Supplemental Data

Asymmetric Regulation of Bipolar Single-stranded DNA Translocation by the Two Motors within *E. coli* RecBCD Helicase

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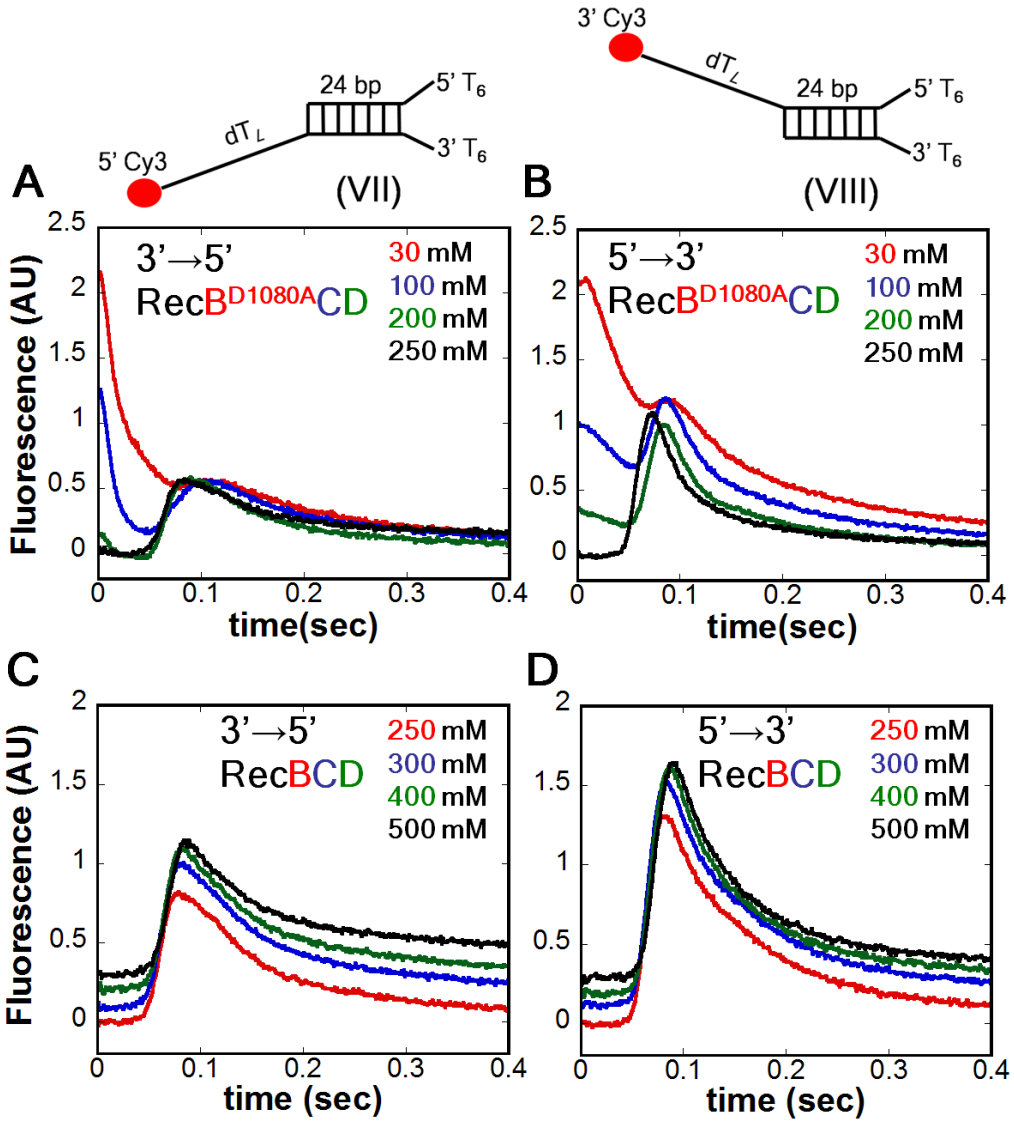


Figure S1. Higher NaCl concentrations increase the specificity of RecBCD binding at the high affinity loading site. All stopped-flow experiments were performed with 5 mM ATP and 7.5 mg/mL heparin in buffer M_{250} containing 10 mM Mg^{2+} at 25°C. Experiments were performed at the NaCl concentration indicated.

(A) (3' to 5) and (B) (5' to 3'). Translocation experiments with $RecB^{D1080A}CD$, a nuclease deficient mutant of RecBCD at low [NaCl] (≤250 mM). At [NaCl] < 250 mM, an exponential decrease in Cy3 fluorescence is superimposed on the expected lag phase time course. This exponential phase is due to random binding of $RecB^{D1080A}CD$ to the ssDNA extension ($(dT)_{60}$), probably via RecD). This phase decreases in magnitude as the [NaCl] increases, becoming negligible at [NaCl] = 250 mM, where only a lag phase time course is observed.

(C) (3' to 5') and (D) (5' to 3'). Translocation time courses with wtRecBCD at high [NaCl] (≥250 mM) show only lag phase time courses with the lag time being independent of [NaCl]. Each time course has been artificially shifted along the ordinate.

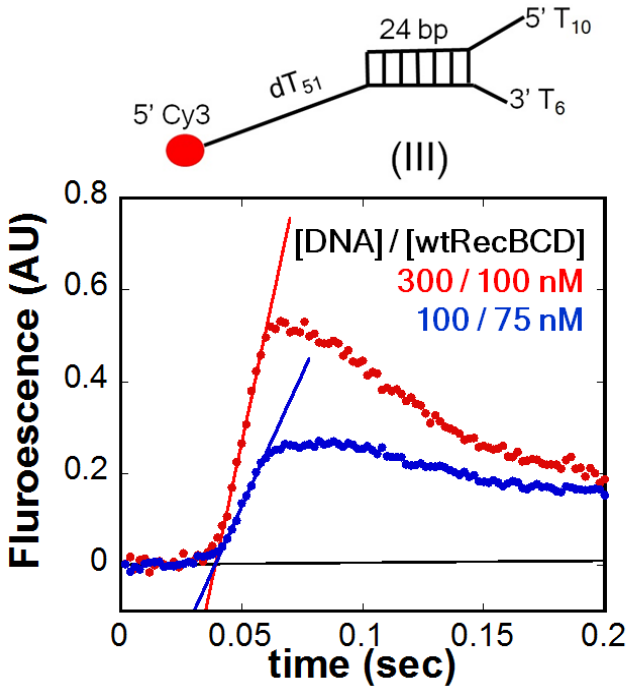


Figure S2. Varying the ratio of the concentrations of wtRecBCD and DNA results in different amplitudes of the Cy3 fluorescence enhancement but yields the same lag phase. Cy3 fluorescence time courses for RecBCD translocation were obtained using the single extension DNA substrates (DNA III, L = 51) with enzyme and substrate concentrations as indicated (before mixing) in buffer M_{250} at 25°C with 5 mM ATP and 7.5 mg/mL heparin. The lag time is the time at the intersection of the two linear fits of the time course. For both time courses, two linear fits were performed using the data points (0～30 ms and 44～60 ms), respectively. Red curve: y=0.011016-0.20436x (0～30 ms) and y=-0.95991+24.516x (44～60 ms, shown as red line), the lag time is 0.0392 sec. Blue curve: 0.0019844+0.043893x (0～30 ms, shown as black line) and y=-0.44983+11.553x (44～60 ms, shown as blue line), lag time is 0.0389 sec.

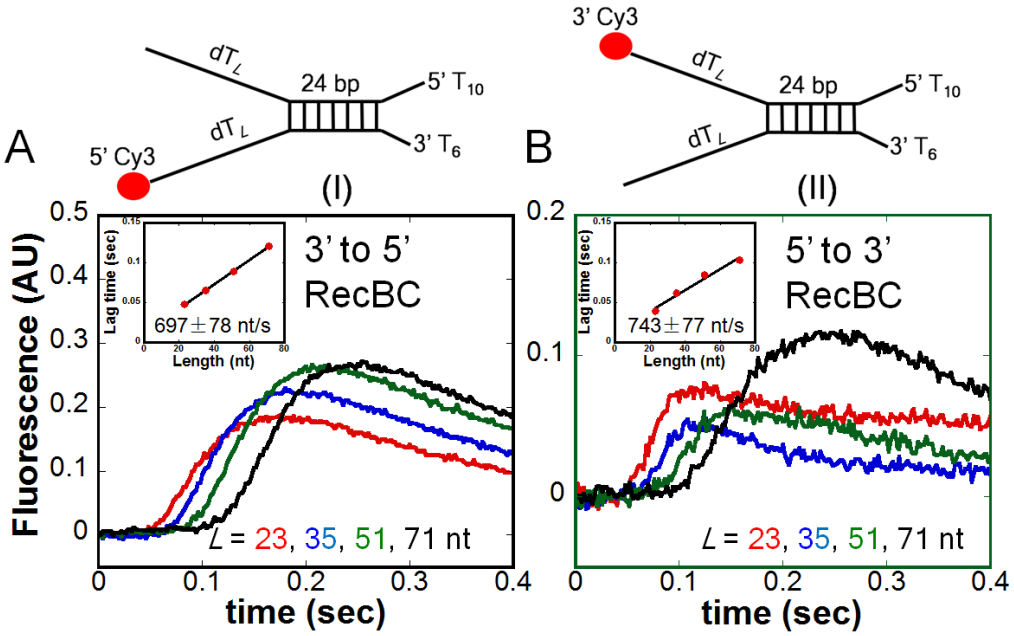


Figure S3. Cy3 fluorescence time courses of RecBC translocation using the single extension DNA substrates (DNA I and II) with a high affinity loading site 5'-dT_{10}-3'-dT_6 tailed end. The stopped-flow experiments were performed in buffer M_{250} at 25°C with 10mM Mg^{2+}, 5 mM ATP and 7.5 mg/mL heparin (concentrations after mixing). Inset shows the linear fit of lag time vs. ssDNA extension length, *L*. The inverse of the slope yields translocation rate.

(A) Time courses monitoring 3' to 5' ssDNA translocation of RecBC using DNA substrate I. Inset: 697±78 nt/s.

(B) Time courses monitoring 5' to 3' ssDNA translocation of RecBC using DNA substrates II. Inset: 743±77 nt/s.

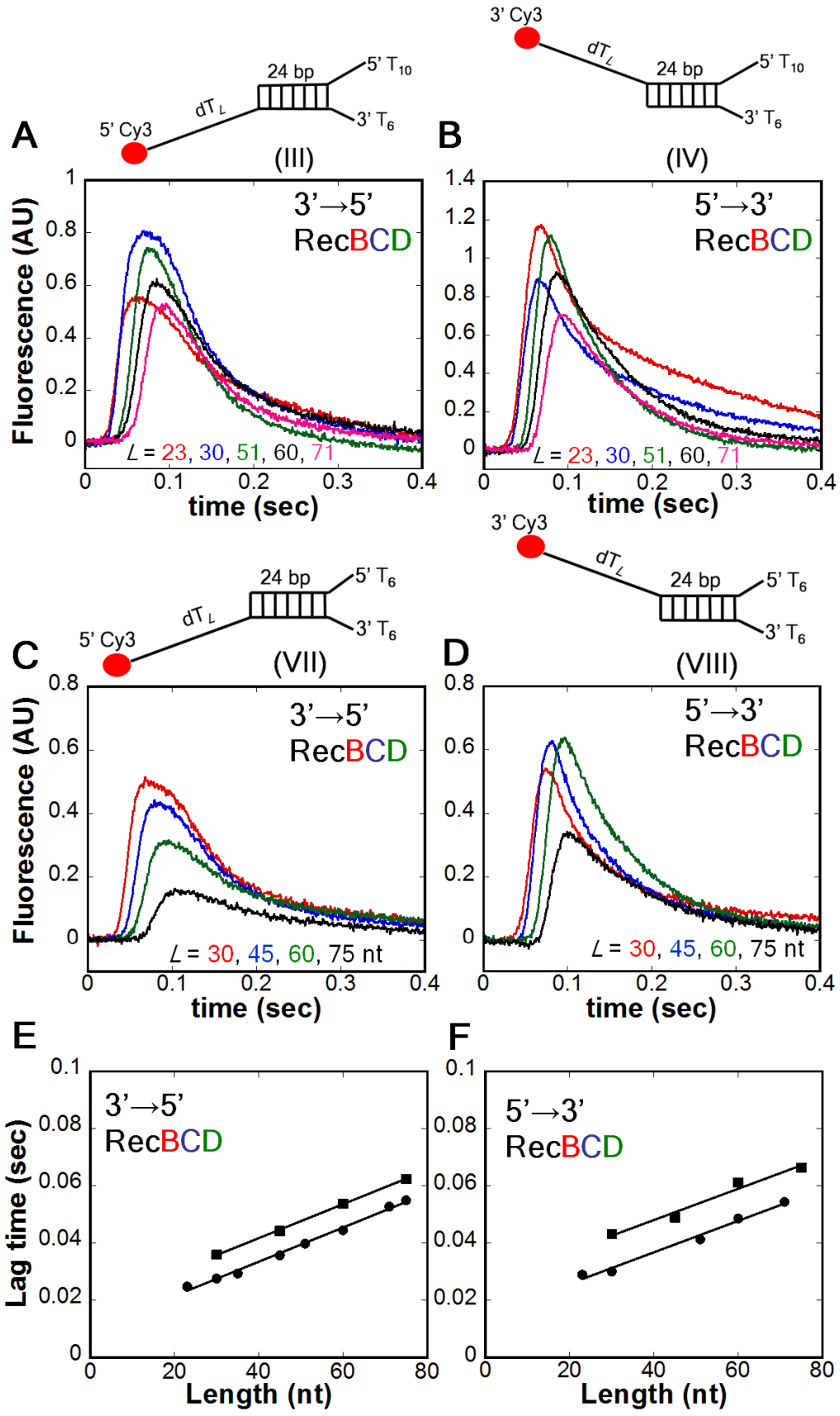

A
B
C
D
E
F
5' Cy3
3' Cy3
dT L
24 bp
5' T10
3' T6
5' T6
(III)
(IV)
(VII)
(VIII)
3'→5'
RecBCD
5'→3'
RecBCD
Fluorescence (AU)
time (sec)
L = 23, 30, 51, 60, 71
L = 30, 45, 60, 75 nt
Lag time (sec)
Length (nt)

Figure S4. RecBCD translocation using single extension DNA substrates. Stopped-flow experiments were performed in buffer M_{250} at 25°C with 5 mM ATP and 7.5 mg/mL heparin (concentrations after mixing).

(A) (3' to 5') and (B) (5' to 3'). Time courses monitoring ssDNA translocation of RecBCD using single extension DNA substrate (DNA III and IV) with a 5'-dT_{10}-3'-dT_6 tailed end (high affinity RecBCD loading site).

(C) (3' to 5') and (D) (5' to 3'). Time courses monitoring ssDNA translocation of RecBCD using single extension DNA substrate (DNA VII and VIII) with 5'-dT_6-3'-dT_6 tailed end (high affinity RecBC loading site).

(E) Linear dependence of lag time on ssDNA extension length, *L*, for translocation time courses (3' to 5') in panel A (solid circles), rate=1627±103 nt/s; and panel C (solid square), rate=1667 nt/s.

(F) Linear dependence of lag time on ssDNA extension length, *L*, for translocation time courses (5' to 3') in panel B (solid circles), rate=1881±33 nt/s; and panel D (solid square), rate=1834 nt/s.

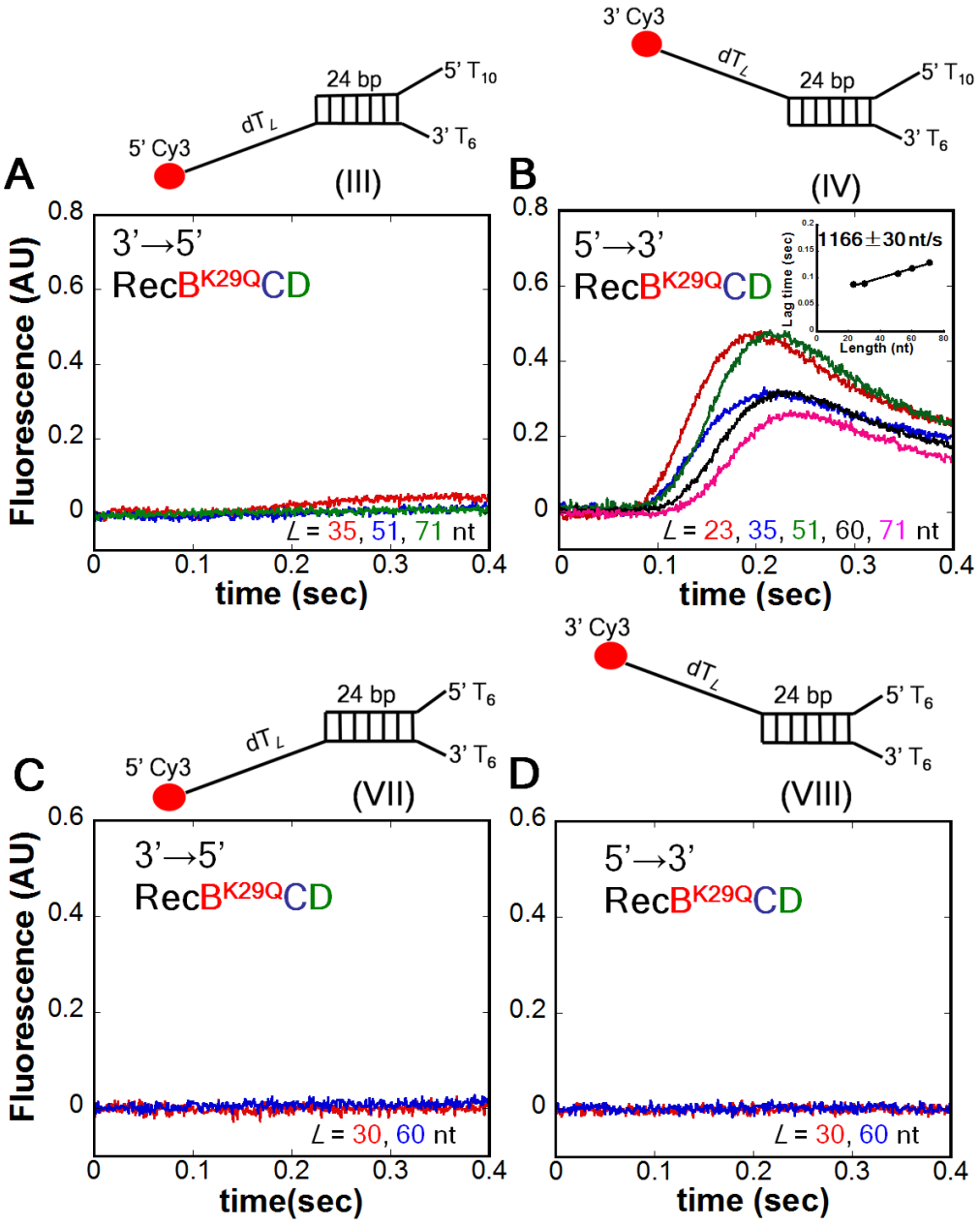


Figure S5. Initiation of RecB^{K29Q}CD unwinding of the 24 bp duplex requires a high affinity loading site possessing a 5'-dT$_{10}$ tail. Stopped-flow experiments were performed in buffer M$_{250}$ at 25°C with 5 mM ATP and 7.5 mg/mL heparin (concentrations after mixing).

(A) No translocation (3' to 5') is detected due to an ATPase inactive RecB^{K29Q}.

(B) Time courses of 5' to 3' translocation of RecB^{K29Q}CD on the Cy3 labeled single extension DNA substrate (DNA IV) containing a 5'-dT$_{10}$-3'-dT$_6$ tailed high affinity RecBCD loading site. Inset: linear dependence of lag time vs. ssDNA extension length, *L*, yields a 5' to 3' translocation rate of 1166±30 nt/s for RecB^{K29Q}CD.

(C) and (D) No translocation activity is observed for RecB^{K29Q}CD on a DNA substrate containing a 5'-dT$_6$-3'-dT$_6$ tailed end.

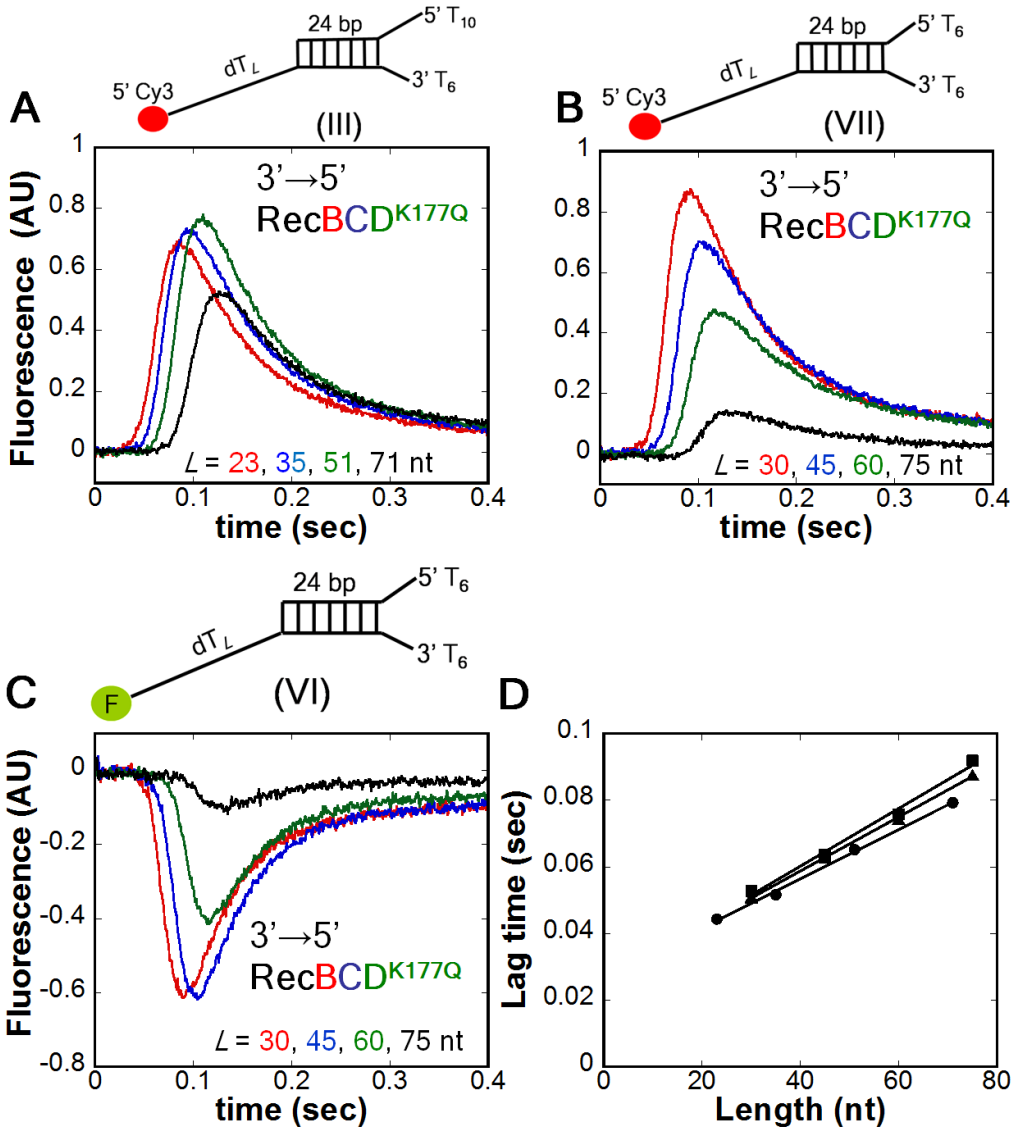


Figure S6. RecBCD^{K177Q} translocation along ssDNA (3' to 5') using Cy3 or fluorescein labeled single extension DNA substrates. Stopped-flow experiments were performed in buffer M_{250} at 25°C with 5mM ATP and 7.5 mg/mL heparin.

(A) Time courses monitoring ssDNA translocation of RecBCD^{K177Q} using Cy3-labeled single extension DNA substrate (DNA III) with a 5'-dT_{10}-3'-dT_6 tailed end.

(B) (Cy3) and (C) (fluorescein). Time courses monitoring ssDNA translocation of RecBCD^{K177Q} using single extension DNA substrate (VII and VI) with a 5'-dT_6-3'-dT_6 tailed end.

(D) Linear dependences of lag time on ssDNA extension length, *L*, for time courses in panel A (solid circle: rate=1380±35 nt/s); panel B (solid triangle: rate=1235 nt/s); panel C (Solid square: rate=1163 nt/s).

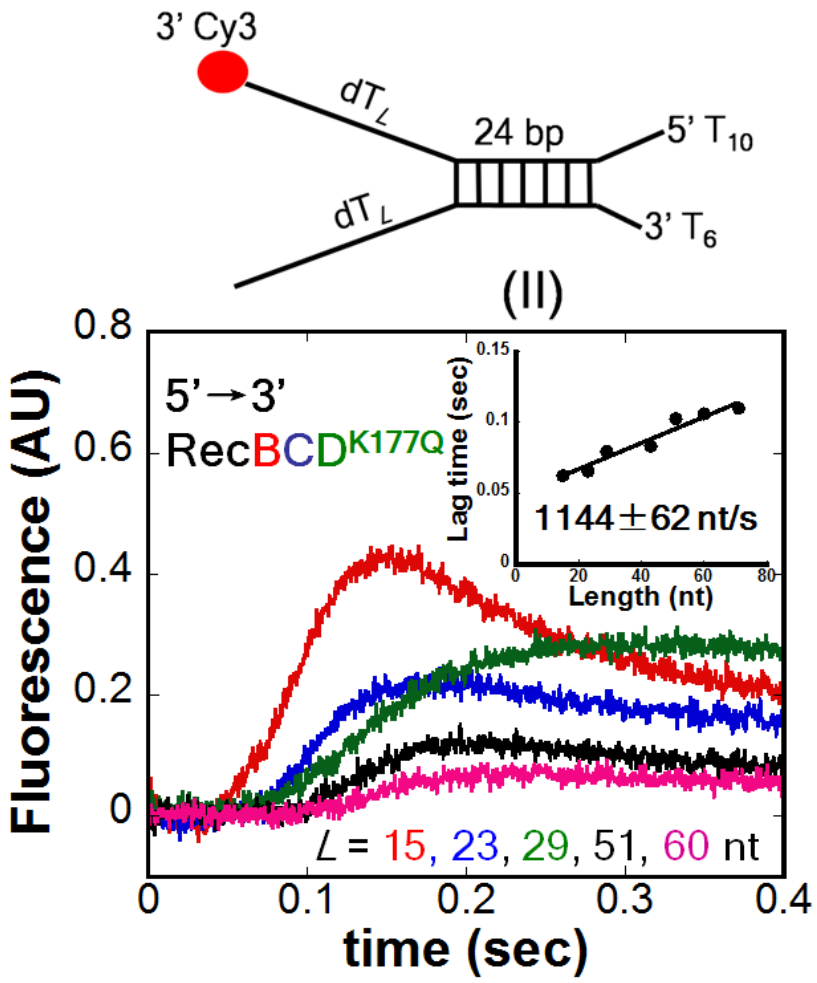


Figure S7. Time courses monitoring 5' to 3' ssDNA translocation of RecBCD^{K177Q} using DNA substrates II. Stopped-flow experiments were performed in buffer M_{250} at 25°C with 5 mM ATP and 7.5 mg/mL heparin. Inset: linear dependence of lag time on ssDNA extension length, *L*, yields a 5' to 3' translocation rate of 1144±62 nt/s for RecBCD^{K177Q}.

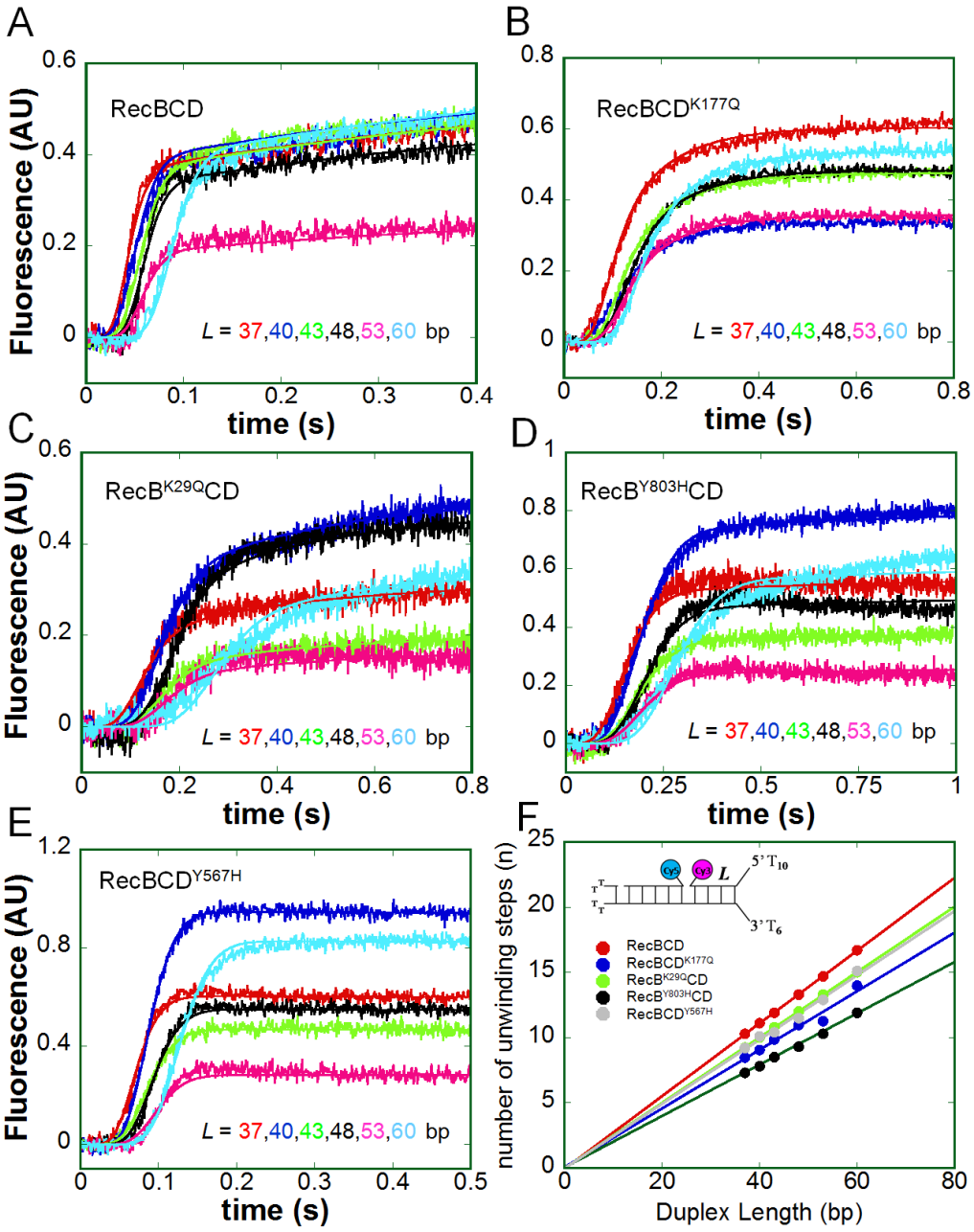


Figure S8. Cy3 fluorescence time courses monitoring DNA unwinding of WT and mutant RecBCD. All DNA unwinding reactions are conducted in buffer M_{250} plus 10 mM Mg^{2+}, 5 mM ATP and 7.5 mg/mL heparin. Smooth curves are fitted time courses according to global NLLS analysis to Scheme 1. (A) WT RecBCD catalyzed DNA unwinding time courses. (B) $RecBCD^{K177Q}$. (C) $RecB^{K29Q}CD$. (D) $RecB^{Y803H}CD$. (E) $RecBCD^{Y567H}$. (F) Linear dependence of DNA unwinding steps (n) vs. duplex length determined by NLLS analysis for corresponding time courses in other panels. Panel A: y = -0.0046+0.28x, yielding step size by the inverse of the slope, m=3.6 bp; panel B: y= -0.07+0.22x, m=4.5 bp; panel C: y= 0.002 + 0.25x, m=4 bp; panel D: y= -0.026 + 0.20x, m=5 bp; panel E: y= -0.013+0.25x, m=4 bp.

Table S1. DNA substrate sequences used for RecBCD translocation experiments.

ssDNA	DNA sequence
A	5'-$(dT)_{10}$CCA TGG CTC CTG AGC TAG CTG CAG-3'
B	5'-CTG CAG CTA GCT CAG GAG CCA TGG $(dT)_6$-3'
C	5'-$(dT)_6$ CCA TGG CTC CTG AGC TAG CTG CAG-3'
a	5'-$(dT)_{10}$CCA TGG CTC CTG AGC TAG CTG CAG $(dT)_L$ XT-3'
b	5'-X $(dT)_L$ CTG CAG CTA GCT CAG GAG CCA TGG $(dT)_6$-3'
c	5'-$(dT)_{10}$CCA TGG CTC CTG AGC TAG CTG CAG $(dT)_L$-3'
d	5'-$(dT)_L$ CTG CAG CTA GCT CAG GAG CCA TGG $(dT)_6$-3'
e	5'-$(dT)_6$ CTG CAG CTA GCT CAG GAG CCA TGG $(dT)_L$ XT-3'
f	5'-$(dT)_{10}$CCA TGG CTC CTG AGC TAG CTG CAG 3'-3'$(dT)_{45}$ TX-5'

DNA substrates formed by annealing top strand with bottom strand. Non-labeled ssDNA is 25% in excess over fluorophore labeled ssDNA. The components of DNA substrates (I-VIII) are listed as below:

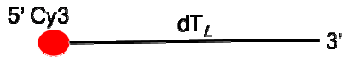
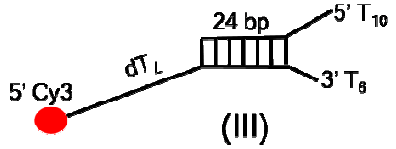
I	c (top) and b (bottom)
II	a (top) and d (bottom)
III	A (top) and b (bottom)
IV	a (top) and B (bottom)
V	f (top) and d (bottom), *L*=45
VI	C (top) and b (bottom), X=fluorescein
VII	C (top) and b (bottom)
VIII	e (top) and B (bottom)

X represents Cy3 or fluorescein which is labeled at either 3' end or 5' end.

X=Cy3 in all substrates except DNA VI.

L =15, 23, 29, 30, 35, 43, 45, 51, 60, 71, 75.

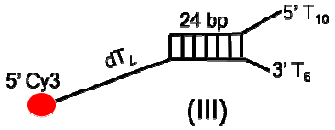
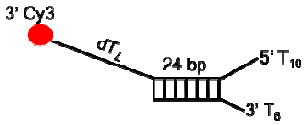
Table S2. Regulation of RecB (3' to 5') translocase activity by RecC and RecD.

Translocase	3' to 5' Translocation Rate (nt/s)	DNA substrate
RecB	803±13[a]	
RecBC	1038±16[b]	
RecBCD	1627±103[b]	
RecBCD^{K177Q}	1380±35[b]	

DNA III is used to measure RecB (3' to 5') translocase activity because RecBC and RecBCD require a loading site to initiate translocation. All translocation rates were determined in buffer M containing 10 mM Mg^{2+} at 25°C. Stopped-flow experiments were performed with 5 mM ATP and 7.5 mg/mL heparin (concentrations after mixing).

a. The rate was determined in buffer M_{30} (19).
b. The rates are determined in buffer M_{250}.

Table S3. Summary of translocation rates (3' to 5' and 5' to 3') of wtRecBCD and mutants determined on the single extension DNA substrates (DNA III and IV).

	Single extension DNA substrate (5'-dT_{10}-3'-dT_6)	
Complex	 (III)	 (IV)
	3' → 5'	5' → 3'
wtRecBCD	1627±103	1881±33
$RecB^{D1080A}CD$	1645±36	1869±42
$RecBCD^{K177Q}$	1380±35	1393±10
$RecB^{K29Q}CD$	No activity	1160±30
$RecB^{Y803H}CD$	611±123	1418±105
$RecBCD^{Y567H}$	1539	1154

All translocation rates were determined in buffer M_{250} containing 10 mM Mg^{2+} at 25°C. Stopped-flow experiments were performed with 5 mM ATP and 7.5 mg/mL heparin (concentrations after mixing).

Table S4. Translocation rates of wtRecBCD under various reaction conditions.

Enzyme	Direction	Translocation rates (nt/s) under [Mg^{2+}] < [ATP]:1 mM Mg^{2+}, 5 mM ATP	
		Buffer T_{250} at 20°C	Buffer M_{250} at 25°C
wt RecBCD	3' → 5'	748	809
wt RecBCD	5' → 3'	974	1298
		Translocation rates (nt/s) under [Mg^{2+}] > [ATP]:10 mM Mg^{2+}, 5 mM ATP	
		Buffer T_{250} at 20°C	Buffer M_{250} at 25°C
wt RecBCD	3' → 5'	1173	1409±109
wt RecBCD	5' → 3'	1523	1922±72

Buffer T_{250} is 250 mM NaCl, 25 mM Tris, pH 7.5 at 20 °C.
Buffer M_{250} is 250 mM NaCl, 20 mM MOPS, pH 7.0 at 25 °C.
Mg^{2+} and ATP concentrations are indicated as the final concentrations after mixing.
All translocation rates were determine on double extension DNA substrates with 5'-dT_{10}-3'-dT_6 tailed end (DNA I and II).

Supplementary Discussion

Salt Dependence of initiation of RecBCD Translocation.

As shown in Figure 1B, when RecBCD binds only at the high affinity loading site of the DNA substrate and initiates unwinding of the 24 bp duplex, a lag phase is observed in the time course. Previous experiments with RecBC using similar DNA substrates were performed in buffer (buffer M_{30}) containing 30 mM NaCl, conditions under which all RecBC molecules were observed to bind only at the high affinity loading site in the presence of excess DNA (19). However, upon performing the same experiments with RecBCD at 30 mM NaCl or $RecB^{D1080A}CD$, containing a mutation (D1080A) in the nuclease active site of RecB that eliminates its nuclease activity (27) in the presence of 10 mM Mg^{2+}, we observed more complex time courses (Figure S1A and S1B). These results suggested that at 30 mM NaCl, RecBCD is able to bind to the $(dT)_{60}$ extension as well as the high affinity loading site and thus interfere with the Cy3 signal resulting from RecBCD that initiates at the high affinity loading site. Experiments performed at higher [NaCl] show that the binding of RecBCD to the $(dT)_L$ extensions is eventually eliminated at a NaCl concentration at or above 250 mM. At [NaCl] $\geq$ 250 mM, the expected lag phase kinetics is indeed observed for RecBCD (Figure S1C and S1D). Furthermore, the value of the lag time is unaffected by increasing the [NaCl] from 250 to 500 mM and the nuclease activity does not interfere with the ssDNA translocation kinetics at 250 mM NaCl or higher. Thus the experiments reported here were performed in buffer containing 250 mM NaCl. The fact that ssDNA is able to compete with the high affinity loading site for RecBCD binding at low [NaCl] likely reflects the ability of RecD to bind ssDNA with high affinity even when part of the RecBCD complex. This is based on the observation that RecBC shows a much higher specificity for its high affinity loading site even at 30 mM NaCl (19).

Processivity of RecBC secondary translocase

We note that the processivity of the RecBC secondary translocase within $RecBCD^{K177Q}$ appears low (compare traces in Figure 4A with those for RecBCD (Supplementary Figure 4B)). This is possibly because the dead $RecD^{K177Q}$ motor is unable to translocate it may also limit the activity of the RecBC secondary translocase. Regardless, these results indicate that the secondary RecBC translocase functions within RecBCD and thus its 5' to 3' translocase activity has contributions from both RecD and the secondary RecBC translocase.