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Kinetics of Presynaptic Filament Assembly in the Presence of SSB and Mediator Proteins[†]

Jie Liu^{‡,¶}, Christopher L. Berger[§], and Scott W. Morrical^{‡,*}

[‡]Department of Biochemistry, University of Vermont College of Medicine, Burlington, VT 05405

[§]Department of Molecular Physiology and Biophysics, University of Vermont College of Medicine, Burlington, VT 05405

Abstract

Enzymes of the RecA/Rad51 family catalyze DNA strand exchange reactions that are important for homologous recombination and for the accurate repair of DNA double-strand breaks. RecA/Rad51 recombinases are activated by their assembly into presynaptic filaments on single-stranded DNA (ssDNA), a process that is regulated by ssDNA-binding (SSB) and mediator proteins. Mediator proteins stimulate strand exchange by accelerating the rate-limiting displacement of SSB from ssDNA by the incoming recombinase. The use of mediators is a highly conserved strategy in recombination, but the precise mechanism of mediator activity is unknown. In this study, the well-defined bacteriophage T4 recombination system (UvsX recombinase, Gp32 SSB, and UvsY mediator) is used to examine the kinetics of presynaptic filament assembly on native ssDNA *in vitro*. Results indicate that the ATP-dependent assembly of UvsX presynaptic filaments on Gp32-covered ssDNA is limited by a salt-sensitive nucleation step in the absence of mediator. Filament nucleation is selectively enhanced and rendered salt-resistant by mediator protein UvsY, which appears to stabilize a pre-nucleation complex. This mechanism potentially explains how UvsY promotes presynaptic filament assembly under physiologically relevant conditions of ionic strength and Gp32 concentration. Other data suggest that presynaptic filament assembly involves multiple nucleation events, resulting in many short UvsX-ssDNA filaments or clusters, which may be the relevant form for recombination *in vivo*. Together, these findings provide the first detailed kinetic model for presynaptic filament assembly involving all three major protein components — recombinase, mediator, and SSB, on native ssDNA.

Keywords

Recombination; presynaptic filament; kinetics; fluorescence; mediator; ssDNA-binding protein

The RecA/Rad51 family of proteins is highly conserved, and its members play central roles in homologous recombination-dependent DNA repair, in support of stalled/collapsed replication forks. RecA/Rad51 enzymes catalyze DNA strand exchange, which is the pairing and physical transfer of strands between homologous DNA molecules. Strand exchange requires the formation of a presynaptic filament consisting of recombinase cooperatively bound to single-stranded DNA (ssDNA[¶]).¹ Errors in presynaptic filament assembly cause

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^{*}To whom correspondence should be addressed at: Department of Biochemistry, University of Vermont College of Medicine, 89 Beaumont Avenue, Burlington, VT 05405. smorrice@uvm.edu; Phone number: +1 802-656-8260.

[¶]Current address: Department of Microbiology and Molecular Genetics, University of California-Davis, Davis, CA 95616

genome instability and sensitivity to DNA damaging agents.² Humans with defective presynapsis are predisposed to cancer.³

The formation of recombinase-ssDNA filaments is regulated by other proteins including ssDNA-binding proteins (SSBs) and recombination mediator proteins (RMPs).⁴ Both protein classes are highly conserved at the functional level. Biochemical studies of different recombination systems have revealed a common mechanism of presynapsis in which (1) an SSB binds to and removes secondary structure from ssDNA and, (2) an RMP then mediates loading of recombinase onto the SSB-ssDNA complex with concomitant release of SSB.⁴⁻⁶ The RMP stimulates DNA strand exchange by accelerating the rate-limiting displacement of SSB from ssDNA by the incoming recombinase.

Studies of the bacteriophage T4 recombination system have provided important insights on the biochemical mechanism of presynaptic filament assembly and the nature of RMP function.⁴⁻¹⁴ UvsX protein, the RecA/Rad51 ortholog of T4 phage, exhibits both ssDNA-dependent ATPase and DNA strand exchange activities. Gp32, the phage SSB, binds tightly and cooperatively to ssDNA. UvsY, the phage RMP, stimulates UvsX activities by overcoming Gp32 inhibition and promoting its assembly onto ssDNA. Protein requirements for DNA strand exchange activity vary as a function of ionic conditions.^{7, 14, 15} Under low-salt conditions, UvsX and Gp32 proteins are sufficient for *in vitro* strand exchange, while UvsY is dispensable. However, under high-salt conditions that approximate *in vivo* ionic strength, UvsY is absolutely required for strand exchange along with UvsX and Gp32. *In vivo*, *UvsY* and *UvsX* mutants are equally deficient in recombination and repair functions, indicating that the mediator activity of UvsY is essential for UvsX biological function.^{16, 17} Previous studies revealed that UvsY successively destabilizes Gp32-ssDNA and stabilizes UvsX-ssDNA interactions.^{8, 9} Results suggest that UvsY-induced changes in ssDNA structure play a major role in modulating the ssDNA-binding activities of UvsX and Gp32.⁷⁻⁹

RMP-dependent assembly of recombinase filaments on SSB-covered ssDNA is a common feature of all recombination systems, but the mechanism by which filaments nucleate and propagate on SSB-ssDNA is poorly understood. To address this problem we developed a real-time assay for the kinetics of T4 presynaptic filament assembly on Gp32-covered ssDNA, based on changes in the fluorescence of a fluorescein-Gp32 conjugate.⁶ In the current study, we use this assay to determine kinetic parameters for the nucleation and growth of UvsX filaments on Gp32-ssDNA, as functions of salt, UvsY, and nucleotide ligands. The data show that UvsY selectively enhances filament nucleation by stabilizing a salt-sensitive pre-nucleation complex, allowing UvsX assembly and Gp32 displacement to occur at higher salt concentrations. The displacement of Gp32 from ssDNA by incoming UvsX appears to be an active process requiring the ATP-bound form of the recombinase. Other results suggest that short UvsX-ssDNA filaments resulting from multiple nucleation events are sufficient for recombination. Collectively, the data shed new light on the mechanism of presynaptic filament assembly during homologous recombination, and the critical role of recombination mediator proteins in this conserved pathway.

MATERIALS AND METHODS

Reagents

T4 proteins and M13mp18 ssDNA were prepared as described.⁶ All ssDNA concentrations are given in nucleotide residues. 6-iodoacetamidofluorescein-labeled Gp32 protein (Gp32F

[¥]Abbreviations: ssDNA, single-stranded DNA; SSB, single-stranded DNA binding protein; RMP, recombination mediator protein; ATP_γS, adenosine-5'-(O)-(3-thio)triphosphate.

€) was prepared as described.⁶ Buffer A contained 20 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, and NaCl as indicated.

Stopped-flow fluorescence assays

Rapid kinetics assays were performed in a μ SFM-20 stopped-flow fluorometer (BioLogic Science Instruments, Claix, France) having a 1.2 ms dead time. This instrument was used for all measurements of Gp32F-ssDNA association kinetics, and for initial studies of Gp32F displacement from ssDNA by UvsX $\pm$ UvsY. All protein and ssDNA concentrations reported are final concentrations after mixing. Reactions were carried out at 25°C in Buffer A plus NaCl as indicated. Excitation wavelength was 460 nm with a 5 nm slit. Emission was monitored at wavelengths > 495 nm using a long-pass-cutoff filter and a 20 nm slit. Each data trace is the average of 10 independent reactions.

Gp32F-ssDNA association kinetics were modeled according to Scheme 1, which parallels the scheme derived previously for native Gp32.¹⁸ The fluorescence traces were fitted to double exponentials according to Eq. 1.

$$F(t) = F_{\infty} - A_1 \exp(-k_{obs1}t) - A_2 \exp(-k_{obs2}t) \quad (\text{Eq. 1})$$

where $F(t)$ is the fluorescence intensity at time t , F_{∞} is the fluorescence intensity at infinite time, A_1 and A_2 are the amplitudes of the first and second relaxation processes, respectively, and k_{obs1} and k_{obs2} are the observed rate constants of the first and second relaxation process, respectively. The standard deviations of k_{obs1} and k_{obs2} reflect the associated uncertainties or fluctuations.

The observed rate constant of the first (fast) relaxation process from the double exponential (Eq. 1) was fitted into Eqs. 2 and 3 to obtain the apparent forward/reverse rate constants and to calculate the equilibrium association constant for Gp32F cluster formation on the ssDNA lattice.

$$k_{obs1} = k_2(\text{app}) \times [\text{ssDNA}] + k_{-2}(\text{app}) \quad (\text{Eq. 2})$$

$$K_2 = k_2(\text{app}) / k_{-2}(\text{app}) \quad (\text{Eq. 3})$$

Gp32F displacement assays for presynaptic filament assembly

The kinetics of Gp32F displacement from ssDNA by UvsX $\pm$ UvsY were studied in a Quantamaster QM-6 steady-state fluorometer (PTI, NJ). The QM-6 yielded higher sensitivity and little information loss compared to identical experiments in a BioLogic μ SFM-20 stopped-flow fluorometer, due to the slow reaction rates involved. Excitation wavelength was 460 nm with 1 nm slit, and emission wavelength was 519 nm with a 5 nm slit. A long-pass-cutoff filter at 495 nm was used for emission data collection. The effects of dilution, solution change, photobleaching, inner filter effects, and intrinsic protein fluorescence on experimental data were evaluated as described,⁶ and were found to be negligible under the experimental conditions employed. Starting solutions containing 0.5 μ M Gp32F and 3.5 μ M ssDNA were pre-incubated at 25°C in Buffer A plus NaCl and

€Morrical, S.W. & Qian, N., US Patent number 7,544,469, "Methods and Compositions for Optical Detection of Single-stranded Polynucleotides". Morrical, S.W. & Qian, N., International Patent Application number PCT/US2004/000665, "Methods and Compositions for Optical Detection of Single-stranded Polynucleotides".

nucleotides as indicated. ATP-containing reactions also contained an ATP-regenerating system (5 mM phosphoenolpyruvate, 2.6 units/mL pyruvate kinase). UvsX ± UvsY proteins were separately preincubated in the same buffer/nucleotide solution then manually mixed into the starting solution to initiate the reaction and data collection (dead-time ≈ 3 s). The final concentrations of UvsX and UvsY in each experiment are indicated. Emission traces were collected at 3 points/s. Reported observed rate constants are averages obtained from 2–3 separate reactions.

Data analysis

All fluorescence traces were fitted to double exponentials using Datafit (Oakdale Engineering, PA) according to Eq. 4

$$F(t) = F_{\infty} - A_1 \exp(-k_{obs1}t) + A_2 \exp(-k_{obs2}t) \quad (\text{Eq. 4})$$

where $F(t)$ is the fluorescence intensity at time t , F_{∞} is the fluorescence intensity at infinite time, A_1 and A_2 are the amplitudes of the first and second relaxation processes, respectively, and k_{obs1} and k_{obs2} are the observed rate constants of the first and second relaxation process, respectively. The standard deviations of k_{obs1} and k_{obs2} reflect the associated uncertainties or fluctuations.

Kinetic traces of Gp32F displacement by UvsX under various conditions were fitted to Eq. 4 to obtain observed rate constants for a fast fluorescence-increasing phase (k_{obs1}) and a slow fluorescence-decreasing phase (k_{obs2}). Both observed rate constants exhibit a hyperbolic dependence on UvsX concentration. A simplified mechanistic model for Gp32F displacement from ssDNA by UvsX is shown in Scheme 2, with two separated phases, filament nucleation (K_1, k_2) and filament growth (K_3, k_4). This simplified model is consistent with the two-step model (nucleation and growth) obtained from a recent single molecule study of RecA filament formation on SSB-covered ssDNA.¹⁹ This model makes the simplifying assumptions that under the experimental conditions used: (1) All UvsX is saturated with ATP (or ATP γ S); (2) isomerization steps k_2 and k_4 are slow and essentially irreversible; and (3) all UvsX is bound to UvsY (when present) and corresponding kinetic parameters are those of the UvsX-UvsY complex (stoichiometry undefined). Kinetic parameters in Scheme 2 were estimated by fitting k_{obs1} and k_{obs2} values obtained at different UvsX concentrations to Eqs. 5 and 6, respectively:

$$k_{obs1} = k_2 \times \frac{K_1[\text{UvsX}]}{1 + K_1[\text{UvsX}]} + k_{-2} \quad (\text{Eq. 5})$$

$$k_{obs2} = k_4 \times \frac{K_3[\text{UvsX}]}{1 + K_3[\text{UvsX}]} + k_{-4} \quad (\text{Eq. 6})$$

Global fitting method in kinetic data analysis

As an independent method for estimating kinetic parameters, kinetic traces obtained at different UvsX concentrations were globally fit to differential equations derived from the model in Scheme 3, using DynaFit (BioKin Ltd., WA).²⁰ Scheme 3 is identical to Scheme 2 but contains term substitutions to facilitate mathematical modeling. The differential equations derived from Scheme 3 are shown in Equations 7.1 – 7.6:

$$d[S1]/dt = -k_1[S1][S2] + k_{-1}[N1] - k_3[S1][N2] + k_{-3}[F1] \quad (\text{Eq. 7.1})$$

$$d[S2]/dt = k_1[S1][S2] + k_{-1}[N1] \quad (\text{Eq. 7.2})$$

$$d[N1]/dt = k_1[S1][S2] - k_{-1}[N1] - k_2[N1] \quad (\text{Eq. 7.3})$$

$$d[N2]/dt = k_2[N1] - k_3[S1][N2] + k_{-3}[F1] \quad (\text{Eq. 7.4})$$

$$d[F1]/dt = k_3[S1][N2] - k_{-3}[F1] - k_4[F1] \quad (\text{Eq. 7.5})$$

$$d[F2]/dt = k_4[F1] \quad (\text{Eq. 7.6})$$

In reactions containing UvsY, all UvsX is assumed to be complexed with UvsY, since the two proteins are pre-incubated together and are known to interact strongly under the range of buffer conditions used in these experiments. Therefore [S1] becomes the concentration of free UvsX-UvsY complex, and all other UvsX-containing terms likewise include UvsY. Scheme 3 (Scheme 2) further assumes that due to the experimental conditions: (1) UvsX is saturated with ATP (or ATP γ S) since nucleotide concentration is higher than K_m or $K_d^{22,23}$, and since experiments with ATP employ a regenerating system to maintain a high ATP/ADP ratio. (2) Free ssDNA in the system is negligible (all of the ssDNA is covered either with Gp32F or with UvsX/UvsY). This assumption is reasonable since Gp32F binds stoichiometrically to mixed sequence ssDNA under the range of buffer conditions used in these experiments⁶, and the concentration of Gp32F is saturating with respect to total ssDNA concentration. (3) Steps k_2 and k_4 are essentially irreversible ($k_{-2} = k_{-4} = 0$) so that once Gp32F is displaced from ssDNA it does not compete with UvsX for ssDNA binding sites. This assumption derives from the pre-steady-state conditions including the fact that UvsX $\pm$ UvsY is added to a pre-assembled Gp32F-ssDNA complex, such that the concentration of free Gp32F is essentially zero at early stages of each reaction and therefore cannot compete. Also it was observed empirically that the re-occupation of ssDNA by Gp32F requires the hydrolytic depletion of ATP by UvsX, which is prevented in these reactions either by using a regenerating system, or by using ATP γ S.

During global fitting using DynaFit, iterative runs for individual isotherms were taken to determine the values and standard errors of rate constants k_2 , k_4 , k_1 , and k_3 sequentially, according to Eq. 7. To facilitate convergence, rate constants k_{-1} and k_{-3} were each assigned a value of 1 s^{-1} and were held constant in all iterations, therefore only the association constants for the equilibrium steps $K_1 (= k_1/k_{-1})$ and $K_3 (= k_3/k_{-3})$ are reported in Table 3. K_1 and K_3 values determined at 50 mM NaCl are similar in magnitude to estimates of UvsX-ssDNA binding affinity under equilibrium binding conditions [4; H. Xu and S. Morrical, unpublished results]. The fitting quality can be judged by Figure 4 and by the standard errors of the kinetic constants in Table 3. In nearly all cases the hyperbolic and global fitting methods yielded similar estimates of kinetic parameters K_1 , k_2 , K_3 , and k_4 .

RESULTS

Kinetic properties of Gp32F-ssDNA interactions

Fluorescein-conjugated Gp32 (Gp32F) exhibits a fluorescence increase upon binding to ssDNA, and its equilibrium ssDNA-binding properties are a reasonable approximation of unlabeled Gp32.⁶ These properties make Gp32F a useful real-time probe for T4 presynaptic filament assembly. To establish the kinetic studies of UvsX-ssDNA assembly on Gp32-

covered ssDNA, we first investigated the basic kinetic properties of Gp32F-ssDNA binding using stopped-flow fluorescence. Gp32F-ssDNA binding follows the same three-step mechanism previously derived for unlabeled Gp32 (Scheme 1, Figure 1A):¹⁸ 1) A very fast nucleation step (too fast to detect) with small amplitude where Gp32F nucleates onto the lattice and forms isolated binding sites. 2) A fast growth step with large amplitude where Gp32F propagates onto the lattice and forms contiguous clusters. 3) A slow isomerization step with small amplitude where the contiguous Gp32F protomers redistribute by facilitated diffusion to form stable clusters on the lattice.

Figure 1A shows a typical kinetic trace when Gp32F is rapidly mixed with excess ssDNA. The time-dependent fluorescence change of Gp32F fits a double exponential function (Eq. 1) similar to wild-type Gp32.¹⁸ The rate constant of the fast phase, k_{obs1} , was assigned to the growth step (Step 2, Scheme 1) based on its large amplitude. To obtain rate constants we performed a series of rapid binding experiments in different salt conditions, with constant Gp32F and variable ssDNA concentrations. In low-salt, strong binding conditions (50 – 100 mM NaCl), the observed growth rate constant k_{obs1} (Step 2 in Scheme 1) increases with increasing salt concentration (Figure 1B). In high-salt, weak binding conditions (100 – 250 mM NaCl), k_{obs1} decreases with increasing salt concentration (Figure 1C). The salt effects on Gp32F-ssDNA binding kinetics parallel those of unlabeled Gp32:¹⁸ Under low salt conditions k_{obs1} increases with increasing salt because Na^+ disrupts electrostatic interactions between the C-terminal and core domains of Gp32 that inhibit DNA binding.²⁷ Under high salt conditions k_{obs1} decreases with increasing salt since higher salt interrupts protein-DNA electrostatic interactions.

As shown in Figure 1B–C, k_{obs1} exhibits linear dependence on [ssDNA] in all salt conditions. Apparent on and off rate constants for the growth step, $k_2(\text{app})$ and $k_{-2}(\text{app})$, were obtained from linear fits of these data to Eq. 2. The apparent association rate has a much stronger dependence on [NaCl] ($\text{dlog}k_2(\text{app})/\text{dlog}[\text{NaCl}] = -0.75 \pm 0.01$) than the apparent dissociation rate ($\text{dlog}k_{-2}(\text{app})/\text{dlog}[\text{NaCl}] = -0.19 \pm 0.01$), when increasing salt from 100 to 250 mM NaCl. In the same salt interval, the equilibrium affinity of Gp32F, K_{ss} , dropped from 1.6×10^5 to $7.1 \times 10^4 \text{ M}^{-1}$, and the $\text{dlog}K_{\text{ss}}/\text{dlog}[\text{NaCl}]$ value is equal to -0.83 ± 0.03 . The substrate for Gp32F filament assembly is natural phage ssDNA under no tension, and our results indicate the salt impact on the electrostatic interactions between Gp32 and ssDNA is through the decrease of association rate, not increase of dissociation rate.

The rate constants and calculated association constants (Eq. 3) are summarized in Table 1 to compare with unlabeled Gp32. Gp32F displays a 50-fold decrease in $k_2(\text{app})$ for binding M13mp18 ssDNA, compared to unlabeled Gp32 binding poly(rEA).¹⁸ We previously reported that Gp32F shows a 12-fold decrease in intrinsic binding affinity for M13mp18 ssDNA compared to unlabeled Gp32.⁶ Additionally, Gp32 displays a lattice bias favoring homopolymers and etheno-modified bases (J. Liu and S.W. Morrical, unpublished result). Overall, the data indicate that Gp32F-ssDNA binding follows the same kinetic mechanism (Scheme 1) as unlabeled Gp32, and that the lower affinity of Gp32F vs. Gp32 for ssDNA is largely due to its slower growth on-rate. For these reasons, following the UvsX-dependent displacement of Gp32F from pre-assembled Gp32F-ssDNA complexes provides relevant information on the kinetics of presynaptic filament assembly on native Gp32-ssDNA.

Presynaptic filament assembly as a function of salt, nucleotide, and UvsY

UvsX-ssDNA filament assembly was monitored by the displacement of Gp32F from ssDNA with concomitant fluorescence decrease.⁶ Pre-assembled Gp32F-ssDNA complexes were rapidly mixed with a stoichiometric amount of UvsX in Buffer A plus 50 mM NaCl and various nucleotides (Figure 2A). Under these conditions filament assembly is UvsY-

independent, due to differential salt effects on Gp32-ssDNA *versus* UvsX-ssDNA interactions (see Discussion). This property is shown clearly by the data in Figure 2A, wherein the addition of UvsX to pre-formed Gp32F-ssDNA in the absence of UvsY leads to a time-dependent decrease in Gp32F fluorescence. The displacement of Gp32F requires ATP binding but not ATP hydrolysis by UvsX, since signal change occurs in the presence of either ATP or ATP γ S, but not in the presence of ADP, AMP, or no nucleotide (Figure 2A). The substrate analog ATP γ S is very slowly hydrolyzed by UvsX, and stabilizes UvsX-ssDNA interactions.^{21, 22} Note that ATP and ATP γ S, when present, were at saturating concentrations,^{22, 23} and that an ATP regenerating system was included to maintain a high ATP/product ratio throughout the timecourse. The latter measure was necessary to prevent UvsX dissociation from ssDNA caused by the build-up of ATP hydrolytic products, which eventually leads to the re-occupation of ssDNA by Gp32F.⁶

Increasing salt concentration inhibits presynaptic filament assembly in the absence of UvsY (Figure 2B). All of the reactions in Figure 2B were performed in the presence of saturating ATP plus an ATP regenerating system. Increasing the NaCl concentration from 50 to 100 mM causes a sharp decrease in the rate of Gp32F displacement, while 200 mM NaCl blocks the reaction entirely. The addition of UvsY protein restores filament assembly at 200 mM NaCl, however (Figure 2B). These observations are consistent with salt and UvsY effects on the *in vitro* DNA strand exchange activity of UvsX protein.^{7, 14} The data may also explain why T4 recombination transactions are co-dependent on UvsX and UvsY *in vivo*,¹⁷ where ionic strength is relatively high.

Filament nucleation is rapid but salt-sensitive

Kinetic traces in the presence of nucleoside triphosphate and at low salt concentration (Figure 2) were fitted accurately by a double exponential function with increasing and decreasing phases (Eq. 4) to obtain the observed rate constants for the fast reaction with small positive amplitude (designated as UvsX nucleation, k_{obs1}) and for the slow reaction with large negative amplitude (designated as UvsX growth, k_{obs2}), as listed in Table 2. In 50 mM NaCl, ATP γ S enhances UvsX's observed nucleation rate about 11 – fold, and observed growth rate about 4 – fold, compared to the natural substrate ATP (Figure 2A and Table 2). This is consistent with the notion that ATP γ S stabilizes the high-affinity ssDNA-binding conformation associated with the ATP-bound form of UvsX.^{21, 22} The data indicate that this form of UvsX is necessary for Gp32 displacement during both nucleation and growth phases of filament assembly. Note that observed nucleation rates are fast compared to growth rates (Table 2), suggesting that presynaptic filaments assemble from many nucleation centers on Gp32-ssDNA (see Discussion).

In the presence of ATP, doubling the NaCl concentration from 50 to 100 mM causes a 2.3-fold decrease in the observed nucleation rate (k_{obs1}) but has little effect (~1.1-fold decrease) on the observed growth rate (Table 2). Therefore salt has a strong effect on nucleation and a weak effect on growth. Thus the inability of UvsX (+ATP) to displace Gp32F from ssDNA in 200 mM NaCl (Figure 2B) likely reflects a failure of UvsX to nucleate onto Gp32F-ssDNA. The addition of UvsY is required for Gp32F displacement under these conditions (Figure 2B). The UvsY-dependent trace is also fit accurately by a double exponential with increasing and decreasing phases (Eq. 4). The observed nucleation rate, k_{obs1} , of the UvsY-dependent reaction in 200 mM NaCl is 2.4- and 5.6-fold higher, respectively, than those of the UvsY-independent reactions in 50 and 100 mM NaCl (Table 2). ATP-binding by UvsX is still required for the UvsY-dependent reaction, however, since no reaction is observed with ADP, AMP, or no nucleotide under these conditions (reference ⁶ and data not shown).

Unique fluorescence signature of filament nucleation

As shown in Figure 2B, the kinetic trace of the reaction containing UvsY shows a significant initial fluorescence increase, which could be fit accurately to Eq. 4. We attribute the initial fluorescence increase to the formation of a pre-nucleation complex with enhanced Gp32F fluorescence (see Discussion). This pre-nucleation complex is formed in the absence of UvsY as well, since these reactions are also fit accurately by Eq. 4 (Figure 2A–B). In buffer containing 200 mM NaCl, where UvsX alone fails to displace Gp32F from ssDNA, the presence of UvsY increases the observed nucleation rate (k_{obs1}) by at least 470-fold over the background rate (Table 2). The observed growth rate (k_{obs2}) is 40-times slower than the observed nucleation rate (Table 2), suggesting that UvsY functions primarily to enhance nucleation rather than growth of UvsX filaments on Gp32-covered ssDNA.

Kinetic model for presynaptic filament assembly

A series of Gp32F displacement assays were conducted at different UvsX concentrations under high- (200 mM NaCl) or low-salt (50 mM NaCl) conditions, and in the presence of either ATP γ S or ATP. In 200 mM NaCl a constant amount of UvsY (stoichiometric w.r.t. ssDNA assuming a binding site size $n = 4$ nucleotide residues per monomer²⁴) was pre-incubated with variable amounts of UvsX. Under all conditions the observed rate constants of both nucleation and growth steps appear to increase with increasing UvsX concentration in a saturable manner (Figure 3). The data can be approximated using a relatively simple four-step model (Scheme 2): 1) A rapid binding step (K_1) where UvsX binds Gp32F-ssDNA and forms a transient pre-nucleation complex (Gp32F retained). 2) A slow and essentially irreversible isomerization step (k_2) where UvsX changes conformation to form a stable nucleation site (Gp32F displaced). 3) Another rapid binding step (K_3) where UvsX forms unstable contiguous clusters (Gp32F retained). 4) Another slow and essentially irreversible isomerization step (k_4) where UvsX clusters redistribute into stable filaments on the lattice (Gp32F displaced). Steps 1 and 2 comprise the nucleation phase defined by k_{obs1} and Eq. 5, whereas steps 3 and 4 comprise the growth phase defined by k_{obs2} and Eq. 6. The assumptions underlying this model are stated in Materials and Methods. Our kinetic model through ensemble studies also agrees well with previous nucleation-growth models from single molecule studies of RecA filament formation on dsDNA or SSB-covered ssDNA.^{19, 25}

Estimates of K_1 , k_2 , K_3 , and k_4 were obtained by fitting hyperbolic Eqs. 2 and 3, respectively, to plots of k_{obs1} or k_{obs2} vs. UvsX concentration (Figure 3). The kinetic mechanism in Scheme 2 was tested independently via global fits of model-derived differential equations (Eq. 7) to raw fluorescence trace data (Figure 4). Kinetic parameters determined from both hyperbolic and global fitting methods are listed in Table 3. In nearly all cases the two fitting methods gave very similar results (~ 2 -fold difference in parameter values; Table 3), indicating that Scheme 2 is a reasonable model for this system. The only exception was a 4.6-fold difference in K_1 obtained from global vs. hyperbolic fitting methods for reactions in 200 mM NaCl plus UvsY and ATP γ S (Table 3). Under these conditions the global fitting method failed to converge at intermediate UvsX concentrations (0.1–0.5 μ M), indicating additional complexity in the kinetic mechanism (see Discussion).

Three major findings emerge from the data in Table 3: First, in reactions at 200 mM NaCl, the presence of UvsY strongly and specifically increases K_1 of the nucleation phase compared to UvsY-independent reactions at 50 mM NaCl. The average increase is 27-fold with ATP, and 11-fold with ATP γ S as cofactor. Therefore UvsY appears to promote presynaptic filament assembly primarily by stabilizing a salt-sensitive pre-nucleation complex. Second, k_4 of the growth phase appears to be rate-limiting for UvsY-dependent filament assembly at high salt (avg. $k_4/k_2 = 0.08$ and 0.2 with ATP and ATP γ S as cofactor,

respectively). The same is true for UvsY-independent reactions at low salt with ATP γ S as cofactor (avg. $k_4/k_2 = 0.2$). Third, in UvsY-independent reactions at low salt, ATP γ S enhances both k_2 of the nucleation phase and K_3 of the growth phase by ~6-fold compared to reactions with ATP as cofactor. The implications of these findings are discussed below.

DISCUSSION

Data from our ensemble measurements of T4 presynaptic filament assembly is described well by a relatively simple, nucleation-growth kinetic model (Figure 5). The model is similar to nucleation-growth models for RecA filament formation on dsDNA or SSB-ssDNA that were derived from single molecule measurements.^{19, 25} The utility of the model is established by the fact that hyperbolic plots of observed rate constants vs. UvsX concentration (Fig. 3), and global fitting of model-derived differential equations to raw fluorescence traces (Fig. 4), both return similar values for kinetic parameters under almost all conditions (Table 3). Under one narrow set of conditions (intermediate UvsX concentrations in 200 mM NaCl plus UvsY and ATP γ S), the global fitting algorithm failed to converge, suggesting additional complexity. One possible reason for the lack of convergence is a competition between the nucleation and growth phases for UvsX under these conditions. UvsY and ATP γ S have been shown to stabilize UvsX-ssDNA interactions synergistically at high salt concentrations.⁸ It is conceivable that this leads to a burst of nucleation events that, at relatively low UvsX concentrations, depletes it from the growth phase. This would be consistent with the idea that UvsY promotes multiple nucleation events leading to many short filaments (see below).

Mediator protein UvsY stabilizes a salt-sensitive pre-nucleation complex

As illustrated in the model (Fig. 5), the critical first step (K_1) of this mechanism is the ATP-dependent formation of a pre-nucleation complex containing UvsX recombinase co-occupying the ssDNA along with the SSB component, Gp32. The pre-nucleation complex is distinguished from subsequent intermediates by its relative salt-sensitivity and by fluorescence enhancement effects on a Gp32F probe. It is worth noting that the T4 helicase loading protein, Gp59, enhances the fluorescence of Gp32F within a tripartite Gp59-Gp32F-ssDNA complex.²⁶ The formation of this complex precedes loading of the replicative helicase, Gp41, which is accompanied by Gp32F displacement. The parallel fluorescence effects on Gp32F seen during UvsX loading and helicase loading suggest that transient intermediates in both processes may involve similar changes in Gp32-ssDNA structure or environment.

The data indicate that UvsY specifically stabilizes the pre-nucleation complex at high salt concentrations (Table 3; Figure 5). By overcoming the salt-sensitivity of presynaptic filament nucleation, UvsY activates UvsX to perform its recombination functions under physiological conditions. The observed salt-sensitivity of filament nucleation is consistent with the observation that the intrinsic affinity parameter of UvsX for ssDNA, K_{ss} , is salt-sensitive.²¹ The isolated binding of UvsX to ssDNA during filament nucleation presumably depends on K_{ss} alone, while contiguous binding of UvsX to ssDNA during filament growth also depends on ω , the salt-insensitive cooperativity parameter. The salt-dependence of UvsX/Gp32 exchange also reflects differential salt effects on the ssDNA-binding activities of Gp32 and UvsX. Over the salt interval covered in this study (50–250 mM NaCl), Gp32-ssDNA binding affinity is only weakly dependent on salt concentration; in fact the affinity decreases slightly at 50 mM NaCl (Table 1; ^{18, 27}). This is attributable to a masking effect caused by the acidic C-terminal domain of Gp32, which has been well-documented elsewhere.²⁷ In contrast, the ssDNA-binding affinity of UvsX increases markedly with decreasing salt concentration over the same interval, and UvsX-ssDNA interactions are

further stabilized by ATP binding.^{8, 28} Therefore at 50 mM NaCl UvsX-ATP possesses sufficient ssDNA-binding affinity to compete with Gp32 for binding sites on ssDNA, while at 200 mM NaCl it does not, and so requires the mediator activity of UvsY.

The observed salt-sensitivity of UvsX-ssDNA filament nucleation is consistent with results of single molecule studies of *E. coli* RecA-dsDNA filaments. The latter revealed a strong impact of ionic strength on nucleation cluster frequency, while the impact is much milder on the average cluster growth rate.²⁵ Somewhat different results were obtained in single molecule studies of RecA filament assembly on SSB-ssDNA complexes, which found relatively modest effects of salt on filament nucleation and growth rates from 50–200 mM NaCl.¹⁹ This difference could be due to the use of ATP γ S in the latter study, since ATP γ S greatly stabilizes RecA-ssDNA filaments in salt concentrations as high as 600 mM NaCl,²⁹ which likely precludes the need for a mediator protein. In contrast, UvsX-ssDNA filaments in the presence of ATP (this study) are significantly more salt-sensitive, and therefore nucleation becomes mediator-dependent with increasing salt.

Growth is rate-limiting in presynaptic filament assembly

UvsX filament assembly on Gp32-ssDNA is characterized by relatively fast nucleation followed by relatively slow growth (Tables 2 and 3). The forward rate constant for the isomerization step of growth, k_4 , appears to be rate-limiting under most conditions. This observation suggests that presynaptic filaments assemble via multiple nucleation events, forming many short filaments rather than a few long ones. The need for many nucleation events, along with the strict requirement for UvsY in filament nucleation at high salt, helps to explain the fact that UvsY is needed in approximately 1:1 stoichiometry with UvsX for optimal strand exchange activity.^{7, 14} It is also consistent with single-molecule studies of human RAD51 and *E. coli* RecA assembly on duplex DNA, which show that these recombinases rapidly form multiple nucleation sites.^{25, 30, 31}

Gp32 displacement from ssDNA by UvsX $\pm$ UvsY proteins is a relatively slow process, yet presynaptic filament assembly occurs rapidly enough to account for observed rates of UvsX-catalyzed DNA strand exchange. At high UvsX concentrations in 50 mM NaCl + ATP, UvsX approaches half-saturation of the ssDNA within 1.5 – 2 min (Figure 4), which corresponds to the earliest times at which recombination intermediates appear in DNA strand exchange reactions performed under similar conditions.^{7, 28} The data suggest that partial saturation of ssDNA by UvsX protein is sufficient to initiate homologous pairing. At high UvsX concentrations in 200 mM NaCl + ATP + UvsY, UvsX approaches half-saturation of the ssDNA within 4–5 min (Figure 4). Early strand exchange and recombination-dependent replication products typically appear within 2–5 min under similar conditions, and require significantly lower concentrations of UvsX than are required under UvsY-independent conditions.^{7, 13} The data suggest that relatively short, UvsY-stabilized filaments are preferred to initiate homologous pairing reactions, which are not limited by slow filament growth rates.

UvsX recombinase actively displaces Gp32 from ssDNA

As illustrated in Figure 5, both nucleation and growth phases include isomerization steps (k_2 and k_4) in which Gp32 is ejected from the ssDNA. In our experiments with the Gp32F probe, values of k_2 and k_4 (Table 3) greatly exceed values of the rate constant for spontaneous dissociation of Gp32F from cooperative clusters (k_{-2} in Table 1) under all conditions examined. Therefore UvsX does not passively occupy binding sites abandoned by Gp32; rather it actively displaces Gp32 from the lattice. Without the challenge from UvsX, pre-assembled Gp32F-ssDNA remains as a stable complex for over ten minutes without detectable dissociation (data not shown). Active displacement requires ATP (or

ATP γ S) binding by UvsX, and also requires UvsY under high-salt conditions. One possible mechanism for active displacement is shown schematically in Figure 5: We propose that isomerization of UvsX not only displaces Gp32 from ssDNA locally, but also remodels and destabilizes adjacent ends of Gp32 clusters, effectively increasing their off-rate from ssDNA.

Implications for recombination in T4 and other systems

Presynaptic filament assembly involves an intricate choreography of protein-ssDNA and protein-protein interactions among recombinase, SSB, and mediator components of the homologous recombination machinery. Each interaction is modulated by solution variables including ion concentrations and nucleotide ligands. These variables determine the efficiency of recombinase/SSB exchange on ssDNA and thereby dictate the requirement for mediator protein activities in filament assembly. In this study, we have quantified key aspects of the salt, nucleotide, and mediator protein effects that influence recombinase/SSB exchange during T4 presynaptic filament assembly. Results indicate that the efficiency of T4 presynaptic filament assembly *in vivo* is controlled by two major kinetic effects: (1) The requirement for UvsY mediator to stabilize the pre-nucleation complex formed by UvsX recombinase on Gp32-covered ssDNA; and (2) The rate-limiting nature of filament growth. The data suggest that T4 *uvsY* mutant strains are recombination-deficient specifically because they cannot form UvsX pre-nucleation complexes under *in vivo* salt conditions. Our model predicts that it should be possible to isolate UvsX or Gp32 mutants, or to identify chemical agents, that suppress *uvsY* mutations by stabilizing the pre-nucleation complex. In this regard, a yeast Rad51-I345T mutant protein shows enhanced binding and stability to ss- and ds-DNA, which can partially bypass the requirement for Rad55-Rad57 mediator proteins in DNA repair.³² Conversely, agents that *destabilize* the pre-nucleation complex would be predicted to inhibit recombination *in vivo*.

Presynaptic filament structure and function are highly conserved among diverse species; likewise SSB and mediator proteins are functionally conserved. Therefore it seems probable that cellular recombination systems follow some of the same kinetic principles as those employed by the T4 system. Our kinetic studies suggest that UvsX/UvsY form many nucleation centers and short segments on Gp32-ssDNA, and the dominance of filament nucleation argues for a kinetically-competent discontinuous filament. A growing body of evidence suggests that discontinuous presynaptic filaments may be a general feature of all homologous recombination systems.³⁶ Single-molecule studies have demonstrated that RecA/Rad51 form multiple nucleation sites on either naked or SSB-covered DNA.^{19, 25, 30, 33} Rad51 forms protein patches with gaps on dsDNA molecules and typically forms irregular structures on ssDNA.^{30, 34} EM structures of human Rad51-Rad52 co-filaments reveal multiple individual Rad52 binding sites on Rad51-covered ssDNA.³⁵ Rad51 paralogs Rad55/Rad57 in yeast, and Rad51B/Rad51C in humans, both appear to integrate into Rad51-ssDNA presynaptic filaments and protect them from disruption by anti-recombination helicases Srs2 and Blm, respectively.^{37,38} We propose that the formation of discontinuous filaments may be advantageous for their regulation by recombination mediator proteins, both at the level of nucleation (e.g. by classic mediators such as T4 UvsY, *E. coli* RecFOR, Rad52, or Brca2^{4,36}), and at the level of segment stabilization (e.g. by Rad51 paralogs such as Rad55/Rad57 or Rad51B/Rad51C). It seems possible that in humans the Rad51-ssDNA filament could be deliberately managed by Brca2 and Rad51B/Rad51C to form relatively short, stable segments that are optimized to promote recombination by a synthesis-dependent strand annealing (SDSA) mechanism that avoids genotoxic crossovers and genomic instability.³⁹ Bacteriophage T4 is proficient in SDSA, which is promoted *in vitro* by purified UvsX, UvsY, Gp32, and replication enzymes.⁴⁰ Mechanistic studies of this

process are likely to inform studies of SDSA and related genome stability processes in humans and other organisms.

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Figure 1.

(A) Time course of rapid association between 25 μM M13mp18 ssDNA and 0.5 μM Gp32F, shown in black. The red curve represents the double exponential fit of the data according to Eq. 1 ($k_{\text{obs}1} = 2.02 \pm 0.02 \text{ s}^{-1}$ and $k_{\text{obs}2} = 0.292 \pm 0.003 \text{ s}^{-1}$), and the residuals are shown in black at the top. The buffer control with only 0.5 μM Gp32F is shown in the black trace on the bottom, containing Buffer A with 100 mM NaCl, $T = 25^\circ\text{C}$. (B–C) Gp32F-M13mp18 ssDNA association as a function of [NaCl] under both strong (B) and weak (C) binding conditions. All the reactions in (B) and (C) contained 0.5 μM Gp32F and various amounts of M13mp18 ssDNA in Buffer A with indicated [NaCl]. The kinetic parameters from fitting (see text) are summarized in Table 1, which reflect the apparent on and off rates in the second step in the Lohman model, as shown in Scheme 1.¹⁸

Figure 2.

The displacement of Gp32F from ssDNA by UvsX is affected by nucleotides, salt, and UvsY. (A) 0.5 μ M Gp32F - 3.5 μ M M13mp18 ssDNA complex was rapidly mixed with 0.875 μ M UvsX in Buffer A + 50 mM NaCl and either no nucleotide, 1 mM AMP, 1 mM ADP, 1 mM ATP + regenerating system (R.S.), or 1 mM ATP γ S, $T = 25^{\circ}\text{C}$. Red and blue curves represent the best fits of Eq. 4 to reactions with 1 mM ATP + R.S. and 1 mM ATP γ S, respectively. (B) 0.5 μ M Gp32F - 3.5 μ M M13mp18 ssDNA complex was rapidly mixed with 0.875 μ M UvsX $\pm$ 0.875 μ M UvsY in Buffer A + 1mM ATP + R.S. + either 50 mM, 100 mM, or 200 mM NaCl, $T = 25^{\circ}\text{C}$. Red, blue, and green curves represent the best fits of Eq. 4 to reactions with 50 mM NaCl, 100 mM NaCl, or 200 mM NaCl + UvsY, respectively. Fitting parameters are shown in Table 2.

Figure 3.

Pre-steady state studies of UvsX-ssDNA binding *via* Gp32F displacement. (A–B) Observed rate constants of (A) UvsX nucleation (k_{obs1}) and (B) UvsX growth (k_{obs2}) on Gp32F-ssDNA as functions of [UvsX] in Buffer A + 50 mM NaCl + either 1 mM ATP + R.S., or 1 mM ATP γ S as indicated. For all reactions in (A) and (B), 0.5 μM Gp32F - 3.5 μM M13mp18 ssDNA complex was rapidly mixed with indicated amount of UvsX at $T = 25^\circ\text{C}$. (C–D) Observed rate constants of (C) UvsX nucleation (k_{obs1}) and (D) UvsX propagation (k_{obs2}) on Gp32F-ssDNA in the presence of UvsY, as a function of [UvsX] in Buffer A + 200 mM NaCl + either 1 mM ATP + R.S., or 1 mM ATP γ S as indicated. For all reactions in (C) and (D), 0.5 μM Gp32F - 3.5 μM M13mp18 ssDNA complex was rapidly mixed with indicated amount of UvsX + 0.875 μM UvsY at $T = 25^\circ\text{C}$. Curves in (A) – (D) represent fits of the data to Eqs. 2–3. Fitting parameters are shown in Table 3.

Figure 4.

Time courses of Gp32F displacement by different concentrations of UvsX. Red curves represents the best stepwise global fit models (Eq. 7). Fitting parameters are shown in Table 3. (A–B) Reactions in Buffer A + 50 mM NaCl + either (A) 1 mM ATP + R.S. or (B) 1 mM ATP γ S. For all reactions in (A) and (B), 0.5 μ M Gp32F - 3.5 μ M M13mp18 ssDNA complex was rapidly mixed with indicated amount of UvsX at T = 25°C. (C–D) Reactions in Buffer A + 200 mM NaCl + 0.875 μ M UvsY + either 1 mM ATP + R.S. (C) or 1 mM ATP γ S (D). For all reactions in (C) and (D), 0.5 μ M Gp32F - 3.5 μ M M13mp18 ssDNA complex was rapidly mixed with indicated amount of UvsX + 0.875 μ M UvsY at T = 25°C. During curve fitting, iterative runs for individual isotherms were taken to determine values and standard errors of kinetic constants.

Figure 5.

A model for UvsX presynaptic filament assembly on Gp32-covered ssDNA. *Left* — Under low-salt conditions in the absence of mediator protein UvsY, ATP-bound UvsX, a high affinity form, binds Gp32-ssDNA rapidly to form an unstable nucleation site or “pre-nucleation complex” (association constant K_1). A slow but almost irreversible conformational change (forward rate constant k_2) is required by UvsX to displace Gp32 and to secure this isolated nucleation site on the lattice. With successful nucleation, more ATP-bound UvsX is recruited to form an unstable cluster (association constant K_3). This rapidly formed UvsX cluster undergoes another slow but almost irreversible conformational change to displace Gp32 and to redistribute into a stable and productive presynaptic filament (forward rate constant k_4). *Right* — Under high-salt conditions the mediator protein, UvsY, facilitates filament nucleation by stabilizing the salt-sensitive pre-nucleation complex (enhanced K_1), by forming a special quaternary complex with UvsX, Gp32, and ssDNA. Filament growth (particularly k_4) is rate-limiting under both UvsY-dependent and – independent conditions. Therefore filament assembly necessary for DNA strand exchange and recombination functions of UvsX likely involves frequent nucleation events leading to many short filaments. See text for details.

Scheme 1.

Model for Gp32F-ssDNA association kinetics.

Scheme 2.

Nucleation-growth kinetic model for UvsX presynaptic filament assembly on Gp32F-covered ssDNA.

Scheme 3.

Symbolic representation of Scheme 2, used for global fitting of model-derived differential equations. Scheme 3 is identical to Scheme 2 but contains the following term substitutions to facilitate mathematical modeling: [S1] represents the concentration of free UvsX protein; [S2] represents the concentration of Gp32F-covered ssDNA which is converted to all available binding sites for UvsX, according to a binding site size $n = 4$ nucleotide residues;²¹ [N1] represents the concentration of an initial pre-nucleation complex Gp32F•ssDNA•UvsX* in which UvsX binds Gp32F-covered ssDNA to form isolated sites; [N2] represents the concentration of Gp32F•ssDNA•UvsX_{isolated}, an isomerized productive form of nucleation complex in which UvsX forms isolated stable sites on Gp32F-covered ssDNA; [F1] represents the concentration of a filament intermediate ssDNA•UvsX*_{cluster}•Gp32F in which UvsX forms initial clusters on Gp32F-covered ssDNA; [F2] represents the concentration of the isomerized productive cluster ssDNA•UvsX_{cluster} in which UvsX forms a productive presynaptic filament on ssDNA.

Table 1

Kinetic parameters of Gp32F binding to ssDNA: Effects of NaCl concentration.

[NaCl]	Gp32F		Gp32 (from ref. ¹⁸)	
	$k_2(\text{app})$ ($\text{M}^{-1}\text{s}^{-1}$)	$k_{-2}(\text{app})$ (s^{-1})	K_2 (M^{-1})	k_2 ($\text{M}^{-1}\text{s}^{-1}$)
50 mM	$4.3 \pm 0.2 \times 10^4$	0.33 ± 0.02	1.3×10^5	1.3×10^6
100 mM	$6.6 \pm 0.2 \times 10^4$	0.41 ± 0.02	1.6×10^5	2.8×10^6
200 mM	$3.7 \pm 0.1 \times 10^4$	0.37 ± 0.02	1.0×10^5	---
250 mM	$2.4 \pm 0.1 \times 10^4$	0.34 ± 0.02	7.1×10^4	---

The apparent rate constants k_2 and k_{-2} and the calculated equilibrium constant K_2 for Gp32F-ssDNA interactions were derived from linear fits of the data in Figure 1B–C as described in Supporting Materials and Methods. Previously published values of k_2 for interactions of unmodified Gp32 with a poly(rεA) lattice are shown for comparison.

Table 2

Observed rates and amplitudes for Gp32F displacement from ssDNA by UvsX recombinase; Effects of nucleotides, salt, and UvsY protein.^a

Nucleotide	[NaCl]	$k_{\text{obs1}} (\times 10^{-3} \text{ s}^{-1})$	$A_1 \times 10^5$	$k_{\text{obs2}} (\times 10^{-3} \text{ s}^{-1})$	$A_2 \times 10^5$
ATP γ S	50	274 $\pm$ 11	2.5 $\pm$ 0.1	37.8 $\pm$ 0.1	19.4 $\pm$ 0.0
ATP/R.S.	50	24.4 $\pm$ 0.4	8.3 $\pm$ 0.2	9.5 $\pm$ 0.0	21.8 $\pm$ 0.2
	100	10.6 $\pm$ 0.8	5.0 $\pm$ 0.1	8.3 $\pm$ 0.5	14.5 $\pm$ 0.1
	200	< 0.125	---	< 0.125	---
ATP/R.S.+UvsY	200	59.1 $\pm$ 1.5	1.4 $\pm$ 0.0	2.4 $\pm$ 0.0	10.0 $\pm$ 0.0
ADP	50	< 0.125	---	< 0.125	---
AMP	50	< 0.125	---	< 0.125	---
None	50	< 0.125	---	< 0.125	---

^a Observed rate constants (k) and amplitudes (A) were derived from double exponential fits of the fluorescence traces in Figure 2 as described in the text. Reactions were carried out in Buffer A containing NaCl concentrations as indicated. Nucleotides, when present, were at a concentration of 1 mM. R.S. denotes the presence of an ATP regenerating system. Concentrations of M13mp18 ssDNA, Gp32F, and UvsX were 3.5, 0.5, and 0.875 μ M, respectively. UvsY, when present, was at a concentration of 0.875 μ M.

Fitted kinetic parameters for the displacement of Gp32F from ssDNA during UvsX-ssDNA filament assembly under low (UvsY-independent) and high (UvsY-dependent) salt conditions.^a

Table 3

Conditions	Fitting Method ^b	Nucleation			Growth	
		$K_1 (\times 10^6 \text{ M}^{-1})$	$k_2 (\times 10^{-3} \text{ s}^{-1})$	$K_3 (\times 10^6 \text{ M}^{-1})$	$k_4 (\times 10^{-3} \text{ s}^{-1})$	
50mM NaCl, no UvsY	hyperbolic	1.4 ± 0.9	53 ± 16	0.7 ± 0.2	30 ± 4	
	global	2.6 ± 1.5	35 ± 4	1.5 ± 0.3	35 ± 4	
	hyperbolic	4.1 ± 1.6	163 ± 13	8.2 ± 1.3	43 ± 1	
	global	3.1 ± 1.2	337 ± 89	4.1 ± 2.7	42 ± 4	
200 mM NaCl + UvsY	hyperbolic	39 ± 26	50 ± 2	4.0 ± 1.5	3.2 ± 0.3	
	global	68 ± 44	47 ± 11	3.0 ± 1.2	4.6 ± 0.6	
	hyperbolic	14 ± 3	49 ± 2	3.5 ± 0.4	8.8 ± 0.2	
	global	64 ± 32	50 ± 7	3.0 ± 1.4	11 ± 2	

^a Reactions were carried out in Buffer A containing 50 mM NaCl (low-salt, UvsY-independent conditions) or containing 200 mM NaCl (high-salt, UvsY-dependent conditions). Nucleotide concentration was 1 mM in all reactions. R.S. denotes the presence of an ATP regenerating system. Concentrations of M13mp18 ssDNA and Gp32F were 3.5 and 0.5 μM, respectively. UvsY, when present, was at a concentration of 0.875 μM. Under each condition, fluorescence traces were obtained at multiple UvsX concentrations (Figure 4A–D).

^b Kinetics were modeled according to Scheme 2 using two independent data-fitting procedures (see Materials and Methods): *Hyperbolic fitting method* — kinetic parameters were determined by fitting hyperbolic functions (Eqs. 2 and 3) to plots of k_{obs1} and k_{obs2} , respectively, vs. UvsX concentration as shown in Figure 3A–D. *Global fitting method* — direct global fits of fluorescence traces to model-derived differential equations (Eqs. 7.1 – 7.6) were performed using DynaFit to obtain kinetic parameters for filament nucleation and growth phases. Scheme 3, a symbolic version of Scheme 2, was used for mathematical modeling. Fitted curves are shown in Figure 4A–D.