In the bacteriophage T4 homologous recombination system, presynaptic filament assembly on single-stranded (ssDNA) DNA requires UvsX recombinase, UvsY mediator, and Gp32 ssDNA-binding proteins. Gp32 exerts both positive and negative effects on filament assembly: positive by denaturing ssDNA secondary structure, and negative by competing with UvsX for ssDNA binding sites. UvsY is believed to help UvsX displace Gp32 from the ssDNA. To test this model we developed a real-time fluorescence assay for Gp32-ssDNA interactions during presynapsis, based on changes in the fluorescence of a 6-iodoacetamidofluorescein-Gp32 conjugate. Results demonstrate that the formation of UvsX presynaptic filaments progressively disrupts Gp32-ssDNA interactions. Under stringent salt conditions the disruption of Gp32-ssDNA by UvsX is both ATP- and UvsY-dependent. The displacement of Gp32 from ssDNA during presynapsis requires ATP binding, but not ATP hydrolysis, by UvsX protein. Likewise, UvsY-mediated presynapsis strongly requires UvsY-ssDNA interactions, and is optimal at a 1:1 stoichiometry of UvsY to UvsX and/or ssDNA binding sites. Presynaptic filaments formed in the presence of UvsY undergo assembly/collapse that is tightly coupled to the ATP hydrolytic cycle and to stringent competition for ssDNA binding sites between Gp32 and various nucleotide-ligated forms of UvsX. The data directly support the Gp32 displacement model of UvsY-mediated presynaptic filament assembly, and demonstrate that the transient induction of high affinity UvsX-ssDNA interactions by ATP are essential, although not sufficient, for Gp32 displacement. The underlying dynamics of protein-ssDNA interactions within presynaptic filaments suggests that rearrangements of UvsX, UvsY, and Gp32 proteins on ssDNA may be coupled to central processes in T4 recombination metabolism.

DNA strand exchange reactions catalyzed by recombinases of the highly conserved RecA family are central to the processes of homologous recombination and DNA double strand break repair. RecA recombinases are enzymatically activated by assembling into presynaptic filaments on single-stranded DNA (ssDNA) (1). Timely and efficient presynaptic filament assembly is promoted by ssDNA-binding proteins (SSBs) and by recombination mediator proteins, which are functionally conserved among diverse species (2). A general model of presynaptic filament assembly has emerged in which SSB sequesters and removes secondary structure from ssDNA, after which a recombination mediator protein mediates loading of recombinase and concomitant displacement of SSB from ssDNA (1–5).

Studies of bacteriophage T4 recombination proteins UvsX (recombinase, 44 kDa), UvsY (recombination mediator protein, 16 kDa), and Gp32 (SSB, 34 kDa) have provided important insights on the biochemical mechanism of presynaptic filament assembly (6–11). UvsX, the RecA ortholog of T4 phage, exhibits both ssDNA-dependent ATPase and DNA strand exchange activities. UvsY, the prototype recombination mediator protein, stimulates enzymatic activities of UvsX by promoting assembly onto ssDNA of the latter protein. Gp32, the prototype SSB, has been characterized extensively (12–14). The primary structure of Gp32 (301 residues, 34 kDa) contains three distinct regions including the N-terminal “basic” (B-) domain (residues 1–22), the ssDNA-binding “core” domain (residues 22–253), and the C-terminal “acidic” (A-) domain (residues 254–301) (15). The x-ray structure of the core domain was determined upon co-crystallization with oligo(dt)$_n$ (14). This domain contains an oligonucleotide/oligosaccharide binding-fold, and its structure is stabilized by a Zn$^{2+}$ atom coordinated to residues His-64, Cys-77, Cys-87, and Cys-90. Molecular studies reveal that the B-domain is the specific region responsible for Gp32 homotypic associations and for the cooperativity of Gp32-ssDNA interactions (16, 17), whereas the A-domain is important for the heterotypic associations of Gp32 with other T4 DNA replication and recombination factors, including UvsX, UvsY, Gp59 (helicase loading/replication mediator protein), Dda (DNA helicase), Gp43 (DNA polymerase), Gp61 (primase), and others (18–21). Equilibrium and kinetic parameters of Gp32-ssDNA interactions, including binding site size $n$, intrinsic binding constant $K$, and cooperativity parameter $\omega$, have been determined for a variety of polynucleotide lattices (22). Gp32-ssDNA interactions are characterized by high cooperat...
Gp32 plays important pre- and post-synaptic roles in DNA strand exchange reactions catalyzed by the UvsX protein in concert with UvsY (6, 8–11, 24). During presynaptic filament formation, Gp32 denatures ssDNA secondary structure and provides an optimum lattice conformation for assembly of UvsX-ssDNA filaments. The net effect of Gp32 on UvsX-ssDNA filament formation is inhibitory, however, because the high affinity of Gp32-ssDNA interactions allows Gp32 to effectively compete with UvsX for binding sites on the ssDNA (25). Proper assembly of presynaptic filaments therefore requires the mediator function of UvsY protein in addition to UvsX and Gp32 (2, 3). UvsX exhibits a strict requirement for UvsY in strand exchange assays performed at salt concentrations approximating physiological ionic strength (11, 26, 27), consistent with the co-dependence of in vivo recombination processes on UvsX and UvsY (28–30). In the current model of T4 presynaptic filament assembly, UvsY weakens Gp32-ssDNA interactions and strengthens UvsX-ssDNA interactions, allowing UvsX filaments to nucleate onto pre-existing Gp32-ssDNA complexes, and ultimately to displace Gp32 from the lattice (27, 31–33). After transiently participating in presynapsis, Gp32 further stimulates DNA strand exchange by binding to the displaced single strand generated during UvsX-catalyzed D-loop formation (24).

The ultimate disruption of Gp32-ssDNA interactions during presynaptic filament assembly has been inferred from protein cross-linking and other data (31), but has never been observed directly. Understanding the dynamics of Gp32 during recombination and other DNA transactions requires a probe to selectively report on Gp32-ssDNA interactions in the presence of other DNA-binding proteins. Here we describe such a probe in the form of a covalent conjugate between Gp32 and 6-iodoacetamidofluorescein (Gp32F) (60, 61). Changes in the fluorescence signal of Gp32F allow for the detection and quantification of Gp32-ssDNA interactions in real time and in the presence of other proteins. Monitoring Gp32F fluorescence allows direct measurements of Gp32 displacement from the ssDNA during UvsX-ssDNA filament formation, and demonstrates the salt, nucleotide binding, and UvsY dependence of Gp32 displacement, in confirmation of the current model of T4 presynapsis. Results indicate that presynaptic filaments formed in the presence of Gp32 undergo assembly and collapse that is tightly linked to the ATP hydrolytic cycle, suggesting that in addition to the stabilizing effects of UvsY, ATP binding by UvsX is necessary for maximum occupancy of the ssDNA lattice by UvsX in the presence of Gp32. Competition for ssDNA binding sites between Gp32 and various nucleotide-ligated forms of UvsX may create opportunities for dynamic instability in presynaptic filaments, which could play important roles in T4 recombination metabolism.

**Experimental Procedures**

**Chemicals and Buffers**—All chemicals were analytical grade. 6-Iodoacetamidofluorescein (6-IAF) was purchased from Molecular Probes. Other chemicals and biochemicals were purchased from Sigma, unless specifically noted. Aqueous buffers and solutions were made with de-ionized, glass-distilled water. Storage buffers for Gp32, UvsX, and UvsY proteins were as previously described (25, 32, 34). Buffer A contained 20 mM Tris-HCl, pH 7.1, 150 mM NaCl, and 10% (w/v) glycerol. Buffer B contained 20 mM Tris-HCl, pH 7.4, 1 mM MgCl2, and the indicated concentration of NaCl. TE buffer contained 10 mM Tris-HCl, pH 8.1, and 1 mM EDTA.

**Proteins and Nucleic Acids**—Recombinant T4 Gp32, UvsX, wild-type UvsY, and two UvsY mutant proteins (UvsYK58A and UvsYK58A,R60A) were purified and stored as previously described (27, 34–36). All proteins were determined to be >98% pure based on SDS-polyacrylamide gels stained with Coomassie Blue. Furthermore, all protein stock solutions were tested and judged to be nuclease-free by previously published criteria (37). Extinction coefficients for Gp32, UvsX, and UvsY sp. at 280 nm were 41,306, 69,760, and 19,180 M–1 cm–1, respectively (38). Circular single-stranded DNA was extracted from purified bacteriophage M13mp18 particles as described (39, 40), and stored at –20 °C in TE buffer. The concentrations of ssDNA stock solutions were determined by the absorbance at 260 nm, using a conversion factor of 33 μg/ml/A260, and are expressed in units of micromoles of nucleotide residues per liter.

**Labeling of Gp32 with 6-IAF**—All steps were carried out in the dark and at 4 °C. 2 mg of Gp32 protein were dialyzed overnight against 1 liter of Buffer A. Meanwhile a 10 mM stock of 6-IAF in Me2SO was prepared. A 3-fold molar excess of 6-IAF was added to the dialyzed Gp32 via drop by drop additions with gentle mixing. The solution was incubated overnight, after which 100 mM 2-mercaptoethanol was added to consume excess 6-IAF. After 1 h of further incubation, the mixture was dialyzed overnight against 3 x 1 liters of Gp32 storage buffer. SDS-PAGE of the resulting fluorescein-Gp32 conjugate (Gp32F) revealed a single yellow band under visible light prior to Coomassie Blue staining, which upon staining appeared to run at approximately the same molecular weight as unmodified Gp32. The labeling stoichiometry of Gp32F was calculated from the absorbance at 492 and 280 nm, respectively, according to manufacturer’s instructions.

**ATPase Assays**—The ssDNA-dependent ATPase activity of UvsX protein in the presence and absence of Gp32 and Gp32F proteins was measured by a modification of the coupled spectrophotometric assay described (37). All reactions contained 20 mM Tris acetate, pH 7.4, 10 mM magnesium acetate, 90 mM potassium acetate, 2 mM ATP, 6 units/ml pyruvate kinase, 6 units/ml lactate dehydrogenase, 2.3 mM phosphoenolpyruvate, 0.23 mM NADH, 4.5 mM M13mp18 ssDNA, 0.5 mM UvsX, and 0–1.4 μM of either Gp32 or Gp32F. Reactions at 37 °C were started by the addition of UvsX protein to a preincubated mixture of the other components. Reaction velocities were calculated from the change in absorbance at 380 nm using the linear portions of time courses.

ATP concentrations present at different times during fluorescence time course experiments were determined using the ATP determination kit from Molecular Probes. Ten-microliter aliquots were removed from reaction mixtures in fluorescence...
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cuvettes at different times and immediately quenched with 10 mM EDTA. Quenched samples then were boiled for 5 min before analysis. Luminescence was read on a FLX-800 Plate Reader (Sunnyvale, CA) in 96-well assay microplates. Values were calculated based on an ATP standard curve. All ATP concentrations were determined in triplicate. The generation of ADP:AMP produced by UvsX protein (6).\(^4\)

ADP concentrations were determined in triplicate. The generation of ADP was calculated based on an ATP standard curve. All ATP concentrations were determined in triplicate. The generation of ADP:AMP produced by UvsX protein (6).\(^4\)

Fluorescence Measurements—All fluorescence data were collected on a Quantamaster QM-4 fluorometer (Photon Technology International, South Brunswick, NJ). This fluorometer was equipped with a 75-watt xenon arc lamp as an excitation source, and excitation/emission monochrometers to measure steady-state fluorescence. All data were collected in the steady-state mode and corrected for effects of dilution, solution change, photobleaching, inner filter effects, and intrinsic protein fluorescence as described (25, 34, 41, 42). The slit widths for excitation and emission were 1 and 5 nm, respectively. The excitation wavelength was 460 nm. A long pass cutoff glass filter at 495 nm was used for all of the emission data collection. Data were corrected for magnesium ion effects using Equations 3 and 4 of Record et al. (47), assuming \(\psi = 0.71\), where \(\psi\) is the fraction of counterions thermodynamically bound to ssDNA, and assuming \(m' = 3\), where \(m'\) is the number of charge interactions formed between Gp32 and ssDNA (22, 46, 47). Ionic effects on Gp32F-ssDNA interactions were estimated according to Equation 2,

\[
d\log K_{\text{Mg-corr}}/d\log [\text{NaCl}] = -m'\psi - a
d\text{Eq. 2}
\]

where \(K_{\text{Mg-corr}}\) is the Mg\(^{2+}\)-corrected association constant, and \(a\) is the number of anions displaced from the protein upon nucleic acid binding (22, 46, 47).

Gp32F-ssDNA Disruption Assays—Competition titrations of pre-formed Gp32F-ssDNA with UvsX protein were performed under steady-state conditions at 25 °C in Buffer B containing 200 mM NaCl, 1 mM ATP, ATP-regenerating system (6 units/ml pyruvate kinase + 2.3 mM phosphoenolpyruvate), 3.5 μM M13mp18 ssDNA, 3.5 μM Gp32F, variable UvsX, and 0 or 1 μM UvsY depending on experiment. Control titrations lacking ssDNA or UvsY contained a volume of TE or UvsY storage buffer, respectively, equal to the volume of the missing component.

Time Resolved ssDNA Binding Competition Assays—The competition between Gp32F and UvsX proteins for a limiting number of ssDNA binding sites was measured as a function of time by adding M13mp18 ssDNA (3.5 μM) to a solution containing both proteins (0.5 μM Gp32F, 0.875 μM UvsX) and monitoring subsequent changes in fluorescein fluorescence. The ssDNA concentration used is stoichiometric with respect to Gp32F alone (binding site size, \(n = 7\) nucleotide residues) or to UvsX alone (\(n = 4\)) but cannot saturate both proteins simultaneously (22, 25). Reactions were carried out at 25 °C in Buffer B containing 200 mM NaCl. To compare the effects of different nucleotides on Gp32F/UvsX competition, some reaction mixtures contained 1 mM AMP, 1 mM ADP, 1 mM ATP, or 1 mM ATP (with or without ATP regenerating system consisting of 2.3 mM phosphoenolpyruvate, and 6 units/ml pyruvate kinase). To study UvsY effects, some reactions contained variable amounts of wild-type UvsY protein (0–0.875 μM), or UvsY\(_{\text{K58A,R60A}}\) (0.875 μM) or UvsY\(_{\text{K58A,R60A}}\) (0.875 μM) mutant proteins. UvsY proteins, when present, were incubated along with Gp32F and UvsX in a buffer/nucleotide mixture prior to the addition of ssDNA. In all reactions, a baseline fluorescence signal was obtained by collecting a 100-s time course (\(\lambda_{\text{ex}} = 460 \text{ nm}, \lambda_{\text{em}} = 519 \text{ nm}\)) prior to ssDNA addition. ssDNA was added with rapid mixing, signal was re-acquired, and a 300–700-s time course (depending on experiment) was collected. For reaction series containing variable protein components, solvent composition was kept constant by adding appropriate amounts of protein storage buffers.

\(^4\) J. Farb and S. Morrical, unpublished results.
RESULTS

Labeling of Gp32 with Fluorescein—Results demonstrate that Gp32 can be site-specifically labeled with fluorescein, and that the resulting conjugate (Gp32F) inhibits UvsX protein activity to a similar degree as unmodified Gp32. Gp32F was produced by treating purified Gp32 protein with the thiol-reactive reagent 6-IAF (6-iodoacetamidofluorescein) as described under “Experimental Procedures.” The labeling stoichiometry of 6-IAF to Gp32 was equal to 1 as determined spectrophotometrically, indicating that only one of the four cysteine residues in Gp32 was covalently modified by 6-IAF. Mass spectrometry indicates that this labeling occurs specifically on Cys-166 (data not shown), consistent with its surface location and with the involvement of the other three cysteine residues in the coordination of Zn$^{2+}$ in the DNA binding core domain of Gp32 (14). Other studies have also reported chemical labeling and/or cross-linking of Gp32 specifically at Cys-166 (36). To test the functional proficiency of Gp32F, its ability to inhibit the ssDNA-dependent ATPase activity of T4 UvsX recombinase was examined. Native Gp32, when present in excess over ssDNA, inhibits this reaction by competing with UvsX for binding sites on the lattice (26, 48). The data in Table 1 demonstrate that excess Gp32F inhibits UvsX-catalyzed ssDNA-dependent ATPase activity to a similar degree as excess native Gp32, suggesting that Gp32F retains ssDNA binding activity with sufficient affinity to compete with UvsX. The ssDNA binding activity of Gp32F is explored in greater detail below. Other results indicate that Gp32F retains the ability to stimulate UvsX/UvsY-dependent DNA strand exchange reactions as well as DNA synthesis reactions catalyzed by T4 DNA polymerase holoenzyme.

ssDNA-induced Changes in Gp32F Fluorescence—Results demonstrate that Gp32F fluorescence is enhanced upon binding to ssDNA, but is not affected by UvsX and UvsY proteins. Fluorescence emission spectra of Gp32F were measured at 200 mM NaCl in the presence/absence of ssDNA, UvsX, or UvsY (Fig. 1). Excited at 460 nm, Gp32F reaches its peak emission at 518 nm in the absence of ssDNA. The addition of M13mp18 ssDNA to the solution causes a ~2-fold enhancement in the intensity of Gp32F fluorescence, with the emission peak slightly red-shifted to 519 nm (Fig. 1). Under identical solution conditions, the addition of double-stranded DNA has no effect on Gp32F fluorescence (data not shown). Therefore Gp32F retains preferential ssDNA binding activity, and the binding reaction alters the environment of the conjugated fluorescein molecule in a way that favors enhanced fluorescence.

The addition of a 2-fold molar excess of UvsY protein had little effect on the fluorescence emission spectra of Gp32F, either in the absence or presence of ssDNA (Fig. 1). Similarly, the addition of a 2-fold molar excess of UvsX protein to Gp32F in the presence of 1 mM ATP had little effect on Gp32F fluorescence, either in the absence or presence of ssDNA (Fig. 1). The salt conditions employed (200 mM NaCl) allow strong interactions of UvsY with Gp32, ssDNA, or Gp32-ssDNA complexes, and weak interactions of UvsX with Gp32 or ssDNA (26, 32, 34, 48). Therefore Gp32F fluorescence is sensitive to ssDNA binding but insensitive to interactions with other recombination proteins, making it an ideal indicator of Gp32-ssDNA interactions during presynaptic filament assembly. The data in Fig. 1 also demonstrate that UvsY and UvsX individually cannot displace Gp32F from ssDNA, because the enhanced fluorescence signal of the Gp32F-ssDNA complex is completely retained in the presence of either recombination protein.

Gp32F Binds to ssDNA with High Affinity and Cooperativity—The ssDNA-induced enhancement of Gp32F fluorescence was used to measure the ssDNA binding parameters of Gp32F, for purposes of comparison with unmodified Gp32. Salt-back titrations (data not shown) demonstrated that Gp32F forms tight, stoichiometric complexes with ssDNA at NaCl concentrations ≥200 mM, whereas higher NaCl concentrations destabilize Gp32F-ssDNA interactions with a dissociation midpoint at ~350 mM NaCl. Reverse titrations of Gp32F with ssDNA (data not shown) under tight binding conditions (50 mM NaCl) demonstrated that Gp32F has a binding site size of 7.6 ± 0.2 nucleotide residues, which is similar to values reported previously for unmodified Gp32 (22). This finding suggests that Gp32F and

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**TABLE 1**

Inhibition of UvsX-catalyzed ssDNA-dependent ATP hydrolysis by Gp32 and Gp32F proteins

All reaction mixtures contained 2 mM ATP, 4.5 μM (nucleotides) M13mp18 ssDNA, and 0.5 μM UvsX protein, in the coupled assay system as described under “Experimental Procedures.” The concentration of Gp32 or Gp32F protein, when present, was 1.4 μM. Gp32 and Gp32F, respectively, were added into the reactions by preincubating with ssDNA prior to the addition of UvsX protein. Data represent averages from triplicate assays.

|                      | UvsX only | UvsX + Gp32 | UvsX + Gp32F |
|----------------------|-----------|-------------|--------------|
| ATP hydrolysis rate  | 5.1 ± 0.4 | 1.7 ± 0.2   | 1.9 ± 0.1    |
| Percentage of UvsX-only rate | 100 | 33 ± 4 | 37 ± 2 |
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FIGURE 2. Forward titrations of ssDNA with Gp32F protein, monitored via the change in Gp32F fluorescence at 519 nm. Theta denotes fractional saturation of the ssDNA lattice with protein. The starting concentration of M13mp18 ssDNA was 3.5 μM. Buffer B contained 260 (filled circles), 300 (open circles), or 340 mM (filled triangles) NaCl as indicated. Other experimental conditions were as described under “Experimental Procedures.” Lines represent theoretical isotherms calculated as described under “Experimental Procedures,” with n (binding site size), K (intrinsic affinity), and ω (cooperativity) parameters as follows: at 260 mM NaCl, n = 7 nucleotide residues/monomer, K = 8.3 × 10^3 M⁻¹, ω = 1000. At 300 mM NaCl, n = 7 nucleotide residues/monomer, K = 5.6 × 10³ M⁻¹, ω = 2000. At 340 mM NaCl, n = 7 nucleotide residues/monomer, K = 2.0 × 10³ M⁻¹, ω = 2000.

Gp32 are equally effective in eliminating ssDNA secondary structure, allowing both to saturate random sequence ssDNA lattices.

The intrinsic binding constant K and cooperativity parameter ω for Gp32F-ssDNA interactions were estimated by performing forward titrations of a fixed amount of M13mp18 lattice with protein under salt conditions that permit relatively weak, non-stoichiometric binding (260, 300, or 340 mM NaCl) (Fig. 2). ssDNA binding parameters were calculated as described under “Experimental Procedures” and are listed in Table 2. All titration curves revealed obvious sigmoidicity, indicative of highly cooperative binding of Gp32F to ssDNA. The data show that the intrinsic affinity K_Mg-corr of Gp32F is 12-fold lower than that of unmodified Gp32. Other aspects of ssDNA binding, including binding site size, cooperativity, and salt dependence of K_Mg-corr, are highly similar between Gp32F and Gp32. The high extrapolated K_Mg-corr values of Gp32F and Gp32 are consistent with the experimentally observed stoichiometric binding of both proteins to ssDNA in 200 mM NaCl. The moderately diminished affinity of Gp32F for ssDNA, compared with unmodified Gp32, is confirmed by the observation that a slight excess of Gp32 can slowly but completely displace Gp32F from ssDNA in 200 mM NaCl.

Disruption of Pre-formed Gp32F-ssDNA Complexes by UvsX + UvsY Proteins—Results demonstrate that UvsX protein displaces Gp32F from ssDNA in a UvsY-dependent manner under stringent salt conditions. In the current model of T4 homologous recombination, presynaptic filament formation involves the displacement of Gp32 protein from ssDNA by the UvsX recombinase, a thermodynamically unfavorable reaction under conditions approximating physiological salt and protein concentrations (25). The UvsY recombinase mediator protein is thought to facilitate this displacement reaction (2). The enhancement of Gp32F fluorescence by ssDNA provides a sensitive and direct means for monitoring the competitive binding of UvsX and Gp32 proteins to the lattice, and the effects of UvsY and nucleotides on this competition. In vivo, T4 homologous recombination events are equally dependent on functional UvsX and UvsY (28, 29, 49, 50). This co-dependence is recreated in vitro by performing UvsX-catalyzed ssDNA-dependent ATPase and DNA strand exchange reactions at moderately high salt concentrations, wherein the reactions are strictly UvsY-dependent in the presence of Gp32 (11, 26, 27). For this reason all Gp32F-ssDNA disruption studies were performed at the moderately high salt concentration of 200 mM NaCl, wherein UvsY mediator effects are maximized. As shown in Fig. 3, titration of a pre-assembled, saturated Gp32F-ssDNA complex with UvsX protein in the presence of UvsY and ATP (+ ATP regenerating system) results in a progressive decrease in Gp32F fluorescence. The UvsY concentration used (1 μM) was in slight excess with respect to Gp32 and potential ssDNA binding sites. The fluorescence decrease is dependent on ssDNA, because a control titration lacking ssDNA starts at a low level of fluorescence and remains essentially unchanged throughout (Fig. 3). The fluorescence decrease is also dependent on UvsY, because a control titration lacking UvsY the Gp32F fluorescence signal remained at its high, ssDNA-enhanced level at all concentrations of UvsX.
that were tested (Fig. 3). Therefore the data in Fig. 3 are consistent with the UvsY-mediated displacement of Gp32F from ssDNA by the UvsX recombinase. The slope of the titration curve decreases and begins to approach a “plateau” in the vicinity of 0.7–0.8 μM UvsX, which is slightly subsaturating with respect to potential ssDNA binding sites. Correspondingly the plateau fluorescence signal attained in the titration is higher than the signal from control reactions lacking M13mp18 ssDNA, 1.0 μM UvsY in Buffer B (+ 200 mM NaCl, 1 mM ATP, and ATP regenerating system. Open squares, same as complete but lacking UvsY protein. Open triangles, same as complete but lacking ssDNA. Solutions were titrated with UvsX, then an excess amount of ssDNA (14 μM) was added to sequester all free protein. Other experimental conditions were as described under “Experimental Procedures.”

Kinetic Competition of UvsX and Gp32F for ssDNA Binding Sites—Results demonstrate that Gp32F rapidly covers ssDNA before being slowly displaced by UvsX in the presence of UvsY. Changes in Gp32F fluorescence allow real-time monitoring of presynaptic filament assembly and collapse by following the dynamics of Gp32F-ssDNA interactions. We developed a simple kinetic assay to measure the competition between UvsX and Gp32F for ssDNA binding sites under pre-steady-state conditions for filament assembly. These two proteins, with or without UvsY depending on the experiment, were preincubated and a baseline fluorescence signal was acquired. Then a limiting amount of M13mp18 ssDNA was added, and the signal was re-acquired immediately and monitored for several minutes. The amount of ssDNA added contained a stoichiometric quantity of potential binding sites for either UvsX or Gp32F, but not for both; therefore the proteins were forced to compete for binding sites. Higher or lower fluorescence signals therefore reflect successful occupation of binding sites by Gp32F or UvsX, respectively, with filament dynamics reflected in the time course.

In the absence of UvsY, the addition of limiting ssDNA to a Gp32F/UvsX mixture in 200 mM NaCl, 1 mM ATP (+ ATP regenerating system) causes a rapid jump in Gp32F fluorescence followed by a slower increase to a plateau signal reflective of a saturated Gp32F-ssDNA complex (Fig. 4). Therefore Gp32F wins the kinetic competition with UvsX for ssDNA binding sites under these conditions. Under otherwise identical conditions, inclusion of UvsY in the protein mixture affords a distinct curve upon ssDNA addition: an immediate fluorescence jump followed by an exponential fluorescence decay (Fig. 4). The initial, acute signal enhancement represents the rapid binding of Gp32F onto ssDNA, whereas the subsequent exponential decay curve represents the slower nucleation and/or propagation of UvsX filaments onto this Gp32F-covered ssDNA in the presence of UvsY, with concomitant displacement of Gp32F. The addition of excess ssDNA at the end of this experiment restored Gp32F fluorescence back to its high ssDNA-enhanced level (data not shown), suggesting that the Gp32F displaced during filament assembly is free in solution and competent to bind free lattice. Thus the pre-steady state kinetic competition data (Fig. 4) are consistent with the steady-state titration data in Fig. 3, both demonstrating that UvsY is required for UvsX to out-compete Gp32F for ssDNA in the presence of 200 mM NaCl and 1 mM ATP. The complete reaction in Fig. 4 reflects the sequential filament assembly process.
that is thought to occur in vivo, details of which will be presented under “Discussion.”

Nucleotides Modulate Presynaptic Filament Assembly and Collapse—Results demonstrate that the displacement of Gp32F from ssDNA by UvsX requires nucleoside triphosphate binding by the enzyme in addition to the mediation effects of UvsY. Results also demonstrate that presynaptic filaments are destabilized by ATP depletion, which allows Gp32F to reoccupy the ssDNA at the expense of UvsX. The effects of various nucleotides on UvsX/UvsY-dependent Gp32F-ssDNA disruption were tested in 200 mM NaCl, using kinetic competition assays similar to those described in Fig. 4. As shown in Fig. 5A, UvsX requires ATP to displace Gp32F from ssDNA, even in the presence of UvsY. The presence of ATP plus an ATP regenerating system leads to a typical competition assay time course wherein fluorescence initially jumps to a higher value as Gp32F binds to ssDNA, followed by an exponential decrease reflecting UvsX/UvsY-dependent displacement of Gp32F from the lattice (Fig. 5A). In contrast, when either ADP or AMP is substituted for ATP plus the regenerating system, fluorescence jumps to a high value but never decreases in the competition assay, even though UvsY is present (Fig. 6). ADP and AMP are the alternative products of UvsX-catalyzed ssDNA-dependent ATP hydrolysis (6). Likewise with no nucleotide present, fluorescence jumps to a high value but never decreases, even though UvsY is present (Fig. 6). Thus the substrate ATP, but not the products ADP or AMP, can induce a conformation of UvsX protein that, with the help of UvsY, displaces Gp32F from ssDNA under stringent salt conditions. The initial displacement of Gp32F appears to require ATP binding but not its hydrolysis by UvsX, because substituting the poorly hydrolyzed analog ATPγS for ATP plus regenerating system generates a qualitatively similar time course (Fig. 5A). UvsY is still required for optimal Gp32F displacement in reactions containing ATPγS.

In reactions containing UvsY and 1 mM ATP but lacking an ATP regenerating system, Gp32F displacement from ssDNA occurs with kinetics that appear identical to reactions containing a regenerating system, but the resulting filaments are unstable (Fig. 5A). The instability is revealed as a time-dependent increase in Gp32F fluorescence following the initial decrease, indicating that Gp32F eventually re-occupies the lattice at the expense of UvsX, even though UvsY is present. The re-establishment of Gp32F-ssDNA interactions correlates with the depletion of the ATP substrate of UvsX with time, as shown in

FIGURE 4. Gp32F/UvsX kinetic competition for ssDNA is affected by UvsY protein. Gp32F-ssDNA association/dissociation was monitored by changes in fluorescence at 519 nm and reactions were carried out in Buffer B plus 200 mM NaCl. Filled squares show the reaction containing 0.5 μM Gp32F and 0.875 μM UvsX preincubated with 1 mM ATP plus ATP regenerating system. Open circles show the reaction containing 0.5 μM Gp32F, 0.875 μM UvsX, and 0.875 μM UvsY preincubated with 1 mM ATP plus the ATP regenerating system. Proteins were added to buffer and a 100-s baseline fluorescence signal was recorded. Reactions were then started by the manual addition and mixing of a limiting amount (3.5 μM) of M13mp18 ssDNA, as indicated by the black arrow. Lines with symbols represent real fluorescence signals and perpendicular lines without symbols represent the interruption of data collection during ssDNA addition (dead-time ~ 3 s). Protein amounts are at 1:1 stoichiometry with respect to ssDNA binding sites assuming a binding site size of n = 7 nucleotide residues per monomer of Gp32F, and of n = 4 nucleotide residues per monomer of UvsX or UvsY.

FIGURE 5. UvsX/UvsY-dependent displacement of Gp32F from ssDNA requires ATP binding. Gp32F-ssDNA association/dissociation was monitored by changes in fluorescence at 519 nm. A, 0.5 μM Gp32F, 0.875 μM UvsX, and 0.875 μM UvsY were preincubated with 1 mM ATP-γS (asterisks), or 1 mM ATP in the presence (open circles) or absence (open squares) of an ATP regenerating system, in Buffer B plus 200 mM NaCl. 3.5 μM M13mp18 ssDNA was added at the time indicated by the black arrow. B, ATP consumption and product generation in the reaction containing 1 mM ATP and no regenerating system (open squares in panel A). ATP concentrations (solid line) were measured directly as described under “Experimental Procedures.” The production of ADP (long dashed line) and AMP (short dashed line) were calculated assuming a fixed ratio of 2:1 for ADP:AMP production.
UvsX increase hyperbolically. 0.875 binding sites), the rate and extent of Gp32F displacement by UvsX, or it could reflect a slower growing type of filament or acts only as a sub-stoichiometric nucleation factor. UvsY remains a stoichiometric component of the presynaptic filament assembly process, presumably by strengthening UvsX-ssDNA interactions. This idea supports previous reports that UvsYK58A protein-protein interactions can promote a limited filament assembly containing sub-stoichiometric amounts of UvsY. Further experimentation will be necessary to resolve these possibilities.

UvsY-ssDNA Interactions Are Required for Normal Presynaptic Filament Assembly—Two UvsY missense mutants, UvsYK58A and UvsYK58A,R60A, exhibit dramatically decreased ssDNA binding ability, whereas retaining proper self- and hetero-protein associations (27, 51). We tested the abilities of these mutants to replace wild-type UvsY in the kinetic competition assay for presynaptic filament assembly in 200 mM NaCl (Fig. 8). Under these salt conditions, neither mutant can effectively bind to ssDNA (51). Compared with wild-type UvsY, both UvsYK58A and UvsYK58A,R60A are severely compromised in their ability to promote UvsX-ssDNA assembly in the presence of Gp32F. Therefore UvsY-ssDNA interactions are extremely important for UvsY-mediated displacement of Gp32F from ssDNA by UvsX. However, comparison with data in Fig. 7 demonstrates that the effects of UvsYK58A and UvsYK58A,R60A are not zero. Competition assay time courses with UvsY mutants (equimolar to UvsX) exhibit rapid increases followed by very slow decreases in fluorescence (Fig. 8), which closely resemble the time course obtained in a reaction with a sub-stoichiometric amount of wild-type UvsY (0.08 μM, shown in Fig. 7, blue line), suggesting that a slow and inefficient displacement of Gp32F from ssDNA by UvsX protein occurs in the presence of UvsY mutants. Because UvsYK58A and UvsYK58A,R60A have essentially no ssDNA binding activity in 200 mM NaCl (27, 51), and because UvsY-mediated destabilization of Gp32-ssDNA interactions is independent of UvsY-Gp32 protein-protein interactions (32), the data in Fig. 8 suggest that UvsY-UvsX protein-protein interactions can promote a limited filament assembly process, presumably by strengthening UvsX-ssDNA interactions. This idea supports previous reports that UvsYK58A and UvsYK58A,R60A slightly stabilize UvsX-ssDNA filaments.
and retain partial activity to stimulate UvsX-catalyzed ssDNA-dependent ATP hydrolysis and DNA strand exchange reactions (27, 33).

**DISCUSSION**

Disruption of SSB-ssDNA interactions during presynapsis is a central tenet of most biochemical models of homologous recombination (1, 2, 4), however, most studies of SSB-ssDNA disruption have been indirect in nature. The extrinsic fluorescence assay for Gp32-ssDNA interactions reported herein provides direct evidence for Gp32 displacement from ssDNA during T4 presynaptic filament assembly, and a unique window on filament dynamics. The data support previous conclusions by Kodadek and co-workers (31), based on changes in chemical cross-linking and protease protection patterns, that Gp32 is released from ssDNA during presynaptic filament assembly. The data are also consistent with the previous observation, based on changes in intrinsic tryptophan fluorescence, that yeast RPA protein is displaced from ssDNA during Rad51-ssDNA filament assembly (52).

An important outcome of this study is the observation that UvsX protein requires both ATP and UvsY to displace Gp32 from ssDNA under stringent salt conditions. The existence of transiently ATP-stabilized UvsX-ssDNA interactions was previously inferred from differential effects of ATP versus ATP on binding isotherms and from the high catalytic turnover of UvsX ATPase activity (25). In this study, direct evidence shows that both ATP and ATP are capable of inducing a form of UvsX that can displace Gp32 from ssDNA. With ATP as substrate, the high-affinity form is maintained under steady-state conditions for ATP hydrolysis, as long as the ATP:product ratio remains high. However, the depletion of ATP substrate and accumulation of ADP/AMP products leads to loss of the high affinity form and ultimately to re-occupation of ssDNA by Gp32 at the expense of UvsX. This is the first direct evidence for modulation of the ssDNA binding state of UvsX during the ATPase cycle, a process that was previously identified for the Escherichia coli RecA protein (46).

Note that although ATP binding by UvsX is necessary for Gp32 displacement, it is not sufficient because all displacement reactions in 200 mM NaCl are co-dependent on UvsY and ATP (ATP$^S$). We recently showed that UvsY globally stabilizes UvsX-ssDNA interactions, with the most stable complex formed in the presence of both UvsY and ATP$^S$ (33). The implication is that Gp32-ssDNA binding energy is surmounted through the synergistic stabilizing effects of UvsY and nucleoside triphosphate ligands on UvsX-ssDNA interactions.

Based on previous studies, a model of UvsY-mediated presynaptic filament assembly was proposed in which a tripartite UvsY-Gp32-ssDNA complex acts as a key intermediate (2, 3, 32). UvsY introduces structural changes in ssDNA that weaken Gp32-ssDNA interactions within this complex, thus “priming” it to accept UvsX-ssDNA filament nucleation events and to initiate the displacement of Gp32. Results of the current study are consistent with this model, reflected in Fig. 9. The data demonstrate that UvsY itself does not displace Gp32 from ssDNA because the Gp32F-ssDNA fluorescence signal is not perturbed by UvsY (Fig. 1) under salt conditions in which both proteins are known to co-occupy the lattice (32, 33). This observation supports previous conclusions that both proteins retain intimate contacts with ssDNA within the UvsY-Gp32-ssDNA intermediate (19, 32).

Results of kinetic competition assays (Figs. 4–8) suggest that there is a highly ordered sequence of protein-ssDNA association and dissociation events during presynaptic filament assembly. A model for this process is shown in Fig. 9. We propose that Gp32 coats the lattice first, removing ssDNA secondary structure (Fig. 9A). UvsY adds next, forming the UvsY-Gp32-ssDNA intermediate (Fig. 9B). UvsY next recruits ATP-bound UvsX subunits to the ssDNA, forming a presynaptic filament nucleation complex with local displacement of Gp32 (Fig. 9C). Further addition of ATP-bound UvsX to an ATP-capped filament end (presumably the 3’ end) leads to directional propagation while progressively displacing more Gp32 (Fig. 9D). This sequence of events is logical given the rapid on-rates observed for the formation of Gp32-ssDNA cooperative clusters (53), the strong affinity of UvsY for Gp32-ssDNA (32), and given the prevailing conditions during T4 infection of E. coli, wherein sufficiently high concentrations of Gp32 are maintained to rapidly saturate any ssDNA that is generated during DNA replication, recombination, and repair transactions (12, 13). Therefore the kinetic competition assay appears to provide a reasonable biochemical model for the kinetics of the presynapsis process as it occurs in vivo. We note that filament assembly in vitro is a relatively slow process, requiring 2–3 min for the ensemble to approach steady-state under the conditions of our experiments (Figs. 4 and 5). Nevertheless, this rate appears to be kinetically competent with respect to rates of UvsX-catalyzed DNA strand exchange (6, 26, 54). The filament assembly rates we observe are also consistent with the slow approach to steady-state rates of ssDNA-depend-
The collapse of presynaptic filaments seen in kinetic competition assays performed in the absence of an ATP regenerating system (Fig. 5) suggests a dynamic instability process that we have incorporated into the model in Fig. 9. UvsX subunits at the relatively aged (presumably 5') filament end have hydrolyzed ATP to ADP, creating a relatively unstable ADP-capped end that is vulnerable to displacement by Gp32 (Fig. 9D). Preferential displacement of Gp32 by UvsX-ATP at one end of the filament concurrent with preferential displacement of UvsX-ADP by Gp32 at the opposite end could set up a classic treadmilling reaction in which a UvsX filament effectively translocates along a UvsY-ssDNA lattice while transiently displacing Gp32 (Fig. 9E). As proposed previously by Alberts and co-workers (6, 54), UvsX treadmilling might be harnessed to drive unidirectional branch migration reactions that occur during DNA strand exchange and during the bubble migration phase of recombination-dependent DNA synthesis reactions. Our studies suggest a new aspect to the old treadmilling model: that the combination of ATP binding and hydrolysis by UvsX coupled to stringent competition between Gp32 and the various nucleotide-ligated forms of UvsX for ssDNA binding sites could be the molecular force that drives directional treadmilling.

Our results indicate that there is a stoichiometric requirement for UvsY protein for optimal assembly of UvsX onto Gp32-saturated DNA. The rate of Gp32F displacement by UvsX/UvsY appears to approach a maximum value as the UvsY concentration approaches 1:1 stoichiometry with respect to UvsX and/or ssDNA binding sites (Fig. 7). The requirement for UvsY clearly is not all-or-none, however, because UvsY/UvsX molar ratios as low as 1:11, monomer:monomer (1:66 hexamer: monomer), are sufficient to promote low rates of Gp32F displacement. These findings are consistent with previous observations that the DNA strand exchange, ssDNA-dependent ATPase, and recombination-dependent DNA synthesis promoting activities of UvsX are optimal in the presence of stoichiometric UvsY, but are also significantly stimulated at lower UvsY/UvsX ratios (8–10). It is unclear from our data whether the low rates of Gp32F displacement observed at low UvsY:UvsX ratios represent the assembly of a few long filaments as opposed to many shorter ones, or if a critical UvsY:UvsX stoichiometry exists within each filament. More detailed kinetic studies (in progress) are needed to distinguish whether UvsY exerts its most important effects on filament nucleation, propagation, or both.

Experiments with UvsY missense mutants defective in protein-ssDNA interactions also demonstrate the key role of these interactions in mediating presynaptic filament assembly. The UvsY K58A and R60A mutations dramatically decrease, but do not totally eliminate, mediated Gp32F-ssDNA disruption by UvsX. Thus our data are consistent with previous observations that both missense mutants weakly stabilize UvsX-ssDNA interactions and retain residual recombination mediator activities in UvsX-catalyzed DNA strand exchange and ssDNA-dependent ATPase reactions (27, 33). The fact that both Gp32-ssDNA destabilization and UvsX-ssDNA stabilization functions depend largely on UvsY-ssDNA interactions (32, 33) suggests that there is linkage between the two processes. It is possible to envision a seamless progression from a pre-existing Gp32-ssDNA complex to a UvsY-Gp32-ssDNA intermediate to a UvsY- and nucleoside triphosphate-stabilized UvsX-ssDNA nucleation complex, with ssDNA structural change as the common denominator. The residual mediator activities of UvsY<sub>K58A</sub> and UvsY<sub>K58A,R60A</sub> mutants presumably result from a relatively weak stabilization of UvsX-ssDNA via protein-protein interactions.

The enhancement of Gp32F fluorescence upon binding to ssDNA reflects an environmental change of the fluorescein moiety. The environmental change could be due to the proximity of bound ssDNA to the fluorophore, contiguous binding of Gp32F molecules in cooperative clusters, an ssDNA-induced conformational change in Gp32F, or some combination of

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5 S. Morrical <em>et al.</em>, unpublished observations.
these effects. The first of these possibilities seems unlikely based on structural considerations. Cysteine 166, the residue to which 6-IAF is covalently conjugated, is not expected to be involved in direct ssDNA contacts. In the x-ray crystallographic structure of the ssDNA-binding core domain of Gp32 (14), Cys-166 is located on the surface of sub-domain II of the core, on a distal turn of the oligonucleotide/oligosaccharide-binding fold motif of the protein and oriented away from the ssDNA binding surface. Cys-166 is ~25 Å away from the nearest residue of the KRKTS sequence (a so-called “LAST” motif) found at positions 110–114 of Gp32, which is the major ssDNA binding surface identified in both x-ray and NMR studies (56, 57). Therefore the fluorescein moiety of Gp32F is probably distant from the bound ssDNA, making it unlikely that interactions with ssDNA directly enhance its fluorescence signal. Instead, evidence suggests that ssDNA-dependent conformational changes in the core domain may be driving the observed changes in Gp32F fluorescence. Cys-166 of Gp32 is located near one of the binding surfaces for Gp59, a T4-encoded helicase loading protein known to modulate the ssDNA binding affinity of Gp32 (21, 58), and can be chemically cross-linked to Gp59 (36). Also, Cys-166 of Gp32 is situated in a hydrogen-bonded turn with residue Asp-163, which is the target for the bacteriophage P2 Tin protein, a molecule that poisons Gp32 function (59). These observations suggest that Cys-166 resides in a region of Gp32 that shifts conformation in response to ssDNA as well as to regulators of ssDNA binding activity, which could also explain why the attachment of 6-IAF at Cys-166 has a moderate weakening effect on Gp32-ssDNA interactions. Other factors including the A- and B-domains of Gp32 may also influence the environment of the fluorescein moiety of Gp32F. These domains are predicted to be distant from Cys-166 in monomeric Gp32 due to chain topology (14), however, in cooperative clusters of Gp32-ssDNA the A- and B-domains could pack against the core domains of neighboring molecules in ways that change the chemical environment surrounding Cys-166. Currently no structural data are available on the inter- or intramolecular interactions of Gp32 core, A-, and B-domains.

The fluorescence properties and strong, cooperative ssDNA binding activity of Gp32F make it a useful reagent for studying a number of DNA transactions. Potential applications include: 1) detection and quantification of ssDNA; 2) measurement of ssDNA-binding parameters for other proteins via competitive binding assays; 3) real-time assays for ssDNA ↔ dsDNA interconversions involved in DNA synthesis, exonuclease, helicase unwinding, and other reactions; and 4) rapid kinetics and single-molecule studies of protein-ssDNA displacement reactions and ssDNA ↔ dsDNA interconversions. Future studies employing Gp32F are likely to yield important new insights on the dynamics of SSB proteins during homologous recombination, DNA replication, and DNA repair processes.

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