | | | | | | | |
| 213 | T | | | | | | | | |
| 214 | G | | | | | | | | |
| 215 | A | | | | | | | | |
| 216 | T | + | | | | 1 | | | |
| 217 | T | | | | | | | | |
| 218 | T | | | | | | | | |
| 219 | T | | | | | | | | |
| 220 | G | | | | | | | | |
| 221 | A | G | | | | | 1 | | |
| 222 | A | | | | | | | | |
| 223 | T | C | | | | | 2 | | |
| 224 | G | | | | | | | | |
| 225 | T | | | | | | | | |
| 226 | A | | | | | | | | |
| 227 | T | | | | | | | | |
| 228 | A | | | | | | | | |
| 229 | G | T | 1 | 2 | | | 2 | | |
| 230 | A | | | | | | | | |
| 231 | G | | | | | | | | |
| 232 | C | T | | | | | 1 | | |
| 233 | G | | | | | | | | |
| 234 | A | | | | | | | | |
| 235 | G | A | | | | | | | 1 |
| | | C | 1 | | | | | | |
| | | T | 1 | | | | 1 | | |
| 236 | G | C | | | | | 1 | | |
| | | T | | 1 | | | | | |
| | | A | | 1 | | | | | 1 |
| 237 | C | | | | | | | | |
| 238 | G | | | | | | | | |
| 239 | C | T | | | | | 1 | | |
| 240 | G | | | | | | | | |
| 241 | G | T | 1 | | | | | | |
| 242 | A | | | | | | | | |
| 243 | G | T | | | | 1 | | | |
| 244 | A | + | | | | | 1 | | |
| 245 | T | | | | | | | | |
| 246 | G | | | | | | | | |
| 247 | G | A | 7 | 22 | 5 | 9 | 5 | 7 | 12 |
| | | T | | | | 1 | | | |
| 248 | C | A | 1 | | | | | | |
| | | T | 3 | 5 | 1 | | | 2 | 1 |
| 249 | A | | | | | | | | |
| 250 | C | T | | 1 | | | 1 | | |
| 251 | A | + | | | | | 1 | | |
| 252 | A | | | | | | | | |
| 253 | T | C | 1 | | | | | | 1 |
| 254 | C | | | | | | | | |
| 255 | A | | | | | | | | |
| 256 | T | C | 1 | | | | | | |
| | | A | | | | | 1 | | |
| 257 | A | G | | 2 | | | 1 | | |
| 258 | C | | | | | | | | |
| 259 | G | | | | | | | | |
| 260 | C | | | | | | | | |
| 261 | T | | | | | | | | |
| 262 | A | | | | | | | | |
| 263 | G | | | | | | | | |
| 264 | A | | | | | | | | |
| 265 | A | | | | | | | | |
| 266 | T | | | | | | | | |
| 267 | T | | | | | | | | |
| 268 | A | | | | | | | | |
| 269 | T | | | | | | | | |
| 270 | G | | | | | | | | |
| 271 | A | | | | | | | | |
| 272 | A | T | | | | 1 | | | |
| 273 | C | | | | | | | | |
| 274 | A | | | | | | | | |
| 275 | T | | | | | | | | |
| 276 | T | | | | | | | | |
| 277 | A | | | | | | | | |
| 278 | A | | | | | | | | |
| 279 | A | | | | | | | | |
| 280 | T | | | | | | | | |
| 281 | T | | | | | | | | |
| 282 | G | | | | | | | | |
| 283 | G | | | | | | | | |
| 284 | A | | | | | | | | |
| 285 | G | | | | | | | | |
| 286 | A | | | | | | | | |
| 287 | C | | | | | | | | |
| 288 | T | | | | | | | | |
| 289 | G | | | | | | | | |
| 290 | A | | | | | | | | |
| 291 | A | | | | | | | | |
| 292 | T | | | | | | | | |
| 293 | G | | | | | | | | |
| 294 | A | | | | | | | | |

| | | | WT | Ps WT | 32 | 41 | X | 32 41 | 32 X |
|---|---|---|---|---|---|---|---|---|---|
| Small (above) | | | 54 | 106 | 19 | 28 | 56 | 39 | 44 |
| Complex HS | | | 6 | 0 | 15 | 7 | 5 | 21 | 3 |
| Other complex | | | 3 | 7 | 2 | 0 | 2 | 5 | 9 |
| Indels ≥2 | | | 3 | 5 | 3 | 2 | 2 | 1 | 3 |
| Total | | | 66 | 118 | 39 | 37 | 65 | 66 | 59 |
| **Arising in** | | | WT | Ps WT | 32 | 41 | X | 32 41 | 32 X |

FIGURE 4.—*Continued.*

far from the *rI* gene, but there is no precedent involving a complex mutation with such a high rate of templated mutagenesis from a distant donor.

**Determinants of replication repair and templated mutagenesis:** Replication repair is a model that invokes template switching to achieve error-free bypass of DNA lesions, for example, by the transient use of the other daughter strand as a template (FUJIWARA and TATSUMI 1976; HIGGINS *et al.* 1976). Experimentally, replication repair has been demonstrated only in phage T4, where it is well defined by an epistasis group whose alleles increase sensitivity to the killing effects of diverse agents that damage DNA (WACHSMAN and DRAKE 1987) and by reconstruction *in vitro* using wild-type and mutant proteins of T4 DNA replication (KADYROV and DRAKE 2003, 2004). Unexpectedly, both the genetic and the biochemical evidence suggests that two different epistasis groups are involved in two different but perhaps parallel biochemical pathways of replication repair.

In the first pathway, gp32 (the SSB protein, encoded by gene *32*) and gp41 (the main helicase of DNA replication, encoded by gene *41*) support replication repair *in vivo*, and certain nonlethal mutations in either gene block this pathway both *in vivo* and *in vitro*. Gp32 binds cooperatively to single-stranded DNA and can thereby enhance its activity as a template and promote either melting or annealing depending on the circumstances, while gp41 is a 5′ → 3′ DNA helicase that unwinds double-stranded DNA and thus also promotes DNA synthesis. Both proteins interact with other proteins of DNA replication and may thereby influence diverse aspects of both normal and perturbed DNA replication (NOSSAL 1994).

In the second pathway, a knockout mutation of the *uvsX* gene (encoding UvsX, a RecA-related recombinase) blocks a pathway classically called "recombination" repair because UvsX is also required for most conventional genetic recombination in T4. UvsX can also mediate replication repair *in vitro*. While the mutant

| *rI* region | Geno-type | No. seen | Models and mutations |
|---|---|---|---|
| | | | **QPs with unchanged spacers:** |
| 36-84 | PsWT | 1 | CTAGGATTGTCATTTGTTTTA...TGAAGCGAATGTCGATCCTCA |
| | | | (red above: ACA) |
| 119-157 | WT | 1 | GCACGTTTATATGCTTTTTGAAAATAAAAGCGTAGAATCGTCT |
| | | | (red above: ATATAAA) |
| | *32 41* | 2 | GCACGTTTATATGCTTTTTGAAAATAAAAGCGTAGAATCGTCT |
| | | | (red above: ATTCTAC) |
| | *32 41* | 1 | GCACGTTTATATGCTTTTTGAAAATAAAAGCGTAGAATCGTCT |
| | *32 X* | 2 | (red above: T A) |
| 153-245 | *32 X* | 1 | GTAGAATCGTCTGAACAAT......GAGGCGGAGATGGCAC |
| | | | (red above: AGAC) |
| 234-243 | PsWT | 1 | GCGAGGCGCGGAGATG......TTACGCTGGGATTTAA |
| | | | (red above: ATCCCAGC) |
| | | | The donor resides at –(85,515-85,506) relative to *rI*. |
| | | | |
| | | | **Palindrome with inverted spacer:** |
| 149-170 | PsWT | 3 | CGTAGAATCGTCTGAACAATTCTATA |
| | *32mms* | 1 | (red above: TGTTCAGACG) |
| | *32 41* | 1 | |
| | *32 X* | 3 | |
| | | | |
| | | | **Imperfect direct repeats:** |
| 97-129 | *32 41* | 1 | AAATTT ATGGAATCTGGTATTAGGCACGTTTATATGCTT |
| | | | (red above: AT) |
| 237-253 | WT | 2 | ACGCGGAGGAGAGCACAATAC......GGCGCGGAG ATG GCACAATC |
| | *32mms* | 1 | (red above: GAGA) |
| | *uvsXam* | 1 | The donor resides at –(40,272-40,255) relative to *rI*. |
| | PsWT | 1 | |
| | *32 uvsX* | 2 | |
| 42-79 | *uvsXam* | 1 | TTGTC ATTTGTTTTATCTCCATCGATTGAA GCGAATGTCGA... |
| | | | (red above: GTTCTATTAAATCCTCTTCAATGAACTAT) |
| | | | ...CGTCGTTCTATTAAATCCTCTTCAATGAACTATGCGAATGTCGCT |
| | | | The donor resides at 56,573-56,614 relative to *rI*. |
| | | | |
| | | | **No obvious donors:** |
| 231-239 | PsWT | 1 | GCGAGGCGC |
| | | | (red above: C T) |
| 231-239 | *32 uvsX* | 1 | GCG A GGCG |
| | | | (red above: GC) |

FIGURE 5.—Other complex mutations. The numbers in the first column indicate the *rI* position from the start of the underlined left repeat to the end of the underlined right repeat. The second column lists the genetic backgrounds in which the mutations arose, where *32 41 = 32mms 41uvs79* and *32 X = 32mms uvsXam*. The mutant sequences are in red letters above the corresponding wild-type sequence and the donor sequences are in blue letters.

alleles of genes *32* and *41* depress overall DNA synthesis only modestly, the *uvsXam* mutation depresses it strongly because the majority of replication is initiated by random recombination, leaving only origin-initiated early DNA synthesis (KREUZER and MORRICAL 1994). A *uvsX* mutation reduces conventional genetic recombination by only about threefold, indicating the operation of other recombination pathways. Another member of the *uvsX* epistasis group, *uvsW*, encodes a DNA helicase (WEBB *et al.* 2007). For replication repair *in vitro*, the UvsX protein also requires a helicase, Dda, yet a third in the T4 repertory. It is not known whether UvsW can replace Dda *in vitro*; a *dda* deletion does not alter the resistance to ultraviolet irradiation of a *uvsW* deletion *in vivo* (G. T. CARVER and J. W. DRAKE, unpublished results).

Because both replication repair and templated mutation proceed via ectopic primer–template pairing, we previously investigated the impact of genetic defects in both pathways of replication repair upon templated mutation. The mutations *32mms*, *41uvs79*, and *uvsXam* each exhibited a modest general mutator activity using the T4 *rI* mutation reporter, but both *32mms* and *41uvs79* exhibited strong mutator activities toward templated complex mutations, particularly at the 146–148 site (SCHULTZ *et al.* 2006); the mutator effect of *uvsXam* was about the same for templated complex mutations as for BPSs and small indels. For ease of comparison and accessibility, we have drawn together all the relevant *rI* mutational spectra into Figures 4 and 5 and Table 4, where we quote our previously published wild-type, *32mms*, *41uvs79*, and *uvsXam* spectra to which we add the PsWT spectrum discussed above plus two new spectra described and discussed below. These spectra are summarized as to kind and rate of mutation in Tables 5

**TABLE 4**

**Tandem duplications and deletions**

| Background genotype | Tandem duplications | Deletions |
|---|---|---|
| Wild type | +31 (8–38), $R = 0$ | −3 (104–106), $R = 0$ |
| | | −3 (104–106), $R = 0$ |
| PsWT | +2 (55–58), $R = $ TC | −9 (−7–8), |
| | +2 (99–101), $R = $ T | $R = $ GGCCTT |
| | +2 (257–258), $R = 0$ | |
| | +130 (116–250), | |
| | $R = $ GGCAC | |
| 32mms | +6 (169–175), | −46 (−12–39), |
| | $R = $ T | $R = $ TAGGA |
| | | −258 (137–396), |
| | | $R = $ AA |
| 41uvs79 | | −6 (104–110), $R = $ G |
| | | −98 (19–120), |
| | | $R = $ GCAC |
| uvsXam | +2 (55–58), T = TC | |
| | +2 (55–58), T = TC | |
| 32mms 41uvs79 | — | −2 (184–185), $R = 0$ |
| 32mms uvsXam | | −153 (−65–96), |
| | | $R = $ TTGATAAA |
| | | −153 (−65–96), |
| | | $R = $ TTGATAAA |
| | | −11 (104–115), $R = $ A |

Numbers in parentheses indicate both the added (or deleted) bases and both repeats ($R$), where $R = 0$ means "no repeat." The mutation adds (or deletes) one copy of the repeat.

and 6. The mutation rates for *32mms* have been revised slightly downward from the previous report as a result of additional sampling.

In addition to these determinants of templated mutations, we also note a role for the T4 DNA polymerase. T4 DNA replication can be conducted either by the cognate gp43 polymerase–exonuclease or by the corresponding protein encoded by the related phage RB69, in which case the overall rate of mutagenesis is unchanged, although site-specific mutation rates show some differences (DRESSMAN *et al.* 1997). With replication driven by the RB69 enzyme, GCG → CTA replacements at 146–148 were 16/79 (20%) of all *rI* mutations at a rate of $(16/79)(4.28 \times 10^{-6}) = 8.9 \times 10^{-7}$, whereas when replication was driven by the T4 enzyme, the hotspot mutations were 6/66 (9%) of all *rI* mutations at a rate of $(6/66)(2.82 \times 10^{-6}) = 2.6 \times 10^{-7}$. This difference has $P = 0.141$ in a $\chi^2$ test in which the genotypes were weighted in proportion to their mutation rates. In addition, various mutator RB69 polymerases show increased frequencies of the hotspot mutations (A. BEBENEK, G. T. CARVER and J. W. DRAKE, unpublished results).

**Rates of templated mutagenesis in the *32mms 41uvs79* double mutant:** These two mutations occupy the same

epistasis group, each single mutant exhibiting sensitivities to irradiation with ultraviolet light (defined by terminal survival slopes on semilog plots) of 1.4 relative to the wild type, while the double mutant has a relative sensitivity of 1.5 (WACHSMAN and DRAKE 1987). If the same pattern of epistasis held for the impact of these mutations on templated mutagenesis, the mutator activity of the double mutant might be similar to that of *32mms*, which has the stronger mutator activity of these two mutations. On the other hand, if local melting of the primer terminus promoted templated mutagenesis, then such melting might be assisted by wild-type gp32 in the presence of mutant gp41 and/or by wild-type gp41 in the presence of mutant gp32, in which case the double mutant might show less melting than either single mutant and also less templated mutagenesis. (The same reasoning applies to any reaction to which both proteins contribute.) As noted above, the mutational spectra of these mutants are shown in Figures 4 and 5 and Table 4, and the kinds and rates of mutations are summarized in Tables 5 and 6. At the 146–148 hotspot, relative rates for *32mms*, *41uvs79*, and the double mutant are 57, 18, and 48 on the basis of 15, 7, and 21 mutations, respectively (Table 6). This trend is approximately maintained for the other complex mutations, where relative rates are 15, ≤5, and 23 on the basis of 2, 0, and 5 mutations, respectively. The combined relative rates are 43, 12, and 40. This pattern is much closer to simple epistasis where, however, the impact of the single mutations on templated mutagenesis is larger in *32mms* than in *41uvs79*, in contrast to their impacts on survival after ultraviolet irradiation. The pattern is inconsistent with any substantial reduction in templated mutagenesis in the double mutant, faulting the hypothesis of impact via local melting of the primer terminus. An alternative interpretation of this result is that the melting that initiates replication repair and templated mutagenesis is promoted neither by gp32 nor by gp41, spontaneous melting perhaps sufficing. Gp32 binds to single-stranded DNA and helps, directly or indirectly, to load several replication proteins and to stabilize the replication fork. Although gp32 does not bind gp41, it does interact with gp59 which in turn loads gp41 (MA *et al.* 2004; DELAGOUTTE and VON HIPPEL 2005; XI *et al.* 2005; ZHANG *et al.* 2005). Thus, when gp32 is partly dysfunctional, as in gp32mms, the primer terminus would more often be exposed, and/or the single-stranded portion of template would more often not be coated with gp32, increasing primer-terminus melting and its mutagenic consequences; the allelic state of gene *41* would not matter very much, as observed (Tables 5 and 6). However, another T4-encoded helicase, Dda, which operates in the $3' \rightarrow 5'$ direction and can displace gp32 from single-stranded DNA (BYRD and RANEY 2006), might also play a role in this initial step.

**Rates of templated mutagenesis in the *32mms uvsXam* double mutant:** These two mutations occupy different

**TABLE 5**

**Kinds and rates of *rI* mutations**

| | Wild type | | PsWT | | *32mms* | | *41uvs79* | | *uvsXam* | | *32 + 41* | | *32 + X* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | μ | N | μ | N | μ | N | μ | N | μ | N | μ | N | μ |
| All mutations | 66 | 2.82 | 118 | 4.01 | 39 | 38.14 | 37 | 24.22 | 65 | 23.79 | 66 | 38.72 | 59 | 20.47 |
| Transitions | 21 | 0.90 | 49 | 1.66 | 10 | 9.78 | 15 | 9.82 | 29 | 10.61 | 15 | 8.80 | 23 | 7.98 |
| A·T → G·C | 5 | 0.21 | 11 | 0.37 | 2 | 1.96 | 2 | 1.31 | 14 | 5.12 | 6 | 3.52 | 5 | 1.73 |
| G·C → A·T | 16 | 0.68 | 38 | 1.29 | 8 | 7.82 | 13 | 8.51 | 15 | 5.49 | 9 | 5.28 | 18 | 6.25 |
| Transversions | 13 | 0.56 | 19 | 0.65 | 7 | 6.85 | 3 | 1.96 | 15 | 5.49 | 9 | 5.28 | 7 | 2.43 |
| A·T → T·A | 0 | ≤0.04 | 0 | ≤0.03 | 4 | 3.91 | 1 | 0.66 | 3 | 1.10 | 2 | 1.17 | 3 | 1.04 |
| A·T → C·G | 1 | 0.04 | 8 | 0.27 | 0 | ≤0.98 | 1 | 0.66 | 5 | 1.83 | 3 | 1.76 | 3 | 1.04 |
| G·C → C·G | 2 | 0.09 | 0 | ≤0.03 | 0 | ≤0.98 | 0 | ≤0.66 | 1 | 0.37 | 0 | ≤0.59 | 0 | ≤0.35 |
| G·C → T·A | 10 | 0.43 | 11 | 0.37 | 3 | 2.93 | 1 | 0.66 | 6 | 2.20 | 4 | 2.35 | 1 | 0.35 |
| BPSs | 34 | 1.45 | 68 | 2.31 | 17 | 16.6 | 18 | 11.8 | 44 | 16.10 | 24 | 14.08 | 30 | 10.4 |
| Small indels | 20 | 0.86 | 38 | 1.29 | 2 | 1.96 | 10 | 6.55 | 12 | 4.39 | 15 | 8.80 | 14 | 4.86 |
| −A·T | 11 | 0.47 | 27 | 0.92 | 1 | 0.98 | 7 | 4.58 | 6 | 2.20 | 10 | 5.87 | 8 | 2.78 |
| −G·C | 1 | 0.04 | 1 | 0.03 | 0 | ≤0.98 | 0 | ≤0.66 | 0 | ≤0.37 | 0 | 0.00 | 2 | 0.69 |
| +A·T | 4 | 0.17 | 6 | 0.20 | 1 | 0.98 | 3 | 1.96 | 5 | 1.34 | 3 | 1.76 | 1 | 0.35 |
| +G·C | 4 | 0.17 | 4 | 0.14 | 0 | ≤0.98 | 0 | 0.00 | 1 | 0.37 | 2 | 1.17 | 3 | 1.04 |
| Larger indels | 3 | 0.13 | 5 | 0.17 | 3 | 2.93 | 2 | 1.31 | 2 | 0.73 | 1 | 0.59 | 3 | 1.04 |
| Tandem duplications | 1 | 0.04 | 4 | 0.14 | 1 | 0.98 | 0 | ≤0.66 | 2 | 0.73 | 0 | ≤0.59 | 0 | ≤0.35 |
| Deletions | 2 | 0.09 | 1 | 0.03 | 2 | 1.96 | 2 | 1.31 | 0 | ≤0.37 | 1 | 0.59 | 3 | 1.04 |
| Complex mutations | 9 | 0.38 | 7 | 0.24 | 17 | 16.62 | 7 | 4.58 | 7 | 2.56 | 26 | 15.25 | 12 | 4.16 |
| Hotspot | 6 | 0.26 | 0 | ≤0.03 | 15 | 14.67 | 7 | 4.58 | 5 | 1.83 | 21 | 12.32 | 3 | 1.04 |
| Other | 3 | 0.13 | 7 | 0.24 | 2 | 1.96 | 0 | ≤0.66 | 2 | 0.73 | 5 | 2.93 | 9 | 3.12 |

*32 + 41 = 32mms 41uvs79. 32 + X = 32mms uvsXam. N*, no. of mutations. μ, mutation rate × $10^6$/genome replication on the basis of 7, 7, 17, 7, 7, 7, 8, and 11 stocks, respectively, for the seven genotypes. When no mutation was observed, the value of μ is less than or equal to the rate for one mutation.

epistasis groups with respect to rates of inactivation by ultraviolet irradiation: *32mms* has a relative sensitivity of 1.4 and *uvsX⁻* (the tested allele having been *uvsX1*) a relative sensitivity of 1.6, whereas the double mutant has a relative sensitivity of 2.1 (WACHSMAN and DRAKE 1987). It is unclear why the survival of DNA damage in T4 has two pathways of apparent replication repair exhibiting multiplicative increases in sensitivity when both are inactivated. We therefore asked whether the effects of the single mutants on templated mutagenesis were also synergistic in the double mutant (Figures 4 and 5 and Tables 4–6). They were not. The relative rates of the hotspot complex mutation for *32mms*, *uvsXam*, and *32mms uvsXam* were 57, 7, and 4, respectively, the two smaller values being indistinguishable because of sample sizes. (The relative rates for the other complex mutations are based on sample sizes too small to justify comparison.) In this double mutant, the relative rate at the complex hotspot was smaller than that of its *32mms* component and resembled that of its *uvsXam* component alone. This observation might be explained if wild-type UvsX initiates or promotes the excess of templated mutagenesis in the *32mms* mutant and this effect is

blocked by wild-type gp32 but not by gp32mms. Interactions between gp32 and UvsX are well documented and can be either cooperative or competitive, depending on the parameter and experimental conditions (VILLEMAIN *et al.* 2000; LIU *et al.* 2006). For instance, when gp32 is defective, UvsX might promote the annealing of a melted primer terminus to the other parental strand, a mutagenic pathway, rather than to the other daughter strand, an antimutagenic pathway. When both proteins are defective, as in the double-mutant *32mms uvsXam*, then UvsX would not promote the mutagenic pathway. Thus, UvsX may control access to each pathway.

There is also the possibility that the effect of mutations in either pathway of replication repair is not only to decrease strand switching substantially but also to allow the residuum to occur promiscuously (in the words of one insightful reviewer), thus simultaneously both reducing survival after DNA damage and increasing the rate of templated mutation.

**Varieties of templated mutations:** Most of the "other" complex mutations could be modeled as templated and are illustrated in Figure 5, including those quoted from SCHULTZ *et al.* (2006). They are instructive. The top

**TABLE 6**

**Mutation rates relative to wild-type T4**

| | Mutation rate relative to wild-type T4 | | | | | | |
|---|---|---|---|---|---|---|---|
| | Wild type | PsWT | *32mms* | *41uvs79* | *uvsXam* | *32mms 41uvs79* | *32mms uvsXam* |
| All mutations | 1.0 | 1.4 | 13.5 | 8.6 | 8.4 | 13.7 | 7.3 |
| Transitions | 1.0 | 1.9 | 10.9 | 10.9 | 11.8 | 9.8 | 8.9 |
| A·T → G·C | 1.0 | 1.8 | 9.2 | 6.1 | 24.0 | 16.5 | 8.1 |
| G·C → A·T | 1.0 | 1.9 | 11.4 | 12.4 | 8.0 | 7.7 | 9.1 |
| Transversions | 1.0 | 1.2 | 12.3 | 3.5 | 9.9 | 9.5 | 4.4 |
| A·T → T·A | ≤1.0 | (ud) | ≥91 | ≥15 | ≥26 | ≥27 | ≥24 |
| A·T → C·G | 1.0 | ≈6 | ≤23 | ≈15 | ≈43 | ≈41 | ≈24 |
| G·C → C·G | 1.0 | ≤0.4 | ≤11 | ≤8 | 4 | ≤7 | ≤4 |
| G·C → T·A | 1.0 | 0.9 | 6.9 | 1.5 | ≈5 | 5.5 | ≈0.8 |
| BPSs | 1.0 | 1.6 | 11.4 | 8.1 | 11.1 | 9.7 | 7.2 |
| Small indels | 1.0 | 1.5 | 2.3 | 7.7 | 5.1 | 10.3 | 5.7 |
| −A·T | 1.0 | 2.0 | ≈2 | 9.7 | 4.7 | 12.5 | 5.9 |
| −G·C | 1.0 | ≈0.8 | ≤23 | ≤15 | ≤9 | ≤14 | ≈16 |
| +A·T | 1.0 | 1.2 | ≈6 | 11.5 | 10.7 | 10.3 | ≈2 |
| +G·C | 1.0 | 0.8 | ≤6 | ≤0.4 | ≈2 | 6.9 | 6.1 |
| Larger indels | 1.0 | 1.3 | 22.9 | 10.2 | 5.7 | ≈5 | 8.1 |
| Tandem duplications | 1.0 | ≈3 | ≈23 | ≤15 | ≈17 | ≤14 | ≤8 |
| Deletions | 1.0 | ≈0.4 | ≈23 | 15.3 | ≤4.3 | 6.9 | 12.2 |
| Complex mutations | 1.0 | 0.6 | 43.2 | 11.9 | 6.7 | 39.6 | 10.8 |
| Hotspot | 1.0 | ≤0.3 | 57.2 | 17.9 | 7.1 | 48.0 | 4.1 |
| Other | 1.0 | 1.9 | 15.2 | ≤5 | 5.7 | 22.9 | 24.3 |

No A·T → T·A transversions were observed in the wild type, and the entries for the other genotypes in that row are based on one mutation in wild-type T4. The value for PsWT, 0/0, is (ud), undefined.

section of Figure 5 involves QPs in which a noncomplementary segment internal to one arm appears to act as the donor for a mutation appearing in the corresponding part of the other arm; the hotspot mutation illustrated in Figure 2 also falls into this category. The second and third entries of this section represent reciprocal transfers exactly of the kind that we sought to detect in the case of the complex hotspot. The fourth entry of this section depicts a partial transfer: inspection of the within-arm noncomplementarities reveals that they are discontinuous: only the two single bases at the ends and the central base are noncomplementary. In the mutation, only two of these three were transferred, suggesting either that the ectopically extending primer returned to its cognate template before extending to the final base or that the third mispair was transported but was removed by proofreading. The fifth example in this section (*rI* region 153–245) is interesting because the putatively transferred bases are flanked by only short regions of complementarity—1 base on one side and 3 on the other—indicating that the flanking complementarities can be quite short, at least anecdotally if not frequently. (The tandem duplications and deletions in

Table 4 often displayed repeats of size 2, 1, or even 0.) In addition, the donor and recipient species are not of identical length, so that the 4 bases replacing "GGA" must have been accommodated by the polymerase and extended without proofreading when bridged by only 1 or 3 correct bases. The final example in this section is interesting in two ways. First, the flanking complementarities are minimal—1 base each; no other direct or indirect donor was found in the T4 genome with two flanking complementarities. Such short patching-in sequences might represent a rare kind of event. Second, the distance between arms was large, >85 kb, compared to the 25, 7, and 76 bases in the previous three QPs. However, because replicating T4 DNA is a pool of many permuted molecules, a wandering primer in principle could find a complement in another genome at any sequence distance from its own.

The second section is a single example in which the palindrome was perfect except for a spacer between its arms and in which the spacer appears to have templated its own replacement. This kind of mutation can arise only when the ectopic template is the other parental strand (Ripley 1982; Schultz *et al.* 2006).

The mutations in the third section are modeled as transfers between direct repeats. Their most striking trait is the very large distance (tens of kilobases) between the repeats in two of the three examples, perhaps for the same reason as mentioned above.

The mutations in the final section are complex, but we could find few or no compelling candidate donors in the T4 genome. The first entry, AGG → CGT, has no potential direct-repeat or QP donor with two or more flanking bases at each end, but does have 36 potential GCGTC direct-repeat donors and 56 potential GACGC QP donors. Perhaps these potential donors are individually poor because of their single flanking bases but donation occurs sporadically because of their high copy number. Alternatively, the mutation may simply represent two close but independent mispairing errors. The second entry, A → GC, might be templated by four copies of the direct repeat CGGCGG in the T4 genome with two flanking bases on each side or by 11 copies of the QP CCGCCG with two flanking bases on each side. Alternatively, this mutation may have been initiated by a slippage error within a G-rich sequence that also replaced an A with a C, the details remaining forever obscure.

Eight of the mutations in Figure 5 were detected only once. The first is structurally similar to the hotspot mutation, but the other six may be anecdotal and individually improbable, surfacing only sporadically from a sea of possibilities.

As noted above, our PsWT construct produced two surprises: there were no examples of either the complex hotspot at 146–148 or the reciprocal mutation. The mutations described in Figure 5 reveal that reciprocal transfer can occur, that an unequal number of bases between donor and recipient sites is not an absolute barrier to transfer, and that a very short complementary flanking sequence is also compatible with templating. We are left with the conclusion that any of these factors, or their combination, might have reduced the frequency of transfer in the PsWT strain sufficiently to render it undetectable among 118 isolates.

All of the spectra contained indels of two or more bases (Table 4), consisting altogether of eight tandem duplications and 11 deletions. Most (14/19) of these arose in association with short, direct sequence repeats with one of the repeat components and all of the intervening bases being duplicated or deleted. Such indels are widely interpreted as arising when a primer terminus melts and reanneals with a complementary sequence nearby (behind in the case of duplications, ahead in the case of deletions). Some of the repeats are of only one or two bases, and in five cases there is no repeat. If only one base can serve as a repeat, then perhaps "no" base can also, a situation that could arise when a mispair is followed by primer-terminus melting and reannealing so that the base in the template strand that is the recipient of the wandering primer terminus is complementary to the incorrectly inserted base. (The misinsertion itself might also promote primer-terminus melting.) Of the five cases for which $R = 0$, two could be mediated by the frequent mispair G·T and three would require pyrimidine–pyrimidine or purine–purine mispairs. Switching to an ectopic template is key to all these errors and thus might be promoted by our gene *32, 41,* and *uvsX* mutations; such is the case (Tables 5 and 6), although usually not as strong a one as are the complex mutations.

**Mutator activities promoting other kinds of mutations:** The three single mutants and the two doubles increased rates not only of apparently templated complex mutations but also of most other kinds of mutations (Tables 5 and 6; Schultz *et al.* 2006). BPSs were enhanced 7- to 11-fold (slightly more for transitions than for transversions) and single-base deletions and additions were enhanced 2- to 10-fold. We have no special insights into the mechanisms involved. Unimpressive speculations include a mispair or slippage that promotes primer melting, plus transiently lower proofreading efficiency upon reannealing with the cognate template, or either direct or indirect interactions between the three mutant proteins and the polymerase that result in increased insertion errors or decreased proofreading.

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