Supporting Information

Jose et al. 10.1073/pnas.1212929109

SI Text

SI Materials and Methods. ***DNA constructs.*** DNA oligonucleotides unlabeled and labeled with 2-aminopurine (2-AP) were purchased from Integrated DNA Technologies. DNA concentrations were determined by UV absorbance at 260 nm (25 °C), based on extinction coefficients furnished by the manufacturer. Base-paired structures were formed by heating equimolar concentrations of the appropriate 2-AP–labeled ssDNA strand with the complementary unlabeled ssDNA at 90 °C for 5 min, and then cooling to room temperature over a period of 3 h. Thermal melting experiments, monitored by tracking $\Delta A_{260\ nm}$, were used to confirm the duplex character of the dsDNA portions of the resulting DNA constructs. The sequences and nomenclature of the DNA constructs used in this study are shown and described in Tables S1 and S2.

Protein purification and properties. The T4-coded helicase (gp41) protein was cloned and overexpressed in *Escherichia coli* strain OR1264/pDH518 (1) and purified as previously described (2). The T4 primase (gp61) protein contains a His-tag at the N terminus and was prepared and purified as described previously (3). Concentrations of purified gp41 and gp61 were determined by UV absorbance at 280 nm, using a molar (per subunit) extinction coefficient ($\varepsilon_{M,280}$) of $7.6 \cdot 10^4\ M^{-1}\,cm^{-1}$ for gp41 and $6.9 \cdot 10^4\ M^{-1}\,cm^{-1}$ for gp61. The extinction coefficients were calculated from amino acid residue composition data (4, 5).

Spectroscopic procedures. CD spectra were measured at wavelengths from 300 to 360 nm using a JASCO model J-720 CD spectrometer equipped with a temperature-controlled sample holder; 10–15 spectra were scanned, averaged, and plotted as graphs of $\Delta\varepsilon_l - \Delta\varepsilon_r$ (the difference in the molar extinction coefficient for left and right circularly polarized light) per mol of 2-AP residue, as a function of wavelength. Fluorescence spectra were measured using either a Jobin-Yvon Fluorolog or Fluoromax spectrofluorometer. Samples were excited at 315 nm and emission spectra were recorded from 330 to 430 nm. The fluorescence intensities reported here were measured at 370 nm and normalized to the fluorescence intensities obtained with the corresponding ssDNA sequence. The error bars shown for the fluorescence measurements represent standard deviations for two to four independent experiments.

Assembly of the T4 primosome helicase from Its components in solution. The determination of the association pathway for the in vitro assembly of the T4 DNA replication primosome from its components (the gp41 helicase, the gp61 primase, DNA constructs, and NTP substrates) has been described elsewhere (6). In all the experiments described in this paper (except for the unwinding assay), helicase complexes were formed by adding 60 μM of GTPγS to 500 nM DNA, followed by addition of gp41 subunit monomers to a final concentration of 3 μM. Gp61 monomers were then added to a final concentration of 500 nM and fluorescence and CD spectra were measured after 3 to 5 min of additional equilibration. For experiments with GTP, a DNA–helicase complex was formed by adding 600-μM concentrations of GTP to 500-nM concentrations of DNA, followed by addition of gp41 to a final monomer concentration of 3 μM.

SI Results. ***T4 helicase activity Is not inhibited by 2-AP base substitutions in fork constructs.*** The 2-AP bases form Watson–Crick base pairs with thymine, and it has been shown by various research groups that 2-AP•T base pairs can substitute for A•T pairs in most biological processes (7). We carried out unwinding experiments with three identical primer/template (P/T) DNA or forked constructs, with none, one, or two (adjacent) adenines replaced by a 2-AP monomer or dimer pair, to determine whether 2-AP substitution has any effect on the activity of the T4 helicase. The constructs used are shown in Table S2.

The lagging ($5' \rightarrow 3'$) strand was labeled with $\gamma^{32}P$ at the 5′ end and was annealed to the leading strand. The DNA fork substrates (5 nM) were incubated with T4 gp41 and gp61 (300 nM and 50 nM, respectively) in reaction buffer and the reaction was initiated by adding ATP and $Mg(OAc)_2$. Reactions were quenched by adding EDTA to a final concentration of 25 mM together with a 500-nM concentration of complementary ssDNA molecules to serve as "trapping strands" for the displaced DNA and thus prevent reannealing of the original construct. The reaction products were subjected to native gel electrophoresis and quantified using the ImageQuant software from Molecular Dynamics.

The unwinding rate was measured for all three DNA fork constructs and the results are plotted in Fig. S1. The rate profiles for the unwinding of all three constructs are clearly superimposable. We conclude from these results that the substitution of 2-AP monomers or dimer pairs for monomer or dimer A residues has no significant effect on the unwinding kinetics of the T4 primosome helicase. In their earlier studies, Raney et al. (7) also concluded that the substitution of monomer 2-AP probes for A residues did not perturb the rates of unwinding of DNA duplexes catalyzed by the ddA helicase of bacteriophage T4.

Tracking helicase-induced dsDNA unwinding using 2-AP dimer probes in the lagging ($5' \rightarrow 3'$) strand. The incorporation of a 2-AP dimer probe instead of a monomer in an oligonucleotide construct has the advantage that the spectroscopic interaction of each 2-AP base is primarily with its dimer partner, rather than with neighboring canonical bases (8, 9). The low-energy CD spectra of such dimer probes can provide information (via exciton coupling interactions) about the relative orientation of this pair of bases within the DNA framework of the construct. In contrast, the fluorescence of a dimer probe pair is particularly weak as a consequence of self-quenching within the dimer. We have used these dimer probes to examine further the conformational changes induced in our DNA construct models by initial strong helicase binding at the replication fork.

Fig. S3 shows the fluorescence and CD spectral changes observed upon primosome helicase binding with 2-AP dimer probes incorporated site specifically in the lagging strand of a forked DNA construct. The fluorescence intensity changes observed are similar to those seen with monomer probes, confirming that conformational changes caused by initial primosome helicase binding extend only up to the third base pair in the dsDNA portion of the construct. The CD signals for ssDNA and the $\{-2, -1\}$ and $\{-1, 1\}$ constructs showed slight decreases in intensity on primosome helicase binding, consistent with the destabilization observed for bases in such partially ssDNA environments (Fig. S3 *B–D*). For the initial (GTPγS-locked) primosome–DNA construct complexes with a 2-AP dimer probe at the 1,2 position, the probes at two base-paired positions showed an increase in fluorescence intensity (Fig. S3*A*), indicating that the same sort of unstacking (or increased exposure to solvent) of these bases observed with monomer probes in the lagging strand occurs also with helicase binding monitored with dimer probes.

In contrast, the CD signal of the {1, 2} construct was essentially unchanged upon helicase binding (Fig. S3*E*), presumably because the CD spectra depend primarily on the relative orientation of the transition dipoles of the two homologous chromophore probes, whereas variations in the fluorescence intensity of 2-AP–labeled constructs reflect primarily dynamic changes in exposure to solvent and hydrogen bonding of adjacent and opposite bases and base pairs. Once the initial primosome helicase binds to the construct, even if some unstacking of the bases at these positions does occur, the primosome complex should stabilize the relative conformational orientations of the base pairs, and this should result in increased fluorescence intensity and an unchanged CD spectrum. The changes observed for other constructs with 2-AP dimer probes in the duplex region are consistent with observations made with the equivalent DNA constructs labeled with monomer probes.

A dangling ssDNA 3′-sequence on the leading strand is required for primosome helicase unwinding activity. Prior studies have shown that a leading strand with a 3′ dangling ssDNA sequence is needed to permit effective helicase-induced unwinding of duplex DNA (10). We have reexamined this issue using our spectroscopic probes to confirm that we get similar results and to further examine the role of the 3′-ssDNA leading strand. Experiments were performed with P/T DNA constructs with no dangling 3′-end and with partial DNA fork constructs with a 3′-dangling ssDNA end 5–7 nt in length (Table S2); 2-AP probes were site specifically introduced into the 5′ (lagging) strand at different positions relative to the ss–dsDNA junction. Fluorescence intensities reflecting the addition of (GTPγS-locked) primosome helicase to leading strand P/T {ptN} and partial fork {pfN} constructs labeled with monomer 2-AP probes are shown, respectively, in Figs. S4 and S5.

Experiments were performed with constructs labeled with 2-AP monomer probes at various positions on the lagging strand and an increase in fluorescence intensity was observed for constructs with 2-AP probes at various positions in the ssDNA portion of the lagging strand, as also observed previously for fully forked constructs, confirming that the primosome helicase can effectively bind to lengthy ssDNA sequences. However, significant changes in fluorescence intensity were not observed for constructs with probes in the duplex region (Figs. S4 and S5), indicating that neither the gp41 hexameric helicase nor the primosome helicase can unwind duplex regions in the absence of a dangling 3′-ssDNA sequence of adequate length on the leading strand. The experiments with DNA constructs [pt1], [pt5], and [pf5] were repeated with GTP to monitor the unwinding of the duplex DNA. Again, the fluorescence signal was unchanged following the addition of the primosome helicase, indicating that even this strongly bound helicase cannot unwind these DNA constructs in the absence of a 3′-ssDNA sequence of sufficient length. These results confirm that the presence of a leading strand 3′-ssDNA dangling sequence of 10–15 nt in length is required to permit the T4 primosome helicase complex to unwind the duplex portion of a DNA construct.

Tracking helicase-induced dsDNA unwinding using 2-AP dimer probes in the leading (3′ → 5′) strand. The 2-AP probes of the [1,2] forked DNA construct are located in the first two base-paired positions near the ss–dsDNA junction of the leading strand where helicase binding should significantly perturb the relative orientations of these bases. Fig. S6*A* shows that the addition of the gp41 helicase hexamer alone resulted in no change in the fluorescence intensity of the complex, consistent with the weak binding of this helicase demonstrated in earlier results. However, the addition of primase resulted in an increase in fluorescence intensity, just as observed with dimer probes in the lagging strand at these same positions, confirming that unstacking (unwinding) of these base pairs does occur with the tight-binding primosome helicase. In addition, we note that the CD spectra of the lagging and leading strands labeled with 2-AP dimer probes at the 1,2 positions were different (compare Figs. S3*E* and S6*E*). Thus, formation of the initial GTPγS-locked primase–helicase complex showed no effect on the CD spectral properties of probes at the 1,2 positions in the lagging strand, whereas a considerable decrease in the size of the CD peak was observed with the probes located at these positions in the leading strand.

This result is consistent with the difference in fluorescence properties observed for leading and lagging strands with 2-AP monomer probes in these positions. In addition, the CD signals of constructs [3,4] and [5,6] did not show any change with addition of the primosome helicase, confirming that binding of the GTPγS-locked primosome helicase complex does not exert an effect beyond three base pairs from the fork junction (Fig. S6 *F* and *G*).

1. Hinton DM, Silver LL, Nossal NG (1985) Bacteriophage T4 DNA replication protein 41. Cloning of the gene and purification of the expressed protein. *J Biol Chem* 260:12851–12857.
2. Dong F, Gogol EP, von Hippel PH (1995) The phage T4-coded DNA replication helicase (gp41) forms a hexamer upon activation by nucleoside triphosphate. *J Biol Chem* 270:7462–7473.
3. Jing DH, Dong F, Latham GJ, von Hippel PH (1999) Interactions of bacteriophage T4-coded primase (gp61) with the T4 replication helicase (gp41) and DNA in primosome formation. *J Biol Chem* 274:27287–27298.
4. Gill SC, von Hippel PH (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem* 182:319–326.
5. Young MC, Kuhl SB, von Hippel PH (1994) Kinetic theory of ATP-driven translocases on one-dimensional polymer lattices. *J Mol Biol* 235:1436–1446.
6. Jose D, Weitzel SE, Jing D, von Hippel PH (2012) Assembly and subunit stoichiometry of the functional helicase-primase (primosome) complex of bacteriophage T4 DNA replication system. *Proc Natl Acad Sci USA*, 10.1073/pnas.1210040109.
7. Raney KD, Sowers LC, Millar DP, Benkovic SJ (1994) A fluorescence-based assay for monitoring helicase activity. *Proc Natl Acad Sci USA* 91:6644–6648.
8. Johnson NP, Baase WA, von Hippel PH (2004) Low-energy circular dichroism of 2-aminopurine dinucleotide as a probe of local conformation of DNA and RNA. *Proc Natl Acad Sci USA* 101:3426–3431.
9. Jose D, Datta K, Johnson NP, von Hippel PH (2009) Spectroscopic studies of position-specific DNA "breathing" fluctuations at replication forks and primer-template junctions. *Proc Natl Acad Sci USA* 106:4231–4236.
10. Richardson RW, Nossal NG (1989) Characterization of the bacteriophage T4 gene 41 DNA helicase. *J Biol Chem* 264:4725–4731.

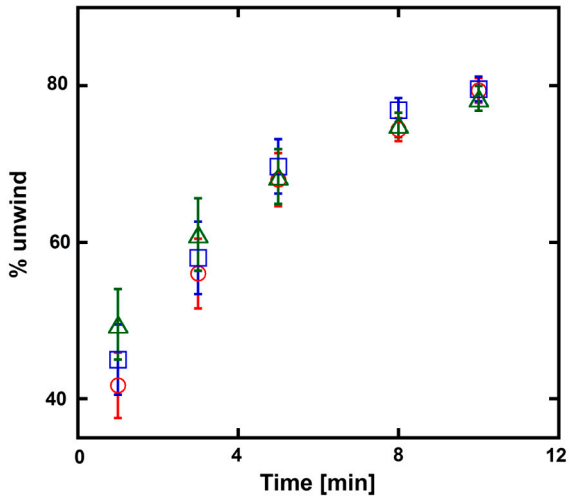

Fig. S1. The helicase activity assay shows that substitution of A residues by either 2-AP monomer or dimer probes does not change the unwinding rate of primosome helicase complex. Helicase unwinding assay with DNA fork constructs (Table S2) containing no 2-AP (red circle), a 2-AP monomer probe (blue square), and a 2-AP dimer probe (green triangle).

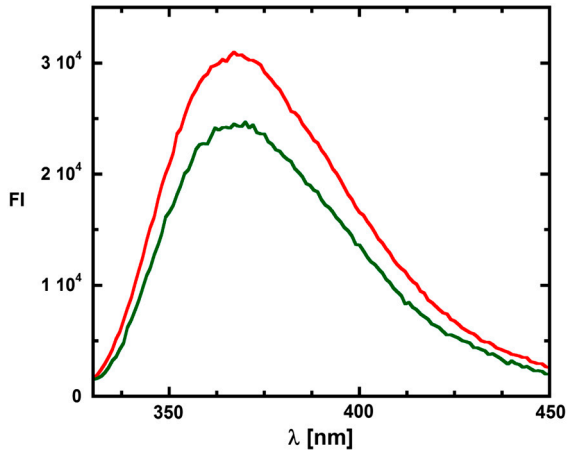

Fig. S2. The fluorescence spectra of forked DNA constructs with unbound and bound helicase do not show a peak shift. The full fluorescence spectrum of the free DNA construct $\{1\}$ is shown in red and that of the construct with the primosome helicase in green. The excitation wavelength was set at 315 nm and emission spectra were measured from 330 to 450 nm. The fact that these peaks do not shift with the binding of the protein components permits us to monitor fluorescence changes at single wavelengths (e.g., Fig. 2, etc).

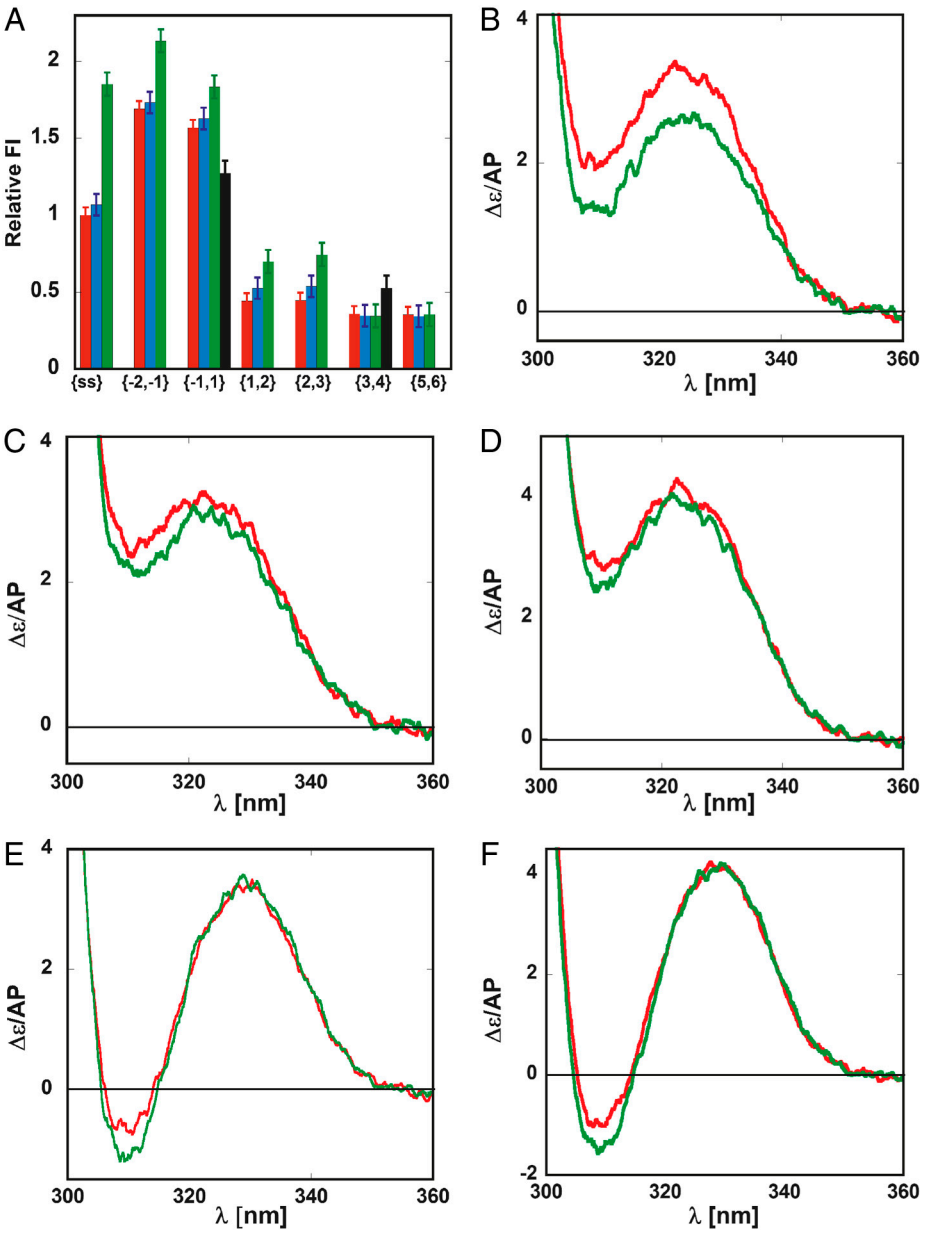

Fig. S3. Fluorescence and CD changes observed for forked DNA constructs with 2-AP dimer probes in the lagging strand. (*A*) The fluorescence intensity changes following the interaction of the T4 helicases (either the gp41 hexamer alone or the gp41–gp61 primosome) for various DNA constructs labeled with 2-AP dimer probes in the lagging strand. Each panel shows bar graphs monitoring the interaction of an individual construct (identified in Table S1) with the two forms of the helicase. The first bar (red) in each panel measures the fluorescence intensity of the free construct; the second (blue) shows the fluorescence intensity of that construct in the presence of gp41 and GTPγS; the third (green) shows the intensity in the presence of gp41, GTPγS, and gp61 (in all panels the green column corresponds to the presence of 6 : 1 subunit ratio of gp41 and gp61); and the fourth column (black, when present) corresponds to the intensity in the presence of gp41 and GTP. Numbers on the *x* axis list the corresponding construct names. (*B*–*G*) CD spectral changes for constructs containing 2-AP dimer probes in lagging strand, with or without primosome binding. Color coding is the same for all panels: red, DNA alone; green, DNA construct and primosome complex. (*B*) The ss construct; (*C*) {−2, −1} construct; (*D*) {−1, 1} construct; (*E*) {1, 2} construct; and (*F*) {5.6} construct.

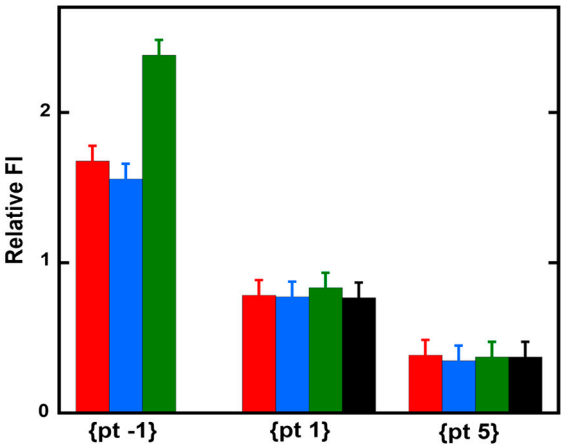

Fig. S4. Fluorescence changes for P/T DNA constructs (no dangling ssDNA sequence on the leading strand) with the lagging strand labeled with 2-AP monomer probes. Fluorescence intensity changes at 370 nm for P/T constructs containing 2-AP monomer probes on the lagging DNA strand, with or without helicase binding. Color coding is the same for all panels: The first bar (red) in each panel measures the fluorescence intensity of the free construct; the second (blue) shows the fluorescence intensity of that construct in the presence of gp41 and GTPγS; the third (green) shows the intensity in the presence of gp41, GTPγS, and gp61 (in all panels the green column corresponds to a 6:1 subunit ratio of gp41 and gp61); and the fourth column (black, when present) corresponds to the intensity in the presence of gp41 and GTP. Numbers along the *x* axis designate the corresponding DNA constructs (see Table S2).

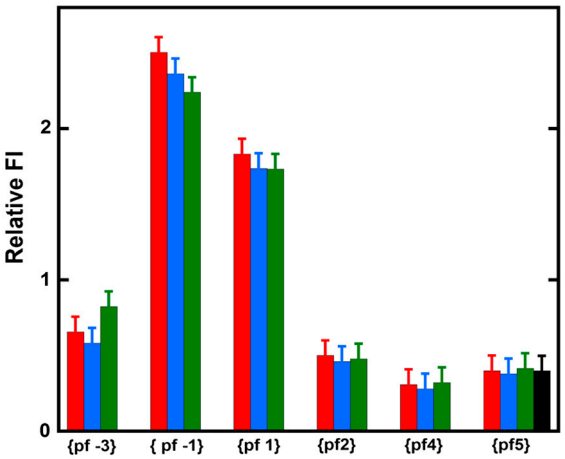

Fig. S5. Fluorescence changes observed for forked DNA constructs labeled with 2-AP monomer probes in the lagging strand (5-nt flanking ssDNA sequence on leading strand). Fluorescence intensity changes at 370 nm for fork constructs, in the presence or absence of helicase, with 2-AP monomer probes inserted in the lagging strand. Color coding for all panels is the same as in Fig. S4.

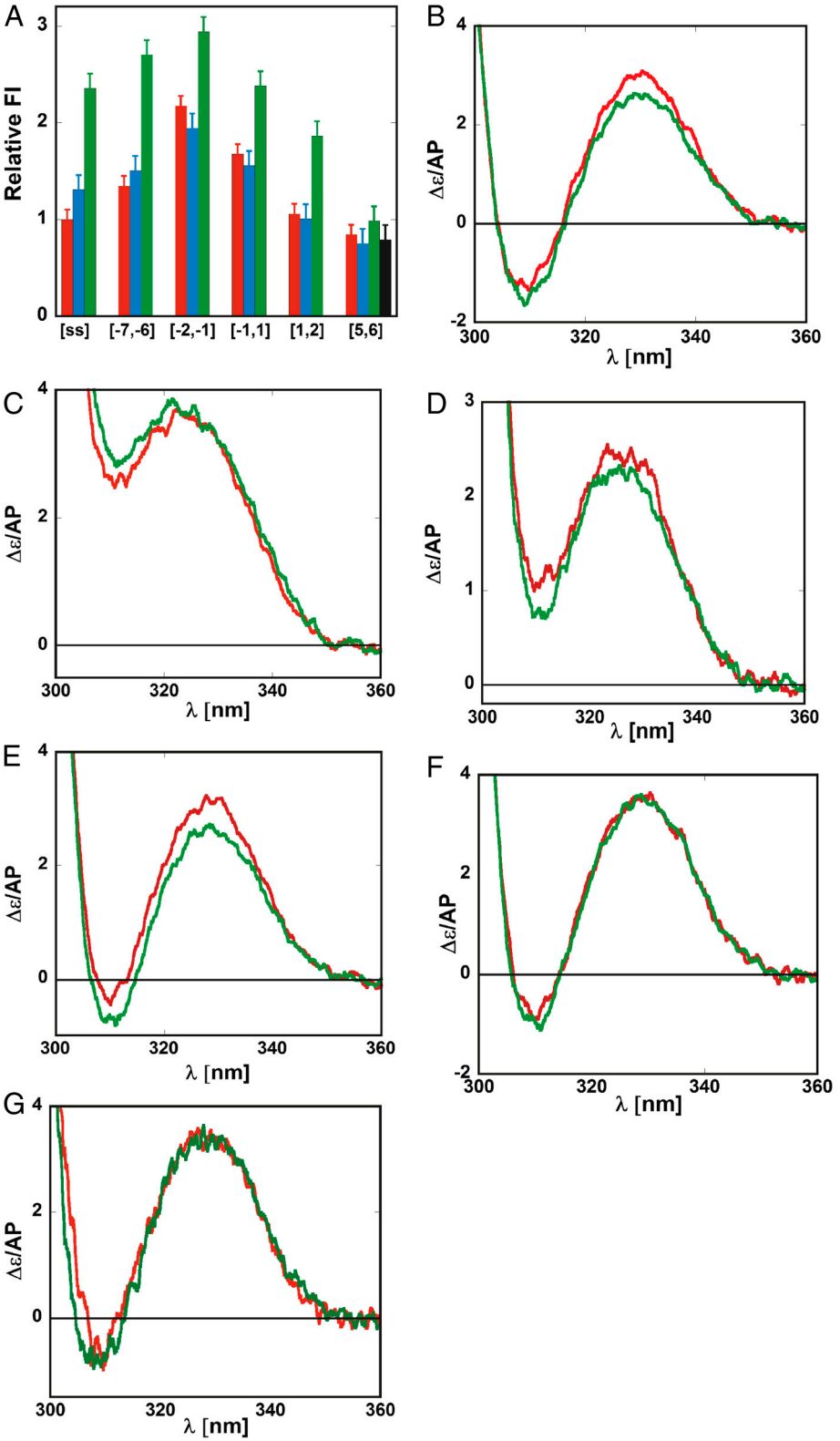

Fig. S6. Fluorescence and CD changes observed for forked DNA constructs with 2-AP dimer probes in the leading strand. The fluorescence intensity changes following the interaction of the T4 helicases (either gp41 hexamer alone or the gp41–gp61 primosome) for various DNA constructs labeled with 2-AP dimer probes in the leading strand. Each panel shows bar graphs monitoring the interaction of an individual construct (identified in Table S1) with various helicase forms. (*A*) The first bar (red) in each set of columns shows the fluorescence intensity of the free construct; the second (blue) shows the fluorescence intensity of that construct in the presence of gp41 and GTPγS; the third (green) shows the intensity in the presence of a GTPγS-locked T4 primosome with a 6:1 gp41:gp61 subunit ratio; and the fourth column (black, when present) corresponds to the intensity in the presence of gp41 and GTP. The numbers along the *x* axis identify the DNA constructs used in each experiment. (*B*–*G*) CD spectral changes for constructs containing 2-AP dimer probes in the leading strand, with or without primosome binding. Color coding is the same for all panels: red, DNA alone; green, DNA construct and primosome complex. (*B*) The ss construct; (*C*) [−2, −1] construct; (*D*) [−1, 1] construct; (*E*) [1,2] construct; (*F*) [3,4] construct; and (*G*) [5,6] construct.

Table S1. Nomenclature and sequences for DNA constructs used in this study (forked DNA constructs with 2-AP monomer or dimer probes incorporated into lagging and leading DNA strands)

Lagging (5′ → 3′) DNA strand

Construct name	DNA sequence
SS 2-AP monomer	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCATATAXTCGCTCGCATATTATGACTG
SS 2-AP dimer	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCATATXXTCGCTCGCATATTATGACTG
{-1},{-2,-1}	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCATATXX**TCGCTCGCATATTATGACTG**
	||||||||||||||||||||
	3′CATTCGTGCACTCACTTTCACCATTTTCGTAGCCTAA**AGCGAGCGTATAATACTGAC**
{1},{−1,1}	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCATATXX**TCGCTCGCATATTATGACTG**
	||||||||||||||||||||
	3′CATTCGTGCACTCACTTTCACCATTTTCGTAGCCTA**TAGCGAGCGTATAATACTGAC**
{2},{1,2}	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCATATXX**TCGCTCGCATATTATGACTG**
	||||||||||||||||||||
	3′CATTCGTGCACTCACTTTCACCATTTTCGTAGCCT**TTAGCGAGCGTATAATACTGAC**
{3},{2,3}	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCATAT**XXTCGCTCGCATATTATGACTG**
	||||||||||||||||||||
	3′CATTCGTGCACTCACTTTCACCATTTTCGTAGCC**ATTAGCGAGCGTATAATACTGAC**
{4},{3,4}	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCAT**ATXXTCGCTCGCATATTATGACTG**
	||||||||||||||||||||
	3′CATTCGTGCACTCACTTTCACCATTTTCGTAGC**TATTAGCGAGCGTATAATACTGAC**
{6},{5,6}	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACC**ATATXXTCGCTCGCATATTATGACTG**
	||||||||||||||||||||
	3′CATTCGTGCACTCACTTTCACCATTTTCGTA**TATATTAGCGAGCGTATAATACTGAC**

Leading (3′ → 5′) DNA strand

Construct name	DNA sequence
SS 2-AP monomer	3′CATTCTGCACTCACTTTCACACTTTTCACCATATAXTCGCTGCGTATAATACTGAC
SS 2-AP dimer	3′CATTCTGCACTCACTTTCACACTTTTCACCATATXXTCGCTGCGTATAATACTGAC
[−6],[−7,−6]	3′CATTCTGCACTCACTTTCACACTTTTCACCATATXXTCGCT**GCGTATAATACTGAC**
	||||||||||||||||||
	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCGTAGCGTAATCGCT**CGCATATTATGACTG**
[−1],[−2,−1]	3′CATTCTGCACTCACTTTCACACTTTTCACCATATXX**TCGCTGCGTATAATACTGAC**
	||||||||||||||||||||
	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCGTAGCGTAA**AGCGACGCATATTATGACTG**
[1],[−1,1]	3′CATTCTGCACTCACTTTCACACTTTTCACCATATXX**TCGCTGCGTATAATACTGAC**
	||||||||||||||||||||
	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCGTAGCGTA**TAGCGACGCATATTATGACTG**
[2],[1,2]	3′CATTCTGCACTCACTTTCACACTTTTCACCATATXX**TCGCTGCGTATAATACTGAC**
	||||||||||||||||||||
	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCGTAGCGT**TTAGCGACGCATATTATGACTG**
[4],[3,4]	3′CATTCTGCACTCACTTTCACACTTTTCACCATATXX**TCGCTGCGTATAATACTGAC**
	||||||||||||||||||||
	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCGTAGC**TATTAGCGACGCATATTATGACTG**
[6],[5,6]	3′CATTCTGCACTCACTTTCACACTTTTCACC**ATATXXTCGCTGCGTATAATACTGAC**
	||||||||||||||||||||
	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCGTA**TATATTAGCGACGCATATTATGACTG**

Complementary sequences are shown in boldfaced letters. X (red) marks the positions of the 2-AP probes. Negative numbers designate residue positions in ssDNA regions, and positive numbers correspond to dsDNA base-pair positions. Thus, the first duplex position at the fork junction is labeled 1, the first unpaired position is −1, and X represents the positions of 2-AP probes in the constructs. Constructs designated by single numbers (e.g., "{4}") define positions that contain a single 2-AP probe. Constructs designated by double numbers (e.g., "{−5, −4}") define the positions of two adjacent 2-AP residue probes (both are listed but only the dimer probe–containing constructs of each type are illustrated). Constructs containing 2-AP probes in the lagging strand are shown in curly brackets ({}) and those with probes in the leading strand in square brackets ([]).

Table S2. Nomenclature and sequences of constructs used in helicase activity assay and pfN and P/T constructs with 2-AP monomer labeled on the lagging strand

Helicase activity assay

Construct	DNA sequence
Unlabeled	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACC**ATATAATCGCTCGCATATTATGACTG** |||||||||||||||||||||||||| 3′CATTCGTGCACTCACTTTCACCATTTTCGTAG**TATATTAGCGAGCGTATAATACTGAC**
2-AP monomer labeled	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACC**ATATAXTCGCTCGCATATTATGACTG** |||||||||||||||||||||||||| 3′CATTCGTGCACTCACTTTCACCATTTTCGTAG**TATATTAGCGAGCGTATAATACTGAC**
2-AP dimer labeled	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACC**ATATXXTCGCTCGCATATTATGACTG** |||||||||||||||||||||||||| 3′CATTCGTGCACTCACTTTCACCATTTTCGTAG**TATATTAGCGAGCGTATAATACTGAC**

pfN with 2-AP monomer labeled on the lagging strand

Construct	DNA sequence
ss	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCATATAXTCGCTCGCATATTATGAG
{pf-3}	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCATATAXTC**GCTCGCATATTA**TGAG |||||||||||| 3′ GTAATC**CGAGCGTATAAT**
{pf-1}	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCATATAX**TCGCTCGCATATTA**TGAG |||||||||||||| 3′ CGTAA**AGCGAGCGTATAAT**
{pf1}	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCATATA**XTCGCTCGCATATT**ATGAG ||||||||||||||| 3′ GCGTA**TAGCGAGCGTATAA**
{pf2}	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCATATA**XTCGCTCGCATAT**TATGAG ||||||||||||||| 3′ TGCGT**TTAGCGAGCGTATA**
{pf4}	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCAT**ATAXTCGCTCGCAT**ATTATGAG ||||||||||||||| 3′ ACTGC**TATTAGCGAGCGTA**
{pf5}	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCA**TATAXTCGCTCGCA**TATTATGAG ||||||||||||||| 3′ CACTG**ATATTAGCGAGCGT**

P/T constructs {ptN} with 2-AP monomer labeled on the lagging strand

Construct	DNA sequence
ss	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCATATAXTCGCTCGCATATTATGAG
{pt-1}	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCATATAX**TCGCTCGCATATTA**TGAG |||||||||||||| 3′ **AGCGAGCGTATAAT**
{pt1}	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCATATA**XTCGCTCGCATATTA**TGAG ||||||||||||||| 3′ **TAGCGAGCGTATAAT**
{pt5}	5′TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACCA**TATAXTCGCTCGCAT**ATTATGAG ||||||||||||||| 3′ **ATATTAGCGAGCGT**

Base pairs are shown in bold letters and connected by a vertical line. X (red) represents the position of 2-AP. N represents the probe position(s) in the ss- or dsDNA regions of the construct. See scheme in the main text and Table S1 for further details of nomenclature conventions.