The rescue of stalled replication forks via a series of steps that include fork regression, template switching, and fork restoration often has been proposed as a major mechanism for accurately bypassing non-coding DNA lesions. Bacteriophage T4 encodes almost all of the proteins required for its own DNA replication, recombination, and repair. Both recombination and recombination repair in T4 rely on UvsX, a RecA-like recombinase. We show here that UvsX plus the T4-encoded helicase Dda suffice to rescue stalled T4 replication forks in vitro. This rescue is based on two sequential template-switching reactions that allow DNA replication to bypass a non-coding DNA lesion in a non-mutagenic manner.

DNA template-strand lesions that prevent the extension of primer strands are usually lethal. Four general mechanisms operate to overcome such lethality: direct repair that regenerates the native structure (e.g. photoreaction and dealkylation), excision repair that replaces damaged bases or deoxyribose residues, translesion synthesis that is usually mutation-prone, and recombination repair that accurately bypasses DNA damage.

The essence of recombination repair is that it derives accurate genetic information from the nascent sister chromatid (or from a homologous chromosome when repairing a double strand break); excision repair, on the other hand, derives the requisite genetic information from the complementary strand of the same double helix. Recombination repair was first characterized in Escherichia coli (1–4). The classical E. coli break-reunion model holds that blocked primer extension is followed by downstream reinitiation, leaving a gap that is then filled at least in part by the physical donation of a strand of appropriate polarity cut from the sister chromatid. The donating strand gap is filled by DNA synthesis, whereas the blocking lesion remains as a problem for the future.

Recent progress in understanding recombination repair, based mainly on work with E. coli, has highlighted its importance for rescuing replication forks stalled at sites of DNA damage or spontaneous fork collapse (8–11). One model of recombination repair (Fig. 1) invokes a topological rearrangement in which the fork regresses into a configuration in which the parental strands reanneal and the two daughter strands anneal. This rearrangement generates a structure that constitutes a Holliday junction and that to some resembles a chicken foot (12). In the case of a primer strand whose extension is blocked by a lesion in the template strand, fork regression provides a template that allows limited primer extension. Subsequent reformation of a conventional replication fork then provides a primer strand that has accurately bypassed the template lesion. This pathway was named replication repair and was initially suggested to occur during mammalian DNA replication (5). It was later validated by genetic and enzymological analyses in phage T4 (6, 7).

In addition to a DNA polymerase, the canonical model of replication repair (Fig. 1) immediately suggests roles for a DNA helicase and a DNA recombinase to drive template switching. Phage T4 encodes a single DNA polymerase, gp43, a single DNA recombinase, UvsX, and three DNA helicases. gp41 is a replicative 5′ → 3′ DNA helicase (13). UvsW is a 3′ → 5′ DNA/RNA and DNA/DNA helicase that is probably involved in DNA recombination and repair (14, 15) and whose biochemical properties are not well understood because a soluble version of the native protein has resisted isolation; nonetheless, a crystallographic structure is available for part of UvsW (16). The third helicase, Dda (17, 18), is well characterized biochemically (19–21), but its biological functions remain unclear. dda mutants show a slightly delayed DNA synthesis (22). When dda is combined with an amber mutation in gene 59, which encodes the gp41 helicase loader, the double mutant cannot synthesize DNA under non-permissive conditions (22). Dda interacts both functionally and physically with UvsX (19, 23).

Here we describe how UvsX recombinase and Dda helicase can rescue stalled replication forks by redirecting DNA synthesis conducted by the T4 DNA polymerase when the polymerase encounters a blocking lesion in the template strand. This redirection is based on two sequential template-switching reactions that allow T4 DNA replication to bypass a non-coding DNA lesion.

**MATERIALS AND METHODS**

**Proteins**—Overproduction and purification of phage T4 gp32, gp43, gp43D219A, gp44/62, gp59, UvsX, and UvsY were as described previously (7, 24). gp41 and gp45 were purified as described previously (24) with modifications to be described elsewhere. Proteins were monitored during purification by SDS-PAGE and Coomassie R-250 staining. T4 Dda helicase was a generous gift from Dr. Kevin Raney (University of Arkansas, Little Rock, AR).

**Replication Repair and Helicase Assays**—Gel-purified oligonucleotides were from Oligos Etc. (Wilsonville, OR) and, when necessary, were 5′-32P-labeled with T4 polynucleotide kinase. To prepare the standard forked substrate for replication repair and helicase assays, 120-mer “1” and 5′-32P-labeled 76-mer “3” were annealed with 94-mer “2” and 120-mer “4”, respectively, in separate 20-µl mixtures containing annealing buffer (80 mM Tris acetate, pH 7.8, 200 mM potassium glutamate, 25 mM magnesium acetate, and 20 mM dithiothreitol) and 75 nM each oligonucleotide at 45 °C for 10 min. The two mixtures were then combined, diluted with annealing buffer to a final concentration of 26.66 nM, and incubated at 40 °C for 5 min; the annealed forked DNA was used
an abasic site that is followed immediately by a template base (G) that cannot easily serve as a template because the reaction mixture is devoid of dCTP (which is needed nowhere else in the reaction). This double lesion effectively abolishes translesion synthesis (7). The other key feature of the DNA substrate is that the second switch is likely to start when the extension of strand 3 reaches or penetrates the template pentanucleotide 5'-rArCrCrUrU-3', which is characteristic of the primers that initiate lagging strands in T4 replication forks (25, 26).

Although strand switching was modestly promoted by the UvsX recombinase in the absence of a helicase (7), exploratory experiments described below revealed that the reaction was not promoted by the T4 gp41 helicase. However, T4 encodes two more helicases. UvsW is an RNA/DNA as well as a DNA/DNA helicase (14, 15). Dda is a DNA/DNA helicase (17, 18) that acts as a monomer and translocates on DNA in the 5'→3' direction (20, 21). DNA synthesis is only slightly delayed in a dda null mutant (22). Gene 59 encodes the gp41 helicase loader (27), and when the dda null mutation is combined with a gene 59 amber mutation, DNA synthesis is blocked (22). Most importantly, UvsX interacts with UvsX; Dda is retained by a UvsX affinity column (23), and it stimulates UvsX-mediated strand exchange (19). We therefore inquired as to whether the T4 Dda helicase might promote template switching catalyzed by UvsX.

When the gp43 DNA polymerase, the gp44/62 clamp loader, and the gp45 clamp are incubated with the artificial replication fork, the extension of strand 3 is blocked by the abasic site, and a 79-nt product accumulates (Fig. 2B, lane 5). Including either UvsX or Dda in the reaction promoted the formation of products containing 90–91 nt. UvsX is more efficient than Dda in promoting this first template switch (Fig. 2B, lanes 3 and 4, and Fig. 2C). Thus, either UvsX or Dda can promote single template switches, but neither promotes the second switch required for replication repair with appreciable efficiency. Because strand 2 contains 89 deoxyribonucleotides followed by five ribonucleotides, the observation of products slightly longer than 89 nt was unexpected and suggests that gp43 has a weak reverse-transcriptase activity. The chance that the pentanucleotide portion of the substrate was contaminated with deoxyribonucleotides at its 3'-end is small because the majority rather than a minority of the products exceeded 89 nt and because the gp43 exonuclease deficiency described below promoted even longer products.

If replication repair occurs, it will generate a 120-nt product via double switching (Fig. 2A). When both UvsX and Dda were included in the reaction, the 120-nt product appeared (Fig. 2B, lane 5, and Fig. 2C). This product was also observed to the same extent when all four dNTPs at 50 μM were included in the reaction, thus reducing the blocking lesion to the abasic site (data not shown). No extension of strand 3 was detected when both UvsX and Dda were included in the absence of the T4 DNA polymerase holoenzyme, excluding the possibility that UvsX and/or Dda preparations contain a contaminating DNA polymerase capable of bypassing the template lesion (data not shown). Thus, UvsX and Dda together promote double template switching by the T4 DNA polymerase holoenzyme to circumvent a template lesion in vitro. The time course of the template-switching reaction showed that the 120-nt product was already observed after 5 min (Fig. 2D). When either Dda or UvsX was omitted, hardly any template switching was observed even after 30 min. These results emphasize that both Dda and UvsX are required to rescue T4 replication forks in vitro by replication repair.

In the reaction with Dda alone, an ~101-nt product was also observed (Fig. 2B, lane 3). This product can form because the pentanucleotide repeat in strand 4 (Fig. 2A) allows 3'-ends of
strand 3 extended to 79 nt to be unwound by Dda and then to reanneal with the downstream copy of the repeat, to be trimmed to 78 nt when the gp43 3'-H11032-exonuclease removes the mispaired terminal T, and then to be extended to 101 nt. The deletion comprises one copy of the 5-nt repeat plus the intervening 14 nt (including the 2-nt blocking lesion) (120/H110025/H1100214/H11005101). (The 101-nt product is not seen with the Exo/H11002polymerase, presumably because the terminal mismatch is difficult to extend.) Thus, Dda may mediate deletion mutagenesis in response to DNA damage, and overproduction of Dda might promote deletions between repetitive sequences. Indeed, overproduction of UvrD, a helicase unrelated to Dda, promotes deletions between tandem repeats via induction of the RecF recombination pathway (28).

We next explored how the composition of the DNA substrate affects replication repair (Fig. 3). As expected, no 120-nt product appeared when the damaged template strand was omitted (Fig. 3, lane 2), when the other daughter strand (the lagging strand) was omitted (lane 3), when both parental strands were omitted (lane 6), when both sister-chromosome strands were omitted (lane 7), or when all but the leading strand were omitted (lane 10). Omitting the lagging strand template increased the yield of the 120-nt product (Fig. 3, lane 4) in accord with previous reports that T4 replication plus recombination proteins can catalyze conservative DNA replication (29, 30). Replacing the 94-nt hybrid RNA-DNA lagging strand with a 90-nt DNA strand did not alter the efficiency of template switching (Fig. 3, lane 8), indicating that a DNA terminus is as efficient as an RNA oligomer in promoting the second switch.

Decreasing the length of the lagging strand from 94 to 86 nt while preserving its RNA-DNA hybrid nature abolished the second switch (Fig. 3, lane 9), suggesting that the 82–84-nt products of the first template switch are not long enough to bridge the two nontemplating residues after residue 79 and then to support the second switch by forming only 1–3 base pairs. Replacing gp43 with the exonuclease-deficient derivative gp43D219A had no strong effects on the reactions (compare the left-hand and right-hand gels in Fig. 3), indicating that the exonuclease activity does not affect replication repair. The only
The difference was that the products of the first switch were somewhat longer than those with the wild-type gp43, which suggests that the proofreading exonuclease restricts the reverse transcriptase activity of gp43. The combined results indicate that the replication-repair reaction absolutely requires the damaged template of the leading strand plus the alternative template lagging strand, which must be sufficiently long to provide more than 3 base pairs to support the second switch.

We next analyzed the effects of Dda and UvsX concentrations on replication repair. The optimal concentration of UvsX was about 375 nM, above which template switching declined sharply (Fig. 4A). The optimal concentration of Dda was reached at about 62.5 nM, and increasing the concentration to 125 nM had no effect (Fig. 4B). When the concentration of the holoenzyme (gp43, gp44/45, and gp44/62) was increased by 4-fold, the yield of the 120-nt double-switch product was increased about 3-fold; however, when the holoenzyme concentration was increased 16-fold, the yield of the 120-nt product decreased sharply (data not shown). We also examined the effects of the concentration of the forked DNA substrate on replication repair. As expected, increasing the concentration of the substrate increased the yield of the double-switch product (Fig. 4C). The yield of the product increased almost linearly with the substrate over the tested range, showing that the double-switch reaction is not forced by high protein/DNA ratios.

We next asked whether template switching occurs while the replication fork retains its four-stranded structure or after its unwinding to the two arms, and the four-stranded structure of the replication fork facilitates template switching.
5B, lane 2). When either Dda or UvsX was present in a reaction, some of the fork was converted into two \(^{32}\)P-labeled sub-structures, a lesion-bearing duplex and a duplex containing the two daughter strands (Fig. 5B, lanes 3 and 4). The same products were also observed when the holoenzyme proteins were omitted from the reaction (data not shown), indicating that Dda and UvsX are sufficient for unwinding the fork. Including both Dda and UvsX in the reaction converted all substrate to the lesion-bearing duplex and the duplex containing the two daughter strands, the products of the first template-switching reaction (Fig. 5B, lane 5). The PAGE mobility of the lesion-bearing duplex composed of strands 1 and 4 is very similar to the mobility of the lesion-bearing duplex composed of strands 3 and 4 (data not shown), so that gel analyses do not clearly distinguish between the fork structures before and after template switching (compare the mobilities of the middle major bands in Fig. 5B, lanes 3 and 4, where there is no template-switching product, with that in lane 5, where template-switching product is present). These results show that although replication repair requires the concerted action of Dda and UvsX, either protein can destabilize or unwind the fork.

UvsY is an accessory protein that loads UvsX onto single-stranded DNA (32, 33), and gp32 contributes to DNA synthesis both by destabilizing DNA secondary structures and by binding to other replication proteins (34). We therefore inquired as to whether UvsY and/or gp32 can modulate the replication-repair reaction. Adding 62.5 nM UvsY, 125 nM gp32, or both to the standard replication-repair mixture inhibited the reaction (data not shown). However, the effects were somewhat different when the UvsX concentration was decreased 2-fold. Under that condition, adding either protein still inhibited the reaction (Fig. 6, lanes 8 and 10), but adding both relieved the inhibition (Fig. 6, lane 9). This result is consistent with previous observations that UvsY helps to load UvsX onto DNA in the presence of competing gp32 (35, 36). We also determined that gp32 cannot replace UvsX in the replication-repair reaction (data not shown).

Finally, we inquired whether the T4 gp41 replicative helicase, alone or together with gp59, can replace Dda in the replication-repair reaction. We included gp59 in the analysis because it loads the gp41 helicase onto DNA (27) and is required for single template switching driven by gp41 (7). When gp41 and gp59 were added to a reaction also containing UvsX and the polymerase holoenzyme proteins, the 120-nt double-switching product accumulated (Fig. 7, lane 5). Omitting either gp41 or gp59 abolished double switching, indicating the importance of both proteins in the reaction (Fig. 7, lanes 6 and 7).

Varying the UvsX concentration at fixed concentrations of gp41 and gp59 and vice versa did not increase the efficiency of the reaction (data not shown). The maximum reaction efficiency in the presence of both gp41 and gp59 was about 5-fold less than in the standard reaction containing Dda. Thus, gp41 plus gp59 cannot efficiently replace Dda in the replication-repair reaction in vitro.

**DISCUSSION**

Using an artificial replication fork, we have shown that UvsX and Dda helicase can rescue stalled phage T4 replication forks in vitro. Rescue is based on a mechanism that allows DNA replication to bypass non-coding DNA lesions via two sequential template-switching reactions (Figs. 1 and 2). When either UvsX or Dda is omitted, no replication repair occurs (Fig. 2): Dda can be replaced by the gp41 replicative helicase plus its gp59 loader, although inefficiently. A similar but single strand-switching process can be driven not by UvsX plus Dda but by the gp41 helicase plus the gp32 single-stranded DNA-binding protein (7). However, this process has been examined so far only using a replication fork designed in a way that prevented the second switch.

A remarkable aspect of our results is that a genetic analysis of T4 survival after DNA damage revealed that the replication-repair alleles of genes 32 and 41 occupy a different epistasis group from the recombination-repair alleles of uvsX (6). However, because dda null alleles do not affect UV sensitivity (37), it would appear that dda is not a member of the uvsX epistasis group. Another member of this epistasis group is uvsW, which encodes another helicase (14, 15). uvsW mutations increase UV sensitivity by only about 1.34-fold, whereas uvsX and uvsY mutations increase sensitivity by about 1.57-fold (6). Therefore, Dda and UvsW may provide redundant helicase functions; in this case Dda might incompletely support replication repair in a uvsW mutant, and perhaps UvsW might completely support replication repair in a dda mutant. Determining whether both UvsX and UvsW support a replication-repair mode of recombination repair in vivo will require greater understanding of the relative roles of UvsW and Dda.

Replication repair and other pathways of recombination repair accurately circumvent DNA lesions. Mutagenic DNA translesion synthesis is often conducted by specialized DNA polymerases, but not in T4, where gp43 seems to do the job (38). However, translesion synthesis is frequently accurate in vitro opposite DNA lesions that retain coding potential (39). Our results suggest that the rigorous characterization of translesion synthesis in vivo requires some understanding of the con-

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**Fig. 6. UvsY and gp32 modulate template switching.** Reactions were conducted under standard conditions with or without Dda, UvsX, UvsY, and/or gp32 at final concentrations of 62.5, 187.5, 62.5, and 125 nM, respectively.

**Fig. 7. Replacing Dda with gp41/gp59 in replication-repair reactions.** Reactions were conducted under standard conditions except that Dda was omitted and that gp59 and the gp41 hexamer, where indicated, were present at final concentrations of 36 and 58 nM, respectively.
comitant contribution of recombination repair to accurate lesion bypass.

What are the relative contributions of the classical break-reunion mode of recombination repair and replication repair to survival? T4 encodes almost all of the proteins required for its own DNA transactions, and these proteins more closely resemble their counterparts in archaea and eukaryotes than in eubacteria (40). Despite several decades of vigorous exploration of T4 repair mechanisms, there is a striking absence of any report of the kind of break-reunion recombination repair so well characterized in E. coli. In phage λ, which relies upon E. coli repair systems, as many as 20 dimers/genome were insufficient to block viral DNA replication in an unirradiated, excision-deficient E. coli cell in which the recA-dependent recombination functions were not induced and in which few recombinational exchanges occurred (41). This high survival seems unlikely to have been the result of simple translesion synthesis because there is little evidence that the host (42); absent the induced level of replication repair, the mechanism may have been distinct from classical recombination repair and may well be replication repair. Further parallels between T4 and E. coli are suggested by studies that implicate the E. coli RegG and PriA helicases in a repair mode that also seems to be distinct from classical recombination repair (43–45). There is a striking lack of published support for a break-reunion mode of recombination repair in yeast and mammalian cells, and at least in yeast, attempts to detect the classical mode did not do so (46, 47). In contrast, as noted previously (7), the yeast RAD5/6 episome group (48) encodes a mode of postreplication repair that is distinct from classical recombination repair and may well be replication repair, and genetical results (49) suggest the operation of a human system for template switching that also resembles replication repair. Taken together, these reports invite the speculation that classical recombination repair is the dominant mode in eubacteria with replication repair playing a secondary role, whereas replication repair is the dominant mode in T4 and in eukaryotes. What, then, is the dominant mode in the archaea?

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