Properties of Bacteriophage T4 Proteins Deficient in Replication Repair*

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An epistasis group of mutations engendering increased sensitivity to diverse DNA-damaging agents was described previously in bacteriophage T4. These mutations are alleles of genes 32 and 41, which, respectively, encode a single-stranded DNA-binding protein (gp32) and the replicative DNA helicase (gp41). The mechanism by which the lethality of DNA damage is mitigated is unknown but seems not to involve the direct reversal of damage, excision repair, conventional recombination repair, or translesion synthesis. Here we explore the hypothesis that the mechanism involves a switch in DNA primer extension from the cognate template to an alternative template, the just-synthesized daughter strand of the other parental strand. The activities of the mutant proteins are reduced about 2-fold (for gp32) or 4-fold (for gp41) in replication complexes catalyzing coordinated synthesis of leading and lagging strands, in binding single-stranded DNA, promoting DNA annealing, and promoting branch migration. In striking contrast, the mutant proteins are strongly impaired in promoting template switching, thus supporting the hypothesis of survival by template switching.

Four general mechanisms that either repair or circumvent potentially lethal damage to DNA have been extensively investigated. These are (i) direct reversal of the damage, as in photoreactivation or dealkylation, (ii) damage excision followed by resynthesis templated by the complementary strand, as in base excision repair and nucleotide excision repair, (iii) recombinational repair, in which the damaged DNA obtains the requisite sequence information from another chromosome or from the sister chromosome in a way that depends on a recombinase and a Holliday-junction resolvase, and (iv) translesion synthesis, which is frequently mutagenic. Strong hints of a fifth mechanism have accumulated over time, but a combined genetic and enzymological demonstration of such a mechanism has not been available. These hints accumulated in two parallel lines of investigation, one using eukaryotic cells and the other using bacteriophage T4. Both lines point toward a repair mechanism based on a template-switching process operating directly at the replication fork.

In a classic model of the fifth mechanism (1), DNA replication on one parental template strand is blocked by a lesion while replication on the other parental strand continues briefly, whereupon strand displacement and branch migration re-associate the two daughter strands into a short duplex (Fig. 1). The blocked daughter strand then continues replication on the alternative template, and then the structure reforms a conventional replication fork with the lesion having been bypassed. This model was supported by experiments in which bromodeoxyuridine was used as a “heavy” label and tritiated thymidine as a “light” label during the replication of DNA in UV-irradiated mammalian cells: a small amount of tritiated DNA of “heavy-heavy” density was observed in DNA from irradiated cells, and this was chased into “heavy-light” DNA. In addition, electron micrographs revealed the predicted four-pronged DNA forks in DNA from irradiated cells. The authors called their model “replication repair.” Shortly thereafter, some effects of post-irradiation caffeine treatments on the survival of mammalian cells and the behavior of the DNA during both alkaline sedimentation and neutral CsCl banding suggested a similar model that was called “replicative bypass repair” (2).

In the meantime, genetic evidence was accumulating for a new kind of repair process in bacteriophage T4. The T4 mutation mms exhibited increased sensitivity to methyl methanesulfonate (MMS) but affected none of the known repair mechanisms (3); this mutation was later mapped to gene 32 (4), which encodes a growth protein, gp32, that binds to single-stranded DNA (ssDNA) and is required for efficient DNA synthesis. The next such mutation to be described, uvs79, exhibited increased sensitivity to both UV and MMS but again did not affect any of the known repair mechanisms and was called a replication repair mutation (5, 6). uvs79 was mapped to gene 41 (7), which encodes gp41, the DNA replicative helicase. The first report in this series (8) to cite the canonical model of replication repair (1) explored an unsatisfactory alternative in which gaps opposite a template lesion would be filled with primer RNA. (Thus, the first use of replication repair in the T4 context was presumably by chance.) A little later, a mode of conservative (rather than semiconservative) T4 DNA replication in vitro was described, which depends on T4 recombination proteins, suggesting a model for translesion synthesis by template switching that depends on the T4 recombinase UvX and would therefore have to be distinct from replication repair (9). Another gene 32 mutation, amA453, was then found to cause UV sensitivity, and extensive tests for epistasis revealed that 41uvs79, 32mms, and 32amA453 are mutually epistatic for.

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1 The abbreviations used are: MMS, methyl methanesulfonate; gp, growth protein; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; nt, nucleotide(s); DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; MOPS, 3-(N-morpholino)propanesulfonic acid; BSA, bovine serum albumin.

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replication repair (10). (The phenotype of 32amA453 is manifest in amber suppressor strains and may therefore be produced by the amber peptide.) Further tests bolstered the conclusion that replication repair is distinct from the classic T4 WXY recombination repair/translesion synthesis systems and showed that mutations disabling replication repair affect survival after treatments with a variety of DNA-damaging agents (11, 12). Another gene 41 allele, rrhI, was added to this epistasis group (11, 12).

In this report we describe the properties of two proteins, gp32mms and gp41uv579, that are associated with defective replication repair. These two proteins turn out to be only slightly defective in an eight-protein in vitro DNA replication system exhibiting coupled leading strand and lagging strand synthesis (13). However, when we then explore their behavior in an assay for template switching, we found that both proteins are markedly defective.

MATERIALS AND METHODS

DNA Preparations—M13mp2 ssDNA and 5'-tailed mp2 double-stranded DNA were prepared as previously described (13). To isolate M13mp2 double-stranded DNA (dsDNA), 2 ml of an overnight culture of E. coli DH5a/pP17 was disrupted with a single phase plaque and grown with moderate shaking in 500 ml of 2 °C. 50 ml of 25 mM Tris-HCl, pH 8.0, 1 mM EDTA). To remove most RNA, and DNA other than supercoiled mp2 dsDNA, CaCl₂ was added to 50 mM, and the precipitate was immediately pelleted by spinning in an Eppendorf 5415C benchtop centrifuge at 10,000 rpm for 1 min. The tube was then held for an additional 5 min in ice, and the new precipitate was pelleted again. More CaCl₂ was then added, to a final concentration of 75 mM, followed by two precipitations as above. Nucleic acids remaining in the supernatant were precipitated with an equal volume of 2-propanol in the presence of 0.15 M sodium acetate. The precipitate was dissolved in 450 μl of TE buffer, and RNase A was added to 0.2 mg/ml. RNA hydrolysis was carried out for 15 min at room temperature, 20% polyethylene glycol 8000 in 2.5 M NaCl was added to a 5.5% final concentration of polyethylene glycol, and precipitation was carried out for 5–15 min in ice. The DNA was pelleted by spinning again at 10,000 rpm for 2 min. The pellet was washed with 70% ethanol and dissolved in 2 ml of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). To remove most RNA, and DNA other than supercoiled mp2 dsDNA, CaCl₂ was added to 50 mM, and the precipitate was immediately pelleted by spinning in an Eppendorf 5415C benchtop centrifuge at 10,000 rpm for 1 min. The tube was then held for an additional 5 min in ice, and the new precipitate was pelleted again. More CaCl₂ was then added, to a final concentration of 75 mM, followed by two precipitations as above. Nucleic acids remaining in the supernatant were precipitated with an equal volume of 2-propanol in the presence of 0.15 M sodium acetate. The precipitate was dissolved in 450 μl of TE buffer, and RNase A was added to 0.2 mg/ml. RNA hydrolysis was carried out for 15 min at room temperature, 20% polyethylene glycol 8000 in 2.5 M NaCl was added to a 5.5% final concentration of polyethylene glycol, and precipitation was carried out for 5–15 min in ice. The DNA was pelleted by spinning again at 10,000 rpm for 2 min. The pellet was washed with 70% ethanol and dissolved in TE buffer. The DNA solution was extracted with phenol/chloroform and then precipitated with ethanol. The pellet was washed with 70% ethanol, dried, and dissolved in 2 ml of TE buffer.

To prepare the substrate for DNA annealing and branch migration assays, 350 μg of mp2 dsDNA was cleaved with 300 units of EcoRI (New England BioLabs) in 1× EcoRI buffer (New England BioLabs) for 1 h at 37 °C, followed by heat inactivation for 20 min at 65 °C, ethanol precipitation, and dissolving the DNA in TE buffer. 100 μg of mp2 dsDNA/EcoRI was cleaved with 200 units of T7 exonuclease (Amersham Biosciences) in 300 μl of 1× T7exo buffer (Amersham Biosciences) for 8 h at 37 °C. EDTA was added to 125 mM, followed by heat inactivation for 15 min at 75 °C and ethanol precipitation in the presence of 0.5 M ammonium acetate. The DNA was dissolved and stored in TE buffer. To estimate the length of T tails produced by T7 exonuclease, the cleaved DNA was separated in a 0.6% agarose gel with 0.1 μg/ml ethidium bromide in Tris-HCl, pH 8.0, 1 M NaCl; and 0.5 μl of 32P-labeled mp2 dsDNA/T7 exonuclease, and 16
and a 200-ml 0.05–1 M linear NaCl gradient in buffer A was applied; UvX was eluted at the end of the gradient. The peak fraction was dialyzed against buffer B devoid of EDTA and loaded onto a 2-ml CHT-21 column (Bio-Rad) equilibrated with buffer C supplemented with 10 mM MgCl₂. The column was washed with 10 ml of the same buffer and proteins were eluted with a 30-ml 0.02–0.8 M potassium phosphate linear gradient supplemented with the same additives; UvX was eluted at about 0.65 M potassium phosphate.

T4 gp32, gp32mms, gp41, gp41uvsX, UvX, and UvY were monitored during purification using SDS-PAGE. All proteins were free of detectable exo- and endo-deoxyribonuclease activities. The final fractions of the purified proteins obtained after the last chromatographic steps were dialyzed overnight against a buffer containing 20 mM Tris–HCl, pH 8.0, 50 mM potassium glutamate, 17.5 mM KCl, 8 mM magnesium acetate, 5 mM DTT, 0.5 mM benzamidine chloride, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA. After dialysis, the fractions were subdivided and stored at −80°C.

Protein concentrations were determined as described previously (17). Concentrations of gp43, gp32, gp61, and gp59 are expressed in monomer molarities. In the case of the gp44/62 heteromultimer, the molar concentration is for a complex of four subunits of gp44 and one of gp62. In the case of gp45 and gp41, the molar concentration is for trimers and hexamers, respectively.

ssDNA Binding—ssDNA binding assays were performed in a final volume of 20 μl of a mixture containing 20 mM Tris acetate, pH 7.8, 50 mM potassium glutamate, 6.25 mM magnesium acetate, 15 mM KC1, 8.7% glycerol (v/v), 25 μg/ml BSA, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 1.5 mM Mg²⁺, 1.5 mM GTP, 0.4 mM CTP, 0.4 mM UTP, 69 μM/ml α-[32P]dGTP (3000 Ci/mmol), and 2 μM 5′-tailed mp2 dsDNA. Unless otherwise indicated, the mixtures were supplemented with 0.4 μM gp43, 16.5 μM gp44/62, 103 μM gp45, 14.2 μM gp41 or gp41uvs97, 500 μM gp32 or gp32mms, 32 μM gp61, and 18 μM gp59. Reaction mixtures without template DNA but with all T4 proteins except gp32 were first incubated at room temperature for 4 min. Then gp32 was added, the mixtures were transferred to a 37°C water bath for 1–1.5 min, pre-warmed template DNA was added, and reactions were run at 37°C. Samples (1 μl) were withdrawn at the indicated times and were mixed with 25 μl of 100 mM EDTA, 30 mM NaOH. Samples (10 μl) of the diluted reaction products were separated in 0.6% alkaline agarose gels in 33 mM NaOH, 1.25× TAE buffer. Gels were stained and visualized using a Storm 850 PhosphorImager and the ImageQuant™ program (Amersham Biosciences). If not otherwise noted, a standard template-switching reaction was carried out at 37°C for 10 min in a 40-μl mixture containing 20 mM Tris acetate, pH 7.8, 50 mM potassium glutamate, 17.5 mM KC1, 8 mM magnesium acetate, 5 mM DTT, 8.7% glycerol (v/v), 50 μg/ml BSA, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 1 mM ATP, 2 nM forked DNA, 1.2 μM gp43, 1.65 μM gp44/62, 10.3 μM gp45, 12 μM gp41 or gp41uvs97, 500 μM gp32 or gp32mms, 32 μM gp61, and 18 μM gp59. Reaction mixtures without template DNA but with all T4 proteins except gp32 were first incubated at room temperature for 4 min. Then gp32 was added, the mixtures were transferred to a 37°C water bath for 1–1.5 min, pre-warmed template DNA was added, and reactions were run at 37°C. Samples (1 μl) were withdrawn at the indicated times and were mixed with 25 μl of 100 mM EDTA, 30 mM NaOH. Samples (10 μl) of the diluted reaction products were separated in 0.6% alkaline agarose gels in 33 mM NaOH, 1.25× TAE buffer. Gels were stained and visualized using a Storm 850 PhosphorImager and the ImageQuant™ program (Amersham Biosciences) as described (18).

DNA Annealing and Unwinding Assays—DNA annealing was performed in a final volume of 20 μl of a mixture containing 20 mM Tris acetate, pH 7.8, 50 mM potassium glutamate, 17.5 mM KC1, 8 mM magnesium acetate, 5 mM DTT, 8.7% glycerol (v/v), 50 μg/ml BSA, 2 mM mp2 ssDNA, 0.2 μM α-[32P]dGMP mp2 dsDNA/T7 exonuclease, 1.8 μM unlabeled mp2 dsDNA/T7 exonuclease, and gp32 or gp32mms at the indicated concentrations. Reaction mixtures were incubated at 37°C for 30 min, and 9-μl samples were mixed with 10 μl of 237.5 mM EDTA, 0.5 mM mg/ml Proteinase K, followed by incubation for an additional 5 min. Samples were analyzed by electrophoresis for 16 h at 20 V at room temperature in 200-ml 0.8% agarose gels in a Wide Cell GT unit (Bio-Rad) filled with 1800 ml of 1.25× TAE buffer. Gels were dried and visualized using a Storm 850 PhosphorImager and the ImageQuant™ program (Amersham Biosciences).

Template Switching Assays—To prepare substrates for template switching assays, we used the oligonucleotides shown later in Fig. 6, which were obtained in gel-purified form from Oligos Etc. 120-mer #1 was annealed with 90-mer #2, and 5′-32P-labeled 70-mer #3 was annealed with 120-mer #4 in a 20-μl mixture containing 20 mM Tris acetate, pH 7.8, 50 mM potassium glutamate, 6.25 mM magnesium acetate, 5 mM DTT, and 150 μM of each oligonucleotide at 40°C for 10 min. Both mixtures were then combined and incubated at 40°C for 10 min, and the annealed forked DNA was used immediately.

RESULTS

The T4 mms and uvs79 mutations produce P88L and A379T changes in the gp32 ssDNA-binding protein and the gp41 replicative helicase, respectively (4, 11). Our 4IUuv579 clone also contained the silent mutation G1239A (lysine codons AAG → AAA). We began our study of these replication repair mutations by cloning the mutant genes into plasmid vector pET-21a under the control of the T7 RNA polymerase promoter. To
confirm that 32mms and 41uvs79 render T4 UV-sensitive, we obtained recombinant phages rT4mms and rT4uvs79. Each increased the terminal inactivation slope about 1.45-fold without affecting the typical T4 1.75-hit shoulder (Fig. 2).

We next overexpressed and purified the two mutant proteins. gp32mms and gp41uvs79 were purified using protocols developed for the corresponding wild-type proteins. gp32mms behaved similarly to gp32 except that, to bind to the ssDNA-cellulose column, it required a lower rate of loading onto the column than did gp32. The purification of the mutant helicase was indistinguishable from that of the wild-type helicase.

**Behavior of the Mutant Proteins in DNA Replication Assays**—32mms and 41uvs79 mutants support DNA replication in vivo and produce viable progeny at levels not much lower than wild-type. To characterize DNA replication with the mutant proteins in vitro, we examined their abilities to catalyze rolling circle DNA replication in concert with seven other bacteriophage T4 replication proteins. Under our standard conditions, these eight proteins catalyze the efficient synthesis of long (>20 kb) leading strands and short (0.6–7 kb) lagging strands when analyzed by denaturing agarose gel-electrophoresis (Fig. 3A, lanes 4–6). (In addition to the bands representing leading strand and lagging strand synthesis, a band of about 8 kb appears. This band represents limited strand displacement synthesis by complexes that have not acquired the primosome.) The mutant proteins increased rates of DNA synthesis 5- to 8-fold compared with a control reaction where the corresponding protein was simply omitted from the reaction mixture (Fig. 3, B and C). However, the mutant replication repair proteins behaved differently from their wild-type counterparts. Approximately two times more gp32mms than gp32 was required to achieve similar rates of DNA synthesis (Fig. 3B). In the case of gp41uvs79, the defect was even stronger (Fig. 3, A and C). At the standard 14.2-nm concentration, long leading strands and short Okazaki fragments appeared only after 4 min, revealing a delay in loading the mutant T4 primosome. The rate of DNA synthesis by the mutant replication complexes increased with increasing gp41uvs79, gp61, and gp59 concentrations. From four to eight times more gp41uvs79 than gp41 was required to achieve similar rates of DNA synthesis.

**Behavior of the Mutant Proteins in DNA Binding Assays**—The purified mutant proteins were then analyzed for their abilities to form complexes with ssDNA. gp32 binds ssDNA strongly and cooperatively (19). Approximately two times more gp32mms was required to form complexes with ssDNA exhibiting a mobility similar to that of complexes formed with wild-type gp32 (Fig. 4A).

The gp41-replicative helicase forms a stable complex with long circular ssDNA in the presence of Mg$^{2+}$, ATP, and the gp61 primase (22). These complexes are displayed in Fig. 4B, lane 4. Including the gp59 helicase loader in the reaction mixture did not affect the mobility of DNA-protein complexes (Fig. 4C).
Neither gp41 (Fig. 4B, lane 3) nor gp41uvs79 (Fig. 4B, lane 6) formed complexes with ssDNA when the gp61 primase was absent. Including the gp59 helicase loader in the DNA-binding reactions decreased the mobility of ssDNA-protein complexes with gp41uvs79 but not with wild-type gp41 (compare the mobility of the complexes in Fig. 4B, lanes 7 and 8, and in lanes 9 and 10). Moreover, a 4-fold molar excess of the mutant over the wild-type enzyme was required to form DNA-protein complexes with a similar mobility. These results suggest that the gp59 helicase loader is required either for efficient loading of the mutant helicase onto ssDNA and/or to stabilize gp41uvs79 already bound to ssDNA. Taken together, the ssDNA binding assays show that the magnitudes of the defects of the two mutant proteins are comparable to those observed in DNA replication.

Behavior of the Mutant Proteins in DNA Annealing and Branch Migration Assays—gp32, gp41, and gp59 can support a recombination pathway in vitro that differs from the uvsWXY recombination pathway (20). In this pathway, gp32 facilitates the annealing of ssDNA with homologous dsDNA bearing 3′-single-stranded tails, and gp41 (with the help of the gp59 helicase loader) drives polar strand exchange between the joint molecules. We therefore examined the ability of gp32mms to promote DNA annealing and the ability of gp41uvs79 to drive polar strand exchange. In these tests, we used M13mp2 circular ssDNA and homologous dsDNA with 3′ tails as substrates. When the DNAs are incubated with gp32 at a concentration sufficient to cover all ssDNA, joint molecules are formed (Fig. 5A, lanes 3 and 4) as a result of the annealing activity of gp32 (19, 20). (The bands migrating just behind the joint molecules, even in the control lane, probably represent spontaneously forming dimers of linear dsDNA.) Unlike wild-type gp32, no joint molecules formed at a 2-fold concentration of gp32mms. However, gp32mms could catalyze efficient DNA annealing at a higher concentration (Fig. 5A, lanes 7 and 8). This result suggests that the P88L replacement in gp32mms compromises
its DNA annealing activity to an extent similar to that observed in DNA replication assays (Fig. 3B) and DNA binding assays (4A).

When joint molecules are incubated with gp32 and the T4 primosomal proteins (gp41, gp61, and gp59), there is a time-dependent formation of gapped circular dsDNA (Fig. 5B). This DNA is a product of polar branch migration driven by the primosome. As expected, the efficiency of branch migration increased with a 2-fold increased concentration of primosomal proteins (compare the intensity of gapped circular dsDNA in lanes 2–5 with those in lanes 6–9 of Fig. 5B). When gp41 was replaced with gp41uvs79, polar branch migration was also observed (Fig. 5B, lanes 10–13). Because the rate of formation of gapped circular dsDNA is similar in reaction mixtures containing 7.1 nM gp41, 8 nM gp61, and 4.5 nM gp59 or 28.4 nM gp41uvs79, 32 nM gp61, and 18 nM gp59, we conclude that about four times more of the mutant primosome is required for a rate of polar branch migration similar to that achieved with the wild-type gp41. Thus, the gp41uvs defect in branch migration is similar to that observed in DNA replication assays.

Template Switching Assays—A mechanism distinct from repair replication was suggested previously for bypassing a block to DNA synthesis during conservative replication by T4 proteins, based on switching a nascent strand from a damaged template to a homologous undamaged one (9). UvsX, the T4 homolog of the host RecA recombinase, would be a key component in this pathway. Such a pathway has recently been reconstituted in vitro (21). However, no data exist to support template switching during semi-conservative replication. To explore whether template switching could occur during semi-conservative replication, we designed a substrate that mimics the synthesis of the leading strand in a replication fork by the T4 polymerase holoenzyme while taking care to optimize the amounts of all proteins. When UvsX was included in the reaction mixtures, template switching was indeed promoted, and a 90-nt product accumulated (Fig. 7, lanes 2–7). Although UvsX promotes template switching in only 2–4% of the substrate, this is 10–15-fold more than in a reaction lacking UvsX. UvsY (a UvsX accessory protein) stimulated strand switching promoted by UvsX when the latter was used at a lower concentration, but gp32 did not stimulate the reaction (Fig. 7).

Because the combined activities of gp32 and gp41 along with the gp61 primase and the gp59 helicase loader support a strand-exchange reaction (Fig. 5 and Ref. 20) similar to that catalyzed by the UvsX recombinase, we inquired next whether the combined activity of these proteins could promote template switching. They do (Fig. 8A). Omitting gp32, gp41, or gp59 prevented most or all template switching, whereas omitting gp61 had no marked effect. We also assayed extension of the 70-mer when one of the other three strands was absent (Fig. 8B). As expected, nearly normal extension occurred when 120-mer #4 was absent and no 90-nt product accumulated when the 90-mer was absent. Omitting 120-mer #1 increased the amount of template switching product about 2-fold, which suggests that the unwinding of this arm of the substrate may be the rate-limiting step for template switching in this reaction. In these assays and those below, we also examined the behavior of an exonuclease-deficient gp43 and obtained identical results.
Note that the absence of any 90-mer product in control reactions with the holoenzyme alone (Fig. 8B) excludes the possibility that the reaction mixtures used in the template transfer experiments (Fig. 8A) contained significant amounts of substrate consisting of a 70-mer annealed to its complementary 90-mer. Because the reaction requires gp32, gp41, and gp59, it is unlikely that the extension of the 70-mer to 90 nt takes place within some other substrate configuration than that shown in Fig. 6.

Finally, we asked whether the mutant proteins are able to promote template-switching reactions. Each mutant protein catalyzes template switching with an efficiency considerably less than that of the corresponding wild-type protein. A 20-fold molar excess of gp32mms over gp32 supported about 1.7-fold less template switching (Fig. 9, A and B), so that gp32mms is about 34-fold less active in this assay than is gp32. A 4- to 8-fold molar excess of gp41uvs over gp41 supported 1.5- to 3.5-fold less template switching (Fig. 9, C and D), so that, using the product of the mid-range values of 4–8 and 1.5–3.5, gp41uvs79 is roughly 6 × 2.5 = 15-fold less active in this assay than is gp41. Thus, the defects of gp32mms and gp41uvs are substantially stronger in template switching than in DNA replication.

**DISCUSSION**

We have examined several properties of two mutant proteins whose gene alleles belong to a bacteriophage T4 epistasis group involved in survival after DNA damage brought about by such diverse agents as UV irradiation, MMS, and photodynamically activated isopsoralen (10). Both proteins are key components of the apparatus that conducts DNA replication: one is a variant of the canonical ssDNA-binding protein gp32 and the other of the replicative DNA helicase gp41. For that reason, among others, the survival process in which they participate was called replication repair. We began the present study by completely removing these alleles from their original, heavily mutagenized backgrounds by cloning them, sequencing to confirm the presence of but a single missense mutation in each, and then recombining them back into T4. These constructs display the same quantitative patterns of survival after UV irradiation as were observed in the mid-1980s (10).

The two mutant proteins display consistent reductions in activity in several assays compared with their wild-type counterparts. The gp32 variant (gp32mms) is about 2-fold less active, and the gp41 variant (gp41uvs79) is about 4-fold less active, in supporting DNA replication in an eight-protein system that synthesizes coupled leading strand and lagging strand DNA synthesis, in DNA-binding assays, and in promoting either DNA annealing (gp32mms) or branch migration (gp41uvs79). (Although plaque sizes and burst sizes are little affected by the corresponding mutant alleles, regulatory adjustments in *in vivo* may circumvent these reduced activities.)

In striking contrast to the above patterns, an assay for template switching (forced by a strong template block to primer strand extension) revealed gp32mms to be about 34-fold less effective

**Fig. 9.** Template switching promoted by mutant T4 replication proteins. A, standard template-switching reactions, including gp43, gp44/62, and gp45, were carried out in the presence of 28.4 nM gp41, 32 nM gp61, 18 nM gp59, and gp32 or gp32mms, except that no proteins were in the reaction for lane 1. The concentrations of gp32 were 50 nM (lane 3), 125 nM (lane 4), 250 nM (lane 5), or 500 nM (lane 6). The concentrations of gp32mms were 50 nM (lane 7), 125 nM (lane 8), 250 nM (lane 9), 500 nM (lane 10), or 1000 nM (lane 11). B, graphic representation of the results shown in A. The vertical axis shows the -fold stimulation of template switching by particular protein concentrations. C, standard template-switching reactions, including gp43, gp44/62, and gp45, were carried out in the presence of 125 nM gp32, except that no proteins were in the reaction for lane 1. The concentrations of gp41 (or gp41uvs79), gp61, and gp59 were: lane 3, 7.1 nM gp41, 8 nM gp61, and 4.5 nM gp59 (0.5×); lane 4, 14.2 nM gp41, 16 nM gp61, and 9 nM gp59 (1×); lane 5, 28.4 nM gp41, 32 nM gp61, and 18 nM gp59 (2×); lane 6, 56.8 nM gp41, 32 nM gp61, and 18 nM gp59 (4×); lane 7, 14.2 nM gp41uvs79, 16 nM gp61, and 9 nM gp59 (1×); lane 8, 28.4 nM gp41uvs79, 32 nM gp61, and 18 nM gp59 (2×); lane 9, 56.8 nM gp41uvs79, 32 nM gp61, and 18 nM gp59 (4×); and lane 10, 113.4 nM gp41uvs79, 32 nM gp61, and 18 nM gp59 (8×). D, graphic representation of the results shown in C.
than wild-type gp32 and gp41uvs79 to be roughly 15-fold less effective than wild-type gp41.

The previous genetic analyses of 32mms and 41uvs79 (Ref. 10 and references therein) had revealed that replication repair promotes survival after DNA damage by a mechanism that is additive to both recombination repair and translesion synthesis mediated by the uvsWXY epistasis group. The uvsWXY system promotes survival after infection by a single phage genome and must therefore be able to act on a single genome and/or its replicative progeny, but the mechanism remains undeciphered. An elegantly simple strand-switching survival mechanism was proposed independently by two groups in 1976 based on results obtained with cultured mammalian somatic cells (1, 2), but this work only slowly came to be cited by students of DNA repair using bacterial and viral systems. In this canonical model for replication repair (Fig. 1), branch migration at the replication fork pairs the terminal portions of the two daughter strands and re-pairs the two parental strands, a realignment that may allow continued synthesis of the previously blocked daughter strand using the other daughter strand as a template. Subsequent reversal of the direction of strand migration regenerates a conventional replication fork except that the previously blocked but now extended daughter strand has bypassed the lesion using accurate genetic information ultimately derived from the undamaged parental strand.

The DNA strand dynamics of a model for the observed strand switching that we observed (Figs. 5–7) are shown in Fig. 10. (To maximize clarity, the eight proteins that drive these steps were omitted from the schematic.) In our construct, the strands in region I have normal base-pairing relationships (strand 1 being complementary to strands 2 and 4), but regions II and III are unusual in that strand 1 is not complementary to strand 4. At the beginning of the reaction, gp43 and gp45 load onto the 3’ end of strand 3 and the helicase preferentially loads onto the 5’ end of strand 2 with the help of gp32 and gp59 (22). In step 1, gp43 extends the strand-3 70-mer to 79 nt, stalling at the double block. In step 2, the helicase unwinds the top duplex at least into region I, and a few bases at the 3’-end of strand 3 melt (either spontaneously or with the assistance of gp32). In step 3, these few bases then anneal with the helicase-generated ssDNA at the 5’-end of strand 2, whereupon strand 3 is further extended to 90 nt. In none of our assays, including those conducted with the recombination proteins UvsX with or without UvsY, did we observe a second switch that returned the primer to the original template strand (which would have resulted in a 120-nt product). This simply reflects the lack of sequence complementarity at the end of the 90-mer that was made by extending the 70-mer. Although the substrate we used to detect template switching certainly differs in several ways from the putative in vivo substrate, it suffices to define a particularly strong switching defect in the two mutant proteins, a result predicted by the template switching hypothesis. It might, with some effort, be possible to design and construct a larger and more complicated substrate in the future to explore conditions that might promote switching back.

As is the case with other proteins involved in T-even phage DNA transactions, both gp32 and gp41 participate in several other DNA transactions besides replication and replication repair, including recombination, recombination repair, and excision repair, and different mutations in the same gene can affect these functions separately. Gene 32 examples were compiled previously (10). Recombination in standard T4 crosses is modestly increased by ucs079 (12), whereas 41rrhl (an allele that also impairs replication repair) severely impairs plasmid-to-phage homologous recombination while modestly reducing recombination in standard crosses (12).

A crystal structure is available for the large ssDNA-binding core of gp32 (residues 22–239 out of 301) complexed with oligo(dT)6 (23). Two or three dT residues are strongly bound by a gp32 zinc finger whose zinc ion is coordinated by His84, Cys77, Cys87, and Cys90. The 32mms alteration converts Pro to Leu at codon 88 (4) and is thus likely to perturb the coordinating function of at least Cys87 and perhaps also Cys90, thus weakening gp32 binding to ssDNA as reported here. A simple hypothesis is that template switching is much more sensitive to modestly reduced ssDNA binding than are the other gp32 functions we assayed, whereas an alternative hypothesis is that this region of the protein is involved in some function specific to template switching. The other gene 32 allele known to block replication repair is 32amA453 (G347A converting Trp116 to an amber codon) (10). The unsuppressed 32amA453 allele is lethal, and a block to replication repair can only be observed when 32amA453 is suppressed as by the insertion of serine or glutamine at codon 116, which occurs in up to half of the
translational supplying and thus produces a mixture of amber peptides and full-length polypeptides. Serine provides a wild growth phenotype; glutamine provides a weakly viable phenotype. Because revertants of 32amA453 bearing either serine or glutamine exhibit normal UV sensitivity, it seems likely that the amber peptide itself binds to ssDNA and blocks replication repair, but we have not tested this hypothesis.

The gene 41 alleles that impair replication repair are 41uss79 (G135A converting Ala<sup>279</sup> to Thr<sup>279</sup>) and 41rrh1 (C1060A converting Ala<sup>354</sup> to Thr<sup>354</sup>) (11). Although no crystallographic structure is yet available for gp41, a partial sequence alignment has been described involving gp41 and several related DnaB-family helicases (24). The alignment is robust in several conserved regions that retain frequent identity, but the degree of identity falls sharply outside of these regions and thus softens the alignment. Crystal structures that rationalize the most strongly conserved regions are available for the phage T7 helicase (24, 25). Gp41 Ala<sup>279</sup> aligns with T7 Gln<sup>494</sup> near the C-terminal end of motif H3, which contributes to the pocket around the bound nucleotide whose hydrolysis drives the helicase reaction. Gp41 Ala<sup>354</sup> aligns with T7 Cys<sup>564</sup> toward the middle of motif H4, close to a loop that is implicated in DNA binding and adjacent to a residue that contacts the nucleotide gamma phosphate. However, this structural information provides no notable insights into the role of gp41 in replication repair, and the same two hypotheses given above for the gp2 mutants have gp41 parallels.

Is there evidence for the operation of replication repair in other organisms? The two canonical reports (1, 2) were based on experiments with cultured mammalian cells and lacked genetic handles. Although the 1976 model has been cited frequently to explain data implying lesion circumvention in bacteria, yeast, or mammalian cells, other explanations (such as translesion synthesis by specialized DNA polymerases, delayed excision repair, and classical recombination repair) were generally not excluded or were even favored, and combined genetic and enzymological methods to resolve the issue were lacking. Recently, however, depletion of the human hMms2 protein was suggested on the basis of a powerful system of genetic markers to specifically block the kind of template switching envisioned for replication repair, rather than template switching to another chromosome (26).

The hMms2 protein was investigated because of the properties of its previously discovered homologue in <i>S. cerevisiae</i>, Mms2. Mms2 is encoded by a gene that resides in an epistasis group whose genes comprise at least MMS2, UBC13, RAD5, RAD6, RAD18, and POL30 (for review see Ref. 27). As in phage T4, the products of several of these genes function in more than one repair pathway, but this yeast epistasis group defines an error-free mode of post-replication repair that is distinct from conventional recombination repair and that may well be replication repair.

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